Copulation Exerts Significant Effects on mRNA Expression of Cryptochrome Genes in a Moth

Jin Xu,1,* Bo Gao,2,* Min-Rui Shi,1 Hong Yu,1 Ling-Yan Huang,2 Peng Chen,3,4 and Yong-He Li1

1Yunnan Academy of Biodiversity, Southwest Forestry University, Kunming 650224, China, 2School of Life Sciences, Yunnan University, Kunming 650091, China, 3Yunnan Academy of Forestry, Kunming 650201, China, 4Corresponding author, e-mail: pengchenn@126.com

*These authors contributed equally to this article.

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Abstract

It is recognized that the behavioral rhythms of organisms are controlled by the circadian clock, while the reverse direction, i.e., whether changes in physiology and behavior react to the internal rhythms, is unclear. Cryptochromes (CRYs) are photolyase-like flavoproteins with blue-light receptor function and other functions on circadian clock and migration in animals. Here, we cloned the full-length cDNA of CRY1 and CRY2 in Spodoptera litura (Fabricius, 1775) (Lepidoptera: Noctuidae). SI-CRYs show high similarity to orthologs from other insects, and their conserved regions contain a DNA photolyase domain and a FAD-binding seven domain. The expression levels of both genes were relatively low during the larval stage, which increased during the pupal stage and then peaked at the adult stage. The expression of SI-CRY1 and SI-CRY2 showed differences between males and females and between scotophase and photophase. Further, our study demonstrated that copulation has a significant effect on the expression of SI-CRYs. More interestingly, the changes in the expression of SI-CRY1 and SI-CRY2 due to copulation showed the same trend in both sexes, in which the expression levels of both genes in copulated males and females decreased in the subsequent scotophase after copulation and then increased significantly in the following photophase. Considering the nature of the dramatic changes in reproductive behavior and physiology after copulation in S. litura, we propose that the changes in the expression of SI-CRYs after copulation could have some function in the reproductive process.

Key words: Spodoptera litura, cryptochrome, CRY1, CRY2, copulation

Cryptochromes (CRYs) are FAD-based blue-light photoreceptors that regulate the circadian clock and magnetosensitivity in animals and the growth, development, and phototropic movement in plants (Ozturk et al. 2007, Cao et al. 2017, Hirano et al. 2017, Ozturk 2017, Zhang et al. 2017, Jiang et al. 2018). Accordingly, CRYs are currently classified as animal CRYs and plant CRYs (Ozturk 2017). Animal CRYs were first classified as Drosophila-type CRYs (type 1) and mammal-type CRYs (type 2). Some insects were later revealed to have a mammal-type CRY, either alone or in addition to a Drosophila-type CRY (Ozturk 2017).

Type 1 CRY is the only CRY possessed by Drosophila, which was mainly expressed in the head and acts as a photoreceptor for resetting of the circadian clock (Emery et al. 1998). Unlike in fruit flies, butterflies contained both CRY1 and CRY2 (Zhu et al. 2005). In the monarch butterfly, Danaus plexippus, CRY1 also functions as a photoreceptor protein for circadian rhythm, whereas CRY2 functions not only as a repressor of the transcriptional feedback loop, but also as a sun compass during the migration of monarch butterflies (Zhu et al. 2008). A recent study (Zhang et al. 2017) using CRISPR/Cas9-mediated mutagenesis in D. plexippus revealed that insect CRY2 regulates circadian transcription via independent repression of CLOCK and BMAL1 activity.

Previous studies have shown that hormone application affected the expression of clock genes in rats and human cells (Karman and Tischkau 2006, Nakamura et al. 2010). In male mice, timed feeding shifted clock gene expression at the RNA and protein level in the gastrointestinal tract but did not shift clock gene expression in the central clock (Hoogerwerf et al. 2007). In female mice, high-fat feeding affected rhythmic expression of the certain clock genes, and in fact the daily rhythmicity in the transcript level of cholesterol 7a-hydroxylase, a hepatic enzyme controlling circadian cholesterol homeostasis, disappeared in the mice on high-fat feeding (Yanagihara et al. 2006). These findings suggest that changes in physiology, metabolism, and behavior can alter the molecular clock system in animals. However, whether copulation will also have such effects is unknown.

Spodoptera litura, also known as tobacco cutworm or cotton leafworm, is one of the world’s major agricultural pests due to its...
wide host range, alternating generations, and strong pesticide resistance (Armes et al. 1997, Zhou and Huang 2002, Ahmad et al. 2009, Rehan et al. 2011). Therefore, environmental friendly management techniques such as light and/or pheromone traps (e.g., Shirvastava et al. 1987, Vernon et al. 2014) and other potential control strategies such as RNAi based control techniques (Terenius et al. 2011) are needed to control this pest. Studies on CRY genes in this pest might provide useful information and target genes for future control techniques (Zhang et al. 2013, Xu et al. 2016, Khyati et al. 2017).

