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[REVIEW]

Photoperiodism of Diapause Induction in the Silkworm, *Bombyx mori*

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The silkworm *Bombyx mori* exhibits a photoperiodic response (PR) for embryonic diapause induction. This article provides a comprehensive review of literature on the silkworm PR, starting from early works on population to recent studies uncovering the molecular mechanism. Makita Kogure (1933) conducted extensive research on the PR, presenting a pioneering paper on insect photoperiodism. In the 1970s and 80s, artificial diets were developed, and the influence of nutrition on PR was well documented. The photoperiodic photoreceptor has been investigated from organ to molecular level in the silkworm. Culture experiments demonstrated that the photoperiodic induction can be programmed in an isolated brain (Br)-subesophageal ganglion (SG) complex with corpora cardiaca (CC)-corpora allata (CA). The requirement of dietary vitamin A for PR suggests the involvement of opsin pigment in the photoperiodic reception, and a cDNA encoding an opsin (Boceropsin) was cloned from the brain. The effector system concerning the production and secretion of diapause hormone (DH) has also been extensively investigated in the silkworm. DH is produced in a pair of posterior cells of SG, transported to CC by nervi corporis cardiaci, and ultimately released into the hemolymph. Possible involvement of GABAergic and corazonin (Crz) signal pathways was suggested in the control of DH secretion. Knockout (KO) experiments of GABA transporter (GAT) and circadian clock genes demonstrated that GAT plays a crucial role in PR through circadian control. A model outlining the PR mechanism, from maternal photoperiodic light reception to DH secretion, has been proposed.

Key words: photoperiodic response, *Bombyx mori*, embryonic diapause, diapause hormone, Boceropsin, GABAergic control, circadian clock

INTRODUCTION

Photoperiodism is the physiological response of organisms, particularly plants and animals, to changes in the length of day or night. These organisms utilize photoperiod as a reliable source of seasonal information to regulate the timing of development and behaviors. The photoperiodic response (PR) in plants was discovered by Garner and Allard (1920), followed by its discovery in insects (aphids) by Marcovitch (1923, 1924). Subsequently, PRs in insects related to diapause, polymorphism, growth rate, and migration have been extensively studied. This response occurs with a time delay after receiving the seasonal signals, indicating that the photoperiodic information is stored in neurons, transmitted to the effector system, and ultimately triggers an adaptive phenomenon through some physiological processes (Košťál, 2011). Numerous textbooks on insect photoperiodism have been authored from various perspectives (Lees, 1955; Danilevsky, 1965; Beck, 1980; Tauber et al., 1986; Danks, 1987; Saunders et al., 2002; Denlinger, 2022).

Erwin Bünning (1936, 1960) proposed a hypothesis stating that the internal circadian rhythm is causally involved in the measurement of day or night length in the photoperiodism (Saunders, 2021). The involvement of the circadian oscillator in the time measurement mechanism of several insect species has been suggested by protocol experiments of the light-dark regime. Recently, numerous studies employing molecular techniques have shown that circadian clock genes, such as *period* (*per*) and *timeless* (*tim*), are essential for the PR of insects (Goto, 2013, 2022; Abrieux et al., 2020).

Diapause represents a programmed developmental state triggered by specific environmental cues. Insects enter diapause at various stages of their developmental cycle as a strategy to withstand hard environmental conditions (Danks, 1987; Denlinger, 2010). The physiological process responsible for initiating diapause by PR comprises four essential functional components: photoreceptor, clock that distinguishes between long day (LD) and short day (SD), counter that accumulates the effects of LD or SD exposure, and an output pathway that involves hormonal secretion and effector (Danks, 2003; Košťál, 2011; Dolezel, 2015). Current understanding suggests that intricate interactions among various genes and signaling pathways contribute to the dia-

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pause regulation of insects.

The silkworm has been domesticated for silk production for over 5500 years (Normile, 2009; Chen et al., 2019). Sericulturists have bred and adapted uni-, bi-, and multivoltine races of this insect to reproduce in various climatic conditions across East Asia (Xiang et al., 2018; Rudramuni et al., 2021). Owing to its operative size, genetic uniformity, and ease of cultivation, the domesticated silkworm has emerged as a valuable organism for experimental purposes. It has significantly contributed to our understanding of genetics, physiology, and biochemistry, leading to noteworthy scientific insights (Tazima, 1978). Serving as a model insect, the silkworm achieved a major milestone in 2008 when the International Silkworm Genome Consortium successfully unveiled its complete genome sequence (Xia and Feng, 2014).

The silkworm enters diapause at a specific embryonic stage, immediately after the formation of the cephalic lobe and mesoderm segmentation (Behrens, 1984; Nakagaki et al., 1991). The diapause eggs accumulate ommochrome pigments in the serosal membrane and become dark-brown several days after oviposition at room temperature, whereas non-diapause eggs develop until hatching without the coloration. The silkworm diapause is determined by hereditary traits and environmental conditions experienced by the mother in her embryonic or larval stage (Morohoshi, 1957; Tazima, 1964). Classical papers written by Japanese sericulture researchers demonstrated that the diapause incidence of the next progeny is affected by humidity (Harada, 1922), temperature (Watanabe, 1924), nutrition (Kitazawa, 1932), and light condition (Nagase and Kobayashi, 1927; Kogure, 1933).

Despite domestication, the silkworm shows robust PR in diapause induction (Kogure, 1933), larval growth rate (Hirasaka et al., 1969), and wing polymorphism (Tsurumaki et al., 1999). Among these, the facultative diapause phenomenon (voltinism) has been the most well documented. In 1933, Kogure presented a pioneering paper about silkworm PR of diapause induction. In 1970s and 1980s artificial diets were developed to rear the silkworm without relying on mulberry leaves, which were in high demand for silk production. The influence of nutritional conditions on PR was well documented during this era (Takamiya, 1974; Tsuchida and Yoshitake, 1983a; Shimizu, 1982a).

The photoreceptor pigment is a promising target for unraveling the molecular mechanism underlying insect PR (Numata, 1997; Saunders, 2012; Goto, 2022), and the PR photoreception in silkworms has been investigated more extensively than that in other insects, from the organs to the molecular level (Shimizu, 2018). In parallel with upstream investigations of PR, biochemical and physiological studies have been performed to reveal the effector system and the control of diapause hormone (DH) secretion (Hasegawa, 1951; Fukuda and Takeuchi, 1967; Sato et al., 1994; Ichikawa, 1995, 1996; Shimizu et al., 1998). In recent years, numerous studies utilizing molecular techniques, including gene knockdown or knockout (KO), have shed light on the PR mechanism, yielding noteworthy discoveries (Sato et al., 2014; Cui et al., 2021; Ikeda et al., 2021; Tsuchiya et

al., 2021). This article provides a comprehensive review of silkworm PR research, encompassing from population-level investigations to the latest breakthroughs exploring the molecular mechanisms. Regarding the abbreviations used in this review refer to Table 1.

PHOTOPERIODIC RESPONSE OF DIAPAUSE INDUCTION

Kogure's pioneering work

Makita Kogure (Fig. 1) was a Japanese entomologist who graduated from Kyushu Imperial University and later served as the director of the Nagano-Prefecture Sericultural Experiment Station (NSES), where he conducted extensive studies on the silkworm PR. After leaving the station, he became a professor at the Tokyo University of Agriculture and Technology.



Fig. 1. Portrait of Dr. Professor Makita Kogure (1899–1964).

Table 1. Abbreviations used in this review.

Br: brain	LD: long day
CA: corpora allata	LL: continuous light
CC: corpora cardiaca	Mrh: metarhodopsin
CDP: carotenoid-dependent process	NCC: nervi corporis cardiaci
Crz: corazonin	PR: photoperiodic response
DAP: dorsal-anterior protocerebrum	PRC: photoperiodic response curve
DD: continuous dark	PTX: picrotoxin
DH: diapause hormone	PUFA: polyunsaturated fatty acid
DIE: diapause inducing event	Rh: rhodopsin
ECM: external coincidence model	SD: short day
GAT: GABA transporter	SG: subesophageal ganglion
KO: knockout	

In 1927 Nagase and Kobayashi, while working as senior technical researchers at the NSES, reported that the light conditions during egg incubation had a significant effect on the diapause incidence of the subsequent progeny. They accidentally stumbled upon this phenomenon. At that time, the production of non-diapause eggs in bivoltine races was generally carried out under continuous dark (DD) conditions at a low temperature (15°C). However, they observed unexpected diapause production when the eggs were inadvertently kept under continuous light (LL) conditions (Nagase and Kobayashi, 1927). Kogure took over their study upon his arrival at the NSES and conducted an extensive investigation of the silkworm PR from the perspective of environmental biology. He examined the effects of day length, photosusceptibility, effective light intensity and wavelength, and temperature influence. Kogure primarily used two bivoltine races, Shohaku (a Japanese race) and Showa (a Cighinese race). He studied the diapause incidence using approximately 65,000 silkworms that had been subjected to various light and temperature conditions during their developmental stages. In 1933, Kogure presented a historic paper on the silkworm PR, which was also his doctoral thesis. This paper is widely cited in textbooks on insect photoperiodism as one of the pioneering works. In the paper, he cited the work of Marchovitch (1924) and used the term “photoperiodism”, which may be the first appearance of this term in zoological literature in Japan. He obtained SD photoperiodic response curves (PRCs) when the eggs were incubated at 20 and 15°C (Fig. 2). This was the first demonstration of PRC in the papers on insect photoperiodism.

