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Research paper

Differential expression of circadian clock genes in two strains of beetles reveals candidates related to photoperiodic induction of summer diapause

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ABSTRACT

Diapause (also known as dormancy) is a state of arrested development induced by photoperiod or temperature that allows insects to survive adverse environmental conditions. By regulating diapause induction, the circadian clock is involved in short-day-induced winter diapause but whether this is also the case in long-day (LD)-induced summer diapause remains unknown. The cabbage beetle *Colaphellus bowringi* could enter summer diapause under LD conditions. However, a non-photoperiodic-diapause (NPD) strain of this species, which was developed in our laboratory by artificial selection, could not enter diapause under LD photoperiod. Therefore, we identified circadian clock genes in this species and measured differences in their expression between a high diapause (HD) strain and the NPD strain to investigate the potential relationship between circadian clock genes and summer diapause induction in *C. bowringi*. We successfully cloned eight circadian clock genes and obtained intact ORFs of four; *cryptochrome2*, *double-time*, *shaggy* and *vrille*. Phylogenetic trees and sequence alignment analyses indicated that these circadian clock genes were conserved across insect taxa. The quantitative real-time PCR indicated that *clock*, *cycle*, *period*, *timeless*, *cryptochrome2*, and *vrille* were differentially expressed between HD and NPD strains reared under LD photoperiod during the diapause induction phase. These findings suggest the potential relationship between circadian clock genes and LD-regulated summer diapause induction in *C. bowringi*.

1. Introduction

Organisms have evolved a number of strategies to survive periodic harsh environmental conditions, such as migration and diapause (also known as dormancy) (Tauber et al., 1986; Hand et al., 2016). Diapause is a genetically pre-programmed developmental response to seasonal change (Hand et al., 2016). In the induction phase of diapause, insects collect and assess environmental signals in order to determine whether they should enter diapause or not (Denlinger, 2002). Many insects use photoperiod, a reliable environmental signal, as the main cue for determining whether to enter diapause (Lees, 1956; Beck, 1962; Kostal, 2006). However, exactly how insects detect photoperiod remains unclear.

The circadian clock in photoperiodism has subsequently been demonstrated in many insects (Saunders et al., 2002; Saunders, 2011). The circadian clock consists of interlocked double feedback loops with positive and negative elements (Williams and Sehgal, 2001). For example, in the fruit fly *Drosophila melanogaster* the circadian clock mainly consists of the *cycle* (*cyc*), *clock* (*clk*), *period* (*per*), *timeless* (*tim*), *cryptochrome1* (*cry1*), *double-time* (*dbt*), *shaggy* (*sgg*), *vrille* (*vri*), *clockwork orange* (*cwo*), and *par domain protein1ξ* (*pdp1ξ*) (Darlington et al., 1998; Emery et al., 1998; Price et al., 1998; Rutila et al., 1998; Cyran et al., 2003). Studies on other insects have discovered additional circadian clock genes, such as *cryptochrome2* (*cry2*) and *timeout* (Rubin et al., 2006; Zhan et al., 2011; Gu et al., 2014). It has been demonstrated that circadian clock genes in insects could regulate short-day-induced winter diapause by modulating photoperiodic diapause induction (Ikeno et al., 2010; Ikeno et al., 2011b; Meuti et al., 2015). However, whether circadian clock genes also relate to long-day (LD)-induced summer diapause needs further study.

The cabbage beetle, *Colaphellus bowringi* Baly (Coleoptera: Chrysomelidae), could enter summer diapause under LD induction (Xue et al., 2002). Resonance experiments suggest that the circadian oscillatory system is involved in photoperiodic diapause induction in wild-type (high diapause strain, HD strain) *C. bowringi* populations (Wang et al., 2004). We have developed and maintained a non-photoperiodic-diapause (NPD) strain of this species in our lab over many years (Tan et al., 2016). This NPD strain has lost the ability to respond to photoperiod at 25 °C, and is consequently reproductive under photoperiods

Abbreviations: *clk*, *clock*; *cry2*, *cryptochrome2*; *cyc*, *cycle*; *dbt*, *double-time*; HD, high diapause; LD, long-day; NPD, non-photoperiodic-diapause; ORF, open reading frames; *per*, *period*; qRT-PCR, quantitative real-time PCR; *sgg*, *shaggy*; *tim*, *timeless*; *vri*, *vrille*; ZT, zeitgeber time.

