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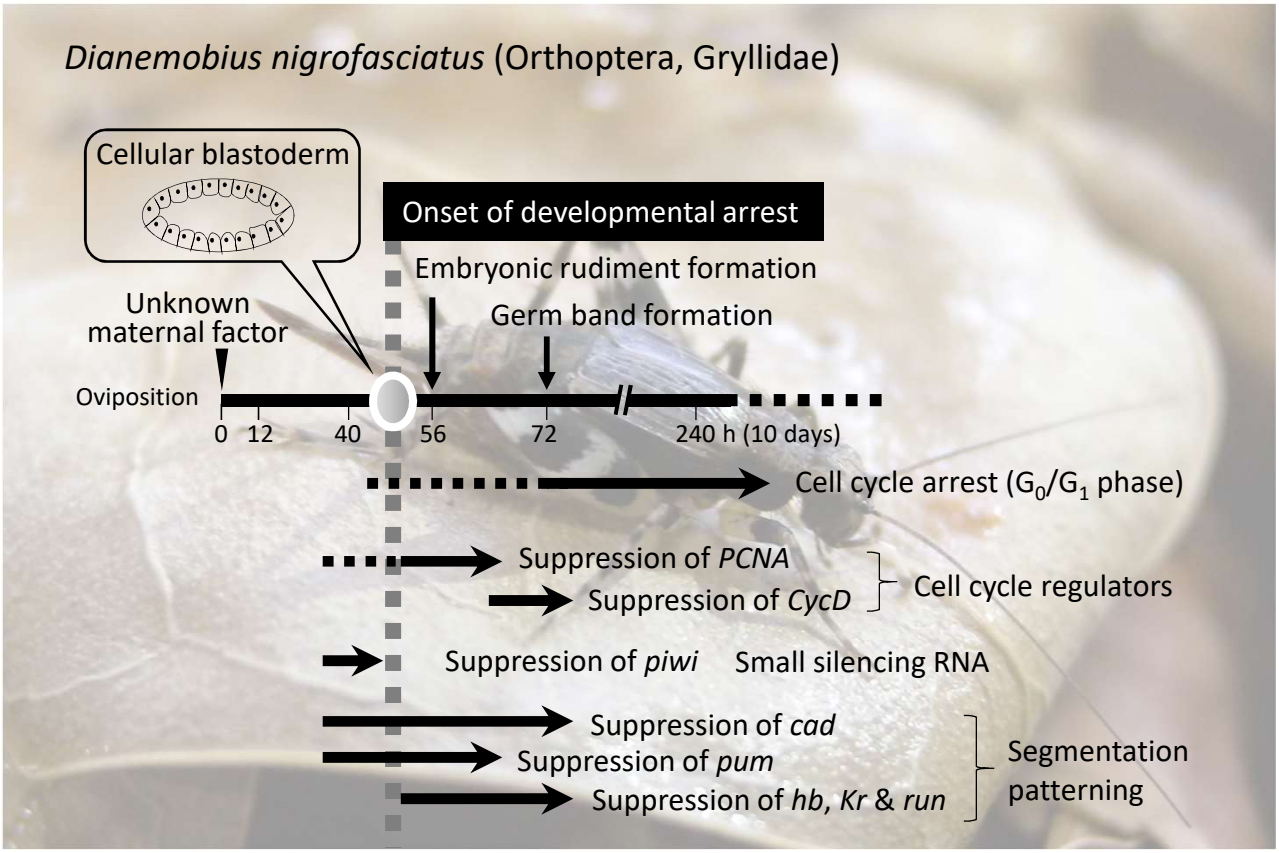

Graphical Abstract

Cell cycle regulator, small silencing RNA, and segmentation patterning gene expression in relation to embryonic diapause in the band-legged ground cricket

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Abstract

Insects enter diapause to synchronize their life cycle with biotic and abiotic conditions favorable for their development, reproduction, and survival. Adult females of the band-legged ground cricket *Dianemobius nigrofasciatus* (Orthoptera, Glyllidae) respond to environmental factors in autumn and lay diapause-destined eggs. The eggs arrest their development and enter diapause at a very early embryonic stage, specifically the cellular blastoderm. To elucidate the physiological mechanisms underlying this very early stage programmed developmental arrest, we investigated the cell division cycle as well as the expression of cell cycle regulators, small silencing RNAs, and segment patterning genes. The diapause embryo arrests its cell cycle predominantly at the G_0/G_1 phase. The proportion of cells in the S phase of the cell cycle abruptly decreased at the time of developmental arrest, but further changes of the G_0/G_1 and G_2/M were later observed. Thus, cell cycle arrest in the diapause embryo is not an immediate event, but it takes longer to reach the steady state. We further elucidated molecular events possibly involved in diapause preparation and entry. Downregulation of *Proliferating cellular antigen* (*PCNA*; a cell cycle regulator), *caudal* and *pumilio* (*cad* and *pum*; early segmentation genes) as well as *P-element induced wimpy testis* (*piwi*) (a small silencing RNA) prior to the onset of developmental arrest was notable. The downregulation of *PCNA*, *cad* and *pum* continued even after entry into developmental arrest. In contrast to upregulation in non-diapause eggs, *Cyclin D* (another cell cycle regulator) and *hunchback*, *Krüppel*, and *runt* (gap and pair-rule genes) were downregulated in diapause eggs. These molecular events may contribute to embryonic diapause of *D. nigrofasciatus*.

Keywords: Cell cycle arrest, developmental arrest, diapause, maternal effect, cellular blastoderm, cricket

1. Introduction

Seasonally recurring environmental stress affects the growth, development, reproduction, and survival of all insects. To overcome harsh seasons, temperate insects enter a specific physiological state called diapause, in which development or reproduction is arrested or greatly suppressed (Koštál, 2006; Denlinger et al., 2011; Goto and Numata, 2014). Diapause insects accumulate additional energy reserves prior to the onset of diapause, and they greatly suppress their metabolic activity during diapause. Sufficient energy reserves and low metabolic activity give rise to a high probability of survival during and after adverse seasons (Hahn and Denlinger, 2011). Although the adaptive significance of diapause has been verified, the molecular mechanisms underlying diapause are still largely obscure.

Among insects, diapause occurs at a specific stage (or at two in some cases) in each species. Embryonic, larval (nymphal), pupal, and adult diapauses have been characterized in various insect species (Denlinger et al., 2011). In Orthoptera, embryonic diapause is relatively common, but their diapause stages vary significantly. Diapause occurs in the late embryo after appendage formation in *Locusta migratoria* and *Melanoplus differentialis*, and in the germ band stage in *Teleogryllus emma*, *Scapsipedus asperses*, and *Homoeogryllus japonica* (Umeya 1950; Masaki 1960). Diapause entry prior to germ band formation is also reported in *Polionemobius mikado*, *Dianemobius nigrofasciatus*, *Pteronemobius ohmachii*, and *Loxoblemmus aomoriensis* (Masaki 1960; Fukumoto et al., 2006).