Kogure investigated the PR of not only the egg stage but also the larval stage. He demonstrated that PR was observed during the larval period when the incubation temperature was below 20°C. LL conditions during the early larval stage, from the 1st to 3rd instar, induced significantly more diapause producers than DD conditions, as observed during the egg stage. In addition, during the late larval stage (4th–5th instar), LL conditions induced significantly more

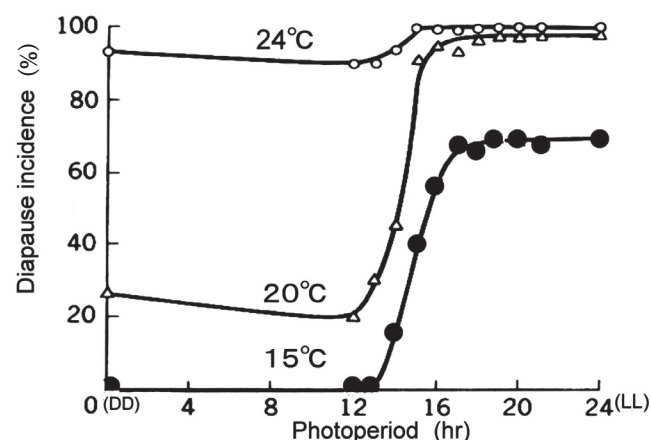


Fig. 2. Photoperiodic response curves (PRCs) for embryonic diapause induction in the silkworm bivoltine race, Shohaku. Three batches of eggs were incubated at 24, 20, and 15°C under different photoperiods during the egg stage. All batches of the larvae were reared under the same condition. Drawn from the data of Kogure (1933).

non-diapause producers than DD conditions. This indicates a change of PR from SD to LD response, both of which produce non-diapause producers under their respective conditions (Saunders, 2002), in the middle of the larval period. Kobayashi et al. (1986a, b; 1990) confirmed this PR change from SD to LD response during the larval stage using the tropical race Ringetsu. The authors discussed the directional change of PR based on transfer experiments of light-dark cycles. In *Heliothis zea*, pupal diapause is induced only by a reverse transfer from LD during the egg stage to SD during the larval stage (Adkisson and Roach, 1971). In the race Ringetsu, SD condition throughout the larval stage induced diapause, and transfer from LD to SD was not required to produce diapause induction. This PR change from SD to LD response differed depending on the silkworm race used. A bivoltine race, Daizo (p50), showed SD response during the egg stage (Egi et al., 2014), but showed LD response throughout the whole larval period, including the 1st instar (Shimizu and Hasegawa, 1988). In this race, exposure to three or four SD cycles during the 5th instar was enough to elicit diapause induction with 100% incidence (Hasegawa and Shimizu, 1987).

Temperature influence

Kogure and his coworker (Kogure and Kobayashi, 1928; Kogure, 1930, 1933) found that ambient temperature remarkably influences the PR in the race Shohaku. Increasing incubation temperatures shifted PRCs of the SD response upward (Fig. 2). The critical day length did not change depending on temperatures. At 24°C incubation, no clear PR was observed with high diapause incidence. The loss of PR by high-temperature incubation was observed in other bivoltine races (Cui et al., 2021). Under LL almost 100% diapause was observed at temperatures examined except 15°C, and under DD the diapause incidence depended on the temperature with 100% diapause at high temperatures (Fig. 3). Kogure (1933) reported that the threshold value of light intensity evoking diapause induction under LL condition depended on the incubation temperature (see the section PHOTORECEPTION MECHANISM). Furthermore, he found

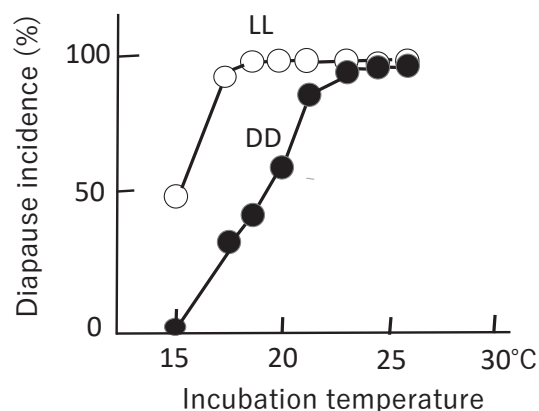


Fig. 3. Effect of incubation temperature on the diapause incidence under LL and DD condition during egg stage. The bivoltine race, Shohaku, was used in the experiments. All batches of the larvae were reared under the same condition. Drawn from the data of Kogure and Kobayashi (1928).

also that high temperature during young larval stages, 1st to 3rd instar, induced significantly more diapause than low temperature, as observed during the egg stage. However, during the 4th and 5th instar of the late larval stage, high temperature induced more non-diapause than low temperature, indicating that the silkworms changed their temperature response for the diapause induction during the middle larval stage. Kobayashi et al. (1986b) confirmed this temperature response change using the Ringetsu race with clearer data.

Nutritional influence

When bivoltine races are incubated at temperatures above 25°C during the egg stage and reared on mulberry leaves, they usually become diapause producers regardless of the photoperiod given during the larval period (Kogure, 1933; Takamiya, 1974; Kinoshita et al., 1982). However, when they were provided with artificial diets in place of mulberry leaves, they exhibited robust LD response throughout the entire larval period, irrespective of the high-temperature incubation (Sumimoto, 1974; Takamiya, 1974; Shimizu, 1982a; Tsuchida and Yoshitake, 1983a). The PRCs obtained by many authors have shown LD response but differed in the shape and critical day length depending on the race and artificial diets they used. The insertion of SD regime into even early larval instars (1st–3rd), produced a significant incidence of diapause (Takamiya, 1974). The replacement of an artificial diet with mulberry leaves during a single instar, along with the insertion of SD regime, induced diapause (Takamiya, 1974; Tsuchida and Yoshitake, 1983a). The replacement of mulberry feed during the 4th instar showed the most pronounced effect on diapause production. Simultaneously replacing mulberry feed and inserting SD regime into a single instar had a synergistic effect on diapause production (Takamiya, 1974). These observations suggest that both dietary chemical influence and photoperiodic signal might bring about the same biochemical impact, thereby integrating diapause induction.

There are many metabolic and physiological differences between silkworms reared on artificial diets and those reared on mulberry leaves (Dong et al., 2017). Many authors (Kato, 1974; Shimizu, 1982a; Tsuchida and Yoshitake, 1983) discussed the possible involvement of chemical compounds of mulberry leaves in this phenomenon. As this was observed, regardless of whether the artificial diets contained mulberry leaf powders or not, the involved compounds are presumed to be easily decomposed by the preparation procedure of the diets, such as autoclaving or heat treatment. In *Pieris brassicae*, Dumortier and Brunnarius (1989) discovered that when artificial diets instead of their host plant were provided to the insects, the photoperiodic clock shifted from a circadian to an hourglass mode. They proposed the potential involvement of dietary substances in their observation.

Bull and Adkisson (1960, 1962) reported that the dietary fat content remarkably affected the diapause incidence of the pink bollworm, *Pectiophora gossypiella*. The author of this review found also that adding 5% of soybean oil (Nacalai Tesque Inc.), which contained abundant polyunsaturated fatty acids (PUFA) of linolenic and linoleic (Ito and Nakasone, 1969), to an artificial diet significantly increased the diapause incidence in a bivoltine race reared under a LD (L20: D4)

condition during the larval stages (unpublished observation), implying that PUFA content in the diets might affect the diapause induction. Silkworm diapause eggs just after oviposition had a fatty acid pattern characteristic of high proportions of PUFAs in the phospholipids compared with non-diapause eggs (Miura and Shimizu, 1989; Shimizu, 1992). Increasing PUFA in fatty acid compositions is considered to be an adaptive component of winter diapause to maintain cell membrane fluidity (Cossins and Bowler, 1987; Khani et al., 2007; Reynolds et al., 2012; Vukašinović et al., 2013). Generally, rearing insects at low temperatures and under SD photoperiods of diapause-inducing conditions increases the ratio of PUFA in lipids (Harwood and Takata, 1965; Valder et al., 1969; Nakasone and Horie, 1978; Behren, 1984; Baldus and Mutchmor, 1988; Hodkova et al., 2002; Hemmati et al., 2017). Some insect clock genes, such as *per* and *tim*, have been suggested to be involved in lipid accumulation (Omura et al., 2016; Zhu et al., 2019). In *Drosophila melanogaster*, composition change of PUFAs in the neurons expressing TRPA1 of a molecular thermosensor, altered the temperature preference behavior by modulating neuronal activity (Zheng, 2013; Suito et al., 2020).