S. litura is a nocturnal moth. Our previous studies have shown that adult males and females exhibit circadian variations with respect to their reproductive physiology and behavior (Li et al. 2012, Li 2014, Yu et al. 2014, Lu et al. 2017). In the present study, we cloned the full-length cDNA of CRY1 and CRY2 in S. litura. To shed more light on the function of these two genes, we further investigated the expression patterns of both genes in relation to development and copulation status in both sexes. The key aim of this study was to test whether and how copulation would affect the expression of CRYs, which may bring us some clues for the understanding of the possible relationship and mechanisms between reproduction and biological clocks.

Material and Methods

Insects

S. litura larvae were reared on an artificial diet (Li et al. 2006) at 25 ± 1°C and a relative humidity of 60–70% with a photoperiod of 14:10 (L:D) h photoperiod regime. Newly eclosed male and female moths were maintained in separate cages to ensure virginity. Adult moths were maintained in the same environment and fed with 10% honey solution.

Molecular Cloning of CRY1 and CRY2

Total RNA was extracted from the head of test moth with Trizol (Takara, China) according to the manufacturer’s protocol, and the purity and concentration of RNA were measured by using a spectrophotometer (NanoDrop 2000, United States).

In our previous transcriptome sequencing and analysis, we obtained partial mRNA sequences of CRY1 and CRY2 of S. litura. Based on the partial sequences, gene-specific primers (GSP) and nested gene-specific primers (NGSP) were designed and synthesized (Table 1). For each 5′-RACE, the first round of amplification was performed on 1 μl of 5′-ready-cDNA with UPM (Universal Primer A Mix, Clontech, United States) and GSPr (Table 1), and then 1 μl of the first round products (1:100 dilutions) were used as templates for nested polymerase chain reaction (PCR) reactions with NUP (Nested Universal Primer A, Clontech) and NGSPr (Table 1). For each 3′-RACE, the first round of amplification was performed on 1 μl of 3′-ready-cDNA with UPM and GSPf; then 1 μl of the first round products (1:100 dilutions) were used as templates for nested PCR reactions with UPM and GSPf. All amplifications were performed with 50 μl reaction mixtures containing 1 μl of template, using Ex Taq HS (Takara) and the same PCR program: 94°C for 5 min, followed by 5 cycles of 94°C for 30 s, 72°C for 4 min, and 5 cycles of 94°C for 30 s, 70°C 30 s, 72°C 4 min, and then 25 cycles of 94°C for 30 s, 68°C 30 s, 72°C 4 min, and a final extension step of 72°C for 10 min. The PCR products were then cloned into DH5α cells using pMD18-T vector systems (TaKaRa). The plasmids were isolated using a SanPrep plasmid DNA extraction kit (Sangon Biotech, China) and were submitted for sequencing in both directions (Sangon Biotech).

Table 1. Primers for RACE and qPCR

| Primer names | Primer sequences (5′ to 3′) | Uses |
|--------------|----------------------------|------|
| CRY1-GSPr    | CCGCATACGGTGTTAGCCACCACCTTT | CRY1 5′-RACE |
| CRY1-NGSPr   | GAAGGTTTGCTGGAGCCTCAATCCG | CRY1 5′-RACE |
| CRY1-GSPI    | CAGAAATGCTATGAAGTGGTTGACGCG | CRY1 3′-RACE |
| CRY1-NGSPI   | TGCTCAACGAGATAATGGCCACGCC | CRY1 3′-RACE |
| CRY2-GSPr    | GCCGGAGAGACAGGACCATGTCGG | CRY2 5′-RACE |
| CRY2-NGSPr   | GGCCTGCTGTCTCGGACCACACAGAA | CRY2 5′-RACE |
| CRY2-GSPI    | GCCATGTCGTTAGCCACCACCTTT | CRY2 3′-RACE |
| CRY2-NGSPI   | CCGCAAGGCACTTGATATCCCAA | CRY2 3′-RACE |
| CRY1-Qf      | CGTGTAGCAGCAGGCTAGAAT | CRY1 qPCR |
| CRY1-Qr      | AGTAAAGGGCTGATATGCCGCC | CRY1 qPCR |
| CRY2-Qf      | GCCGGTGACTATCGGTTACC | CRY2 qPCR |
| CRY2-Qr      | TCAATGTGGCTTTCGCTTATA | CRY2 qPCR |
| Actin-Qf     | CATCTAGAAGGGTACGCGCCT | qPCR |
| Actin-Qr     | AGCGGTGGTGTGAAAGATA | qPCR |

CRY1 and CRY2 Expression in Relation to Development

The development duration of S. litura larvae and pupae is about 18 and 11 d, respectively (Li 2014). Adult moths can live up to 10 d but most copulations and ovipositions occurred in the first 3 d after eclosion (Li 2014). Therefore, in the present study, we collected samples from 1-, 9-, and 18-d-old larvae, 0-, 6-, and 11-d-old pupae, and 0-, 1-, and 2-d-old adult male and female virgin moths at midnight (5 h into the scotophase) and midday (7 h into the photophase), respectively. Total RNA was extracted from the heads (10 heads were used for each sample) of each sample using an RNA prep pure Tissue kit (TianGen, China). The purity and concentration of the RNA were assessed as above. Three samples were used for each category.

