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# **Cell cycle arrest in the jewel wasp *Nasonia vitripennis* in larval diapause**

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## Highlights

- Developmental and cell cycle arrests occur in diapause larvae of the jewel wasp.
- Cell cycle arrest occurs primarily in G<sub>0</sub>/G<sub>1</sub> and secondarily in G<sub>2</sub>.
- Downregulation of housekeeping genes and cell cycle regulatory genes is evident.



## **Abstract**

Insects enter diapause to synchronise their life cycle with biotic and abiotic environmental conditions favourable for their development, reproduction, and survival. One of the most noticeable characteristics of diapause is the blockage of ontogeny. Although this blockage should occur with the cessation of cellular proliferation, i.e. cell cycle arrest, it was confirmed only in a few insect species and information on the molecular pathways involved in cell cycle arrest is limited. In the present study, we investigated developmental and cell cycle arrest in diapause larvae of the jewel wasp *Nasonia vitripennis*. Developmental and cell cycle arrest occur in the early fourth instar larval stage of *N. vitripennis* under short days. By entering diapause, the S fraction of the cell cycle disappears and approximately 80 % and 20 % of cells arrest their cell cycle in the G0/G1 and G2 phases, respectively. We further investigated expression of cell cycle regulatory genes and some housekeeping genes to dissect molecular mechanisms underlying the cell cycle arrest.

## **Keywords**

Cell cycle arrest, developmental arrest, diapause, ontogeny, gene expression

## 1. Introduction

Diapause is an adaptive strategy to synchronise the insect's life cycle with biotic and abiotic environmental conditions favourable for development, reproduction, and survival (Danks, 1987; Tauber et al., 1986). Diapause elicits a number of behavioural, physiological, morphological, and molecular modifications, and one of the most noticeable characteristics is the blockage of ontogeny at a fixed and species-specific stage from the early embryo to adult (Danks, 1987; Denlinger, 2002; Košťál, 2006). Blockage of ontogeny is most likely based on the cessation of cellular proliferation, i.e. cell cycle arrest. However, it was confirmed in only a few insect species (Košťál et al., 2009; Nakagaki et al., 1991; Tammariello and Denlinger, 1998). Although we have accumulated much information on diapause (Denlinger et al., 2012; Hahn and Denlinger, 2011; Košťál, 2006), we know surprisingly little about cell cycle arrest during diapause. In addition, molecular pathways involved in regulation of the cell cycle arrest were investigated in only a few species (Fujiwara et al., 2006; Fujiwara and Shiomi, 2006; Kidokoro et al., 2006; Košťál et al., 2009; Tammariello and Denlinger, 1998).

Nakagaki et al. (1991) first focused on the cell cycle arrest during diapause in the silkworm *Bombyx mori*, which enters diapause at a specific embryonic stage (a stage before the dermal differentiation is completed; Yamashita, 1996). Cell divisions and the morphological development of diapause embryos cease as long as the eggs are incubated at 25 °C. Before entering diapause, the number of cells in G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M phases of the cell division cycle were 10, 35, and 55%, respectively, whereas after entering diapause almost all cells were arrested at the G<sub>2</sub> phase (Nakagaki et al., 1991). In contrast, in two dipteran species, the drosophilid fly *Chymomyza costata* and the flesh fly *Sarcophaga crassipalpis*, which enter diapause at the larval and pupal stages, respectively, the majority of cells in the central nervous

system (CNS) halted their cell cycle in the G0/G1 phase during diapause (Košťál et al., 2009; Tammariello and Denlinger, 1998). These studies further compared the expression of several cell cycle regulatory genes, and found that expression of *proliferating cell nuclear antigen* (*pcna*), one of the pivotal players in cell cycle progression, is significantly suppressed in diapause individuals.

