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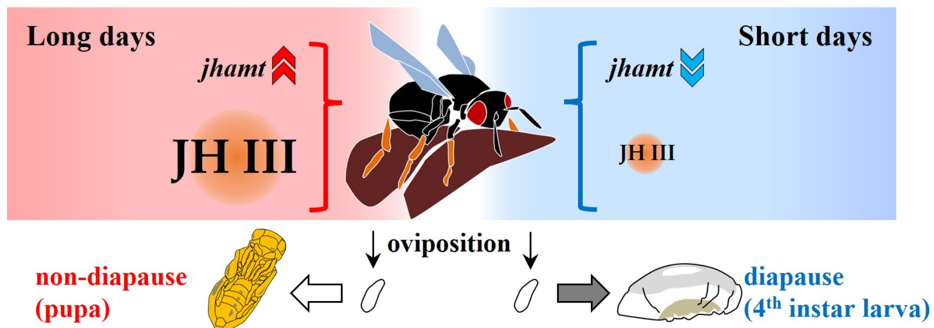
Maternal regulation of diapause in Nasonia vitripennis
Long days
jhamt
JH III
Short days
jhamt
JH III
oviposition
non-diapause
(pupa)
diapause
(4th instar larva)

Juvenile hormone as a causal factor for maternal regulation of diapause in a wasp

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Abstract

Most temperate multivoltine insects enter diapause, a hormonally controlled developmental suspension, in response to seasonal photoperiodic and/or thermal cues. Some insect species exhibit maternal regulation of diapause in which developmental trajectories of the offspring are determined by mothers in response to environmental cues that the mother received. Although maternally regulated diapause is common among insects, the maternal endocrinological mechanisms are largely veiled. To approach this issue, we used the parasitic jewel wasp *Nasonia vitripennis*, which produces non-diapause-destined offspring under long days and diapause-destined offspring under short days or low temperatures. Comparative transcriptomics of these wasps revealed possible involvement of the juvenile hormone (JH) biosynthetic cascade in maternal diapause regulation. JH acid methyltransferase was typically downregulated in short-day wasps, and this was reflected by a reduction in haemolymph JH concentrations. RNAi targeted at *jhamt* reduced haemolymph JH concentration and induced wasps to produce diapause-destined offspring even under long days. In addition, topical application of JH suppressed the production of diapause-destined offspring under short days or low temperatures. These results indicate that diapause in *N. vitripennis* is determined by maternal *jhamt* expression and haemolymph JH concentration in response to day length. We, therefore, report a novel role for JH in insect seasonality.

Keywords (maximum 6): *Nasonia vitripennis*, photoperiodism, maternal effect, *jhamt*, RNAi, parasitic jewel wasp

1. Introduction

Diapause is a hormonally controlled cessation of development or reproduction and a strategy that allows many insects to synchronize their life cycle with the biotic and/or abiotic environment (Denlinger et al., 2012). Multivoltine temperate insects may enter diapause in response to various environmental factors, but photoperiod is the most widespread cue (Danks, 1987). The life stage sensitive to photoperiod (during which an insect receives the daylength cues triggering a physiological response) typically occurs in advance of the actual diapausing life stage. However, the stage can also occur in the previous generation and determine whether diapause occurs in the offspring, e.g. a maternal effect (Mousseau and Dingle, 1991).

Maternal regulation of diapause is known across many insect orders, including Hymenoptera (Saunders, 1966; Zaslavski and Umarova, 1990), Diptera (Anderson, 1968; Henrich and Denlinger, 1982), Orthoptera (Masaki 1972; Hakomori and Tanaka, 1992), Lepidoptera (Kogure, 1933; Raina and Bell, 1974), Hemiptera (Hokyo and Suzuki, 1983; Groesters and Dingle, 1988), and Psocodea (Glinyanaya, 1975; Eertmoed, 1978). The most classical example of this diapause is found in the silk moth *Bombyx mori*, in which photoperiodic signals experienced by the embryos and larvae of one generation determine the developmental fate of the embryos of the next generation (Kogure, 1933; Shimizu and Hasegawa, 1988). In another example, female parasitic jewel wasps *Nasonia vitripennis* (the focal species of this study) produce offspring destined to enter diapause in their larval stage when exposed to short days (Saunders, 1965, 1966). The offspring has no ability to respond to environmental factors to avoid diapause entry (Schneiderman and Horwitz, 1958).

Much of our current knowledge about the maternal endocrinological mechanisms underlying maternal regulation of diapause was gained from studies in *B. mori.* Diapause hormone (DH) is a member of the FXPRL-amide peptide family and is released from the oesophageal ganglion of female *B. mori* during their egg maturation, acting upon the ovarioles to produce diapause-destined eggs (Tsuchiya et al., 2021). A recent study also revealed the significance of maternal regulation of DH expression in the production of diapause-destined eggs in the locust *Locusta migratoria* (Hao et al., 2019). However, the involvement of DH in maternal regulation of diapause for other insect species has not been reported, and the endocrinological mechanisms underlying maternal regulation of diapause remain largely veiled in general (Denlinger et al., 2012). Juvenile hormone (JH) is an acyclic sesquiterpenoid that is synthesized at and released from an endocrine organ, the corpora allata (CA), and is important for insect metamorphosis and reproduction (Tobe and Stay, 1985; Goodman and Cusson, 2012). Although its involvement in maternal regulation of diapause was proposed, causal relationships between diapause regulation and JH concentration have yet to be verified (Flavell, 2017).

In the present study, we focus on the role of JH in maternally-regulated diapause of *N. vitripennis*. Using adult females, we compare the expression patterns of genes involved in the JH biosynthetic pathway under long and short days by RNA-seq and RT-qPCR. By using ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS), we found JH III is the JH of this species. We then link JH biosynthetic gene expression to haemolymph JH concentration and the diapause status of the offspring. Further, we examine the effect of *juvenile hormone acid O-methyl transferase* (*jhamt*) knockdown on haemolymph JH concentration and diapause

status of the offspring. To clarify the causal effect of JH in maternally-regulated diapause, we determine whether topical application of JH to mothers alters the developmental status of their eggs. Overall, we verified JH as the causal factor regulating the maternal effect in *N. vitripennis*.

2. Materials & methods

2.1 Insect rearing

Nasonia vitripennis adults were collected in Osaka City, Japan (34.6 °N, 135.5 °E) in 2011. The wasp stock culture was maintained under long-day conditions (16-h light and 8-h dark; abbreviated as 16L:8D) at 18 ± 1 °C. Newly emerged adult wasps were transferred to a glass vial (at a density of 50–80 individuals per vial) and fed on a 10% (w/v) sucrose solution in a cotton-plugged 0.6 mL tube. Approximately 100 non-diapause flesh fly pupae (*Sarcophaga similis*) were supplied as hosts 12–14 days after adult wasp emergence. Adult wasps of the next generation emerged from the fly pupae after 28-32 days.

2.2 Wasp photoperiodic response

To determine the proportion of females producing non-diapause- or diapause-destined offspring under long and short day lengths, female wasps were isolated and each provided with two *S. similis* pupae. One day later, the parasitized fly pupae were

collected and maintained under the same environmental conditions as the female wasp. Each fly puparium was opened 21 days after parasitism and the number of wasp larvae (those in diapause) and wasp pupae (those that had not entered diapause) were counted. In most cases, over 90% of the brood was in diapause under short days but avoided diapause under long days. Female wasps were considered 'non-diapause producers' if <50% of their brood entered diapause. In contrast, females were considered 'diapause producers' when ≥50% of their brood had entered diapause. It is important to note that the proportion of diapause producers was variable, especially for young adult wasps (days 0-8). To minimize this variation, we used only females that had emerged on the same day for a given experiment.

