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THE INVOLVEMENT OF CIRCADIAN CLOCK GENE PERIOD IN PHOTOPERIODIC TIME MEASUREMENT OF THE FALL WEBWORM, *HYPHANTRIA CUNEA*

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ABSTRACT

Photoperiodic response is a crucial adaptation for many insects in temperate regions to survive stressful seasons, typically winter. Mechanisms underlying photoperiodism have not completely been elucidated but the involvement of circadian system has been conventionally conjectured. To examine this possibility, the involvement of *period* (*per*) was examined in photoperiodic control of diapause termination in the fall webworm, *Hyphantria cunea* (Lepidoptera: Arctiidae). Pupal diapause in *H. cunea* is induced under short days LD 14:10 but can be terminated by exposure to LD 16:8. RNAi by injecting double stranded RNA corresponding to the partial sequence of *per* stimulated termination of diapause and adult emergence even under LD 14:10, while the $dsRNA^{PER}$-injected pupae kept under LD 16:8 showed no difference compared with the control pupae. Thus, the present results suggest that the circadian clock regulates photoperiodism of *H. cunea* where *per* plays an active role in circadian system.

Keywords: *Hyphantria cunea*, diapause, period, photoperiodism

1. INTRODUCTION

The fall webworm, *Hyphantria cunea* (Drury) (Lepidoptera: Arctiidae) is originally distributed in North America, has invaded and colonized Europe and East Asia(Umeya and Itô 1977, Warren and Tadić 1967). It is a top quarantine species still expanding its distribution such as to Iran and New Zealand (Kean and Kumarasinghe 2007; Rezaei et al. 2003).

H. cunea enters facultative diapause as a diapause in response to short-day conditions (Gomi 1997). As in many living organisms, diapause is defined as a physiological and developmental state of arrest in which metamorphosis and metabolism are

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suppressed (Saunders 2002; Danks 2013). The reason behind this is regulated photoperiodically (Danilevskii 1965).Photoperiodic system consists of several functional elements; aphotoreceptor, a clock/timer, a summation mechanism countingeffective photoperiodic cycles and an endocrine switch (Danks 2013). The molecular mechanism of the circadian clock behind photoperiodism has not been completely elucidated, however, molecular studies on circadian oscillation have proceeded rapidly (Bünning 1960). The best-studied model system of circadian oscillation is the fruit fly *Drosophila melanogaster* (Meigen), where a number of circadian clock genes have been cloned and genetically analyzed for their functions (Hall 2003). *Period* (*per*) and other clock genes such as Cycle (*cyc*), Clock (*clk*) and Timeless (*tim*) and their protein products form a feed-back loop comprised of transcriptional activators or repressors (Goto and Denlinger 2002).

Per was extensively characterized both structurally and functionally, which suggests that the molecular mechanism of both *D.melanogaster* clocks and mammalian ones were based on negative transcription/ translation feedback loops (Konopka and Benzer, 1971; Sandrelli et al. 2008; Zylka et al. 1998).Hence, RNAiof *per* is performed in pupae of *H. cunea* to confirm general importance of per in photoperiodic time measurement, and to investigate effects on thephotoperiodic regulation of diapause.

2. MATERIALS AND METHODS

2.1 Insects

Ten laboratory-reared egg masses were provided by Chinses Academy of Forestry Science and were personally carried by researchers from Beijing, China, to Japan, which was authorized by the Plant Quarantine Service (ex., No. 25-Kobe-546). The egg masses were kept under light/dark (LD) 16:8h at 25°C until hatching. After that, larvae were reared on artificial diets (InsectaLF, Nihon Nosan Kogyo, Co. Ltd) in constant temperature cabinets (SANYO Incubator, MIR-553, Osaka, Japan) under designated photoperiods at 25°C until pupation.

