Supporting Information

Bajgar et al. 10.1073/pnas.1217060110

SI Methods

Sequences and Cloning. Genes concerned in this study were isolated by RT-PCR with degenerate primers corresponding to conserved protein motifs, followed by extension of the obtained products by using 3′ and 5′ RACE (Ambion). Some sequences were retrieved by using BLAST from a 454 sequencing run (GATC Biotech), PCR-amplified, and verified by sequencing. Sequences are available from GenBank under the following accession nos.: *cryptochrome2 (cry2)* (1562936), *Par domain protein1 (Pdp1)*$_{iso1}$ (1563012), *Pdp1*$_{iso2}$ (1563013), *cycle (cyc)* (1562940), *Clock (Clk)* (1562939), *Clockwork orange (cwo)* (1563010), *tango (tgo)* (1563014), *taiman (tai)* (1562945), *lipase (lip)* (1563016), *defensin (def)* (1563019), *esterase (est)* (1563020), *superoxide dismutase (sod)* (1563024), *transferrin (tf)* (1563021), *Methoprene tolerant (Met)* (JN416984), and *Krüppel-homolog 1 (Kr-h1)* (JN416987).

Northern Blot Hybridization. Total RNA was isolated from guts of *Pyrrhocoris apterus* females by using TRIzol reagent (Invitrogen), and transferred with the NorthernMax kit (Ambion). The membrane was hybridized overnight at 65 °C with a *cry2* antisense RNA probe that was generated from a PCR-amplified cDNA fragment (primers shown in Table S3) with the T7/T3 MaxiScript system (Ambion) using digoxigenin-conjugated dUTP (Roche). Chemiluminescent signal was detected by the Luminoimage analyzer LAS-3000 (Fujifilm). Band intensities were determined densitometrically with Advanced Image Data Analyzer software (Raytest), with relative expression ratio being calculated for each band.