2.3 Transcriptomic profiles of JH biosynthetic genes

Mass-reared wasps under 16L:8D were separated just after adult eclosion (the day of adult eclosion is defined as day 0) into two groups; one group maintained under 16L:8D and the other transferred to 10L:14D. On day 2, females were placed singly into glass vials and provided with two *S. similis* pupae for oviposition every two days until day 6. At Zeitgeber time (ZT) 22-24 on day 7, the female wasps were snap-frozen in liquid nitrogen and stored separately at -80°C. Only those females that had deposited eggs on day 6 and produced all non-diapause offspring under long-day conditions (n = 90) or at least one diapause offspring under short-day conditions (n = 27) were used for comparative transcriptomics.

Cryopreserved adult female wasps were grouped into three pools (biological replicates) per treatment (n = 30 individuals per replicate under long-day conditions, n =

9 individuals per replicate under short-day conditions). To extract total RNA, the heads of the wasps were homogenized in TRIzol reagent with a PureLink RNA micro kit (Thermo Fisher Scientific, Waltham, MA, USA). RNA quality control, cDNA library preparation, and 100 bp paired-end read sequencing on the Illumina HiSeq 4000 (Illumina, San Diego, CA) were performed by Macrogen Corp. (Tokyo, Japan). A total of 396 million reads were generated across the six cDNA libraries, with a mean GC content of 48.5%. We used the Galaxy platform (Goecks et al. 2010; usegalaxy.org) to process the sequence data. Within Galaxy we assessed library quality using FastQC (v0.72; Andrews 2018) and performed adapter sequence trimming and quality control using Trimmomatic (v0.38; Bolger et al. 2014), discarding sequences with unknown bases and those shorter than 35 bases after a 15 base head crop. Approximately 317 million sequences remained after trimming, with a mean GC content of 48%. We used HISAT2 (v2.1; Kim et al. 2019) to align our sequences against an *N. vitripennis* reference genome generated by Caltech ('Nvit_psr_1.1' GenBank assembly accession: GCA_009193385.2; alignment rate: 96.7%) and used the 'feature Counts' tool (v1.6.4; Liao et al. 2014) to assign annotations and estimate gene expression. We manually searched the transcriptomes for select genes involved in the JH biosynthetic cascade (Fig. S1), retaining those with at least 10 reads in three of the six libraries. Fold change differences between long- and short-day conditions were reported.

2.4 Verification of JH biosynthetic gene expression by RT-qPCR

To verify transcriptome results (above), we performed RT-qPCR for JH biosynthetic genes in adult female wasps under short- (8L:16D) and long-day (16L:8D)

conditions. The focal genes were: farnesol dehydrogenase (*FOHSDR*), farnesal dehydrogenase (also known as aldehyde dehydrogenase, *ALDH*), *jhamt*, and methyl farnesoate epoxidase (*Cyp15a1*) (Noriega, 2014). As we found multiple transcripts of these genes by RNA-seq, we used the Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.genome.jp/kegg) to define focal transcripts and design primers for RT-qPCR (Table 1). For example, three out of five *N. vitripennis ALDH* genes appear in the 'insect hormone biosynthesis' KEGG pathway (ko00981), therefore we designed primer pairs to discriminate between these three genes.

Mass-reared wasps under 16L:8D were separated just after adult eclosion and transferred to 16L:8D or 8L:16D. They were flash-frozen in liquid nitrogen at ZT 2-4 on days 1, 6, and 11. Total RNA was isolated from the heads of five females per photoperiod with TRIzol Reagent (Thermo Fisher Scientific). Each sample was subjected to a DNase I (Thermo Fisher Scientific) to eliminate any DNA contamination. cDNA was synthesised with a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). RT-qPCR was performed in a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with each reaction consisting of 10 μL of 2x GoTaq qPCR Master Mix (Promega, Madison, WI, USA), 1 μL of each primer (1 μM), 1 μL of 25x diluted cDNA product, and nuclease-free water for a total volume of 20 μL. Each reaction was performed in duplicate. *Ribosomal protein L32* (*RpL32*), also known as *ribosomal protein 49* (*rp49*), was used as a housekeeping gene. Gene expression values were compared to a standard curve created from a serial cDNA dilution. The two-way ANOVA followed by the Tukey-Kramer test was used to compare the expression of JH biosynthetic genes among photoperiods and days.

2.5 Effect of jhamt RNAi

To generate DNA template for double-stranded RNA (dsRNA), total RNA was extracted from the whole bodies of adult female wasps using TRIzol Reagent (Thermo Fisher Scientific). cDNA was synthesized with M-MLV reverse transcriptase and random hexamers (Thermo Fisher Scientific). PCR was conducted with Pho DNA Polymerase (NIPPON GENE, Tokyo, Japan) and primers (see Table 1) to generate T7-promoter-attached templates. A T7 Ribomax Express RNAi System (Promega) was used for dsRNA synthesis. As a control, dsRNA of *β-lactamase* (*bla*), which provides bacteria with ampicillin resistance, was synthesized using a pTA2 vector as a template (Toyobo, Osaka, Japan). The dsRNA was dissolved in nuclease-free water and stored at −20 °C until use. Injection of dsRNA into *N. vitripennis* pupae was performed according to Lynch and Desplan (2006). In brief, yellow pupae (17-18 days after hatching, which are ideal for dsRNA injection) were fixed on a glass slide using double-sided tape, and approximately 0.5 μg of dsRNA (a volume of ~0.1 μL) was injected into each pupa using a glass capillary (4748, World Precision Instrument, Sarasota, FL). Talcum powder was used to prevent any emerging adult wasps from adhering to the tape.

Wasps were maintained at 16L:8D and 18 °C until the day of eclosion, at which half of the individuals were transferred to 8L:16D, and thereafter all wasps were provided with hosts every day. We examined the effect of *jhamt* RNAi on the photoperiodic response by determining the number of days required to switch the type of offspring produced by these wasps (non-diapause- vs diapause-destined), termed the 'switching day', after changing the photoperiod (Saunders 1966, 1974). Friedman's test was used to compare the switching day among intact and dsRNA treatments. The

Tukey-Kramer test was also used to compare fecundity (the total number of larvae and pupae) among intact and dsRNA treatments. Knockdown efficiency (*jhamt* expression) was verified at ZT 2-4 on day 12. Total RNA isolation, genomic DNA elimination, cDNA synthesis, and RT-qPCR were performed according to the methods described in section 2.4. The Mann-Whitney *U* test was used to compare the *jhamt* expression among dsRNA treatments.

2.6 Haemolymph JH quantification and maternal status

We measured the concentration of JH III, the JH of the focal species (see results), in the haemolymph of intact wasps under long and short days, as well as in ds*bla* and ds*jhamt* injected wasps under long days. Intact short-day wasps were transferred from long days at the yellow pupal stage. Two days before haemolymph collection (day 10), wasps were isolated and provided with *S. similis* pupae to allow for assessment of the offspring type produced (non-diapause- or diapause-destined). We also assessed the relationship between haemolymph JH concentration and fecundity by comparing the number of offspring produced by wasps in the above treatment groups.

