# Role of the clock gene *period* in the circadian rhythm and photoperiodism of the silkmoth *Bombyx mori*

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## General introduction

The daily rotation of the earth and the annual revolution of the earth around the sun make daily and annual environmental changes, respectively. Organisms have abilities to adapt these changes. The circadian clock drives daily rhythms in behavioral, physiological and developmental activities, and is entrained to daily environmental cues. Photoperiodism is a response to the day or night length in a day. These abilities allow organisms to anticipate the daily or seasonal environmental changes and to prepare accordingly (Tauber et al., 1986; Danks, 1987; Yerushalmi and Green 2009).

Molecular models of the clock that produces circadian rhythms have been postulated in various organisms (Bell-Pedersen et al., 2005). The first molecular model of the circadian clock was proposed in the fruit fly *Drosophila melanogaster* (Hardin et al., 1990; Hardin, 2005). This clock model is described as an ensemble of interlocked negative transcription/translation feedback loops. In each loop, positive elements, such as *cycle* and *Clock*, drive the transcription of negative elements, such as *period* (*per*) and *timeless* (*tim*), which rhythmically feedback to inhibit the action of the former (Sandrelli et al., 2008; Tomioka and Matsumoto, 2015). In Lepidoptera, although the presence of *per* has been shown, the PERIOD temporal localization pattern is different among several species and from that of *D. melanogaster* (Kotwica et al., 2009; Kobelkova, 2015; Sauman and Reppert, 1996; Sehadova et al., 2004; Wise et al., 2002). In the monarch butterfly, *Danaus plexippus*, *cryptochrome-2* (*cry-2*), instead of *per* in *D. melanogaster*, is likely the important negative regulator of the circadian clock (Zhu et al., 2008). Thus, it remains necessary to examine the molecular mechanisms of the circadian clock in Lepidoptera further, with special reference to the role of *per*.

For photoperiodism, organisms must measure the length of day or night in a day. Bünning (1936) first proposed a hypothesis that a circadian clock is involved in photoperiodic time measurement. Later, experimental results to support the involvement of a circadian clock in photoperiodic time measurement have been accumulated and this concept is now wildly accepted (Vaz Nunez and Saunders, 1999; Numata et al., 2015). Involvement of clock genes in photoperiodism has been demonstrated in several insects. Results that suppression or dysfunction of clock genes disrupts photoperiodism have been obtained exclusively in insects with photoperiodism controlling larval (nymphal) diapause (Sakamoto et al., 2009; Mukai et al., 2016) or adult diapause (Ikeno et al., 2010, 2011ab, 2013; Meuti et al., 2015; Tamai et al., 2019; Kotwica-Rolinska, 2017; Iiams et al., 2019). In general, the juvenile hormone (JH) plays important roles in larval and adult diapause (Denlinger et al., 2012). In fact, JH has been shown to be involved in diapause in some of the above species (Spielman, 1974; Hodkova, 1976; Herman, 1981; Numata and Hidaka, 1984; Kotaki and Yagi, 1989; Miki et al., 2020). Moreover, Bajgar et al. (2013ab) reported that clock genes have a role in peripheral tissues at the downstream of the JH signaling pathway in the linden bug, *Pyrrhocoris apterus*. Emerson et al. (2009) pointed out that pleiotropic effects of clock gene might induce loss of photoperiodism. Therefore, it is still unclear whether pleiotropic effects of clock genes or a circadian clock consisting of these genes causes the loss of photoperiodism by knockdown or knockout of the genes.

The silkmoth *Bombyx mori* is a domesticated species in invertebrates, and extensive studies were performed in this species over a century for sericultural industry. In these studies, both the circadian rhythm and photoperiodism were reported (Kogure, 1933; Takamiya 1974; Kobayashi et al., 1986; Hasegawa and Shimizu 1987; Oshiki and

Watanabe, 1978ab; Sakamoto and Shimizu, 1994; Sakamoto et al., 2003; Shimizu and Matsui, 1983; Tanaka 1966abc). In *B. mori*, the maternal photoperiod controls the induction of embryonic diapause, which is regulate by diapause hormone secreted by the subesophageal ganglion of the mother (Yamashita and Hasegawa, 1985; Yamashita, 1996; Denlinger 2002; Saunders 2020). Furthermore, highly efficient genome editing techniques have been established in this species (Daimon et al., 2014). Thus, *B. mori* is one of useful insects to examine a role of clock genes in the circadian rhythm and photoperiodism.

I established *per* knockout strains using TALEN in a nondiapause strain *pnd w-1* in Chapter 1 and a bivoltine strain Kosetsu in Chapter 2. In Chapter 1, I compared eclosion and hatching rhythms between the *per* knockout and wild type strains to examine whether *per* is actually involved in these rhythms. In Chapter 2, I reared larvae of the *per* knockout and wild type strains under long-day and short-day conditions and compared the occurrence of embryonic diapause and larval development between the two strains to examine whether *per* is involved in photoperiodism.

## Chapter 1

## Involvement of the clock gene *period* in the circadian rhythm of the silkmoth *Bombyx mori*

### Introduction

In Lepidoptera as in other insects, circadian rhythms have been reported both in behavioral activities and developmental events, such as hatching and adult eclosion (Broadhead et al., 2017; Kamimura and Tatsuki, 1994; Sakamoto et al., 2003; Sakamoto and Shimizu, 1994; Shimizu and Matsui, 1983; Suszczynska et al., 2017). However, the molecular clock producing these rhythms has not been clarified yet. The expression of *per* has been reported in several lepidopteran species, including the silkmoth *Bombyx mori* (Kotwica et al., 2009; Kobelkova, 2015; Sauman and Reppert, 1996; Sehadova et al., 2004; Wise et al., 2002). Unlike in other insects, including *D. melanogaster*, temporally regulated movement of the *per* product protein PERIOD (PER) into the nucleus is not observed in the brain of the Chinese tussar moth, *Antheraea pernyi* (Sauman and Reppert, 1996), and also, the tobacco hornworm, *Manduca sexta*, shows no rhythmic expression of *per* (Wise et al., 2002). Moreover, even though many studies in Lepidoptera have shown temporally regulated expression of *per* (Iwai et al., 2006; Kotwica et al., 2009; Kobelkova, 2015; Sauman and Reppert, 1996; Tao et al., 2017), the role of *per* in a behavioral or developmental rhythm has been unequivocally demonstrated only in the sperm release rhythm of moths (Kotwica et al., 2009). Thus, it remains necessary to examine the circadian clock in Lepidoptera further, with special reference to the role of *per*.

Suppressing mRNA expression by RNAi is an effective means of studying clocks in some insects (Moriyama et al., 2008, Uryu et al., 2013). Although RNAi has been applied to study the circadian clock in Lepidoptera also, the knockdown of *per* by RNAi continued for less than 24 h producing a delay of sperm release and only a slight effect on behavioral rhythms (Kotwica et al., 2009; Sandrelli et al., 2007). It is thus difficult to examine the mechanism of the circadian clock over the long term using RNAi in Lepidoptera. As an alternative to RNAi, genome editing techniques can be applied to Lepidoptera to establish specific gene knockout strains. Indeed, knockout strains of *Clock* and *cry-2* have been described for the monarch butterfly, *Danaus plexippus*, with the circadian rhythm in eclosion was shown to be disrupted in both (Merkert et al., 2016; Merlin et al., 2013). Thus, genome editing techniques can help to reveal the connection between clock genes and behavioral rhythms.

In *B. mori*, clear circadian rhythms are observed in adult eclosion and hatching (Oshiki and Watanabe, 1978ab; Sakamoto and Shimizu, 1994; Sakamoto et al., 2003; Shimizu and Matsui, 1983; Tanaka 1966abc). Because highly efficient genome editing techniques have been established in *B. mori* (Daimon et al., 2014), in Chapter 1, I investigated the effect of *per* knockout on eclosion and hatching rhythms in *B. mori*. The results demonstrated that the circadian rhythms in eclosion and hatching were disrupted in the *per* knockout strain, which indicates that the negative feedback loop of the circadian rhythm involving *per* is required for the production of behavioral rhythms in *B. mori*.

## Materials and methods

### *Insects*

The strain *pnd w-1* (*pnd, pigmented and non-diapausing egg; w-1, white egg 1*), a standard strain for transgenesis of *B. mori* (Tamura et al. 2000), was used as the wild type strain. Larvae were reared on an artificial diet (Kuwano-hana, JA Zennoh Gunma, Maebashi, Japan) under a LD 12:12 h cycle at 25.0 ± 1.0°C. The light intensity in the photophase produced by a daylight fluorescent lamp was 870 to 1450 mW/m. Knockout *B. mori* were generated using TALENs, as described previously (Takasu et al., 2013; Suppl. Fig. S1). The left and right TALEN binding sites are 5'-CATCGCTGACGGCTAC-3' and 5'-CCCGATCCACATGTCCT-3', respectively. TALEN mRNAs (400 ng; 200 ng + 200 ng for left and right TALEN mRNA, respectively) were injected into preblastoderm embryos. Established *per* knockout lines were not outcrossed to other standard strains so that I could compare the phenotypes and gene expression levels in the same genetic background, i.e., *pnd w-1*.

### *Genotyping*

For genotyping, the heads of moths or whole bodies of first-instar larvae were crushed in alkaline solution (50 mM NaOH) and then heated at 95°C for 10 to 15 min. After neutralization by the addition of an equal volume of 0.2 M Tris-HCl (pH 8.0), the supernatants were used as templates for PCR. PCR was performed using ExTaq (TaKaRa, Kusatsu, Japan) or Paq5000 (Agilent Technologies, Tokyo, Japan). Primers

used for genotyping were per-F (5'-ATGACTGCATGACGGCAACT-3') and per-R (5'-CTCTTCGACA AAGGATACGTAGC-3'). These primers were designed to hybridize to sequences upstream and downstream of the TALEN target site.

*Recording of hatching and eclosion rhythms*

To examine the hatching rhythm, each egg mass was divided into batches of approximately 20 eggs and kept under LD 12:12 h until recording. To examine the eclosion rhythm, pupae were removed from cocoons 6 days after pupation and kept under LD 12:12 h until recording. Hatching and eclosion rhythms were examined under LD 12:12 h, constant light (LL) or constant darkness (DD) at 25.0 ± 1.0℃. Recording commenced at lights-on (LL) or lights-off (LD 12:12 h and DD) before the predicted time of hatching. Hatching or eclosion was recorded using a digital camera (EX-ZR3100, CASIO, Tokyo, Japan or D5100, Nikon, Tokyo, Japan) at 30-min intervals. In darkness, red LED light (Kaito Denshi, Hasuda, Japan, 660 nm) was used as the light source for recording. The LED was wrapped with white tape to reduce the light intensity to approximately 0.3 $mW/m^2$.