First strand cDNA synthesis was performed using a PrimeScript RT reagent Kit (Perfect Real Time) (TaKaRa). Real-time PCR was performed with GSP for CRY1 and CRY2 (Table 1) using the SYBR Premix Ex Taq II (TaKaRa) in a volume of 25 μl. Actin was used as a reference gene (Li et al. 2014a, Lu et al. 2015). Reactions were run in triplicate on the QuantStudio 7 Flex (Thermo Fisher Scientific, United States) using the following program: 95°C for 5 min followed by 40 cycles of 95°C for 30 s, 60°C for 34 s. Analysis of the dissociation curves for the target and reference genes showed a single melt peak. The efficiencies of the target and reference genes were similar,
and the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) was used to calculate the relative quantities of the target genes.

**Effect of Copulation on the Expression of CRY1 and CRY2**

To determine whether and how copulation affects the expression of CRY1 and CRY2, we paired 1-d-old virgin adults during 4–5 h after lights off in the second scotophase after emergence, with one pair in each box. Copulation (two insects engaged at the tip of the abdomen) events were recorded. The copulated moths were then individually caged and sampled at midnight (5 h into the scotophase) of the subsequent night (about 24 h after copulation) and at midday (7 h into the photophase) of the following day (about 36 h after copulation). Copulations were verified by dissecting the females to check for the presence of a spermatophore in the mating sac (Li 2014). Virgin males and females of the same age were used as controls. Total RNA was extracted from the heads (10 heads were used for each sample) of each sample as above. Three samples were used for each category. Real-time PCR and relative expression calculation followed the same procedure outlined above.

**Statistics**

Two-way ANOVAs were used to analyze the effect of development (three levels: larvae, pupae, and adults) and photoperiod (two levels: scotophase and photophase) on the expression of CRY1 and CRY2. Three-way ANOVAs were conducted to analyze the effect of sex (two levels: males and females), age (three levels: 0-d-old, 1-d-old and 2-d-old) and photoperiod (two levels: scotophase and photophase) on the expression of CRY1 and CRY2 during the adult stage. For simplicity, only those significant effects from the above analyses are reported.

The data on the effects of copulation on the expression of CRY1 and CRY2 were analyzed by one-way ANOVA followed by Fisher’s Least Significant Difference (LSD) test for multiple comparisons. All analyses were conducted using SPSS 16.0. The rejection level was set at $\alpha < 0.05$. All values are reported as mean ± SE.

**Results**

**Molecular Cloning and Phylogenetic Analysis of CRY1 and CRY2**

Based on sequencing and analysis, the full-length cDNA of the putative CRY1 and CRY2 were cloned from *S. litura*. The CRY1 cDNA of *S. litura* (SI-CRY1; GenBank accession: MH814726) contained an ORF of 1,647 nucleotides encoding a 548-amino acid protein, flanked by a 5′-UTR of 264bp and a 3′-UTR of 3208bp. The CRY2 cDNA of *S. litura* (SI-CRY2; GenBank accession: MH814727) contained an ORF of 2,376 nucleotides encoding a 791-amino acid protein, flanked by a 521bp-long 5′-UTR and a 469bp-long 3′-UTR.

BLAST analysis showed that SI-CRY1 has an obvious high identity to CRY1 sequences from other insects, such as *S. exigua* (ADY17887.1; 99% identity) and *Agrotis segetum* (AUG44605.1; 91% identity). Similarly, SI-CRY2 showed significant homology with the CRY2 sequences identified from other insects, such as *Helicoverpa armigera* (ADN94465.2; 95% identity), *Bombyx mori* (NP_001182627.1; 81% identity) and *Anopheles gambiae* (ABR29887.1; 75% identity). Multiple alignments indicated that the N-terminus of CRYs showed high conservation, while the C-terminal showed lower conservation (Fig. 1). The conserved regions of CRY1 and CRY2 contained a DNA photolyase domain and a flavin adenine dinucleotide (FAD) binding seven domain, which play important roles in the function of CRY proteins.

A phylogenetic tree based on the protein sequences of known CRYs was constructed by the Neighbor-Joining method with MEGA6.0 software (Fig. 2). The phylogenetic analysis indicated that CRYs were grouped into two clusters (CRY1 and CRY2). SI-CRY1 and CRY1s from other lepidopteran species were clustered together with 100% bootstrap support, while SI-CRY2 was clustered together with CRY2s of other lepidopteran species with 100% bootstrap support, each forming a monophyletic clade.

**CRY1 and CRY2 Expression in Relation to Development**

Two-way ANOVA indicated a significant development × photoperiod interaction for the expression of SI-CRY1 ($F_{2,4} = 3.57, P < 0.05$) (Fig. 3a). Post hoc simple main effect and LSD analyses indicated that the expression levels were significantly higher for adults compared with larvae and pupae both in scotophase and photophase ($P < 0.05$) and the expression levels were significantly different between scotophase and photophase during the adult stage ($P < 0.05$). Furthermore, statistical analysis using three-way ANOVA of data for adults indicated a significant sex × age × photoperiod interaction for the expression of SI-CRY1 during the adult stage ($F_{2,4} = 458.44, P < 0.0001$). Post hoc analyses indicated that the expression levels were significantly different between 1) scotophase and photophase in 0- and 1-d-old males and females, as well as in 2-d-old males ($P < 0.05$), 2) males and females of 0- and 1-d-old adults under either scotophase or photophase, and of 2-d-old adults under scotophase ($P < 0.05$), and 3) different ages either in males or females under scotophase ($P < 0.05$). The expression levels of 1-d-old adults were significantly higher than 0- and 2-d-old adults under photophase ($P < 0.05$).