Haemolymph was collected from wasps according to Demontis and Perrimon (2010). In brief, 23-25 decapitated females were added to a silicon-coated 200 µL micropipette tip (Watson, Tokyo, Japan) on ice. The micropipette tip was then placed within a 1.5 mL silicon-coated microcentrifuge tube (Watson) and centrifuged at 1,500 × *g* at 4 °C for 12 min. After centrifugation, 1.5-2.0 µL of haemolymph was collected from the tube. To this haemolymph, we added 10 µL of 2.5% phenylthiourea in EDTA

(pH 5.2) to prevent melanisation and 300 µL of methanol to denature proteins. The haemolymph samples were stored at -80 °C until use.

Haemolymph JH purification was performed according to Kotaki et al. (2011). To calibrate the amount of JH lost during the purification process, we added authentic hemipteran JH III skipped bisepoxide ($JHSB_3$) (Kotaki et al. 2009) as an internal standard because it was not detected in *N. vitripennis* haemolymph (data not shown). The samples were mixed with 500 µL of 2 % NaCl solution and the JH-containing hydrophobic fraction was extracted with 200 µL of hexane three times. To purify JH, the hexane extract was applied to 1 g of neutral alumina (activity III) in a glass Pasteur pipette column (6 mm diameter). After washing the alumina with 2 mL of 10 % ether in hexane, 2 mL of 50% ether in hexane was applied and the eluate was collected. The solvent was dried with a vacuum evaporator and 30 µL of methanol was added to each sample.

We detected haemolymph JH III and $JHSB_3$ *via* UPLC-MS/MS (ACQUITY UPLC H-Class, Xevo TQ-S micro, Waters, Milford, MA, USA) with a C-18 column (ACQUITY UPLC BEH C18 Column, 2.1 mm × 100 mm, 1.7 µm particle size; Waters) according to Ando et al. (2020). Samples were eluted with 80% methanol in water at a flow rate of 0.2 $mL \cdot min^{-1}$. The MS/MS analysis of the authentic JH III showed the $[M +H]^+$ ion at *m*/*z* 267.3 and its product ion at *m*/*z* 43.0 was used as a monitor ion. The MS/MS analysis of $JHSB_3$ showed the $[M +H]^+$ ion at *m*/z 283.2 and its product ion at *m*/*z* 233.2 was used as a monitor ion. The MS/MS analysis of JH I showed the $[M +H]^+$ ion at *m*/z 317.1 and this was used as a monitor ion. The Student's *t*-test was used to compare the haemolymph JH concentration between photoperiods and dsRNA

treatments. The Mann-Whitney *U* test and Student's *t*-test were used to compare the fecundity between photoperiod and dsRNA treatments.

2.7 Effect of topically applied JH

We assessed the effect of topically applied JH on the maternal switching day of offspring type (non-diapause- vs diapause-destined). Adult females reared under 16L:8D were transferred to 8L:16D on the day of eclosion. Females were provided with *S. similis* hosts every day from days 1 to 12. On day 4 approximately 3 h before hosts were provided, wasps were chilled on ice for 20 min to immobilize them and 1 μL of JH III in acetone (0.1, 1 or 10 ng/μL) was applied to the ventral side of the thorax and abdomen using a glass syringe (Ito Corporation, Shizuoka, Japan). A control group received 1 μL of acetone. The proportion of diapause-destined offspring was assessed for each wasp each day (i.e. before and after JH application). A two-way ANOVA after arcsine transformation was used to compare the switching day, and Tukey-Kramer test was used to compare the fecundity among 0.1-10 ng JH or solvent applied wasps.

Because exogenous JH appeared to be degraded rapidly, we performed a second, modified experiment with higher JH III concentrations applied to wasps receiving a 'diapause producer' stimulating pre-treatment. Wasps reared under 16L:8D were transferred to 8L:16D at an earlier (yellow pupa) stage. One day after adult emergence, female wasps were chilled for three days at 10 °C under constant darkness (DD), then transferred back to 8L:16D conditions (at 18 °C). This 'chilling treatment' rapidly induces wasps to deposit diapause-destined offspring (Rivers et al., 2000; Mukai and Goto, 2016). We provided *S. similis* pupal hosts on days 4, 6, and 8. On day 6

approximately 3 h before hosts were provided, we applied 1 μL of JH III in acetone (1, 10, or 100 ng/μL) or acetone controls as described above. To assess whether topical JH application affects fecundity, we calculated the fecundity ratio of each wasp by dividing the number of offspring in the host provided on day 6 (after JH application) by the number of offspring in the host provided on day 4 (before JH application). Tukey-type multiple comparisons for proportions were used to compare the proportions of diapause producers. The Steel-Dwass test was used to compare fecundity ratios.

2.8 Statistical analyses

All analyses were performed in R software, version 3.1.2 (R core team 2017). Data are reported as mean ± standard deviation (or median with interquartile range).

3. Results

3.1 Transcriptomic profiles of JH biosynthetic genes

Despite small short day vs. long day differences in the diapause response (0 out of 116 females under short days and 28 out of 168 females under long days), the JH pathway differences were substantial: expression patterns for 18 genes encoding JH biosynthesis-associated enzymes (*jhamt*, *FOHSDR*, *ALDH*, and *Cyp15a1*) reflected a change in maternal status for diapause regulation. For the majority of these genes (15 out of 18), expression in short-day females was reduced (albeit often subtly, < 2 fold)

compared to long-day females (Supplementary Table S1). However, expression of *jhamt* was 3.8-fold lower in short-day females compared to long-day females.

3.2 Photoperiodic and temporal expression profiles of JH biosynthetic genes

Expression of *RpL32* was unaffected by photoperiod and time (two-way ANOVA, $p > 0.05$, data not shown) in the experimental schedule (Fig. 1A). *FOHSDR* expression was highest on day 1, irrespective of photoperiod (Tukey-Kramer test, $p < 0.05$) (Fig. 1B). No significant differences were detected in *ALDH like-1*, *-2*, *-3*, and *Cyp15a1* expression between photoperiods or among days (Tukey-Kramer test, $p > 0.05$). Expression of *jhamt* did not change over time under long days but gradually decreased under short days. On day 11, *jhamt* expression was significantly lower (approximately 10-fold less) under short days compared to long days (Tukey-Kramer test, $p < 0.05$). Thus, *jhamt* expression appears to be photoperiodically regulated.

3.3 Haemolymph JH concentration and maternal status

Downregulation of *jhamt* expression under short days suggests a lower haemolymph JH concentration in short-day wasps. To verify this, we investigated the proportion of diapause producers and JH concentrations in the haemolymph of long- and short-day females (Fig. 2A). UPLC-MS/MS detected a clear peak of JH III in both groups (Fig. S2), while no peaks corresponding to JH I, JHB_3, and $JHSB_3$ were detected (data not shown). Haemolymph JH concentration in long-day wasps (18.8 ± 10.9 pg/μL, $n = 3$) was significantly higher than that of short-day wasps (3.2 ± 0.60 pg/μL, $n = 4$)

(Student's *t*-test, $p < 0.05$; Fig. 2B). Thus, downregulation of *jhamt* under short days was reflected by a reduction in haemolymph JH and an increase in the proportion of diapause producers (0% and 96.9% in long and short days, respectively). Fecundity was greater in short-day wasps (28.1 ± 8.1 offspring, $n = 96$) compared to long-day wasps (22.2 ± 6.9 offspring, $n = 67$) (Student's *t*-test, $p < 0.05$; Fig. 2C).

