Knockouts of positive and negative elements of the circadian clock disrupt photoperiodic diapause induction in the silkworm, *Bombyx mori*

Hisashi Tobita[1] and Takashi Kiuchi[1]*

[1]Department of Agricultural and Environmental Biology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

*Corresponding Author:

Takashi Kiuchi, kiuchi@g.ecc.u-tokyo.ac.jp

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Abstract

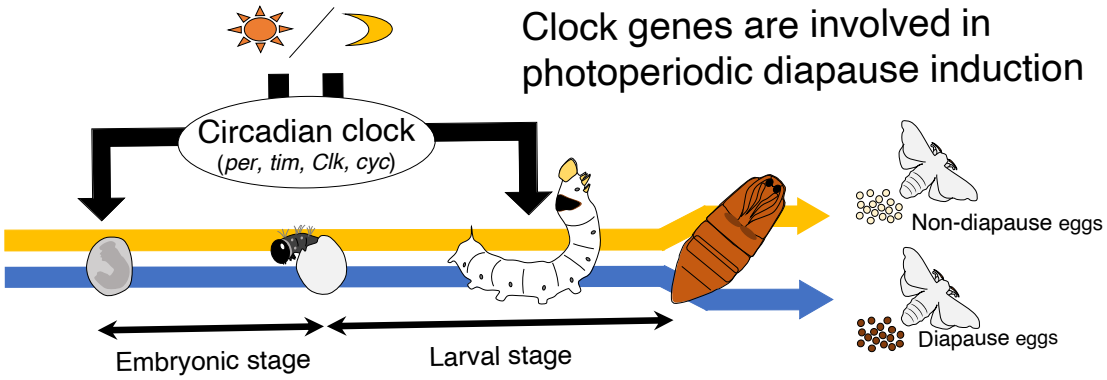
Diapause is one of the most important traits that have sustained insects to thrive. Insects can prospectively arrest their development and increase environmental stress-resistance by entering diapause. The photoperiod is the signal that indicates insects the proper timing to enter diapause. Circadian clock genes are shown to be involved in photoperiodic diapause induction in various insect species. The silkworm, *Bombyx mori*, enters diapause at the embryonic stage. In bivoltine strains, diapause determination is affected by embryonic temperature and photoperiodic conditions during embryonic and larval stages. Two independent studies showed that knocking out the core clock gene, *period*, perturb photoperiodic diapause induction. However, whether the circadian clock as whole or individual clock genes are responsible for the photoperiodic diapause induction remains unknown. In this study, using CRISPR/Cas9 we knocked out negative (*period* and *timeless*) and positive elements (*Clock* and *cycle*) in p50T, a bivoltine strain which exhibits photoperiodic diapause induction during both embryonic and larval stages. The temporal expression patterns of clock genes changed in each core clock gene knockout strain, suggesting disruption of normal feedback loops produced by circadian clock genes. Furthermore, photoperiodic diapause induction during both embryonic and larval stages was lost in all knockout strains. Our results indicate the involvement of circadian clock in photoperiodic diapause induction in *B. mori*.

Keywords: *Bombyx mori*, clock genes, CRISPR/Cas9, diapause, photoperiodism

Highlights

- We established knockout *Bombyx mori* strains of four core clock genes
- The temporal expression patterns of clock genes changed in knockout strains
- Photoperiodic diapause induction was not observed in any knockout strains

Graphical abstract



1. Introduction

Winter is a severe season for temperate-zone insects because of the cold temperature, drought, and food shortage. To overcome such a difficult season, most insects arrest their development and enter diapause, an inactive physiological state that increases environmental stress-resistance (Denlinger, 1991). Developmental life stages of diapause are diverse among insect species even within orders and sometimes even within genus, suggesting that diapause has evolved multiple times in insects (Meuti and Denlinger, 2013; Nylin, 2013). For example, in Lepidoptera, diapause is induced at the embryonic stage in the silkworm, *Bombyx mori* (Kogure, 1933), at the larval stage in the European corn borer, *Ostrinia nubilalis* (Beck and Hanec, 1960), at the pupal stage in the oak silkworm, *Antheraea pernyi* (Williams and Adkisson, 1964), and at the adult stage in the monarch butterfly, *Danaus plexippus* (Herman, 1981). While diapause stages are genetically fixed, insects can properly time diapause thanks to environmental information, such as the photoperiod and temperature (Denlinger et al., 2012). Although the endocrine control of diapause induction is well known (Denlinger et al., 2012), how insects accept those environment signals, translate the information, and timely activate the endocrine system is not fully understood.

In widely distributed insect species, there is geographical variation in the critical photoperiod (the incidence of diapause is 50% of its maximal level) for diapause induction

(Bradshaw and Holzapfel, 1975; Bradshaw and Lounibos, 1977). The critical photoperiod generally increases with latitude among populations of the same species, because of longer summer day length and earlier winter arrival in higher latitudes. Several reports indicate that the critical photoperiod variation is caused by polymorphism in circadian clock genes (Paolucci et al., 2016; Pruisscher et al., 2018; Yamada and Yamamoto, 2011). Clock genes control rhythmic gene expression through autoregulatory feedback loops producing circadian rhythms (Hardin, 2005). These autoregulatory mechanisms are well studied in the fruit fly, *Drosophila melanogaster* (Beer and Helfrich-Förster, 2020). The protein products of the clock genes, *Clock* (*Clk*) and *cycle* (*cyc*), heterodimerize with each other activating the transcription of *period* (*per*) and *timeless* (*tim*) (Allada et al., 1998; Rutila et al., 1998). Then, their protein products, PER and TIM, heterodimerize with each other, translocate to the nucleus, and suppress their own transcriptional activation by inhibiting CLK/CYC (Darlington et al., 1998). This core feedback loop produces the rhythmic expression of *per*, *tim*, and clock-controlled genes that mediate downstream signaling cascades. It is also known that other circadian clock genes, such as *clockwork orange* (*cwo*), *Par-domain protein 1* (*Pdp1*), and *vrille* (*vri*) participate in the core feedback loop producing interlocked feedback loops (Cyran et al., 2003; Glossop et al., 2003; Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007). In addition, TIM is degraded by blue-light dependent binding of the photoreceptor CRYPTOCHROME (CRY) (Ceriani et al.,

1999). Therefore, PER/TIM heterodimerization and subsequent CLK/CYC suppression only occur at night. Although the essential features are conserved among insects, the circadian clock components are slightly different (Sandrelli et al., 2008; Tomioka and Matsumoto, 2015). For example, almost all non-drosophilid insects possess *mammalian type cry* (*cry-m* or *cry2*), which has no photoreception ability, in addition to *Drosophila* type *cry* (*cry-d* or *cry1*) (Sandrelli et al., 2008; Tomioka and Matsumoto, 2015). In the monarch butterfly, *Danaus plexippu*, CRY2 functions as a negative element by forming a complex with PER and TIM to inhibit CLK/CYC-mediated transcription (Zhu et al., 2008).

Reduction or loss of core clock genes is reported to perturb normal photoperiodic diapause induction in insects (Table S1). However, it is difficult to distinguish whether the circadian clock as a whole or individual clock genes are responsible for photoperiodic diapause induction (Emerson et al., 2009). To investigate this, the effects of the loss-of-function of multiple single clock genes on photoperiodic diapause induction have been evaluated in the past. In the bean bug, *Riptortus pedestris*, the involvement of core clock genes *cry-m* (*cry-2*), *per*, *Clk,* and *cyc* in photoperiodic regulation of ovarian development (*i.e.,* reproductive diapause) was investigated with RNA interference (RNAi) (Ikeno et al., 2010, 2011, 2013). In the Northern house mosquito, *Culex pipiens*, RNAi against negative elements, *cry2*, *per*, and *tim* averted reproductive diapause phenotypes under diapause-inducing conditions. In addition, in *D. plexippus*, genetic

106 knockouts by genome editing tools revealed that *Clk*, *cyc,* and *cry2* are necessary for the
107 photoperiodic responses of oocyte maturation (Iiams et al., 2019). These reports support that
108 circadian clock feedback loops participate in photoperiodic diapause induction in various insect
109 orders. However, since diapause has evolved multiple times in insects, it is noteworthy to
110 examine whether the involvement of circadian clock genes in diapause induction is widely
111 conserved using insects that enter diapause in different stages.

