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# The *Cryptochrome1* (*cry1*) Gene has Oscillating Expression Under Short and Long Photoperiods in *Sesamia nonagrioides*

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## Abstract

A In order to understand whether and how the circadian system is connected to photoperiodism, an important piece of information is whether clock genes products oscillate and how they react to a changing photoperiod. In the moth *Sesamia nonagrioides,* which undergoes a facultive diapause controlled by photoperiod, we isolated the clock gene *cryptochrome1* (*cry1*), named *Sncry1. Sncry1* consists of 1762 bp encoding a polypeptide of 528 amino acid residues. SnCRY1 presented two characteristic conserved domains: the DNA photolyase and the Flavin-Adenine Dinucleotide (FAD) binding domain, which been demonstrated to be sufficient for light detection and phototransduction in *Drosophila*. SnCRY1 had significant homology with the CRY1 sequences identified from other insects. We also investigated the expression patterns of *Sncry1* in brain of larvae growing under long-day 16L: 8D (LD), constant darkness (DD) and short-day 10L: 14D (SD) conditions using qRT-PCR assays. The mRNAs of *Sncry1* expression was rhythmic in LD, DD and SD cycles. *Sncry1* abundance tended to decrease during the day and then increase in the night. It is remarkable that the photoperiodic conditions affected the expression patterns and/or amplitudes of circadian clock gene *Sncry1*. Our data indicate that this gene may be associated with diapause in *S. nonagrioides*, because under SD (diapause conditions) the photoperiodic signal altered mRNA accumulation.

**Keywords:** Circadian clock; *Cryptochrome*; Photoperiodism; Diapause; *Sesamia nonagrioides*;

## Introduction

There are two major rhythms of the biosphere, a daily cycle of night and day, and an annual seasonal cycle marked by changes in day and night length. The daily cycle is traced by an internal circadian clock that rules a large array of daily biochemical and physiological responses, while the seasonal cycle motivates photoperiodic responses that can be critical to survival, as in the case of insect diapause [1]. Circadian (≅24 h) clock is a core molecular mechanism that allows organisms to forestall daily environmental changes and adapt the timing of behaviors to maximize their efficacy. The basic circadian clock of insects functions as a light-delicate molecular oscillator, including a light-sensitive protein known as CRYPTOCHROME (CRY) and various feedback loops with positively and negatively substitute elements. The circadian clock in *Drosophila* is designed by co-operating molecular feedback loops containing of central clock and linked genes [2]. The expression of the two clock genes, *period* (*per*) and *timeless* (*tim*), is controlled by transcriptional activators, encoded by *Clock* (*Clk*) and *cycle* (*cyc*). This initiation leads to periodic growth in the levels of *per* and *tim* mRNA tailed by the increase of PER and TIM proteins in cell nuclei, where PER actions as a repressor of CLK/CYC leading to overthrow of *per* and *tim* transcription. The photoreceptive CRY protein, encoded by the *cryptochrome* gene, intermediates the degradation of TIM, coordinating the clock to LD cycles. On contact to light, *Drosophila*-type *cryptochrome* (dCRY) encourages fast degradation of TIM that reduces PER unbalanced. PER is ultimately degraded, releasing the reserve of transcription [3]. Animal CRY proteins are phylogenetically separated into two groups: one comprises *Drosophila*-type CRY (CRY1) and the other contains all the vertebrate CRY (CRY2). Owing to ancestral gene duplication, there may be two cryptochrome genes in any certain insect species -*cry1* and *cry2* [4].

Photoperiodism, a reaction to the size of the light or dark period in a day, has been recognized in many insects, and controls diapause, seasonal morphs, growth rate, migration strategy, and a variability of related physiological states [5]. Diapause is a programmed stopped state of development that permits insects and other arthropods to continue opposing seasonal conditions either by becoming resting locally, or wandering to a more advantageous environment [6].

In order to comprehend whether and how the circadian system is associated to photoperiodism, a significant piece of evidence is whether clock genes products oscillate and how they respond to a varying photoperiod [7-9]. The connection of circadian clock genes in diapause initiation has been debated [1, 10-12].

The Mediterranean corn stalk borer, *Sesamia nonagrioides* (MCSB) undergoes facultative diapause and photoperiod rules the

beginning of larval diapause. When diapause has been induced, larvae persist to grow and molt without pupating and up to six additional instars, elsewhere the normal five or six have been noticed, when larvae are continuous in diapausing conditions [11]. To accomplish indications to the link among the molecular mechanism of circadian and photoperiod clocks, in recent works we studied the expression of the clock genes *period, timeless* and *cycle* and the results suggested that transcriptional regulations of these clock genes act in the diapause programming in MCSB [11, 12]. In the current study, we examined the contribution of *cryptochrome1* (*cry1*) gene in the circadian rhythm and in the photoperiodic regulation of diapause in *S. nonagrioides.*