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Table 1
Blastp analyses of putative *Colaphellus bowringi* circadian clock genes.

Gene	Acc. number[a]	Length (bp)	Closest relative	Intact ORF (aa)[b]	Identity	Location (aa)[c]
clk	KX602314	1885	*L. decemlineata* (AKG92749.1)	601	77%	8 to 601
cyc	KX602315	2038	*L. decemlineata* (AKG92783.1)	633	90%	10 to 632
per	KX602316	2337	*T. castaneum* (EFA04566.2)	1109	60%	368 to 1106
tim	KX602317	2109	*T. castaneum* (NP 001106936.1)[d]	1079	68%	346 to 844
cry2	KX602318	1617	*T. castaneum* (NP 001076794.1)	535	86%	6 to 534
dbt	KX602319	1245	*T. castaneum* (XP 015838159.1)[e]	430	88%	1 to 430
sgg	KX602320	1263	*L. decemlineata* (ALE20559.1)[f]	419	96%	1 to 419
vri	KX602321	1176	*T. castaneum* (EFA11543.1)	338	69%	1 to 338

[a] GenBank accession number.
[b] The intact ORF length of the corresponding gene in related species.
[c] The location of *C. bowringi* circadian clock gene amino acid fragments on the ORF of corresponding gene in closest relative.
[d] Peptide listed as "timeless isoform B" in GenBank.
[e] Peptide is a predicted protein homologous with *dbt* and listed as "predicted: case in kinase I isoform delta-A isoform X2" in GenBank.
[f] Peptide listed as "Gsk3b" in GenBank and homologous with *sgg*.

that induce diapause in the HD strain (Xue et al., 2002; Ma et al., 2011). This suggests that the HD and NPD strains may differ in the mechanism used to detect photoperiod (Tan et al., 2016). We hypothesized that, if circadian clock genes are involved in detecting photoperiod in *C. bowringi*, they should have differential expression patterns in HD and NPD strains during the photoperiod-sensitive stage of diapause induction under LD conditions, the diapause-inducing photoperiod. Previous research demonstrated that 3- to 5-day-old larvae were the most sensitive to photoperiod at temperatures under 25 °C (Xue et al., 2002). Therefore, we systematically identified circadian clock genes based on the transcriptome database (SRP026471) for *C. bowringi*, and compared their 24-hour expression patterns in 4-day-old larvae of the HD and NPD strains under LD photoperiod. The results suggest that the *C. bowringi* circadian clock is similar to that of insects that only possess *cry2*, and that the expression patterns of the *clk*, *cyc*, *per*, *tim*, *cry2* and *vri* were different in the HD and NPD strains. To our knowledge, this is the first report which investigates the relationship between circadian clock genes and LD-induced summer diapause in insects.

2. Materials and methods

2.1. Insects and sample collection

The HD strain of *C. bowringi* was descended from individuals collected from a natural, wild population in Xiushui and had been maintained in our laboratory since 2008 (Ma et al., 2011). The NPD strain was produced by artificial selection from individuals collected from the same wild population (Tan et al., 2016). The HD strain displays a clear photoperiodic response with the incidence of diapause varying with daylength at 25 °C, whereas the NPD strain is always reproductive at 25 °C, irrespective of photoperiod (Liu et al., 2009; Tan et al., 2016). Newly hatched larvae from each strain were collected in the morning and separated into two treatment groups. Both groups were kept at 25 °C and 70% relative humidity under a 16L: 8D photoperiod; conditions that reliably induce diapause in the HD strain (Wang et al., 2004; Tan et al., 2015). The heads of 4-day-old larvae were collected at zeitgeber time (ZT) 0 (light on), ZT 4, ZT 8, ZT 12, ZT 16 (light off), ZT 20 and ZT 24. These samples were placed in RNase-free microtubes, then immediately frozen in liquid nitrogen and stored at −80 °C until required for further analyses. Each treatment had three replicates; each ZT comprised of a pooled sample of 200 heads of newly hatched larvae.