2.2 Immunohistochemistry

Immunohistochemistry was performed following Wang et al. (2013). The brain- subesophageal complex (BR-SOG) was dissected from last instar larvae and adults reared at 25°C under LD 16:8. Dissection were carried out from the water-anesthetized animals in sterile saline and the brain-SOG complex was fixed overnight at 4°C in Bouin solution. Then, dehydration was sequentially conducted with 80% ethanol 3 times for 15 min, 90% ethanol (15 min), 95% ethanol (15 min), 100% ethanol (15 min), 100 % ethanol (30 min) and xylene (10 min, 2 min) for dealcoholation. Embedded tissues in paraffin were cut at 8 μm. After deparaffinization (Mohamed et al. 2014), the sections were blocked against non-specific adsorption with 1.5% normal goat serum in Tris-buffered saline (TBS) for 30 min at room temperature. Subsequent overnight incubation with anti-*Periplanetaamericana*PER antiserum (1:10,000) (340 amino acids, GenBank accession no. U12772) was conduct in a humidified chamber at 4°C. After washing 3 times in TBS for 15 min, the sections were incubated in the biotinylated secondary antibody for 1 hour using the Vectastain ABC peroxidase kit (Vector Laboratories, Burlingame, CA, USA). After washing 3 times in TBS each for 10 minutes and once with 0.1 M Tris-HCl, pH7.5 (5 min), the sections were stained with DAB solution (pH 7.2, 0.1 M Tris-HCl, 0.1% DAB, containing 0.02% H_2O_2) for 5 min.

2.3 RNAi

dsRNAs were synthesized from a 461 bp PCR product of *H. cuneaper* as the template (DDBJ/Genebank: accession number LC179545), which were prepared by gene- specific primers (forward 5'-TAATACGACTCACTATAGGGAGATCACATCTGGATTCAGTGTTAAAGA-3' and reverse 5'-TAATACGACTCACTATAGGGAGAGATGAATTCAGCTTTTTAGACCAA-3'). The 5'end of these primers were attached by T7 promoter and the dsRNA synthesis was conducted withMEGAscript RNAi kit (Ambion, CA, USA) according to the manufacturer's instructions. The dsRNA and Metafectene PRO (Biontex, Planegg, Germany) were mixed at a ratio of 1:1 (v:v) before injection. Larvae were reared under LD 14:10 at 25°C until silk- spinning pharate pupal stage to ensure individuals entering pupal diapause. Four experimental groups (40-45 for each group) of diapausing pupae were prepared. Five hundred nanograms of $dsRNA^{PER}$ or $dsRNA^{GFP}$ (as control) was injected into abdominal intersegmental membrane of each pupa that was then kept under LD 16:8 or LD 14:10 at 25°C.

2.4 qRT-PCR

The brain-subesophageal ganglion complex (Br-SOG) of *H. cunea* was dissected and immediately transferred to liquid nitrogen and total RNA was extracted and purified by using the RNAiso Plus reagent (Takara, Japan). Five hundred nanograms of total RNA with

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primers using ReverTra Ace kit (Toyobo Co. Ltd., Osaka, Japan) was used for synthesizing the cDNA. Quantitative real-time PCR (qPCR) was performed with the SYBR® Green and ThunderbirdTM qPCR Mix (Toyobo Co. Ltd., Osaka, Japan), using the software ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers of *Hcper*for qRT-PCR were designed outside the region of knocking down by RNAi; forward 5'-GTCCAATATTATGACCATTGGTAGC-3' reverse 5'-CTTCAATAACTCCTTCTCCATCTCA-3' (118-bp product). For a standard as expression levels of each transcript, the*gapdh* (DDBJ/Genebank: accession number LC179544) mRNA served as a control for RNA content in each sample (forward 5'-GGTATCTCTCTGAATGACAATTTCG-3' and reverse 5'-AGGAAACAGCTATGACCATGATTAC-3'). Cycling parameters were 95°C for 1 min to activate DNA polymerase, and then 40 cycles of the following PCR amplification with primers were performed using the following temperature program; 95°C for 15 sec and 60°C for 1 min. To confirm the specificity of PCR products, melting curves were determined. Amounts of amplified products were calculated from cDNA standard curves generated for each PCR run.

2.5 Statistical analysis

The results are presented as mean ± S.E.M. Mean values were statistically analyzed using one-way ANOVA (Fisher's LSD) and Kaplan-Meier. A probability value of $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1 PER-like immunohistochemicalreactivities (ir) in the brain of larva and adult of *H. cunea*

A pair of large PER-ir neurosecretory cells were observed in the pars lateralis of protocerebrum (PL) of each hemisphere of brain in the last instar larva (Fig. 1A) and adult incubated under LD 16:8 (Fig. 1B).