Kogure's pioneering work showed that the embryonic diapause in the silkworms is determined by light and temperature experienced at each developmental stage. Textbooks describing insect photoperiodism have identified the silkworm as a SD response insect with photosensitivity during the egg and young larval stages (Danilevsky, 1965; Beck, 1980; Danks, 1987; Saunders et al., 2002; Denlinger, 2022). However, Kogure (1933) demonstrated not only the SD response during the early larval stages but also the LD response during the later larval stages. Few insects change the PR during the same developmental stage, though there are reports of PR changes during different periods (Saunders, et al., 2002). Kogure (1933) also found the change of low and high temperature effect on diapause incidence during the larval period, which was confirmed by Kobayashi et al. (1986b).

These changes appear to be reasonably adaptable to adjust the life cycle of bivoltine races to the seasonal transition in the temperate zone. In bivoltine races, SD and low temperature (early spring information) during the egg and early larval stage, followed by LD and high temperature (early summer information) during the late larval stage, induce non-diapause progeny of the next generation. Furthermore, LD and high temperature (summer information) during the egg and early larval stage, followed by SD and low temperature (autumn information) during the late larval stage, induce diapause progeny of the second generation. The mechanism behind the PR change during the larval period remains unknown. Regrettably, research on silkworm PR at the population level has shown no progress since Kogure's initial work. Further investigation into the dominance and accumulation of PR at various developmental stages is required.

PHOTORECEPTION MECHANISM

Light sensitivity and effective wavelength

Kogure (1933) studied the light sensitivity for diapause induction and found that the photosensitivity was observed just after blastokinesis during embryonic development. He

observed that the threshold light intensity that produces LL response was between 0.04 and 0.06 lux at 20°C incubation and between 0.1 and 0.14 lux at 15°C at egg stage. Shimizu and Kato (1984) found that it was between 0.1 and 1.0 lux during the larval period when a bivoltine race was reared on an artificial diet. The threshold value for the induction of LD response was between 0.0084 and 0.039 $\mu\text{W}/\text{cm}^2$ with irradiation of monochromatic light of 525 nm under a photoperiod of L17:D7 (Nakajima et al., 1972).

Although strict action spectra recorded according to the proper procedure have not been obtained for the silkworm, several researchers have investigated the spectral sensitivity of PR. Kogure (1933) used colored glass plates and found that red and orange-yellow light had little or no effect on diapause incidence, whereas violet light showed a distinct effect compared with other light. Nakajima and his coworkers (1972, 1981) used modern optical equipment to investigate the effects of different wavelengths on diapause incidence. They found that wavelengths in the blue-green (411–547 nm) region were effective, with a maximum sensitivity peak at 527 nm. The sensitivity falls rapidly above 588 nm, with red light being virtually ineffective. Tsuchida and Yoshitake (1979) also showed that light wavelengths from 400 to 540 nm were effective for LD response, and those above 580 nm were ineffective. Although these reports did not include any strict action spectrum, such as that shown in *Megoura viciae* (Lees, 1981), the involvement of a receptor pigment with blue-green absorption properties has been considered.

Localization of photoreceptor

The photoreception pathway of light input for PR has been found to differ among insect species, without any phylogenetic relationship (Numata et al., 1997; Saunders, 2012). In the silkworm, it has been shown that the photoperiodic receptor resides in the larval head, rather than the stemmata,

using black cover painting and local supplementary illuminations with chemiluminescent paste (Shimizu and Hasegawa, 1988). The possibility of a translucent clypeus acting as a window to admit light to the pupative cerebral photoreceptor in the 1st and 2nd instar larva has been discussed. Claret (1966) described a similar idea of a window function of the clypeus in a cabbage butterfly, *Pieris brassicae*.

Bowen et al. (1984) demonstrated that excised Br-retrocerebral complexes of the hornworm moth, *Manduca sexta*, can be photoperiodically programmed under in vitro culture conditions, suggesting that both the photoreceptor and clock system of diapause induction are Br-centered. Soon after Bowen's report, in vivo and in vitro culture experiments were conducted by Hasegawa and Shimizu (1987) using Br-subesophageal ganglion (SG) complexes with endocrine glands, corpora cardiaca (CC)-corpora allata (CA), in the silkworm (Fig. 4). The results demonstrated that photoperiodic induction of the silkworm can be programmed under in vivo and in vitro culture conditions, implying that the components of the photoreceptor, clock, and counter system are located in the Br-SG complex.

As mentioned below, the silkworm Br has been found to control the secretion of DH from SG (Fukuda, 1952; Matsutani and Sonobe, 1987; Shimizu et al., 1997). It has been suggested that extra-retinal photoperiodic receptors of insects are closely associated with the regulatory site of hormone secretion in the Br (Steel and Lees, 1977; Shiga and Numata, 2007), and therefore, it is likely that the photoreceptor of the PR resides in the silkworm Br, not in SG. In lepidopterans, many reports have shown Br-centered photoreception in PR (Williams et al., 1965; Claret, 1966; Segue and Veith, 1976; Bowen et al., 1984).

Vitamin A requirement

When silkworms were reared on a vitamin A-deprived

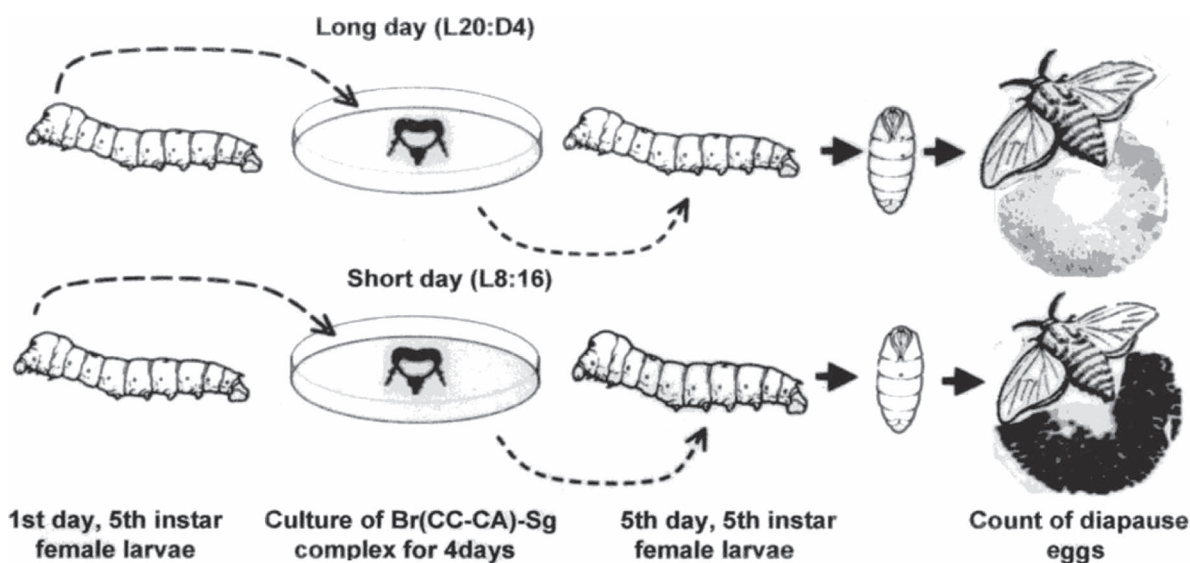


Fig. 4. In vitro photoperiodic programming of diapause induction in the silkworm. Pairs of Br-SG complexes with CC-CA were isolated from 5th instar-day 1 females destined to become non-diapause producers and cultured for 4 days under LD of L20:D4 or SD of L8:L16. Then, the complexes were transferred into 5th instar-day 5 females destined to become non-diapause producers. The moths receiving SD-exposed complexes produced significantly more diapause eggs than those receiving LD-exposed complexes (Hasegawa and Shimizu, 1987). This illustration was redrawn from Shimizu (2018) with permission of Japanese Society of Sericulture Science.

artificial diet, they exhibited a loss of larval phototaxis and ERG (electroretinograph) response in both their stemmata and compound eyes (Shimizu et al., 1981). Furthermore, the rearing of larvae on the vitamin A-deprived diet resulted in the disappearance of PR in the larval stage. However, the introduction of β -carotene (provitamin A) or vitamin A to the diet restored the PR (Shimizu and Kato, 1984; Hasegawa and Shimizu, 1988). The impacts of carotenoid and vitamin A deficiency were only noticeable under a low light intensity of 1 lux, as opposed to higher intensities exceeding 5 lux (Shimizu, 1982a; Shimizu and Kato, 1984). This correlation between light intensity and the effects of vitamin A deficiency implies that this phenomenon is associated with the photoreception process itself, rather than downstream of diapause induction.

