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Circadian clock genes link photoperiodic signals to lipid accumulation during diapause preparation in the diapause-destined female cabbage beetles *Colaphellus bowringi*

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ABSTRACT

Many organisms have evolved a series of adaptions, such as dormancy or diapause in insects that enable them to withstand seasonally adverse conditions. In insects, photoperiodic signals received during the diapause induction phase have irreversible effect on diapause initiation. Insects continue to be exposed to diapause-inducing photoperiod after the diapause induction phase during diapause preparation before they enter diapause. However, how photoperiodic signals experienced during the diapause preparation phase (DPP) regulate diapause remains largely unclear. In this paper, we investigate this in the cabbage beetle *Colaphellus bowringi*. The cabbage beetle is in many respects an ideal experimental model in which to investigate the effect of photoperiodic signals on the DPP because it has facultative reproductive diapause induced by long-day length and has differentiable diapause induction and preparation phases. We found that the lipid content of female cabbage beetles decreased after diapause-destined (DD) individuals were exposed to a diapause-inhibiting photoperiod during the DPP. Two circadian clock negative regulators, *per* and *tim*, were probably involved in the photoperiodic response of beetles during the DPP. *Per* and *tim* presented obvious oscillation of circadian rhythm and photoperiodic response during the DPP in DD females and knock-down of these genes in DD females caused their lipid content to decrease. *Per* and *tim* probably promote lipid accumulation by regulating the expression of genes that regulate lipogenesis and lipolysis. Moreover, decreased lipid accumulation caused by exposure to different photoperiods during the DPP was independent of juvenile hormone. In summary, these results suggest that photoperiodic signals received during the DPP influence lipid accumulation in DD insects. DD insects still have some ability to monitor photoperiodic changes during the DPP and *per* and *tim* are probably involved in regulating physiological responses to photoperiodic signals during diapause preparation. These results shed light on the relationship between photoperiodic signals and diapause preparation, and may provide new insights on both how to better utilize insects as resources and for pest management.

1. Introduction

To coordinate their development and physiology with annual changes in environmental conditions, organisms that inhabit temperate regions have evolved seasonal strategies, such as migration (Jenni and Kery, 2003), dormancy (seeds) (Bewley, 1997), and hibernation (mammals) (Ruf and Geiser, 2015). Many insects cope with adverse environmental condition by entering diapause (Danks, 1987). The initiation of diapause is programmed by environmental stimuli which trigger endocrine responses that lead to reduced metabolic activity, developmental arrest and high stress resistance (Denlinger, 2002). Better understanding of diapause could therefore provide important

Abbreviations: ADH, alcohol dehydrogenase NADP(+); ALDH1, retinal dehydrogenase 1; DAPI, 4',6-diamidino-2-phenylindole; DD, diapause-destined; DPP, diapause preparation phase; dsRNA, double-stranded RNA; *FABP, fatty acid binding protein*; *FAS, fatty acid synthase*; GFP, green fluorescent protein; JH, Juvenile hormone; GPO-PAP, glycerol-3-phosphate oxidase phenol + aminophenazone; *JHAMT, juvenile hormone acid methyltransferase*; *JHE, JH esterase*; *Kr-h1, Krüppel homolog 1*; *per, period*; *tim, timeless*; LD, long-day; *Met, methoprene-tolerant*; qRT-PCR, quantitative real-time PCR; RNAi, RNA interference; *RPL19, ribosomal protein L19*; SD, short-day; TG, triglyceride; *TGL1, triacylglycerol lipase 1*; *TKT2, transketolase 2*; ZT, zeitgeber time

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insights into manipulating it to improve both the utilization of insects as resources and pest management (Denlinger, 2008).

The environmental stimuli experienced in the pre-diapause phase are important for diapause initiation (Danks, 1987). Insects collect and assess environmental stimuli primarily during the diapause induction phase, which is the period in which they are most sensitive to environmental stimuli. They then begin the diapause preparation, including increasing their food intake to store energy and migrating to suitable microhabitats. These processes are part of a pre-programmed developmental destiny (Denlinger, 2002; Koštál, 2006). It has been shown that this developmental destiny is irrevocable and that changing environmental conditions during the diapause preparation phase (DPP) does not prevent many insects entering diapause. However, insects are not entirely insensitive to environmental stimuli during the DPP (Danks, 1987) and further research is required to clarify how, and to what extent, environmental stimuli regulate diapause during the DPP.

Lipid accumulation is one of the most important physiological activities during diapause preparation. Accumulating sufficient lipids is essential for insects to successfully complete diapause (Hahn and Denlinger, 2007). Diapause-inducing environmental stimuli received by diapause-destined (DD) individuals during the DPP probably influence the lipid accumulation. Photoperiod is a reliable environmental trigger for insect facultative diapause (Danks, 1987). It has been suggested that the trigger effect of photoperiod on diapause has a circadian basis (Stehlik et al., 2008; Ikeno et al., 2010; Meuti et al., 2015). However, the circadian clock genes can also participate in facultative diapause regulation in a manner distinct from their clock function (Bradshaw and Holzapfel, 2010; Bajgar et al., 2013a, 2013b). *Period* (*per*) and *timeless* (*tim*) are the core negative regulators of the insect circadian clock (Lee et al., 1999) and are known to promote the occurrence of diapause in some insects (Stehlik et al., 2008; Ikeno et al., 2010, 2011; Meuti et al., 2015). In both vertebrates and invertebrates, *per* and *tim* are closely associated with lipid synthesis and metabolism (Grimaldi et al., 2010; Asher and Schibler, 2011; Meuti et al., 2015; Omura et al., 2016). Therefore, if the DD individuals can continue to respond to photoperiodic signal during the DPP, we hypothesize that *per* and *tim* play a role in the responses of insects to photoperiod during the DPP.

To investigate the role of photoperiodic signals and the mechanisms through which these might influence diapause preparation during the DPP, we needed a study species with distinct eco-physiological phases of diapause. The cabbage beetle *Colaphellus bowringi,* a serious pest of cruciferous crops, is widely distributed in mountainous areas of China (Xue et al., 2002). The Xiushui population of *C. bowringi* can be reliably induced to undergo facultative reproductive diapause by exposure to long-day length at 25 °C (Xue et al., 2002; Wang et al., 2004). Before diapause, beetles are continuously exposed to the diapause-inducing photoperiod. In the larval stage, which is the developmental stage most sensitive to photoperiod, individuals pre-program their developmental destiny according to the photoperiodic signals they received (Xue et al., 2002; Wang et al., 2004). The circadian clock genes *per* and *tim* appear to be involved in the reception of photoperiodic signals (Zhu et al., 2017a). DD beetles enter the DPP after eclosion. In this period, due to absence of juvenile hormone (JH) signaling, DD female *C. bowringi* arrest the development of their reproductive system, accumulate substantial lipid reserves and burrow into the soil after having fed intensively for 4 days (Xue et al., 2002; Tan et al., 2016; Liu et al., 2016, 2017). However, whether diapause preparation can be affected by exposure to a diapause-inducing photoperiod during the DPP is unknown. To investigate this question, we analyzed the effect of photoperiod on the developmental destiny, lipid accumulation, and expression of JH-related genes during DPP in DD female *C. bowringi*. Then daily oscillations and responses to photoperiodic transformation during DPP were performed to investigate the roles of *per* and *tim* in the photoperiodic response during this phase. To further investigate the roles of *per* and *tim* we knocked down these genes and measured the expression levels of genes associated with lipid accumulation. Our results highlight the influence of photoperiodic signals during the DPP on insect diapause and provide new insights that may benefit both the utilization of insect resources and pest management.