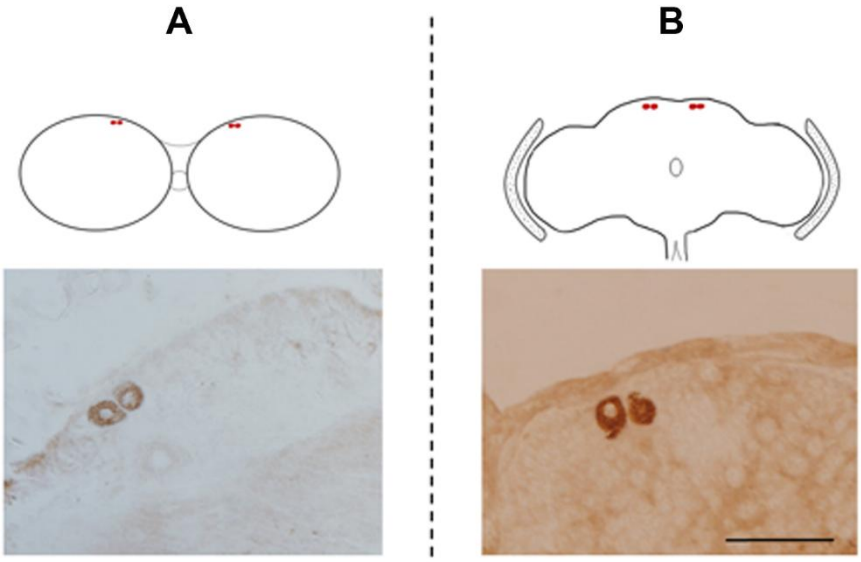

Figure 1 Two large PER-ir neurons in the pars lateralis (PL) of the protocerebrum of brain in the last instar larva (A) and adult (B) in *H. cunea*. Dilution of primary antibody was: 1:10,000. Scale bar=100μm.

3.2 Photoperiodic response

To examine photoperiodic response on pupae of *H. cunea*, two group of larvae (N=35 for one group) were reared under LD 14:10 at 25°C until silk- spinning pharate pupal stage, then one group were transferred to long-day photoperiod (LD 16:8). Thereafter, the number of adult emergence of each group was counted. Pupal diapause was terminated when diapause pupae were exposed to LD 16:8, and the percentage of emerged individuals was 92.0% in 5 months. Conversely, larvae consistently kept in LD 14:10 at 25°C mostly (91.4%) stayed in pupal diapause (Fig. 2).

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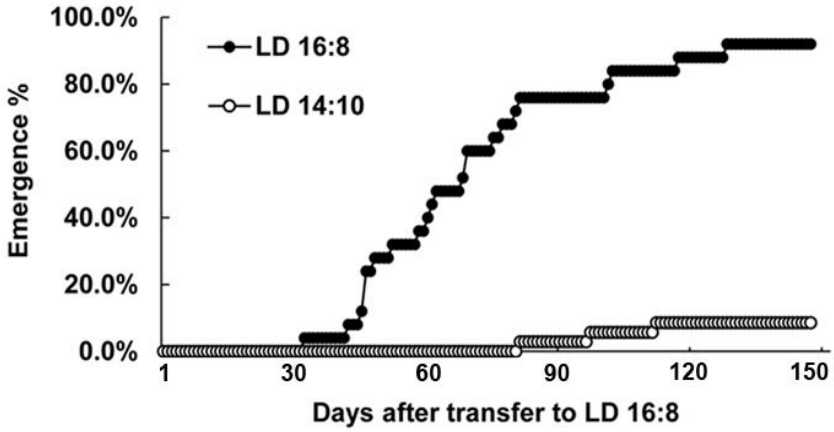

Figure 2 Post- diapause adult emergence of *H. cunea*. Larvae were reared under LD 14:10 at 25°C till silk-spinning pharate pupal stage, and then one group (N = 35) was consistently kept in LD 14:10, while another group (N = 35) was transferred to LD 16:8 at 25°C. Newly emerged moths were counted daily, and the photoperiodic response was represented as the cumulative percentage (%) of emergence from each group within 150 days at 25°C.