3.4 Effect of jhamt RNAi

Knockdown of *jhamt* by RNAi was investigated under long days to examine the causal relationship between *jhamt* expression, haemolymph JH concentration, and maternal status (non-diapause producer vs diapause producer) (Fig. 3A). The relative expression of *jhamt* was lower in ds*jhamt*-injected wasps compared to control (ds*bla*) wasps (Mann-Whitney *U* test, $p < 0.05$; Fig. 3B). Wasps with *jhamt* knockdown also had lower haemolymph JH III concentrations (7.04 ± 1.21 pg/μL, $n = 4$) compared to control wasps (20.0 ± 9.42 pg/μL; $n = 4$) (Student's *t*-test, $p < 0.05$; Fig. 3C). In addition, most *jhamt* knockdown wasps became diapause producers even under long-day conditions (96.7%, $n = 89$), in stark contrast to controls (0%, $n = 76$) (Fisher's exact test, $p < 0.05$; Fig. 3C). RNAi of *jhamt* also affected fecundity; the number of offspring produced by ds*jhamt*-injected wasps (median = 18, interquartile range = 13-21, $n = 89$) was significantly less than for control wasps (median = 23, interquartile range = 17-28, $n = 76$) (Mann-Whitney *U* test, $p < 0.05$; Fig. 3D).

We also investigated the effects of *jhamt* RNAi on temporal change in the proportion of diapause producers (Fig. 4A). In intact and ds*bla*-injected wasps, the proportion of diapause producer was continuously low under long days but gradually

increased under short days; i.e., we observed a clear photoperiodic response (Fig. 4B, C). In contrast, a high proportion of ds*jhamt*-injected wasps was diapause producers under both long and short days throughout the experimental period (Friedman's test, $p < 0.05$). The number of offspring produced by ds*jhamt*-injected wasps (230.4 ± 51.6) was also significantly less than for control wasps (351.1 ± 34.6 in intact and 329.9 ± 64.8 in ds*bla*-injected wasps) under long days (Tukey-Kramer test, $p < 0.05$; Fig. 4D). Under short days, the number of offspring produced by intact, ds*bla*-injected, and ds*jhamt*-injected wasps was 324.3 ± 49.1, 269.6 ± 74.5, and 261.4 ± 72.3, respectively (Fig. 4E). A Tukey-Kramer test detected no difference in fecundity among these groups ($p > 0.05$).

3.5 Effect of topically applied JH

We applied JH III to wasps under short days to see whether exogenous JH affects maternal status (non-diapause vs diapause producer) (Fig. 5A). In response to short days, control wasps gradually became diapause producers (Fig. 5B). Topical application of small amounts of JH III (0.1 and 1 ng) did not significantly alter this pattern. Although topical application of 10 ng JH III delayed the switching day, a two-way ANOVA after arcsine transformation detected no statistical significance among treatments ($p > 0.05$). The number of offspring produced by control (solvent applied) wasps (300.9 ± 69.3) was higher than for wasps receiving 1 ng (230.7 ± 82.0) or 10 ng (235.4 ± 75.9) JH III (Tukey-Kramer test, $p < 0.05$). A similar number of offspring were produced by 0.1 ng applied wasps (261.7 ± 77.1) and control wasps (Tukey-Kramer test, $p > 0.05$) (Fig. 5C).

To verify the effect of JH III on diapause producers, we applied JH on females that were pre-chilled for three days in constant darkness (Fig. 6A). On day 4, almost all females deposited diapause-destined offspring, indicating the pre-chilling was effective. On day 6, wasps that received 100 ng JH III were less likely to be diapause producers than intact or control (solvent applied) wasps (Tukey-type multiple comparisons for proportions, $p < 0.05$). This difference disappeared on day 8 (Fig. 6B). The fecundity ratio (number of offspring on day 6 relative to day 4) was significantly lower in the 100 ng JH III wasps (median = 1.2, interquartile range = 1.0-1.6 in) compared to intact wasps (1.8, 1.5-2.9), but no difference was detected among solvent (1.4, 1.0-1.8), 1 ng JH III (1.5, 1.2-1.9), or 10 ng JH III (1.6, 1.3-2.1) groups (Steel-Dwass test, $p > 0.05$) (Fig. 6C).

4. Discussion

Although the JH activity of the extracts of *N. vitripennis* larvae and pupae was investigated by a classic JH titre assay (the *Galleria* assay) (De Loof et al., 1979), the chemical structure of JH has not been identified in this species. The present study aided with the UPLC-MS/MS revealed that the JH of *N. vitripennis* is JH III as found in other hymenopterans (Hagenguth and Rembold, 1978; Burns et al., 2002; Dong et al., 2009).

In the present study, we investigated the possible involvement of JH in the maternal regulation of diapause for *N. vitripennis*. Maternal short day length reduced the expression of *jhamt* [which supports similar findings by Flavell (2017)], reduced haemolymph JH concentration, and induced adult females to produce diapause-destined offspring. A close correlation between *jhamt* expression and haemolymph JH

concentration has also been reported in other insect species (Xu et al., 2017; Ishimaru et al., 2016; Ueda et al., 2009; Fu et al., 2014; Marchal et al., 2011).

Reduction in haemolymph JH concentration is a typical response to short day length in insects with reproductive diapause (Denlinger et al., 2012). However, *N. vitripennis* does not enter reproductive diapause (the present study); ovarian development in female *N. vitripennis* is not suppressed by short days and low haemolymph JH concentrations. Rather, these wasps are highly reproductive, and the number of offspring is comparable to that of long-day females. Although some statistical differences were detected, the effects of *jhamt* RNAi and JH application were inconsistent. The role of JH in the ovarian development of *N. vitripennis* is therefore unclear, but ovarian development may be independent of JH as was reported in a locust, moth, and wasp (Bradley et al., 1995; Dong et al., 2009; Tsuchida et al., 1987).

In the present series of experiments with *jhamt* RNAi and topical JH application, we show that JH is causally involved in the maternal regulation of diapause for *N. vitripennis*. Flavell et al. (2017) reached a similar conclusion via RNAi, but here we strengthen our understanding of this process by assessing the effect of topical JH application and demonstrating that haemolymph JH concentration reflects changes in *jhamt* expression. De Loof et al. (1979) reported the effect of topically applied JH I, which is absent in this species (the present study), on the proportion of diapause producers. JH I application changed the diapause induction curve from the typical short-day pattern to a long-day pattern, but the application of the solvent (acetone) also showed the same result. Thus, the involvement of JH in the maternal regulation of diapause has been overlooked in *N. vitripennis*, but we clearly demonstrate that JH III is the causal factor regulating the maternal effect in this species.

JHs are among the most versatile insect hormones, playing a role in almost every aspect of development and reproduction, including metamorphosis, caste determination in the social insects, regulation of behaviour, polyphenisms, larval and adult diapause regulation, vitellogenin synthesis, ovarian development, and various aspects of metabolism associated with these functions (Nijhout 1994). Here we add a new role for JH: the maternal regulation of diapause. However, such a role may be only applicable to species in which ovarian development is independent of JH, and involvement of JH in the maternal regulation of diapause was rejected for the flesh fly, *Sarcophaga bullata* (Rockey et al., 1989).

The upstream cascade of JH should be investigated in the future. We know that *N. vitripennis* assesses photoperiods by two circadian oscillators (dawn and dusk oscillators), i.e., the internal coincidence model (Saunders, 1974). One or both of the oscillators is comprised of the clock gene *period* (Mukai and Goto, 2016; Della Benetta et al., 2019), but it is still unknown how the oscillators regulate the CA. Short neuropeptide F, allatostatin C, and myoinhibitobory peptide might be the mediators that link the circadian clock and the photoperiodic regulation of the CA activity (Yamanaka et al., 2008; Yao and Shafer, 2014; Tamai et al., 2018; Zhang et al., 2021). Another area of future work could involve the investigation of the downstream cascade of JH. In *N. vitripennis*, DNA methylation through DNA methyltransferase (Dnmt) is crucial for maternal regulation of diapause (Pegoraro et al., 2016). It is therefore important to determine if and how DNA methylation and Dnmt levels are regulated by JH.