Adult females of the band-legged ground cricket *D. nigrofasciatus* in southern Japan, including Osaka, lay eggs destined to develop into nymphs without developmental interruption (non-diapause) in response to long day length. However, females lay eggs destined to enter diapause in response to short day length; i.e., photoperiodically- and maternally-induced diapause (Kidokoro & Masaki, 1978; Shiga & Numata 1997). Tanigawa et al. (2009) assessed ultra-thin sections of eggs and found that diapause eggs arrest their development at the cellular blastoderm stage 40–56 h after egg laying at 25°C. At this stage, any organs as well as distinct tissues have not

been formed yet. This is one of the earliest stages of embryonic diapause among insects. How development is arrested at such an early stage before cell differentiation is a key area of interest.

In the present study, we focused on cell (or nuclear) division cycles and gene expression before and after entry into programmed developmental arrest in *D. nigrofasciatus*, to unveil the physiological mechanisms underlying this event. We first investigated cell division. Although cell cycle arrest is generally accepted as a hallmark of insect diapause (Koštál, 2006), cell division phases in diapause have only been investigated in a few species (Tammariello et al., 1998; Koštál et al., 2009; Nakagaki et al., 1991; Champlin and Truman, 1998; Shimizu et al., 2018). In *D. nigrofasciatus*, many nuclei in diapause eggs are immunoreactive to phosphorylated and acetylated histone H3, which involves mitotic chromosome condensation or mitogen-induced gene expression (Tanigawa et al., 2009). This indicates possible mitotic activity even during embryonic diapause in this species. We tested this hypothesis by estimating the relative number of nuclei in a single egg. In addition, to elucidate the molecular mechanisms underlying the programmed developmental arrest, we assessed the expression of four cell cycle regulators [cyclin E/Cdk2 inhibitor gene *p53*, *Cyclin D* (*CycD*), *Cyclin E* (*CycE*), and *Proliferating cell nuclear antigen* (*PCNA*)], and three small silencing RNAs [*P-element induced wimpy testis* (*piwi*), *spindle E* (*spn-E*), and *Argonaute2* (*AGO2*)], of which roles in diapause have been paid attention in previous studies (Tammariello et al., 1998; Koštál et al., 2009; Reynolds et al., 2013). We also focus on five segment patterning genes, which include two maternal effect genes [*caudal* (*cad*) and *pumilio* (*pum*)], two gap genes [*hunchback* (*hb*), and *Krüppel* (*Kr*)], and one pair-rule gene *runt* (*run*). We assumed that their suppression plays a pivotal role in diapause entry at very early embryonic stage. The present study revealed distinct gene expression patterns of some of these genes between non-diapause-destined and diapause-destined *D. nigrofasciatus* eggs. We discuss roles of these genes in diapause entry.

2. Materials and methods

2.1. Insects

D. nigrofasciatus adults and nymphs were collected from the grassy field on the campus of Osaka City University (34°59′N, 135°50′E) and from the bank of the Yamato-gawa river (34°59′N, 135°51′E), Japan, from June to October 2014–2016. Approximately 40–60 insects were reared in a plastic case (29.5 cm width, 19.0 cm depth, and 17.0 cm height) at 25.0 ± 1.0°C and were fed an artificial insect diet (Oriental Yeast, Tokyo, Japan), fresh carrots, and water.

Female adults of *D. nigrofasciatus* under short-day conditions (LD 12:12 h) lay eggs destined to enter diapause, whereas those under long-day conditions (LD 16:8 h) lay eggs destined to direct development (non-diapause) (Goto et al., 2008). In the present study, eggs laid by females under short-day and long-day conditions were called short-day and long-day eggs, respectively. Eggs deposited within a 2–3 h period were collected and used for experiments, unless otherwise indicated. These eggs were continuously maintained under the photoperiodic conditions.

2.2. Flow cytometric analysis

Long-day and short-day eggs were collected 1.7 (40 h), 3, 5, and 10 days after egg laying. Short-day eggs were also collected 20 and 30 days after egg laying. In this experiment, we used eggs laid within 12 h for the 40-h sample, because of the difficulty in collecting enough eggs for experimentation.

Eggs were soaked in 12% sodium hypochlorite solution (Wako Pure Chemical Industries, Osaka, Japan) for 2 min at room temperature (20-25°C). The eggs were then transferred to citrate buffer solution, and their chorion was removed using fine forceps. The BD Cycletest Plus DNA Reagent Kit (BD Biosciences, San Jose, CA, USA) was used to prepare uniform suspensions of single nuclei using a homogenizer (Bio Masher II; Nippi, Tokyo, Japan). After trypsin and RNase A treatments, the samples were incubated with propidium iodide for 10 min in the dark at 4°C. Finally, the sample was filtered through a 50-μm nylon mesh. Cellular DNA content was assessed on the BD Accuri C6 Flow Cytometer (BD Biosciences). The obtained data were analyzed using

the FCS Express software (De Novo Software, Glendale, CA, USA) and the cells were classified in G_0/G_1, S, and G_2/M phases according to the intensity of fluorescence peaks. The relative number of nuclei in a single egg were also estimated. In the present study, the extraction efficiency of nuclei from eggs was not introduced.

2.3. RNA sequencing

There are very few studies on genes and their nucleotide sequences in *D. nigrofasciatus*. Therefore, we developed an RNA sequencing database of this species with the aid of high throughput sequencing technology.

We reared pairs of a female and a male under LD 16:8 h or LD 12:12 h at 25°C and collected eggs every 2 days. Ovaries were dissected out from females at zeitgeber time (ZT) 6 (light on is defined as ZT0) on day 10 (day 0 is defined as the day of adult emergence). Four or five ovaries were then stored in RNA*later* solution (Thermo Fisher Scientific, Waltham, MA, USA). After confirming that females reared under long days and short days laid non-diapause and diapause eggs, respectively, total RNA was purified with TRIzol Reagent and a PureLink RNA Micro Scale kit (Thermo Fisher Scientific) after homogenizing with the BioMasher II tissue grinder. RNA integrity was confirmed using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). RNA was prepared as tagged cDNA libraries for each sample using a TruSeq RNA sample prep kit (Illumina, San Diego, CA, USA) in accordance with the manufacturer's instruction. The cDNA libraries were sequenced using a Hiseq 2500 (Illumina) sequencer based on the paired-end method and a read length of 100 bp. Reads were assembled using the Trinity platform (Grabherr et al., 2011).

2.4. Cloning of caudal gene fragment from D. nigrofasciatus

The nucleotide sequence of the gene homologous to *cad* was not found in our RNA sequencing database. Therefore, we cloned the gene from *D. nigrofasciatus* using a conventional method. Total RNA was extracted from the whole body of adult females using TRIzol Reagent. cDNA was synthesized with M-MLV reverse transcriptase and

oligo(dT)$_{12-18}$ primer (Thermo Fisher Scientific). PCR was then performed using GoTaq DNA Polymerase (Promega, Madison, WI, USA). The primers, which were designed from the *Gryllus bimaculatus cad* sequence (DDBJ/GenBank/EMBL accession No. AB191008.1), were as follows (from 5′ to 3′): AAG ACG CGC ACC AAG GAC AAG T and GCT TGC GCT CCT TGG CGC G. The amplified DNA fragment was purified using the Wizard Plus SV Minipreps DNA Purification System (Promega) and sequenced on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) with BigDye Terminator v3.1 Cycle Sequence Kit (Applied Biosystems). The nucleotide sequence of *D. nigrofasciatus cad* was deposited in the database (DDBJ/GenBank/EMBL accession No. LC279612).