3.3 Effects of dsRNAPER on diapause

Function of *per* in the photoperiodic regulation of diapause is shown in Figs. 3 and 4. The levels of per mRNAs is significantly suppressed in 72 hours following the injection of dsRNAPER under LD 16:8 and 14:10 (Fisher's LSD, P<0.01) in comparison with the control groups (Figs 3A, 4A). It suggested that RNAi efficiently suppressed per transcription. Under LD 16:8, no significant difference was detected in the groups treated with dsRNAPER from dsRNAGFP (Fig. 3B, C). Under LD 14:10, however, pupae injected with dsRNAGFP stayed in diapause, but pupae injected with dsRNAPER terminated diapause and eclosed (Fig. 4B, C). Short day effect was turned over.

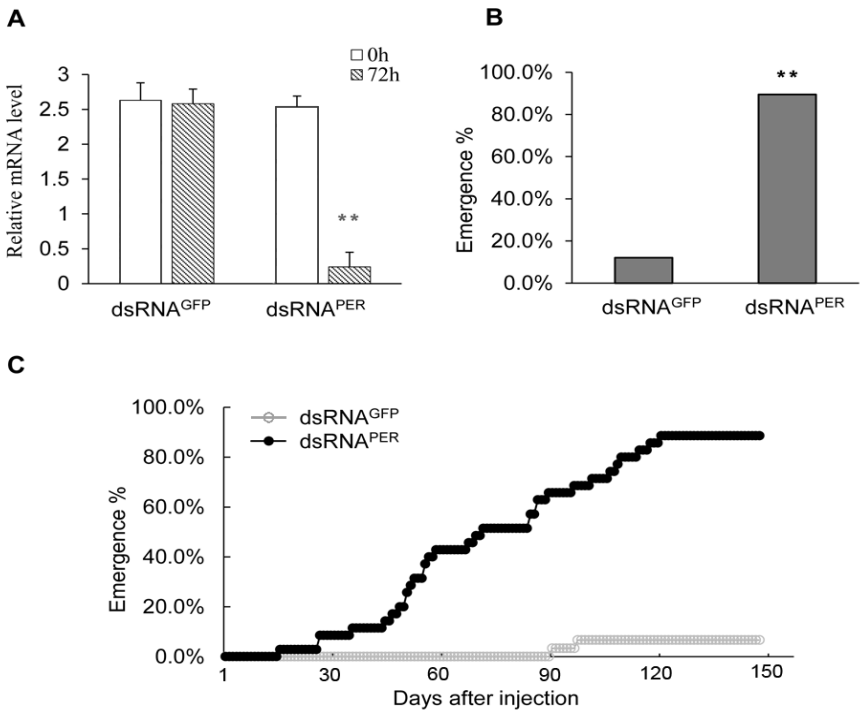

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Figure 3 RNAi for per in *H. cunea* under LD 16:8 at 25°C. The larvae reared under LD 14:10 at 25°C till pharate pupae and then were transferred to LD 16:8. Diapausing pupae were injected with dsRNAGFP and dsRNAPER. Relative mRNA level of PER was measured by qPCR with total RNA extracted from Br-SOGs collected at 0 and 72 h under LD 16:8 at 25°C. N=8-10 for each time point (A). (B) No significant difference in percentage between the pupae injected with dsRNAPER and dsRNAGFP ($p>0.05$). (C) Cumulative percentage emergence from dsRNAGFP- or dsRNAPER- injected diapause pupae under LD 16:8 at 25°C.