In the present study, we provide an additional example of cell cycle arrest and the expression of cell cycle regulatory genes in a hymenopteran insect, the jewel wasp *Nasonia vitripennis*, in relation to diapause initiation. This species exhibits a clear photoperiodic larval diapause (Saunders 1965, 1966). Adult females under long-day conditions lay eggs that develop into adults without developmental interruption (non-diapause), whereas those reared under short-day conditions lay eggs in which development is arrested at the final (4<sup>th</sup>) instar larval stage (diapause). The physiological mechanisms underlying photoperiodic induction of diapause have been extensively studied in this species (Mukai and Goto, 2016; Pegoraro et al., 2016; Saunders, 1965, 1966, 1974; Schneiderman and Horwitz, 1958; Wolschin and Gadau, 2009). In addition, genomic information is available and the RNAi technique is applicable (Lynch and Desplan, 2006; Werren et al., 2009, 2010), which makes this species an attractive model organism for the elucidation of the molecular mechanisms underlying diapause. In the present study, we found that the S fraction of the cell cycle disappears and approximately 80 % and 20 % of cells arrest their cell cycle in the G0/G1 and G2 phases, respectively, in the brain of *N. vitripennis* larvae in diapause. Such high proportion of the subdominant cell cycle phase during diapause has not been reported in other insect species. We further investigated expression of cell cycle regulatory genes (*pcna*, *cyclin D*, *cyclin E*, and *p53*) and housekeeping genes (*ribosomal protein L32* [*RpL32*] and *elongation factor-1 $\alpha$*  [*EF-1 $\alpha$* ]) to dissect molecular mechanisms underlying the cell cycle arrest.

## 2. Materials and methods

### 2.1. Insects

*N. vitripennis* adults were collected from the pupae of the flesh fly *Sarcophaga similis* in Osaka, Japan (34.6°N, 135.5°E) in 2011. The wasp stock culture was maintained under a stationary photoperiod of light: dark (LD) 16: 8 h at  $20.0 \pm 1.0$  °C. Approximately 50–80 newly emerged adult wasps were transferred to a glass vial (30 mm in diameter, 100 mm in height) and fed a 10% (w/v) sucrose solution. Approximately 20–50 non-diapause pupae of *S. similis* were supplied as a host for wasps in each vial 12–14 days after adult emergence. Wasps emerged from the fly pupae 20–22 days after parasitisation.

Newly emerged adult wasps were mass-reared under diapause-averting long-day conditions (LD 16:8 h) or diapause-inducing short-day conditions (LD 12:12 h) at  $20.0 \pm 1.0$  °C. Approximately 30 females were individually transferred into each of the vials 12 days after adult emergence, and two non-diapause pupae of *S. similis* were supplied as a host for each female for 24 h. The parasitised fly pupae were maintained under the same photoperiodic conditions. In this study, the larvae derived from adults that were reared under long-day conditions were referred to as long-day larvae, which were destined to avert diapause. Similarly, larvae derived from adults that were reared under short-day conditions were referred to as short-day larvae, which were destined to enter diapause. Day zero was defined as the day of oviposition.

It is important to note that there is a clear sexual dimorphism in the larval size in *N. vitripennis*. At a late larval stage (fourth instar), females are clearly larger than males (Schneiderman and Holowitz, 1958), whereas such a size difference is not detectable in earlier



larval instars. Therefore, we sexed and sampled only females after day 7 or later, whereas larvae on day 5 were not sexed because of the difficulty in discrimination.

## 2.2. Ontogeny

We investigated the progress in ontogeny at  $20.0 \pm 1.0$  °C under long- and short-day conditions, by opening fly puparia once a day from days 2 to 10 and on days 13 and 16. The wasp larval stage was classified into five stages (1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup>, early 4<sup>th</sup>, and late 4<sup>th</sup> instars) based on their morphological features according to Azab et al. (1967). The first instar larva is translucent and mobile on the surface of the host fly. The second instar larva is larger, cloudy translucent, and is arched dorsally. The third instar larva is opaque, yellowish, and is crescent-shaped. The early fourth instar larva is milky white or yellow and is elongate and cylindrical, with an arched back. Although the late fourth instar is similar to the early fourth instar, its body is white and the internal organs can be observed through the epidermis. We did not identify sex of the larvae in this ontogeny analysis.

## 2.3. Longitudinal length of the brain

Larval brains were dissected in 0.9% NaCl solution under a stereomicroscope at room temperature on days 5, 7, and 10 in long-day larvae, and on days 5, 7, 10, 20, 30, and 40 in short-day larvae. The longitudinal length of the brain was measured with Nikon NIS Elements BR 3.0 software (Nikon, Tokyo, Japan). For each data point, 10–20 brains were measured.