2.2. RNA extraction, cDNA synthesis and gene cloning

Total RNA was isolated from the heads of larvae using Trizol (Takara, D9108A, Japan). cDNA was synthesized from 1 μg total RNA using the PrimeScript RT reagent Kit with gDNA Eraser (Takara, DRR047A, Japan). Fragments of circadian clock genes were obtained from the transcriptome database of *C. bowringi* which was published in 2015 (Tan et al., 2015). In order to identify circadian clock gene fragments, primers were designed by NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table S1). The melting temperature of the primers was as close to 58 °C as possible. PCR was performed with LA Taq (Takara, DRR002A, Japan) according to the supplier's instructions. To obtain complete sequences of circadian clock genes, 3′ and 5′ RACEs were performed with the 3′ Full RACE Core Set Ver 2.0 (Takara, D314, Japan) and the 5′ Full RACE Kit (Takara, D315, Japan), according to the supplier's instructions. PCR products were inserted into plasmids with the 18-T Vector (Takara, D101A, Japan) and sequenced by Genscript Company (Nanjing, China).

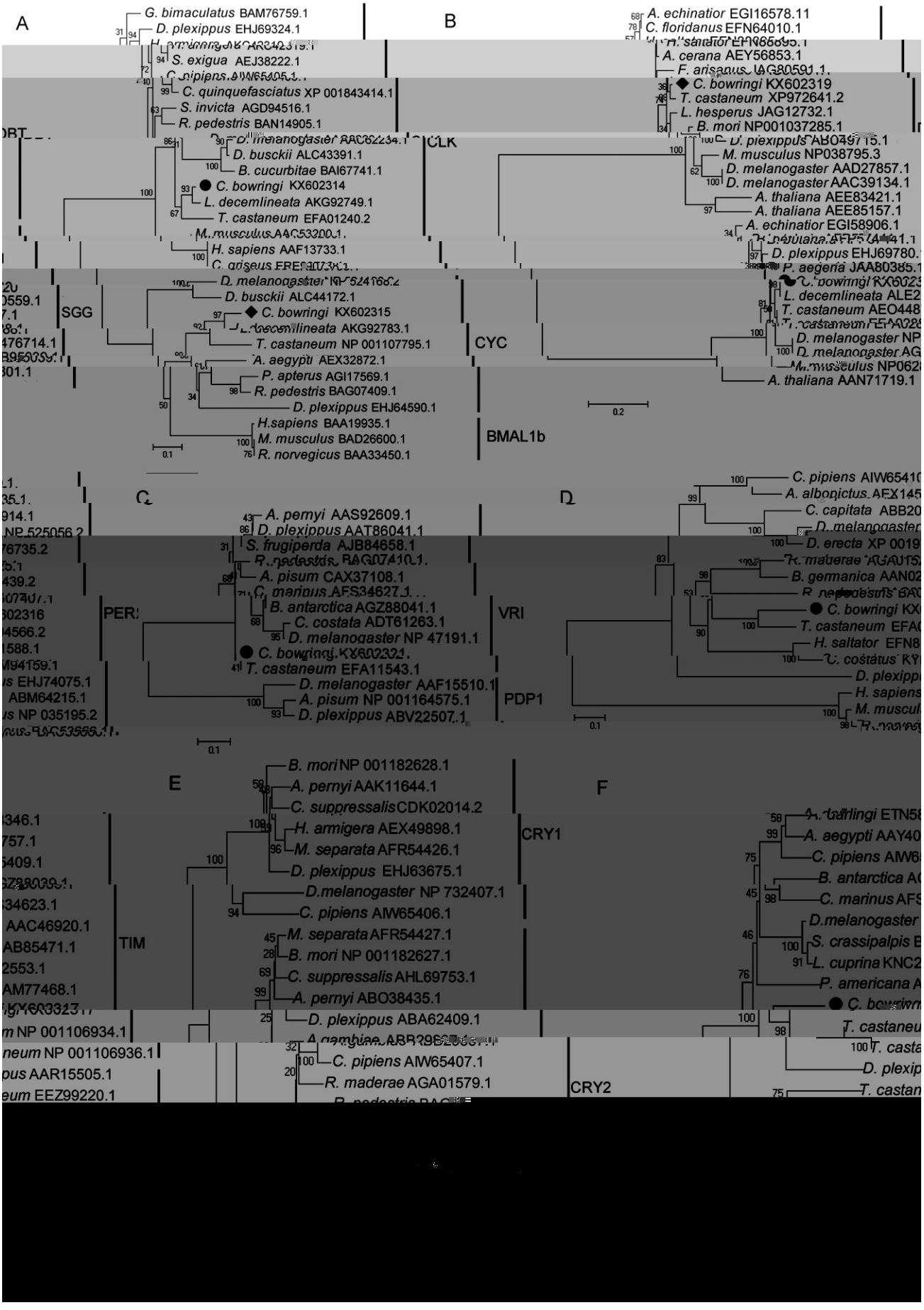
2.3. Domain inference and phylogenetic analysis