Two-way ANOVA indicated a significant development main effect for the expression of SI-CRY2 ($F_{2,4} = 6.69, P < 0.01$) (Fig. 3b). A post hoc LSD test indicated that the expression levels were significantly higher for adults than for larvae and pupae under scotophase ($P < 0.05$). Three-way ANOVA indicated a significant sex × age × photoperiod interaction for the expression of SI-CRY2 during the adult stage ($F_{2,4} = 1379.08, P < 0.0001$). Post hoc analyses indicated that the expression levels were significantly different between 1) scotophase and photophase in 0- and 2-d-old adult males or females, and in 1-d-old adult males ($P < 0.05$), 2) males and females of 0- and 2-d-old adults under either scotophase or photophase, and of 1-d-old adults under photophase ($P < 0.05$), and 3) different ages for both adult males and females no matter if tested under scotophase or photophase ($P < 0.05$).

**Effect of Copulation on the Expression of CRY1 and CRY2**

One-way ANOVAs showed that copulation has significant effects on the expression of SI-CRY1 and SI-CRY2 in both sexes ($F_{2} = 10.66, P < 0.005$ for female SI-CRY1; $F_{2} = 274.60, P < 0.0001$ for male SI-CRY1; $F_{2} = 1927.17, P < 0.0001$ for male SI-CRY2; $F_{2} = 9510.21, P < 0.0001$ for male SI-CRY2) (Fig. 4). Post-hoc LSD tests indicated that the expression levels of the two genes in copulated males and females, compared to virgin individuals of the same age, decreased significantly ($P < 0.05$ in most cases (except a slight but not significant decrease of CRY1 in copulated females during scotophase, $P > 0.05$) in the subsequent scotophase after copulation and then all increased significantly ($P < 0.05$) in the following photophase (Fig. 4a–d).
Discussion

There are more than a million species of insects recognized worldwide, and they are found in nearly everywhere on earth. Insects have adapted to a wide array of environments and show different daily rhythms at the biochemical, physiological and behavioral levels (Goto 2013). Adapting to different environmental conditions may have also diversified the underlying clock machinery (Tomoka and Matsumoto 2015). Previous studies have shown that Drosophila possess only one CRY type (CRY1) (Emery et al. 1998), whereas in some other insects, such as butterflies and moths, both types of CRY genes are present, i.e., CRY1 and CRY2 (Zhu et al. 2005, Yan et al. 2013).

In the present study, we cloned two potential CRY genes, Sl-CRY1 and Sl-CRY2, from the head of S. litura. Online BLAST indicated that the putative amino acid sequences of Sl-CRY1 has an obvious higher similarity (≥78%) to orthologs from Lepidoptera, but has...
a relative lower identity (≤60%) to those from other insect orders including Coleoptera and Diptera. Moreover, Sl-CRY2 also showed high identity (>72%) to orthologs from Diptera, Hymenoptera, and Lepidoptera. The homology is 42% between Sl-CRY1 and Sl-CRY2, as compared to 43% in H. armigera (Yan et al. 2013).

Phylogenetic analysis showed that Sl-CRY1 and Sl-CRY2 were clustered together with CRY1 and CRY2 from other insects, respectively. The phylogenetic tree also indicated that S. litura had a shorter genetic distance to lepidopterans than insects from other orders, which was consistent with traditional taxonomy. Multiple alignments indicated that the N-terminus of CRYs showed high conservation, while the C-terminal showed lower conservation (Fig. 1). CRYs contain a C-terminal domain and a photolyase homology domain. The photolyase homology domain contains a DNA photolyase domain and a FAD-binding seven domain (Todo 1999, Cashmore 2003, Merlin et al. 2006, Ozturk 2017). The photolyase homology domain function in light detection and phototransduction, while the C-terminal domain may function in the regulation of CRY stability, CRY-TIM interaction and circadian photosensitivity (Busza et al. 2004). The structural conservation of the DNA photolyase domains and the FAD-binding seven domains in CRYs from different insect species suggests common mechanistic features of CRY, such as in photoreception (Merlin et al. 2006, Yan et al. 2013).

A few studies have tested the expression frame of CRYs in relation to the insect’s developmental stage (Yan et al. 2013, Xu et al. 2016, Chang et al. 2017). In H. armigera, the expression of CRY1 and CRY2 in the head is lower in adults than in larvae and pupae (Yan et al. 2013). A possible explanation for this is that the larvae and pupae lack compound eyes, CRY1 can act as a photoreceptor for photic entrainment (Yan et al. 2013). In the present study, however, we found that although the expression of Sl-CRY1 and Sl-CRY2

in the head fluctuated among different developmental stages, both genes showed similar trends in expression where the levels were relatively low during the larval stage, increased during the pupal stage and peaked in the adult stage for both males and females. Similar results also have been found in the brown planthopper, Nilaparvata

