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Photoperiodic gene expression of insulin receptor is associated with diapause regulation in silkworm

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ABSTRACT: Bivoltine silkworms (*Bombyx mori*) are destined to respectively produce diapause or non-diapause eggs when they are reared under short or long days during the larval stage. The insulin signaling pathway is thought to play an essential role in regulating diapause in various insect species, but its involvement in silkworm diapause programming has not been investigated in detail. Therefore, we examined day-night expression of the insulin receptor (*InR*) gene in the silkworm larval brain under different photoperiods or in night interruption experiments in which larvae were exposed to light for 2 h during the nighttime of short days. Expression of the *InR* gene was photoperiod-dependent and *InR* mRNA levels decreased with increasing daylength. As the daylength increased, expression during the nighttime decreased to lower stable levels earlier than that during the daytime. During night interruptions that induced non-diapause, the nighttime expression of *InR* decreased to low levels like those during long days, although daytime expression was only moderately decreased. Nighttime *InR* expression was downregulated in silkworms reared under non-diapause-inducing conditions (long days and night interruptions). In contrast, abundant *InR* was expressed during the day and night in short days that induced diapause. Our findings suggested that *InR* expression in the larval brain is associated with programming the diapause status in the next generation of silkworms. Downregulated *InR* might suppress the insulin signaling pathway and cause non-diapause induction in the next generation.

Keywords: *Bombyx mori*; Diapause; Gene expression; Insulin receptor; Photoperiod.

1. INTRODUCTION

Diapause is a programmed, hormonally regulated arrest of development or reproduction to cope with harsh environmental conditions [1, 2]. Insects have evolved diapause as an adaptive strategy to unfavorable seasons. Diapause enables insects to expand their habitat in temperate zones. Diapause occurs at a species-specific ontogenetic stage. Some species of insects enter genetically programmed diapause regardless of the prevailing environmental inputs. Others determine whether to enter diapause or develop without diapause depending on seasonal cues. However, the mechanisms that program insect diapause depending on environmental factors have not been fully elucidated.

The insulin signaling pathway (ISP) seems to play essential roles in regulating diapause and overwintering in various insect species [2, 3] such as nymphal overwintering in the cricket *Modicogryllus siamensis* [4], pupal diapause in the cotton bollworm *Helicoverpa armigera* [5], and reproductive diapause in

the fruit fly *Drosophila melanogaster* and the mosquito *Culex pipiens* [6, 7]. The ISP appears to regulate metabolism and growth through downstream targets that lead insects to the diapause phenotype [3, 8]. Transcriptomic findings have identified the diapause-distinct expression of genes involved in the ISP [9]. However, little is known about the daily variations in ISP gene expression during insect diapause regulation [10], that would facilitate further understanding of diapause-regulatory mechanisms.

The domestic silkworm *Bombyx mori* L. (Lepidoptera: Bombycidae) has served as a model system for studying diapause regulation in insects [11-15]. Embryonic diapause in this species is maternally controlled. Bivoltine silkworms are destined to produce diapause or non-diapause eggs depending on environmental cues such as temperature and photoperiod (length of day) during the egg and larval stages [12, 16]. This program of diapause in the next generation is generated and retained in the silkworm brain [17]. However, involvement of the ISP in diapause programming of silkworm larvae has not been investigated in detail.

Here, we examined day-night expression of the insulin receptor (*InR*) gene that is essential to the ISP, under photoperiods that program diapause or non-diapause in silkworms. We reared silkworm larvae under short or long days to direct them towards producing diapause or non-diapause eggs, respectively, then assessed *InR* mRNA levels in their brains during the day and night using real-time quantitative PCR (RT-qPCR). We also assessed the effects of night interruptions (under short days) with 2 h of light on larval *InR* expression because such disturbances can inhibit diapause induction in some insects [18]. In the present study, *InR* expression was examined only in the brain. This is because the brain has been shown to be the diapause programming center in the silkworm [17], as in other insects [2].