## Methods

### Biological material and experimental schedules

An established laboratory colony of *S. nonagrioides*, derived from field-collected larvae in Kopais (Latitude 38o 14′, Central Greece) was used as experimental biological material. Larvae were reared on artificial diet and were grown under photoperiods of 16 h light (L): 8h dark (D) at 25±1°C and 65% relative humidity [11]. Photoperiods of 16L: 8D and continuous dark (DD) were used to encourage normal development while photoperiods of 10L: 14D were used to encourage diapause conditions. For quantitative real-time RT-PCR analyses (see below), larvae were reared until the 5th instar for long-day (16L: 8D) and constant darkness (DD) conditions while for short day conditions (10L: 14D) were reared until the 8th instar (day 55). Samples were collected every 3 hours starting at the zeitgeber time (ZT) 0 or circadian time (CT) 0. The zeitgeber time (ZT) 0 was the time for the beginning of the light phase to which the phase associations were linked under two (long- and short- day) photoperiods while the circadian time (CT) 0 corresponded to the time schedule "lights-on" and circadian time (CT) 12 to the time schedule "lights-off" in DD conditions.

### cDNA synthesis, cloning and sequencing

Total RNA was isolated according to the supplier's instructions from larvae using TRIzol® reagent (Gibco BRL, Carlsbad, CA, USA). RNA concentration was estimated using a spectrophotometer (Spectronic model 21D, Carlsbad, CA, USA). Genomic DNA was removed using DNAse I (Invitrogen, Carlsbad, CA, USA). Partial clones of *cryptochrome1* (*cry1*) from *S. nonagrioides* were isolated by RT-PCR from an RNA pool of nondiapausing larvae (5th instar). Total RNA was incubated with RNase-free DNAse I (Promega, Southampton, UK). Two μg were used as template for the synthesis of the first strand cDNA synthesis with Superscript TM II RNase H–Reverse Transcriptase (Invitrogen). Degenerate primers were designed using conserved sequences from several Lepidoptera (Table 1). cDNA was used as template together with 200 μM of each dNTP, 20 pmol of each primer, 2 U of DNA polymerase (Expand-High Fidelity, Roche, Mannheim, Germany) and amplification was achieved in a thermal cycle (Model PTC-200, M.J. Research, Waltham, MA), using a denaturation step (94 °C for 2 min), and 35 cycles were run each with 94 °C for 30 sec, 55 °C for 30 sec, and 74 °C for 45 sec. PCR amplified fragment sizes approximately 800 bp were gel extracted and sequenced. The 3′- and 5′-ends of the cDNA fragment were amplified according to Frohman [13]. For 3′-RACE, the first-strand cDNA was primed off with the T17XhO primer. Based on sequence information of the cDNA, two specific forward primers were designed: Cry Forward 1 and Cry Forward 2. PCR conditions were: 30 s at 94 °C, 1 min at 57 °C and 2 min at 72 °C, for 30 cycles followed by a final extension at 72 °C for 7 min. For 5′-RACE, the synthesis of the first-strand cDNA was accomplished using the reverse specific primer Cry Reverse 1, was dA-tailed and amplified with the nested primer Cry Reverse2 followed by the primer Cry Reverse 3 in combination with T17XHO. PCR conditions were: 30 s at 94 °C, 1 min at 55 °C and 2 min at 72 °C, (30 cycles) followed by a final extension of 72 °C for 7 min. Sequences for *Sncry1* are deposited on EMBL/GENBANK as the accession number DQ243705.

### Sequencing and Phylogenetic analysis

The amino acid sequence of Sncry1 was predicted using the DNAman v.5.2.2 software (Lynnon Biosoft, Quebec, Canada) and was submitted to the NCBI website (http://www.ncbi.nlm.nih.gov). The pI and MW were computed using the Compute pI/MW software (http://www.expasy.ch/tools/pi_tool.html). The post-translational modification sites were predicted using the Prosite Scan software (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_prosite.html), while secondary structures were determined using the SOPMA software (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html). The 3D structures were constructed using the Swiss Model Workspace (http://swissmodel.expasy.org). Phylogenetic trees and P-Distances were constructed using the neighbour-joining method with the MEGA5 software [14, 15]. To construct the phylogenetic tree, known sequences of predicted CRY proteins from other insect species were attained from GenBank. Bootstrapping was used to estimate the reliability of phylogenetic reconstructions (1000 replicates) [16]. Abbreviations and accession numbers of genes in the phylogenetic trees are listed in the figure legends.