112 The embryonic diapause of *B. mori* is induced by a maternal diapause hormone,
113 synthesized in the suboesophageal ganglion and secreted from the corpus cardiacum into the
114 hemolymph during the pupal stage(Sato et al., 1998; Takeda and Ogura, 1976). In bivoltine
115 strains, its secretion is determined by environmental conditions, such as temperature and
116 photoperiod. Temperatures >21°C during the late embryonic stage are sensed by a
117 thermoreceptor, transient receptor potential ankyrin 1 (TRPA1), which suppresses diapause
118 hormone secretion (Sato et al., 2014). It is unclear how the “thermal-memory” during the late
119 embryonic stage suppresses diapause hormone secretion during the pupal stage, but the cerebral
120 γ-aminobutyric acid (GABA) ergic and corazonin pathways modulate diapause hormone release
121 via temperature-dependent expression of a plasma membrane GABA transporter (Tsuchiya et al.,
122 2020). Diapause induction in *B. mori* is also affected by the photoperiod during the late
123 embryonic and late larval stages. Diapause induction is promoted by long-day conditions during

124 the late embryonic stage and short-day conditions during the late larval stage (Kogure, 1933).
125 Recently, *per* and *tim* knockout mutants were generated using the genome editing tools TALENs
126 and CRISPR/Cas9, respectively (Ikeda et al., 2019; Nartey et al., 2021). Both mutants lost
127 eclosion and hatching rhythms, indicating that core clock genes are indispensable for circadian
128 rhythms in *B. mori*. In addition, two groups independently described *per's* involvement in
129 photoperiodic diapause induction (Cui et al., 2021; Ikeda et al., 2021). Ikeda et al. demonstrated
130 that *per* knockout in the Kosetsu strain abolishes photoperiod sensitivity during the larval stage
131 and inhibits diapause egg production. Cui et al. (2021) showed knocking out *per* in the Dazao
132 strain attenuates the effects of temperature and photoperiod on diapause induction during the
133 embryonic stage thorough GABAergic signals. Since both studies only focused on a single
134 negative element, it is difficult to conclude whether clock genes control photoperiodic diapause
135 induction as a unit. In this study, we performed CRISPR/Cas9-mediated gene knockouts of both
136 negative, *per* and *tim*, and positive elements, *Clk* and *cyc*, using a bivoltine strain and
137 investigated the effects on photoperiodic diapause induction at both embryonic and larval
138 stages.
139
140 **2. Materials and methods**
141 *2.1. Silkworm strains*

142 A bivoltine inbred strain p50T (Daizo) was used in this study. The strain was maintained in
143 our laboratory with mulberry leaves or artificial diet SilkMate PS (NOSAN) under a daily 12 h
144 light:12 h dark (12L12D) cycle at 25℃.
145
146 *2.2. CRISPR/Cas9-mediated gene knockouts*
147 CRISPR/Cas9 was used to knockout *B. mori* core clock genes. A unique single guide RNA
148 (sgRNA) was designed for the target genes, *per* (gene model, KWMTBOMO00426), *tim* (gene
149 model, KWMTBOMO01950), *Clk* (full-length cDNA clone, AK380522), and *cyc* (gene model,
150 KWMTBOMO00654) using CRISPRdirect (Naito et al., 2015: https://crispr.dbcls.jp/) based on
151 their coding sequence. sgRNA synthesis was conducted according to the method of Bassett et al.
152 (2014) using the primers listed in Table S2. Cas9 protein (600 ng/μL; NIPPON GENE) and
153 sgRNA (150 ng/μL) were mixed in injection buffer (100 mM KOAc, 2 mM Mg $(OAc)_2$, 30 mM
154 HEPES-KOH; pH 7.4) and injected into non-diapause eggs within 2–4 h after oviposition.
155
156 *2.3. Establishment of knockout strains*
157 Hatched G_0 (injected generation) larvae were raised to adulthood and crossed with
158 wild-type moths to obtain G_1 eggs. Genomic DNA was briefly extracted from a leg of each G_1
159 adult moth by the HotSHOT method (Truett et al., 2000). DNA fragments containing sgRNA

target sequence were amplified by genomic PCR using KOD One (TOYOBO) with the primers listed in Table S2. The PCR reaction was conducted as follows: 35–40 cycles at 98°C for 10 s, 60°C for 5 s, and 68°C for 5 s/kb. Mutations were detected by Heteroduplex Mobility Assay using the MultiNA (SHIMADZU) microchip electrophoresis system (Ansai et al., 2014; Ota et al., 2013). G_1 moths with an identical mutation were intercrossed with each other to generate a homozygous knockout strain. PCR products were also sequenced using the ABI3130xl genetic analyzer (Applied Biosystems) to confirm the mutation sequence.

2.3. Quantitative real-time PCR

Non-diapause eggs were maintained at 25°C under continuous darkness (DD) until hatching. Hatched larvae were reared with artificial diets under 12L12D during the larval stage. Day 3 5th-instar larvae were instantly frozen at Zeitgeber time (ZT) 1, ZT 5, ZT 9, ZT 13, ZT 17, and ZT 21 under 12L12D conditions and stored at −80°C until use. Total RNA was extracted from a whole head using TRIzol reagent (Invitrogen) and then used to synthesize cDNA with TaKaRa RNA PCR™ Kit (AMV) Ver.3.0 (TaKaRa). Quantitative real-time PCR (qPCR) was carried out using a KAPA™ SYBR FAST qPCR Kit (KAPA Biosystems) and StepOnePlus™ Real-Time PCR System (Applied Biosystems), and the transcript levels of core clock genes were calculated by the $2^{-\Delta\Delta Ct}$ method. *actin A3* and *A4* genes were used as reference

178 to normalize the mRNA level of each gene. The primers used for qPCR are listed in Table S2.

179 One-way ANOVA was used to examine the statistical significance of temporal differences
180 in clock gene expression in each strain. Two-way ANOVA was used to detect the statistical
181 significance of differences in clock gene expression between wild-type and each knockout
182 strain.

183

184 *2.4. Diapause phenotyping*

185 For diapause phenotyping, non-diapause eggs from all strains were used; larvae were fed
186 with artificial diet SilkMate PS (NOSAN) to arrange nutritional conditions. To verify the effects
187 of clock gene knockouts on photoperiodic diapause induction during the larval stage, eggs were
188 incubated at 25°C under DD and the hatched larvae were reared under long-day (non-diapause)
189 (20L4D) or short-day (diapause) conditions (8L16D). On the other hand, to verify the effects
190 during the embryonic stage, eggs were incubated at 18°C under DD or continuous light (LL)
191 until hatching and the larvae reared at 25°C under 12L12D. The resultant females were crossed
192 with males, and the oviposited eggs were maintained at 25°C for 7–8 days.
193 Ommochrome-pigmented (colored) eggs were judged as diapause eggs, whereas uncolored and
194 developed eggs until the head pigmentation stage were considered non-diapause eggs.
195 Uncolored and undeveloped eggs were judged as unfertilized and excluded from the calculation.

196 The ratio of diapause and non-diapause eggs was counted in each batch. When >90% eggs were
197 diapause eggs, the batch was considered as “diapause.” On the other hand, if >90% eggs were
198 non-diapause eggs, the batch was considered as “non-diapause.” When ≥10% eggs were mixed
199 in the same batch, the batch was considered as “mix.” Then, the diapause phenotype of each
200 strain was evaluated by the ratio of diapause, non-diapause, and mix batches to the total batch
201 number.
202
203 **3. Results and discussion**
204 *3.1 Establishment of core clock gene knockout strains*
205 To investigate the involvement of circadian clock in photoperiodic diapause induction in *B.*
206 *mori*, we knocked out four core clock genes using CRISPR/Cas9. The inbred strain p50T was
207 used in this study, because our group already published high-quality genome assembly and
208 predicted gene models on this strain (Kawamoto et al., 2019). In addition, the original bivoltine
209 strain “Daizo” exhibits photoperiodic diapause induction during both embryonic and larval
210 stages (Shimizu and Hasegawa, 1988; Egi et al., 2014). We designed sgRNAs targeting the 7th
211 and 6th exon of the negative elements, *per* and *tim*, respectively (Fig. 1A, B). We also designed
212 sgRNAs targeting the 7th exon of the positive elements, *Clk* and *cyc* (Fig. 1C, D). We injected
213 each sgRNA with Cas9 protein into non-diapause eggs and successfully established four core

clock gene knockout strains. In each strain, a premature stop codon was generated by site specific random mutations produced by non-homologous end joining and a frame shift, resulting in C-terminally truncated proteins. As all truncated proteins lacked some functional domains, protein function was probably disrupted in all knockout strains.

The negative element PER possesses two PER-ARNT-SIM (PAS) domains (PAS-A and PAS-B) involved in heterodimerization with TIMELESS and cytoplasmic localization domain (CLD, Fig. 1A; Iwai et al., 2006). The $per^{\Delta 2}$ strain had a 2-bp deletion in the targeting site of exon 7 and potentially produced truncated PER protein lacking the two PAS domains and the CLD. Ikeda et al. (2019, 2021) also established two *per* knockout strains using TALENs targeting the same exon and observed circadian rhythm disruption. On the other hand, Cui et al. (2021) targeted just downstream of the translation start site to establish a *per* knockout strain using TALENs.