Various arthropods, including three mites, *Tetranychus urticae* (Veerman and Hell, 1978; Veerman, 1980; Bosse and Veerman, 1996), *Amblyseius potentiiae* (Veerman et al., 1983) and *A. cucumeris* (Overmeer et al., 1989), and three insects, *B. mori* (Shimizu and Kato, 1984), *Apanteles glomeratus* (Veerman et al., 1985), and *P. brassicae* (Claret and Volkoff, 1992), have demonstrated dependencies on carotenoids or vitamin A in their PRs. Additionally, liams et al. (2019) demonstrated that retinal and its derivatives are essential for photoreception in the monarch butterfly, *Danaus plexippus*, by knocking out *NINAB1*, which produces the enzyme that converts carotenoids into retinal. Hasegawa and Shimizu (1988) found that the silkworm Br of 5th instar larvae contained cis- and all-trans 3-OH-retinal (0.01 pmol per brain in combination) and all-trans-retinal (0.03 pmol per brain), which are chromophores of the insect visual pigments (Seki and Vogt, 1998). Furthermore, cis- and all-trans 3-OH-retinal were found in the Br and the compound eyes, whereas only all-trans-retinal was detected in the Br. These pieces of evidence suggest that 3-OH-retinal in the larval brain might be formed from all-trans retinal and function as the chromophore of opsin-like pigment (Gartner and Towner, 1995; Seki et al., 1998).

Boceropsin: A candidate of photoperiodic receptor pigment

Shimizu et al. (2001) hypothesized that an opsin-like pigment expressed in the silkworm Br could be a photoperiodic receptor and attempted to clone the cDNA encoding the opsin protein from the organ. They successfully cloned a cDNA (Genbank Accession No. AB064496) encoding an opsin protein consisting of 381 amino acids, which was conserved in insect visual pigments. This protein was predicted to be a G-protein-coupled opsin with seven transmembrane segments, and it had the 327th lysine (K)-residue that presumably bound the chromophore by a Schiff base (Gartner and Towner, 1995). Southern blotting experiments indicated that this gene is present in a single copy, and it was designated Boceropsin (*Bombyx* cerebral opsin). Miao et al. (2005) showed that this *Boceropsin* gene is located on the 15th chromosome. This was the first finding of an opsin expressed in the central nervous system of an insect. Molecular phylogenetic analysis revealed that this novel opsin belonged to the group of long-wavelength (green) photoreceptors. A database search showed that it had the highest degree of homology (88%) with a long-wavelength receptor

(Bomopsin 1: BAB12276) expressed in the silkworm compound eyes. It also had a high degree of homology (84%) with Manopin 1 (L78080) from the sphingid moth *M. sexta*, which has an absorption peak at 520 nm (Chase et al., 1997; Briscoe, 2008).

RT-PCR analysis revealed that *Boceropsin* was expressed in the larval Br, but not in SG, first thoracic ganglia, or compound eye. Whole-mount immunohistochemistry was conducted using mouse antiserum to locate the cells that produce Boceropsin in the Br (Shimizu et al., 2001). The results showed that some cells (15–20 μ m in diameter) in the Br exhibited clear immunoreactivity. Specifically, two types of bilateral Boceropsin immunoreactive cells were found in the dorsal-anterior protocerebrum (DAP) cells and ventral-anterior protocerebrum cells (VAP). The DAP cells had large somata and exhibited more intense immunostaining compared with other immunoreactive cells. Boceropsin immunoreactivity was also observed bilaterally in dorsal-posterior protocerebrum cells and lateral-posterior tritocerebrum cells, but not in SG or thoracic ganglia. A detailed histochemical observation by confocal laser microscopy confirmed these results (Shimizu, 2018).

Yu et al. (2008) conducted a systematic analysis of microRNAs (miRNAs) within the silkworm genome sequence. They successfully identified and cloned 35 previously undiscovered miRNAs. Among these, Bmo-miR-92 was identified as an ortholog of *Drosophila* dme-miR-92a expressed in the fly's Br primordium. Furthermore, they found that *Boceropsin* is a potential target of Bmo-miR-92, and noted a relatively low expression of bmo-miR-92 during the trachea-appearing embryonic stage. The authors speculated that this particular stage might hold essential implications for the functioning of Boceropsin. Notably, Kogure (1933) previously observed that the onset of light-sensitivity related to diapause induction occurs during the trachea differentiation stage following blastokinesis. This parallel is worth considering regarding Boceropsin's function.

In addition to Boceropsin, the author of this review cloned the cDNA encoding the long-wavelength receptor opsin in the larval Br of a wild moth, *Antheraea pernyi*, and cabbage butterfly, *P. rapae*. The deduced amino acid sequence of the new opsin of *A. pernyi* was 381 (BAC66474), and that of *P. rapae* was 382 (BAD06459), among acids long, exhibiting homology with Boceropsin of 87% and 76%, respectively. Since the discovery of Boceropsin, the expression of some opsins has been reported in other insect Brs. Velarde et al. (2005) reported pteropsin, which is expressed in the lateral and dorsal central portion of the anterior cerebral region of *Apis mellifera*. This non-visual opsin of honeybees belongs to the Opn3 family, including mosquito Opn3, but its function remains unknown (Terakita and Nagata, 2014). Craig Montell and his coworkers found that three rhodopsins, Rh5, Rh6, and Rh7, were expressed in the Br neurons of *D. melanogaster* (Sokabe et al., 2016; Ni et al., 2017). *Drosophila* Rh7 with a broad spectrum with a longer wavelength tail reaching 500 nm was found to function as a circadian photoreceptor, as well as CRY1 (Ni et al., 2017; Sakai et al., 2017; Sokabe et al., 2017). In an aphid, *Acyrtosiphon pisum*, Collantes-Alegre et al. (2018) found that three species of opsin (Ap-Anthropsn, Ap-SWO4, Ap-C-Ops) were expressed in several cells of the protocerebrum

of the insect. Among these cerebral opsins, *Drosophila* RH6 showed a relatively high homology (58%) with Boceropsin in terms of amino acid sequence, but the other opsins exhibited a lower homology of about 30%.

Studying the photoreceptor in PR of organisms appears to be an effective strategy to uncover the enigmatic mechanism of the photoperiodism. Several authors (Goto and Numata, 2009; Saunders, 2012, 2020; Barbera et al., 2021; Goto, 2022) have suggested that two types of photopigments may be involved in insect photoperiodic reception based on the external coincidence model (ECM) proposed by Pittendrigh and Minis (1964). One is Cryptochrome 1 (CRY1), a blue-light photopigment of the circadian system, and the other is the opsin pigment involved in the photoinductive process of diapause or non-diapause determination. Regarding *Bombyx cry1*, it has only been reported that KO of *Bombyx cry1* does not affect diapause induction at 25°C incubation (Homma et al., 2022), while some pieces of evidence have suggested the involvement of opsin pigment in the photoreception. Boceropsin, expressed in the defined cells of Br, has been proposed as a likely candidate of photoperiodic receptor pigment (Shimizu et al., 2001).

In the silkworm, genome analysis identified six homologs of opsin (Velarde et al., 2005): BmUVop (UV-sensitive opsin of *Drosophila* Rh3 ortholog), BmBLop (blue-sensitive opsin, Rh5), BmLop1 (long wavelength1 opsin, Rh6), BmLop2 (long wavelength2 opsin, Rh6), BmUnop (broadband opsin, Rh7), and *Bombyx* pteropsin (a homolog of Opn3 group opsin). Among these, BmLop1 corresponds to Bomopsin1, a long-wavelength receptor of the compound eyes (Shimizu et al., 1998), and BmLop2 corresponds to Boceropsin. Based on the insight into the location of other insects' cerebral opsins, BmBLop, BmUnop, and *Bombyx* pteropsin are considered to be potent candidates for cerebral photoperiodic pigments in addition to Boceropsin, although the expression of these opsins has not yet been demonstrated in the silkworm. In the cricket *Modicogryllus siamensis*, three opsins that are expressed in the compound eyes were suggested to play roles in photoperiodic regulation (Tamaki et al., 2013).

It is generally accepted that the dorsal protocerebrum of the insect Br is an essential region that regulates endocrine control for diapause induction (Raabe, 1989; Ichikawa, 1991; Shiga and Numata, 2007; Shiga, 2023). The protocerebrum of the silkworm Br was found to control the release of DH from the CC-CA complex (Matutani and Sonobe, 1987; Shimizu et al., 1997). In the anterior region of protocerebrum, there are neurons expressing corazonin (Crz) (Tsuchiya et al., 2021) and clock-related proteins (Sehadová et al., 2004), which are suggested to be important molecules involved in the diapause induction, as described below. Reliable classical experiments suggest that the photoperiodic receptors are restricted to the dorsal protocerebrum region of *A. pernyi* (Williams, 1969) and the dorsal anterior region of *M. vicina* (Lees, 1964; Steel and Lees, 1977; Gao et al., 1997). Consequently, it is reasonable to speculate that the DAP cells expressing Boceropsin are the most likely photoreceptor cells involved in the silkworm photoperiodism. Further investigations, including neurophysiological and genetic experiments, are required to verify the role of Boceropsin.