## 2.4. Flow cytometry

Brains of five female *N. vitripennis* individuals were dissected in 0.9% NaCl under a stereomicroscope at room temperature. Brains were transferred to a microtube and gently

homogenised using Bio Masher II (Nippi, Tokyo, Japan). Uniform suspensions of single nuclei were prepared with the BD Cycletest Plus DNA Reagent Kit (BD Biosciences, Tokyo, Japan). Cellular DNA contents were analysed using a BD Accuri C6 Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The obtained data were analysed with FCS Express software (De Novo Software, Glendale, CA, USA), and cells were classified in G0/G1, S, and G2/M phases depending on the intensity of fluorescence peaks.

Contamination of male samples with females was detected in the flow cytometry analysis because female and male Hymenoptera are diploid and haploid, respectively. Despite thorough sampling, we were not able to collect male-free samples on day 5 because of the difficulty of sexing. Thus, we excluded the data on day 5, and only the brain samples taken on days 7 and 10 from long-day female larvae, and on days 7, 10, 20, 30, and 40 from short-day female larvae, were further analysed.

## 2.5. RNA extraction and quantitative real-time PCR

Total RNA was extracted from the whole body of *N. vitripennis* using the TRIzol RNA Isolation Reagents (Life Technologies, Carlsbad, CA, USA) and purified with PureLink PCR Micro Kit (Life Technologies) according to the manufacturer's instructions. cDNA was synthesised from 1 µg of total RNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies) in a 20-µL system. Relative abundance of *pcna*, *cyclin D*, *cyclin E*, and *p53* transcripts was estimated by quantitative PCR (qPCR) using a 7500 Real-Time PCR system (Life Technologies). One microlitre of cDNA was used at a final concentration of 1 × Go Taq qPCR Master Mix (Promega, Madison, WI, USA) with 0.2 µM of each primer. Each reaction was performed in duplicate. Primers used were 5'-GTG GAT AGC CCT GAC AAC ATT A-3' and 5'-CCA GGT CCA TGT TGA TGA GTT-3' for *pcna*, 5'-CTA CAC CGA CAA CTC CAT

CAC-3' and 5'- CCA GGA TGT AGA GGA GGA AGT-3' for *cyclin D*, 5'- CAA CAA AGT TCC TGC CCT TTC-3' and 5'- TGG ATG GTT GAG GTG TGT ATG-3' for *cyclin E*, and 5'- CCA GTG CAA AGA TGC CAT AAT C-3' and 5'- CAT GCA CCA TGC ATA CCT CTA-3' for *p53*. Relative RNA abundance of *RpL32*, *EF-1 $\alpha$* , and *18S ribosomal RNA (18S rRNA)* were also investigated. Primers used were 5'-AGA AAT TGC CCA TGG AGT TAG C-3' and 5'-CTG CTG GGC ACG TTC GA-3' for *RpL32*, 5'-CAC TTG ATC TAC AAA TGC GGT G-3' and 5'- CCT TCA GTT TGT CCA AGA CC-3' for *EF-1 $\alpha$* , and 5'-AAG ACG GAC AGA AGC GAA AG-3' and 5'-GGT TAG AAC TAG GGC GGT ATC T-3' for *18S rRNA*. Accession numbers of sequences that were used for designing primers are as follows; XM\_001605149 for *pcna*, NM\_001159333 for *cyclin D*, XM\_001599633 for *cyclin E*, XM\_001606850 for *p53*, XM\_001601472.3 for *RpL32*, NM\_001172756.1 for *EF-1 $\alpha$* , and GQ410677.1 for *18S rRNA*. Amplified fragments in all PCRs were approximately 100 bp in length. In all the reactions, generation of a single expected amplicon was confirmed by melting curve analysis, and quantification was performed by the standard curve method. Five independent RNA samples were used for each condition as biological replicates.

### **3. Results**

#### **3.1. Developmental arrest**

Early instars of long- and short-day larvae developed similarly until the early 4<sup>th</sup> instar larval stage on days 7 or 8 (Fig. 1). Long-day larvae became the late 4<sup>th</sup> instar stage on day 10 and completed pupation on day 13. In contrast, short-day larvae remained in the early 4<sup>th</sup> instar stage beyond day 40, entering diapause.