Another negative element, TIM, possesses two PER interaction sites (PIS) and CLD (Fig. 1B; Iwai et al., 2006). The tim^{In11} strain had a 6-bp deletion and a 17-bp insertion in the targeting site of exon 6 and potentially produced a truncated protein lacking a part of PIS1, the whole PIS2, and the C-terminal CLD. Nartey et al. (2021) obtained *tim* knockout mutants by CRISPR/Cas9 targeting two sgRNA target sites located in adjacent exons and observed circadian rhythm disruption.

232 The positive element CLK possess a basic helix-loop-helix (bHLH) domain and two PAS
233 domains involved in heterodimerization with CYC (Fig. 1C; Allada et al., 1998). The $Clk^{\Delta 5}$
234 strain lacked most of the PAS-B domain due to a premature stop codon generated by a 5-bp
235 deletion in the target site of exon 7.

236 CYC, another positive element, has a bHLH domain and two PAS domains (Fig. 1D;
237 Markova et al., 2003). The cyc^{In2} strain lacked the whole PAS-B domain due to a premature stop
238 codon generated by a 1-bp deletion and 3-bp insertion in the target site of exon 7.

239

240 *3.2 Expression analysis of core clock genes*

241 The temporal expression patterns of core clock genes under 12L12D were examined by
242 qPCR using heads of 5th-instar larvae on the 3rd day after ecdysis (Fig. 2, 3). In the wild-type
243 strain, weak but clear temporal changes in the expression of the negative elements, *per* and *tim*,
244 were observed, although there were no significant differences in *tim* expression in two of the
245 four trials (*per*: $P < 0.001$, *tim*: $0.0133 \leq P \leq 0.242$, one-way ANOVA). Ikeda et al. (2021) also
246 analyzed *per* and *tim* expression under almost the same conditions using another bivoltine strain,
247 Kosetsu. However, the expression patterns of both negative elements differed between p50T and
248 Kosetsu. This may be caused by differences in *B. mori* strains since previous studies showed
249 differing *per* and *tim* expression rhythms among tissues, stages, and strains (Cui et al., 2021;

Ikeda et al., 2019, 2021; Iwai et al., 2006; Tao et al., 2017). In the $per^{\Delta 2}$ strain, *tim* expression was upregulated at all time points ($P < 0.001$, two-way ANOVA; Fig. 2A). This result indicates that knocking out *per* increased *tim's* transcriptional level through the lack of negative feedback. In other words, this result suggests that PER is involved in the repression of *tim's* CLK/CYC-mediated transcriptional activation in *B. mori*. On the other hand, although *tim* mRNA levels decreased in tim^{In11} strain ($P < 0.001$, two-way ANOVA), knocking out *tim* did not increase *per's* transcriptional levels ($P = 0.3835$, two-way ANOVA; Fig. 2B). This might be due to the loss of CLK/CYC-mediated transcriptional activation in the p50T strain, as described below.

Clk and *cyc*, positive elements of circadian clock, showed rhythmic expression peaking in the early photophase in the wild-type strain ($P < 0.001$, one-way ANOVA; Fig. 2, 3). The expression patterns of clock genes differ among insect species (Tomioka and Matsumoto, 2015). In *D. melanogaster*, *Clk* is expressed rhythmically, whereas *cyc* expression is constant (Rutila et al., 1998). On the other hands, in honeybees, aphids, crickets, and firebrats, *Clk* is constitutively expressed and *cyc* rhythmically expressed (Cortés et al., 2010; Kamae et al., 2010; Moriyama et al., 2012; Rubin et al., 2006; Uryu et al., 2013). Interestingly, both *Clk* and *cyc* exhibit rhythmic expression patterns in sandflies and mosquitoes (Meireles-Filho and Kyriacou, 2013; Tomioka and Matsumoto, 2015).

In both $Clk^{\Delta 5}$ and cyc^{In2} strains, *tim* mRNA expression levels were extremely low ($P < 0.001$, two-way ANOVA), while *per* expression was not downregulated, but rather upregulated during the scotophase ($P < 0.001$, two-way ANOVA; Fig. 3A, B). These results differ from previous studies in *D. melanogaster* and *D. plexippus*, which showed extremely low mRNA levels of *tim* and *per* mRNA in loss-of-function mutants of positive elements (Allada et al., 1998; Markert et al., 2016; Rutila et al., 1998). Our results suggest that CLK/CYC is involved in transcriptional activation of *tim*, but not *per*. However, it is unclear whether this is a common feature within *B. mori* strains, as this is the first time that *per* and *tim* expression was examined in knockout strains of positive elements.

Clk and *cyc* expression levels were significantly higher in cyc^{In2} and $Clk^{\Delta 5}$ strains, respectively ($P < 0.001$, two-way ANOVA; Fig. 3A, B). In *D. melanogaster*, CLK/CYC activates *Pdp1* and *vri* transcription; subsequently, the resultant PDP1 and VRI proteins activate and repress *Clk* transcription, respectively (Cyran et al., 2003; Glossop et al., 2003). The *cyc* and *Clk* upregulation in knockout strains $Clk^{\Delta 5}$ and cyc^{In2} suggests the presence of interlocked feedback loops via *vri* like in *D. melanogaster* and the fall armyworm *Spodoptera frugiperda* (Hänniger et al., 2017; Rego et al., 2021).

3.3 Effects of core clock gene knockout on photoperiodic diapause induction

286 The inbred strain p50T exhibits clear photoperiodic diapause induction during both the
287 embryonic and larval stages (Egi et al., 2014). Using p50T-derived knockout strains, we
288 examined the involvement of core clock genes in photoperiodic diapause induction at each stage.
289 When wild-type larvae were reared under the short-day condition (8L16D) at 25℃, all female
290 moths produced diapause eggs (Fig. 4). On the other hand, under the long-day condition
291 (20L4D), they all produced non-diapause eggs. All female moths produced non-diapause eggs
292 in all core clock gene knockout strains, $per^{\Delta 2}$, tim^{In11}, $Clk^{\Delta 5}$, and cyc^{In2}, regardless of
293 photoperiodic conditions (Fig. 4). Our results agree with Ikeda et al. (2021) that found that
294 knocking out *per* in Kosetsu, another bivoltine strain, inhibits the diapause egg production
295 induced by a short-day condition during the larval stage. Recently, Homma et al. (2022)
296 reported that core clock genes regulate temperature-dependent diapause induction in *B. mori*
297 using TALEN-mediated knockouts in the Kosetsu strain. Although the hatching condition in this
298 experiment (25°C DD) is also affected by temperature-dependent diapause induction during the
299 embryonic stage, our results support that core clock genes are involved in photoperiodic
300 diapause induction during the larval stage.

301 Next, we examined the involvement of core clock genes in photoperiodic diapause
302 induction during embryonic stages. Under low temperature (18°C), continuous light during the
303 embryonic period induced diapause egg production in all wild-type females, whereas

304 continuous darkness decreased the number of female moths which produced diapause eggs to
305 40% (Fig. 5). When the eggs of all core clock gene knockout strains, $per^{\Delta 2}$, tim^{In11}, $Clk^{\Delta 5}$, and
306 cyc^{In2} were maintained under continuous light or darkness conditions, knockout females only
307 produced non-diapause eggs. This means that the photoperiodic diapause induction was
308 disrupted by knocking out core clock genes. Cui et al. (2021) also established a *per* knockout in
309 the Dazao strain and found that lack of PER protein suppressed the diapause egg production
310 induced by continuous light condition during the embryonic stage.

311 Our results together with previous evidence demonstrate that feedback loops involving
312 core clock genes, that is, the circadian clock, participate in photoperiodic diapause induction in
313 *B. mori*. In the monarch butterfly, *D. plexippus*, reproductive diapause is induced by short-day
314 conditions during the larval stage (Goehring and Oberhauser, 2002); the photoperiodic diapause
315 induction is abolished in *Clk* or *cyc* loss-of-function mutant females. In contrast, the
316 reproductive diapause is strongly induced in loss-of-function mutant females for *cry2*,
317 regardless of day length. Although diapause stage and the endocrine system controlling
318 diapause differ between both lepidopteran species, core clock genes are necessary for
319 photoperiodic diapause induction in both. In the bean bug *R. pedestris*, RNAi of the negative
320 elements, *cry-m* (*cry2*) and *per*, causes the insect to avert reproductive diapause under a
321 diapause-inducing photoperiod (short-day) whereas RNAi of the positive elements, *Clk* and *cyc*,

induces reproductive diapause under a diapause-averting photoperiod (long-day). These results indicate that circadian activators and repressors may function in a coordinated fashion to sense the photoperiod, probably through a feedback loop. In contrast to previous results, *B. mori* females bearing non-functional positive and negative elements constantly produced non-diapause eggs. These differences among insect species after clock gene perturbation strongly support a conserved involvement of circadian clock feedback loop in photoperiodic diapause induction, despite its independent evolution in each insect species. Since circadian clocks control diverse physiological process, such as behavior, learning, feeding, metabolism, chemosensation, and immunity (Allada and Chung, 2009), we cannot conclude whether circadian clock or non-clock function of clock genes is related to photoperiodic diapause induction in *B. mori*. However, temperature-dependent diapause induction can be rescued by injection of picrotoxin, an ionotropic GABA receptor antagonist, and diapause hormone into female pupae, suggesting that the GABAergic and diapause hormone signaling pathways are intact in clock gene knockouts (Homma et al., 2022). Since diapause hormone release from the corpus cardiacum is suppressed by GABAergic signaling pathway (Homma et al., 2022; Shimizu et al., 1989), our next goal is to reveal how circadian clock genes are influenced by the photoperiod and how the circadian clock controls the GABAergic signaling pathway and diapause hormone secretion.