2.5. RT-qPCR

Total RNA was extracted from whole eggs 12, 24, 40, 56, and 72 h after egg laying using TRIzol Reagent. Total RNA was dissolved in 20 μL of nuclease-free water and genomic DNA in the sample was digested with Deoxyribonuclease (RT Grade) for Heat Stop (Nippon gene, Tokyo, Japan). cDNAs were synthesized from 200 ng of total RNA using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The relative abundance of mRNA transcripts was estimated by reverse transcription quantitative PCR (RT-qPCR) using the 7500 Real-Time PCR system (Thermo Fisher Scientific). cDNA (1 μL) was used at a final concentration of 1x Go Taq qPCR Master Mix (Promega) and 0.2 μM of each primer according to the supplier's instructions, and each reaction was performed in duplicate. As a control, the relative amount of *18S* ribosomal RNA (*18S* rRNA) was used for normalization. Amplified fragments were approximately 100 bp in length. In all the reactions, the generation of only a single expected amplicon was confirmed by melting analysis. Quantification was performed based on the standard curve methodology. Four or five independent RNA samples were used for each condition as biological replicates. Primers used for qPCR are shown in Table S1.

In the relative amount of *18S* rRNA to the amount of total RNA, two-way ANOVA detected a significant difference among chronological sample ($P < 0.05$).

However, no significant difference between photoperiods ($P > 0.05$) and a marginal interaction between photoperiods and hours after egg laying ($P = 0.05$) were detected. Therefore, we used *18S* rRNA as an internal control.

2.6. Statistical analysis

The numbers of nuclei were logarithmically-transformed and were analyzed by one-way ANOVA for each photoperiod. Only the time points where data were obtained for both photoperiodic regimes were included into two-way ANOVA. The proportions of cells in different phases of the cell cycle were arcsine-square-root-transformed and were analyzed by one-way ANOVA for each photoperiod. Only time points where data were obtained for both photoperiods were included into two-way ANOVA. The gene expression data were statistically analyzed using two-way ANOVA. When significant difference was not detected in photoperiod, no further analysis was executed. When significant difference was detected in interaction between photoperiod and time, Tukey-Kramer post-hoc multiple comparisons were executed. In this paper, we present only the ANOVA *P* values, which allow estimating the significance of the effects of time, photoperiod and interaction of both on the observed response ($P < 0.05$, significant effect). In addition, we provide the results of multiple comparisons in the form of letters flanking the means. Values with the same letters indicate no significant difference among them.

3. Results

3.1. The number of nuclei in eggs and their division phases

The relative numbers of nuclei in a long-day and short-day egg were estimated based on the count number in a fixed volume of suspensions (Fig. 1). We were unable to estimate the number of nuclei in eggs 40 h after egg laying due to their small count numbers. The number of nuclei significantly increased with the days after egg laying in both photoperiodic conditions (one-way ANOVA for each, $P < 0.05$). Two-way ANOVA detected significant differences in photoperiod, time, and interaction of them ($P < 0.05$). Tukey-Kramer multiple comparisons detected significant difference in the number of

nuclei on days 3, 5 and 10 in long-day eggs ($P < 0.05$), but not in short-day eggs ($P > 0.05$). These results indicate that cell division stops or significantly slows down in short-day eggs from day 3 or possibly earlier.

Ontogenetic changes of the relative proportions of cells (or nuclei, in the case of 1.7-day samples, of which stage is prior to cellularization) in the G_0/G_1, S, and G_2/M phases in long-day or short-day eggs are shown in Figure 2. Long-day eggs demonstrated distinct changes in the relative proportions of cells in each cell division cycle in correspondence with their development; i.e., the S and G_2/M fractions decreased and G_0/G_1 fraction increased (one-way ANOVA, $P < 0.05$ for all). Although the patterns were generally identical to those in short-day eggs (one-way ANOVA, $P < 0.05$ for all), large differences between photoperoids were detected on days 3 and 5 in the G_0/G_1 and S phases (Tukey-Kramer multiple comparisons, $P < 0.05$). Abrupt reduction of the S fraction and incremental increase of the G_0/G_1 fraction in short-day eggs indicates that cell cycle arrest occurs mainly at the G_0/G_1 phase in diapause eggs, starting between days 1.7 and 3. The proportion of eggs in the G_0/G_1 and G_2/M phases gradually reached the steady phase 10 days after egg laying, which is much later than the onset of developmental arrest (40-56 h after egg laying). It is important to note that approximately 5% of the S fraction remained in diapause eggs.

3.2. Expression of genes potentially involved in diapause entry

Through RNA sequencing, we obtained 466,566,600 reads, which were trimmed and assembled into 235,995 contigs with an average length of 771 bases and N50 of 1,516 bases (DDBJ/GenBank/EMBL Accession No. IADE01000001-IADE01235995). The raw reads of the transcriptome are publicly available in the DNA Data Bank of Japan (DDBJ) under Bioproject ID PRJDB5888, Biosample accession numbers SAMD00128720 and SAMD00128721. Assembled sequences are also available in the DDBJ database.

We selected four cell cycle regulators (*p53*, *CycD*, *CycE* and *PCNA*), three small silencing RNAs (*piwi*, *spn-E*, and *AGO2*), four genes involved in segment patterning (*pum*, *hb*, *Kr*, and *run*), and *18S rRNA* gene from our RNA sequencing database (Table

1). These genes demonstrated high similarity with the respective genes in other organisms. The nucleotide sequence of *cad*, which was obtained by a conventional cloning method, also showed high similarity with the *cad* gene in other insect species (Table 1).

Relative amounts of *p53*, *CycD*, *CycE*, and *PCNA* mRNAs are shown in Figure 3. Two-way ANOVA detected significant photoperiodic effects in *CycD*, *CycE*, and *PCNA* ($P < 0.05$). Significant differences among hours after egg laying were detected in all the genes and significant interactions between hours after egg laying and photoperiodic conditions were detected in *CycD* and *PCNA* ($P < 0.05$). Relative amounts of *p53* mRNA were stable until 40 h after egg laying and gradually decreased thereafter, irrespective of photoperiod. *CycD* mRNA levels gradually decreased toward 56 h after egg laying, irrespective of photoperiod. Thereafter, the *CycD* was upregulated in long-day eggs but not in short-day eggs. Tukey-Kramer multiple comparisons detected a significant photoperiodic difference at 72 h after egg laying ($P < 0.05$). *CycE* mRNA levels abruptly increased at 24 h but gradually decreased thereafter and the photoperiodic differences were very small. A significant photoperiodic effect was observed in *PCNA* and the short-day eggs always showed lower levels of mRNA than the long-day eggs. However, Tukey-Kramer multiple comparisons detected a significant photoperiodic effect only at 56 h after egg laying.