*Analysis of rhythmicity*

The degree of rhythmicity in eclosion was measured by the parameter R (Winfree, 1970). Eclosion data from several days were pooled to calculate the total number of eclosions for each hour of the day. The 8-h period (gate) of the day with the highest number of eclosions was then determined. The parameter R was calculated by

dividing the number of eclosions outside this 8-h period by the number of eclosions within it and multiplying by 100. The theoretical range of R is from 0, if all moths emerge within the gate, to 200, if eclosion is distributed uniformly throughout the day. R values of 150 or greater show statistically uniform eclosion (Winfree, 1970). R values of 60 or less represent rhythmic eclosion, those between 60 and 90 represent weakly rhythmic eclosion, and those greater than 90 represent arrhythmic eclosion (e.g., Saunders, 1979; Smith, 1985; Watari, 2005).

The free-running period was defined as the mean interval between the medians of eclosion/hatching peaks. To examine the arrhythmicity in the knockout strain within the range of the free-running period in the wild type under the same conditions ±4 h (test range), the Rayleigh test was used with the "circular" package (Version 1.1.3) in R (Version 3.3.0) (Lund and Agostinelli, 2013; R Development Core Team 2013). This mode of analysis was selected because the present data of eclosion and hatching were not quantitatively sufficient for a chi-square periodogram. Before the Rayleigh test, time series data were converted to angle data using a test period chosen from the test range. Accepting the null hypothesis of uniformity ($p > 0.05$) indicated arrhythmicity during the chosen test period. The test period was chosen in turn from the test range, and the Rayleigh test was repeatedly carried out.

*Quantitative real-time PCR*

Quantitative real-time PCR (qPCR) was used to investigate the temporal expression patterns of *per* and *tim* mRNAs. Eggs were incubated under LD 12:12 h until sampling. Sampling was started at lights-off one day before hatching, when

embryos were at the body pigmentation stage. Sampling was performed every 4 h. Manipulation of the eggs under darkness was conducted under dim red light. Total RNA from an egg was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), treated with DNase I (Invitrogen), and then used to synthesize cDNA with High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). Quantitative PCR assays were performed using SYBR Green I (Roche, Basel, Switzerland) and a Light Cycler 96 system (Roche, Basel, Switzerland). The reaction conditions were as follows: 95℃ preinitiation heating for 1 min, followed by 45 cycles of 95℃ for 10 s, 58℃ for 10 s and 72℃ for 10 s. After PCR, a melting curve analysis was used to confirm the amplification of specific products. The *BmRp49* gene was used as a reference. The standard curve method was used to determine the expression levels of samples. The primers used for qPCR were the same as those used previously (Iwai et al. 2006): *per*-F (GTAATGCTCGGCGGGATATC) and *per*-R (AGCGG TGTTTCTGTGCTTGA) for *per, tim*-F (CTTGAAGTTT CTGCAGCTGTTACAC) and *tim*-R (AAACACTTT CCCGGAATCGA) for *tim*, and Rp49-F (ATGACGGG TCTCTTTGTTGGAA) and Rp49-R (CAGGCGATTC AAGGGTCAAT) for *rp49*. Two-way ANOVA was used to examine statistically significant differences in gene expression between the 2 strains, whereas one-way ANOVA was used to examine statistically significant temporal differences in each strain.

## Results

*Establishment of a period knockout strain*

To establish a *per* knockout strain, I used a TALEN-mediated gene-targeting approach, because TALENs have been shown to induce highly efficient targeted gene disruption in *B. mori* (Daimon et al., 2014; Takasu et al., 2013). I designed a target site in exon 7 of the *per* gene, an exon that encodes a PAS domain, which is known to interact with other proteins (Fig. 1). I injected TALEN mRNA into early embryos of the *pnd w-1* strain. The hatched larvae were reared, and adults were crossed with the parental strain (*pnd w-1*). In the next generation (G1), I screened for the induced mutations using the CEL-I assay (Daimon et al., 2014) and identified adults carrying mutant alleles of *per*. From the recovered *per* mutant adults with various mutant alleles, I selected an adult with a large (64-bp) deletion within exon 7 that produced a premature stop codon downstream of the target site (Fig. 1). This allele was considered a null allele, as it encoded an extensively truncated form of PER (198 aa in the protein from the 64-bp deletion allele; 1113 aa in the wild type protein). This mutant adult was crossed to an adult of the parental strain, and the 64-bp deletion allele was genetically fixed in the next generation (G2). The G2 adults were crossed to siblings and, in the next generation, I established a *per* knockout line. As *per* is located on the Z chromosome (ZW for females and ZZ for males in *B. mori*), females are hemizygous and males are homozygous for the *per* knockout allele. In Chapter 1, I used this knockout strain for further experiments and named it $per^{\Delta 64}$. In my rearing conditions

and under the *pnd w-1* genetic background, viability and fertility of $per^{\Delta 64}$ appeared to be normal without any apparent external phenotype.

*Eclosion rhythm*

Under LD 12:12 h, eclosion was observed in the middle of scotophase in the wild type, whereas in $per^{\Delta 64}$, a steep peak of eclosion occurred 1 h after lights-on, with eclosion also observed in scotophase (Fig. 2A, B). Under DD conditions, eclosion of the wild type showed a free-running rhythm with a period of approximately 23.5 h (Fig. 2C). The parameter R was 4.76 and the Rayleigh tests showed $p < 0.01$ at any chosen test period from the test range, indicating strong rhythmicity. In $per^{\Delta 64}$, however, eclosion was continuously observed from 17 h after lights-off (Fig. 2D), and the parameter R of 167 and $p > 0.1$, as shown by Rayleigh tests at any chosen test period from the test range, indicated arrhythmicity. Under LL conditions also, eclosion of the wild type showed a free-running rhythm but the period was approximately 16 h, which was much shorter than that under DD conditions (Fig. 2E). In $per^{\Delta 64}$ under LL conditions, eclosion was observed immediately after lights-on and continuously from 20 h after lights-on (Fig. 2F). In the Rayleigh test, the immediate peak after lights-on was excluded because this peak was considered a direct response to light. The parameter R of 153 and $p > 0.4$ shown by the Rayleigh tests at any chosen test period from the test range indicated arrhythmicity. Therefore, I concluded that $per^{\Delta 64}$ has lost circadian rhythm in eclosion.

*Hatching rhythm*

Wild type eggs hatched mostly around the time of lights-on, and the hatching peak occurred 1 h after lights-on under LD 12:12 h (Fig. 3A). In $per^{\Delta 64}$, however, hatching was observed even at other times of day, even though a steep peak was observed at 1 h after lights-on (Fig. 3B). Hatching of the wild type showed a free-running rhythm with a period of approximately 24.5 h under DD conditions. The parameter R was 10.78 and the Rayleigh tests showed $p < 0.01$ at any chosen test period during the test range (Fig. 3C), indicating strong rhythmicity, as seen in eclosion. Hatching of $per^{\Delta 64}$ was observed continuously from 2 h after lights-off (Fig. 3D), and the parameter R of 123 and $p > 0.03$, as shown by Rayleigh tests at any chosen test period during the test range, indicated arrhythmicity. Under LL conditions also, hatching of the wild type was observed as 3 groups, with median times of 1 h, 23 h, and 37 h after the start of the experiment. Although the third hatching group consisted of only a small number of larvae and its peak is unclear, the free-running period seemed to shorten gradually. The time intervals between the hatching groups were much shorter than those under DD conditions (Fig. 3E). Hatching of $per^{\Delta 64}$ was observed continuously from 6.5 h after the start of the observation (Fig. 3F). The Rayleigh tests indicated rejection of the null hypothesis of uniformity ($p < 0.01$ during 16-22 h), probably because hatching was restricted mostly to the first half of the observation period. However, the parameter R of 94 indicated arrhythmicity, and apparently hatching occurred sporadically in the first half of the observation period. Therefore, I concluded that $per^{\Delta 64}$ has lost circadian rhythm in hatching.

*Expression of clock genes*

To examine the effect of *per* knockout on the molecular clockwork, I performed qPCR of 2 clock genes, *per* and *tim*, in embryos at the body pigmentation stage. In the wild type, *per* expression showed temporal changes ($p < 0.05$, one-way ANOVA), whereas there was no significant difference in *per* expression over time in $per^{\Delta 64}$ ($p > 0.05$) (Fig. 4A). The results of the two-way ANOVA showed that *per* expression was significantly lower in $per^{\Delta 64}$ than in the wild type ($p < 0.01$) (Fig. 4A). In the wild type, *tim* expression also changed temporally ($p < 0.05$, one-way ANOVA), but did not significantly change in $per^{\Delta 64}$ ($p > 0.05$) (Fig. 4B). In $per^{\Delta 64}$, *tim* expression was significantly higher than in the wild type ($p < 0.05$) (Fig. 4B).

**Discussion**

I established a *per* knockout strain using TALEN in *B. mori*, and this is the first *per* knockout strain in insects excluding $per^{0}$ strain of *D. melanogaster* (Konopka and Benzar, 1971). The *per* knockout strain of *B. mori* produced in the present study showed periodic patterns in hatching and eclosion under LD 12:12 h conditions, but did not show free-running rhythms under LL or DD conditions. From these results, I concluded that *per* is indispensable for circadian rhythms in this species. In the *per* knockout strain, moreover, no temporal change in expression was observed for *per* or *tim*, and the expression levels of *per* and *tim* were lower and higher, respectively, than those of the wild type used in the present study (*pnd w-1*). Moreover, Tao et al. (2017) showed entry of PER into the nucleus in *B. mori*, suggesting a molecular feedback loop

that includes *per* in the circadian hatching rhythm of this insect. Therefore, I suggest that the molecular feedback loop involving *per* and *tim* was disrupted in the *per* knockout strain, and that this disruption probably caused the arrhythmicity of behavioral rhythms.

The level of *per* expression was extremely low at all time points in the *per* knockout strain. If this strain lacks negative feedback by PER/TIM, one could have anticipated that the *per* knockout strain would show high expression levels of *per* at all time points, like those of *tim*. However, the results contradicted this anticipation. In the $per^0$ mutant of *D. melanogaster*, in which the negative feedback loop of PER/TIM was established, the *per* expression level showed no consistent rhythmic fluctuation and was approximately 50% of that in the wild type at its peak (Hardin et al., 1990; van Gelder and Krasnow, 1996); thus, the results in *D. melanogaster* also cannot be simply explained by the lack of the negative feedback. Instead, the low expression levels of *per* can be explained by nonsense-mediated mRNA decay (NMD), which is known to occur generally for mRNA surveillance in eukaryotes: most mRNAs that cannot be translated along their full length are rapidly degraded (Cullbertson, 1999). Expression depression and disappearance of mRNA by NMD have been reported in *B. mori* (Komoto et al., 2009) and therefore *per* mRNA was probably degraded by NMD in the *per* knockout strain, because the *per* mRNA in this strain cannot be translated along its full length.