### Quantitative Real-Time PCR (qRT-PCR)

Larvae heads were dissected and used to isolate total RNA. Larvae were reared under long-day, constant dark or short-day conditions. For quantitative real time RT-PCR (qRT-PC), we used specific primers (Table 1) and the incorporation of the fluorescent dye SYBR Green Brilliant (Stratagene, LA Jolla, CA, USA) into double-stranded PCR products was estimated. Plasmids were constructed into pGEM T-easy vector (Promega, Madison, WI, USA) by inserting a ~200 bp fragment from the coding region of each gene amplified from individual cDNA with gene specific primers Cry RT forward and Cry RT reverse (Table 1). We designed real-time PCR primers from the Sncry1 sequences at 3′-UTR (Table 1). The recombinant plasmids were used as template to produce standard curves using a Stratagene MX3005PTM Real Time PCR system (Stratagene) at concentrations ranging from 1

| **Table 1:** Primers of sequence used in this study | |
|---|---|
| **Oligonucleotides** | **Sequence (5´→3´)** |
| **Degenerate PCR** | |
| Cry forward: | C T ( C / G ) A C C T A C C A ( A / G ) ATGTTC(C/T)TGCA |
| Cry reverse: | A(C/T)ACCCACATCCA(A/G)TT(A/G) CC(A/G)GC |
| **RACE PCR** | |
| 3´ – Cry Forward 1: | AACTACGGACAGATGAGTGGCAA |
| 3´ – Cry Forward 2: | AACACTCTGGCTGTCGTGGGAA |
| 5´ – Cry Reverse 1: | TAAGGAAGAACGCCACAGTGTTGC |
| 5´ – Cry Reverse 2:<br>5´ – Cry Reverse 3: | TGATGCACCTCACGGAATAGGTCC<br>ATCTGTTTAAGCGCAGTGGTCTC |
| **Real-time PCR assays** | |
| Cry RT forward: | AGAGCGTGCGTCCTGTATAATAG |
| Cry RT reverse: | CCGTAAGTCATTACTCGTTGAGC |
| *Sn* RT-TubF | GAGCAGTTCACCGCTATGTTC |
| *Sn* RT-TubR | GGTGTGAGTGCTTTAGTTGTCC |

ng to 10 fg . The amplification cycle was: 95 °C for 10 s, 56 °C for 30 s and 72 °C for 30 s (35 cycles) using 5 pmol of each primer. The mRNA levels was normalized with *S. nonagrioides* β-tubulin (GENBANK accession no. DQ147771) gene and quantified in the same manner. Data are expressed as means ± SEM of 4 independent biological replicates and 3 technical replicates (N = 12/time point) and estimated by ANOVA and the Tukey's post hoc test using the IBM SPSS software (IBM Analytics).

## Results

### Sequence of *Sncry1*

The full-length nucleotide sequence of *cry1* cDNA of *S. nonagrioides* (*Sncry1*, GenBank Accession no. DQ243705) consists of 1762 bp. Conceptual translation of the cDNA sequence yields a 5' untranslated region (UTR) of 102 bp, a 3´-terminal UTR of 76 bp with a poly (A) tail, and an open reading frame (ORF) of 1584 bp, encoding a polypeptide of 528 amino acid residues (Figure 1). The molecular mass of the deduced SnCRY1 protein was predicted to be 59.6 kDa, and the calculated isoelectric point (pI) was 8.33. Comparing the deduced amino acid sequence of SnCRY1 (NCBI, BLAST) revealed that SnCRY1 had significant homology with the CRY1 sequences identified from other insects, such as *Mamestra bracicae* (AY947639, 86%), *Helicoverpa armigera* (AEX49898, 86% identity), *Mythimna separate* (AFR54426, 86% identity), *Antheraea pernyi* (AAK11644.1, 80% identity), *Bombyx mori* (NP_001182628.1, 75% identity). SnCRY1 presented two characteristic conserved domains: the DNA photolyase and the Flavin-Adenine Dinucleotide (FAD) binding domain (Figure 1), which been demonstrated to be sufficient for light detection and phototransduction in *Drosophila* [17]. Multiple alignments (Figure 2) revealed that the N-terminus of the proteins showed the highest conservation in analyzed SnCRY1 sequences, whereas the C-terminal had lower levels of conservation. The conserved regions of SnCRY1 and contained DNA photolyase domains and FAD binding domains, which were highly conserved among the different CRY1s (Figure 2).

The secondary structures were constructed by SOPMA, and the rates determined were 37.31% of alpha helix, 9.85% of beta turn, 14.58% of extended strand and 38.26% of random coil. The crystallographic structures of CRY1 are very important for their functions. The structures of SnCRY1 (Figure 3) was predicted, based on the three-dimensional structure of *Drosophila melanogaster* (6-4) photolyase 3cvvA 224 (2.10 A). Monomer structure comparison of 4jzy.1.A. template (*D. melanogaster*), show Seq Identity 52.67% and Description as Cryptochrome 1. Also identifired Ligads: 1XFAD and matching prediction 1x FLAVIN-ADENINE DINUCLEOTIDE. Ligand 1 in contact with: Chain A : R229, S257, L258, S259, L262, Q300, L301, W303, R304, F307, W364, L365, H367, R370, N371, F393, D399, A400, D401, V404, C405, N408, W409, F523.