2. Materials and methods

2.1. Insects

Our *C. bowringi* colony was founded from nearly 1000 beetles collected in Xiushui County (29°10' N, 114°40' E), Jiangxi Province, China in 2015. Offspring of beetles that terminated diapause in 2017 were used in this study. These beetles were fed radish leaves (*Raphanus stativus var. longipinnatus*) and kept at 25 °C and 70% RH in an incubator (HP-250-GS; Wuhan Ruihua Instrument & Equipment, Hubei, China) (Xue et al., 2002). DD adults were obtained by consistently rearing insects at 25 °C under a photoperiod of 16 L: 8D. Newly emerged DD adults fed intensively for 4 days during diapause preparation at 25 °C after which they burrowed into the soil to commence diapause (Wang et al., 2004; Tan et al., 2016). Newly emerged DD (day 0) females were used in all experiments.

2.2. cDNA synthesis and qRT-PCR

qRT-PCR was performed according to our published protocol (Liu et al., 2016). Briefly, total RNA was isolated from different treatments, each with three replicates, using Trizol (Takara, D9108A, Japan). One μg of total RNA was used for cDNA synthesis as the PrimeScript RT reagent Kit with gDNA Eraser (Takara, DRR047A, Japan) instructions. qRT-PCR was then performed with the corresponding primers (Table S1) and SYBR Premix Ex Taq II (Takara, DRR081A, Japan) using an ABI Quant Studio6. *Rpl19* and *actin1* were used as endogenous reference genes (Tan et al., 2015). Each qRT-PCR reaction was replicated three times. The $2^{-\Delta\Delta CT}$ method was used to analyze qRT-PCR data (Livak and Schmittgen, 2001).

2.3. Experimental procedures

2.3.1. Investigating the development destiny and lipid accumulation after photoperiodic transformation during DPP in DD female C. bowringi

Reproductive diapause can be induced in *C. bowringi* by a 16 L: 8D photoperiod and inhibited by a 12 L: 12D photoperiod since the beetles hatched out. To determine whether photoperiodic signals during the DPP affect females' diapause preparation we exposed DD female *C. bowringi* to diapause-inhibiting photoperiod and compared the proportion of females that subsequently burrowed into the soil, ovarian development and lipid deposition between treatment groups. A schematic description of our experimental design is shown in Fig. 1. Larvae were randomly assigned to three photoperiod treatment groups and each treatment group contained three replicates; a short-day (SD; 12 L: 12D, diapause-inhibiting) treatment group, a long-day (LD; 16 L: 8D, diapause-inducing) treatment group, and a long-day + short-day (LD-SD) treatment group in which larvae and pupae were kept under a diapause-inducing 16 L: 8D photoperiod but newly emerged females were switched to a diapause-inhibiting 12 L: 12D photoperiod. LD treatment group and SD treatment group served as controls. Ovarian development and burrowing behavior were regarded as diagnostic indicators of preparation for diapause and were respectively recorded on the 4th and 7th day after eclosion (Xue et al., 2002; Wang et al., 2004). The degree of ovarian development was assessed from the length and width of the ovaries, which was measured with ImageJ2x software (National Institutes of Health, USA). The amount of accumulated lipid, including intracellular lipid droplets and triglyceride (TG), were measured on the 4th day after eclosion. TG content was estimated using a glycerol-3-phosphate oxidase phenol + aminophenazone (GPO-PAP) system according to our previous protocol (Tan et al., 2016). The prepared solutions were added to 96-well plates and compared using a microplate

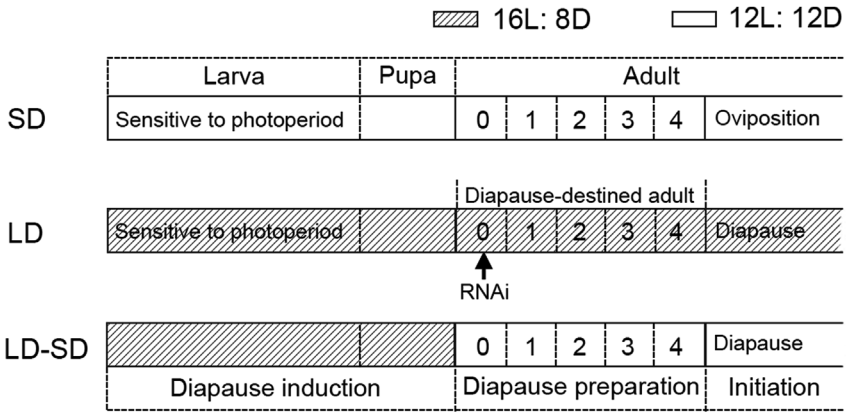

Fig. 1. Schematic depiction of experimental design. *C. bowringi* were randomly assigned to one of three different photoperiod treatments; a short-day (SD, 12 L: 12D, diapause-inhibiting photoperiod) treatment, a long-day (LD, 16 L: 8D, diapause-inducing photoperiod) treatment and a combined LD-SD treatment in which larvae and pupae were kept under the LD photoperiod but newly emerged females were kept under the SD photoperiod. Female beetles kept under the SD photoperiod did not enter the diapause preparation phase after eclosion but became sexually mature, reproductive adults. Female beetles kept under the LD photoperiod entered the diapause preparation phase after eclosion, feeding intensively for 4 days before burrowing into soil to begin diapause. Each segment of the diapause preparation phase indicates a single day within this 4-day period. Day 0 was the day on which a female emerged. RNAi was administered to newly emerged (day 0) females to knockdown the clock genes *per* and *tim*. White and hatched frames indicate diapause-inhibiting (12 L: 12D), and diapause-inducing (16 L: 8D), photoperiods, respectively.

absorbance reader (Tecan V2.2, Switzerland) under A_{510}. Intracellular lipid droplets were visualized by staining female beetle fat bodies (FBs) with Nile red (Sigma, 19123, USA) (Tian et al., 2011; Wang et al., 2017). FBs were washed two times with 1 × PBS and then fixed in 4% paraformaldehyde for 30 min at room temperature. After two further washings with 1 × PBS, FBs were incubated for 90min in 1 μg/mL Nile red at room temperature for lipid droplet staining. For nucleus staining, FBs were incubated for 15min in 1 μg/mL DAPI (Sigma, D8417, USA) at room temperature after two washings with 1 × PBS. Finally, FBs were washed twice more with 1 × PBS and then mounted on microslides and examined under a Leica TCS SP8. The intensity of fluorescence of the intracellular lipid droplets was measured with ImageJ2x software.