Longitudinal lengths of the brain were nearly identical between the 2<sup>nd</sup> instar long-day and short-day larvae on day 5 (Fig. 2). On day 7, the brain was enlarged more than twice in the early 4<sup>th</sup> instar long-day larvae, whereas that of the short-day larvae, although enlarged, was significantly smaller than that of long-day larvae. On day 10, the brain size of long-day larvae further increased, especially because of the development of the optic lobe. By contrast, development was suppressed in short-day larvae, and thereafter, the longitudinal length showed little change. Thus, the developmental arrest occurred on day 10 in short-day larvae.

### 3.2. Cell cycle arrest

Relative proportions of cells in each phase of the cell division cycle are presented in Fig. 3. From days 7 to 10, the proportion of cells in the G0/G1 phase markedly increased in long-day larvae. In the short-day larvae, the proportions increased only slightly, though significantly, from days 7 to 10 and remained at a constant level thereafter. The proportion of cells in the S phase significantly decreased in both long-day and short-day larvae from days 7 to 10. Thereafter, further decrease was observed in short-day larvae, and the proportion reached extremely low levels by days 30 and 40. The proportion of cells in the G2/M phase was nearly identical on day 7 in both larvae. Long-day larvae decreased the G2/M proportion towards day 10, whereas short-day larvae maintained the same proportion. The proportions of cells in G0/G1, S, and G2/M phases were approximately 80 %, < 1 %, and 20 %, respectively, in short-day larvae in diapause on day 40.

### 3.3. Housekeeping genes and cell cycle regulatory genes

We investigated relative RNA levels of three genes, *RpL32* (also known as *rp49*), *EF-1 $\alpha$* , and *18S rRNA*, to  $\mu$ g of total RNA (Fig. 4-A). Relative abundance of *RpL32* and *EF-1 $\alpha$*  mRNA

markedly decreased along with larval development. One-way ANOVA detected significant differences in *RpL32* and *EF-1 $\alpha$*  ( $P < 0.05$ ) among samples, whereas levels of *18S rRNA* remained comparatively constant ( $P > 0.05$ ). Thus, *18S rRNA* was used as a reference for normalisation in the present study.

We measured mRNA levels of cell cycle regulatory genes, *pcna*, *cyclin D*, *cyclin E*, and *p53*, which are involved in the G1–S phase transition (Fig. 4-B). Relative levels of *pcna*, *cyclin E*, and *p53* mRNA decreased in long-day larvae along with their development, whereas relative levels of *cyclin D* remained relatively constant. Expression of all the genes was also decreased in short-day larvae from days 5 to 10, and thereafter, low levels were maintained in individuals in diapause. No significant differences were detected between long-day and short-day larvae on each day.

#### **4. Discussion**

Obvious differences in ontogenetic phases (larval instars) were not detected between long-day and short-day larvae until day 9. Thereafter, long-day larvae developed to pupae, whereas short-day larvae remained in the early 4<sup>th</sup> instar larval stage. Longitudinal lengths of the brains were nearly identical between long-day and short-day larvae on day 5; the brains were enlarged in both larvae, but the growth rate decelerated in short-day larvae and their growth stopped on day 10. Proportions of cells in each cell division phase were nearly identical between long-day and short-day larvae on day 7, although the brain is slightly, but significantly, larger in long-day larvae than in short-day ones. This is likely to be based on cellular differentiation onset along with optic lobe development; neuroepithelium (rectangular

cells) will start to be converted into neuroblasts (circular cells), which generate neurons in the optic lobe (neurogenic phase) in mature larvae (Lanet and Maurange, 2014). Differences in the proportion of cells in the G0/G1, S, and G2/M phases of the cell division cycle were clearly detected on day 10 between long-day and short-day larvae, and short-day larvae maintained these same proportions thereafter (Fig. 3). Thus, the developmental and cell cycle arrests occur on day 10 in diapause larvae of *N. vitripennis*, and the CNS cells in diapause larvae arrested their cell cycle at the G0/G1 phase (80%) in addition to the G2 phase (20%).

Cell cycle arrest in insects in diapause has been reported in only three species, but the results differ among species. More than 97% of the brain cells in diapause pupae of *S. crassipalpis* were arrested in the G0/G1 phase and only 2% or less were in the G2 phase (Tammariello and Denlinger, 1998). In diapause larvae of *C. costata*, 87% of cells in the CNS arrested in the G0/G1 phase and the remaining 13% were in the G2 phase (Košťál et al., 2009). The present study provides an additional example of a cell cycle arrest in the G0/G1 phase in most cells of the CNS in *N. vitripennis*. On the other hand, 98 % of the cells in diapause embryos of *B. mori* were arrested at the G2 phase (Nakagaki et al., 1991). Košťál et al. (2009) proposed the significance of their difference to be based on the developmental stages at which diapause occurs. It is of interest to investigate the cell cycle arrest in other insect species which undergo diapause at various developmental stages.