A phylogenic tree (Figure 4) based on the CRYs from different insects was constructed using the neighbor-joining method. Phylogenetic analysis showed that CRYs in insects could be classified into two cluster CRY1 and CRY2. The result of the phylogenetic analysis agreed with the structure and distribution of these CRYs. The SnCRY1 belongs to the CRY1 cluster. Moreover, the phylogenetic tree demonstrated that *S. nonagrioides* had a shorter genetic distance to Lepidopteran species than other insects, which was consistent with the traditional taxology.

### Expression patterns of *Sncry1* under different photoperiods

To determine if *Sncry1* transcripts oscillate, we examined the levels of gene mRNAs in the head of larvae under 16L: 8D (long day, LD), constant darkness (DD) and 10L:14D (short day, SD) by performing Real-Time PCR assays and showed that the expression was rhythmic. Under 16L: 8D conditions a clear diel rhythm of *cry1* mRNA levels was detected (Figure 5A): the expression of *Sncry1* mRNA appeared to cycle with a peak during the night (ANOVA, $p<0.01$). The peak value at ZT 21 (5 h after onset of scotophase) was about 7 times higher than the trough level (at ZT 15) and the difference was statistically significant (Tukey's test, $p<0.01$). The oscillation of *Sncry1* mRNA under LD also appeared in three days observations (data not shown). The rhythmic *cry1* mRNA expression persisted in DD (Figure 5B) with a peak at late subjective night (CT21; ANOVA, $p < 0.01$), and the peak was about 5-fold of the trough level. Under 10L: 14D conditions, a clear diel rhythm of *Sncry1* mRNA levels was also with two peaks: one during the day (at ZT3) and the other during the night (at ZT12) (Figure 5C). The oscillation of *Sncry1* mRNA under SD photoperiod was quite different than LD: the peak value was shifted to early in the scotophase at ZT 12 (2 h after onset of the scotophase), and the peak value was about 4 times higher than the trough level. The oscillation of *Sncry1* mRNA under SD appeared also in three days observations (data not shown). By