Fig. 2. Phylogenetic analysis of CRYs. The phylogenetic tree was constructed using the Neighbor-Joining method based on amino acid sequences with 1,000 bootstrap replicates. The percentage bootstrap support was presented by the number above the branches. The scale bar indicates the amino acid substitution rate. The GenBank accession numbers for CRY1 sequences from 12 insect species are as follows: S. exigua (ADY17887.1), Antheraea pernyi (NP_001182628.1), Amyelois transitella (XP_013199860.1), Chilo suppressalis (CDK20014.2), Papilio machaon (XP_014354985.1), Plutella xylostella (XP_015559253.1), A. gambiae (ABB29886.1), Bactrocera dorsalis (JAC52130.1), and Aegilus planipennis (XP_018322589.1). The GenBank accession numbers for CRY2 sequences from 9 insect species are as follows: H. armigera (ADN94465.2), M. separata (AFR54427.1), A. segetum (AUG44606.1), A. pernyi (ABO38435.1), B. mori (NP_001182627.1), P. machaon (XP_014360422.1), P. xuthus (XP_013166823.1), A. gambiae (ABB29887.1), and Bombus impatiens (NP_001267051.1).

Fig. 3. The expression levels of Sl-CRY1 (a) and Sl-CRY2 (b) in the head of S. litura in relation to developmental stages. L1, L9 and L18 refer to 1-, 9- and 18-d-old larvae; P0, P6, and P11 refer to 0-, 6- and 11-d-old pupae; A0♀, A1♀, and A2♀ refer to 0-, 1- and 2-d-old adult virgin females; and A0♂, A1♂, and A2♂ refer to 0-, 1-, and 2-d-old adult virgin males. Calibrator: Sl-CRY1 of 2-d-old virgin females in scotophase.
Fig. 4. Effect of copulation on the mRNA expression of Sl-CRY1 and Sl-CRY2 in the head of S. litura adults. (a) Sl-CRY1 in females; (b) Sl-CRY2 in females; (c) Sl-CRY1 in males; (d) Sl-CRY2 in males. Calibrator: Sl-CRY1 of virgin females in scotophase. For each parameter, bars with different letters are significantly different (P < 0.05).

lugens (Xu et al. 2016), and the black cutworm, A. ipsilon (Chang et al. 2017). It is interesting to note that N. lugens, A. ipsilon, and S. litura (Murata and Tojo 2004) all are long-distance migrants. The higher expression of CRYs in adults of these three insects may reflect that both CRY1 (Gegear et al. 2010) and CRY2 (Zhu et al. 2008) likely function as a sun compass and magnetic biocompass for successful navigation.

In H. armigera, S. littoralis, and A. ipsilon, expression levels of CRY1 reached a maximum during photophase and a minimum during scotophase (Merlin et al. 2007, Yan et al. 2013, Chang et al. 2017). These results support that CRY1 transcription is induced by light, and CRY1 rhythmicity might reflect the ancestral photolyase activity or circadian photoreception of the proteins. For a photolyase, a higher DNA repair activity might be advantageous during the photoperiod, and the expression of certain photolyase genes could be induced by light. In Bactrocera cucurbitae, individuals from the S strain mate early during the day, whereas individuals from the L strain mate later in the day; the expression pattern of CRY1 in the heads of flies taken from the S strain (were higher in the photophase but lower in the scotophase) significantly differed from adults in the L strain (lower in the photophase but higher in the scotophase) (Fuchikawa et al. 2010). These results suggest that CRY1 may have an important role in reproductive isolation (Fuchikawa et al. 2010). In Sarcophaga crassipalpis, CRY1 mRNA oscillation in the heads was fairly constant when reared either under 12:12 (L:D) h and 15:9 (L:D) h at 25°C or under 12:12 (L:D) h at 20°C (Goto and Denlinger 2002). These differences in the expression of CRY1 between different species imply CRY1 possesses multiple functions and its role may be species specific.

It has commonly been reported that the expression level of CRY2 is higher in the scotophase but lower in the photophase (Rubin et al. 2006, Merlin et al. 2007, Ikeno et al. 2008, Gentile et al. 2009, Yan et al. 2013, Zhang et al. 2017), which is consistent with oscillations of other clock genes, such as PER and VR1 (So and Rosbash 1997, Cyran et al. 2003, Rubin et al. 2006). These results support that CRY2 is the main repressor of CLK-CYC mediating transcription (Zhang et al. 2017).