2. MATERIALS AND METHODS

2.1. Animals

Eggs of p50 silkworms were incubated at 25°C under continuous darkness. Hatched larvae were reared at 25°C under a daily 12 h light-12 h dark cycle (LD12:12, short day; lights on 5:00-17:00), LD16:8 (long day; lights on 3:00-19:00), or LD20:4 (long day; lights on 1:00-21:00). In night interruption experiments, hatched larvae were reared under LD12:12 (lights on 5:00-17:00), then exposed to light for 2 hours at night (19:00-21:00) to determine the effects of such interruption.

When the larvae started to spin cocoons, silkworms of all groups were maintained at 25°C under LD12:12 until they became adult moths and laid eggs. Thereafter, the diapause status of eggs produced by resultant female moths was confirmed as described [19]. Briefly, laid eggs were stored at 25°C, then non-diapause eggs hatched within 14 days whereas diapause eggs produced ommochrome serosal membranes and remained at the embryonic stage.

A FL10EX-D-Z white fluorescent lamp (Toshiba Corporation, Tokyo, Japan) provided 100 lux of illumination at the level of the animals. Larvae were fed with the Silkmate PS artificial diet (Nosan Corporation, Yokohama, Japan).

2.2. Tissue collection and RNA extraction

We analyzed the brains of female larvae collected at midday (11:00) and midnight (23:00) on day 2 of the fifth instar using RT-qPCR. The corpora allata and corpora cardiaca were carefully dissected away from the brains. Five or six pools containing five brains were collected per experimental group. Total RNA was extracted from each tissue pool using RNeasy Plus Micro Kits (Qiagen GmbH, Hilden, Germany).

2.3. Real-time quantitative PCR

First-strand cDNA was reverse-transcribed from RNA samples using ReverTra Ace® qPCR RT Kits (Toyobo, Osaka, Japan), then RT-qPCR proceeded using THUNDERBIRD® Next SYBR® qPCR Mix (Toyobo) and a Thermal Cycler Dice Real Time System TP800 (Takara Bio Inc., Kusatsu, Japan) with the following primers:

- *Bombyx mori insulin receptor* (*InR*; Genbank Accession number NM_001043546.1) forward, 5'-acaacaggccgcagatactt-3' and reverse 5'-aagcgcaaacaccatttttc-3' (147 bp product);
- *Bombyx mori ribosomal protein 49* (*rp49*; GenBank accession number AB048205.1) forward, 5'-tcaatcggatcgctatgaca-3' and reverse, 5'-atgacgggtcttcttgttgg-3' (136 bp product).

The reaction parameters were 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Amounts of *InR* transcripts were normalized to that of *rp49*.

2.4. Statistical analysis

The incidence of diapause defined as the ratio (%) of female moths (n = 24–36) that laid diapause eggs [20], was compared among experimental groups using Fisher's exact test with Benjamini-Hochberg multiple testing correction. The abundance of mRNA measured by RT-qRCR is expressed as means ± S.E.M. (n = 3–6 independent samples). Data were analyzed using a one-way ANOVA followed by the Tukey-Kramer test. Values with $P < 0.05$ were considered significant.

3. RESULTS

We initially examined the incidence of diapause in the next generation when larvae were reared under photoperiods of LD12:12, LD16:8 or LD20:4, or night interruptions in which animals were exposed to 2 h of light during the nighttime under LD12:12 throughout the entire larval period (Fig. 1A). The diapause incidence was 100% in the LD12:12 group and 0% in the other three groups. These results indicated that LD12:12 (short days) stimulated the induction of diapause, whereas LD16:8 and LD20:4 (long days), and night interruptions stimulated that of non-diapause.