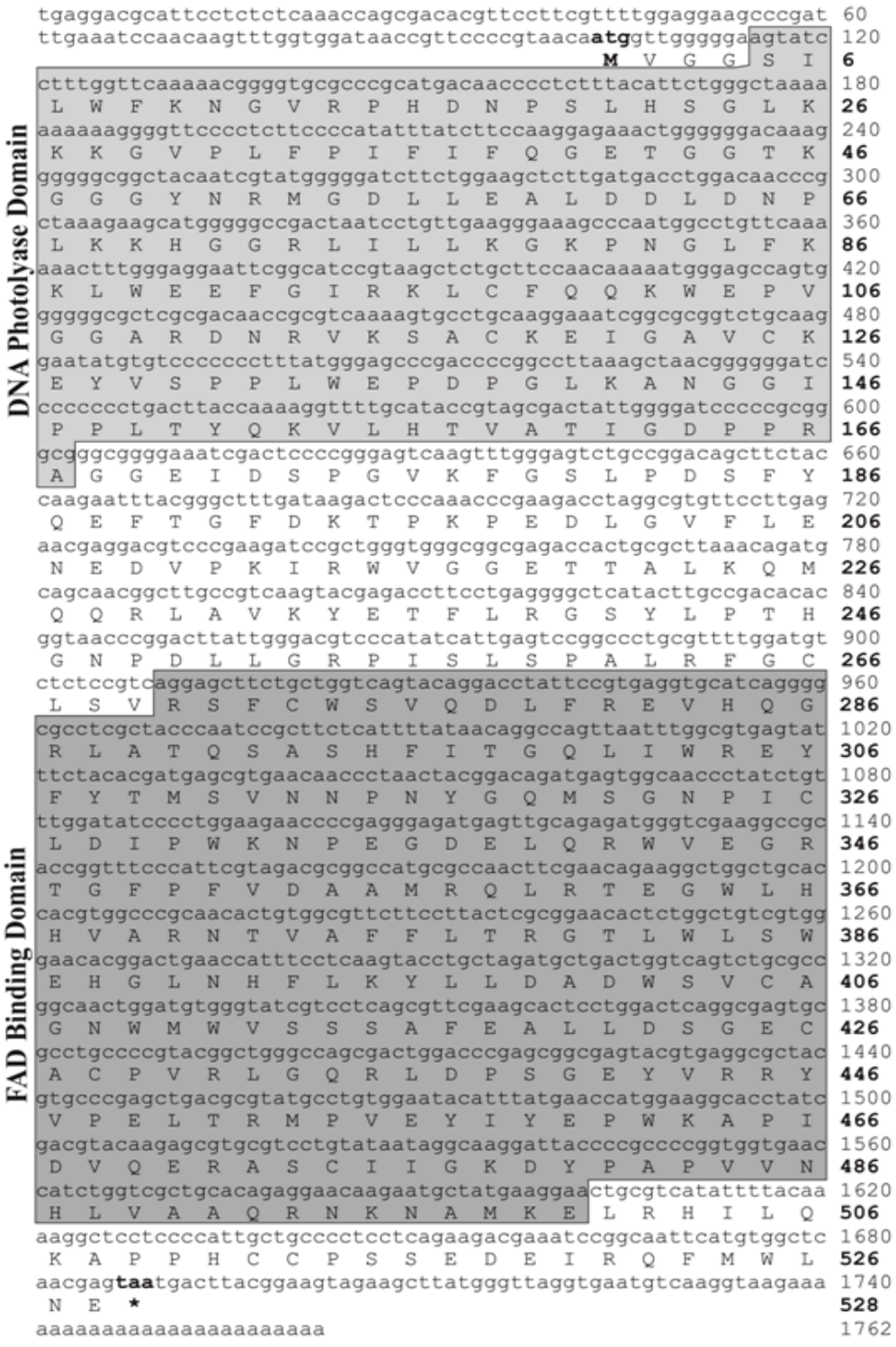


**Figure 1:** The nucleotide and deduced amino acid sequences of *Sncry1*. The positions of the nucleotides and amino acids were indicated in the right margin. The termination codon was marked with a star. Faction motif of *cryptochrome* correspondence of *D. melanogasterare* marked: DNA Photolyase Domain; FAD (Flavin-Adenine Dinucleotide) binding domain of DNA photolyase.

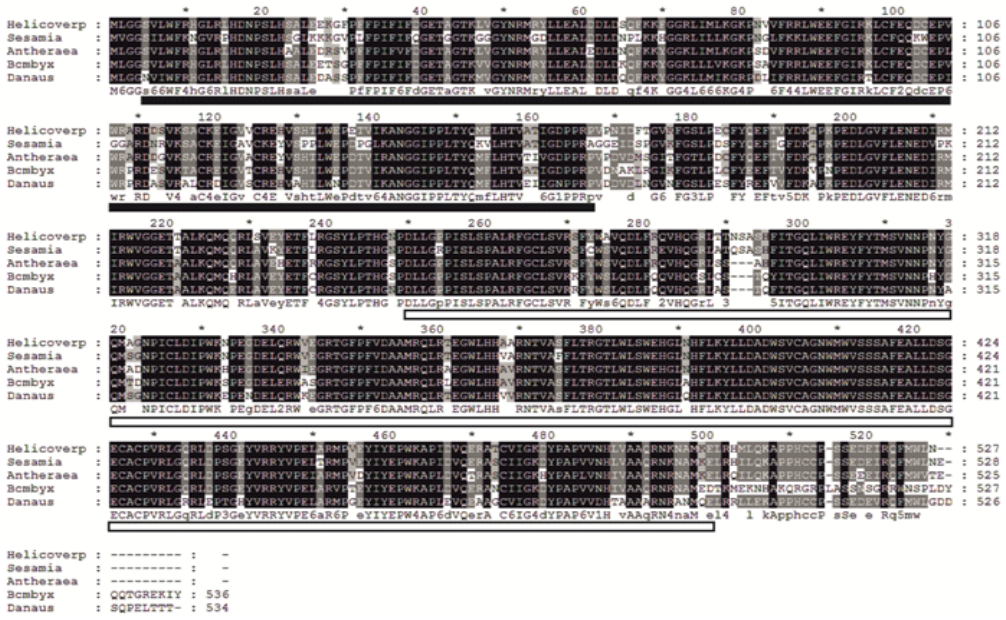

**Figure 2:** Alignment of the SnCRY1 amino acid sequence with sequences of other lepidopteran CRY1s: numbers on the right side of the alignment indicate the position of residues in the sequence of each protein. Conserved residues of the five sequences are shaded in black. The DNA-photolyase domain was indicated by black bar under the alignment (position 5-167 in *S. nonagrioides*) while the white bar indicates the FAD binding domain (position 250-500 in S. nonagrioides). The GenBank accession numbers: Antheraea: *A. pernyi* (AAK11644.1); Bombyx: *B. mori* (NP_001182628.1); Danaus: D. plexippus (AAX58599.1); Helicoverpa: *H. armigera:* (ADN94464.1); Sesamia: *S. nonagrioides* (ABB52818.2).