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Competing interests

The authors declare no competing interests.

Authors' contributions

SGG conceived the study, designed the study, coordinated the study, helped draft the manuscript, and critically revised the manuscript; AM participated in the design of the study, performed RNAi, RT-qPCR, diapause assessment, and haemolymph extraction, carried out the statistical analyses, drafted the manuscript, and critically revised the manuscript; GM performed quantification of JH by UPLC and provided critical advice for experiments; LDM performed RNA-seq analysis and critically revised the manuscript; TS synthesized the authentic JHs and critically revised the manuscript. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

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Figure legends

Fig. 1 Photoperiodic and temporal profiles of JH biosynthetic gene expression in adult female *Nasonia vitripennis*. (A) Experimental schedule. (B) Amounts of mRNA of JH biosynthetic genes (*FOHSDR*, *ALDH like-1*, *-2*, *-3*, *jhamt* and *Cyp15a1*) on days 1, 6 and 11 under long-day (16L:8D, open circles) and short-day (8L:16D, closed circles) conditions. The amount of target gene mRNA is shown relative to the amount of *RpL32* (housekeeping gene) mRNA. Different letters indicate significant differences (Tukey-Kramer test, $p < 0.05$). No significant difference was detected in *ALDH like-1*, *-2*, *-3*, and *Cyp15a1*. $n = 3$ for each.

Fig. 2 Haemolymph JH III concentration and maternal status of *Nasonia vitripennis* in response to photoperiod. (A) The experimental schedule. (B) The haemolymph JH III concentration (primary vertical axis) and proportions of non-diapause and diapause producers (secondary vertical axis) under long-day and short-day conditions. Haemolymph JH III concentrations are shown as open diamonds and error bars (mean ± SD; $n = 3$ and 4 for long and short days, respectively). Open and shaded columns indicate proportions of non-diapause and diapause producers, respectively ($n = 67$ and 96 in long and short days, respectively). (C) The number of offspring under long and short days (mean ± SD, $n = 67$ and 96, respectively).

Fig. 3 Effect of *jhamt* silencing on haemolymph JH III concentration and number of offspring produced under long-day conditions in *Nasonia vitripennis*. (A) The experimental schedule. (B) Amounts of *jhamt* mRNA relative to *RpL32* mRNA in ds*bla* (control) or ds*jhamt* injected wasps (n = 7 for each condition). (C) Effect of *jhamt* RNAi on haemolymph JH III concentration (primary vertical axis) and proportions of non-diapause and diapause producers (secondary vertical axis). The haemolymph JH III concentrations are shown as open diamonds and error bars (mean ± SD, n = 4 in each condition). Open and shaded columns indicate proportions of non-diapause and diapause producers, respectively. (D) The number of offspring in ds*bla*- or ds*jhamt*-injected wasps (n = 76 and 89, respectively). Columns and error bars indicate median and interquartile range, respectively.

Fig. 4 Effect of *jhamt* silencing on the switching day and the number of offspring under long and short days in *Nasonia vitripennis*. (A) The experimental schedule. (B, C) The proportion of diapause producers in intact (closed circles), ds*bla*-injected (open squares) and ds*jhamt*-injected (open circles) females (n = 5-15). (D, E) The number of offspring produced by intact and dsRNA-injected females (mean ± SD, n = 5-15). Tukey-Kramer test detected a significant difference (shown in different letters, $p < 0.05$) under long days, but not under short days. Data on days 1-3 were excluded because parasitism scarcely occurred. B and D are the data under long days, and C and E are under short days.

Fig. 5 Effects of JH III topical application on the switching day in *Nasonia vitripennis*. (A) Experimental schedule. (B) The temporal proportions of diapause producers of solvent (acetone, closed diamonds), or JH III-applied groups (0.1 ng, open circles; 1 ng, open diamonds; 10 ng, open triangles) are shown. Each plot shows the data from 7-18 females. (C) The number of offspring produced by solvent- or JH III-applied females (mean ± SD). Different letters indicate statistical differences according to the Tukey-Kramer test ($p < 0.05$).

Fig. 6 Effects of JH III topical application on the proportion of diapause producers directly induced by chilling in *Nasonia vitripennis*. (A) Experimental schedule. (B) The temporal proportions of diapause producers of intact (closed circles), solvent (acetone, closed diamonds), or JH III-applied groups (1 ng, open diamonds; 10 ng, open triangles; 100 ng, open squares) are shown. Each plot shows the data from 16-25 females. Different letters on day 6 indicate statistical differences according to Tukey-type multiple comparisons for proportions ($p < 0.05$). (C) The fecundity ratio of intact, solvent- or JH III-applied females (median and the interquartile range). Different letters indicate statistical differences according to the Steel-Dwass test ($p < 0.05$).

Table 1. Primers used in the present study.

Gene	Primer	Sequence (from 5' to 3')	Accession no.[#]
FOHSDR			XM_001605397.6
	qPCR-F	CGT GAC GAA CGA AGA GAA CGT	
	qPCR-R	CAG CAC AAG GCC GTC ATG	
ALDH-like1			XM_001603399.4
	qPCR-F	TCC AGG CGT TGT CAA TGT TC	
	qPCR-R	GGA AAT AGC CGC ACC TGT AGA T	
ALDH-like2			XM_031928648.2
	qPCR-F	TTG GTG TTG TCG GCC AGA T	
	qPCR-R	CCA GGC GGT CAT CAT GAT C	
ALDH-like3			XM_031925691.2
	qPCR-F	CAC CAT GGA GGA CGA TGG AT	
	qPCR-R	CAA ATC TGC CAA CCT GTG TAA CA	
jhamt			XM_001604413.4
	qPCR-F	CCA ACC AGC ACC AGC GTC GT	
	qPCR-R	CGC CAA TGA GAT GCG GCG GA	
	RNAi-F	GAA CGA CTC ATG CTG ACG AA	
	RNAi-R	TCC ATT TTC TGC TTT CAC GA	
	RNAi-F-T7	TAA TAC GAC TCA CTA TAG GGA ACG	
		ACT CAT GCT GAC GAA	
	RNAi-R-T7	TAA TAC GAC TCA CTA TAG GTC CAT	
		TTT CTG CTT TCA CGA	
Cyp15a1			XM_001605535.4
	qPCR-F	CCC AGA GAT TCC TCG ACG AA	
	qPCR-R	CCA TAG CGC CGG TGG AT	
RpL32			
	qPCR-F	AGA AAT TGC CCA TGG AGT TAG C	XM_001601472.6
	qPCR-R	CTG CTG GGC ACG TTC GA	
*β-lactamase**			
	RNAi-F	TCG CCG CAT ACA CTA TTC TC	
	RNAi-R	TAC GAT ACG GGA GGG CTT AC	
	RNAi-F-T7	TAA TAC GAC TCA CTA TAG GTC GCC	
		GCA TAC ACT ATT CTC	
	RNAi-R-T7	TAA TAC GAC TCA CTA TAG GTA CGA	
		TAC GGG AGG GCT TAC	

[#]DDBJ/GenBank/EMBL accession no.