Figure 1B shows the day-night expression of *InR* in the brains of fifth instar under these diapause-controlling conditions. The expression of *InR* was abundant during the daytime and nighttime under LD12:12, and during the daytime under LD16:8. But the nighttime expression under LD16:8 was ~50% of the daytime value. Expression under LD20:4 was reduced during the daytime and nighttime to the levels found during the nighttime under LD16:8. The expression of *InR* decreased during the nighttime in the night interruption group to a level similar to that under LD16:8 and LD20:4, whereas daytime expression was only moderately decreased compared with that under LD12:12.

We analyzed the relationship between nighttime *InR* expression and the number of days that larvae were exposed to interruption at night (Fig. 2). The larvae were reared under LD12:12, then exposed to interruption for 0, 1, 3 or 11 consecutive days immediately before dissection at midnight on day 2 of the fifth instar. Exposure for 1 day did not significantly affect *InR* expression, whereas exposure for 3 and 11 days decreased nighttime expression to about half the level of the day 0 control. Exposure for 2 or 3 days was sufficient to lower nighttime *InR* expression to stable low levels.

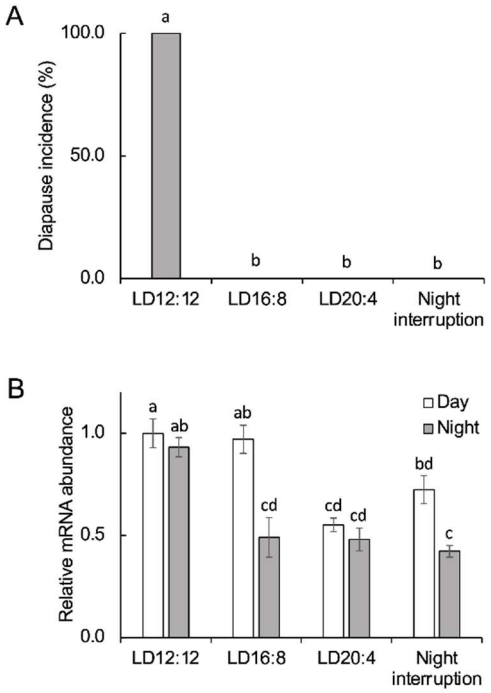

Figure 1. Effects of photoperiod and night interruptions during larval stage on (A) incidence (%) of diapause in next generation and (B) amounts of insulin receptor mRNA in larval brain.

Incidence of diapause was compared among groups ($P < 0.05$, Fisher's exact test with Benjamini-Hochberg multiple-testing correction). Relative amounts of insulin receptor mRNA were measured at midday (11:00, white column) and midnight (23:00, shaded column). Data are shown as means ± S.E.M. Value at day in the LD12:12 group is expressed as 1.0 ($P < 0.05$, one-way ANOVA followed by the Tukey-Kramer test). Superscript letters indicate significant differences among groups.

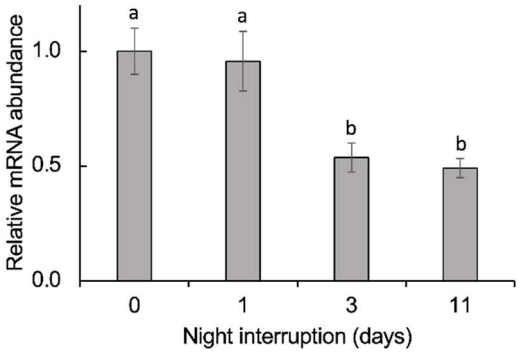

Figure 2. Effects of larval exposure to night interruptions on amounts of insulin receptor mRNA in larval brains over time.

Larvae were exposed to light for 2 h at night in short days for 0, 1, 3 or 11 consecutive days immediately before dissection. Data are shown as means ± S.E.M. Value of day 0 group is expressed as 1.0 ($P < 0.05$, one-way ANOVA followed by the Tukey-Kramer test). Superscript letters indicate significant differences among groups.