Relative amounts of *piwi*, *spn-E*, and *AGO2* mRNAs are shown in Figure 3. Two-way ANOVA detected significant differences between photoperiods in *piwi* and *AGO2* ($P < 0.05$), but not in *spn-E* ($P > 0.05$). Significant time differences were detected in all the genes, and a significant interaction between photoperiod and time was detected in *piwi* only. Relative amounts of *piwi* mRNA gradually increased toward 40 h after egg laying, but they decreased thereafter, in both photoperiodic conditions. Significant difference was detected between long-day and short-day eggs only at 36 h after egg laying, prior to the onset of developmental arrest (Tukey-Kramer multiple comparisons, $P < 0.05$). Relative amounts of *spn-E* mRNA increased toward 24 h after egg laying and gradually decreased thereafter, irrespective of photoperiod. *AGO2*

mRNAs gradually increased with hours after egg laying in both photoperiodic conditions. The photoperiodic differences were very small.

It is important to note that significant increase in mRNA levels was detected in the maternal effect genes, *cad* and *pum* (one-way ANOVA, $P < 0.05$ for both photoperiodic conditions), with increased time after egg laying (Fig. 4). These results indicate that we estimated the amounts of mRNAs produced by zygotes themselves (zygotic expression) in addition to the mRNAs maternally transferred to zygotes. For both genes, two-way ANOVA detected significant differences in photoperiod, time, and their interaction ($P < 0.05$ for all). *cad* mRNA levels were low at 12 h and 24 h after egg laying but abruptly increased at 40 h in long-day and short-day eggs. Zygotic expression of *cad* may occur at this time. Its levels increased toward 56 h and then decreased at 72 h after egg laying in long-day eggs. Short-day eggs showed a similar expression pattern, but its upregulation was not pronounced (Tukey-Kramer multiple comparisons, $P < 0.05$). *pum* expression was similar between long-day and short-day eggs at 12 and 24 h but its expression was subsequently suppressed in short-day eggs (Tukey-Kramer multiple comparisons, $P < 0.05$).

For *hb*, *Kr* and *run*, two-way ANOVA detected significant differences in photoperiod, time, and their interaction ($P < 0.05$ for all). The relative amounts of *hb* mRNA abruptly increased 24 h after egg laying and remained at a high level in long-day eggs. Short-day eggs also showed a similar pattern, but *hb* was downregulated from 56 h after egg laying (Tukey-Kramer multiple comparisons, $P < 0.05$). *Kr* expression was distinct between short- and long-day eggs after onset of developmental arrest. Its mRNA level was low until 40 h after egg laying, irrespective of the photoperiod. Long-day eggs abruptly increased their *Kr* mRNA levels thereafter but short-day eggs did not (Tukey-Kramer multiple comparisons, $P < 0.05$). Similar distinct differences after the onset of developmental arrest were detected in the pair-rule gene *run* (Tukey-Kramer multiple comparisons, $P < 0.05$).

4. Discussion

We summarized results of the present study in Fig. 5. The present study reveals that diapause eggs of *D. nigrofasciatus* arrest their cell cycle predominantly (> 90%) at the G_0/G_1 phase. Although significant information on diapause has accumulated in the literature (Denlinger et al., 2011; Hahn and Denlinger, 2011; Koštál, 2006), very little is known about cell cycle arrest during diapause. Diapause pupae of the flesh fly *Sarcophaga crassipalpis* and diapause larvae of the drosophilid fly *Chymomyza costata* and the jewel wasp *Nasonia vitripennis* arrest their brain cells at the G_0/G_1 phase. However, diapause embryos of the silk moth *Bombyx mori* and the optic lobe of the tobacco hawk moth *Manduca sexta* in pupal diapause arrest their cells at the G_2/M phase (Nakagaki et al., 1991; Tammariello and Denlinger, 1998; Champlin and Truman, 1998; Koštál et al., 2009; Shimizu et al., 2018). These results clearly indicate that there is no correlation between the developmental stages in diapause and the phases of cell cycle arrest.

The present study further revealed that diapause eggs of *D. nigrofasciatus* have some fraction of their cells in the S phase (approximately 5%), which has not been detected in other insect species (Nakagaki et al., 1991; Tammariello and Denlinger, 1998; Koštál et al., 2009; Shimizu et al., 2018). This suggests active mitotic activity even during diapause in *D. nigrofasciatus.* Tanigawa et al. also detected many nuclei that are immunoreactive to phosphorylated and acetylated histone H3, one of the S-phase markers, in diapause eggs (Tanigawa et al., 2009). However, the present study clarified that there was no increase in the relative numbers of nuclei after onset of developmental arrest. Therefore, although continuous cell division may occur during diapause in this species, it is very slow and does not promote significant increase in the number of cells. This type of diapause is very different from that commonly presented in the literature. However, very slow but continuous morphological development (i.e., no cessation of morphological development) has been shown in the pea aphid *A. pisum* in embryonic diapause (Shingleton et al., 2003).

The expression of cell cycle regulatory genes, in relation to diapause initiation, has been investigated in various insect species (Koštál et al., 2009; Tammariello and

Denlinger, 1998; Bao and Xu, 2011; Ragland et al., 2011; Huang et al., 2015; Poupardin et al., 2015; Shimizu et al., 2018). Among them, suppression of *PCNA*, an essential component of the eukaryotic chromosomal DNA replisome (Maga and Hübscher, 2003), appears to be a common feature in cell cycle arrest during diapause. We detected significant suppression of *PCNA* after onset of developmental arrest and large but insignificant suppression prior to this in *D. nigrofasciatus* short-day eggs. In contrast to upregulation in long-day eggs, *CycD* in short-day eggs was suppressed after the onset of developmental arrest. These changes may contribute to cell cycle arrest in short-day eggs of *D. nigrofasciatus*.

Small silencing RNAs, including microRNAs (miRNAs), small-interfering RNAs (siRNAs), and *piwi*-related RNAs (piRNAs) are key for posttranscriptional gene regulation. They interfere with the expression of target genes by binding to their target mRNA with complementary nucleotide sequences (Ghildiyal and Zamore, 2009). Recently, these small RNAs have received significant attention with regards to diapause regulation (Reynolds, 2017). Elevated RNA levels of *piwi* and *spn-E*, as well as *AGO2* were observed in photosensitive first instar larvae under pupal diapause-inducing conditions in the flesh fly *Sarcophaga bullata* (Reynolds et al., 2013). These are key players in small silencing RNA processes: SPN-E is the DEAD box RNA helicase that plays an important role in piRNA production, AGO2 is selectively associated with siRNAs, and PIWI load mature piRNAs as an element of the RNA-inducing silencing complexes (RISC) (Ghildiyal and Zamore, 2009; Ryazansky et al., 2016. Tomari et al., 2007; Handler et al., 2013). The results in *S. bullata* suggest involvement of the piRNA and siRNA pathways in programming developmental trajectories. In contrast to *S. bullata*, *AGO2* is downregulated in larvae exposed to diapause-inducing short-day conditions compared to larvae under diapause-averting long-day conditions in *C. costata* (Poupardin et al., 2015). In this case, reduction of the piRNA pathway could be important for the switch from direct development to diapause. In addition, *AGO2* RNA is drastically upregulated in postdiapause larvae in the leaf-cutting bee *Megachile rotundata* (Yocum et al., 2015). We assumed some difference between long-day and

short-day eggs of *D. nigrofasciatus* in expression of these small RNAs. A significant difference in *piwi* expression prior to the onset of developmental arrest may indicate its role in diapause preparation. It is important to investigate expression of other small RNAs, to clarify their roles in embryonic diapause of *D. nigrofasciatus* (see, Reynolds et al., 2017).