The wild type strain used in Chapter 1 showed circadian rhythms in hatching and eclosion, as reported in other strains (Tanaka, 1966abc; Oshiki and Watanabe, 1978ab; Sakamoto and Shimizu, 1994; Sakamoto et al., 2003; Shimizu and Matsui, 1983). In developed embryos of this strain, temporal changes in the expression of *per* and *tim* were observed during the day. Expression peaks occurred in the early

scotophase, and expression levels were low in the photophase. Similar expression trends were reported in the adult brain and larval midgut of *B. mori* (Iwai et al., 2006; Nobata et al., 2012). Tao et al. (2017) showed in the embryo of this species that the expression peak of *per* also resides in the earlier scotophase but that of *tim* occurred in the later scotophase. The differences between the results of Tao et al. (2017) and Chapter 1 in *tim* expression may be due to the difference in the strains.

In the *per* knockout strain under LD 12:12 h, there was an eclosion peak immediately after lights-on, but this peak was not observed in the wild type. In the eclosion rhythm of other strains previously studied, there were 2 peaks: one in the scotophase and the other immediately after lights-on under LD 12:12 h (Shimizu and Matsui, 1983; Ohsiki and Watanabe, 1978). Shimizu and Matsui (1983) concluded that the eclosion peak immediately after lights-on is produced by a masking effect: a direct response to light. I suggest, therefore, that, in *pnd w-1*, this direct response to light is concealed by a strong suppressive effect on circadian rhythm. In the *per* knockout strain, however, suppression by the circadian rhythm is negated and the direct response to light becomes obvious.

In the hatching rhythm of *B. mori*, Sakamoto et al. (2003) hypothesized that a carotenoid-dependent hourglass mechanism suppresses hatching after lights-off. This mechanism suppresses hatching on the first day under DD conditions and in the scotophase under LD 12:12 h. In the present study, however, this suppression was not observed in either the wild type or the *per* knockout strain. This difference might be due to the use of strains with different genetic backgrounds: I used a strain without a diapause due to its *pnd* background, whereas Sakamoto et al. (2003) used a diapause strain and artificially terminated the diapause by acid treatment.

In the present study, I established a *per* knockout strain in *B. mori*, and showed that *per* is essential for the expression of circadian rhythms at the behavioral and molecular levels. In the monarch butterfly, *Danaus plexippus*, the circadian eclosion rhythm was inhibited by the knockout of 2 other clock genes, *Clock* and *cryptochrome-2* (*cry-2*) (Markert et al., 2016; Merlin et al., 2013). These results show that the knockout method is a powerful tool to investigate connections between clock genes and behavior in Lepidoptera, and demonstrate that the negative feedback loop of circadian rhythm previously established in various insects, including *D. melanogaster* (Sandrelli et al., 2008; Tomioka and Matsumoto, 2015), also functions in Lepidoptera. In the circadian clock of *D. melanogaster, per* and *tim* are the predominant negative elements in the negative feedback loop: PER and TIM form heterodimers and enter the nucleus to suppress their own transcription (Hardin, 2005). In the honeybee *Apis melifera*, however, there is no *tim*, and *cry-2*, which is different from *Drosophila* type *cryptochrome* (*d-cry* or *cry-1*), plays a similar role to that played by *tim* in *D. melanogaster* (Rubin et al., 2006). Many other insects have both *tim* and *cry-2*, and their roles vary by species (Tomioka and Matsumoto, 2015). In *A. pernyi* and *D. plexippus* (Lepidoptera), entry of PER into the nucleus has not been observed (Sauman and Reppert, 1996; Sauman et al., 2005). Zhu et al. (2008) suggested that *cry-2* functions as a negative element without involvement of *per* in the feedback loop of the circadian clock in *D. plexippus*, in which the role of *per* as a negative element in the feedback loop was not shown. In the present study, I showed that *per* plays an essential role in the circadian clock of *B. mori*, and suggest that *tim* is also involved in the negative feedback loop because *per* knockout produced stable high expression of *tim*. In *B. mori*, however, it has not been examined whether PER is involved in the regulation of *cry-2* expression

or CRY-2 nuclear entry; although, *cry-2* does exist in this species (Tao et al., 2017). The role of *cry-2* in the circadian clock of *B. mori* is still unclear, and I cannot deny the possibility that the loss of circadian behavioral rhythms and *tim* oscillations in the *per* knockout strain in *B. mori* is intervened by the role of *cry-2*. Further studies are needed to clarify the complete molecular clockwork of Lepidoptera. The next step is to examine the temporal localization of clock proteins, e.g., CRY-2, in the *per* knockout strain of *B. mori*.

## Chapter 2

## Involvement of the Clock Gene *period* in the Photoperiodism of the Silkmoth *Bombyx mori*

### Introduction

For a decade, several authors have attempted to clarify the role of the circadian clock produced by the molecular mechanism in photoperiodism by reducing the expression of clock genes with the RNAi technique in some insects, i.e., the cricket, *Modicogryllus siamensis* (Sakamoto et al., 2009; Ueda et al., 2018), the bean bug, *Riptortus pedestris* (Ikeno et al., 2010, 2011ab, 2013), the northern house mosquito, *Culex pipiens* (Meuti et al., 2015), the jewel wasp, *Nasonia vitripennis* (Mukai et al., 2016), the brown-winged green bug, *Plautia stali* (Tamai et al., 2019), and the linden bug, *Pyrrhocoris apterus*, (Kotwica-Rolinska, 2017). Moreover, knockout experiments of clock genes were performed in the monarch butterfly, *Danaus plexippus* (Merlin et al., 2013; Markert et al., 2016; Iiams et al., 2019). All these results showed that suppression or dysfunction of clock genes disrupted photoperiodism, suggesting the involvement of the circadian clock consisting known clock genes in the photoperiodic clock. The results in a drosophilid fly *Chymoyiza costata* that a mutant strain of a clock gene *tim* has lost photoperiodism also support the above suggestion (Pavelka et al., 2003; Stehlik et al., 2008). However, Emerson et al. (2009) claimed that even if suppression of clock genes disrupted photoperiodism, the results might be caused by pleiotropic effects of the genes. In *P. apterus*, in fact, clock genes in peripheral tissues play a role in an organ-autonomous regulatory mechanism that is independent of

circadian clock at the downstream of juvenile hormone (JH) signaling (Bajgar et al., 2013ab).

The above results that suppression or dysfunction of clock genes disrupted photoperiodism have been obtained exclusively in insects with photoperiodism controlling larval (nymphal) diapause (Sakamoto et al., 2009; Mukai et al., 2016) or adult diapause (Ikeno et al., 2010, 2011ab, 2013; Meuti et al., 2015; Tamai et al., 2019; Kotwica-Rolinska, 2017; Iiams et al., 2019). In general, JH plays important roles in larval and adult diapause (Denlinger et al., 2012). In fact, JH has been shown to be involved in diapause in some of the above species (Spielman, 1974; Hodkova, 1976; Herman, 1981; Numata and Hidaka, 1984; Kotaki and Yagi, 1989; Matsumoto et al., 2013; Miki et al., 2020). Therefore, we cannot eliminate the possibility of pleiotropic effects of clock genes, such as non-lock roles in the JH signaling pathway on photoperiodism.

In the silkmoth *Bombyx mori*, maternally-induced embryonic diapause has been examined extensively. Kogure (1933) first reported that progeny diapause is determined by photoperiod and temperature during embryonic development of the mother. Later, a photoperiodic response was reported in the larval stage of the maternal generation also (Takamiya, 1974; Kobayashi et al., 1986; Hasegawa and Shimizu, 1987). In *B. mori*, clock genes have been identified and knockout of *period* (*per*) or *timeless* (*tim*) resulted in loss of circadian rhythms (Iwai et al., 2006; Nartey et al., 2021; Chapter 1). Therefore, it is possible to examine the relationship between clock genes and photoperiodism in *B. mori*. In this species, embryonic diapause is regulated by diapause hormone, a peptide synthesized in the subesophageal ganglion and GABAergic and corazonin pathway in the brain of the mother (Yamashita and

Hasegawa, 1985; Yamashita, 1996; Tsuchiya et al., 2021) and ERK/MAPK signaling cascade in the embryo (Fujiwara et al., 2006ab). Thus, the regulatory mechanism of this diapause is quite different from that for larval and adult diapause. If knockout of a clock gene results in the loss of photoperiodism in *B. mori*, the results further support that the circadian clock is involved in photoperiodism at the upstream of hormonal output.

In Chapter 1, I produced a *per* knockout strain of *B. mori* that had lost circadian rhythms, but this strain originated from a nondiapause strain *pnd w-1*. In this chapter, therefore, I established a new *per* knockout strain using a bivoltine strain Kosetsu, which has been used for a genetic examination of diapause using a knockout technique (Shiomi et al., 2015). I confirmed that the strain lost circadian rhythms in eclosion and hatching, and then reared wild type and the *per* knockout strains under long-day or short-day conditions to examine their photoperiodism. The results showed that the photoperiodism was disrupted in the *per* knockout strain, indicating that the circadian clock involving *per* is required for photoperiodism in *B. mori*.

## Materials and methods

### *Insects*

A bivoltine strain (Kosetsu) of *B. mori* was used as the wild type in Chapter 2. Diet and the light intensity were same as those in Chapter 1. Knockout *Bombyx mori* were generated using TALENs, as described previously (Takasu et al., 2013). TALEN binding sites and mRNA were same as Chapter 1. Established *per* knockout lines were

not outcrossed to other standard strains so that I could compare the phenotypes and gene expression levels in the same genetic background, i.e., Kosetsu.

*Genotyping*

Genotyping was performed according to Chapter 1.

*Recording of hatching and eclosion rhythms*

Larvae were reared under a LD 12:12 h cycle at 25.0 ± 1.0℃. To examine the hatching rhythm, an egg mass was divided into batches of approximately 20 eggs, and kept under LD 12:12 h until recording. To examine the eclosion rhythm, pupae were removed from cocoons 6 days after pupation and kept under LD 12:12 h until recording. Hatching and eclosion rhythms were examined under the following two conditions: LD 12:12 h and constant darkness (DD) at 25.0℃. Recording was started at light-off (LD 12:12 h and DD) before the predicted time of hatching. Recording was performed according to Chapter 1.