It is clear that many behavioral rhythms of organisms are controlled by the circadian clock (Tomioka and Matsumoto 2015). An interesting question is whether and how significant changes in physiology, metabolism, or behavior will react to or even disrupt the internal rhythms? In the present study, to seek some enlightenment on this question, we further tested whether copulation can exert any effects on the expression of CRY genes. Indeed, our results demonstrated that copulation significantly affected the expression rhythms of CRYs. And of more interest, copulation caused similar changes in the expression of Sl-CRY1 and Sl-CRY2 in both sexes, where the expression levels of both genes in copulated males and females decreased significantly in most cases (except for Sl-CRY1 in...
copulated females where it tended to decrease but not significantly) in the subsequent scotophase after copulation and then all increased significantly in the following photophase (Fig. 4). In insects, copulation often leads to dramatic changes in the physiology and behavior of females and males (Gillott 2003). In addition, some studies found that copulation causes significant changes (up- or down-regulated) in expression of many genes, such as transcription factors, metabolic enzymes, immune response-related genes (such as attacin A, cecropin A1), egg maturation-related genes (such as yolk protein 1, yellow-g and chiorion protein 15), and other genes related to nutritional allocation (such as fit, LSP 2), behavior (such as takeoff) and aging (such as SMP 30) (Yanagihara et al. 2006, Hoogerwerf et al. 2007, Dalton et al. 2010, Goto 2013). However, it is still unclear why do these genes, including CRYs (as reported in this study), change after copulation and how are these changes linked to physiological and behavioral changes are still largely considered. Considering the nature of the significant changes on reproductive behavior and physiology before and after copulation in S. littura (Li et al. 2012, 2014a,b; Yu et al. 2014; Lu et al. 2015, 2017), we propose that the changes in the expression of CRYs due to copulation likely has a reproductive function. In S. littura, reproductive behavior of virgin males and females, including female calling, mate courtship and mating, occur mostly around midnight; females start to lay eggs in the subsequent night after copulation and egg laying can last to the late of the night; remarriages in these females usually occur in the late time of the nights; on the other hand, males also adjusted their reproductive behavior accordingly (Li et al. 2012, 2014a). In our previous study on S. littura females it was shown that copulation also changed the rhythms of sex pheromone biosynthesis and releasing (Lu et al. 2017). Therefore, the changes in mRNA expression in CRYs induced by copulation are consistent with the changes in behavioral and physiological rhythms due to copulation, suggesting a link between the CRYs and reproduction. In insects, copulation may change the production and titer of neuropeptides and hormones, such as the steroid hormones and juvenile hormone (e.g., Cole et al. 2003, Sugime et al. 2017, Diener et al. 2018). Other studies have shown that some hormones can affect the expression of clock genes (Karman and Tischkau 2006, Nakamura et al. 2010). Therefore, it is possible that copulation could regulate the synthesis and releasing of hormones, with the changes in hormone titer affecting the expression of CRYs and other clock genes in some way, which will then affect the insect's behavioral and physiological rhythm. Further studies are needed to clarify the possible regulation pathway and mechanism between reproduction and molecular clockwork by considering not only CRYs but also other clock genes.

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References Cited

Ahmad, M., M. A. Saleem, and A. H. Sayeed. 2009. Efficacy of insecticide mixtures against pyrethroid- and organophosphate-resistant populations of Spodoptera litura (Lepidoptera: Noctuidae). Pest Manag. Sci. 65: 266–274.

Armes, N. J., J. A. Wightman, D. R. Jadhav, and G. V. R. Rao. 1997. Status of insecticide resistance in Spodoptera litura in Andhra Pradesh, India. Pestic. Sci. 50: 240–248.

Busza, A., M. Emery-Le, M. Rosbash, and P. Emery. 2004. Roles of the two Drosophila cryptochrome structural domains in circadian photoresponse. Science. 304: 1503–1506.

Cao, Q., X. Zhao, J. Bai, S. Gery, H. Sun, D. C. Lin, Q. Chen, Z. Chen, L. Mack, H. Yang, et al. 2017. Circadian clock protein cryptochromes regulate autophagy. Proc. Natl. Acad. Sci. USA 114: 12548–12553.

Cashmore, A. R. 2003. Cryptochromes: enabling plants and animals to determine circadian time. Cell. 114: 537–543.

Chang, H., X. W. Fu, S. Y. Zhao, L. M. He, Y. M. Hou, and , K. M. Wu. 2017. Molecular characterization, tissue, and developmental expression profiles of mag and cryptochrome genes in Agrigota iphlon (Lepidoptera: Noctuidae). Ann. Entomol. Soc. Am. 110: 422–432.

Cole, T. J., P. Eggelston, and H. Hurd. 2003. Juvenile hormone titre and egg production in Tenebrio molitor infected by Hymenolepis diminuta: effect of male and/or female infection, male age and mating. J. Insect Physiol. 49: 583–590.

Cyran, S. A., M. A. Buchsbaum, K. L. Reddy, M. C. Lin, N. R. Glossop, P. E. Hardin, M. W. Young, R. V. Storti, and J. Blau. 2003. vriP, Pdp1, and dClock form a second feedback loop in the Drosophila circadian clock. Cell. 112: 329–341.

Dalton, J. E., T. S. Kacheria, S. R. Knott, M. S. Lebo, A. Nishitani, L. E. Sanders, E. J. Stirling, A. Winbush, and M. N. Arbeinman. 2010. Dynamic, mating-induced gene expression changes in female head and brain tissues of Drosophila melamogaster. BMC Genomics 11: 541.

Diener, M., A. Gallot, H. Binz, C. Gaertner, S. Veitcek, J. Kahnt, J. Schachtner, E. Jacquin-Joly, and C. Gadenne. 2018. Matin-induced differential peptidomics of neuropeptides and protein hormones in Agrigota iphlon moths. J. Pept. Res. 17: 1397–1414.

Emery, P. W. V. So, M. Kaneko, J. C. Hall, and M. Rosbash. 1998. CRV, a Drosophila clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell. 95: 669–679.

