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

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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.

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; Koštál, 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 (Koštál 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; Koštál, 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; Koštál 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 G0/G1, S, and G2/M phases of the cell division cycle were 10, 35, and 55%, respectively, whereas after entering diapause almost all cells were arrested at the G2 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 (Koštál 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 (4th) 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;

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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-1α* [*EF-1α*]) 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 ± 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 ± 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 Horwitz, 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 ± 1.0 °C under long- and short-day conditions, by opening fly puparia once a day from days 2–10 and on days 13 and 16. The wasp larval stage was classified into five stages (1st, 2nd, 3rd, early 4th, and late 4th 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. Width 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 width of the brain (length from right edge of the right brain hemisphere to left edge of the left brain hemisphere) 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-1α*, 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-1α,* 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-1α*, and GQ410677.1 for *18S rRNA*. Amplified fragments in all PCRs were

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