It is also noteworthy that the proportion of phases at which cell cycle arrest occurs is greatly biased in *S. crassipalpis* and *B. mori* (Nakagaki et al., 1991; Tammariello and Denlinger, 1998), unlike *C. costata* and *N. vitripennis* (Košťál et al., 2009; the present study). In the former, 97–98% cells were in the G0/G1 or G2 phases, whereas a subdominant phase was observed (13–20% of cells in G2) in addition to the dominant phase (80–87% in G0/G1) in the latter two species. These results indicate a mechanism promoting cell cycle arrest at two distinct phases

(G0/G1 and G2) exist in the latter species. Alternatively, species exhibiting cell cycle arrest only in a dominant phase may have a unique mechanism to set specific phases of the cell cycle during diapause. In general, larval and pupal diapauses are induced by a failure of ecdysteroid production (Denlinger et al., 2012). Lack of ecdysteroids is also known to be the key in *N. vitripennis* diapause (de Loof et al. 1979). Ecdysteroids are the key elements in development, metamorphosis, and moulting, and therefore, inhibition of their production promotes developmental arrest. Effects of 20-hydroxyecdysone (20E), the primary ecdysteroid among insects, on the cell division cycle were investigated in the accessory glands of pupae of the mealworm *Tenebrio molitor*, which does not have a diapause (Yaginuma et al., 1988). Immediately after pupal ecdysis, the percentage of cells in the G2 phase (> 60%) was greater than twice that in the G1 phase (25-30%) and less than 12% of the cells were in S phase. During pupal development, the S fraction decreased gradually, and thereafter 97% of cells reached the G2 phase, and cell cycling stopped 8 days after pupal ecdysis. This cell cycle arrest is caused by a low titre of 20E. When the accessory glands were isolated and cultured with 20E, a significant fraction of cells (20-40%) were in the S phase throughout the experimental period, and the proportions of cells in G1 and G2 phases were 30% and 60%, respectively. When the glands were cultured without 20E, the S fraction gradually decreased, 20-30% of cells remained in G1 with the balance in G2, and finally cell cycling stopped (Yaginuma et al., 1998). These results indicate that the absence of 20E promotes cell cycle arrest; however, the absence of 20E does not promote cell cycle arrest in only a dominant phase. To set some phases into a dominant phase, some additional mechanism, such as impeding cellular proliferation at a certain cell cycle checkpoint (Johnson and Walker, 1999), is likely to play a role. If this were applicable to diapause, *S. crassipalpis* and *B. mori* would possess certain unique mechanisms.

The expression of cell cycle regulatory genes, in relation to diapause initiation, is

investigated only in a few insect species (Košťál et al., 2009; Tammariello and Denlinger, 1998). In *S. crassipalpis*, the expression of *pcna* was significantly suppressed in the whole body of diapause pupae, whereas expression of *cyclin E*, the tumour suppressor gene *p21*, and the cyclin E/Cdk2 inhibitor gene *p53* was unaffected (Tammariello and Denlinger, 1998). In diapause larvae of *C. costata*, *pcna* expression was slightly but significantly suppressed in the CNS, whereas the expression of *cyclin D*, *cyclin E*, stimulatory phosphatase Cdc25 gene *string*, and inhibitory kinase genes *myt1* and *wee1* showed no or minimal changes, irrespective of developmental destination. In *C. costata*, expression of these genes in the whole body was also compared with that of the transcriptome analysis (Poupardin et al., 2015), which identified significant downregulation of genes involved in cell cycle regulation, such as *cyclin E*, *cyclin B* and *Cdks 1, 2, and 7*. Other sequences coding for cell cycle regulators such as *myt1*, *string*, *wee*, *fizzy*, and *polo* were also downregulated in response to a short-day photoperiod (Poupardin et al., 2015). Similarly, positive cell cycle regulators and DNA replication transcripts, particularly *pcna*, were downregulated in the adult females of the mosquito *Aedes albopictus* during the maternal diapause induction phase (Huang et al., 2015). Similar results were obtained for the cotton bollworm *Helicoverpa armigera* and the apple maggot *Rhagoletis pomonella* in diapause (Bao and Xu, 2011; Ragland et al., 2011). We also investigated expression of several cell cycle regulatory genes in the whole body of *N. vitripennis* in the present study. Relative mRNA levels of cell cycle regulatory genes (*pcna*, *cyclin E*, and *p53*) and housekeeping genes (*RpL32* and *EF-1 $\alpha$* ) decreased in long-day larvae when most cells have entered the G0/G1 phase on day 10. Downregulation of these genes would be related to cellular differentiation at this stage (neurogenic phase). Relative RNA levels of these genes also decreased in short-day larvae. However, this event is not for cellular differentiation, but quite probably for developmental and cell cycle arrest. Wolschin and Gadau (2009) focused on the proteomic signature of early