Molecular mechanisms underpinning insect segmentation patterning have been extensively investigated, demonstrating that a few dozen genes are required for the process. They are classified into 4 groups based on their roles: maternal effect, gap, pair-rule, and segment polarity genes (Jaeger et al., 2012). Maternal factors encoded by maternal effect genes specify the antero-posterior (A-P) axis and regulate zygotic downstream expression of gap genes by their gradients. Each of the gap genes regulate the formation of a contiguous set of segments through the pair-rule gene expression. The segment polarity genes are expressed in the embryo following expression of the gap and pair-rule genes (Akam, 1987). Most of the segmentation genes identified by studies of *D. melanogaster* development are likely to be found in other insects (Sommer et al., 1992). However, *Drosophila* exhibits an evolutionarily derived mode of development (a long germ-band insect), and thus, molecular mechanisms of its segmentation may be unrepresentative for arthropods. In long germ-band insects including *Drosophila*, all segments are specified simultaneously during the blastoderm stage. However, short or intermediate germ-band insects, to which many insect species belong, only specify the anterior segments leaving the posterior segments to be specified through a secondary growth process (Mito and Noji, 2006; Damen, 2007). Crickets belong to the intermediate germ-band group.

In *Drosophila*, the mRNA of the maternal effect genes *bicoid* (*bcd*) and *nanos* (*nos*) are loaded into the oocyte and are specifically targeted to the anterior and posterior poles of eggs, respectively. However, no *bcd* gene has been isolated from non-Diptera species (McGregor, 2005; Demuth and Wade, 2007). Instead of the *bcd* system, the primary role of *cad* in the segmentation process has been proposed. *cad* is a maternal effect gene but it is also expressed zygotically. Its zygotic expression induces the

expression of a series of segment patterning genes, and is therefore crucial for posterior patterning in various insect species including *G. bimaculatus* (Shimyo et al., 2005; Deaarden and Akam, 2001; Olesnicky et al., 2006; Schultz et al., 1998; Xu et al., 1994). Thus, *cad* is regarded as a master organizer of early patterning (Copf et al., 2004; Olesnicky et al., 2006). *pum* is the ortholog of the *D. melanogaster* maternal effect gene and is specifically required for abdominal formation, similar to the maternal effect gene *nos* (Barker et al., 1992). In *T. castaneum*, *nos* and *pum* crucially contribute to posterior segmentation by preventing *hb* and *Kr* expression directly or indirectly (Schmitt-Engel et al., 2012). *hb* and *Kr* are orthologs of the *D. melanogaster* gap genes and also act as gap genes in *G. bimaculatus* (with some modifications in *hb*) (Mito et al., 2005, 2006). Shinmyo et al. (2005) clearly demonstrated that *hb* and *Kr* are under the control of *cad* in the cricket. *run* is a primary pair-rule gene that plays a significant role in segmentation in *T. castaneum* and *B. mori* (Choe et al., 2006; Nakao, 2015). Quantitative real-time PCR analyses in the present study revealed that the relative expression levels of *cad* and *pum* significantly decreased 40 h after egg laying. Moreover, the relative expression levels of *hb*, *Kr,* and *run* decreased from 56 h after egg laying in *D. nigrofasciatus* short-day eggs. It is noteworthy that suppression of *cad* and *pum* occurs earlier than the onset of developmental arrest in short-day eggs. These results suggest that developmental arrest in short-day eggs might be caused by decreased expression of these segmentation genes. Otherwise, developmental arrest may occur at the very early stage preceding *cad* and *pum* upregulation.

Further studies are needed to determine what elements precede *cad* and *pum* expression. A high concentration of sorbitol in diapause eggs of *Bombyx mori* functions as an arrestor of embryonic development (Horie et al., 2000). However, its levels do not vary between long-day and short-day eggs in *D. nigrofasciatus*, suggesting that sorbitol is not essential for their developmental arrest (Goto et al., 2008). The gypsy moth *Lymantria dispar* enters diapause as a pharate first instar larva, and an elevated level of ecdysteroids titer is a key for the induction of developmental arrest (Lee and Denlinger, 1997ab), which has also been proposed in the cricket *Allonemobius socius*

(Reynolds and Hand, 2009). In contrast, decreased ecdysteroids levels can also induce embryonic diapause as in the case of the Australian plague locust *Chortoicetes terminifera* (Gregg et al., 1987) and *L. migratoria* (Tawfik et al., 2002). It is of great interest to investigate the ecdysteroid concentration in *D. nigrofasciatus* embryos in future studies. It is also noticeable that *D. nigrofasciatus* eggs respond to the environmental temperature before they reach the cellular blastoderm stage, to change their diapause destiny. When non-diapause-destined eggs are exposed to a low-temperature pulse (10°C, 24 h) on the day of deposition (day 0), the incidence of diapause increases. The low-temperature pulse on day 1 does not increase the incidence. In contrast, when the eggs of short-day adults are exposed to a high-temperature pulse (35°C, 24 h) on day 0 or day 1, the incidence of diapause decreases (Fukumoto et al., 2006). These results indicate that maternal information can be overridden even in embryos with no organs or tissues. The temperature-sensitivity of the maternal information determining diapause destiny would be the clue to elucidate the molecular mechanisms underlying diapause entry.

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Figure legends

Fig. 1. Ontogenetic change in the relative number of nuclei in *Dianemobius nigrofasciatus* eggs. Long-day and short-day eggs were collected 3, 5, or 10 days after egg laying. Short-day eggs were also collected 20 or 30 days after egg laying. The relative numbers of nuclei in a single egg were calculated. Open and closed circles indicate long-day and short-day eggs, respectively. Data from 3-4 samples are plotted. Lines are drawn through the means. Results of two-way ANOVA are also shown (PP, photoperiod; Int., interaction). The same letters indicate no significant statistical difference among samples (Tukey–Kramer multiple comparisons, $P > 0.05$).

Fig. 2. The relative proportions of cells (mean ± SD) in G_0/G_1, S, and G_2/M phases in long-day (open circles) and short-day (closed circles) *Dianemobius nigrofasciatus* eggs. Long-day and short-day eggs were collected 1.7 (40 h), 3, 5, and 10 days after egg laying. Short-day eggs were also collected 20 and 30 days after egg laying. Data from 3-4 samples are plotted. Lines are drawn through the means. Results of two-way ANOVA are also shown (PP, photoperiod; Int., interaction). The same letters indicate no significant statistical difference among samples (Tukey–Kramer multiple comparisons, $P > 0.05$).