2.3.2. Exploring the role of JH signaling in the photoperiodic response during the DPP

Low JH levels are believed to be the primary cause of reproductive diapause (Danks, 1987; Denlinger, 2002). To determine whether different photoperiods during DPP can regulate diapause phenotypes through JH signaling we measured and compared the expression levels of genes related to JH biosynthesis and signaling in all three treatment groups. The expression level of *juvenile hormone acid methyltransferase* (*JHAMT1*, the core gene in the JH synthesis process) (Shinoda and Itoyama, 2003) was measured in beetles' heads and the expression levels of *Krüppel homolog 1* (*Kr-h1*, a marker gene for JH signaling) (Kayukawa et al., 2012), *JH esterase* (*JHE*) *1* and *JHE2* (both of which can be induced by JH signaling in *C. bowringi*) (Zhu et al., 2017b) were measured in the fat body. It has been reported that JH can regulate downstream events through a JH receptor methoprene-tolerant (Met) (Konopova and Jindra, 2007; Smykal et al., 2014; Jindra et al., 2015). Hence, to examine function of JH signaling in lipid accumulation, we injected dsRNA against *Met* to block JH signaling and then measured the level of lipid accumulation on day 4 of the DPP in the DD females with or without photoperiodic transformation.

2.3.3. Investigating the function of per and tim in lipid accumulation

To determine the role of *per* and *tim* in lipid accumulation, the 24 h rhythmic oscillations and expression levels after photoperiodic transformation during DPP of these genes were investigated. Entire 4 day-old females from the LD and SD groups were collected at zeitgeber times (ZT) 0 (light on), ZT 4, ZT 8, ZT 12, ZT 16 (light off), ZT 20, and ZT 24, and the heads, fat bodies, ovaries and midguts of 4-day-old LD and LD-SD females were collected at ZT 4. The expression levels of *per* and *tim* in these different samples was determined with qRT-PCR. To verify the function of *per* and *tim* in lipid accumulation during the DPP, RNA interference (RNAi) was used to suppress the expression of these genes in newly emerged DD females. This was done using two independent fragments of double-stranded RNA (dsRNA) to knock down the *per* (iPer1 and iPer2) and *tim* (iTim1 and iTim2) genes. dsRNA against *green fluorescent protein* (dsGFP) was the control (iGFP). The primers used for dsRNA synthesis are shown in Table S1 dsRNA was synthesized from 1 μg DNA template using a T7 transcription kit (Fermentas, Lithuania). Two μg of dsRNA in 200 nL were injected into each newly emerged DD female adult. Total RNA was then extracted from the head and fat body of these individuals after 4 days to determine the effectiveness of gene knockdown and the diapause status and lipid accumulation of these females as quantified. Ovarian development and the proportion of females that burrowed into soil were recorded on the 4th and 7th day, respectively, after eclosion. Intracellular lipid droplets and TG content were measured on the 4th day after eclosion. To identify downstream lipid regulated genes that could respond to the photoperiod during the DPP, the expression levels of various candidate genes were detected in the fat bodies of females in the LD and LD-SD treatments. Lipid regulated genes that responded to photoperiod during DPP were also identified in the fat bodies of females in the iGFP, iPer1 and iTim1 treatments.

2.4. Statistical analysis

Statistical analyses were conducted in SPSS 22 (IBM SPSS Statistics, USA). Results are presented as means ± standard deviation. Levene's tests were used to confirm homogeneity of variance and nonparametric tests (Kolmogorov-Smirnov test, K−S test) to confirm normality. The statistical significance of differences in diapause status, lipid accumulation and expression levels of JH-related genes among treatment groups was analyzed using one-way ANOVA followed by Tukey's LSD tests ($\alpha = 0.05$). Independent-Samples *t*-tests were used to assess the significance of differences between experimental groups in other experiments (*P < 0.05, **P < 0.01). ANOVAs followed by Bonferroni's tests to analyze the statistical significance of between-group differences in the expression of *per* and *tim* over 24 h (Goto and Denlinger, 2002; Zhu et al., 2017a).

3. Results

3.1. Photoperiod during the DPP affects lipid accumulation in DD female C. bowringi

It has been suggested that the developmental destiny of insects is preprogrammed before they enter the DPP (Koštál, 2006). However, DD individuals remain exposed to, and potentially influenced by, the diapause-inducing photoperiod during the DPP. To clarify the importance of continued exposure to a diapause-inducing photoperiod for diapause preparation, we investigated the effects of photoperiod on ovarian development, the proportion of females that burrowed into soil to begin diapause, and lipid accumulation, during the DPP. SD females had abundant yolk deposition whereas LD and LD-SD females had none

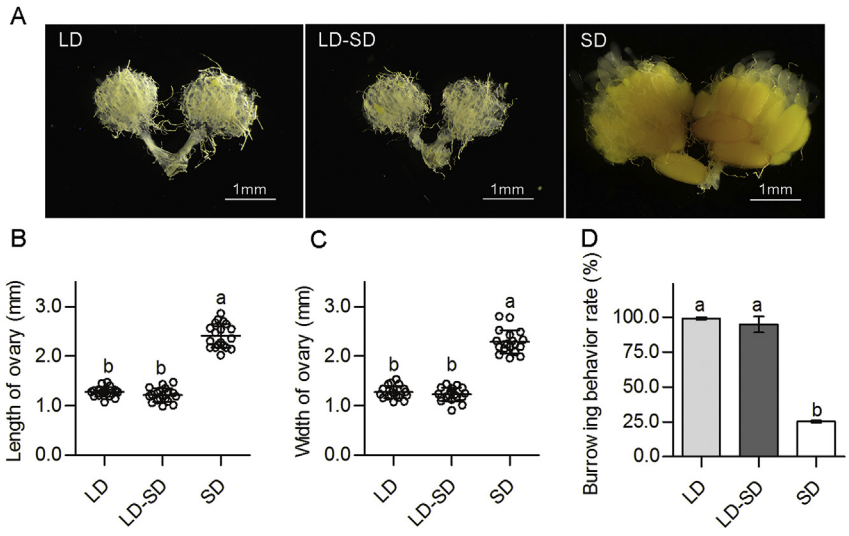

Fig. 2. Transforming the photoperiod during *C. bowringi* DPP does not change diapause destiny. Effect of exposing female *C. bowringi* to either a continuous diapause-inhibiting photoperiod (SD), continuous diapause-inducing photoperiod (LD), or a combined LD-SD photoperiod in which larvae and pupae were kept under the LD photoperiod but newly emerged DD females were kept under the SD photoperiod, on (A) yolk deposition, ovary length (B), ovary widths (C) and (D) the proportion of females that subsequently burrowed into soil to commence diapause. Values in B and C are means ± standard deviation (n = 19). Values in D are means ± standard deviation of three independent biological replicates (one-way ANOVA followed by LSD test, α = 0.05).