Fuchikawa, T., S. Sanada, R. Nishio, A. Matsumoto, T. Matsuyma, Y. Yamagishi, K. Tomioka, T. Taninura, and T. Miyatake. 2010. The clock gene cryptochrome of Bactrocera cucurbitae (Diptera: Tephritidae) in strains with different mating times. Hereditas (Edinb). 104: 387–392.

Gegear, R. J., L. E. Foley, A. Casselman, and S. M. Reppert. 2010. Animal cryptochromes mediate magnetoreception by an unconventional photochemical mechanism. Nature. 463: 804–807.

Gentile, C., G. B. Rivas, A. C. Meireles-Filho, J. B. Lima, and A. A. Peixoto. 2009. Circadian expression of clock genes in two mosquito disease vectors: cry2 is different. J. Biol. Rhythms 24: 444–451.

Gillott, C. 2003. Male accessory gland secretions: modulators of female reproductive physiology and behavior. Annu. Rev. Entomol. 48: 163–184.

Goto, S. G. 2013. Roles of circadian clock genes in insect photoperiodism. Entomolom. Sci. 16:1–16.

Goto, S. G., and D. L. Denlinger. 2002. Short-day and long-day expression patterns of genes involved in the flesh fly clock mechanism: period, timeless, cycle and cryptochrome. J. Insect Physiol. 48: 803–816.

Hirano, A., D. Braas, Y. H. Fu, and L. J. ÐiÄeAÁek. 2017. FAD regulates cryptochrome protein stability and Circadian clock in mice. Cell Rep. 19: 255–266.

Hoogerwerf, W. A., H. L. Hellmich, G. Cornelissen, F. Halberg, V. B. Shahnain, J. Bostwick, T. C. Savidge, and V. M. Cassone. 2007. Clock gene expression in the murine gastrointestinal tract: endogenous rhythmicity and effects of a feeding regimen. Gastroenterology. 133: 1250–1260.

Ikeno, T., H. Numata, and S. G. Goto. 2008. Molecular characterization of the circadian clock genes in the bean bug, Riptortus pedestris, and their expression patterns under long- and short-day conditions. Gene. 419: 56–61.

Jiang, Y. D., X. Yuan, W. W. Zhou, Y. L. Bai, G. Y. Wang, and Z. R. Zhu. 2018. Cryptochrome regulates circadian locomotor rhythms in the small brown planthopper Laodelphax straetelli (Fallén). Front. Physiol. 9: 149.

Karman, B. N., and S. A. Tischkau. 2006. Circadian clock gene expression in the ovary: effects of late-juvenile hormone. Biol. Reprod. 73: 624–632.

Khyati, I. Malik, and R. K. Seth. 2017. Insect clocks: implication in an effective pest management. Biol. Rhythm. Res. 48: 777–788.

Li, C. 2014. Molecular characterization and functional analysis of the sex-peptide receptor in the tobacco cutworm Spodoptera littura. Southwest Forestry University, Kunming, China. pp. 64.
Li, C., J.-F. Yu, J. Xu, J.-H. Liu, and H. Ye. 2012. Reproductive rhythms of the tobacco cutworm, Spodoptera litura (Lepidoptera: Noctuidae). GSTF J. BioSci. 2: 25–29.

Li, C., J.-F. Yu, Q. Lu, J. Xu, J.-H. Liu, and H. Ye. 2014a. Molecular characterization and functional analysis of a putative sex-peptide receptor in the tobacco cutworm Spodoptera litura (Fabricius, 1775) (Lepidoptera: Noctuidae). Aust. Entomol. 53: 424–431.

Li, Y. Y., J.-F. Yu, Q. Lu, J. Xu, and H. Ye. 2014b. Female and male moths display different reproductive behavior when facing new versus previous mates. PLoS One 9: e109654.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 25: 402–408.

Lu, Q., L.-Y. Huang, P. Chen, J.-F. Yu, J. Xu, J.-H. Liu, and H. Ye. 2015. Identification and RNA interference of the pheromone biosynthesis activating neuropeptide (PBAN) in the common cutworm moth Spodoptera litura (Lepidoptera: Noctuidae). J. Econ. Entomol. 108: 156–162.

Merlin, C., M. C. François, I. Queguiner, M. Maïbèche-Coisné, and E. Jacquin-Joly. 2006. Evidence for a putative antennal clock in Mamestra brassicae: molecular cloning and characterization of two clock genes–period and cryptochrome–in antennae. Insect Mol. Biol. 15: 137–145.

Merlin, C., P. Lucas, D. Rochat, M. C. François, M. Maïbèche-Coisné, and E. Jacquin-Joly. 2007. An antennal circadian clock and circadian rhythms in peripheral pheromone reception in the moth Spodoptera littoralis. J. Biol. Rhythms 22: 502–514.

Murata, M., and S. Tojo. 2004. Flight capability and fatty acid level in triacylglycerol of long-distance migratory adults of the common cutworm, Spodoptera litura. Zoolog. Sci. 21: 181–188.