diapause in *N. vitripennis* and observed lower levels of ribosomal proteins, elongation factors, and histones in early diapause larvae. Maintaining the lower RNA levels of the housekeeping genes as well as the cell cycle regulatory genes support an idea that the silencing of these genes would promote developmental and cell cycle arrest effectively in this species.

In summary, in the diapause of *N. vitripennis*, we observed that developmental arrest and cell cycle arrest occur at the early 4<sup>th</sup> larval instar under short-day conditions. Cell cycle arrest occurs primarily in the G0/G1 phase and secondarily in the G2 phase. Expression of not only cell cycle regulatory genes but also housekeeping genes was downregulated in diapause larvae.

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## Figure legends

**Fig. 1.** Ontogeny of *Nasonia vitripennis* at 20 °C.  $n = 16-60$ .

**Fig. 2.** Longitudinal length (mean  $\pm$  SD) of the brains of larval *Nasonia vitripennis* ( $n = 10-20$ ). The same letters indicate no significant statistical difference among samples (One-way ANOVA followed by Tukey–Kramer test,  $P > 0.05$ ).

**Fig. 3.** Relative proportions of brain cells in G0/G1, S, and G2/M phases of long-day and short-day larvae of *Nasonia vitripennis* (mean  $\pm$ SD,  $n = 3-6$ ). The same letters indicate no significant statistical difference among samples (One-way ANOVA followed by Tukey–Kramer test,  $P > 0.05$ ).

**Fig. 4.** Relative levels of *RpL3* and *EF-1 $\alpha$*  mRNA and that of *18S rRNA* normalized by  $\mu\text{g}$  of total RNA (A) and relative mRNA levels of *pcna*, *cyclin D*, *cyclin E*, and *p53* normalized by *18S rRNA* (B) in *Nasonia vitripennis*. Closed and open circles indicate long-day and short-day larvae, respectively (mean  $\pm$  SD,  $n = 5$ ). The same letters indicate no significant statistical difference among samples (One-way ANOVA followed by Scheffé's  $F$  test,  $P > 0.05$ ).