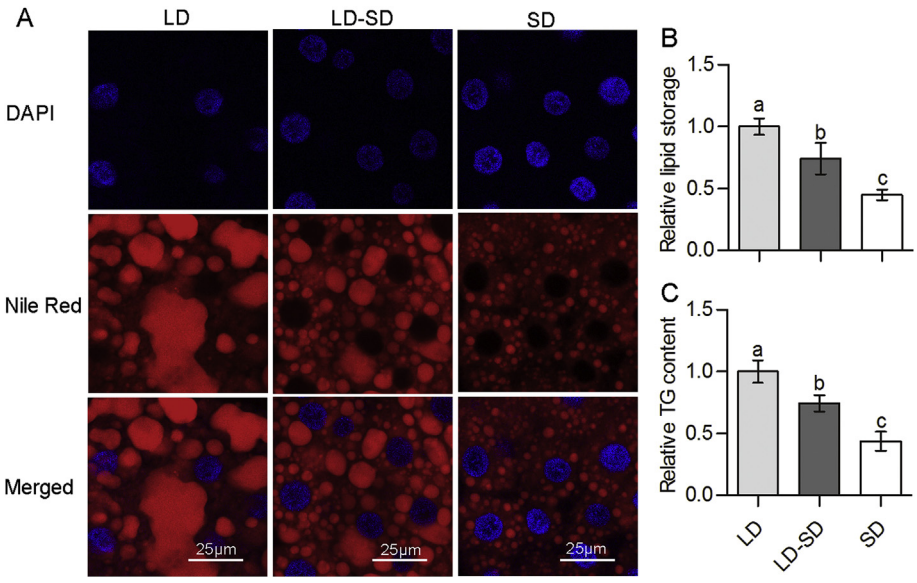

Fig. 3. The lipid accumulation of DD female *C. bowringi* decreased after photoperiodic transformation during DPP. (A) Size of intracellular lipid droplets, (B) relative intensity of fluorescence of lipid droplets after Nile red staining, (C) relative TG content. All data were collected on day 4 of the DPP (see Fig. 1 for a schematic depiction of the experimental design). Cell nuclei were stained by DAPI and intracellular lipid droplets were stained by Nile red. All values in B-C are means ± standard deviation measured from three independent biological replicates. Different letters above bars indicate significant differences determined by a one-way ANOVA followed by Tukey's LSD test (α = 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Fig. 2A). There was no difference in the ovarian length and width (Fig. 2B and C), or the rate of burrowing behavior (Fig. 2D), between LD and LD-SD females. The results suggest that the change from a diapause-inducing to diapause-inhibiting photoperiod did not change females' diapause status, as the DD females still entered diapause maintenance phase regardless of the photoperiod condition during the DPP. However, although LD-SD females had more intracellular lipid droplets and TG content than SD females, they had markedly less than LD females (Fig. 3A–C). Therefore, although the switch to the diapause-inhibiting photoperiod during the DPP did not change females' diapause status it did affect their lipid accumulation.

3.2. The effect of photoperiod on lipid accumulation during DPP may be independent of JH signaling

It has been reported that the JH signaling inhibits lipid accumulation in *C. bowringi* (Liu et al., 2016, 2017; Tan et al., 2017b). This suggests that the reduction in lipid storage following the switch to the diapause-inhibiting photoperiod during the DPP could be related to JH signaling. To verify this, we examined the expression levels of the *JHAMT1* gene, which is involved in JH biosynthesis, and the JH signaling genes *Kr-h1*, *JHE1* and *JHE2*. The expression of all four JH-related genes was highest in the SD group (Fig. 4A), suggesting that JH levels in the LD group were downregulated by diapause-inducing photoperiod. The expression of JH-related genes the LD group was, however, not obviously different to that in the LD-SD group (Fig. 4A), indicating that the switch to the diapause-inhibiting photoperiod during the DPP did not alter JH levels. Beyond that, we blocked the JH signaling by knocking down *Met* and then measured the lipid content during the DPP (Fig. 4B and C). The results showed that the lipid content decreased when the individuals injected with dsGFP were transformed to short-day condition during DPP (Fig. 4D–F). The lipid content also decreased in individuals injected with dsMet after the beetles were transformed to short-day condition during DPP (Fig. 4D–F). Thus, the decreased lipid accumulation in the LD-SD group appears to be independent of JH-Met signaling.

3.3. Per and tim link photoperiodic signals to lipid accumulation during DPP

Two circadian clock genes, *per* and *tim*, could be involved in the regulation of photoperiod-induced diapause (Stehlik et al., 2008; Ikeno et al., 2010; Zhu et al., 2017a). To test this, we investigated the function of *per* and *tim* in DD females exposed to different photoperiods during the DPP. *Per* and *tim* were highly expressed, and underwent obvious circadian oscillation, in LD females (Fig. 5A). Both genes were expressed in the fat bodies, midguts, ovaries and heads, of LD females