340

341 **Acknowledgments**

342 We thank the Institute for Sustainable Agro-ecosystem Services, The University of Tokyo, for
343 facilitating the mulberry cultivation and the Biotron Facility at the University of Tokyo for
344 rearing the silkworms. We also thank Dr. Susumu Katsuma for useful advice. We are grateful to
345 Wakako Saito and Natsuki Nakashima for technical assistance for silkworm maintenance. This
346 study was supported by JSPS KAKENHI grant numbers JP17H05047, JP20H02997 to TK and
347 JST SPRING (JPMJSP2108) to HT.

348

349 **Author contributions**

350 TK supervised and designed this study. HT conducted most of the experiments. TK and HT
351 analyzed the data and wrote the manuscript.

352

353 **References**

354 Allada, R., Chung, B.Y., 2009. Circadian organization of behavior and physiology in
355 *Drosophila*. Annu. Rev. Physiol. 72, 605–624.
356 https://doi.org/10.1146/annurev-physiol-021909-135815

357 Allada, R., White, N.E., So, W.V., Hall, J.C., Rosbash, M., 1998. A mutant *Drosophila*
358 homolog of mammalian *Clock* disrupts circadian rhythms and transcription of *period* and
359 *timeless*. Cell 93, 791–804. https://doi.org/10.1016/S0092-8674(00)81440-3

360 Ansai, S., Inohaya, K., Yoshiura, Y., Schartl, M., Uemura, N., Takahashi, R., Kinoshita, M.,
361 2014. Design, evaluation, and screening methods for efficient targeted mutagenesis with
362 transcription activator-like effector nucleases in medaka. Dev. Growth Differ. 56, 98–107.
363 https://doi.org/10.1111/dgd.12104

364 Bassett, A.R., Tibbit, C., Ponting, C.P., Liu, J.L., 2014. Highly Efficient Targeted Mutagenesis
365 of *Drosophila* with the CRISPR/Cas9 System. Cell Rep. 6, 1178–1179.
366 https://doi.org/10.1016/j.celrep.2014.03.017

367 Beck, S.D., Hanec, W., 1960. Diapause in the European corn borer, *Pyrausta nubilalis* (Hübn.).
368 J. Insect Physiol. 4, 304–318. https://doi.org/10.1016/0022-1910(60)90056-1

369 Beer, K., Helfrich-Förster, C., 2020. Model and Non-model Insects in Chronobiology. Front.
370 Behav. Neurosci. 14, 1–23. https://doi.org/10.3389/fnbeh.2020.601676

371 Bradshaw, W.E., Holzapfel, C.M., 1975. Biology of tree-hole mosquitoes: photoperiodic
372 control of development in northern *Toxorhynchites rutilus* (Coq.). Can. J. Zool. 53, 889–
373 893.
374 Bradshaw, W.E., Lounibos, L.P., 1977. Evolution of dormancy and its photoperiodic control in
375 pitcher-plant mosquitoes. Evolution (N. Y). 31, 546–567.
376 https://doi.org/10.1111/j.1558-5646.1977.tb01044.x
377 Ceriani, M.F., Darlington, T.K., Staknis, D., Más, P., Petti, A.A., Weitz, C.J., Kay, S.A., 1999.
378 Light-dependent sequestration of TIMELESS by CRYPTOCHROME. Science. 285, 553–
379 556. https://doi.org/10.1126/science.285.5427.553
380 Cortés, T., Ortiz-Rivas, B., Martínez-Torres, D., 2010. Identification and characterization of
381 circadian clock genes in the pea aphid *Acyrthosiphon pisum*. Insect Mol. Biol. 19, 123–
382 139. https://doi.org/10.1111/j.1365-2583.2009.00931.x
383 Cui, W.Z., Qiu, J.F., Dai, T.M., Chen, Z., Li, J.L., Liu, K., Wang, Y.J., Sima, Y.H., Xu, S.Q.,
384 2021. Circadian Clock Gene *Period* Contributes to Diapause via GABAeric-Diapause
385 Hormone Pathway in *Bombyx mori*. Biology, 10,
386 842. https://doi.org/10.3390/biology10090842
387 Cyran, S.A., Buchsbaum, A.M., Reddy, K.L., Lin, M.-C., Glossop, N.R.J., Hardin, P.E., Young,
388 M.W., Storti, R. V, Blau, J., 2003. *vrille*, *Pdp1*, and *dClock* Form a Second Feedback

389 Loop in the *Drosophila* Circadian Clock. Cell 112, 329–341.

390 Darlington, T.K., Wager-Smith, K., Ceriani, M.F., Staknis, D., Gekakis, N., Steeves, T.D.L.,
391 Weitz, C.J., Takahashi, J.S., Kay, S.A., 1998. Closing the circadian loop: CLOCK-induced
392 transcription of its own inhibitors *per* and *tim*. Science. 280, 1599–1603.
393 https://doi.org/10.1126/science.280.5369.1599

394 Denlinger, D.L., 1991. Relationship between Cold Hardiness and Diapause, in: Lee, R.E.,
395 Denlinger, D.L. (Eds.), Insects at Low Temperature. Springer, Boston, MA, pp. 174–198.

396 Denlinger, D.L., Yocum, G.D., Rinehart, J.P., 2012. Hormonal Control of Diapause, in: Gilbert,
397 L.I. (Ed.), Insect Endocrinology. Academic Press, San Diego, pp. 430–463.

398 Egi, Y., Akitomo, S., Fujii, T., Banno, Y., Sakamoto, K., 2014. Silkworm strains that can be
399 clearly destined towards either embryonic diapause or direct development by adjusting a
400 single ambient parameter during the preceding generation. Entomol. Sci. 17, 396–399.
401 https://doi.org/10.1111/ens.12073

402 Emerson, K.J., Bradshaw, W.E., Holzapfel, C.M., 2009. Complications of complexity:
403 integrating environmental, genetic and hormonal control of insect diapause. Trends Genet.
404 25, 217–225. https://doi.org/10.1016/j.tig.2009.03.009

405 Glossop, N.R.J., Houl, J.H., Zheng, H., Ng, F.S., Dudek, S.M., Hardin, P.E., 2003. VRILLE
406 feeds back to control circadian transcription of *Clock* in the *Drosophila* circadian oscillator.

Neuron 37, 249–261. https://doi.org/10.1016/S0896-6273(03)00002-3

Goehring, L., Oberhauser, K.S., 2002. Effects of photoperiod, temperature, and host plant age on induction of reproductive diapause and development time in *Danaus plexippus*. Ecol. Entomol. 27, 674–685. https://doi.org/10.1046/j.1365-2311.2002.00454.x

Hänniger, S., Dumas, P., Schöfl, G., Gebauer-Jung, S., Vogel, H., Unbehend, M., Heckel, D.G., Groot, A.T., 2017. Genetic basis of allochronic differentiation in the fall armyworm. BMC Evol. Biol. 17, 1–14. https://doi.org/10.1186/s12862-017-0911-5

Hardin, P.E., 2005. The circadian timekeeping system of *Drosophila*. Curr. Biol. 15, 714–722. https://doi.org/10.1016/j.cub.2005.08.019

Herman, W.S., 1981. Studies on the Adult Reproductive Diapause of the Monarch Butterfly, *Danaus Plexippus*. Biol. Bull. 160, 89–106. https://doi.org/10.2307/1540903

Homma, S., Murata, A., Ikegami, M., Kobayashi, M., Yamazaki, M., Ikeda, K., Daimon, T., Numata, H., Mizoguchi, A., Shiomi, K., 2022. Circadian Clock Genes Regulate Temperature-Dependent Diapause Induction in Silkworm *Bombyx mori*. Front. Physiol. 13, 1–9. https://doi.org/10.3389/fphys.2022.863380

Iiams, S.E., Lugena, A.B., Zhang, Y., Hayden, A.N., Merlin, C., 2019. Photoperiodic and clock regulation of the vitamin A pathway in the brain mediates seasonal responsiveness in the monarch butterfly. Proc. Natl. Acad. Sci. U. S. A. 116, 25214–25221.

https://doi.org/10.1073/pnas.1913915116

Ikeda, K., Daimon, T., Sezutsu, H., Udaka, H., Numata, H., 2019. Involvement of the Clock Gene *period* in the Circadian Rhythm of the Silkmoth *Bombyx mori*. J. Biol. Rhythms 34, 283–292. https://doi.org/10.1177/0748730419841185