Nakamura, T. J., M. T. Sellix, T. Kudo, N. Nakao, T. Yoshimura, S. Ebihara, C. S. Colwell, and G. D. Block. 2010. Influence of the estrous cycle on clock gene expression in reproductive tissues: effects of fluctuating ovarian steroid hormone levels. Steroids. 75: 203–212.

Ozturk, N. 2017. Phylogenetic and functional classification of the photolyase/cryptochrome family. Photosom. Photochem. 93: 104–111.

Ozturk, N., S. H. Song, S. Ozgür, C. P. Selby, L. Morrison, C. Parth, D. Zhong, and A. Sancar. 2007. Structure and function of animal cryptochromes. Cold Spring Harb. Symp. Quant. Biol. 72: 119–131.

Rehan, A., M. A. Saleem, and S. Freed. 2011. Baseline susceptibility and stability of insecticide resistance of Spodoptera litura (Lepidoptera: Noctuidae) in the absence of selection pressure. Pak. J. Zool. 43: 973–978.

Rubin, E. B., Y. Shemesh, M. Cohen, S. Elgavish, H. M. Robertson, and G. Bloch. 2006. Molecular and phylogenetic analyses reveal mammalian-like clockwork in the honey bee (Apis mellifera) and shed new light on the molecular evolution of the circadian clock. Genome Res. 16: 1352–1365.

Shrivastava, S. K., B. C. Shukla, and A. S. R. A. S. Shastri. 1987. Effect of lunar cycle on light trap catches of Spodoptera litura Fabricius. Indian J. Agr. Sci. 57: 117–119.

So, W. V., and M. Rosbash. 1997. Post-transcriptional regulation contributes to Drosophila clock gene mRNA cycling. EMBO J. 16: 7146–7155.

Sugime, Y., D. Watanabe, Y. Yasuno, T. Shinada, T. Miura, and N. K. Tanaka. 2017. Upregulation of juvenile hormone titer in female Drosophila melanogaster through mating experiences and host food occupied by eggs and larvae. Zoolog. Sci. 34: 52–57.

Terenius, O., A. Papanicolaou, J. S. Garbutt, I. Eleftherianos, H. Huvonen, S. Kanginakudru, M. Albrechtsen, C. An, J. L. Aymeric, A. Barthel, et al. 2011. RNA interference in Lepidoptera: an overview of successful and unsuccessful studies and implications for experimental design. J. Insect Physiol. 57: 231–245.

Tod, T. 1999. Functional diversity of the DNA photolyase/blue light receptor family. Mutat. Res. 434: 89–97.

Tomioka, K., and A. Matsumoto. 2015. Circadian molecular clockworks in non-model insects. Curr. Opin. Insect Sci. 7: 58–64.

Vernon, R. S., R. P. Blackshaw, W. G. Van Herk, and M. Clodius. 2014. Mass trapping wild Agriotes obscurus and Agriotes lineatus males with pheromone traps in a permanent grassland population reservoir. Agr. Forest. Entomol. 16: 227–239.

Yu, J. J., G. J. Wan, D. B. Hu, J. He, F. J. Chen, X. H. Wang, H. X. Hua, and W. D. Pan. 2016. Molecular characterization, tissue and developmental expression profiles of cryptochrome genes in wing dimorphic brown planthoppers, Nilaparvata lugens. Insect Sci. 23: 805–818.

Yan, S., H. Ni, H. Li, J. Zhang, X. Liu, and Q. Zhang. 2013. Molecular cloning, characterization, and mRNA expression of two Cryptochrome genes in Helicoverpa armigera (Lepidoptera: Noctuidae). J. Econ. Entomol. 106: 450–462.

Yanagihara, H., H. Ando, Y. Hayashi, Y. Obi, and A. Fujimura. 2006. Fat food exerts minimal effects on rhythmic mRNA expression of clock genes in mouse peripheral tissues. Chronobiol. Int. 23: 903–914.

Yu, J. F., C. Li, J. Xu, J.-H. Liu, and H. Ye. 2014. Male accessory gland secretions modulate female post-mating behavior in the moth Spodoptera litura. J. Insect. Behav. 27: 105–116.

Zhang, H., H. C. Li, and X. M. Xiao. 2013. Feasibility, limitation and possible solutions of RNAi-based technology for insect pest control. Insect Sci. 20: 15–30.

Zhang, Y., M. J. Markert, S. C. Groves, P. E. Hardin, and C. Merlin. 2017. Vertebrate-like CRYPTOCHROME 2 from monarch regulates circadian transcription via independent repression of CLOCK and BMAL1 activity. Proc. Natl. Acad. Sci. USA 114: E7516–E7525.

Zhou, X. M., and B. Q. Huang. 2002. Insecticide resistance of the common cutworm (Spodoptera litura) and its control strategies. Entomol. Knowl. 59: 98–102.

Zhu, H., Q. Yuan, A. D. Briscoe, O. Froy, A. Casselman, and S. M. Reppert. 2005. The two CRYS of the butterfly. Curr. Biol. 15: R953–R954.

Zhu, H., I. Sauma, Q. Yuan, A. Casselman, M. Emery-Le, P. Emery, and S. M. Reppert. 2008. Cryptochrome defines a novel circadian clock mechanism in monarch butterflies that may underlie sun compass navigation. PLoS Biol. 6: e4.