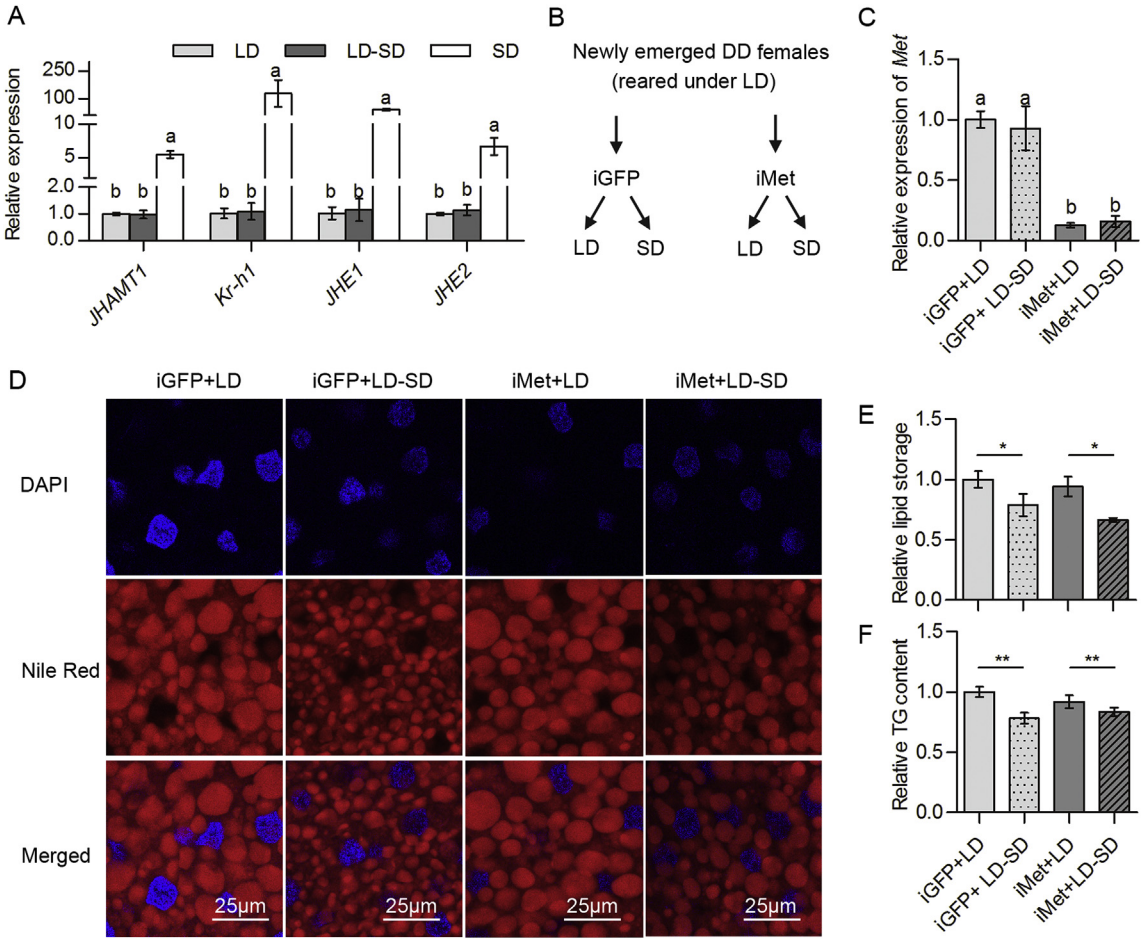

Fig. 4. The decreased lipid accumulation caused by transforming DD female to diapause-inhibiting short-day condition during DPP is independent of JH signaling. (A) The photoperiodic response of JH-related genes after photoperiodic transformation during DPP. cDNAs extracted from beetles' heads were used for detecting the expression level of *JHAMT1*, and cDNAs extracted from the fat bodies were used for detecting the expression level of *Kr-h1*, *JHE1,* and *JHE2*. (B) Schematic depiction of experimental design. We injected dsRNA against *Met* to block the JH signaling in DD females, the beetles injected with dsRNA against GFP were served as control. Then the beetles injected with dsGFP and dsMet were divided into two groups, and rearing under LD and SD condition respectively. iGFP + LD, the DD females were continuously reared under LD condition after dsGFP injection; iGFP + LD-SD, the DD females were transformed to SD condition after dsGFP injection; iMet + LD, the DD females were continuously reared under LD condition after dsMet injection; iMet + LD-SD, the DD females were transformed to SD condition after dsMet injection. (C) RNAi efficiency of knockdown of *Met* in the entire body of DD females. Effect of photoperiodic transformation on the (D) size of intracellular lipid droplets, (E) relative intensity of fluorescence of lipid droplets after Nile red staining, (F) relative TG content in the beetles injected with dsGFP and dsMet. All data were collected on day 4 of the DPP. Cell nuclei were stained by DAPI, and intracellular lipid droplets were stained by Nile red. All values are means ± standard deviation measured from three independent biological replicates. Different letters above bars indicate significant differences determined by a one-way ANOVA followed by Tukey's LSD test ($\alpha = 0.05$). Asterisks indicate significant differences determined by an Independent-Samples *t*-test (*P < 0.05, **P < 0.01). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Fig. 5B). We further investigated tissue-specific photoperiod responses in the head (where environmental information is collected), and in the fat body (where lipid is accumulated). The expression of *per* in the head was significantly lower in LD-SD females than in LD females but the expression of *tim* in the fat body was higher in LD-SD females and lower in the head (Fig. 5C). The circadian oscillation in the expression levels of these genes, and changes in their expression profiles, in the different treatment groups suggests that they may be involved in the response to photoperiod during DPP.

The LD-SD group accumulated fewer lipids than the LD group and differed in *per* and *tim* expression in the head and fat body. To verify whether this difference in *per* and *tim* expression could be responsible for the reduced lipid accumulation in the LD-SD group, we knocked down *per* and *tim* in newly emerged DD female beetles and compared the amount of lipid these accumulated to that in control females treated with iGFP. The injection of two independent dsRNAs against *per* or *tim* significantly reduced the expression of these genes relative to the control (Fig. 6A) and lipid accumulation (Fig. 7), but had no significant effect on ovarian development or the proportion of females that burrowed into soil (Fig. 6C–E).

The fat bodies of dsRNA-treated females became detached, whereas those of iGFP females were clumped and hypertrophic (Fig. 7A). After 4 days, the abundance of intracellular lipid droplets and the TG content were significantly less in females in which *per* and *tim* had been knocked down than in the iGFP group (Fig. 7A–C). We conclude that *per* and *tim* play a key role in lipid accumulation in response to photoperiodic signals during the DPP in *C. bowringi*.

3.4. *Per and tim regulate lipid accumulation by mediating the expression of genes associated with fatty acid synthesis and lipolysis during the DPP*

To understand how *per* and *tim* regulate lipid accumulation, we attempted to identify genes that mediate fatty acid synthesis and fat degradation during the DPP. *Fatty acid binging-protein* (*FABP*) is a key gene involved in fatty acid transport, whereas *alcohol dehydrogenase NADP* (+) (*ADH*), *retinal dehydrogenase 1* (*ALDH1*), *fatty acid synthase*

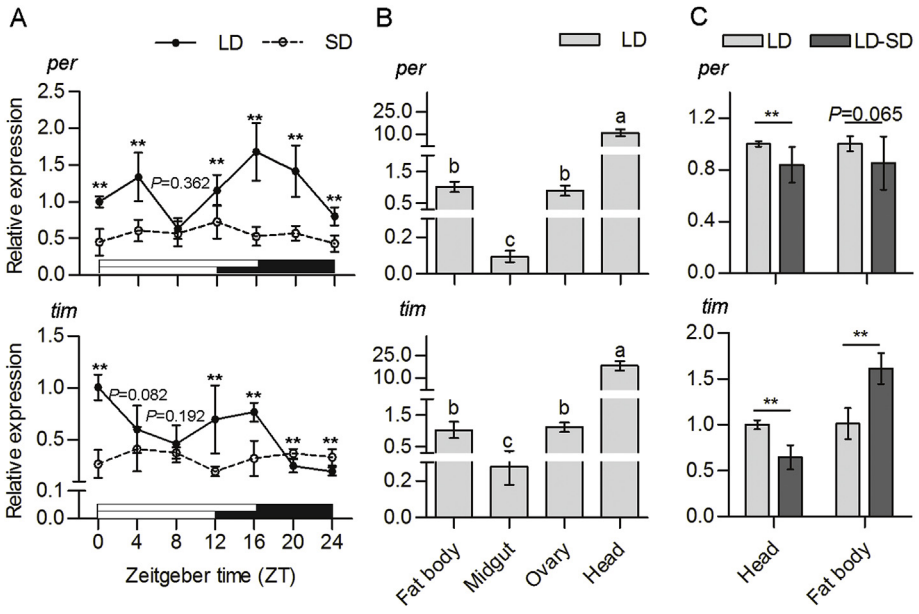

Fig. 5. *Per* and *tim* respond to photoperiodic signal during DPP in DD female *C. bowringi*. (A) Expression of *per* and *tim* in the entire body over 24-h (white and black bars indicate the duration of the photophase and scotophase, respectively), (B) *per* and *tim* transcript abundance in different organs of LD females (C) comparison of *per* and *tim* expression in the head and fat body of the LD and LD-SD treatment groups. All data were collected on day 4 of the DPP. Expression levels of B and C were measured at Zeitgeber time 4. All values are means ± standard deviation measured from three independent biological replicates. Different letters above bars indicate significant between-group differences determined by one-way ANOVA followed by Tukey's LSD test ($\alpha = 0.05$). Asterisks indicate significant differences determined by an Independent-Samples *t*-test (*P < 0.05, **P < 0.01).