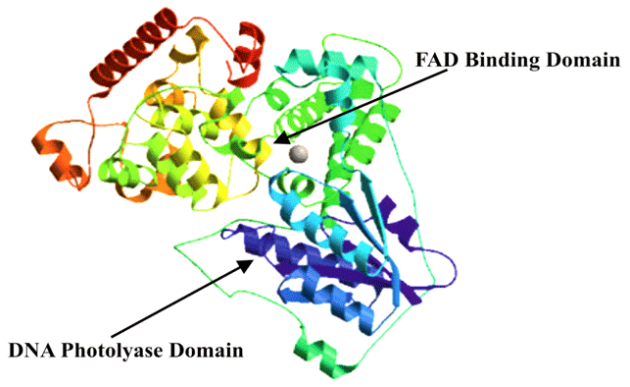


**Figure 3:** Molecular modeling into the structural feature of *S. nonagrioides* CRY1.

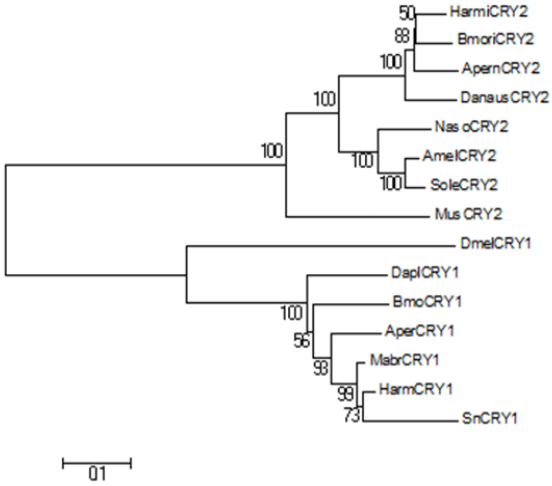


**Figure 4:** Phylogenetic analysis based on CRY1 and CRY2 amino acid sequences. The numbers at the nodes indicated the bootstrap. The GenBank accession numbers used: DmelCRY1(*D. melanogaster*: NP_732407.1); BmoCRY1 (*B. mory*: NP_001182628.1); AperCRY1 (*A. pernyi:* AAK11644.1); MbrCRY1 (*M. brassicae*: AAY23345.1); Dapl-CRY1 (D. plexippus: AAX58599.1); HarmCRY1 (*H. armigera*: ADN94464.1); SnCRY1(*S. nonagrioides*: ABB52818.2); HamCRY2 (*H. armigera*: ADN94465.1); BmorCRY2 (*B. mori*: ADM86935.1); AperCRY2 (*A. pernyi:* ABO38435.1), DanausCRY2 (*D. plexippus:* ABA62409.1); NasCRY2 (*Nasonia vitripennis*: XP_001606405.2); AmelCRY2 (*A. mellifera* ABO38437.1); SoleCRY2 (*Solenopsis invicta*: JX948389); MusCRY2 (*Mus musculus*: NP031797).

comparing the ratios between the peak: trough levels under short and long day conditions, it seems that the amplitude of the *Sncry1* mRNA expression was weaker at SD than at LD.

## Discussion

In the current study, we reported on the structure and expression profile of the clock gene *cryptochrome1* (*Sncry1*) in the moth Mediterranean corn stalk borer (MCSB), *S. nonagrioides.* This insect undergoes a facultative diapause characterized by prolonged larval duration in reaction to short-day conditions. The nucleotide and amino acid sequences of *Sncry1* (Figure 1)

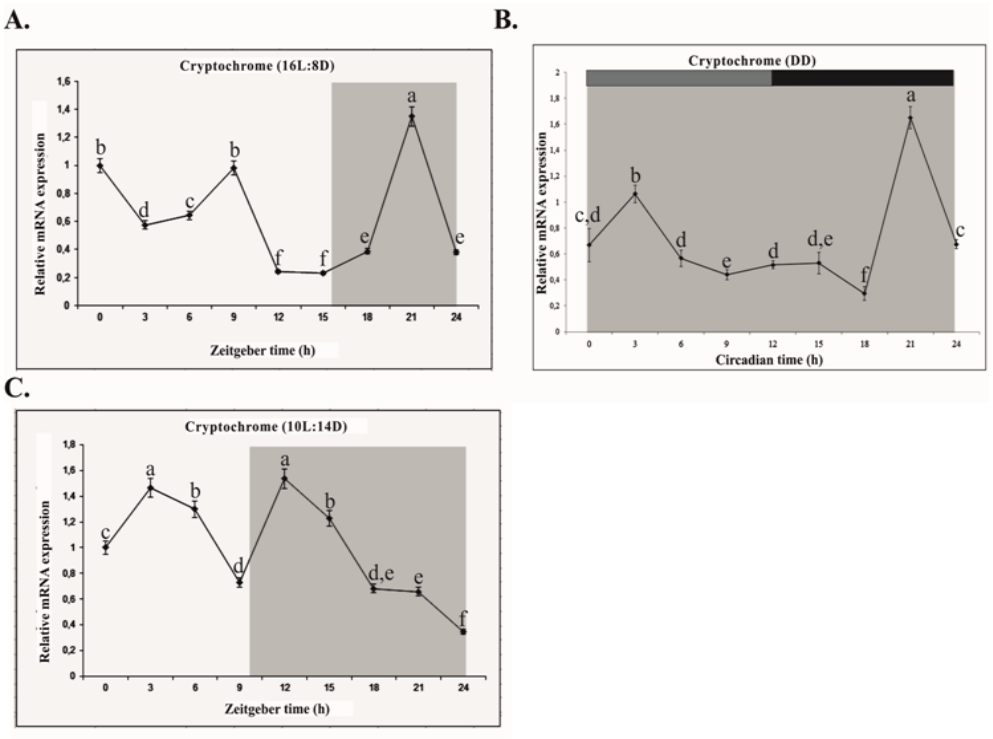


**Figure 5:** Daily and circadian expression patterns of *Sncry1* mRNA in the heads of 5th instar MCSB larvae in: A: 16L: 8D (lights on at ZT0 and lights off ZT16), B: constant dark DD (lights off) and C: 10L: D14D (lights on at ZT0 and lights off ZT10). Larvae were entrained in light–dark cycles for 25 days and the tissues were collected at ZT 0, ZT3, ZT6, ZT9, ZT12, ZT15, ZT18, ZT21, and ZT24. Shaded area shows the scotophase. Bars represent Mean± SEM of 4 independent biological replicates plus 3 technical replicates (N=12/time point). Letters indicate statistical significance (One way ANOVA followed by the Tukey's *post hoc* test).