Fig. 3. Ontogenetic profiles of mRNA levels of cell cycle regulators (*p53*, *CycD*, *CycE*, *PCNA*; left column) and small silencing RNAs (*piwi*, *spn-E*, *AGO2*; right column) in long-day (open circles) and short-day (closed circles) eggs of *Dianemobius nigrofasciatus* (mean ± SD). Eggs were collected 12, 24, 40, 56, and 72 h after egg laying (n = 4-5). Lines are drawn through the means. The highest average value of each gene was set at 1.0. Vertical grey bars indicate the onset of developmental arrest. Results of two-way ANOVA are also shown (PP, photoperiod; Int., interaction). The same letters indicate no significant statistical difference among samples (Tukey–Kramer multiple comparisons, $P > 0.05$).

Fig. 4. Ontogenetic profiles of mRNA levels of genes involved in segmentation patterning, i.e., maternal effect genes (*cad*, and *pum*), gap genes (*hb* and *Kr*), and a pair rule gene (*runt*) in long-day (open circles) and short-day (closed circles) eggs of *Dianemobius nigrofasciatus* (mean ± SD) (n = 4-5). Lines are drawn through the means. The highest average value of each gene was set at 1.0. Vertical grey bars indicate the onset of developmental arrest. Results of two-way ANOVA are also shown (PP, photoperiod; Int., interaction). The same letters indicate no significant statistical difference among samples (Tukey–Kramer multiple comparisons, $P > 0.05$).

Fig. 5. Processes potentially involved in embryonic diapause of *Dianemobius nigrofasciatus*.

Fig. S1. The relative amount of *18S* rRNA relative to the total amount of RNA in *Dianemobius nigrofasciatus*. Open and closed circles indicate long-day and short-day eggs, respectively. mean ± SD, n = 4-5. Lines are drawn through the means. The highest average value was set at 1.0.

Table 1. Genes used in RT-qPCR analyses.

Gene	Contig ID or DDBJ ID	Length (bases)	Hit gene ID in Blast seach[a]	Descriptions	E-value[a]	% Identity[a]
18SrRNA	comp162879_c2	3216	KM853307.1	*Phaloria* sp. BYU-IGC-OR447 18S ribosomal RNA gene, partial sequence	0	89
p53	comp160048_c0	1919	XP_002422610.1	Cellular tumor antigen p53, putative [*Pediculus humanus corporis*]	6E-58	39
CycD	comp131110_c0	1721	XP_018326730.1	PREDICTED: G1/S-specific cyclin-D2-like [*Agrilus planipennis*]	4E-89	44
CycE	comp160952_c2	2949	XP_021938255.1	G1/S-specific cyclin-E [*Zootermopsis nevadensis*]	2E-90	48
PCNA	comp150704_c0	1248	XP_021934966.1	Proliferating cell nuclear antigen [*Zootermopsis nevadensis*]	5E-176	89
piwi	comp147944_c0	798	AFV31611.1	piwi-like protein, partial [*Gryllus bimaculatus*]	1E-134	68
spn-E	comp1240669_c0	229	XP_021940704.1	probable ATP-dependent RNA helicase spindle-E [*Zootermopsis nevadens*	2E-34	71
AGO2	comp148467_c0	2831	AGO85972.1	argonaute 2, partial [*Locusta migratoria*]	0	55
cad	LC279612	93	BAD51739.1	caudal [*Gryllus bimaculatus*]	4E-19	100
pum	comp162032_c0_seq10	6607	KDR20699.1	Maternal protein pumilio [*Zootermopsis nevadensis*]	0	90
hb	comp141143_c0	1223	BAD12839.1	hunchback [*Gryllus bimaculatus*]	5E-112	87
Kr	comp797386_c0	822	XP_017757074.1	PREDICTED: protein krueppel-like [*Eufriesea mexicana*]	2E-81	92
run	comp163306_c0	1642	XP_971415.2	PREDICTED: runt-related transcription factor 3 [*Tribolium castaneum*]	7E-27	72

[a]Blastn for *18S* rRNA and Blastx for others

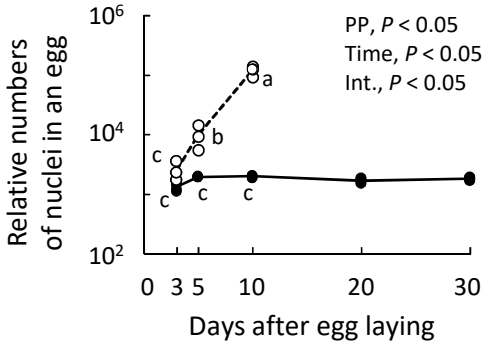
Relative numbers of nuclei in an egg
10^6
10^4
10^2
PP, $P < 0.05$
Time, $P < 0.05$
Int., $P < 0.05$
a
b
c
c
c
c
0 3 5 10 20 30
Days after egg laying

Fig. 1

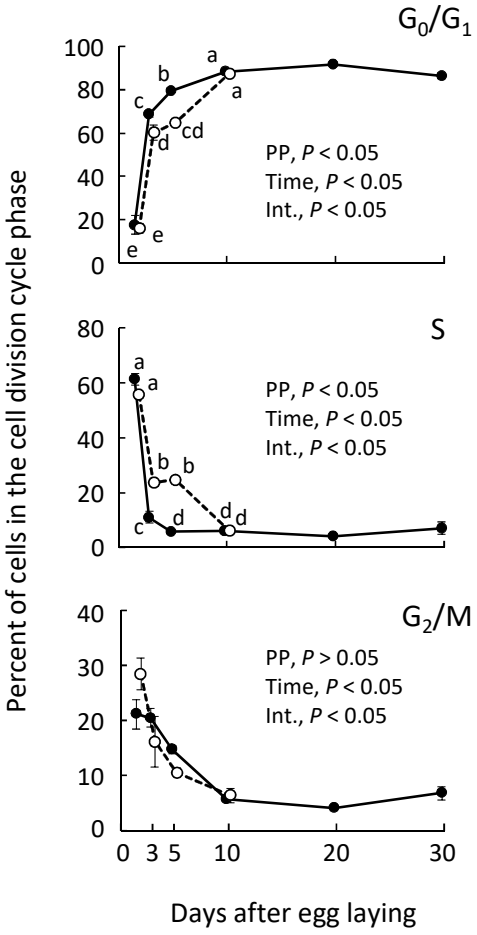
G0/G1
PP, P < 0.05
Time, P < 0.05
Int., P < 0.05
S
PP, P < 0.05
Time, P < 0.05
Int., P < 0.05
G2/M
PP, P > 0.05
Time, P < 0.05
Int., P < 0.05
Percent of cells in the cell division cycle phase
Days after egg laying
0
20
40
60
80
100
0
10
20
30
40
0 3 5 10 20 30