*https://lifescience.toyobo.co.jp/user_data/pdf/products/manual/TAK.pdf

Figure 1

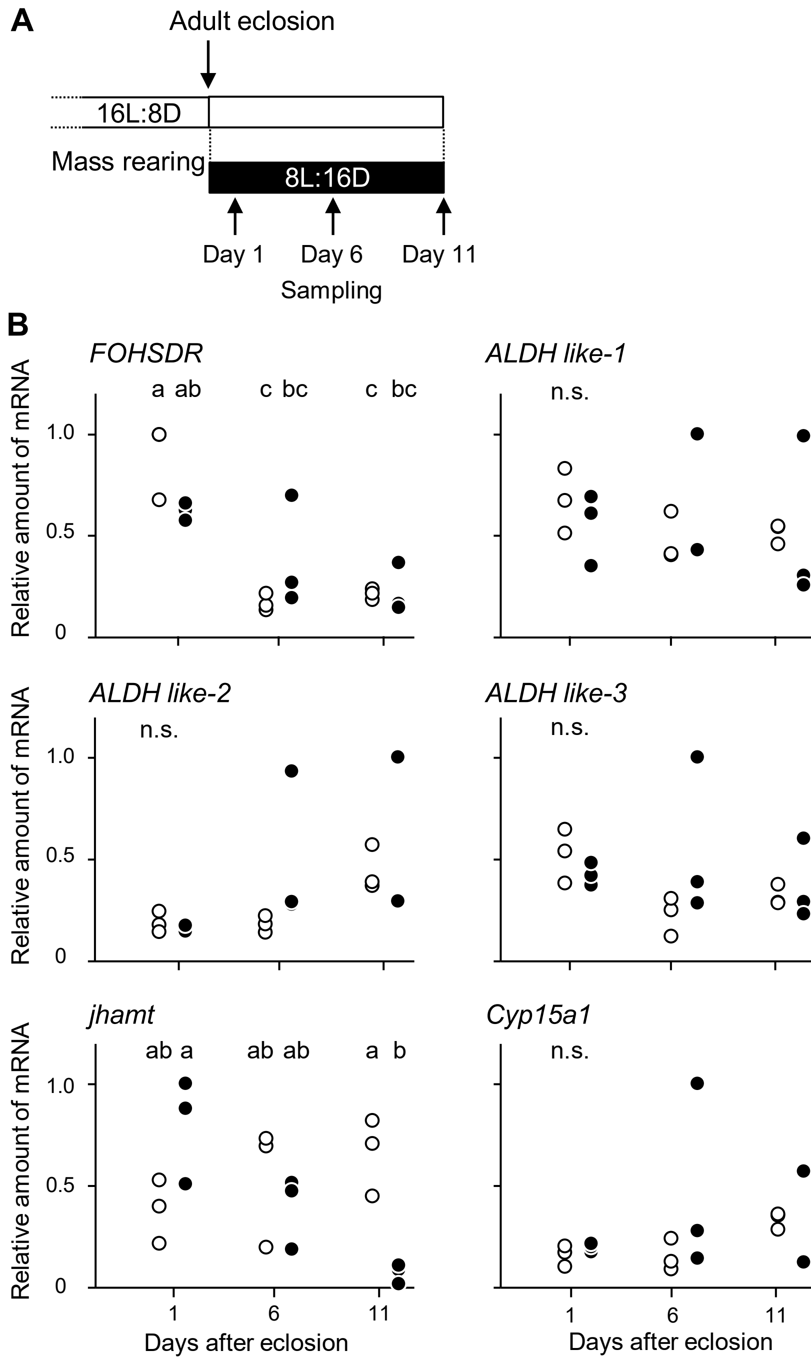


Figure 2

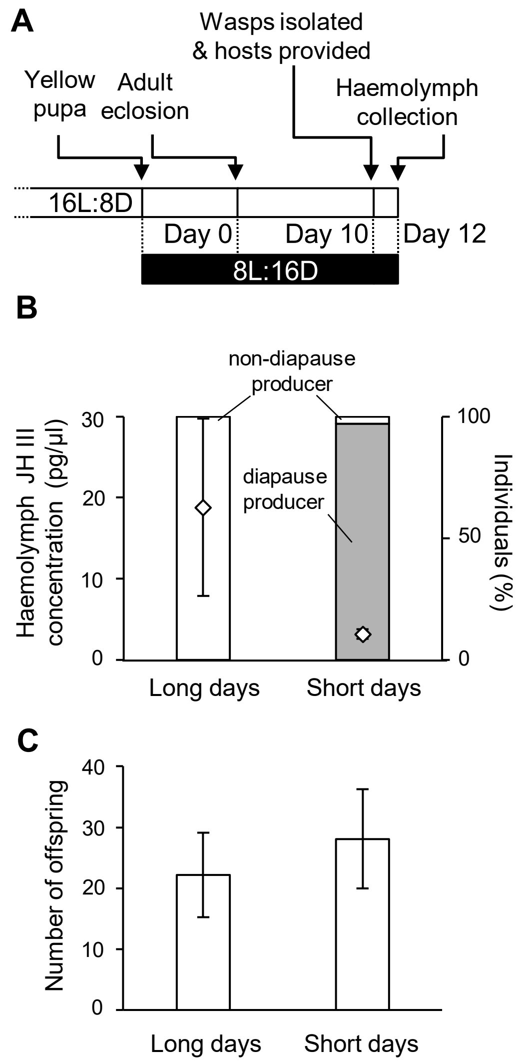


Figure 3

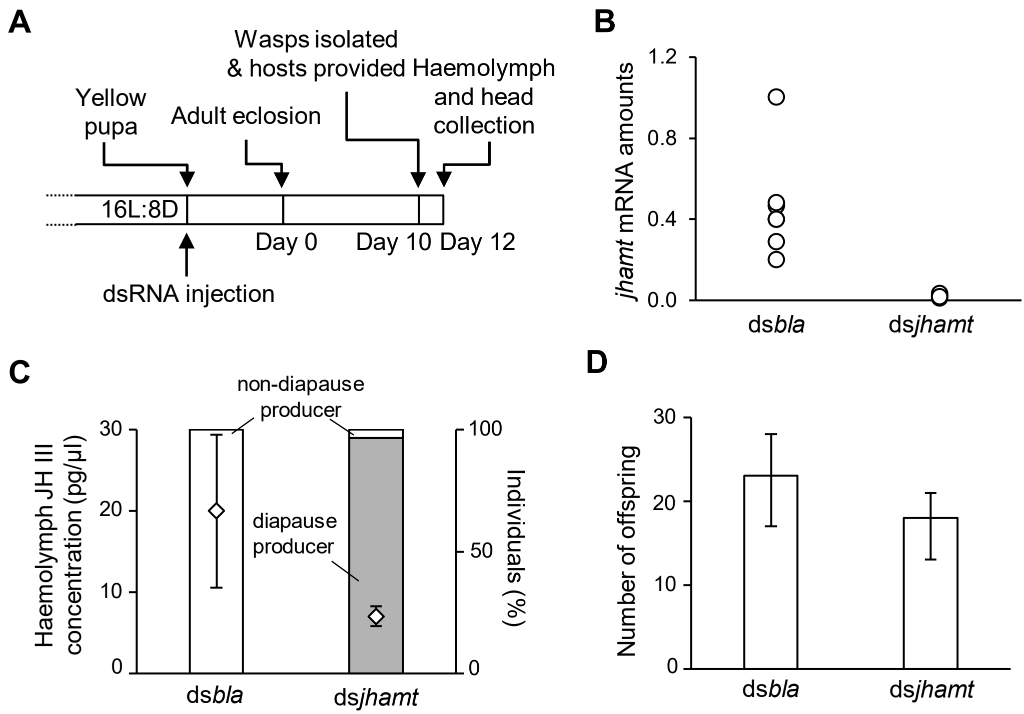


Figure 4

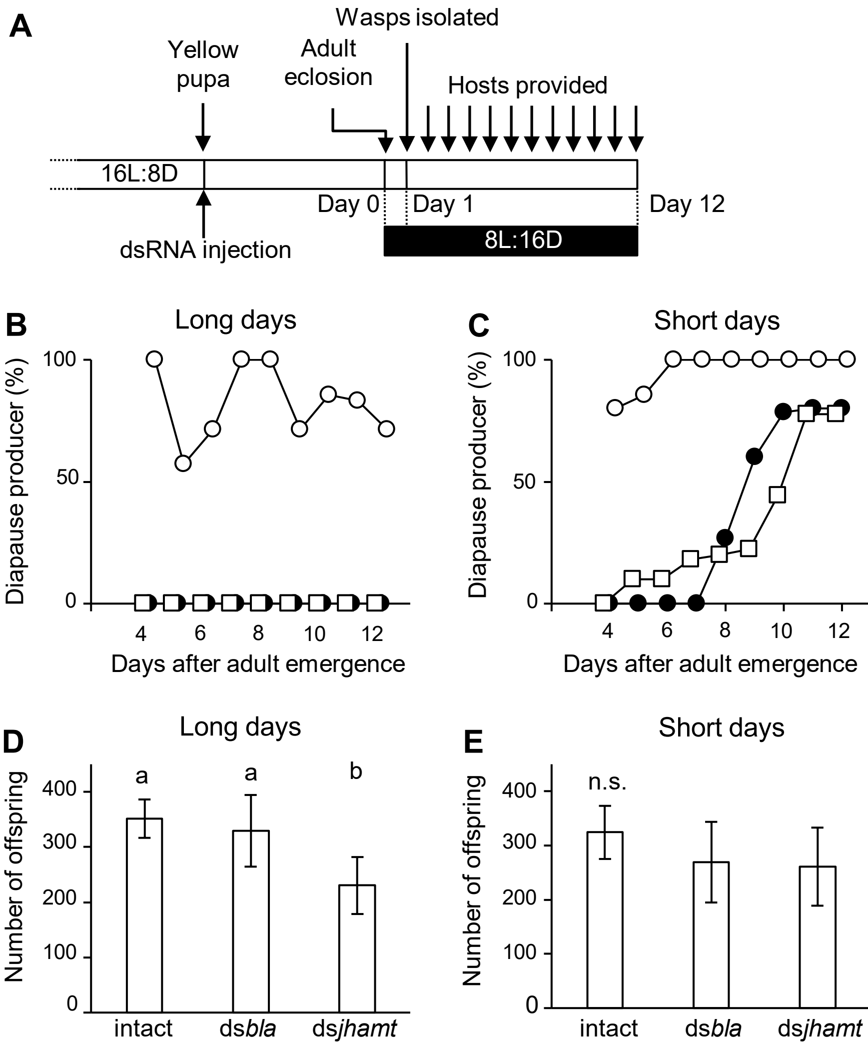