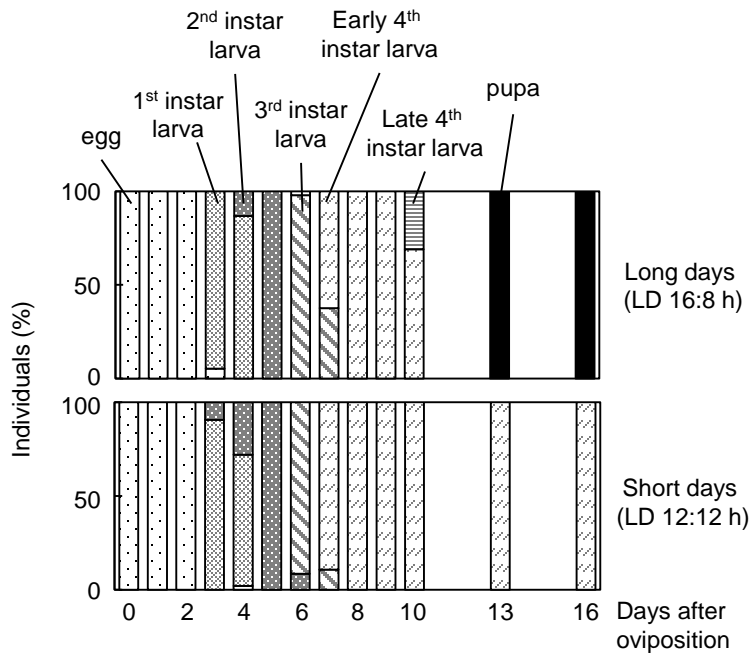


Fig. 1

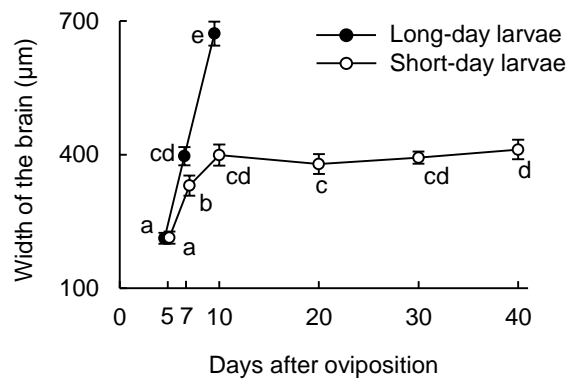


Fig. 2