(*FAS*) *1*, *FAS2*, and *transketolase 2* (*TKT2*) are key genes involved in fatty acid synthesis in both vertebrates and invertebrates (Ziouzenkova et al., 2007; Arrese and Soulages, 2010; Majerowicz and Gondim, 2013; Liu et al., 2017). The expression levels of *FABP* and *ADH* were significantly higher in the LD group relative to the LD-SD group (Fig. 8A) whereas that of *triacylglycerol lipase 1* (*TGL1*), a gene facilitates lipolysis, was significantly less (Fig. 8A). This leads us to suspect that *ADH*, *FABP*, and *TGL1* are involved in photoperiod-mediated changes in lipid accumulation during the DPP and that these genes are regulated by *per* and *tim*. Indeed, knockdown of both *per* and *tim* decreased *ADH* and *FABP* expression, whereas knockdown of *tim* significantly increased *TGL1* expression (Fig. 8B). This suggests that *per* and *tim* promote lipid accumulation by facilitating fatty acid transport, and that *tim* may also promote lipid accumulation by upregulating fatty acid synthesis and downregulating lipolysis.

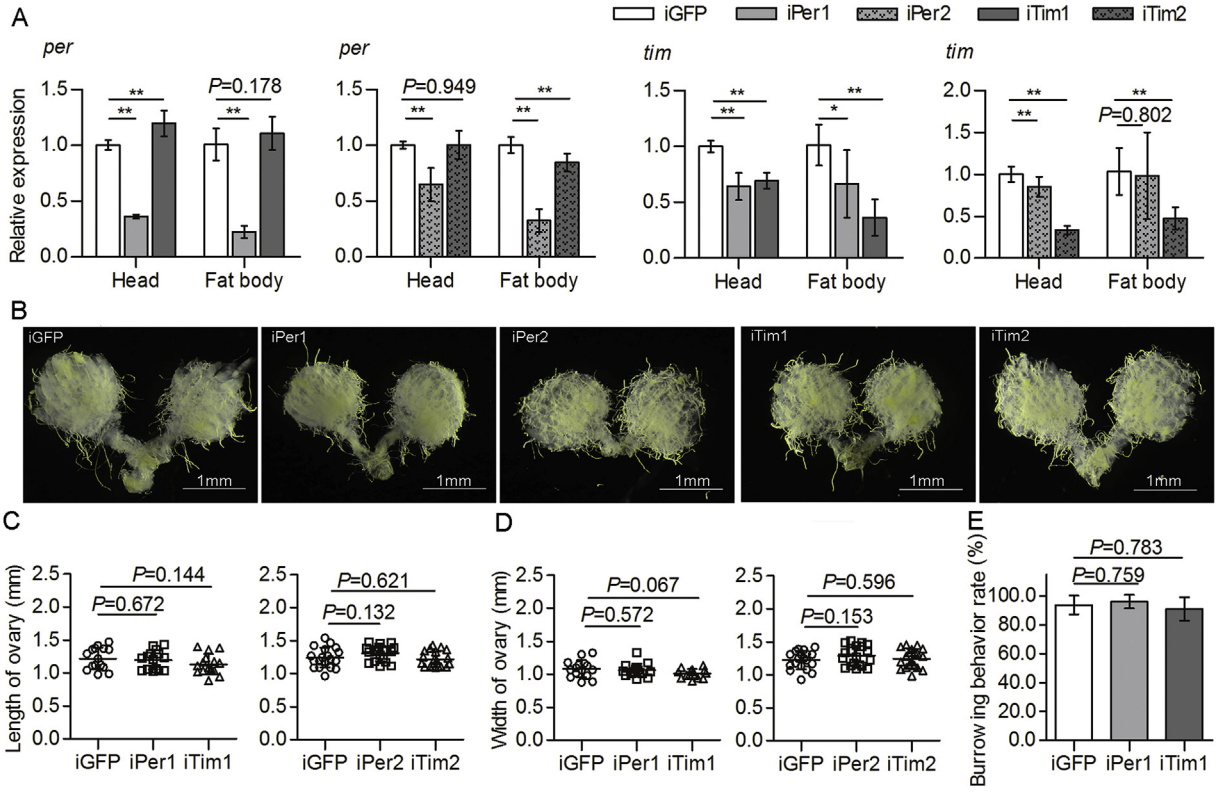

Fig. 6. Knocking down *per* or *tim* does not change the diapause destiny of DD female *C. bowringi*. Effect of knockdown of *per* and *tim* in female *C. bowringi* on (A) *per* and *tim* expression relative to the control (iGFP), (B) ovarian development, (C) ovary length, (D) ovary width and (E) the proportion of females that had burrowed into soil to begin diapause 7 days after the dsRNA treatment. The ovary lengths and widths of iPer1 and iTim1 are means ± standard deviation for 15 females. The ovary lengths and widths of iPer2 and iTim2 are means ± standard deviation for 19 females. Values in E are means ± standard deviation, $n_{iGFP} = 35, 26, 31$, $n_{iPer} = 37, 33, 26$, $n_{iTim} = 33, 33, 35$).*P < 0.05, **P < 0.01 (Independent-Samples *t*-test).