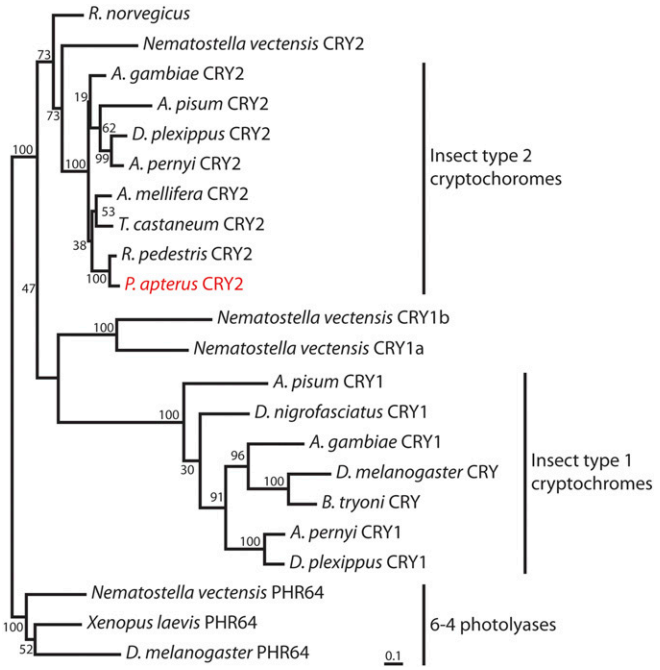


Fig. S1. A phylogenetic tree showing relationships among cryptochromes (Crys). Cry proteins were aligned by using the ClustalW method in Mega software (1), and this alignment was analyzed in the RAxML program (2) to construct a maximum likelihood tree (−ln = 12550.9981; WAG substitution matrix). Bootstrap values are given at the nodes (500 replicates). Sequences are unambiguously clustered into three groups: (*i*) type 1 cryptochromes containing *Drosophila* Cry and insect Cry1 genes, (*ii*) type 2 cryptochromes containing insect Cry2 and mammalian Crys, and (*iii*) 6-4 photolyases. The *P. apterus* cryptochrome sequence clearly belongs to the Cry2 group, with the closest relative being from the bean bug *Riptortus pedestris*. The following protein sequences were used: *P. apterus* (1562936), *R. pedestris* (BAG07408), *Tribolium castaneum* (EFA04537), *Apis mellifera* (NP_001077099), *Anopheles gambiae* Cry2 (ABB29887), *Danaus plexippus* Cry2 (ABA62409), *Antheraea pernyi* Cry2 (EF117813), *Acyrthosiphon pisum* Cry2 (FN377570), *Rattus norvegicus* Cry2 (AAK61419), *A. pisum* Cry1 (FN377569), *D. plexippus* Cry1 (EHJ63675), *A. pernyi* Cry1 (AAK11644), *Dianemobius nigrofasciatus* (BAF45421), *A. gambiae* Cry1 (ABB29886), *Bactrocera tryoni* (AAU14170) and *Drosophila melanogaster* Cry (NP_732407), *Nematostella vectensis* Cry1a (XP_001631029), Cry1b (XP_001632849), Cry2 (XP_001623146), 6-4 photolyase (PHR64, XP_001636303), *D. melanogaster* 6-4 photolyase (PHR64, NP_724274), and *Xenopus laevis* 6-4 photolyase (PHR64, NP_001081422).

1. Tamura K, et al. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28(10): 2731–2739.
2. Stamatakis A (2006) RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22(21):2688–2690.

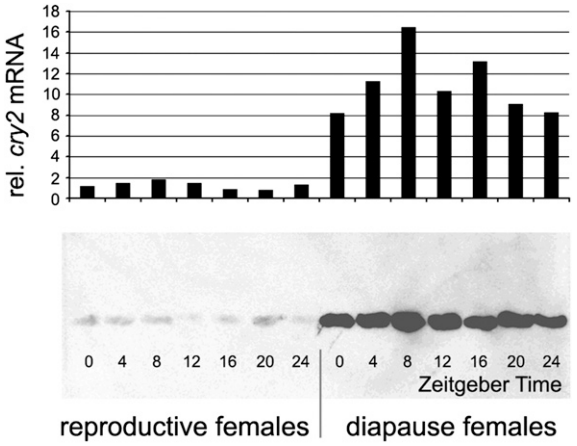


Fig. S2. Northern blot hybridization of *P. apterus cry2* transcripts. The relative mRNA expression levels (*Upper*) were calculated from band intensities in the Northern blot (*Lower*) and are shown in arbitrary units. Hybridization confirmed six- to tenfold up-regulation of *cry2* mRNA in the gut of diapause females relative to reproductive ones.

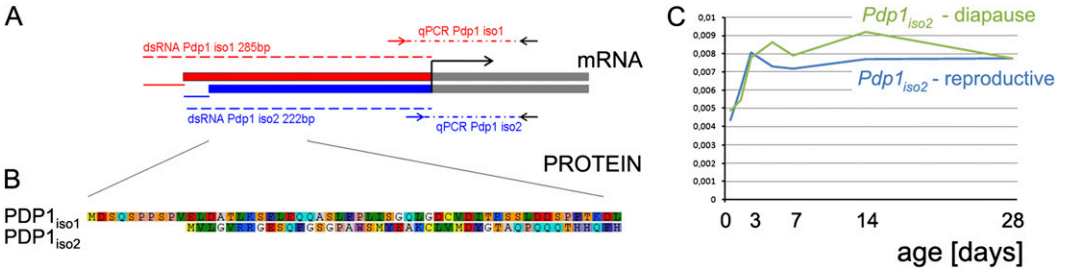


Fig. S3. *Pdp1* mRNA isoforms in *P. apterus*. (*A* and *B*) Alternative transcription start sites produce two *Pdp1* mRNA isoforms, which encode two proteins with different N termini. Broken lines delimit isoform-specific sequences that were used to generate dsRNAs for RNAi experiments. Short arrows indicate positions of isoform-specific forward primers that were combined with a common-region reverse primer in quantitative RT-PCR (qRT-PCR) experiments. (*C*) qRT-PCR with RNA from guts of diapause and reproductive females kept under short and long day length, respectively, show that expression of $Pdp1_{iso2}$ is unaffected by the photoperiod, but, unlike $Pdp1_{iso1}$, it is also independent of the reproductive state over a period of 4 wk.