*Analysis of rhythmicity*

The degree of rhythmicity in eclosion and hatching was measured by the parameter R (Winfree, 1970). In this study, I modified this method. Eclosion and hatching data from several days were pooled to calculate the total number of eclosions and hatchings for each hour of the free-running period. The 1/3 period (gate) of the free-

running period containing the highest number of eclosions was then determined. The parameter R was calculated by dividing the number of eclosions outside this gate by the number of eclosions within it and multiplying by 100. The theoretical range of R is from 0, if all moths emerge within the gate, to 200, if eclosion is distributed uniformly throughout the day. R values of 150 or greater show statistically uniform eclosion (Winfree, 1970). R values of 60 or less represent rhythmic eclosion, those between 60 and 90 represent weakly rhythmic eclosion, and those greater than 90 represent arrhythmic eclosion (e.g., Saunders, 1979; Smith, 1985; Watari, 2005).

Defining free-running periods and the Rayleigh test were performed according to Chapter 1.

*Quantitative real-time PCR*

Quantitative real-time PCR (qPCR) was used to investigate the temporal expression patterns of *per* and *tim* mRNAs. Larvae were reared under two different photoperiods: LD 12:12 h and LD 16:8 h at 25.0°C. Sampling of the head was started at 1 h after light-on, when larvae were 5th stage at 3 days after molting. Sampling was performed every 4 h. The manipulation of the larvae under darkness was conducted under dim red light. Extraction of Total RNA, synthesizing cDNA and Quantitative PCR were performed according to Chapter 1.

To examine statistically significant differences in gene expression between the two strains, two-way ANOVA for the strain and the time point was used, and to examine the statistical significance of temporal differences in each strain one-way ANOVA was used.

*Recording of development time and photoperiodic response*

Eggs were incubated under constant light at 20℃, and hatched larvae and pupae were reared under two different photoperiods: LD 16:8 h (long-day) and LD 12:12 h (short-day) at 25℃. Larval instars were examined every day, and pupae were weighed 8 days after spinning.

Eclosed moths were allowed to oviposit overnight and these eggs were incubated at 25℃. Eggs were kept at 25℃ for 14 days and determined as diapause or nondiapause. Diapause eggs were identified as those that were colored dark brown and remained unhatched at 14 days after oviposition, at which time all nondiapause eggs had hatched. Females laying only diapause eggs and those laying only nondiapause eggs were judged to be diapause-egg and nondiapause-egg producers, respectively, in addition to those of mixed-egg producers laying both diapause and nondiapause eggs.

In *B. mori*, low temperature in the larval stage induces diapause (Takizawa and Kato 1984). Therefore, to examine whether the *per* knockout strain retains the ability to lay diapause egg, larvae of the *per* knockout strain were reared under LD 6:18 h at 20°C, and diapause incidence of their progeny was examined.

**Results**

*Establishment of a period knockout strain*

I used a TALEN-mediated gene-targeting approach to establish a *per* knockout line according to Chapter 1 using a bivoltine strain, Kosetsu. I selected a 13-bp insertion in the targeted exon 7. This allele was considered a null allele, because a new stop

codon was come up in the insertion allele (Fig. 5). I used this *per* knockout strain for further experiments.

*Eclosion and hatching rhythms*

To examine whether the *per* knockout strain has lost circadian rhythms, I recorded eclosion and hatching rhythms under LD 12:12 h and constant darkness. Under LD 12:12 h, eclosion was observed in the latter half of scotophase in the wild type, whereas in the *per* knockout strain, a steep peak of eclosion occurred 1.5 h after lights-on, with eclosion also observed even at other times of day (Fig. 6A, B). Under constant darkness, eclosion of the wild type showed a free-running rhythm with a period of approximately 30 h (Fig. 6C). The parameter R was 17.8 and the Rayleigh tests showed $p < 0.01$ at any chosen test period from the test range, indicating strong rhythmicity. In the *per* knockout strain, however, eclosion was continuously observed (Fig. 6D), and the parameter R of 165.6 and $p > 0.4$, as shown by Rayleigh tests at any chosen test period from the test range, indicated arrhythmicity.

Wild type eggs hatched mostly around the time of lights-on, and the hatching peak occurred 1 h after lights-on under LD 12:12 h (Fig. 7A). In the *per* knockout strain, however, hatching was observed even at other times of day, even though a steep peak was observed at 1 h after lights-on (Fig. 7B). Hatching of the wild type showed a free-running rhythm with a period of approximately 24.5 h under DD conditions. The parameter R was 9 and the Rayleigh tests showed $p < 0.01$ at any chosen test period during the test range (Fig. 7C), indicating strong rhythmicity, as seen in eclosion. Hatching of the *per* knockout strain was observed continuously (Fig. 7D), and the parameter R of 148.9 and $p > 0.03$, as shown by Rayleigh tests at any chosen test period

during the test range, indicated arrhythmicity.

Therefore, I concluded that the *per* knockout stain has lost circadian rhythms both in eclosion and hatching.

*Expression of clock genes*

I measured *per* and *tim* mRNA levels in the heads of 5th instar larvae 3 days after 4th ecdysis under long-day and short-day conditions by qPCR to examine whether clock gene expression shows temporal expression changes. Under the long-day condition, the wild type showed a clear temporal expression change in *per* and *tim* expression ($p < 0.05$, one-way ANOVA) (Fig. 8B and 8C), whereas there was no significant difference in *per* and *tim* expression of the *per* knockout strain among the six time points ($p > 0.3$) (Fig. 8A and 8C). Under the short-day condition, the wild type show a weak temporal expression change but the difference was not statistically significant (*per*, $p = 0.08$; *tim*, $p = 0.12$), but the *per* knockout strain showed no significant difference in *per* and *tim* expression among the six time points ($p > 0.3$) (Fig. 8B, and 8D). Under both conditions, the results of the two-way ANOVA showed a significant difference of *per* and *tim* expression in the *per* knockout and wild type strains ($p < 0.01$). Only under the long-day condition, the results of two-way ANOVA showed a significant difference in the time points and in the interaction between the strains and time points ($p < 0.01$). In the wild type, the highest time of *per* and *tim* mRNA levels was ZT17 under both conditions. However, the lowest time was different between the long-day and short-day conditions, i.e., ZT9 and ZT5, respectively.

*Photoperiodic response*

To investigate photoperiodism of Kosetsu and the *per* knockout, I reared larvae under long-day or short-day conditions at 25℃. All wild type moths laid only nondiapause eggs under a long-day condition, whereas 94% of moths laid diapause egg under the short-day condition, indicating a clear photoperiodic response (Tukey-type Multiple comparison for proportions, $p < 0.05$, Zar 2010) (Fig. 9A). In the *per* knockout strain, 96% of moths laid only nondiapause eggs even under the short-day condition (Fig. 9A). No significant difference was observed in the proportions of moths laying diapause eggs among the long-day condition of the wild type and both conditions of the *per* knockout strain ($p > 0.05$).

Thus, most adults of the *per* knockout strain laid only nondiapause eggs both under long-day and short-day conditions. To examine whether the *per* knockout strain retains the ability to lay diapause egg, I reared larvae of the *per* knockout under LD 6:18 h at 20°C. Under these conditions, 23% females laid only diapause egg and 46% females produced both nondiapause and diapause eggs (Fig. 9B).

The larval duration from hatching to spinning was significantly shorter under long-day than under short-day condition both in the wild type and the *per* knockout strains (Tukey test, $p < 0.05$) (Table 1). Moreover, the larval period was significantly shorter in the *per* knockout strain than the wild type under both conditions ($p < 0.05$). The pupal weight was significantly less under long-day than under short-day conditions in both sexes of both strains ($p < 0.05$). However, the difference in the pupal weight between the two strains in each sex was not statistically significant ($p < 0.05$).

## Discussion

The present results show that knockout of *per* disrupted the behavioral circadian rhythms and temporal expression change of clock genes, and photoperiodism controlling the induction of embryonic diapause in *B. mori* (Figs. 6, 7, 8 and 9). I conclude that *per* plays important roles both in the circadian rhythm and photoperiodism in this species. Involvement of clock genes in photoperiodism has been shown by knockdown and knockout experiments in insects with photoperiodism controlling larval or adult diapause (Sakamoto et al., 2003; Ikeno et al., 2010, 2011ab, 2013; Bajgar et al., 2013ab; Meuti et al., 2015; Mukai et al., 2016; Kotwica-Rolinska., 2017; Ueda et al., 2018; Tamai et al., 2019; Iiams et al., 2019). Generally, larval and adult diapause is regulated by the JH pathway: JH maintains diapause in the larval stage and avert diapause in the adult stage (Denlinger et al., 2012). In contrast, embryonic diapause in *B. mori* is regulated by a different hormonal pathway in the mother (Yamashita and Hasegawa, 1985; Yamashita, 1996; Tsuchiya et al., 2021), and ERK/MAPK signaling cascade in the embryo (Fujiwara et al., 2006ab). Thus, suppression or dysfunction of clock genes disrupted photoperiodism for diapause controlled by different hormonal effectors. It is more probable that the clock gene *per* plays a role as a component of the circadian clock at the upstream of the hormonal effector rather than that *per* brings out a pleiotropic (non-clock) effect on the photoperiodic induction of diapause suggested by Bradshaw and Holzapfel (2010). The opposite effects by RNAi of positive and negative elements on photoperiodic diapause in *R. pedestris* also support that the cyclic phase change of the circadian clock is required for photoperiodism (Ikeno et al., 2010, 2011ab, 2013, see Numata et al., 2015

also). Even in the *per* knockout strain established in the present study, moreover, when larvae of the maternal generation were reared under LD 6:18 h at a lower temperature of 20°C, some progeny embryos entered diapause (Fig. 9B). The results indicate that the neural and endocrine mechanisms for diapause induction are retained in this strain and further support that *per* is necessary for the upstream photoperiodic clock.

Kosetsu, a strain of *B. mori* used in Chapter 2, showed a longer free-running period for circadian eclosion and hatching rhythms (Figs. 6 and 7) than *pnd w-1* used in Chapter 1 and other strains previously reported (Chapter 1; Shimizu and Miura 1987; Nartey et al., 2021; Oshiki and Watanabe, 1978a; 1978b; Sakamoto and Shimizu, 1994; Sakamoto et al., 2003). Diversity of free-running periods between geographic strains has been reported in several insects (Lankinen 1986; Sawyer et al., 1997; Pivarciova et al., 2016). Xiang et al. (2018) surveyed the genes selected in domestication of *B. mori* and pointed out that circadian clock related genes were selected in domesticated groups. Especially, *cry-2* and *shaggy* (*sgg*) showed selection signatures among the Chinese wild silkmoth, *Bombyx mandarina*, and various local strains of *B. mori*. In *D. melanogaster*, *sgg* regulates the period length of the circadian clock by phosphorating TIM (Martinek et al., 2001). By the genome-wide association study, Kumar et al (2021) listed the genes that affect the circadian period in *D. melanogaster* and showed that *Kairos* plays a critical role in determining the period length. Although the gene responsible for the longer circadian period in Kosetsu is unclear, *sgg*, *Kairos*, or other genes listed by Kumar et al (2021) might be involved.