Ikeda, K., Daimon, T., Shiomi, K., Udaka, H., Numata, H., 2021. Involvement of the Clock Gene *period* in the Photoperiodism of the Silkmoth *Bombyx mori*. Zoolog. Sci. 38, 523–530. https://doi.org/10.2108/zs210081

Ikeno, T., Ishikawa, K., Numata, H., Goto, S.G., 2013. Circadian clock gene *Clock* is involved in the photoperiodic response of the bean bug *Riptortus pedestris*. Physiol. Entomol. 38, 157–162. https://doi.org/10.1111/phen.12013

Ikeno, T., Numata, H., Goto, S.G., 2011. Photoperiodic response requires *mammalian-type cryptochrome* in the bean bug *Riptortus pedestris*. Biochem. Biophys. Res. Commun. 410, 394–397. https://doi.org/10.1016/j.bbrc.2011.05.142

Ikeno, T., Tanaka, S.I., Numata, H., Goto, S.G., 2010. Photoperiodic diapause under the control of circadian clock genes in an insect. BMC Biol. 8. https://doi.org/10.1186/1741-7007-8-116

Iwai, S., Fukui, Y., Fujiwara, Y., Takeda, M., 2006. Structure and expressions of two circadian clock genes, *period* and *timeless* in the commercial silkmoth, *Bombyx mori*. J. Insect

443 Physiol. 52, 625–637. https://doi.org/10.1016/j.jinsphys.2006.03.001

444 Kadener, S., Stoleru, D., McDonald, M., Nawathean, P., Rosbash, M., 2007. *Clockwork Orange*
445 is a transcriptional repressor and a new *Drosophila* circadian pacemaker component.
446 Genes Dev. 21, 1675–1686. https://doi.org/10.1101/gad.1552607

447 Kamae, Y., Tanaka, F., Tomioka, K., 2010. Molecular cloning and functional analysis of the
448 clock genes, *Clock* and *cycle*, in the firebrat *Thermobia domestica*. J. Insect Physiol. 56,
449 1291–1299. https://doi.org/10.1016/j.jinsphys.2010.04.012

450 Kawamoto, M., Jouraku, A., Toyoda, A., Yokoi, K., Minakuchi, Y., Katsuma, S., Fujiyama, A.,
451 Kiuchi, T., Yamamoto, K., Shimada, T., 2019. High-quality genome assembly of the
452 silkworm, *Bombyx mori*. Insect Biochem. Mol. Biol. 107, 53–62.
453 https://doi.org/10.1016/j.ibmb.2019.02.002

454 Kogure, M., 1933. The Influence Of Light And Temperature On Certain Characters Of The
455 Silkworm, *Bombyx Mori*. J. Dep. Agric. Kyushu Imp. Univ. 4, 1–93.
456 https://doi.org/10.5109/22568

457 Lim, C., Chung, B.Y., Pitman, J.L., McGill, J.J., Pradhan, S., Lee, J., Keegan, K.P., Choe, J.,
458 Allada, R., 2007. *clockwork orange* Encodes a Transcriptional Repressor Important for
459 Circadian-Clock Amplitude in *Drosophila*. Curr. Biol. 17, 1082–1089.
460 https://doi.org/10.1016/j.cub.2007.05.039

461 Markert, M.J., Zhang, Y., Enuameh, M.S., Reppert, S.M., Wolfe, S.A., Merlin, C., 2016.
462 Genomic access to monarch migration using TALEN and CRISPR/Cas9-mediated
463 targeted mutagenesis. G3 Genes, Genomes, Genet. 6, 905–915.
464 https://doi.org/10.1534/g3.116.027029
465 Markova, E.P., Ueda, H., Sakamoto, K., Oishi, K., Shimada, T., Takeda, M., 2003. Cloning of
466 *Cyc* (*Bmal1*) homolog in *Bombyx mori*: Structural analysis and tissue specific distributions.
467 Comp. Biochem. Physiol. - B Biochem. Mol. Biol. 134, 535–542.
468 https://doi.org/10.1016/S1096-4959(03)00004-6
469 Matsumoto, A., Ukai-Tadenuma, M., Yamada, R.G., Houl, J., Uno, K.D., Kasukawa, T.,
470 Dauwalder, B., Itoh, T.Q., Takahashi, K., Ueda, R., Hardin, P.E., Tanimura, T., Ueda,
471 H.R., 2007. A functional genomics strategy reveals *clockwork orange* as a transcriptional
472 regulator in the *Drosophila* circadian clock. Genes Dev. 21, 1687–1700.
473 https://doi.org/10.1101/gad.1552207
474 Meireles-Filho, A.C.A., Kyriacou, C.P., 2013. Circadian rhythms in insect disease vectors.
475 Mem. Inst. Oswaldo Cruz 108, 48–58. https://doi.org/10.1590/0074-0276130438
476 Meuti, M.E., Denlinger, D.L., 2013. Evolutionary links between circadian clocks and
477 photoperiodic diapause in insects. Integr. Comp. Biol. 53, 131–143.
478 https://doi.org/10.1093/icb/ict023

479 Moriyama, Y., Kamae, Y., Uryu, O., Tomioka, K., 2012. *Gb'clock* is expressed in the optic lobe
480 and is required for the circadian clock in the cricket *Gryllus bimaculatus*. J. Biol. Rhythms
481 27, 467–477. https://doi.org/10.1177/0748730412462207
482 Naito, Y., Hino, K., Bono, H., Ui-Tei, K., 2015. CRISPRdirect: software for designing
483 CRISPR/Cas guide RNA with reduced off-target sites. Bioinformatics 31, 1120–1123.
484 https://doi.org/10.1093/bioinformatics/btu743
485 Nartey, M.A., Sun, X., Qin, S., Hou, C.X., Li, M.W., 2021. CRISPR/Cas9-based knockout
486 reveals that the clock gene *timeless* is indispensable for regulating circadian behavioral
487 rhythms in *Bombyx mori*. Insect Sci. 28, 1414–1425.
488 https://doi.org/10.1111/1744-7917.12864
489 Nylin, S., 2013. Induction of diapause and seasonal morphs in butterflies and other insects:
490 Knowns, unknowns and the challenge of integration. Physiol. Entomol. 38, 96–104.
491 https://doi.org/10.1111/phen.12014
492 Ota, S., Hisano, Y., Muraki, M., Hoshijima, K., Dahlem, T.J., Grunwald, D.J., Okada, Y.,
493 Kawahara, A., 2013. Efficient identification of TALEN-mediated genome modifications
494 using heteroduplex mobility assays. Genes to Cells 18, 450–458.
495 https://doi.org/10.1111/gtc.12050
496 Paolucci, S., Salis, L., Vermeulen, C.J., Beukeboom, L.W., van de Zande, L., 2016. QTL

497 analysis of the photoperiodic response and clinal distribution of *period* alleles in *Nasonia*
498 *vitripennis*. Mol. Ecol. 25, 4805–4817. https://doi.org/10.1111/mec.13802
499 Pruisscher, P., Nylin, S., Gotthard, K., Wheat, C.W., 2018. Genetic variation underlying local
500 adaptation of diapause induction along a cline in a butterfly. Mol. Ecol. 27, 3613–3626.
501 https://doi.org/10.1111/mec.14829
502 Rego, N. de F.C., Chahad-Ehlers, S., Campanini, E.B., Torres, F.R., de Brito, R.A., 2021.
503 VRILLE shows high divergence among Higher Diptera flies but may retain role as
504 transcriptional repressor of *Clock*. J. Insect Physiol. 133.
505 https://doi.org/10.1016/j.jinsphys.2021.104284
506 Rubin, E.B., Shemesh, Y., Cohen, M., Elgavish, S., Robertson, H.M., Bloch, G., 2006.
507 Molecular and phylogenetic analyses reveal mammalian-like clockwork in the honey bee
508 (*Apis mellifera*) and shed new light on the molecular evolution of the circadian clock.
509 Genome Res. 16, 1352–1365. https://doi.org/10.1101/gr.5094806
510 Rutila, J.E., Suri, V., Le, M., So, W.V., Rosbash, M., Hall, J.C., 1998. CYCLE is a second
511 bHLH-PAS clock protein essential for circadian rhythmicity and transcription of
512 *Drosophila period* and *timeless*. Cell 93, 805–814.
513 https://doi.org/10.1016/S0092-8674(00)81441-5
514 Sandrelli, F., Costa, R., Kyriacou, C.P., Rosato, E., 2008. Comparative analysis of circadian

clock genes in insects. Insect Mol. Biol. 17, 447–463. https://doi.org/10.1111/j.1365-2583.2008.00832.x

Sato, A., Sokabe, T., Kashio, M., Yasukochi, Y., Tominaga, M., Shiomi, K., 2014. Embryonic thermosensitive TRPA1 determines transgenerational diapause phenotype of the silkworm, *Bombyx mori*. Proc. Natl. Acad. Sci. U. S. A. 111. https://doi.org/10.1073/pnas.1322134111

Sato, Y., Shiomi, K., Saito, H., Imai, K., Yamashita, O., 1998. Phe-X-Pro-Arg-Leu-NH_2 peptide producing cells in the central nervous system of the silkworm, *Bombyx mori*. J. Insect Physiol. 44, 333–342. https://doi.org/10.1016/S0022-1910(97)00140-6

Shimizu, I., Hasegawa, K., 1988. Photoperiodic induction of diapause in the silkworm, *Bombyx mori*: location of the photoreceptor using a chemiluminescent paint. Physiol. Entomol. 13, 81–88. https://doi.org/10.1111/j.1365-3032.1988.tb00911.x

Shimizu, I., Matsui, T., Hasegawa, K., 1989. Possible Involvement of GABAergic Neurons in Regulation of Diapause Hormone Secretion in the Silkworm, *Bombyx mori*. Zoolog. Sci. 6, 809–812.