The putative amino acid sequences of *C. bowringi* circadian clock genes were deduced by ExPASy (http://web.expasy.org/translate/). We searched for related protein sequences of other insects, the mouse *Mus musculus* and the plant *Arabidopsis thaliana* in NCBI (http://www.ncbi.nlm.nih.gov/protein/). The domains of circadian clock genes were predicted by NCBI and confirmed by comparison to the SMART (http://smart.embl-heidelberg.de) and ProSite (http://prosite.expasy.org/) databases. Protein alignment and phylogenetic analyses were performed in Mega 4.0. Additional multiple domain sequence alignments were performed using GeneDoc.

2.4. Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was used to investigate the temporal expression patterns of circadian clock genes. Total RNAs

Fig. 1. Neighbor-joining trees of the circadian clock genes *clk* and *cyc* (A), *sgg* and *dbt* (B), *vri* (C), *per* (D), *cry2* (E), and *timeless* (F). Included species: *Acromyrmex echinatior*, *Acyrthosiphon pisum*, *Aedes aegypti*, *Aedes albopictus*, *Anopheles darling*, *Anopheles gambiae*, *Antheraea pernyi*, *Apis cerana*, *Apis mellifera*, *Arabidopsis thaliana*, *Bactrocera cucurbitae*, *Belgica antarctica*, *Biston betularia*, *Blattella germanica*, *Bombus impatiens*, *Bombyx mori*, *Camponotus floridanus*, *Ceratitis capitate*, *Chilo suppressalis*, *Chymomyza costata*, *Clunio marinus*, *Colaphellus bowringi*, *Cricetulus griseus*, *Culex pipiens*, *Culex quinquefasciatus*, *Cyphomyrmex costatus*, *Danaus plexippus*, *Drosophila busckii*, *Drosophila melanogaster*, *Gryllus bimaculatus*, *Harpegnathos saltator*, *Helicoverpa armigera*, *Homo sapiens*, *Leptinotarsa decemlineata*, *Lucilia cuprina*, *Lygus hesperus*, *Mus musculus*, *Mythimna separate*, *Periplaneta americana*, *Rattus norvegicus*, *Riptortus pedestris*, *Rhyparobia maderae*, *Sarcophaga crassipalpis*, *Solenopsis invicta*, *Spodoptera exigua*, *Spodoptera frugiperda* and *Tribolium castaneum*. Outgroups were *M. musculus*, *C. griseus*, *H. sapiens*, *R. norvegicus* and *A. thaliana*.