When a negative element of the circadian clock is disabled, higher expression of the genes, such as *per* and *tim*, regulated by positive elements, CYC and CLK, is expected. In Chapter 2, however, the expression levels of *per* and *tim* were constantly

low (Fig. 8). Low expression levels of the target gene of knockout were explained as nonsense-mediated mRNA decay (Chapter 1; Nartey et al., 2021). However, this cannot explain the low expression levels of *tim* that was not the target of knockout in Chapter 2. In another *per* knockout strain derived from *pnd w-1* of *B. mori*, *tim* was highly expressed (Fig. 4B). However, in a *tim* knockout strain established in the strain *Nistari* of *B. mori*, *per* showed low expression as in the *per* knockout strain in this chapter (Nartey et al., 2021). In the monarch butterfly, knockout of *cry-2* showed high expression of *per* and *tim* (Merlin et al., 2013). Zhu et al (2008) showed that *cry-2* is the dominant repressor in circadian clock and *per* is important for stabilizing *cry-2*. In the present study, it is unclear why expression of *tim* was suppressed by *per* knockout in Kosetsu.

The temporal expression changes of *per* and *tim* under LD 12:12 h in Chapter 2 (Fig. 8B and 8D) were different from those in the previous studies (Iwai et al., 2006; Tao et al., 2017; Nartey et al., 2021) and Chapter 1 (Fig. 4). Both the tissues and strains used for the expression analysis are different between the present and previous studies: The expression was examined in the head, antenna and flight muscle of adults of *N4* (Iwai et al., 2006), the whole embryo of *c108* (Tao et al., 2017), *pnd w-1* (Chapter 1) and *Nistari* (Nartey et al., 2021), and the larval head of Kosetsu in Chapter 2. The peak time of *per* expression was shown at the middle of the photophase (*Nistari*), lights-off (*N4* and *c108*) or the early scotophase (*pnd w-1* and Kosetsu). Some strains (*Nistari*, *c108*, Kosetsu and *N4*) continued high expression levels of *per* after the peak time. It seems that the difference of the expression pattern is caused by the difference of strains or developmental stages because the difference was observed even in the same developmental stage, embryo, between different strains (*pnd w-1*, *Nistari*, and *c108*).

Moreover, the timing of eclosion was also different among strains. Eclosion occurred in the middle of the scotophase in *pnd w-1*(Fig. 2A), throughout scotophase in *Nistari* (Nartey et al., 2021) and in the later scotophase in Kosetsu (Fig. 6A). These results show diversity of the circadian clock among strains in *B. mori*. However, it is difficult to explain the relation between the expression patterns of clock genes and the timing of eclosion, because in these studies temporal expression changes of *per* were not examined in the pupal brain.

In Chapter 2, both the wild type and *per* knockout strains of *B. mori* showed a photoperiodism in the larval development (Table 1). In both strains, larval duration was significantly shorter and the pupal weight was significantly less under long-days than short-days. To explain that the *per* knockout strain showed photoperiodism, I propose two hypotheses: The first is that a *per* independent circadian clock is involved in the photoperiodism, and the other is that no circadian clock is involved in it. In *D. melanogaster*, the existence of *per* independent circadian clocks has been shown (Helfrich and Engelmann, 1987; Helfrich-Förster 2001; Ito et al., 2008), and $per^0$ mutant also shows photoperiodism for ovarian development (Saunders et al., 1989; Saunders, 1990). It is a subject of a future study which is applicable to the photoperiodism in the larval development of *B. mori.*

**General discussion**

In Chapter 1 and Chapter 2, *pnd w-1* and Kosetsu were used to establish *per* knockout strains, respectively. Behavioral rhythms and temporal expression changes of clock genes were disrupted in both *per* knockout strains. This repeatability convinced that *per* is involved in the circadian clock for the production of circadian rhythms in *B. mori*.

What is the role of *per* in generating the circadian clock? In *D. melanogaster*, PER forms a heterodimer with TIM, and inhibits the function of the CYC-CLK complex that promotes the expression of genes including *per* and *tim* (Sandrelli et al., 2008; Tomioka and Matsumoto, 2015). In Chapter 1, the *per* knockout strain from *pnd w-1* showed high levels of *tim* expression at all time points. This result can be expected from the function of *per* and *tim* in *D. melanogaster*. Therefore, *per* of *pnd w-1* may have the same function as that in *D. melanogaster*. However, expression of *tim* was maintained at low levels in the *per* knockout strain from Kosetsu. Low expression of genes regulated by CYC-CLK was reported in the *tim* knockout strain from *Nistari* also (Nartey et al., 2020). Translocation of PER into the nucleus was not observed in the adult brain of *p50* but observed in the embryo head of *c108* (Sehadova et al., 2004; Tao et al., 2017). These results suggest different roles of *per* for organizing the circadian clock between the strains.

The genes selected in domestication of *B. mori* were analyzed by the genome-wide association study. The results of the genome-wide association study mention that circadian clock related genes were selected in domesticated groups (Xiang et al., 2018). In the present study, Kosetsu has a longer free-running period than *pnd w-1*. Moreover,

*tim* expression levels of the *per* knockout strains were different between *pnd w-1* and Kosetsu. This finding suggests diversity of the circadian rhythm and the circadian clock mechanisms between the strains. Thus, further studies comparing genome and behavioral rhythm between the strains of *B. mori* might reveal the stability and transition of the circadian clock in evolution.

In the present study, the *per* knockout strain from Kosetsu produced nondiapause eggs under diapause-inducing short-day conditions. In *D. plexippus*, a knockout strain of a negative element, *cry-2*, showed no mature oocytes under nondiapause inducing long-day conditions. The results were opposite in terms of diapause induction between *B. mori* and *D. plexippus*. Here I propose a hypothesis to explain this difference: an output signal of the circadian clock for photoperiodism is reversed between the two species. Kawahara et al (2018) estimated that 75-85% species are nocturnal and the others are diurnal in Lepidoptera. *Bombyx mori* belongs to Bombycoidea in which most species exhibit nocturnal activity, whereas *D. plexippus* belongs to Papilionidea (Butterfly), a diurnal group. In a recent phylogenomics study, butterflies are considered to have become diurnal from the other Obtectomera in the Late Cretaceous Period (98.3 million years ago; CI, 110.3 to 86.9 million years ago) (Kawahara et al., 2019). Hence, I point out a possibility, that the output signal of the circadian clock is reversed simultaneously with the change of the activity time from nocturnal to diurnal. Knockout experiments of clock genes in other family or superfamily are needed to examine this hypothesis.

In the study of the molecular mechanisms in insect photoperiodism, extensive studies have been performed by knockdown and knockout of *per*, *tim*, *cycle*, *Clk*, *cry1* and *cry-2* (Sakamoto et al., 2009; Mukai et al., 2016; Ikeno et al., 2010, 2011ab, 2013;

Meuti et al., 2015; Tamai et al., 2019; Kotwica-Rolinska, 2017; Iiams et al., 2019). In these studies, suppression or dysfunction of clock genes disrupted photoperiodism. These results have been obtained in insects in which diapause is regulated by the juvenile hormone. The present study showed the involvement of *per* in photoperiodism in *B. mori* in which diapause is regulated by the diapause hormone. Therefore, I conclude that the circadian clock is involved in photoperiodism at the upstream of hormonal regulation of diapause. However, the whole picture of the molecular mechanisms of photoperiodism is still unclear. Recently, Iiams et al. (2019) reported that the vitamin A pathway is changed by experienced photoperiod in *D. plexippus* and involved in photoperiodism. Knockout of *ninaB1* in this species resulted in development of mature oocytes under diapause-inducing short-day conditions although the circadian rhythm in eclosion remained. Therefore, the vitamin A pathway is at the downstream of the circadian clock (Iiams et al., 2019). Opsins, vitamin A-based pigments, were conceived as photoperiodic photoreceptors (Saunders, 2012). In *B. mori*, an opsin named BOCEROPSIN was considered to be involved in photoperiodism because its estimated wavelength of absorption was similar to the effective wavelength in photoperiodism (Shimizu et al., 2001). Shimizu et al. (1984) showed the involvement of vitamin A in photoperiodism in *B. mori*. Further study is needed to clarify the whole molecular mechanism in photoperiodism in this species. A next step is to examine the genes involved in the photoreception for photoperiodism, e.g., *boceropsin* knockout from Kosetsu.

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**Figures**

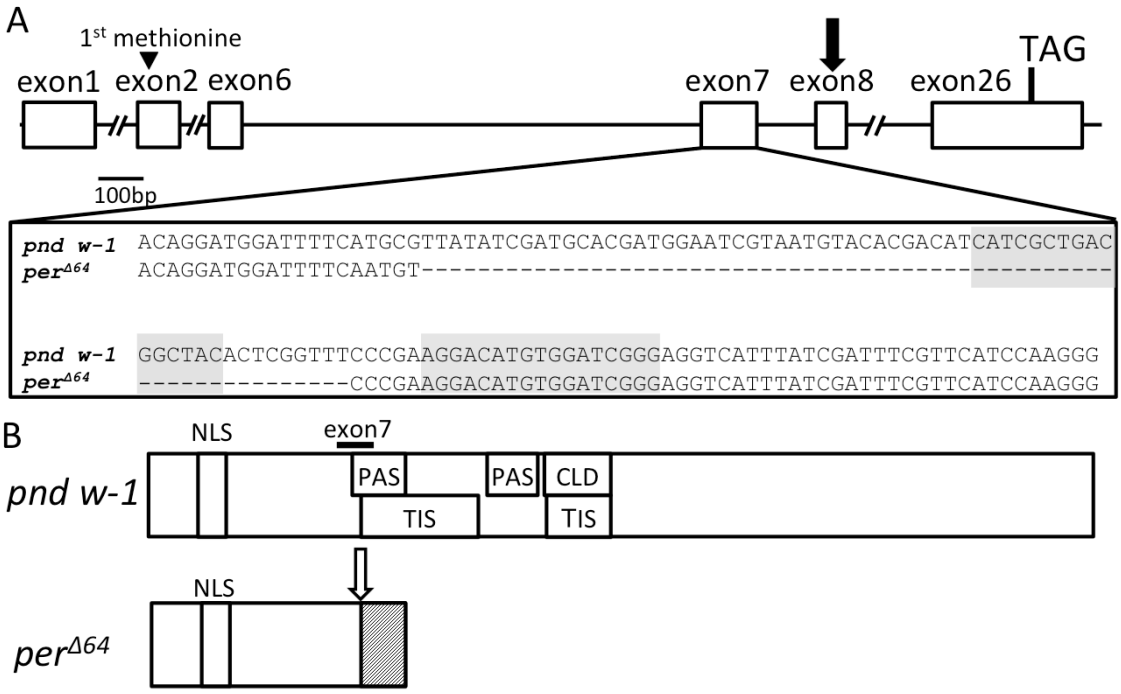


Figure 1. Schematic representation of the *per* knockout allele generated in Chapter 1. (A) Genomic structure of the *period* gene of *Bombyx mori*. The black arrow indicates the premature stop codon in the 64-bp deletion allele, and TAG shows the original stop codon. The nucleotide sequence of exon 7 with the TALEN targeting site and the introduced mutation are shown at the bottom. The gray boxes indicate the TALEN binding sites. (B) Structure of PERIOD protein of *B. mori*. Motifs corresponding to the functional domains in PERIOD of *Drosophila melanogaster* are shown: NLS (nuclear localization signal), PAS (Per-Arnt-Sim) domain, TIS (TIM interaction site) and CLD (cytoplasmic localization domain) (Iwai et al 2006). The white arrow and the downstream hatched box indicate the mutation site and truncation, respectively, of PERIOD resulting from genome editing.