Takeda, S., Ogura, N., 1976. Induction of egg diapause by implantation of corpora cardiaca and corpora allata in *Bombyx mori*. J. Insect Physiol. 22, 941–944. https://doi.org/10.1016/0022-1910(76)90075-5

533 Tao, H., Li, X., Qiu, J.F., Liu, H.J., Zhang, D.Y., Chu, F., Sima, Y., Xu, S.Q., 2017. The light
534 cycle controls the hatching rhythm in *Bombyx mori* via negative feedback loop of the
535 circadian oscillator. Arch. Insect Biochem. Physiol. 96, 1–14.
536 https://doi.org/10.1002/arch.21408
537 Tomioka, K., Matsumoto, A., 2015. Circadian molecular clockworks in non-model insects. Curr.
538 Opin. Insect Sci. 7, 58–64. https://doi.org/10.1016/j.cois.2014.12.006
539 Truett, G.E., Heeger, P., Mynatt, R.L., Truett, A.A., Walker, J.A., Warman, M.L., 2000.
540 Preparation of PCR-quality mouse genomic dna with hot sodium hydroxide and tris
541 (HotSHOT). Biotechniques 29, 52–54. https://doi.org/10.2144/00291bm09
542 Tsuchiya, R., Kaneshima, A., Kobayashi, M., Yamazaki, M., Takasu, Y., Sezutsu, H., Tanaka,
543 Y., Mizoguchi, A., Shiomi, K., 2020. Maternal GABAergic and GnRH/corazonin pathway
544 modulates egg diapause phenotype of the silkworm *Bombyx mori*. Proc. Natl. Acad. Sci. U.
545 S. A. 118. https://doi.org/10.1073/pnas.2020028118
546 Uryu, O., Karpova, S.G., Tomioka, K., 2013. The clock gene *cycle* plays an important role in
547 the circadian clock of the cricket *Gryllus bimaculatus*. J. Insect Physiol. 59, 697–704.
548 https://doi.org/10.1016/j.jinsphys.2013.04.011
549 Williams, C.M., Adkisson, P.L., 1964. Physiology of Insect Diapause. XIV. An Endocrine
550 Mechanism for the Photoperiodic Control of Pupal Diapause in the Oak Silkworm,

551 *Antheraea pernyi*. Biol. Bull. 127, 511–525.

552 Yamada, H., Yamamoto, M., 2011. Association between circadian clock genes and diapause

553 incidence in *Drosophila triauraria*. PLoS One 6.

554 https://doi.org/10.1371/journal.pone.0027493

555 Zhu, H., Sauman, I., Yuan, Q., Casselman, A., Emery-Le, M., Emery, P., Reppert, S.M., 2008.

556 Cryptochromes define a novel circadian clock mechanism in monarch butterflies that may

557 underlie sun compass navigation. PLoS Biol. 6, 0138–0155.

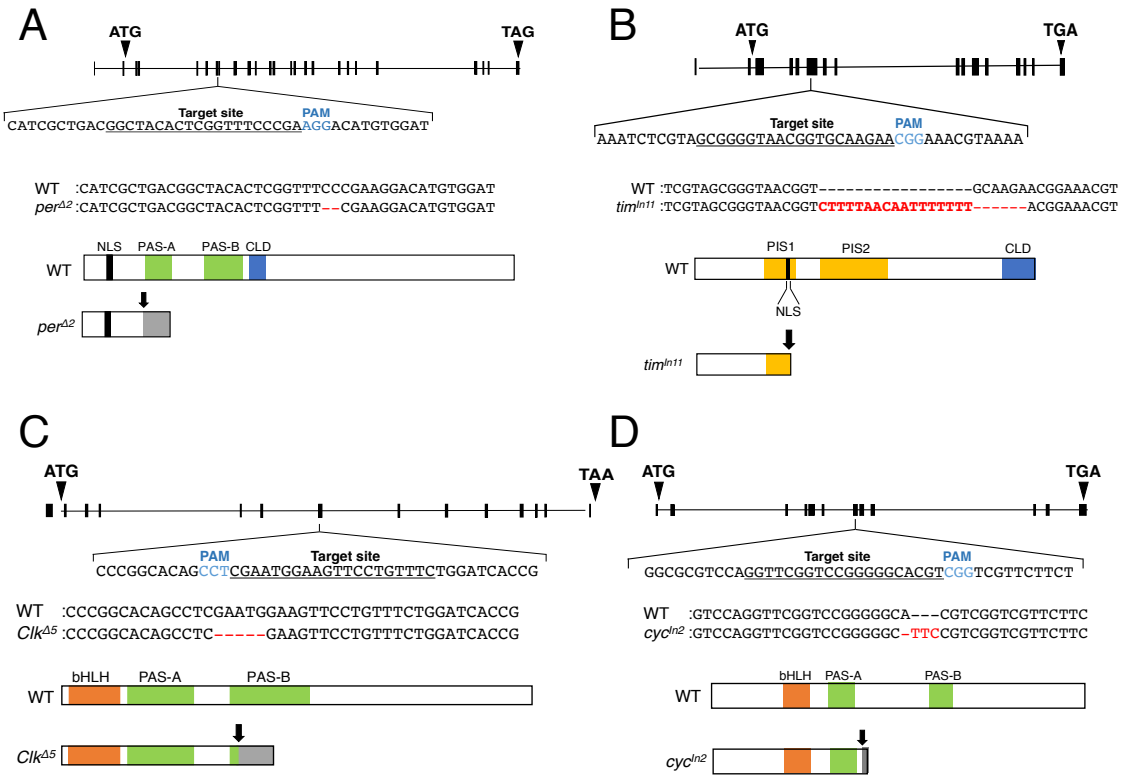
558 https://doi.org/10.1371/journal.pbio.0060004