Fig. 2

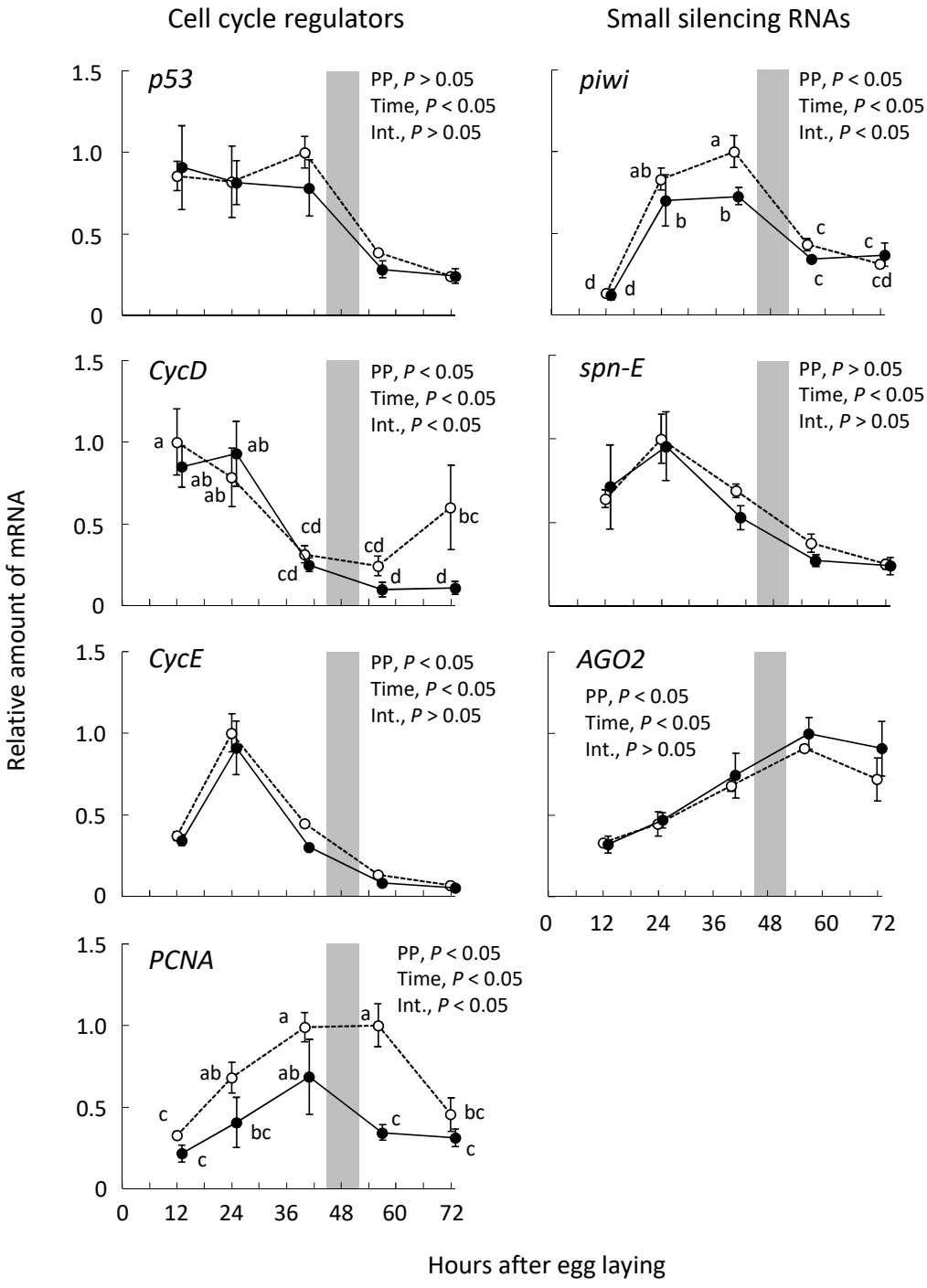
Cell cycle regulators
Small silencing RNAs
p53
PP, P > 0.05
Time, P < 0.05
Int., P > 0.05
piwi
PP, P < 0.05
Time, P < 0.05
Int., P < 0.05
CycD
PP, P < 0.05
Time, P < 0.05
Int., P < 0.05
spn-E
PP, P > 0.05
Time, P < 0.05
Int., P > 0.05
CycE
PP, P < 0.05
Time, P < 0.05
Int., P > 0.05
AGO2
PP, P < 0.05
Time, P < 0.05
Int., P > 0.05
PCNA
PP, P < 0.05
Time, P < 0.05
Int., P < 0.05
Relative amount of mRNA
Hours after egg laying
0
0.5
1.0
1.5
0
12
24
36
48
60
72

Fig. 3

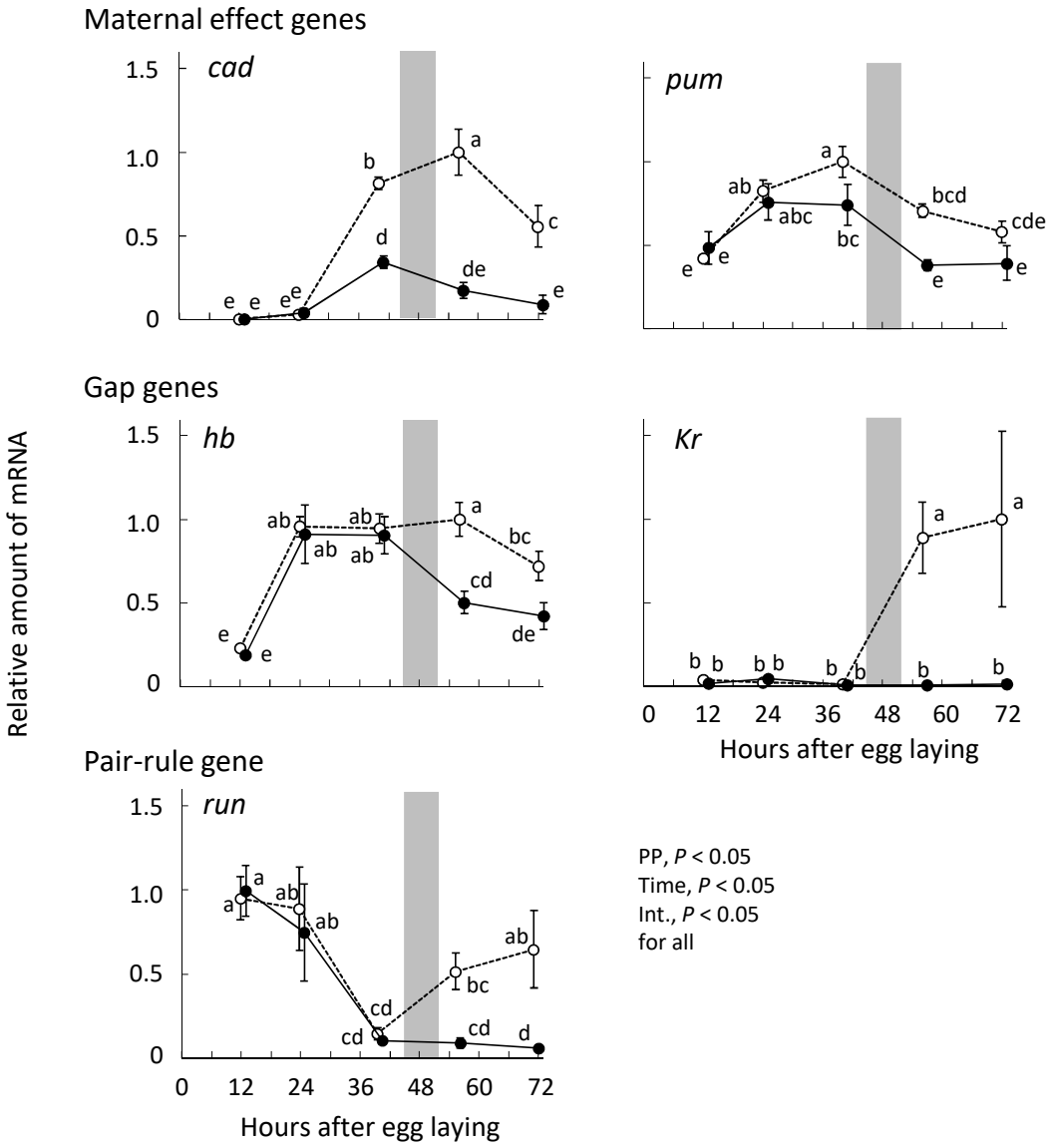
Maternal effect genes
cad
pum
Gap genes
hb
Kr
Pair-rule gene
run
Relative amount of mRNA
Hours after egg laying
PP, P < 0.05
Time, P < 0.05
Int., P < 0.05
for all