A
G. bimaculatus BAM76759.1
D. plexippus EHJ69324.1
S. exigua AEJ38222.1
C. quinquefasciatus XP 001843414.1
S. invicta AGD94516.1
R. pedestris BAN14905.1
D. busckii ALC43391.1
B. cucurbitae BAI67741.1
C. bowringi KX602314
L. decemlineata AKG92749.1
T. castaneum EFA01240.2
H. sapiens AAF13733.1
SGG
D. busckii ALC44172.1
C. bowringi KX602315
T. castaneum NP 001107795.1
A. aegypti AEX32872.1
P. apterus AGI17569.1
R. pedestris BAG07409.1
D. plexippus EHJ64590.1
H.sapiens BAA19935.1
M. musculus BAD26600.1
R. norvegicus BAA33450.1
B
A. echinatior EGI16578.11
C. floridanus EFN64010.1
A. cerana AEY56853.1
C. bowringi KX602319
T. castaneum XP972641.2
L. hesperus JAG12732.1
B. mori NP001037285.1
M. musculus NP038795.3
D. melanogaster AAD27857.1
D. melanogaster AAC39134.1
A. thaliana AEE83421.1
A. thaliana AEE85157.1
A. echinatior EGI58906.1
D. plexippus EHJ69780.
CYC
A. thaliana AAN71719.1
BMAL1b
C
A. pernyi AAS92609.1
D. plexippus AAT86041.1
S. frugiperda AJB84658.1
A. pisum CAX37108.1
B. antarctica AGZ88041.1
C. costata ADT61263.1
D. melanogaster NP 47191.1
T. castaneum EFA11543.1
D. melanogaster AAF15510.1
A. pisum NP 001164575.1
D. plexippus ABV22507.1
VRI
PDP1
D
C. pipiens AIW65410
A. albopictus AEX145
C. capitata ABB20
D. erecta XP 0019
B. germanica AAN02
C. bowringi KX
T. castaneum EFA
H. saltator EFN8
D. plexipp
H. sapiens
M. muscul
E
B. mori NP 001182628.1
A. pernyi AAK11644.1
C. suppressalis CDK02014.2
H. armigera AEX49898.1
M. separata AFR54426.1
D. plexippus EHJ63675.1
D.melanogaster NP 732407.1
C. pipiens AIW65406.1
M. separata AFR54427.1
B. mori NP 001182627.1
C. suppressalis AHL69753.1
A. pernyi ABO38435.1
D. plexippus ABA62409.1
C. pipiens AIW65407.1
R. maderae AGA01579.1
TIM
CRY1
CRY2
F
A. aegypti AAY40
C. pipiens AIW6
B. antarctica A
C. marinus AFS
D.melanogaster
S. crassipalpis E
L. cuprina KNC2
P. americana A
T. castaneu
D. plexip
T. castan

were isolated from the heads of 4-day-old larvae from each strain that had been collected at ZT 0, 4, 8, 12, 16, 20 and 24. cDNA was synthesized from 1 μg total RNA with a PrimeScript RT reagent Kit with gDNA Eraser (Takara, DRR047A, Japan) according to the manufacturer's instructions. For qRT-PCR analysis, 10% of the cDNA and 0.4 μM of each primer were used in a final concentration of 2 × SYBR Premix Ex Taq II (Takara, DRR081A, Japan), and each reaction was duplicated three times. Primers for qRT-PCR were also designed by NCBI (Table S2). The melting temperature of the primers was as close to 60 °C as possible, and PCR efficiency ranged from 94.1% to 103.6%. Following previous research published by our lab, we used *β-tubulin* as an endogenous reference gene (Tan et al., 2015). All qRT-PCR data were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The statistical significance of differences in the real-time relative expression of circadian clock genes was analyzed using ANOVAs followed by Bonferroni's test (Goto and Denlinger, 2002; Goto et al., 2006).

3. Results

3.1. Identification of C. bowringi circadian clock genes

Eight circadian clock genes were discovered, based on the *C. bowringi* transcriptome database (SRP026471) and biological pathways analysis, and there were seven genes (*clk*, *cyc*, *per*, *tim*, *dbt*, *sgg* and *vri*) mapped onto the fly circadian clock pathway in Kyoto Encyclopedia of Genes and Genomes. After analyzing and sorting we obtained 8 circadian clock genes, which were comprised of 16 fragments in total (Table S3). Through cloning and splicing, we successfully obtained 8 circadian clock genes; *clk* (KX602314), *cyc* (KX602315), *per* (KX602316), *tim* (KX602317), *cry2* (KX602318), *dbt* (KX602319), *sgg* (KX602320) and *vri* (KX602321) (Table 1).