559

560

561 **Figures and supplementary tables**

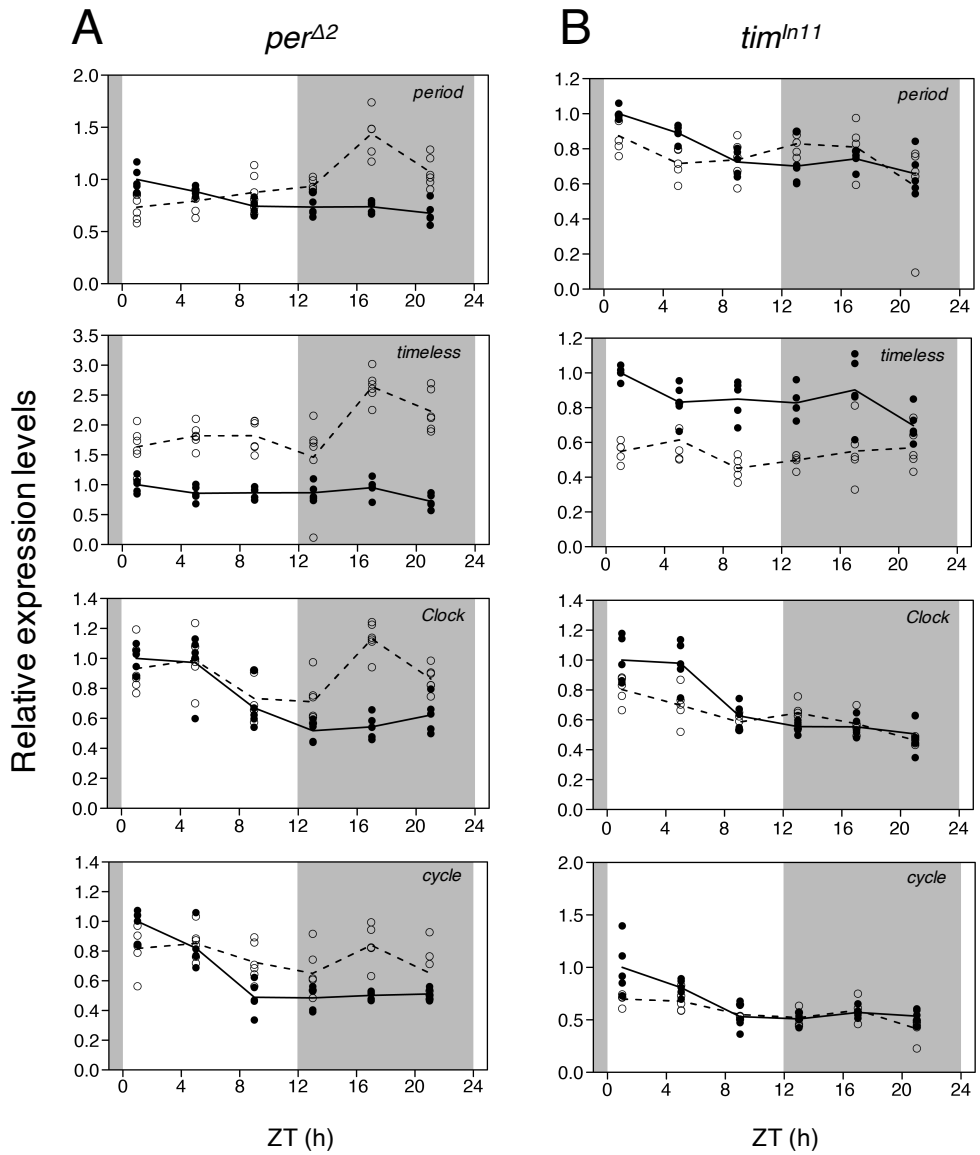
562



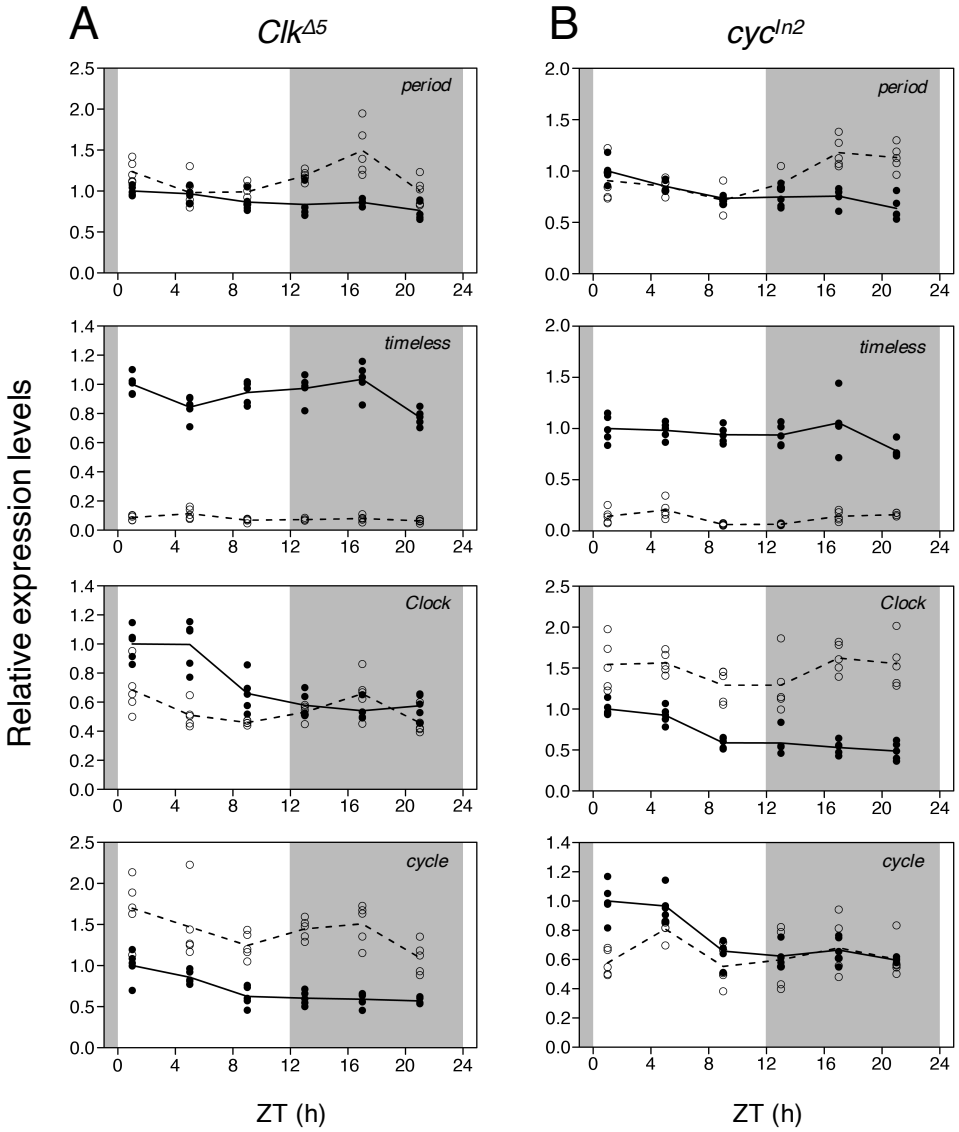
563

564 **Figure 1**. CRISPR/Cas9-mediated knockouts of core clock genes. Genomic structures of *period*
565 (A), *timeless* (B), *Clock* (C), and *cycle* (D) with the sgRNA target site (underlined) and the
566 protospacer adjacent motif (PAM) site (blue letters) at the top. Alignments of nucleotide
567 sequences surrounding the sgRNA target site from wild-type (WT) and knockout strains at the
568 center. Mutations are indicated by red letters. Predicted protein structures are shown at the
569 bottom. Black arrows indicate the mutation site of each clock gene. Functional domains are
570 shown by colored boxes. Nuclear localization signal (NLS; black), PER-ARNT-SIM (PAS;
571 green), cytoplasmic localization domain (CLD; blue), PER interaction site (PIS; yellow), and
572 basic helix-loop-helix (bHLH; orange).

573

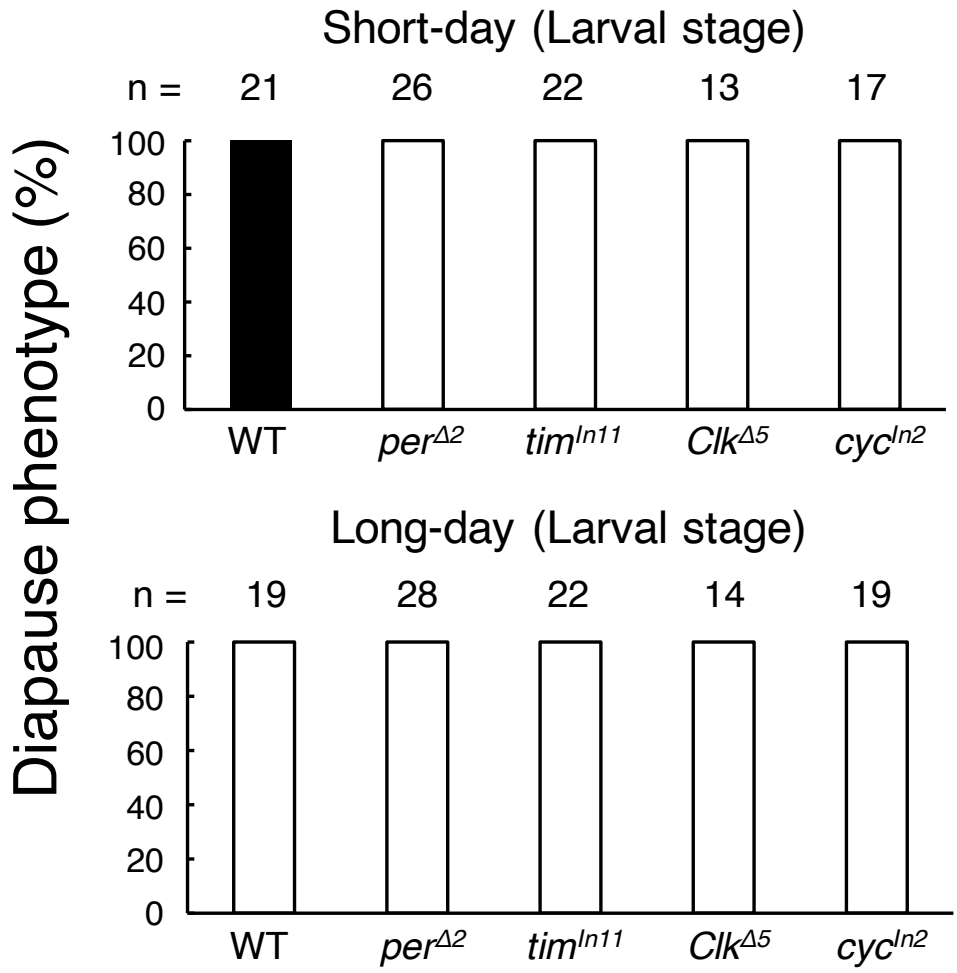


574 **Figure 2**. Temporal expression patterns of core clock genes in knockout strains of negative
575 elements. The expression levels of *per*, *tim*, *Clk*, and *cyc* in heads of day 3 5th-instar larvae
576 under 12L12D were examined by qPCR. The relative expression levels (the means of wild-type
577 at Zeitgeber time (ZT) 1 = 1) of wild-type (closed circles) and knockout strains (open circles),
578 $per^{\Delta 2}$ (A) and tim^{In11} (B), are shown by solid and broken lines, respectively (n = 5 or 6). *actin A3*
579 and *A4* genes were used as reference to normalize the mRNA level of each clock gene. Shaded
580 areas indicate scotophase.