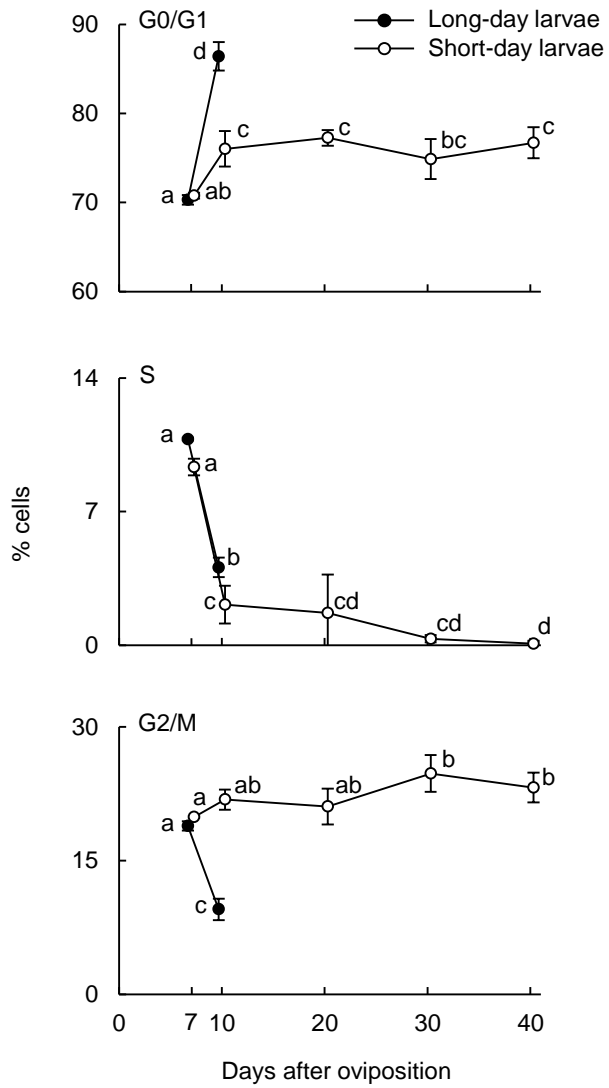


Fig. 3

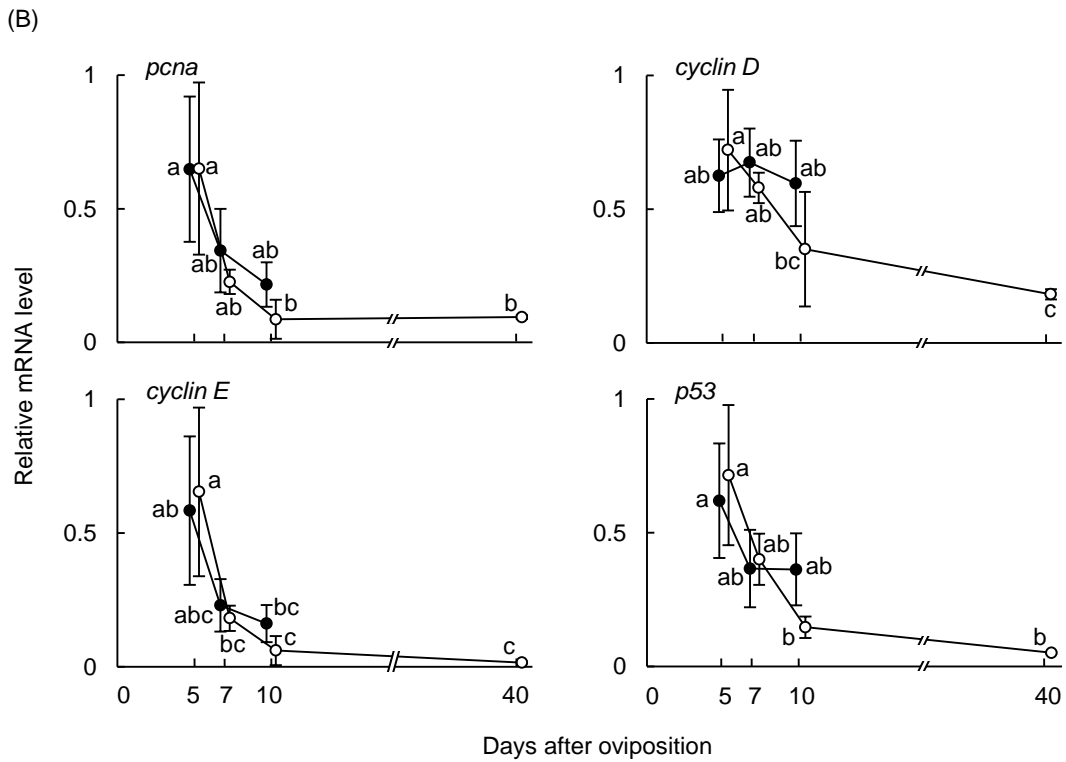
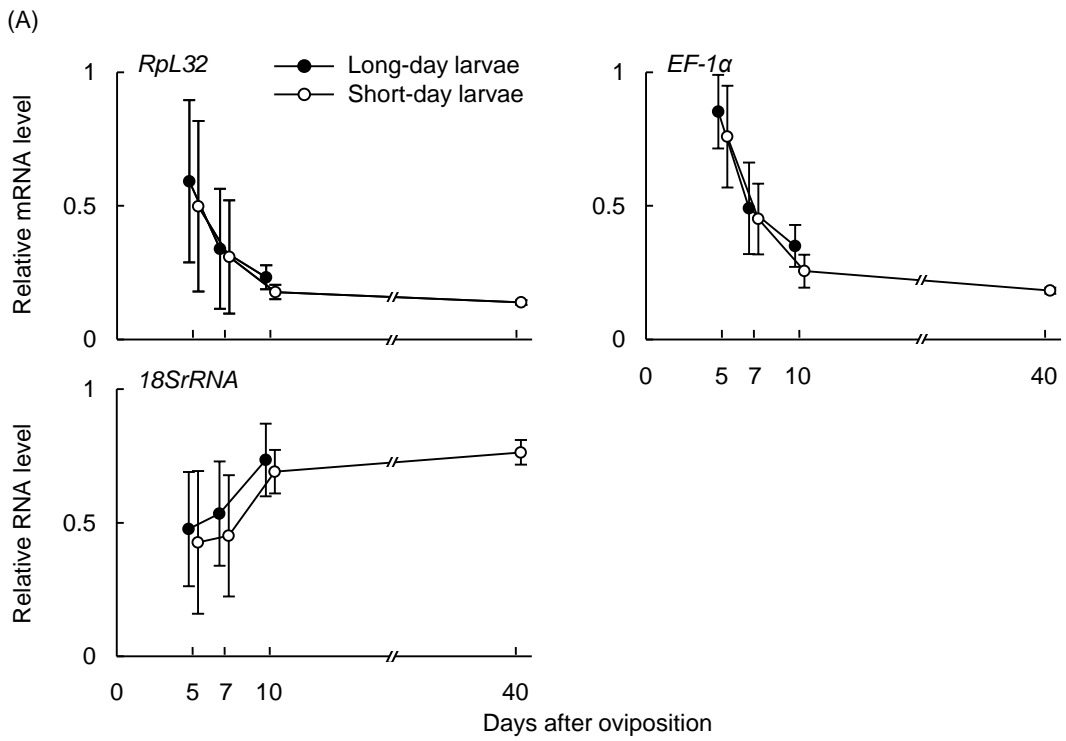


Fig. 4