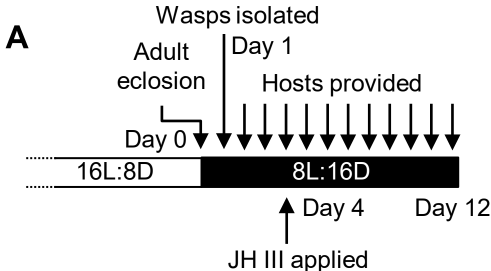
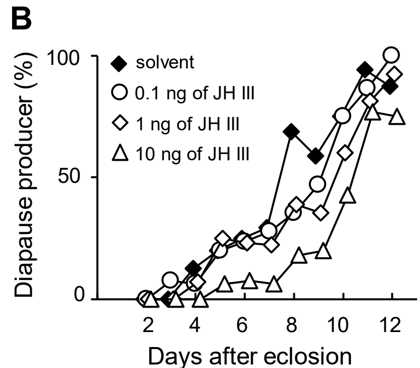


Figure 5





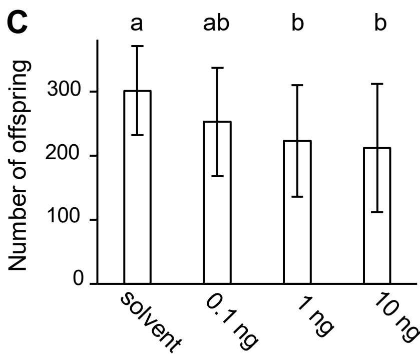


Figure 6

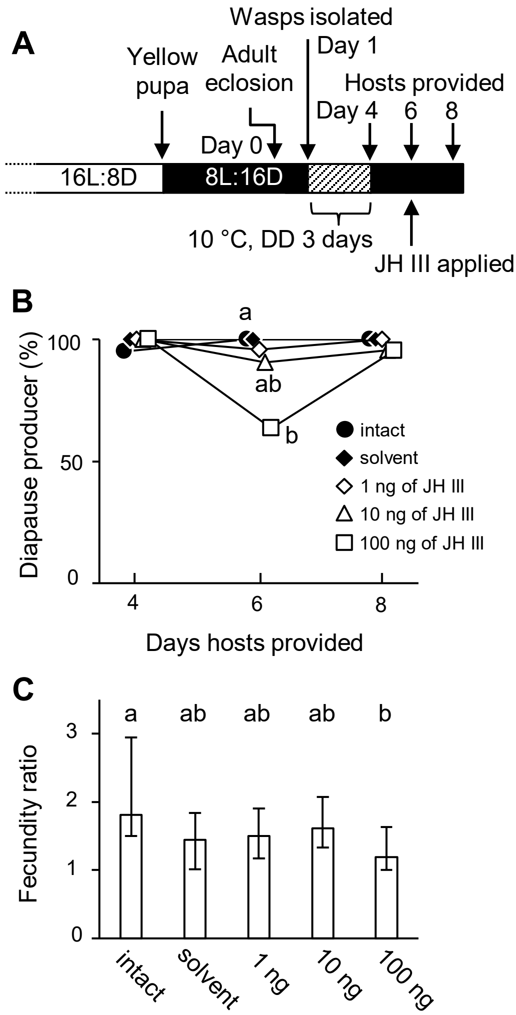


Table S1. Transcriptomic profiles of juvenile hormone (JH) biosynthetic genes under long (L) and short (S) days.