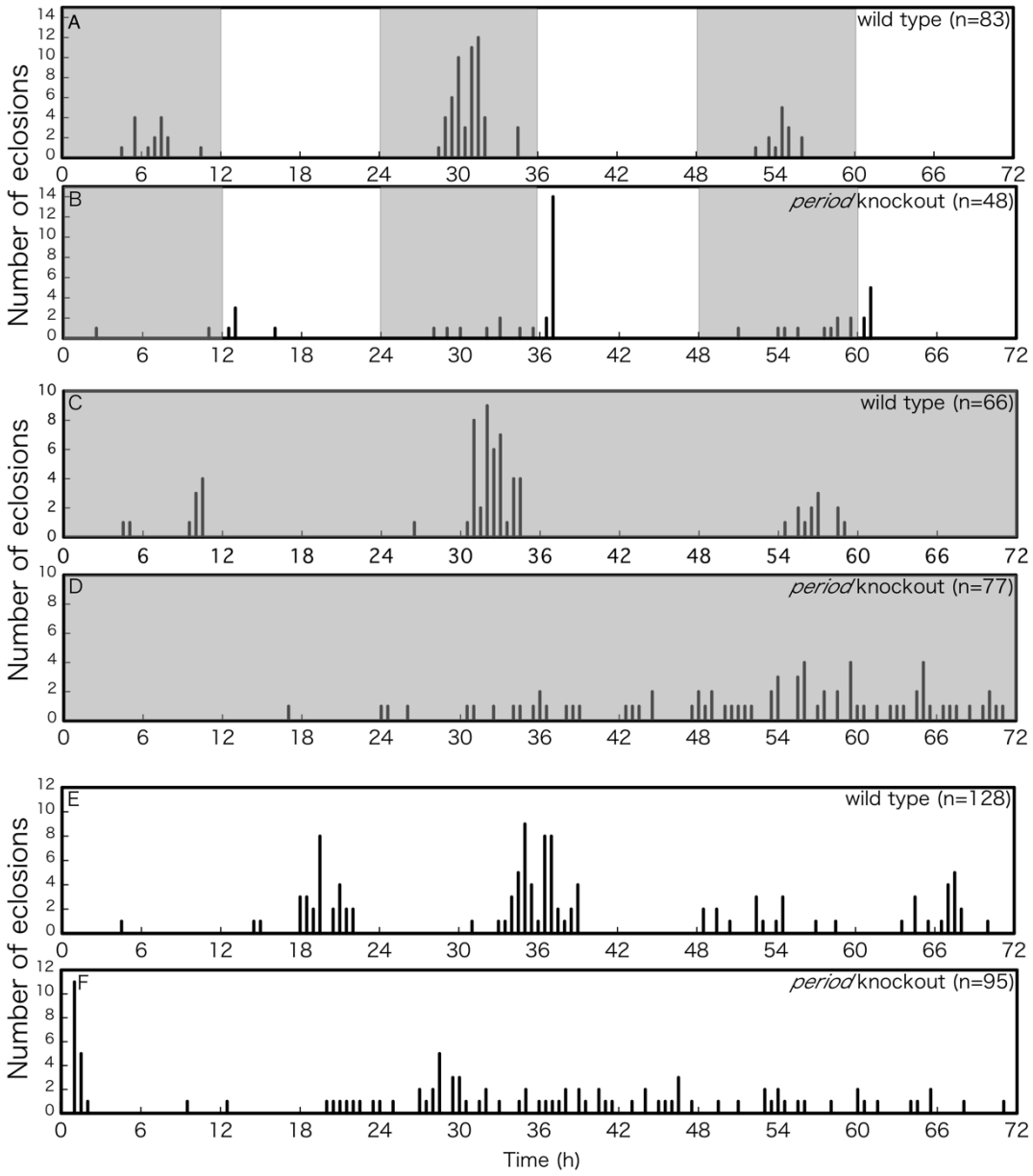


Figure 2. Distribution of eclosions in the wild type and *per* knockout strains of *Bombyx mori* (*pnd w-1*) under LD 12:12 h, DD, and LL at 25°C. Pupae were kept under LD 12:12 h before observation. Shaded areas show dark periods.

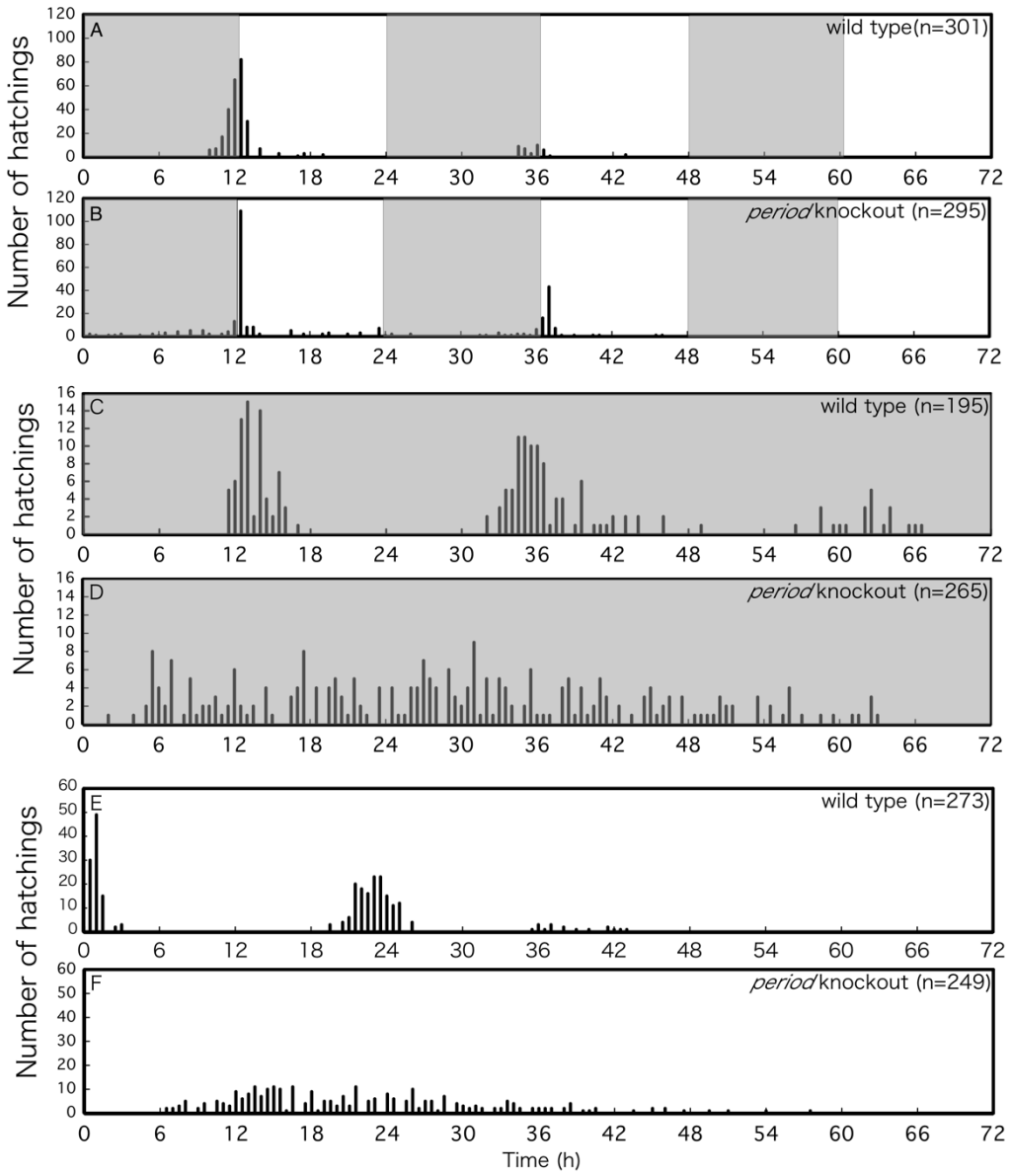


Figure 3. Distribution of hatchings in the wild type and *per* knockout strains of *Bombyx mori* (*pnd w-1*) under LD 12:12 h, DD, and LL at 25°C. Eggs were kept under LD 12:12 h before observation. Shaded areas show dark periods.

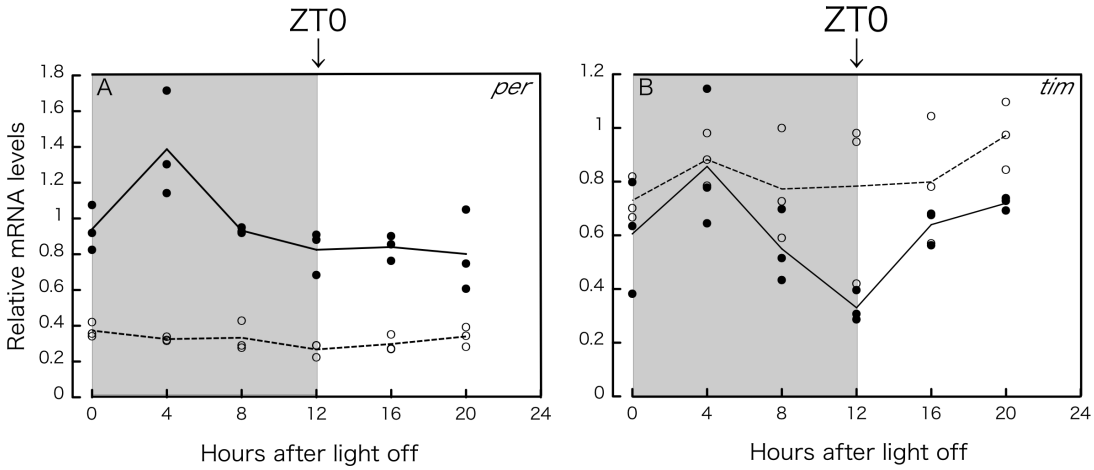


Figure 4. Temporal expression patterns of clock genes *period* and *timeless* in the eggs of the wild type (closed circles) and *per* knockout (open circles) strains of *Bombyx mori* (*pnd w-1*) under LD 12:12 h at 25°C. Eggs were kept under LD 12:12 h until the start of sampling. mRNA expression was analyzed by performing quantitative real-time PCR. Relative levels were standardized against mRNA levels of *Bmrp49*. Solid and broken lines show the mean of relative expression levels designated as closed and open circles, respectively (n = 3) in the wild type and *per* knockout strains, respectively. Shaded areas show dark periods.

