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Does DNA methylation play a role in photoperiodic diapause of moths?

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Abstract
The present study pharmacologically investigated the involvement of DNA methylation in photoperiodic diapause of the domestic silkworm *Bombyx mori* (Lepidoptera: Bombycidae) and the Chinese giant silkmoth *Antheraea pernyi* (Lepidoptera: Saturniidae). The DNA methyltransferase inhibitor 5-azacytidine was dietarily given to larvae of *B. mori* under short days or long days. However, regardless of the drug administration, most resultant moths that had been exposed to short days during the larval stage laid diapause eggs and those to long days laid nondiapause eggs. 5-azacytidine did not significantly affect the diapause status of eggs. In addition, 5-azacytidine was intraperitoneally injected into diapause pupae of *A. pernyi*. The drug treatment caused no significant effect on diapause maintenance during short days or adult emergence under long days. We could not find evidence that DNA methylation plays a role in photoperiodic diapause of moths.

Keywords: 5-azacytidine, DNA methylation, moth, photoperiodic diapause.

1. Introduction
A variety of organisms including insects are able to sense the season of the year by measuring changes in the length of day or night, or photoperiod [1, 2]. Seasonal timing allows organisms to adjust their lifestyles and developmental destiny predictively to cope with coming harsh seasons. In insects, photoperiodism provokes diapause (arrest of development or reproduction) and seasonal morph determination at species-specific ontogenetic stages [3, 4]. Several studies have revealed that the circadian system plays an important role in photoperiodic time measurement [5-8]. However, the molecular mechanisms that control insect photoperiodism are largely unknown.
DNA methylation controlling gene expression is a key mechanism underlying epigenetic regulation and has been shown to be critical for seasonal regulation [9]. For example, DNA methylation functions in measuring photoperiod in the jewel wasp *Nasonia vitripennis* [10], as well as in the Siberian hamster *Phodopus sungorus* [11]. But, to our knowledge there are no epigenetic studies on photoperiodic responses in insects other than *Nasonia*.
The domestic silkworm *Bombyx mori* (Linnaeus) and the Chinese oak silkmoth *Antheraea pernyi* (Guérin-Méneville) have been used for studies of photoperiodic diapause [12-14]. These species show an evident photoperiodic response in diapause regulation and the large body size facilitates surgical procedures. In *B. mori*, embryonic diapause is under maternal control. Silkworms reared under short days during the larval stage produce diapause eggs and those under long days produce nondiapause eggs in some bivoltine strains [15, 16]. On the other hand, *A. pernyi* enters diapause at the pupal stage. Pupal diapause is maintained during short days and can be terminated by exposure to long days [14, 17].
The present study pharmacologically investigated the involvement of DNA methylation in the photoperiodic response of diapause regulation in *B. mori* and *A. pernyi*. The pyrimidine analogue 5-azacytidine was used as a DNA methyltransferase inhibitor. We reared *B. mori* larvae on an artificial diet with 5-azacytidine under short days or long days, and then observed the diapause state of eggs laid by resultant moths. Furthermore, we injected 5-azacytidine into diapause pupae of *A. pernyi* to examine the effect on diapause maintenance during short days and adult emergence (diapause termination) under long days.

2. Materials and Methods
2.1.1 Animals
The entire study was carried out from January 2014 to April 2016. All individuals of *B. mori* and *A. pernyi* were kept at 25 °C throughout the experiment. A white fluorescent

lamp was the source of illumination (0.3-0.6 W/m^2 at the level of the animals). The Animal Care and Use Committee of Kobe University approved all experiments and procedures.

2.1.2 Bombyx mori

Diapause eggs of the bivoltine strain p50 provided by Kyushu University, Japan (http://www.shigen.nig.ac.jp/silkwormbase/index.jsp) were incubated at 25 °C under continuous darkness. Hatched larvae were reared under a daily 12 h light–12 h dark cycle (LD12:12; lights-on 8:00-20:00 h) as a short-day photoperiod or LD20:4 (lights-on 4:00-0:00 h) as a long-day photoperiod until the day before the 4th larval molt. During this period, larvae were fed with a 5-azacytidine-added artificial diet. From the day of the 4th molt, silkworms in any experimental group were fed with a normal artificial diet without 5-azacytidine and kept in long days until they reached adulthood and laid eggs. Then, we observed the diapause status of eggs laid by resultant female moths (n = 19 - 51 individuals in each experimental group). Diapause incidence was defined as the ratio (%) of moths that produced diapause eggs, as described [15].

2.1.3 Antheraea pernyi

Diapause pupae of the univoltine strain of *A. pernyi* were provided by Shinshu University, Japan (http://shigen.nig.ac.jp/wildmoth/). Pupae arrived were stored at 25 °C under a short-day photoperiod (LD12:12, lights-on 8:00-20:00 h) for 16 weeks and divided into two groups. Then, one was kept in short days and the other was transferred to a long-day photoperiod (LD16:8, lights on 6:00-22:00 h). We started 5-azacytidine injections from the first day after the transfer. All experiments were conducted on male pupae. To determine the effect of drug treatments on the diapause state of pupae, we counted the number of moths that emerged every day, and the incidence of diapause termination was expressed as the ratio (%) of the cumulative number of adults that emerged per group (n = 13-15 individuals in each experimental group).