581 **Figure 3**. Temporal expression patterns of core clock genes in knockout strains of positive
582 elements. The expression levels of *per*, *tim*, *Clk*, and *cyc* in heads of day 3 5th-instar larvae
583 under 12L12D were examined by qPCR. The relative expression levels (the means of wild-type
584 at Zeitgeber time (ZT) 1 = 1) of wild-type (closed circles) and knockout strains (open circles),
585 $Clk^{\Delta 5}$ (A) and cyc^{In2} (B), are shown by solid and broken lines, respectively (n = 5). *actin A3* and
586 *A4* genes were used as reference to normalize the mRNA level of each clock gene. Shaded areas
587 indicate scotophase.

588



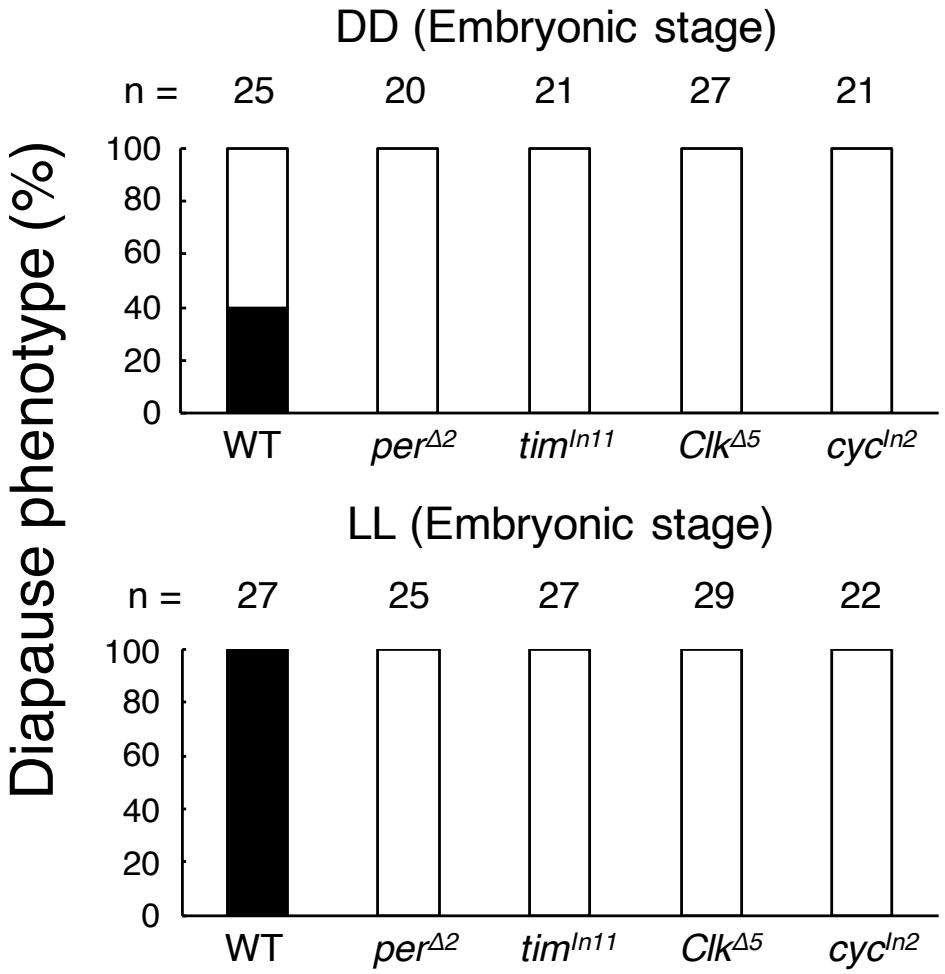
589 **Figure 4**. Effects of core clock gene knockouts on photoperiodic diapause induction during
590 larval stages. Larvae were reared under short-day (8L16D; top) or long-day (20L4D; bottom)
591 conditions. Closed and open bars show the proportions of females that oviposited diapause and
592 non-diapause eggs, respectively. No female oviposited both diapause and non-diapause eggs in
593 the same batch.

594

595

596

597



598 **Figure 5**. Effects of core clock gene knockouts on photoperiodic diapause induction during
599 embryonic stages. Eggs were incubated under continuous darkness (DD; top) or continuous
600 light (LL; bottom) conditions until hatching. Closed and open bars show the proportions of
601 females that oviposited diapause and non-diapause eggs, respectively. No female oviposited
602 both diapause and non-diapause eggs in the same batch.

603

604

605

606

Supplementary tables

Table S1. Summary of results of clock gene disruption in insects and its effects on photoperiodic diapause induction.

Insects	Disrupted clock genes		Diapause phenotype	References
Bombyx mori	negative elements	*period, timeless*	Non-diapause	Cui *et al*. (2021); Ikeda *et al*. (2021); This study
	positive elements	*Clock, cycle*	Non-diapause	This study
Culex pipiens	negative elements	*period, timeless, cryptochrome2*	Non-diapause	Meuti *et al*. (2015)
	positive elements			
Danaus plexippus	negative elements	*cryptochrome2*	Diapause	Iiams *et al*. (2019)
	positive elements	*Clock, Bmal1* (*cycle*)	Non-diapause	Iiams *et al*. (2019)
Dianemobius nigrofasciatus	negative elements			
	positive elements	*Clock*	Non-diapause	Goto & Nagata (2022)
Nasonia vitripennis	negative elements	*period*	Non-diapause	Mukai & Goto (2016)
	positive elements			
Riptortus pedestris	negative elements	*period, mammalian-type cryptochrome*	Non-diapause	Ikeno *et al*. (2010, 2011)
	positive elements	*Clock, cycle*	Diapause	Ikeno *et al*. (2010, 2013)

Table S2. List of primers.

Primer name	Primer sequence (5'-3')	Purpose
*per*_CRF	GAAATTAATACGACTCACTATAggctatactcggtttcccgaGTTTTAGAGCTAGAAATAGC	sgRNA template
*tim*_CRF	GAAATTAATACGACTCACTATAgggggtaacggtgcaagaaGTTTTAGAGCTAGAAATAGC	sgRNA template
*Clk*_CRF	GAAATTAATACGACTCACTATAgaaacaggaacttccattcgGTTTTAGAGCTAGAAATAGC	sgRNA template
*cyc*_CRF	GAAATTAATACGACTCACTATAggttcggtccgggggcacgtGTTTTAGAGCTAGAAATAGC	sgRNA template
sgRNA-R	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	sgRNA template
per-F	CATGCGTTATATCGATGCACG	HMA*, sequencing
per-R	CCCTTGGATGAACGAAATCGA	HMA, sequencing
tim-F	CGATGACGAACTACCCTCTGA	HMA, sequencing
tim-R	CAGCCCTTGTACGTTGATTCT	HMA, sequencing
*Clk*_F	CAGGTCACAACCTTTTCCAGA	HMA, sequencing
*Clk*_R	GGTCGTCTGTTCACACCTCAT	HMA, sequencing
*cyc*_F	TACCTTTGACTCGATGCTTCC	HMA, sequencing
*cyc*_R	AATTTGCACTTGATCCGACAG	HMA, sequencing
RT_*per*_F	TGACCAAGTTGCTCGTTCAC	qPCR
RT_*per*_R	GACGTCAGAGCTCGTCTACA	qPCR
RT_*tim*_F	CCTGGCACTTCATTCGGTA	qPCR
RT_*tim*_R	CGACGGCAACTTCATCACTA	qPCR
RT_*Clk*_F	GTTGCAGTATACCAAGCCTG	qPCR
RT_*Clk*_R	CCTCTACCGATCATCGTGTC	qPCR
RT_*cyc*_F	GAGTTGCTGGGCACTAGTCTG	qPCR
RT_*cyc*_R	GTTTGTATTCTGGCCATCGAG	qPCR
RT_*actin A3*/4_F1	ATCGAACACGGAATCGTCACTA	qPCR
RT_*actin A3*/4_R1	GAATGTTTCGAACATGATCTGG	qPCR
*Heteroduplex Mobility Assay		