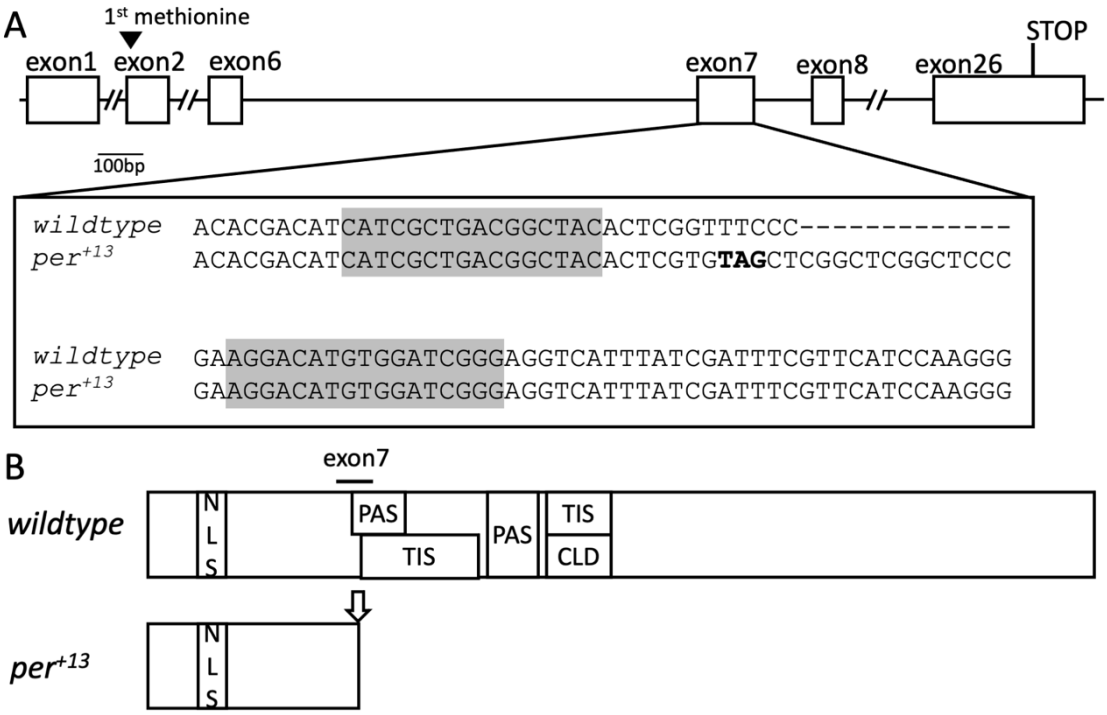


Figure 5. Schematic representation of the *per* knockout allele generated in Chapter 2. (A) Genomic structure of the *period* gene of *Bombyx mori*. Bold word indicates the premature stop codon in the 13-bp insertion allele, and STOP shows the original stop codon. the nucleotide sequence of exon 7 with the TALEN targeting site and the introduced mutation are shown at the bottom. The gray boxes indicate the TALEN binding sites. (B) Structure of PERIOD protein of *B. mori*. Motifs corresponding to the functional domains in PERIOD of *Drosophila melanogaster* are shown: NLS (nuclear localization signal), PAS (Per-Arnt-Sim) domain, TIS (TIM interaction site) and CLD (cytoplasmic localization domain) (Iwai et al 2006). The white arrow and the downstream hatched box indicate the mutation site and truncation, respectively, of PERIOD resulting from genome editing.

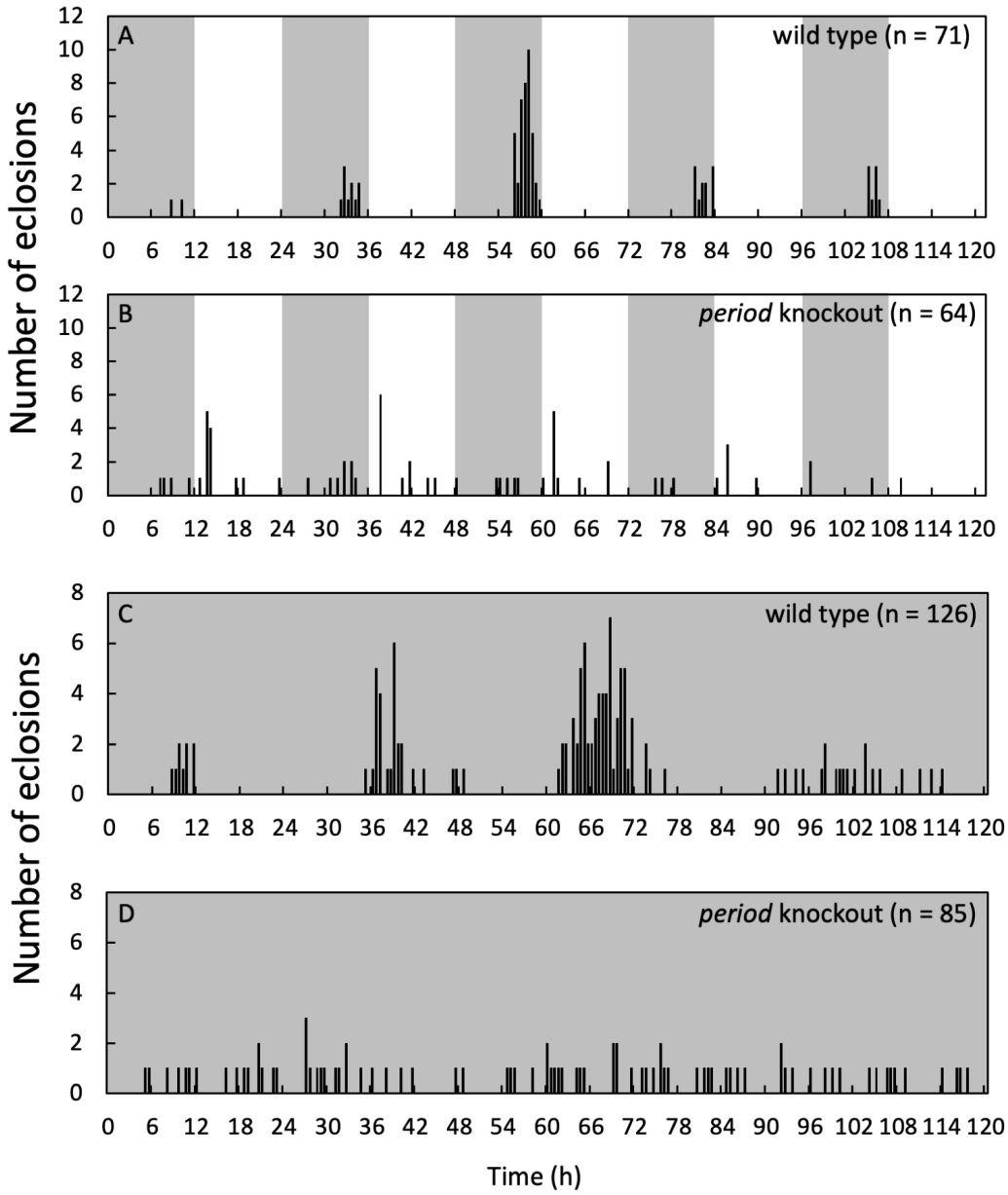


Figure 6. Distribution of eclosion in the wild type and *per* knockout strains of *Bombyx mori* (Kosetsu) under LD 12:12 h and DD at 25°C. Pupae were kept under LD 12:12 h before observation. Shaded areas show dark periods.