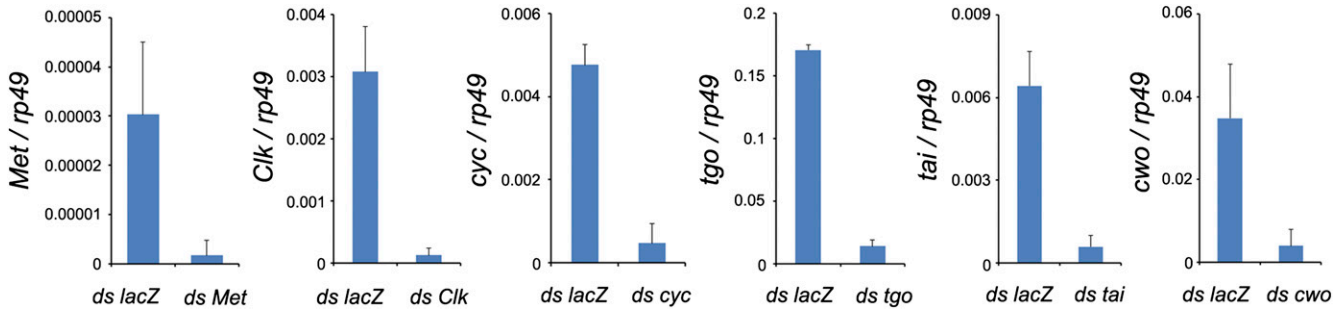


Fig. S4. Efficiency of RNAi-mediated depletion of targeted mRNAs. Decrease in abundance of *Met*, *Clk*, *cyc*, *tgo*, *tai*, and *cwo* transcripts in female guts 4 d after injection with respective dsRNAs was measured by using qRT-PCR relative to mRNA levels in controls injected with *lacZ* dsRNA. The relative mRNA levels were normalized to *rp49* expression. Data are mean ± SEM from three independent experiments.

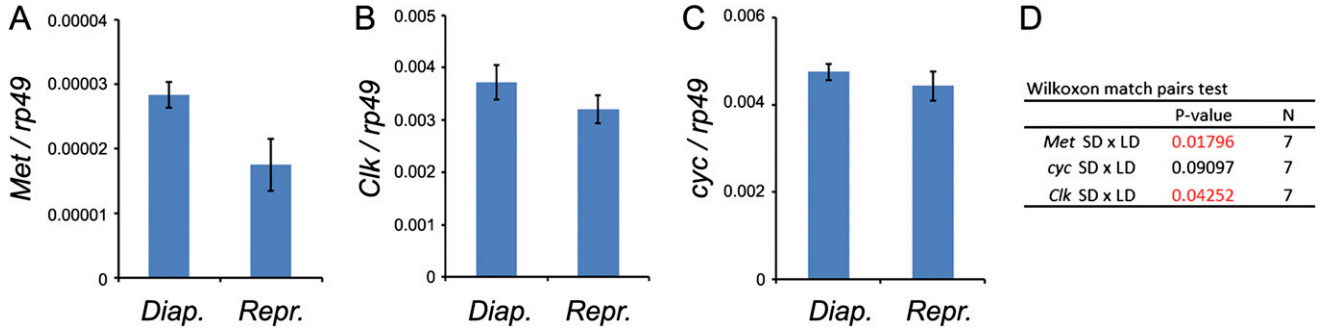


Fig. S5. Expression of *Met* (*A*), *Clk* (*B*), and *cyc* (*C*) in the guts of 7-d old diapause and reproductive females. Levels of *Met* (*A*) and *Clk* (*B*) are significantly higher in diapause females compared with reproductive ones (statistical values are shown in *D*). Relative mRNA levels were normalized to *rp49* expression. Data are mean ± SEM from seven independent samples.