CONTROL OF DIAPAUSE HORMONE SECRETION

Identification, production, and secretion of DH

Alongside upstream studies of the silkworm PR, Japanese researchers conducted systematic investigations of the hormonal effector system in the PR. Kinsaku Hasegawa (1951) and Soichi Fukuda (1951a, b) presented papers in the same volume of the Proceedings of the Japan Academy, which demonstrated the release of DH (factor) from SG into the hemolymph. Although they both contended for the priority of this discovery, Hasegawa attempted to identify the chemical structure of DH, and Fukuda aimed to reveal the control mechanism of DH secretion (Yaginuma, 2015). Fukuda was an insect endocrinologist who discovered the function of the prothoracic gland by studying the silkworm (Fukuda, 1940a, b; Ohnishi and Ishizaki, 1990).

With great effort, using a large number of silkworm pupae, the structure of DH has been identified as an amidated peptide of 24 amino acid residues, belonging to the insect FXPRL amide peptide family (Imai et al., 1991; Sato et al., 1992; Shen et al., 2018). It shares the same C-terminus five amino acid sequence with several other neuropeptides, including pheromone biosynthesis activating encoded in a single reading frame (Kawano et al., 1992; Sato et al., 1993). In situ hybridization with the cDNA encoding the common polypeptide precursor was performed, and 12 cells from three clusters were stained in SG: the three clusters were localized in the mandibular (anterior), maximal (medial), and labial (posterior) neuromere (Sato et al., 1994). Shiomi et al. (2015) established a TALEN-based KO mutant of *DH-PBAN* and showed that DH signaling is essential for embryonic diapause induction. Morphological polymorphism of the moth wing is determined also by DH action during the early pupal developmental stage (Tsurumaki et al., 1999; Yamanaka et al., 2000). A gene encoding DH was identified within other Lepidopterans lacking the embryonic diapause, and various physiological actions of DH were reported (Denlinger, 2022).

Fukuda and Takeuchi (1967) performed histological and surgical experiments and demonstrated that a pair of putative DH-producing cells were located on both sides of the midventral line of SG at a level approximately two-thirds from its anterior end. Immunocytochemical studies using DH and PBAN antiserum revealed three clusters of neurosecretory cells located on the ventral midline of SG: four cells in the anterior, six cells in the medial, and two cells in the posterior cluster (Ichikawa et al., 1995). The number, size, and position of the cells were identical to those observed by cDNA hybridization experiments reported by Sato et al. (1994). Surgical removal of the cluster cells revealed that the posterior cells had diapause induction activity, whereas median cells had pheromonotropic activity (Ichikawa et al., 1996). These posterior cells (hereafter called DH cells) corresponded to the neurosecretory cells that Fukuda and Takeuchi (1967) observed as being the pupative DH cells.

The level of DH in SG gradually increased during larval development and peaked at the early pupal stage in both diapause and non-diapause-producing silkworms (Kitagawa et al., 2005). It decreased markedly in diapause producers compared with non-diapause producers at the middle pupal stage, implying the release of DH into the hemolymph in diapause producers. Additionally, DH was found to act on

developing ovaries through a G protein-coupled receptor during pupal-adult development to produce the diapause eggs (Homma et al., 2006). It was reported that DH activates trehalose in the ovary cells and the oocytes (Kamei et al., 2011; Cui et al., 2021).

Lucifer yellow-filled DH cells projected bilaterally symmetric dendritic branches to the anterior half of SG, and the axon of the cell passed through the Br and then entered the nervus corporis cardiaci3 (NCC3) to spread varicose terminal branches in CC-CA (Ichikawa et al., 1995). Immunocytochemical studies performed by Sato et al. (1998) showed the same results. Shimizu et al. (1997) confirmed the DH secretion from CC-CA by conducting anatomical experiments, including the expiration of SG and Br-SG complexes with CC-CA. In summary, these observations demonstrated that DH produced in the somata of the DH cells located in SG is transported to CC-CA via NCC3, and finally released into the hemolymph.

Long-term chronic recordings were also made from the axonal tract NCC-3 of DH cells during pupal-adult development (Ichikawa, 2003; Ichikawa and Kamimoto, 2003; Ichikawa and Suenobu, 2003). DH cells in the diapause producers showed firing activities throughout pupal-adult development, whereas the same cells in the non-diapause producers maintained an inactive state until the last quarter of the pupal period. The ionic mechanism of excitability in DH cells was analyzed with intrasomatic recordings (Miyazaki, 1980). The action potential in the soma is primarily dependent on the presence of calcium (Ca) ions, and the excitability of the soma is characterized by the less pronounced potassium (K) conductance, which causes the large and prolonged Ca-dependent action potential. Injection of a Ca-chelating agent, ethylene glycol bis tetraacetic acid (EGTA), into the pupae of diapause producers induced non-diapause eggs (Xian et al., 1997), whereas the injection of KCl in non-diapause producers induced diapause eggs (Yoshitake, 1954). The hemolymph K concentration of the silkworm was reported to rapidly elevate during the larval-pupal development (Shimizu, 1982b). These observations suggest that developmental changes in hemolymph cations concentration might affect DH secretion (Kitagawa et al., 2005).

Control of DH secretion by brain

Fukuda (1952) conducted some surgical manipulation experiments using pupae and concluded that, in non-diapause producers, the release of DH was inhibited by the Br through the esophageal connectives, but in diapause producers, it was stimulated by the Br: he proposed both inhibiting and stimulating action of the Br in DH secretion (Raabe, 1989). Moreover, Tsuchida and Yoshitake (1983b) proposed inhibiting action of the Br, and Morohoshi and Oshiki (1969) stimulating action by performing translation experiments of the Br-SG complex. Matsutani and Sonobe (1987) carried out microsurgical manipulation of the Br to reveal the controlling region of DH secretion. They found that cortices lateral to the median line on the dorsal side of the protocerebrum were related to the inhibitory control of DH secretion and that cortices of anterolateral areas on the ventral side of the protocerebrum participated in the stimulatory control. Shimizu et al. (1997) demonstrated that microsurgical transection of the protocerebrum from other cerebral parts induced diapause

egg production in non-diapause producers, whereas the operation in diapause producers did not affect diapause production. From these observations, the authors concluded that the protocerebrum of the Br plays a crucial role in the inhibitory action on the DH secretion in non-diapause producers.

GABAergic control of DH secretion

The GABAergic control of DH secretion was demonstrated in pharmacological studies by Shimizu and his colleagues (Shimizu et al., 1989; Hasegawa and Shimizu, 1990; Shimizu et al., 1997). They used *in vitro* culture experiments to demonstrate the pharmacological effects of γ -aminobutyric acid (GABA) and picrotoxin (PTX), a GABA antagonist, on DH secretion from isolated Br-SG complexes or SG. Ichikawa (2003) observed the inactivity of firing from the axonal tract (NCC-3) of DH cells in non-diapause producer pupae. Further, he found that the injection of PTX into non-diapause producer young pupae induced firing excitation in the inactive DH cells, supporting the hypothesis that DH cells are regulated by GABAergic signals. One Br of the Daizo race was reported to contain approximately 0.32 nmol of GABA just after pupation (Yang et al., 1992). A considerable number of neurons of Br and SG were immunoreactive to a GABA-antiserum (Shimizu, 1998). There have been some reports describing the involvement of GABAergic regulation in the diapause induction of other insects (Webb and Denlinger, 1998; Zhang et al., 2013; Nikonorov et al., 2018).

Recently, Tsuchiya et al. (2021) proposed a hierarchical pathway consisting of GABAergic and subsequent Crz signals that modulate diapause induction. Crz is an undecapeptide involved in various physiological functions, including cardiostimulation, regulation of silk spinning rate and diapause induction in insects (Tanaka et al., 2002; Shiga et al., 2003; Predel et al., 2007). They reported that the injection of Crz into pupae of non-diapause producers induced diapause eggs. In addition to this noteworthy finding they observed that the expression of the plasma membrane GABA transporter (GAT) gene was 10–100 fold higher in the Br-SG complex in diapause producers than in non-diapause producers. GAT transports GABA from the synaptic cleft back into the presynaptic neuron to terminate GABAergic inhibition (Umesh and Gill, 2002). Dorsolateral Crz neurons were found to express PTX-sensitive GABA receptors in the Br. Furthermore, they discovered that KO mutants of GAT became mostly non-diapause producers, regardless of the diapause-producing condition. As described below, the expression of GAT is regulated by the circadian clock in the silkworm (Cui et al., 2021). These observations strongly suggest that GAT is a crucial factor in the diapause production of the silkworm through the circadian control.