4. DISCUSSION

We found that *InR* gene expression in the silkworm larval brain was photoperiod-dependent. Interestingly, the daily feeding pattern of silkworm larvae also varies with daylength [21]. Such photoperiod-dependent feeding behavior might be associated with daily changes in *InR* expression in the brain, as food intake affects insulin signaling [22] and, conversely, insulin signaling influences feeding behavior [23]. In studying the regulatory mechanisms of diapause, it may be important to investigate correlations with feeding behavior.

We also found that *InR* mRNA levels decreased with increasing day length. Furthermore, the length of the day at which *InR* expression switched to stable low levels was shorter for nighttime than daytime expression. Therefore, as the photoperiod increased, nighttime expression decreased to stable low levels earlier than daytime expression. A common feature in silkworms reared under non-diapause-inducing conditions (LD16:8, LD20:4 and night interruptions) was downregulated nighttime *InR* expression, whereas daytime expression varied between low and high levels. In contrast, *InR* expression was high during the day and night under LD12:12 that induced diapause. These findings suggested that *InR* expression in the larval brain is associated with programming the diapause status of the next silkworm generation. Downregulated *InR* expression might suppress the ISP and cause non-diapause induction of the next generation. It is plausible that *InR* downregulation only at night could be sufficient to switch from diapause to non-diapause induction.

Our findings suggested that downregulated *InR* expression in silkworm larvae is associated with non-diapause induction. This is similar to pupal diapause in the flesh fly *Sarcophaga crassipalpis* [24], where most ISP genes are more abundantly expressed in diapausing than non-diapausing pupae. However, ISP suppression seems to be involved in diapause induction in other species including flies, mosquitoes, moths and grasshoppers [5, 7, 25-27]. Species-dependent strategies for regulating ISP gene expression appear to provoke similar diapause responses [24]. Further studies are needed to elucidate these mechanisms.

A previous study has suggested that the ISP is involved in photoperiodic regulation of seasonal shifts of reproductive modes in the aphid *Acyrthosiphon pisum* [10]. The expression of *InR* in this insect is high in the daytime and low in the nighttime under long days, and constantly low in short days. This response of *InR* expression to photoperiods is opposite that of silkworms. The daily expression of *InR* appears to be species-specific. We believe that investigating the ISP relative to daily rhythmicity is important for advancing understanding of the diapause-regulatory mechanisms in insects.

The present findings indicated that night interruptions decreased nighttime *InR* expression to low levels similar to those in long days that induced non-diapause. We found that three days of night interruptions decreased nighttime *InR* expression to stable low levels, whereas one day did not. This indicated that only 2 or 3 days are required for the *InR* gene to change its expression in response to a shift between stimuli that induce diapause and non-diapause. However, although night interruptions stimulated the induction of non-diapause, three days of interruption was not sufficient to significantly decrease the incidence of diapause (data not shown). Thus, *InR* mRNA levels might not be directly related to the incidence of diapause, but might mediate information about diapause-controlling stimuli that induce diapause or non-diapause. To our knowledge, this is the first investigation of the effects of nighttime interruptions on the expression of ISP genes associated with diapause regulation in insects. Night interruption might be a useful strategy for studying the mechanisms of diapause regulation in insects.

5. CONCLUSION

The present findings suggested that photoperiod-dependent expression of the *InR* gene in the larval brain is associated with programming the diapause status of the next generation in silkworms. We speculate that downregulating the *InR* gene could suppress the insulin signaling pathway and cause non-diapause induction in the next generation. We also found that only 2-3 days were required for the expression of the *InR* gene to change in response to a shift between stimuli that induce diapause and non-diapause. Thus, *InR* might be associated with mediating information about diapause-controlling stimuli.

Authors Contributions: KS supervised the study. AI and KS designed the research. AI carried out the experiments. YE analysed the data and made figures. KS wrote the manuscript. All authors read and approved the final manuscript.

Conflict of Interest: The authors declare no potential conflict of interest.

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