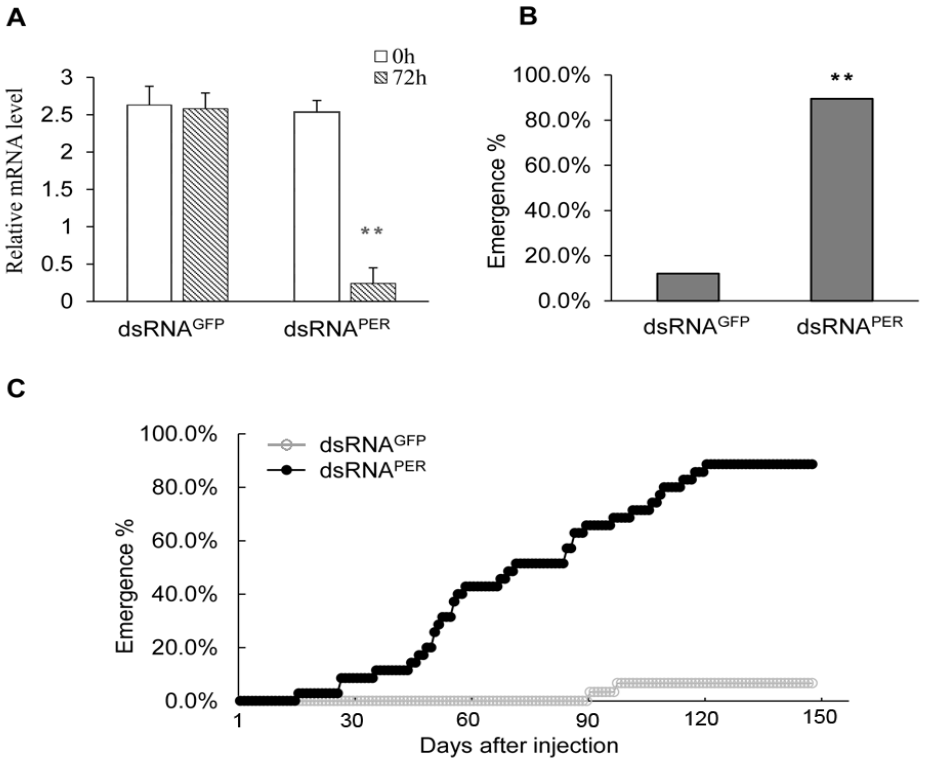

Figure 4 Injection of dsRNAPER in *H. cunea* under LD 14:10 at 25°C. (A) Larvae were reared under LD 14:10 at 25°C until pupation. Diapausing pupae were injected with dsRNAGFP, dsRNAPER. Relative mRNA level of PER measured by qPCR with total RNA extracted from Br-SOGs collected at 0 and 72 h under LD 14:10 at 25°C. N=8-10 for each time point. (B) Percentage adult emergence from each group after injection of dsRNAGFP, dsRNAPER. Differences were highly significant ($p<0.01$). (C) Cumulative percentage emergence from dsRNAGFP- or dsRNAPER-injected diapause pupae under LD 14:10 at 25°C.

4. DISCUSSION

Clock neurons have been immunohistochemically investigated in some lepidopteran insects, including *Bombyx mori* (Linnaeus) (Sehadová et al. 2004), *Antheraea pernyi* (Levine et al. 1995, Wang et al. 2013), *Manducasexta* (Linnaeus) (Wise et al. 2002) and *Danausplexippus* (Linnaeus) (Sauman et al., 2005). CYC-, CLK- and PER-ir neurons with exclusively cytoplasmatic staining were found in the dorsolateral protocerebrum (DL) of some species such as *B. mori*, *A. pernyi* and M. sexta, suggesting that this brain region represents a conserved site of the circadian clock in lepidopteran species (Závodská et al. 2003). Mohamed et al. (2014) reported for *A. peryni* where antigen of PER-, CYC-, CLK-, HIOMT-, melatonin- and arylalkylamine N-acetyltransferase (aaNAT)-ir were colocalized in the DL cells, confirming these neurons function as circadian clock neurons. In the cephalic ganglia of *H. cunea*, we detected PER-ir in the pars lateralis. PER-ir were also found in the pars intercerebralis or lateralis in other species such as the monarch butterfly, *D. plexippus* (Sauman et al. 2005) and *Apismellifera* (Linnaeus) (Bloch et al. 2003) and more scattered in still other species such as cricket *Dianemobiusnigrofasciatus* (Matsumura) (Shao et al., 2008) and cockroaches *Blattellagermanica* (Linnaeus) and *Blattellabisignata* (Brunner) (Wen and Lee 2008). Such variability occurs in the neuroanatomical architecture in different insects.

H. cunea showed a clear photoperiodic response and pupal diapause was induced by a short photoperiod during the larval stage. Masaki et al. (1968) reported that the critical photoperiod that induced diapause in 50% of pupae from Yokohama was 14 h 35 min at

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25°C. Chen et al. (2014) also reported photoperiodic response curves in a population of H. cunea from China showing that short day lengths of 8–13 h resulted in more than 80% of individuals entering diapause. This response occurred in a broad range of high temperatures (22–28°C). Our results showed that pupal diapause induced by a short photoperiod was more promptly terminated when exposed to LD 16:8.