indicated high similarities to previous identified CRY1s. SnCRY1 contains a C-terminal domain and the photolyase homology domain consists of a DNA-Photolyase domain and a FAD binding7 domain [18, 19]. Busza et al. establish that the photolyase homology domain was for light discovery and phototransduction, whereas C-terminal domain regulated CRY solidity, CRY-TIM contact and circadian photosensitivity [17]. The structural protection of the DNA photolyase and the FAD binding domains among the different CRYs might be the suggestion of common mechanistic features, principally in photoreception [19]. Many organisms display circadian rhythms generated by the circadian clock; the photoreceptor is essential for the clock to coordinate the light-dark cycles and photic entrainment depends on the function of CRY. Phylogenetic analyses appearance at least two rounds of gene duplication at the base of the metazoan radiation, as well as several losses, offered growth to two *cryptochrome* (*cry*) gene families in insects, a *Drosophila-like cry1* gene family and a vertebrate-like *cry2* family. These genes are similar in their nucleotide and amino acid sequences, but paralogous and apparently different by phylogenetic analyses [4]. The CRY1 acts as a blue-light photoreceptor for photic entrainment; the CRY2 roles as a major transcriptional repressor but not as a circadian photoreceptor [20, 21]. The predicted crystallographic structures of SnCRY1 (Figure 3) presented that it is a blue-light photoreceptor. The phylogenetic tree in our study (Figure 4) discovered that SnCRY1 belongs to the cry1 family. As established by the phylogenetic tree, the CRY1 sequence of *S. nonagrioides* was closer to those of *Helicoverpa armigera* and *Mamestra bracicae* than those of non-Lepidopteran insects. This result adapted well to the traditional classes of these species.

Expression analysis of *Sncry1* using qRT-PCR was rhythmic in LD, DD and SD cycles in the larvae head of MCSB (Figure 5). Cycling of Sncry1 under DD condition (Figure 5B) insisted with phases and amplitudes similar to that observed in LD condition, demonstrating that it was under circadian regulation. In *Drosophila, cry* mRNA cycle under a light-dark cycle (LD), with high levels in light and low levels in the dark. This cycling persists in continuous darkness (DD), but with reduced amplitude [22]. Unlike, in MCSB to LD condition, *Sncry1* abundance have a tendency to decrease during the day, then increase in the night. An analogous result is observed in *H. armigera* moths under LD conditions for *Hacry2*, whereas *Hacry1* abundance tended to increase during the day and then decrease in the night [23]. The dCRY1 structure reveal that the tail residue Cys523 plays key roles in the dCRY photoreaction [24]. The 3D structure of SnCRY1 (Figure 3) show similarities with dCRY1 in this location but details of the role that photoreceptors play in the photic entrainment of MCSB need to be additional investigated.

In our study (Figure 5) we showed that the *Sncry1* mRNA peaked in the mid night comparable to *Snper* [11]. The discovery of the existence of two *cry* genes in moths and butterflies suggested that the clockwork mechanism of Lepidopteran insects differ from that of *D. melanogaster* [25]. In species where *cry2* is existing, its oscillation matches that of *per*, as revealed in our study, with a trough in the light phase and a highest in the dark phase [26-28]. In *Drosophila*, PER is the key negative regulator of clock function, but until now, in all insects where *cry2* is present and irrespective of *cry1*'s presence, CRY2 shows this role [29]. In a previous work we found that the chronological pattern of gene expression in MCSB brain is amazingly distinct from that of *Drosophila* [12]. In the MCSB, *Sncyc* and *Snper* mRNA levels oscillate with a similar phase, but in *Drosophila* they are in anti-phase [30, 31]. The ancestral circadian clock maybe involved CYC as the positive-acting transcriptional activator, CRY1 as the main photoreceptor, and CRY2 as the light in sensitive, negative acting transcriptional regulator [25]. *Drosophila* expresses CRY1 only, while some insects, like mosquitos and butterflies, express both CRY1 and CRY2 [32]. The honeybee *Apis mellifera* and the beetle *Tribolium castaneum* contain only CRY2 [4, 32]. This proposes two significant options. First, the core oscillator in insects has itself evolved such that at least three kinds of clocks exist, those having only CRY1 as in *Drosophila*, those enclosing CRY1 and CRY2 as in monarch and mosquito, and those containing CRY2 only as in beetle and honeybee. Second, in insects enclosing only CRY2, the cryptochrome may service dual functions, as both a transcriptional repressor and a photoreceptor [25]. In a previous work we found that the *cycle* gene in MCSB show interesting changes related to *Drosophila,* proposing that this species is a remarkable new model to study the molecular control of insect biological clocks [12]. We speculate that in insects maybe there is another possibility, such as in MCSB, where it seems that CRY1 alone, could evolve dual functions as transcriptional repressor and a photoreceptor. An interesting observation is that *Snper, Sntim, Sncyc* and *Sncry1* synchronously peaked at midnight, revealing that MCSB has a distinct circadian cycle when compared to Drosophila (Figure 6). In *Drosophila, cry* expression is regulated at the transcriptional level by the clocks and at the translational and posttranslational level by the light [22]. In our case light and clock regulates the transcriptional regulation of *Sncry1* gene (Figure 6). The transcript accumulation profile suggests that *Snper, Sntim, Sncyc* and *Sncry1* genes are coordinately regulated by light. While in Drosophila, CRY intermediates the degradation of TIM, in MCSB the coordinated expression of *Sntim* and *Sncry*1 suggests that cry activation is regulated at translational and/or posttranslational level [3]. This conjecture is still unclear and needs further investigation.