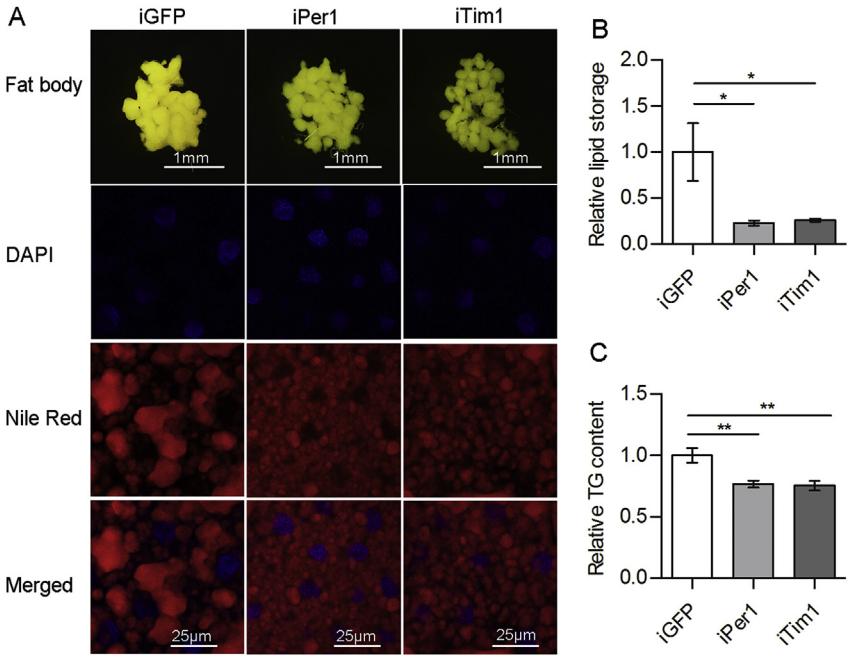

Fig. 7. Roles of *per* and *tim* in lipid accumulation during DPP in DD female *C. bowringi*. Effect of knockdown of *per* and *tim* in female *C. bowringi* on lipid accumulation during the diapause preparation phase 4 days after dsRNA injection on, (A) Fat body morphology (Row 1) and intracellular lipid droplets observed after Nile red staining (Rows 2–4) photographed under a scanning laser confocal microscope, (B) the relative intensity of fluorescence of lipid droplets after Nile red staining (three independent biological replicates measured) and (C) TG content (three independent biological replicates comprised of five individuals were measured). Values are means ± standard deviation. *P < 0.05, **P < 0.01 (Independent-Samples *t*-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

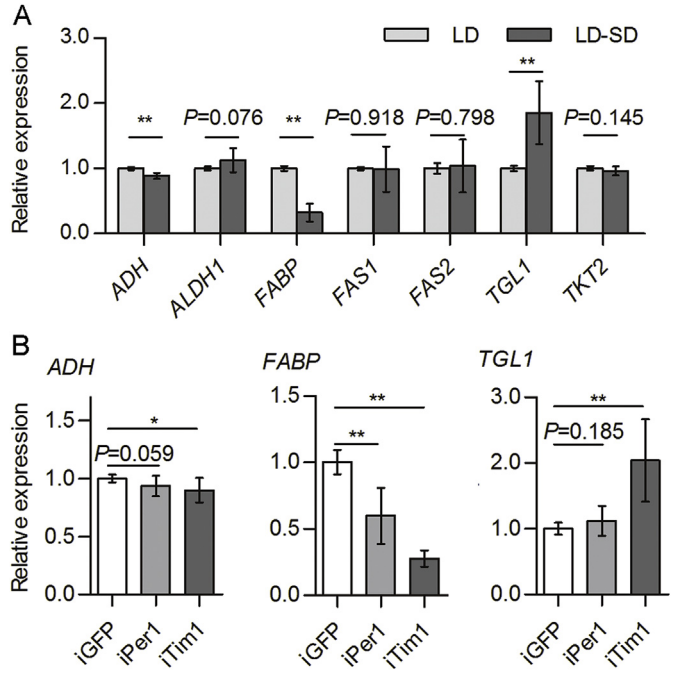

Fig. 8. Knocking down *per* and *tim* affects the expressions of the genes related to lipogenesis and lipolysis. (A) Comparison of post-knockdown expression of genes related to lipid metabolism in the fat body of female adults that had been kept under a long-day length (LD) with those kept under a combined LD-SD photoperiod (larvae and pupae kept under the LD photoperiod but newly emerged females kept under the SD photoperiod). (B) Effect of *per* and *tim* knockdown on the expression of *ADH, FABP* and *TGL1* in the fat body. Values are means ± standard deviation of three independent biological replicates. *P < 0.05, **P < 0.01 (Independent-Samples *t*-test).

4. Discussion

The pre-diapause phase is comprised of the diapause induction and preparation phases (Koštál, 2006). Since insects are most sensitive to photoperiodic signals during the diapause induction phase, most research on photoperiodic response mechanisms has focused on that phase (Saunders et al., 2004; Ikeno et al., 2010; Koštál et al., 2017). Consequently, very little is known about the extent to which DD insects are affected by photoperiod during the DPP. We investigated this in the cabbage beetle *C. bowringi*, a facultative, reproductive diapause insect with differentiable diapause induction and preparation phases. Interestingly, we found that DD females exposed to a diapause-inhibiting photoperiod during the DPP accumulated fewer lipids than those that were continually kept under a diapause-inducing photoperiod. Our results suggest that exposure to a diapause-inhibiting photoperiod during the DPP inhibits lipid accumulation by inhibiting the expression of *per* and *tim*, rather than by JH signaling. This suggests that continuous exposure to a diapause-inducing photoperiod is important for normal diapause preparation in *C. bowringi*, and that *per* and *tim* mediate lipid accumulation in response to photoperiodic signals during the DPP.

Photoperiod plays an important role in the regulation of insect diapause. The appropriate photoperiod can determine the developmental destiny of insects during the diapause induction and diapause termination phase (Danks, 1987; Koštál, 2006; Koštál et al., 2017). Although insects normally continue to be exposed to the diapause-inducing photoperiod during the DPP (Denlinger, 2002; Koštál, 2006), few studies have investigated the effects of photoperiod during this period. We found that DD female *C. bowringi* continued to respond to photoperiod during the DPP and that exposing these to a diapause-inhibiting photoperiod prevented them accumulating the same amount of lipid reserves as females kept under a diapause-inducing photoperiod. These findings provide a link between photoperiodic signals and energy reserve accumulation during the DPP. Xu et al. (2012) and Poelchau et al. (2013) suggested that there could be an interaction between energy reserves and diapause regulation. Since the photoperiodic signal received during the diapause induction phase is irrevocable, the DD females only decreased the lipid accumulation but still entered the diapause maintenance phase when they were exposed to diapause-inhibiting photoperiod during DPP. Evidence has shown that insufficient energy reserves during the DPP may lead to premature termination of diapause or even death (Hahn and Denlinger, 2007, 2011). It is therefore possible that continued exposure to diapause-inducing photoperiods during the DPP is essential for female *C. bowringi* to accumulate sufficient fat to complete diapause successfully. However, high individual variation in duration of the diapause maintenance phase in *C. bowringi* (Wang et al., 2006) makes this hypothesis difficult to test.

Determining the effect of photoperiod on the diapause maintenance period should therefore be investigated in an insect species with less individual variation in the duration of the diapause maintenance period.

JH, which is secreted by the corpus allatum, is considered an essential hormone regulating reproductive diapause (Smykal et al., 2014) and its absence is a hallmark of reproductive diapause (Denlinger, 2002). With the discovery of *Met*, an intracellular JH receptor (Charles et al., 2011; Jindra et al., 2015), more studies suggest that JH-Met signaling plays an important role in the lipid metabolism of insects (Baumann et al., 2013; Wang et al., 2017). In *C. bowringi*, it has been demonstrated that JH can inhibit both lipid accumulation and the expression of *FAS1*, *FAS2* and *ALDH1* during diapause preparation through *Met* (Liu et al., 2016, 2017; Tan et al., 2017b). We consequently attempted to find a link between lipid accumulation induced by exposure to a diapause-inducing photoperiod and JH signaling during the DPP. However, we found that the expression levels of genes related to JH biosynthesis and action were not affected by photoperiod during the DPP. The reduced lipid storage caused by transforming DD females from LD to SD during DPP was not changed by the knockdown of *Met*. This suggests that photoperiod regulates lipid accumulation independently of JH signaling during this phase. It also suggests that abundant lipid accumulation in diapausing adult bean bug *Riptortus pedestris* may not have been caused by inactivity of the corpus allatum (Morita et al., 1999), and implies that a diapause-inducing photoperiod can promote lipid accumulation by other pathways (Morita et al., 1999; Omura et al., 2016). In both invertebrates and vertebrates, 20-hydroxyecdysone signaling (Sieber and Spradling, 2015; Cara and King-Jones, 2016), insulin signaling (Dillin et al., 2002; Sim and Denlinger, 2013), and mTOR pathway (Ramanathan et al., 2018) display obvious circadian rhythms, and could potentially be involved in lipid metabolism (Hahn and Denlinger, 2011; Majerowicz and Gondim, 2013). The potential involvement of these pathways in photoperiod-related lipid metabolism during the DPP requires investigation.