We obtained the intact open reading frames (ORF) of 4 circadian clock genes after RACEs, namely *cry2* (1617 bp), *dbt* (1245 bp), *sgg* (1263 bp) and *vri* (1176 bp), which respectively encoded 538, 414, 420, and 391, amino acids. The ORFs of the other 4 circadian clock genes; *clk* (1885 bp), *cyc* (2038 bp), *per* (2337 bp) and *tim* (2109 bp), were partial cDNA that respectively encoded 627, 679, 779 and 703 amino acids. Phylogenetic analyses revealed that *C. bowringi* circadian clock genes are similar to those in other Coleoptera, such as the red flour beetle *Tribolium castaneum* and Colorado potato beetles *Leptinotarsa decemlineata* (Fig. 1). Domain inference suggested that the domain types of *C. bowringi* circadian clock genes are consistent with those of the corresponding genes in *T. castaneum* and *D. melanogaster*, and even show remarkable homology with those of *M. musculus* (Table S4). Further multiple domain sequence alignments indicate that the core domains of CLK, CYC, PER and CRY2 show little variation between species, and that the core domain sequences of DBT, SGG and VRI are well conserved among different species (Figs. S1–S7). These results suggest that *C. bowringi* circadian clock genes are homologous to those of other Coleopteran species, that the domains of these genes are conserved, and that they therefore are likely to perform similar functions as the clock genes of other Coleopteran species.

3.2. Differential expression patterns between the HD and NPD strains under the diapause-inducing LD conditions

Because the 4-day old larval stage is the photoperiod-sensitive stage for diapause induction, we compared the expression of circadian clock genes over a 24 h period in the heads of 4-day old HD and NPD strain larvae that had been raised under a photoperiod of 16L: 8D, a diapause-inducing LD photoperiod. The expression patterns of *clk*, *cyc*, *per*, *tim*, *cry2* and *vri* were obviously different in the HD and NPD strains (Fig. 2). *Clk*, *cry2* and *vri* were more strongly expressed around the transformation of photoperiod, and *cyc*, *per* and *tim* relatively weakly expressed during the photophase, in the NPD strain. *Clk* was up-regulated from ZT 4 to ZT 16, and peaked at ZT16 ($df = 6, 20$; $P = 0.003$), in the NPD strain, but showed downregulation ($df = 6, 20$; $P = 0.005$) in expression in the HD strain (Fig. 2A). In the HD strain, *cry2* was downregulated and was suppressed in the scotophase ($df = 6, 20$; $P = 0.013$). However, in the NPD strain, *cry2* was up-regulated during the day with peak expression occurring in the scotophase ($df = 6, 20$; $P = 0.000$) (Fig. 2E). *Vri* was highly expressed from ZT 0 to ZT 8 in the HD strain ($df = 6, 20$; $P = 0.000$), but was highly expressed during the entire photophase in the NPD strain ($df = 6, 20$; $P = 0.029$) (Fig. 2H). *Cyc* expression increased during the photophase, peaking with the onset of the scotophase in the HD strain ($df = 6, 20$; $P = 0.003$), but expression of this gene fluctuated and remained low during the day in the NPD strain ($df = 6, 20$; $P = 0.009$) (Fig. 2B). *Per* expression was similar between the HD and NPD strains; up-regulated during the photophase and with peak expression at ZT 16 ($df = 6, 20$; $P_{HD} = 0.003$; $P_{NPD} = 0.000$), but its expression in the HD strain was slightly higher than in the NPD strain (Fig. 2C). In the HD strain, *tim* expression was irregularly up regulated in the photophase, peaking at ZT 12, then downregulated ($df = 6, 20$; $P = 0.000$). *Tim*

(df = 6, 20; $P = 0.007$). However, there was no temporal variation in *tim* expression in the NPD strain (Fig. 2D). There was no difference in the expression of *dbt* between strains at ZT0 (df = 1, 5; $P = 0.062$), but *dbt* continued to be downregulated from ZT4 with minimum expression at ZT12 (df = 6, 20; $P = 0.243$) in the HD strain (Fig. 2F). The expression of *sgg* was steadily expressed in both strains (df = 6, 20; $P_{HD} = 0.097$; $P_{NPD} = 0.144$) (Fig. 2G). Collectively, the differential expression profiles suggest the potential roles of circadian clock genes in diapause induction in *C. bowringi*.