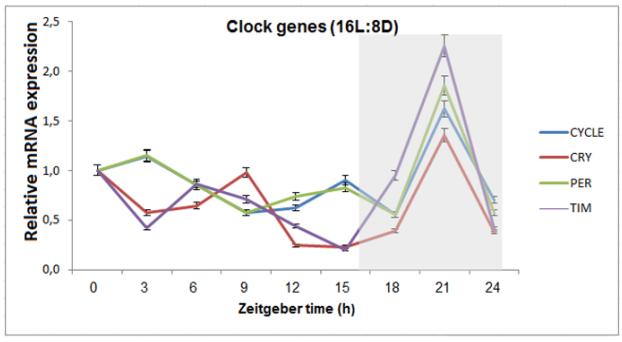


**Figure 3:** MDaily and circadian expression patterns of *Snper, Sntim, Sncyc* and *Sncry1* mRNA in the heads of 5th instar MCSB larvae in: A: 16L: 8D (Lights on at ZT0 and lights off ZT16). Larvae were entrained in light–dark cycles for 25 days and the tissues were collected at ZT 0, ZT3, ZT6, ZT9, ZT12, ZT15, ZT18, ZT21, and ZT24. Shaded area shows the scotophase. Data of *Snper, Sntim* and *Sncyc* are from [11, 12].

In our study the expression of the circadian gene *Sncry1* was directly related to diapause-inducing photoperiod. The *Sncry1* mRNA oscillation exhibited a peak 5 h after onset of the scotophase under long days and shifted 2 h after onset of the scotophase under short days. *Sncry1* showed association with diapause in *S. nonagrioides*, since under 10L: 14D (diapausing conditions) the photoperiodic signal produced alteration of mRNAs. Under short-day conditions, higher levels of *Sncry1* where detected in larval heads than those reared under long-day conditions. In addition, a difference exhibited in the *Sncry1* mRNA oscillation after onset of the scotophase (Figure 5C). Possibly, the alteration of mRNA levels of *Sncry1*, is necessary for the expression of diapause in *S. nonagrioides.* The connection of circadian clock genes in diapause induction has been discussed and some circadian clock genes have been recommended to be linked with diapause in insects [7, 6, 11, 12, 32]. In the parasitic wasp, *Nasonia vitripennis,* the circadian oscillation of *per* and *cry* mRNAs in the heads of *Nasonia* females, also kept under short and long photoperiods [33]. It is generally accepted that a circadian clock is involved in the photoperiodic response and photoperiods often modulate the circadian parameters and the waveform of the clock [34, 35]. Thus, if the circadian clock were involved, the photoperiodically moderated waveform would have some roles in the photoperiodic time measurement [36]. Interestingly, the *S. nonagrioides* showed strong modulated waveform in the clock gene *Sncry1* expression and a drastic difference in the expression level of *Sncry1* was observed between different photoperiodic conditions (LD and SD). At present, few studies have studied cry expression during different photoperiods. Here, our results show that transcriptional regulation of *Sncry1* acts in diapause programming in MCSB and may be essential for daily rhythms and photoperiodic diapause.

In order to realize whether and in what way the circadian system is associated to photoperiodism, a significant part of information is whether clock genes products fluctuate and how they respond to a changing photoperiod. Our data on the clock genes *Snper, Sntim, Sncyc* and *Sncry1* revealed that in the MCSB the expression patterns of these oscillate and affected by photoperiod [11, 12]. Since our experiments were done under LD, DD and SD conditions, the data reflect how the molecular clock adapts to photoperiodic changes. Our results show that

transcriptional regulation of these four clock genes maybe play important roles in the diapause programming in MCSB.

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