Because animals use their circadian clock to assess day or night length, various authors have suggested that circadian clock genes could be involved in the perception of photoperiodic signals during diapause induction (Saunders et al., 2002; Emerson et al., 2009; Ikeno et al., 2010; Denlinger et al., 2017). We found that the circadian clock genes *per* and *tim* continuously responded to changes in photoperiod in DD female *C. bowringi*. Exposure to a diapause-inhibiting photoperiod during the DPP reduced both lipid accumulation and *per* and *tim* expression. This raises the possibility that the reduction in lipid accumulation may have been caused by the down-regulation of *per* and *tim*. A crucial link between circadian clock genes and lipid metabolism is known to exist in both vertebrates and invertebrates (Xu et al., 2008; Grimaldi et al., 2010; Meuti et al., 2015; Omura et al., 2016). In PER2-deficient mice, circadian clock genes regulate TG levels, fatty acid transport, biosynthesis and fatty acid β oxidation (Grimaldi et al., 2010). We found that *per* and *tim* knockdown changed the expression of *C. bowringi* genes involved in fatty acid transport (*FABP*), lipogenesis (*ADH*) and lipolysis (*TGL1*). Moreover, the expression of *FABP*, a gene that encodes a fatty acid transport protein (Chmurzynska, 2006), was significantly higher in females that were continuously exposed to a diapause-inducing photoperiod than in those exposed to a diapause-inhibiting photoperiod. *FABP* expression during the DPP was also significantly reduced by the knockdown of both *per* and *tim*. Reduced *FABP* expression reduced females' lipid content and caused the fat body to become detached (Tan et al., 2017a). In addition to transporting fatty acids, *FABP* can also act as a signaling molecule. In fruit fly *Drosophila melanogaster*, *FABP* plays an essential role in modulating and enhancing long-term memory (Gerstner et al., 2011). Therefore, *FABP* could be regulated by *per* and *tim*, the expression of which is in turn regulated by exposure to a diapause-inducing photoperiod during the DPP. The increase in *FABP* caused by exposure to a diapause-inducing photoperiod, and the decrease in *FABP* caused by the knockdown of *per* and *tim*, could regulate lipid accumulation in DD female insects.

Previous studies suggest that circadian clock may exert its effect on insect diapause through modular and gene pleiotropy (Emerson et al., 2009; Bradshaw and Holzapfel, 2010; Denlinger et al., 2017). It is difficult to distinguish between modular and gene pleiotropy of circadian clock genes in *C. bowringi* diapause because no obvious circadian behaviors could be currently observed in this beetle. However, our results support that the two circadian genes, *per* and *tim*, regulate lipid accumulation of *C. bowringi* during the DPP likely through gene pleiotropy. Silencing *per* and *tim* could both reduce lipid accumulation during DPP (Fig. 7), while the expression of *tim, per* and lipid-related genes were different after silencing. *per* RNAi not only reduced *per* expression but also decreased *tim* expression (Fig. 6A). However, *tim* RNAi had no considerable effect on *per* expression (Fig. 6A). Moreover, *tim* had a more significant influence on the expression of *ADH* and *TGL1* compared to *per* (Fig. 8B). Thus, we speculate that *per* and *tim* regulate lipid accumulation in a non-clock role during DPP of *C. bowringi*.

Our results indicate that the photoperiod experienced during the DPP affects lipid accumulation in DD female *C. bowringi*. This suggests that DD females continue to monitor photoperiod during the DPP and adjust their diapause preparation strategy in response to photoperiodic cues before entering diapause. We hypothesize that exposure to a diapause-inducing photoperiod during the diapause induction phase leads to low JH levels via an unknown mechanism (Fig. 9). This step determines whether an individual will subsequently enter diapause or not. Low JH levels then arrest ovarian development and induce burrowing behavior and lipid accumulation (Fig. 9). The expression of the circadian clock genes *per* and *tim* are influenced by the photoperiod experienced during the DPP (Fig. 9). Exposure to a diapausing-inducing photoperiod during this period promotes lipid accumulation by upregulating the expressions of the lipogenesis genes (*ADH* and *FABP*), and downregulating the expression of the lipolysis gene (*TGL1*). Thus, the photoperiod experienced during the DPP can promote lipid accumulation independently of JH. These results provide new information on the molecular mechanisms underlying insect diapause. In addition, the finding that DD insects continue to monitor and respond to photoperiodic signals during the DPP provides new insights for the more

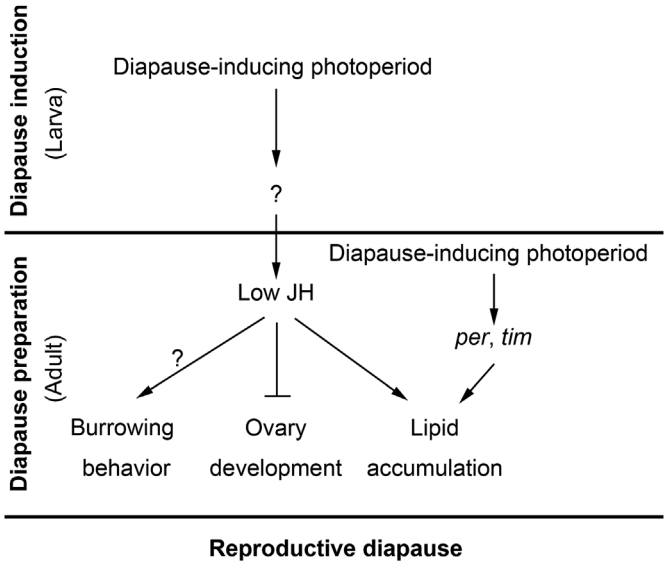

Fig. 9. Model of how photoperiod regulates lipid accumulation during diapause preparation in female *C. bowringi*. Diapause-inducing conditions (25 °C, 16 L: 8D) during the diapause induction phase induce *C. bowringi* to induced to undergo facultative reproductive diapause. However, the photoperiod experienced during the diapause preparation phase can affect lipid accumulation by altering the expression of the circadian clock genes *per* and *tim*. JH signaling is not affected by the photoperiod during the diapause preparation phase.

effective utilization of beneficial insect resources and for improving pest management.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2018.11.001.

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