INVOLVEMENT OF CIRCADIAN CLOCK GENES AND RELEVANT GENES

Circadian clock genes

Numerous studies have investigated the role of circadian clock genes in the photoperiodism of insects (Ikano et al., 2011; Goto, 2013; Meuti et al., 2015; Mukai and Goto, 2016). Clock genes have been identified in the silkworm (Iwai et al., 2006; Xiang et al., 2018), and KO of *per* and *tim* genes was reported to cause the loss of circadian behav-

ioral activities (Ikeda et al., 2019; Nartey et al., 2021).

Recently, three groups reported the effects of KO of the *per* gene on silkworm PR using different races (Cui et al., 2021; Ikeda et al., 2021; Tobita and Kiuchi, 2022). Ikeda et al. (2021) generated a *per* KO strain using TALEN-mediated genome editing technology with the Kosetsu race. They observed the loss of the circadian rhythm for hatching and eclosion in the KO strain and a concurrent disruption of the temporal expression pattern of *per* and *tim* in the larval head. They also observed that the wild strain showed LD PR during the larval stage, whereas the KO strain showed 100% non-diapause production under both LD and SD conditions (Fig. 5A). Cui et al. (2021) also generated a KO strain of the *per* gene using the TALEN method with the Dazao race. They found that the KO disrupted the temporal expression patterns of the circadian clock genes compared with those of the wild-type strain, and reduced diapause induction under LL conditions during the embryonic stage at 20°C (Fig. 5B). Another research group, Tobita and Kiuchi (2022), generated KO mutants of *per* gene using the CRISPR/Cas9 method with the Daizo race. In the KO mutant, the diapause-producing action of SD during the larval stage and that under LL conditions during the embryonic stage were completely lost (Fig. 5C, D). In summary, the KO of *per* caused non-

diapause production regardless of the diapause-inducing photoperiodic conditions and prevented the silkworm PR. *Per* KO mutants are unable to distinguish not only light condition but also temperature condition (Homma et al., 2022).

Similar results were obtained by KO of other core circadian genes such as *tim*, *Clock*, and *cycle* (Tobita and Kiuchi, 2022). This evidence suggests that the circadian clock is not just related to the PR induction through the pleiotropic function of one or a few genes, but is fully integrated into the mechanism (Denlinger, 2017). In the *per* KO mutant, Cui et al. (2021) reported that the transcription levels of *GAT* and *GABA* transaminase (*GABAT*) in the pupal Br-SG were downregulated compared with those in the wild-type strain. Notably, the level of *GAT* gene transcription was remarkably downregulated to approximately one-tenth in the *per* KO silkworm. The authors also observed upregulation of the transcription of the glutamic acid decarboxylase (*GAD*) gene and ionotropic *GABA* receptor (*GRD*) gene in the mutant. These alterations in the transcription of the genes resulted in an elevated amount of *GABA* released into the synaptic cleft.

Among the core genes of the circadian clock in the silkworm, *per*, *tim*, and *cry2* are known as negative regulators, while *Clock* and *cycle* are known as positive regulators in

the feedback loop, based on the transcription and translation of the clock genes (Brady et al., 2021; Goto, 2022). Cui et al. (2021) reported that the circadian clock system directly upregulated the expression of ionotropic *GABA* receptor (*GRD*) in the pupal stage through *Cycle*.

In bivoltine races, incubation at a high temperature (25°C) resulted in 100% diapause occurrence irrespective of the light conditions. Nonetheless, both the group of Cui et al. (2021) and that of Homma et al. (2022) observed that KO mutants of the *per* gene exhibited nearly 100% non-diapause even at the high temperature incubation under DD. Homma et al. (2022) obtained similar results in the KO strains of other clock genes *tim*, *Clock*, *cycle*, and *cry2*. This diapause disappearance was significant in KO mutants of *per* and *time*. Further, they found that both PTX and DH injection into the pupae of these KO strains restored the diapause production. These observations suggest that the circadian clock,

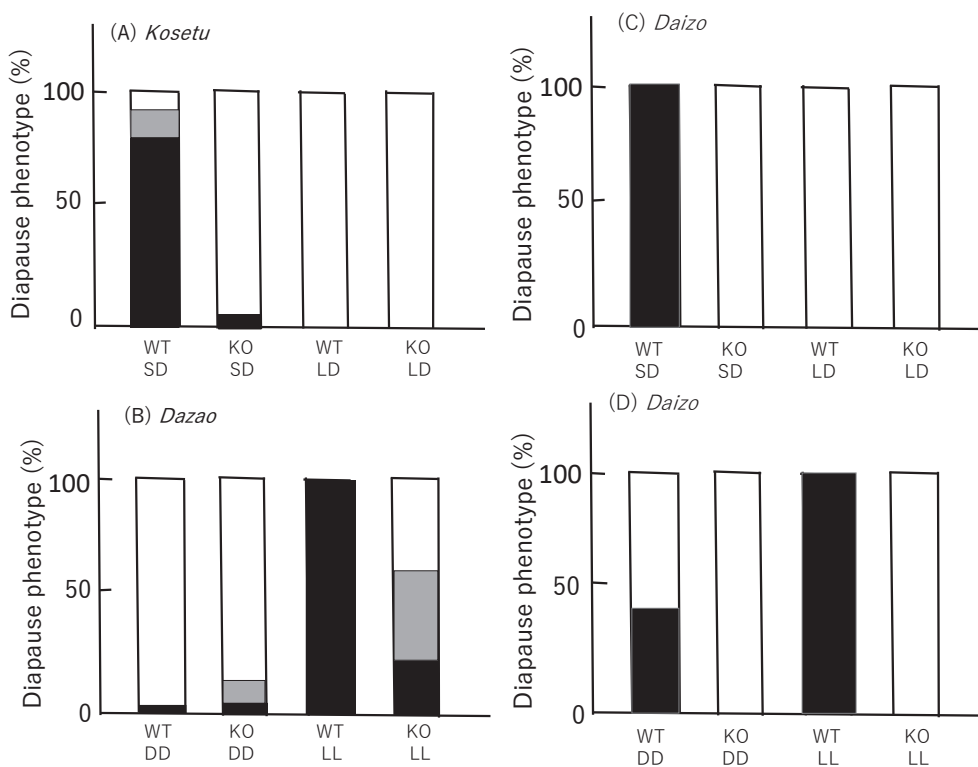


Fig. 5. Effect of *per* gene KO on the PR. (A, B) were redrawn from the data of Ikeda et al. (2019) and Cui et al. (2021), respectively, and (C, D) were both redrawn from the data of Tobita and Kiuchi (2022). In each experiment, the silkworm race (R), embryonic stage conditions (ESC) and larval stage condition (LSC) were as follows. In experiment (A), R: the Kosetsu race, ESC: under LL at 20°C, LSC: under SD (L12:D12) or LD (L16:8) at 25°C. In experiment (B), R: the Dazao race, ESC: under DD or LL at 20°C, LSC: under 12L:12D at 25°C. In experiment (C), R: the Daizo race, ESC: under DD at 25°C, LSC: under SD (L8:D16) or LD (L20:D4). In experiment (D), R: the Daizo race, ESC: under DD or LL at 18°C, LSC: under 12L:12D at 25°C. Open, hatched, and closed bars show proportion of moths that oviposited non-diapause, mixed, and diapause eggs in the same batch, respectively. WT: wild-type strain, KO: knockout strain.

which is involved in diapause induction, is hierarchically located upstream of the GABAergic pathway. Liu et al. (2021) found that bmo-miR-6497-3p, one of the miRNAs, may be involved in the diapause induction of the silkworm by affecting the expression of the circadian clock genes during the blastokinesis phase. They discussed the epigenetic regulation of the circadian clock genes mediated by the miRNA in the diapause induction under different temperatures.

Relevant genes

The molecular receptor responsible for temperature cues involved in insect dormancy and PR remains unclear. Shiomi and his coworkers proposed that *Bombyx* (*Bm*) transient receptor potential ankyrin 1 (TRPA1) may function as a thermosensor in the diapause induction (Sato et al., 2014). A hypothetical model representing the hierarchical pathway from the perception of temperature cue by BmTRPA1 to DH release through GABAergic regulation has been proposed by Tsuchiya et al. (2021). In insects, TRPA1 is a Ca²⁺-permeable, non-selective cation channel localized to the plasma membranes and found to be expressed in nociception receptor neurons and Br (Kang et al., 2019). They reported that the knockdown of *BmTRPA1* through embryonic RNAi influenced diapause incidence and DH release during pupal-adult development in the Kosetsu race. Additionally, they found that the KO of *BmTRPA1* induced LD response during the larval stage even at a high temperature incubation at 25°C under DD, which caused 100% diapause in the wild-type strain (Yokoyama et al., 2021). This means BmTRPA1 is not necessary for the function of the circadian clock underlying the PR during the larval period (Ikeda et al., 2021; Tobita and Kiuchi, 2022). On the other hand, in the KO strain, there were significant temporal changes in the expression profiles of the clock genes compared to the wild strain and no daily rhythm was observed, suggesting an interaction between BmTRPA1 and the circadian clock (Homma et al., 2022). Thermoperiod can synchronize the circadian clocks as a Zeitgeber, and substitute for photoperiod in the diapause induction of insects (Saunders, 2014). The clarification of BmTRPA1's role in these phenomena can be expected.