4. Discussion

We successfully identified eight circadian clock genes and compared their expression patterns in HD and NPD strains of *C. bowringi* under LD conditions, the diapause-inducing photoperiod. Our results showed that the expression patterns of *clk*, *cyc*, *per*, *tim*, *cry2* and *vri* were significantly different in the HD and NPD strains, suggesting the potential relationship between these circadian clock genes and LD-regulated diapause induction in *C. bowringi*.

Phylogenetic analysis indicates that the *C. bowringi* circadian clock genes we identified are homologous to corresponding genes in other Coleopteran insects. Many of the deduced amino acid sequences of protein domains were highly conserved among different species, especially *dbt*, *sgg* and *vri*. It has been reported that the *dbt*, *sgg* and *vri* genes of the copepod *Calanus finmarchicus* show extensive sequence conservation with the corresponding genes in *D. melanogaster*, especially in functional domains (Christie et al., 2013). Many studies also suggest a high degree of evolutionary conservation between *D. melanogaster dbt* and *sgg* genes and those of vertebrates (Ruel et al., 1993; Fan et al., 2009). However, some circadian clock genes do display species-specific variation, such as those in the *cry*-family (Rubin et al., 2006). There are two kinds of CRYs in insects: CRY1 which functions as a blue-light photoreceptor, and CRY2, which is not light sensitive (Emery et al., 1998; Kume et al., 1999). It had been reported that some insects possess both *cry1* and *cry2*, whereas others only have either *cry1* or *cry2* (Emery et al., 1998; Zhu et al., 2005; Rubin et al., 2006; Yuan et al., 2007). We confirmed that *C. bowringi* has *cry2*, but whether it has *cry1* requires further investigation.

The *clk*, *cyc*, *per*, *tim*, *cry2*, and *vri* were differentially expressed in the HD and NPD strains. The differential expression of circadian genes between wild-type and NPD strain were also found in the linden bug *Pyrrhocoris apterus*. In *P. apterus*, diapause-inducing photoperiod induced high expression of *per* and *clk* in the wild-type strain, whereas expression of *per* and *clk* remained low in non-diapause individuals in both the wild-type and a photoperiod insensitive strain (Hodkova et al., 2003; Syrová et al., 2003). Besides *P. apterus*, it has been reported that *tim* was suppressed in the brain of a drosophilid fly *Chymomyza costata* NPD strain (Stehlik et al., 2008). Further studies showed that this *tim*-deficiency caused this *C. costata* NPD strain to be insensitive to photoperiod and also influenced the expression of *tim*, *per* and *vri* in the NPD strain (Stehlik et al., 2008; Kobelkova et al., 2010). Besides *tim*, the function of other circadian clock genes in short-day-regulated diapause induction had been demonstrated in *R. pedestris* and northern house mosquito *Culex pipiens*. For example, RNAi *cyc* and *clk* induced individuals of these species to enter diapause, even under photoperiods that normally induce reproduction (Ikeno et al., 2010; Ikeno et al., 2011a; Ikeno et al., 2013). Similarly, RNAi *cry2* and *tim* both induced ovary development under diapause-inducing photoperiods, and the RNAi of one circadian clock gene could influence the expression patterns of other circadian clock genes (Meuti et al., 2015). But there is still a hot debate over it is modular pleiotropy or gene pleiotropy of the circadian clock in insects (Bradshaw and Holzapfel, 2010; Kostal, 2011; Meuti and Denlinger, 2013). It still needs further efforts to investigate the involvement of circadian clock in photoperiodism (Saunders and Bertossa, 2011). Interestingly, we found *clk*, *cyc*, *per*, *tim*, *cry2*, and *vri* exhibited differential expression during the LD-regulated diapause induction in *C. bowringi*. Our results not only provide the new evidence of the potential function of *clk*, *cyc*, *tim*, and *cry2* in photoperiodic insect diapause, but also give us a cue that circadian clock genes may participate in the photoperiodic diapause induction in both winter and summer diapause. However, it needs to be further studied.

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Appendix A. Supplementary data

Supplementary material

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