2.2 Chemical applications

5-azacytidine (DNA methyltransferase inhibitor; Tokyo Chemical Industry Co., Ltd, Tokyo, Japan) was dissolved (40 mM) in distilled water. We added this solution to an artificial diet (Silkmate PS, Nihonnousan Kogyo Co. Ltd., Kanagawa, Japan) to make a 5-azacytidine-added (2 mM) diet for *B. mori*. In *A. pernyi*, the 40 mM 5-azacytidine solution (50 µL) was intraperitoneally injected into each pupa twice with an interval of 4 days using Hamilton syringes. The negative control was distilled water in experiments on both *B. mori* and *A. pernyi*.

2.3 Statistical analysis

Diapause incidences in *B. mori* were compared using Fisher's exact test and the incidences of diapause termination in *A. pernyi* using Kaplan-Meier analysis with the log-rank test between the 5-azacytidine-treated and control groups under each experimental condition. *P* values < 0.05 were considered to represent significance.

3. Results and Discussion

The present study examined the effects of 5-azacytidine administrations on the photoperiodic response in diapause regulation of moths. In *B. mori*, either with or without 5-azacytidine, most resultant moths that had been exposed to short days during the larval stage laid diapause eggs and those to long days laid nondiapause eggs (Table 1). Diapause incidences did not significantly differ between the 5-azacytidine-treated and control groups under each condition (Fisher's exact test, $P > 0.1$). In *A. pernyi*, pupal diapause was maintained during short days but terminated under long days (Figure 1). 5-azacytidine caused no significant effects on the timing of adult emergence (Kaplan-Meier analysis with the log-rank test, $P > 0.1$). We could not find evidence that DNA methylation plays a role in photoperiodic diapause of moths.

Table 1: Effect of 5-azacytidine administration during the larval stage on diapause incidence in the next generation of *Bombyx mori*

Larval stage (1st - 4th instar)		No. of resultant moth		Diapause incidence (%)
Photoperiod	Diet	Diapause-egg producer	Nondiapause-egg producer	
Short day	Control	18	1	94.7
	5-azacytidine	47	1	97.9
Long day	Control	0	27	0.0
	5-azacytidine	1	50	2.0

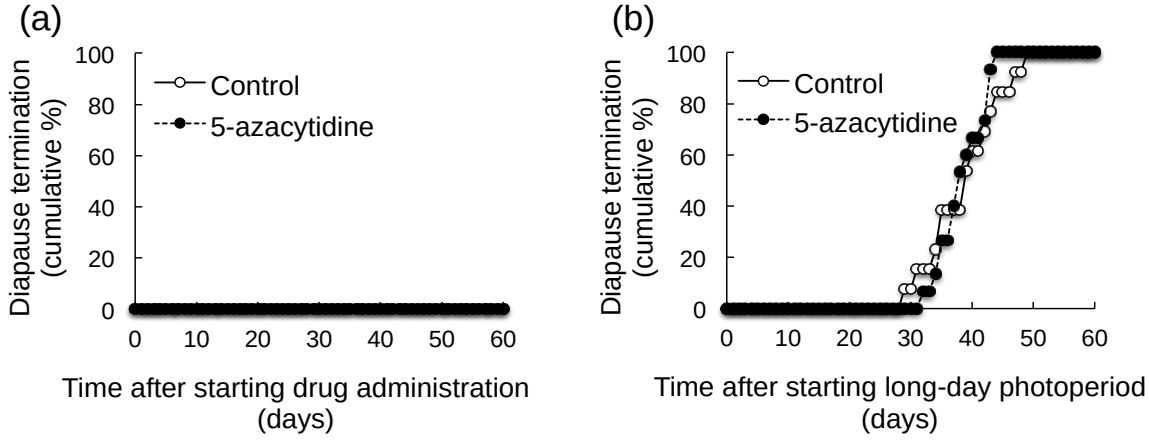

Fig 1: Effect of administration of 5-azacytidine on diapause pupae of *Antheraea pernyi* (a) during short days and (b) during long days.

Recent research has indicated both by knocking down *DNA methylaransferase* genes and by blocking DNA methylation pharmacologically that DNA methylation functions in measuring photoperiod in the jewel wasp *Nasonia vitripennis* [10]. The regulatory mechanisms of photoperiodism might be different between Hymenoptera and Lepidoptera species. In

invertebrates including insects, DNA methylation preferentially targets gene bodies or transcribed genic regions in the genome [18, 21]. Actually, 30% of cytosines were methylated in CpG dinucleotides of genes examined in *N. vitripennis* [22]. On the other hand, cytosine methylation levels in CpGs were low (<4%) in genes of *B. mori* [20]. DNA methylation might not contribute much to regulation of gene expression in the silkworm.

However, there still remains a possibility that the dosages of 5-azacytidine administrated in this study were too low to affect the photoperiodic responses. But, only a few larvae (<10%) survived until the 4th molt in the 5-azacytidine-treated group of *B. mori*. And, 5-azacytidine solution injected into *A. pernyi* pupae was almost saturated. So, it was difficult to use 5-azacytidine at higher doses for drug administration. After all, further studies knocking down *DNA methyltransferase* genes should clarify the functional involvement of DNA methylation in photoperiodic timing of moths.

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