Fig. 4

Dianemobius nigrofasciatus (Orthoptera, Gryllidae)

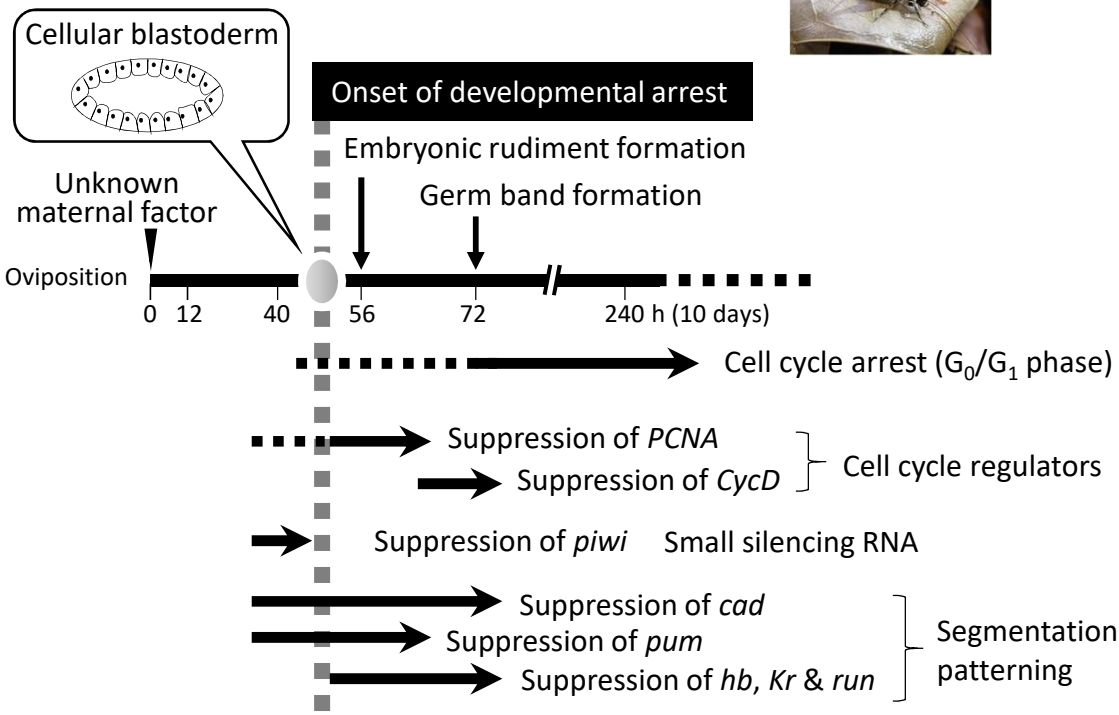

Fig. 5

Table S1. Primers, their amplification efficiency, and coefficient of determination (R^2) values of the standard curve for quantification

Gene	Direction	Sequence (5' - 3')	Efficiency	R^2
18S rRNA	Forward	AAG ACG GAC AGA AGC GAA AG	0.927	0.996
	Reverse	GGT TAG AAC TAG GGC GGT ATC T		
p53	Forward	TGT GGT TGT TCC TCT TGA TCC	1.158	0.998
	Reverse	GCC GTG TTC CGT CTA TTC AT		
CycD	Forward	GCC TAC ACA GAT CAC AGC ATA A	0.959	0.995
	Reverse	GAA ATA TGC GCT AAG ACG AAA TCC		
CycE	Forward	AAG CCA ATC AAG GAG GAT AGC	1.250	0.997
	Reverse	GTG TGC CAA GGA TGA GGA TAA		
PCNA	Forward	AAG GAA GAA GAA GCT GTC ACT G	1.152	0.996
	Reverse	GCG AAA GTG ATG TAG CCT TAG T		
piwi	Forward	CCC AGT CAA TCT ACA CAG TTC A	1.096	0.997
	Reverse	CAA CCA ACC TCT GCT TGT TTC		
spn-E	Forward	GAT CCA AAC ACA AGG CAA GAA G	1.118	0.999
	Reverse	TGT CCT CTG GAC CGA GTA AT		
AGO2	Forward	GTG AGT CGT CCT ACC AAA TAC C	1.049	0.995
	Reverse	GCA TAA TAC GTA GGA GCA GGA TAG		
cad	Forward	CAG CCG CTA CAT CAC CAT AC	1.083	0.998
	Reverse	GGT TCT GGA ACC AGA TCT TCA C		
pum	Forward	CTG CCT TTG GCT CTT CAA ATG	1.054	0.994
	Reverse	CCA TCC AGT TCT CTC ACG ATT T		
hb	Forward	GTC ACA GAG TAC AAA CAC CAT CT	0.970	0.998
	Reverse	GAC TTG TTG ACG CAG GTG TA		
Kr	Forward	TGT CCC GAA TGC CAC AAG	1.035	0.995
	Reverse	TCG CAA TGC GTA CAG TGA TAG		
run	Forward	GAT CGG CAG GGA CTT GTT	0.928	1.000
	Reverse	CAC GAA GCC CTT CAA GAG TA		

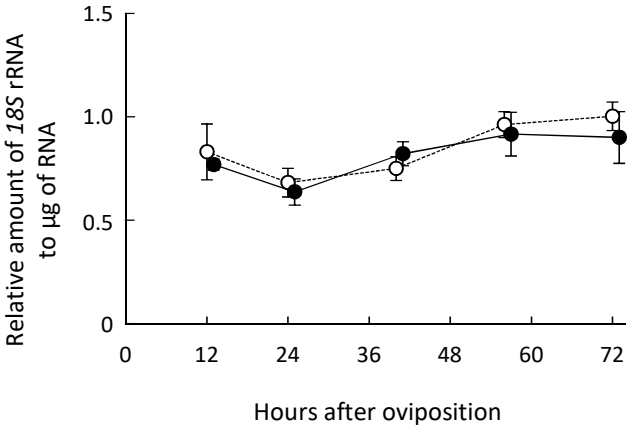
Relative amount of 18S rRNA
to µg of RNA
1.5
1.0
0.5
0
0
12
24
36
48
60
72
Hours after oviposition

Fig. S1