Protein	Synonyms	EC	Accession	Chromosome	Locus	Protein product	A.A. Length	Protein Name	Expression (counts) by replicate						Mean count		Fold change
									L1	L2	L3	S1	S2	S3	L	S	L vs S (>1 = higher in L)
Farnesol dehydrogenase	FOHSDR / NADP+-dep. farnesol dehydrogenase	1.1.1.216	NC_045760.1	chromosome 4	LOC100117064	XP_001599715.1	247	farnesol dehydrogenase	14772	14821	13509	14105	10795	11948	14367	12283	1.17
			NC_045759.1	chromosome 3	LOC100116955	XP_001604944.4	286	farnesol dehydrogenase	16026	19776	15394	17312	12961	14032	17065	14768	1.16
			NC_045757.1	chromosome 1	LOC100115586	XP_001600270.1	247	farnesol dehydrogenase	18	16	15	14	24	20	16	19	0.84
			NC_045757.1	chromosome 1	LOC100113904	XP_001599160.3	247	farnesol dehydrogenase	811	771	776	592	518	721	786	610	1.29
			NC_045758.1	chromosome 2	LOC100118150	XP_008209485.1	243	farnesol dehydrogenase	128	139	147	114	92	115	138	107	1.29
			NC_045758.1	chromosome 2	LOC100120166	XP_016838044.1	247	farnesol dehydrogenase	75	57	42	72	33	26	58	44	1.33
			NC_045759.1	chromosome 3	LOC100121837	XP_008216554.1	284	farnesol dehydrogenase isoform X1	3340	3550	2875	2703	2309	2112	3255	2375	1.37
			NC_045757.1	chromosome 1	LOC100116170	XP_032452066.1	247	farnesol dehydrogenase isoform X1	95	68	30	60	36	40	64	45	1.42
			NC_045760.1	chromosome 4	LOC100120547	XP_001604180.2	245	farnesol dehydrogenase-like	8	14	10	4	16	12	11	11	1.00
Farnesal dehydrogenase / Aldehyde dehydrogenase	ALDH	1.2.1.94	NW_022279927.1	Un	LOC100499205	NP_001180248.1	530	aldehyde dehydrogenase family 7-like	12580	23087	20032	20820	13805	19383	21461	18003	1.19
			NC_045760.1	chromosome 4	LOC100119754	XP_031784508.1	487	aldehyde dehydrogenase X, mitochondrial	3616	4623	3902	3978	2866	3732	4047	3525	1.15
			NC_045760.1	chromosome 4	LOC100113596	XP_031785480.1	656	aldehyde dehydrogenase, dimeric NADP-preferring isoform X1	11278	11192	8642	9543	7037	8440	10371	8340	1.24
			NW_022279623.1	Un	LOC100120559	XP_031781551.1	511	aldehyde dehydrogenase, mitochondrial	4867	4743	4089	4439	3099	3634	4566	3724	1.23
			NC_045760.1	chromosome 4	LOC100119724	XP_001603449.1	494	retinal dehydrogenase 1 isoform X1	1533	1842	1563	874	731	997	1646	867	1.90
Juvenile hormone acid O-methyltransferase	JHAMT	2.1.1.325	NC_045759.1	chromosome 3	LOC100117819	XP_001601958.2	286	juvenile hormone acid O-methyltransferase	10	13	11	11	8	15	11	11	1.00
			NC_045758.1	chromosome 2	LOC100120870	XP_001604463.1	278	juvenile hormone acid O-methyltransferase	3175	4041	2879	846	635	1144	3365	875	3.85
Methyl farnesoate epoxidase	CYP15A1	1.14.14.127	NC_045757.1	chromosome 1	LOC100115268	XP_001600039.1	505	methyl farnesoate epoxidase	8	15	22	6	12	13	15	10	1.45
			NC_045757.1	chromosome 1	LOC100121983	XP_001605585.1	496	methyl farnesoate epoxidase	4260	6779	4918	6215	7804	9385	5319	7801	0.68

Figure S1

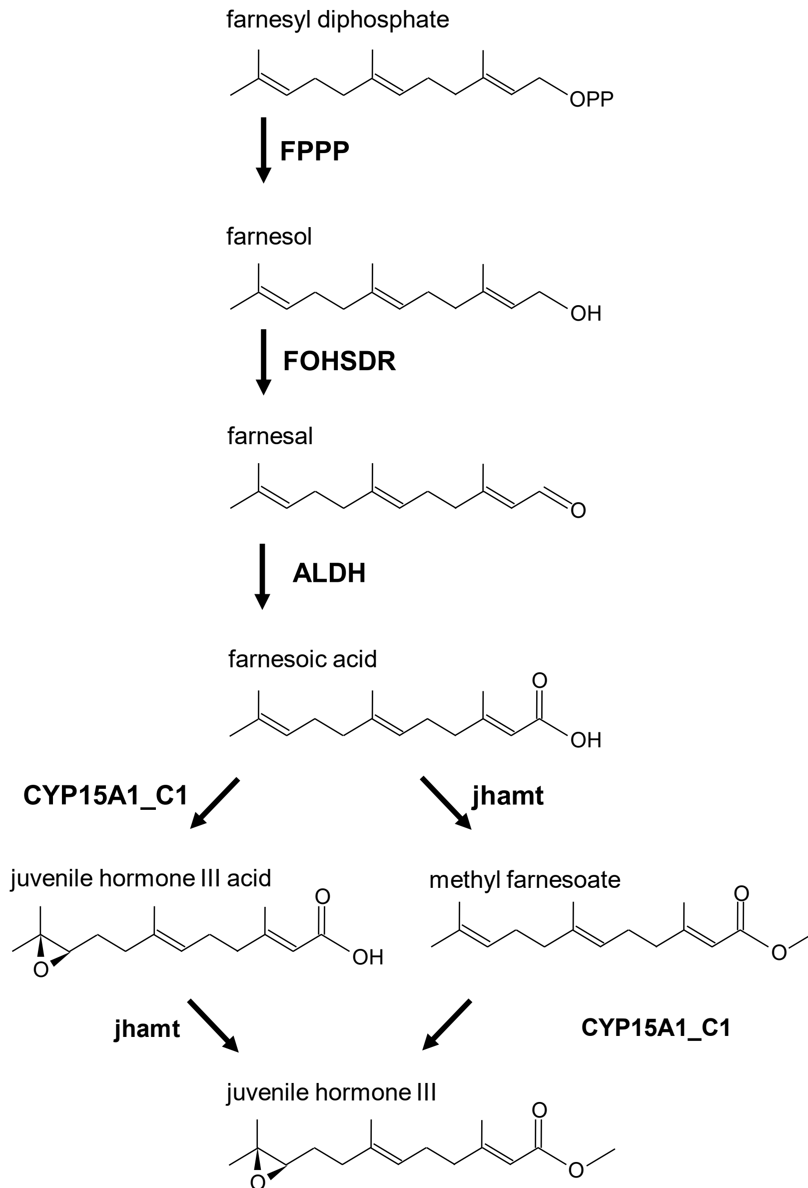


Figure S1. The biosynthetic cascade of juvenile hormone III (JH III) in insects. Note that *Nasonia vitripennis* FPPP was not found in GenBank and our present RNA-seq data. Two patterns of catalyzation on farnesoic acid are known in insects; one is epoxidation following esterification, the other is the converse pattern.

Figure S2

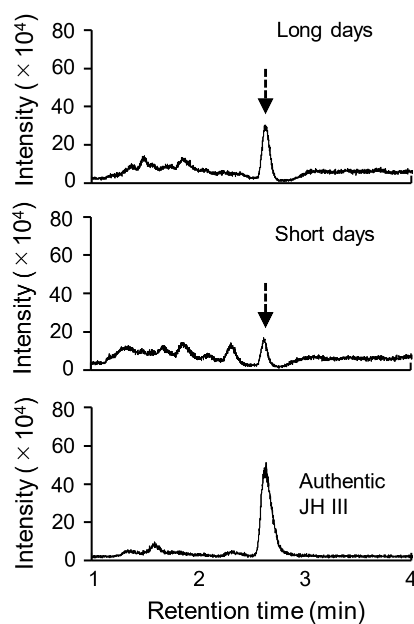


Figure S2 The representative C-18 UPLC-MS/MS results of haemolymph juvenile hormone (JH) III in females of *Nasonia vitripennis* under long-day (16L:8D, top) and short-day (8L:16D, middle) and authentic JH III (bottom). The signals of the product ion at *m/z* 43.0 from the precursor ion at *m/z* 276.3 are shown. Dotted arrows indicate peaks corresponding to the authentic JH III peak.