Earlier surgical studies in *A. pernyi* has demonstrated that the critical brain region for photoperiodic termination of pupal diapause is located in the DL brain region (Truman 1973). Critical role of circadian system in photoperiodism has been demonstrated in *Antheraea pernyi* (Guérin-Méneville) (Mohamed et al. 2014; Wang et al. 2013). RNAi of per terminated diapause also resulting in a high percentage of emergence under short day conditions at 25°C in *A. pernyi* (Mohamed et al. 2014). This similar result suggest *per* is a fundamental component in photoperiodism in both *Antheraea* and *Hyphantria* and the circadian clock is involved in the common mechanism governing photoperiodic responses.

Life-history traits have changed in the *H. cunea* with its expanding to southern region in Japan, and the critical photoperiod was shorter in populations south of 36°N than in populations north of 36°N resulting in bivoltine in the north and trivoltine in the south (Gomi and Takeda 1990). These results suggest that *H. cunea* adjust their life cycles to climatic conditions and shift over time in the colonized habitats. This is shifts in generation number made primarily by the photoperiodic response that controls diapause, which may be results of long-term colonization and adaptation (Gomi and Takeda 1991; Gomi and Takeda 1996). Levy et al. (2015) found that the differences in voltinism in European corn borer moth (*Ostrinianubilalis*) are associated with changes in the circadian clock pathway. Per showed variation between the voltinism ecotypes as well as variation within the bivoltine ecotype, producing a significant cyclical pattern. Thus, the involvement of per in photoperiodic response of *H. cunea* will lay a foundation for future series of studies, where we suppose circadian gene correlates with the adjustment for the shift from a bivoltine to trivoltine for ecological and evolutionary adjustment. Further research is required to support this prediction.

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6. REFERENCES

[1] Bünning E (1960). Circadian rhythms and the time measurement in photoperiodism. *Cold Spring Harbor Symposia on Quantitative Biology***25**, 249–256.

[2] Bloch G, Solomon SM, Robinson GE, Fahrbach SE (2003) Patterns of PERIOD and pigment−dispersing hormone immunoreactivity in the brain of the European honeybee (*Apis mellifera*): Age- and time-related plasticity. *Journal of Comparative Neurology* **464,** 269–284.

[3] Chen C, Wei X, Xiao H, He H, Xia Q, Xue F (2014) Diapause induction and termination in *Hyphantria cunea* (Drury)(Lepidoptera: Arctiinae). *PloS one***9,** e98145.

[4] Danilevskii AS (1965) *Photoperiodism and seasonal development of insects*.Oliver and Boyd, Edinburgh, UK.

[5] Danks HV (2013) Studying insect photoperiodism and rhythmicity: components, approaches and lessons. *European Journal Entomology***100,** 209–221.

[6] Gomi T (1997) Geographic variation in critical photoperiod for diapause induction and its temperature dependence in *Hyphantria cunea* Drury (Lepidoptera: Arctiidae). *Oecologia*,**111,** 160–165.

[7] Gomi, T. and M. Takeda. 1990. The transition to a trivoltine life cycle and mechanisms that enforce the voltinism change in *Hyphantria cunea* Drury (Lepidoptera: Arctiidae) in Kobe. *Applied Entomology and Zoology* 25:483-489.

[8] Gomi, T. and M. Takeda. 1991. Geographic Variation in Photoperiodic Responses in an Introduced Insect, *Hyphantria cunea* DRUTY (Lepidoptera: Arctiidae) in Japan. *Applied Entomology and Zoology* 26:357-363.

[9] Gomi, T. and M. Takeda. 1996. Changes in life-history traits in the fall webworm within half a century of introduction to Japan. *Functional Ecology*:384-389.

[10] Goto SG, Denlinger DL (2002) Short-day and long-day expression patterns of genes involved in the flesh fly clock mechanism: period, timeless, cycle and cryptochrome. *Journal of Insect Physiology***48,** 803–816.

[11] Hall JC (2003) Genetics and molecular biology of rhythms in Drosophila and other insects. *Advances in genetics***48,** 1–280.