Akitomo et al. (2017) conducted a microarray analysis of expressed genes in the larval Br and found that the *Crp18a1* (cytochrome P450 18a1) and *Kr-h1* (Krüppel homolog 1) genes were upregulated in diapause-producing silkworms compared with non-diapause producers. The *Crp18a1* gene encodes a key enzyme for steroid hormone inactivation (Guittard et al., 2011), and *Kr-h1* encodes a zinc-finger-type transcription factor that mediates the repression of metamorphosis (Kayukawa et al., 2014). In a more recent study, Egi and Sakamoto (2022) proposed that the programming process of diapause induction by maternal photoperiodic and thermal conditions may involve juvenile hormone acid methyltransferase (Jhamt) and proton-coupled folate transporter (Pcft).

The epigenetic control of PR in insects has also been observed recently (Trionnaire et al., 2013; Reynolds, 2017). For example, in *Nasonia vitripennis*, knocking down DNA methyltransferase (*Dnmt1a*) or pharmacologically blocking DNA methylation disrupted PR of diapause induction, suggesting that DNA methylation is associated with the photoperiodism of the wasp (Pegoraro et al., 2016). In the silkworm,

there has been one pharmacological study examining epigenetic control, in which the dietary administration of 5-azacytidine, a DNA methyltransferase inhibitor, did not affect PR during the larval stage (Egi et al., 2017).

TIME MEASUREMENT MECHANISM IN THE PHOTOPERIODIC CLOCK

The mechanism by which the photoperiodic clock measures the length of day or night has been studied through various experimental protocols that manipulate light and dark conditions. Based on the results, various models have been proposed (Saunders et al., 2002). Studies on the clock mechanism in the silkworm are scarce, but some authors have reported results related to this issue. Kogure and Kobayashi (1930) investigated the diapause incidence under abnormal light and dark cycles (L1:D3, 5, 7, 9, 11) at 19°C incubation using the Shohaku race. They found that more than 7 hours of scotophase induced SD response and stated that the light (photo) period was important for diapause induction, and its effect was prolonged for at least 7 hours during the dark period. Hirasaka and Koyama (1970, 1972) investigated the relationship between non-daily photoperiodic regimes and larval growth rate, and discussed a possible involvement of the circadian system in the larval development.

Nakajima (1981) conducted a night interruption experiment by exposing light-pulse to eggs of a bivoltine hybrid race (J106 × Daizo) during scotophase. When the eggs were incubated under SD (L10:D14) at 20°C, almost all resultant moths became non-diapause producers. He interrupted the scotophase using monochromatic blue-green light with 4 μW/cm² for 1 hour. The interruptions at 3 and 11 hours after scotophase onset produced remarkable diapause production, with two maximum peaks. Short light pulses of 30 sec were also effective in the diapause production. These results can be explained by ECM (Pittendrigh and Minis, 1964; Saunders, 2020). The ECM appears to be an adequate representation of the photoperiodic mechanism in holometabola insects, in which Br-centered photoreception has been suggested (Saunders, 2021).

Sakamoto et al. (2003) observed that under a light-dark cycle of L20:D4, the larval hatching peak was observed at the early part of the photophase. In contrast, under L4:D20, it occurred at the late part of the scotophase. This scotophase peak was only observed in carotenoid-deficient silkworms but not in carotenoid-supplemented ones. The first peak of the circadian hatching rhythm, induced by the transfer from constant light (LL) to constant darkness (DD), was significantly reduced in carotenoid-supplemented silkworms compared to carotenoid-deficient ones, without affecting the subsequent circadian oscillation. Thus, despite carotenoids not being essential for the photoreception, formation, and entrainment of the circadian hatching rhythm (Sakamoto and Shimizu, 1994), the carotenoid-dependent process (CDP) affecting hatching pattern was identified. This suppression phenomenon by carotenoid was absent in the silkworm eclosion rhythm (Shimizu and Miura, 1987), and its physiological mechanism is not known. Drawing upon these observations, a theoretical framework for time measurement in the silkworm PR is proposed in Fig. 6. It is based on ECM and assumes the existence of two independent components that interact to induce the diapause. The first component is a diapause-inducing

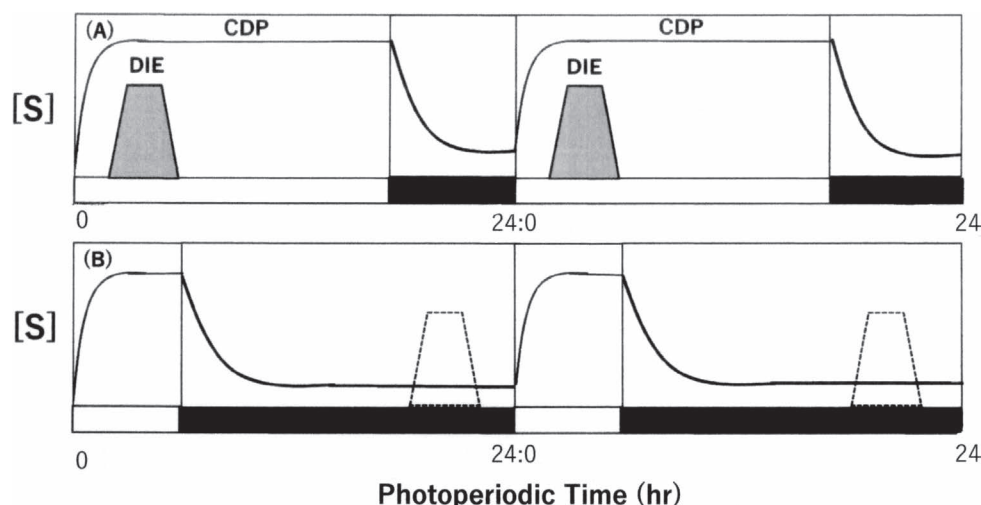


Fig. 6. Hypothetical model for photoperiodic time measurement in the silkworm PR of diapause induction. In this model, interaction between carotenoid-dependent process (CDP) represented by virtual kinetic curves of substance [S] and diapause inducing event (DIE) determines the diapause induction. White and dark bars represent photophase and scotophase, respectively. Solid trapezoidal symbols represent the expression of DIE, and dashed ones represent its depression. **(A)** and **(B)** represent LD and SD photoperiodic condition during the embryonic stage, respectively. See the text for details.

event (DIE), such as gene transcription, which occurs during a restricted phase (represented by trapezoidal symbols in Fig. 6) within the light-dark cycles. It corresponds to the photoinducible phase ψ in ECM (Saunders, 2020). In the figure, the tentative phases of DIE are drawn at those of hatching peaks, as night interruption experiments demonstrated that ψ occurs in the late part of the scotophase in L10:D14 cycle, nearly coinciding with the hatching peak (Nakajima, 1981). The second component is a CDP, in which the concentration of a hypothetical substance, denoted as [S], begins to increase in response to the dawn's light-on signal, reaching a plateau value, and subsequently decreases when exposed to the dusk's light-off signal, reaching its lowest level: CDP behaves like a discontinuous hourglass mode by light-on and off signal. In the proposed model, a high concentration of [S] induces the expression of DIE during the photophase of LD condition thereby promoting diapause production (Fig. 6A). Conversely, a low concentration of [S] does not induce its expression during the scotophase of SD condition, thus favoring non-diapause production (Fig. 6B). This concept resembles Truman's model (1971), which aimed to elucidate the photoperiodic control of diapause termination in the silkworm, *A. pernyi*. Truman proposed an interaction between a circadian clock responsible for triggering the release of PTTH (prothoracicotropic hormone), crucial for diapause termination, and an hourglass mechanism that restrained this release.

Anthony Lees (1981) investigated the action spectra for the photoperiodic control of polymorphism in *M. viciae* and pointed out the possible involvement of the slow regeneration of rhodopsin in time measurement of the critical night length in the darkness: the photoreceptor pigment itself functions as the hour glass timer. Recent research has unveiled that insect opsins have thermally bistable forms of rhodopsin (Rh) and metarhodopsin (Mrh), which is a physiologically active form (Minke, 2012; Tsukamoto, 2014).