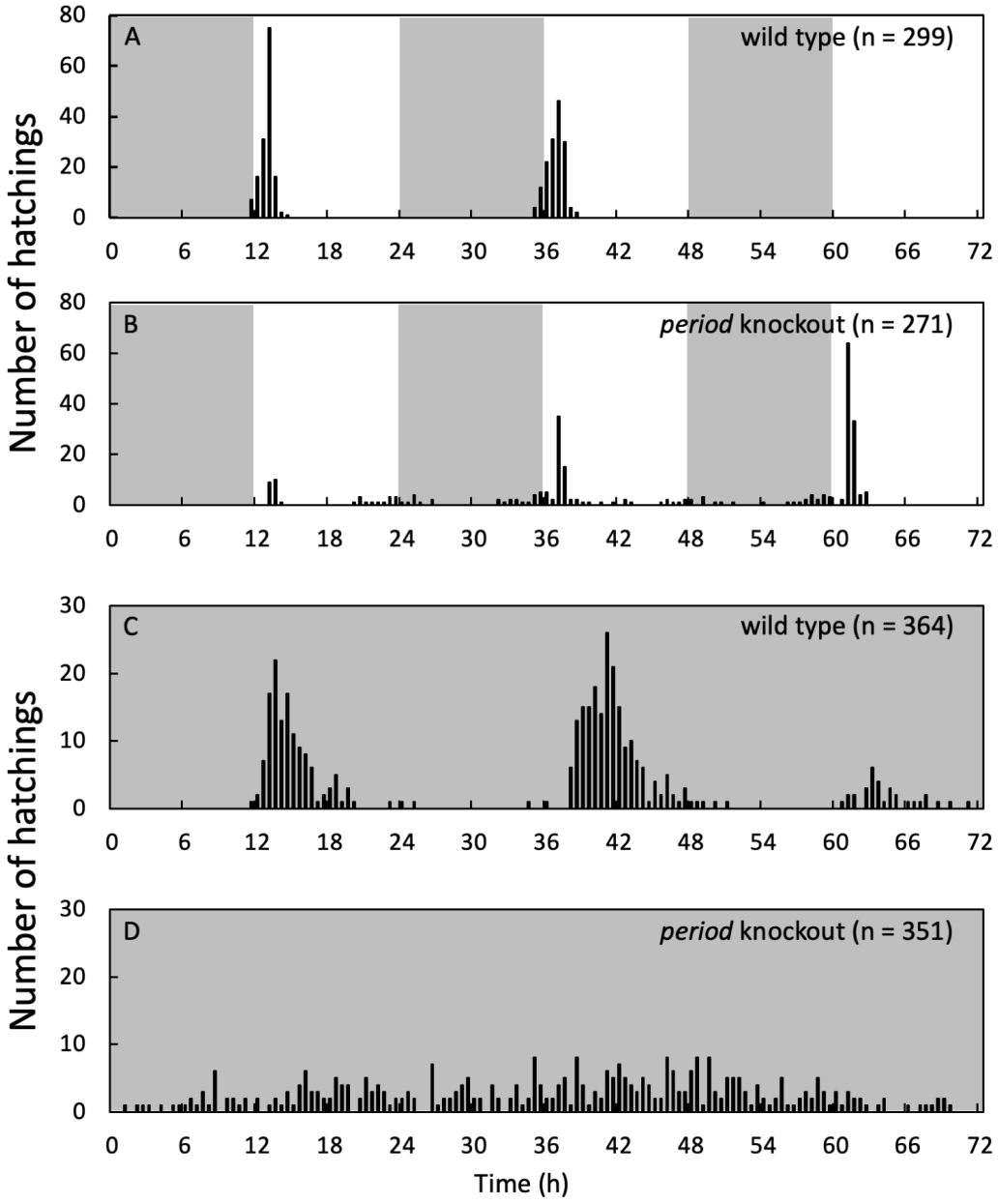


Figure 7. Distribution of hatchings in the wild type and *per* knockout strains of *Bombyx mori* (Kosetsu) under LD 12:12 h and DD at 25°C. Eggs were kept under LD 12:12 h before observation. Shaded areas show dark periods.

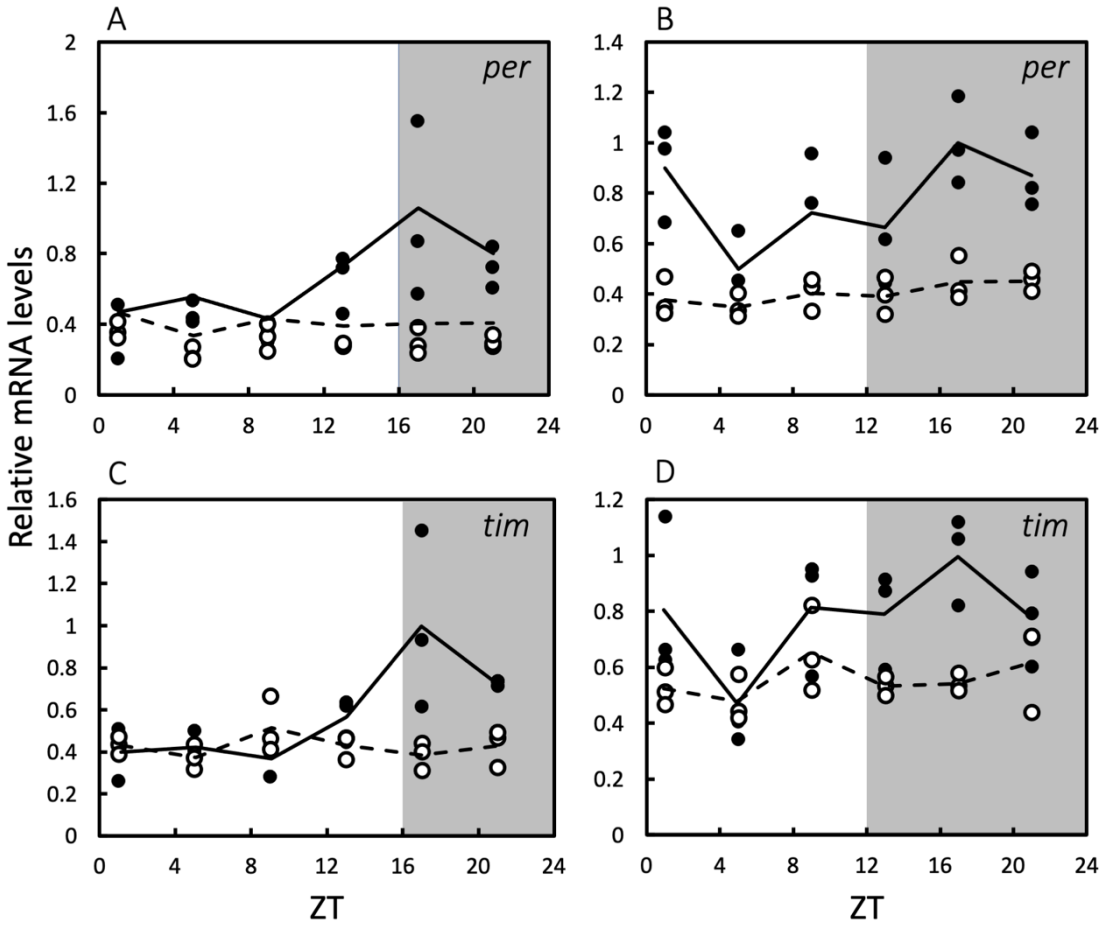


Figure 8. Temporal expression patterns of clock genes *period* (A and B) and *timeless* (C and D) in the head of larvae in the wild type (closed circles) and *per* knockout (open circles) strains of *Bombyx mori* (Kosetsu) under LD 16:8 h (A and C) and LD 12:12 h (B and D) at 25°C. Larvae were maintained under each condition until the start of sampling. Expression levels were analyzed by performing quantitative real-time PCR. Relative levels were standardized against mRNA levels of *Bmrp49*. Solid and broken lines show the mean of relative expression levels designated as closed and open circles, respectively (n = 3) in the wild type and *per* knockout strains, respectively. Shaded areas show dark periods.

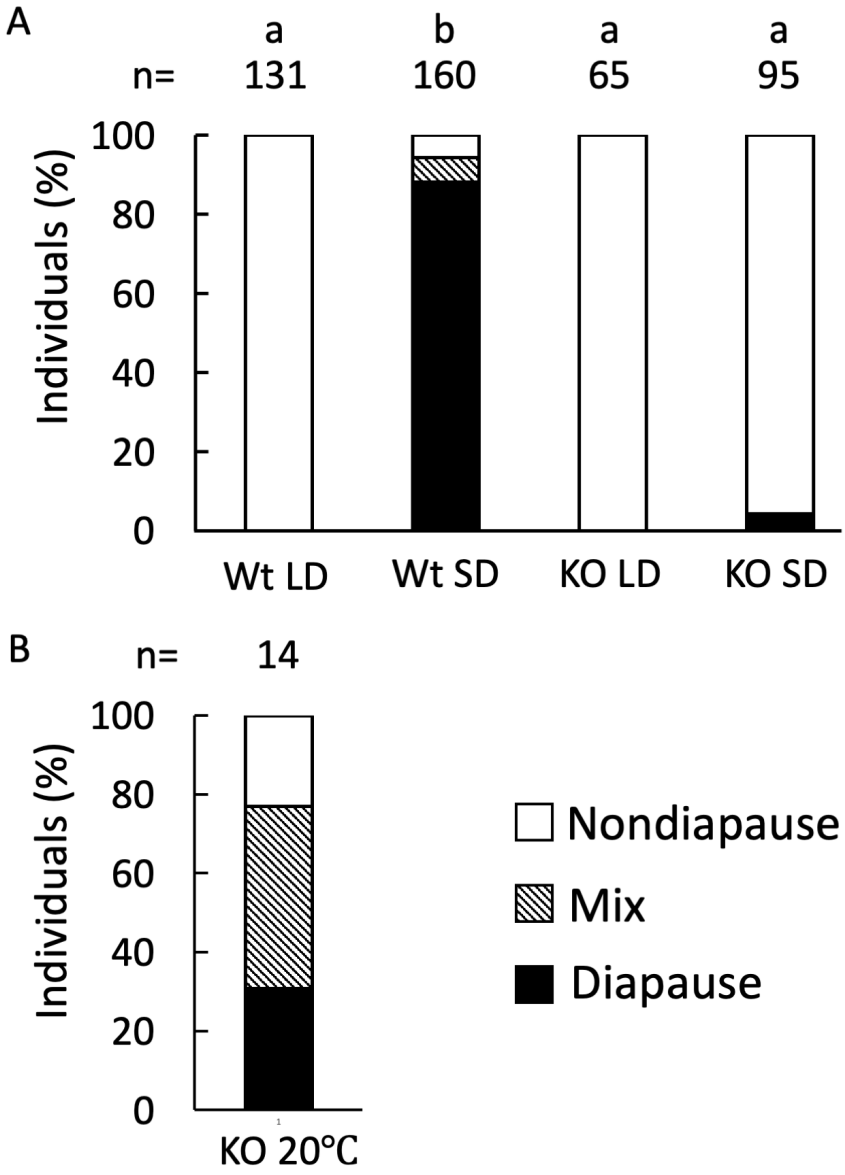


Figure 9. Effect of maternal photoperiod and temperature on the induction of embryonic diapause in wild type (Kosetsu, WT) and *per* knockout (KO) strains of *Bombyx mori*. Larvae were reared under LD 16:8 h (LD), LD 12:12 h (SD) at 25°C (A), or under LD 6:18 h at 20°C (B). Open, hatched and closed bars show the proportions of nondiapause egg-, mixed egg- and diapause egg-producers. Different lowercase letters indicate statistically significant difference (Turkey-type Multiple comparison for proportions, $p < 0.05$).

**Table**

Table1. Effect of photoperiod on the larval duration and pupal weight in the wild type and *per* knockout strains of *Bombyx mori* (Kosetsu) at 25°C.

| Strain | Photoperiod | Days from hatching to spinning (mean ± SD) | Pupal weight (g, mean ± SD) | |
|---|---|---|---|---|
| | | | Male | Female |
| wild | LD16:8 h | 22.7 ± 1.1 (100) a | 0.678 ± 0.106 (52) ab | 0.808 ± 0.136 (40) ab |
| | LD12:12 h | 24.2 ± 0.8 (100) b | 0.776 ± 0.072 (47) c | 0.996 ± 0.081 (44) c |
| *per* knockout | LD16:8 h | 22.0 ± 1.3 (95) c | 0.618 ± 0.113 (45) a | 0.781 ± 0.173 (50) a |
| | LD12:12 h | 23.0 ± 1.1 (97) a | 0.751 ± 0.119 (45) bc | 0.892 ± 0.148 (46) bc |

Different lowercase letters in the same column indicate statistically significant difference (Tukey-Kramer test, $p < 0.05$).