GLOBAL JOURNAL OF ADVANCED RESEARCH
(Scholarly Peer Review Publishing System)

[12] Kean J, Kumarasinghe L (2007) Predicting the seasonal phenology of fall webworm (*Hyphantria cunea*) in New Zealand. *New Zealand Plant Protection***60,** 279.
[13] Konopka RJ, Benzer S (1971) Clock mutants of Drosophila melanogaster. *Proceedings of the National Academy of Sciences***68,** 2112–2116.
[14] Levine JD, Sauman I, Imbalzano M, Reppert SM, Jackson FR (1995) Period protein from the giant silkmoth *Antheraea pernyi* functions as a circadian clock element in Drosophila melanogaster. *Neuron***15,** 147–157.
[15] Levy, R., G. Kozak, C. Wadsworth, B. Coates, and E. Dopman (2015) Explaining the sawtooth: latitudinal periodicity in a circadian gene correlates with shifts in generation number.Journal of evolutionary biology 28:40-53.
[16] Masaki S, Umeya K, Sekiguchi Y, Kawasaki R-I (1968) Biology of *Hyphantria cunea* DRURY (Lepidoptera: Arctiidae) in Japan: III. Photoperiodic Induction of Diapause in Relation to the seasonal Life Cycle. *Applied Entomology and Zoology***3,** 55–66.
[17] Mohamed AA, Wang Q, Bembenek J, Ichihara N, Hiragaki S, Suzuki T, *et al*. (2014) N-acetyltransferase (nat) is a critical conjunct of photoperiodism between the circadian system and endocrine axis in *Antheraea pernyi*. *PloS one***9,** e92680.
[18] Rezaei V, Moharramipour S, Talebi A (2003) The first report of *Psychophagus omnivorus* (Walker) and *Chouioia cunea* (Yang) parasitoid wasps of American white webworm *Hyphantria cunea* Drury (Lep.: Arctiidae) from Iran. *Applied Entomology and Phytopathology***70,** En33, Pe137–138.
[19] Sandrelli F, Costa R, Kyriacou CP, Rosato E (2008) Comparative analysis of circadian clock genes in insects. *Insect molecular biology***17,** 447–463.
[20] Sauman I, Briscoe AD, Zhu H, Shi D, Froy O, Stalleicken J, *et al.* (2005) Connecting the navigational clock to sun compass input in monarch butterfly brain. *Neuron***46,** 457–467.
[21] Saunders DS (2002) *Insect clocks*.Elsevier Science, Amsterdam.
[22] Sehadová H, Markova EP, Sehnal F, Takeda M (2004) Distribution of circadian clock-related proteins in the cephalic nervous system of the silkworm, *Bombyx mori*. *Journal of biological rhythms***19,** 466–482.
[23] Shao Q-M, Bembenek J, Hiragaki S, Takeda M (2008) Molecular structure, expression patterns, and localization of the circadian transcription modulator CYCLE in the cricket, *Dianemobius nigrofasciatus*. *Journal of insect physiology***54,** 403–413.
[24] Truman JW (1973) How moths"turn on": A study of the action of hormones on the nervous system. *American Scientist***61**, 700–706
[25] Umeya K, Itô Y (1977) Invasion and establishment of a new insect pest in Japan. In:Hidaka (ed) *Adaptation and speciation in the fall webworm*, pp1–12. Kodansha, Tokyo, Japan.
[26] Wang Q, Mohamed AA, Takeda M (2013) Serotonin receptor B may lock the gate of PTTH release/synthesis in the Chinese silk moth, *Antheraea pernyi*; a diapause initiation/maintenance mechanism? *PloS one***8,** e79381.
[27] Warren L, Tadić M (1967) The fall webworm, *Hyphantria cunea*, its distribution and natural enemies: a world list (Lepidoptera: Arctiidae). *Journal of the Kansas Entomological Society***,** 194–202.
[28] Wen CJ, Lee HJ (2008) Mapping the cellular network of the circadian clock in two cockroach species. *Archives of insect biochemistry and physiology***68,** 215–231.
[29] Wise S, Davis NT, Tyndale E, Noveral J, Folwell MG, Bedian V, *et al.* (2002) Neuroanatomical studies of period gene expression in the hawkmoth, *Manduca sexta*. *Journal of Comparative Neurology***447,** 366–380.
[30] Závodská R, Šauman I, Sehnal F (2003) Distribution of PER protein, pigment-dispersing hormone, prothoracicotropic hormone, and eclosion hormone in the cephalic nervous system of insects. *Journal of Biological Rhythms***18,** 106–122.
[31] Zylka MJ, Shearman LP, Weaver DR, Reppert SM (1998) Three period homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. *Neuron***20,** 1103–1110.