These bistable pigments, each with distinct absorption spectra, can shift between two forms upon exposure to specific light wavelengths, as observed in phytochrome which has interconvertible forms of Pr (inactive) and Pf (active) (Izawa et al., 2002). Prolonged exposure to white light leads to the conversion of Rh to Mrh, establishing a photosteady state influenced by the relative absorption coefficients of these two states. Subsequently, in the absence of light, Mrh reverts back to Rh in a reversible manner, ultimately reaching an equilibrium state (Tsukamoto, 2014). Carotenoid (provitamin A) is essential for CDP, and so one conceivable mechanism of CDP is Rh -Mrh conversion: the hypothetical substance [S] in Fig. 6 corresponds to Mrh.

As for DIE, GAT transcription, the levels of which were found to be regulated by the circadian clock, appears to be the most likely candidate (Cui et al., 2021; Tsuchiya et al., 2021). This is also likely involved in regulating the secretion of eclosion hormone evoking the larval hatching (Fugo et al., 1985).

GENERAL DISCUSSION AND SUMMARY

The silkworm PR is influenced by temperature at the developmental stages in different manners. During egg stage low temperature and SD conditions act together jointly to contribute to reducing diapause, while high temperature and LD act together to enhance it. One focus for researchers studying insect photoperiodism is the integration of signals from temperature and light into the diapause induction process (Beck, 1980; Saunders et al., 2002; Denlinger, 2022). At the molecular level, an opsin pigment (Boceropsin) and BmTRPA1 have been proposed as the light and temperature sensor, respectively, in the diapause induction of the silkworm (Shimizu et al., 2001; Sato et al., 2014). Investigating the link between photoperiodic and thermoperiodic responses appears to be a reasonable approach to unveil the interaction mechanism (Beck, 1991; Saunders, 2014). However, a thermoperiodic experiment for diapause has not been conducted in the silkworm, despite the clear entrainment of adult eclosion rhythm under temperature cycles (Takahashi et al., 2020).

An attractive hypothesis for the integration mechanism is that the light and temperature signals are received by a common sensor, and opsin is the prime candidate. This idea has been suggested first in the context of a predatory mite, *Amblyseius potentillae* (van Houten et al., 1987; van Houten and Veerman, 1990). While the silkworm lacks definitive evidence to substantiate this hypothesis, the temperature dependency of diapause incidence under DD (Fig. 3) and that of threshold light-intensity of the diapause induction

under LL serves to support this idea (Kogure, 1933). The requirement of clock genes for both PR (Cui et al., 2021; Ikeda et al., 2021; Tobita and Kiuchi, 2022), and thermal control (Homma et al., 2022) suggests that these environmental cues are integrated in one shared signal.

Recently, opsins have been found to have diverse functions, including thermo-, mechano-, and chemosensory roles (Frentiu et al., 2015; Leung and Montell, 2017; Feuda et al., 2022; Vöcking et al., 2022). Consequently, they can no longer be exclusively classified as mere light-sensing molecules. In a study by Shen et al. (2011), it was demonstrated that a deficiency in dietary vitamin A impaired the temperature discrimination ability of *Drosophila*. Moreover, the rhodopsin1 (Rh1) protein, coded by the *nina E* gene, was shown to play a role in the thermotactic response. Essential components of the signal cascade in the visual perception of fly eyes, Rh1, phospholipase C (norpAp24), and the TRPA1 channel, were found to be integral for the thermal response (Kwon et al., 2008; Shen et al., 2011). Furthermore, in *Drosophila*, Rh5 and Rh6 were identified as functioning thermosensors in TRPA1-expressing Br neurons (Leung and Montell, 2017; Sokabe et al., 2017). Recent research on *D. melanogaster* unveiled an intriguing role for the Eyes Absent (EYA) protein, known for its significance in various tissue morphogeneses, including the compound eye. This protein acts as a seasonal sensor, interpreting both photoperiodic and temperature signals through its interaction with TIM (Abrieux et al., 2020). However, no analogous information is available in the silkworm.

It has been revealed that the regulation of DH secretion is mediated through GABAergic control involving GAT expression (Shimizu et al., 1989; Tsuchiya et al., 2021). Further it was found that the expression of GAT was downregulated in the *per* KO mutant (Cui et al., 2021). These observations strongly suggest the crucial role of GAT in silkworm PR of diapause induction through the circadian clock system. The daily bio-synthetic accumulation of GAT in the plasma membrane of GABA neurons under circadian control might be the molecular mechanism underlying the silkworm PR, considering that the cellular mechanism of memory and conditioning generally involves synaptic modifications (Martin et al., 2000). Chronobiological investigation of GAT expression in the plasma membrane of GABA neurons could provide valuable insights into this perspective. Ramos et al. (2003) reported that, in *Acyrtosiphon pisum*, the ApSDI-1 protein, which is assumed to be a transporter of neutral amino acids in the aphid GABAergic neurons, might be involved in the transduction of the photoperiodic message in the process that triggers PR. A systematic study analyzing genes of domestic and wild silkworms revealed that circadian clock genes are associated with the domestication process of the silkworm through daily and seasonal adaptation (Xiang et al., 2018). The difference in the PR mode between the silkworm races might be due to the variation of GABAergic control caused by the genetic variance of the circadian system.

Figure 7 presents a hypothetical model outlining the pathway in the silkworm PR, spanning from the maternal perception of photoperiodic signals (SD or LD) during the egg stage to the diapause eggs production. This scheme encompasses several key components, including the photoreceptors, circadian clock, photoperiodic clock, GABAergic

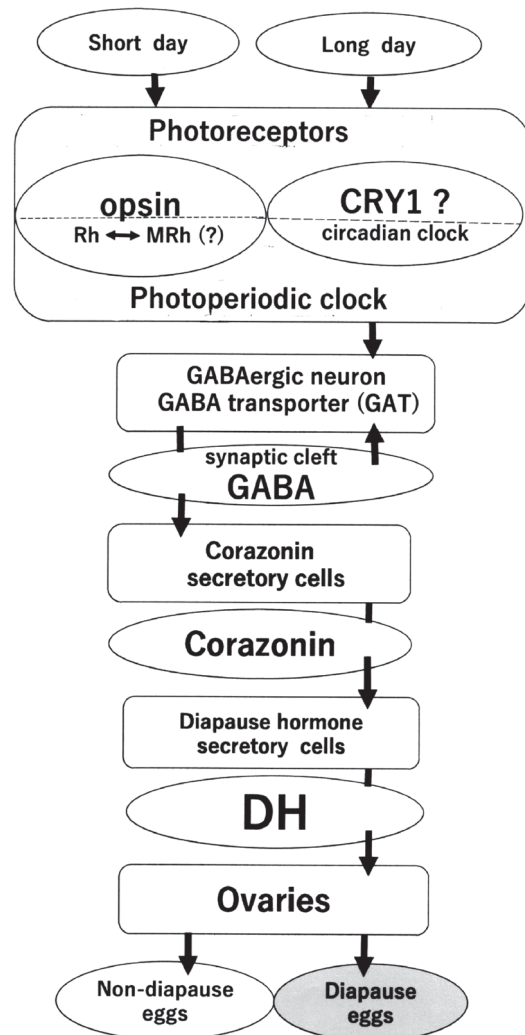


Fig. 7. Schematic representation of a hierarchical pathway in the silkworm photoperiodic response, starting from maternal photoreception of SD or LD cues during the egg stage and leading to the deposition of diapausing or non-diapausing eggs by moths, through the involvement of photoreceptors, circadian clock, photoperiodic clock, GABAergic, corazonin, and diapause hormone signal pathways. Arrows indicate signal and substance flows. Upward arrow shows the back flow of GABA from the synaptic cleft to presynaptic neurons by GAT.

neurone, Crz neural cells, and DH cells. The photoperiodic clock distinguishes between SD and LD condition and sends corresponding signals to downstream of the PR induction pathway. While the exact mechanism of this photoperiodic clock remains unknown, the hypothesis put forth by Sakamoto et al. (2003), which proposed an interaction between the circadian clock and an hourglass-like timer involving an interconversion of Rh and Mrh, underlies the foundation. In this model, under SD, the signal from the photoperiodic clock potentially leads to the suppression of GAT expression in GABAergic neurons. This, in turn, could result in the prolonged influence of GABAergic signals on Crz neurons, thereby continuously inhibiting the release of Crz from the somata. Subsequently, this extended inhibition could trigger the suppression of DH release from DH secretory

cells, and result in the production of non-diapause eggs. In contrast, under LD, the photoperiodic clock elevated the expression of GAT, which transports GABA from the synaptic cleft back to the presynaptic neurons (upper arrow in Fig. 7) to terminate GABAergic inhibition. The termination induces Crz release followed by the stimulation of DH release and diapause eggs production. Based on these insights, further comprehensive investigations including molecular and neuroanatomical studies will clarify the mechanism of the silkworm photoperiodic response.

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COMPETING INTERESTS

The author declares no competing financial interests.

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