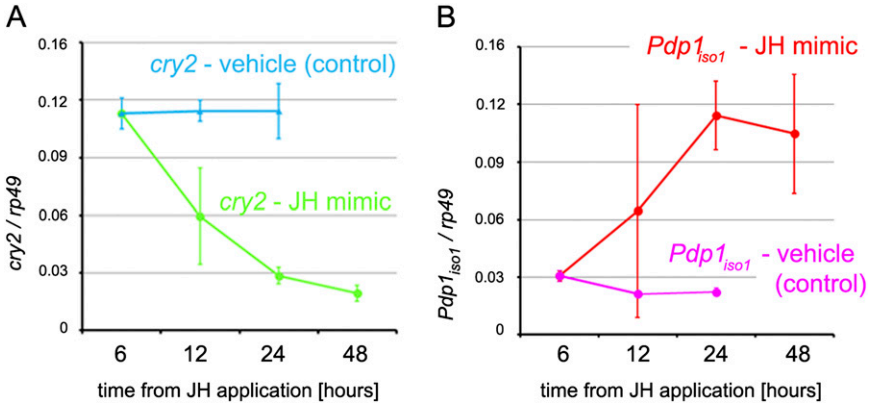
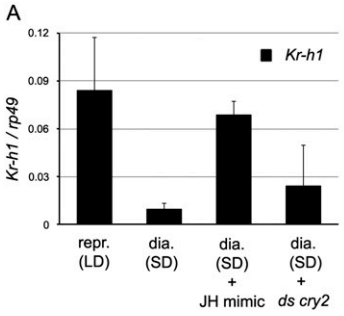


Fig. S6. The juvenile hormone (JH) mimic methoprene switches *cry2* and $Pdp1_{iso1}$ expression patterns in the gut from the diapause to the reproductive mode. Guts were dissected from 4- to 7-d old adult diapause females and cultured in 100 μL of Grace insect medium supplemented with antibiotics. After addition of 4 μL of acetone (control) or 4 μL of acetone-diluted 0.3 mM methoprene (final concentration of approximately 12 μM), the tissues were incubated and sampled for qRT-PCR at the indicated time points. The JH mimic suppressed the normally high *cry2* mRNA levels (*A*) and enhanced the normally low $Pdp1_{iso1}$ expression (*B*) occurring in the gut of diapause females. Each value is a mean ± SEM from three independent gut culture experiments.



B

	Tukey HSD test; Experimental samples, Post Hoc Tests, Error: Between MS = ,00000, df = 22,000			
	LD (repr.)	SD (diapause)	SD + JH analog	SD - cry2 RNAi
LD (reproductive)		0.000184	0.995286	0.000299
SD (diapause)	0.000184		0.003101	1.000000
SD + JH analog	0.995286	0.003101		0.005757
SD - cry2 RNAi	0.000299	1.000000	0.005757	

Fig. S7. Expression of the JH-dependent *Kr-h1* gene in the gut. (*A*) Transcription of *Kr-h1* was high in the gut of reproductive females that naturally produce JH, and low in diapause females that lack JH. The JH mimic methoprene induced *Kr-h1* when applied to diapausing animals. However, expression of *Kr-h1* in the gut of *cry2* RNAi females remained at levels comparable to those in untreated diapause females, suggesting that the loss of *cry2* function did not increase JH titer. Values are mean ± SEM from three independent experiments. (*B*) Red color marks statistically significant differences between values in *A* as assessed by the Tukey honestly significant difference test.

Table S1. Statistical significance of correlation between *cry2*, *Pdp1*$_{iso1}$, and downstream gene expression in Fig. 3

Gene	*Pdp1*$_{iso1}$	*cry2*
cry2	−0.56*	+1.00
lip	+0.64*	−0.61*
def	+0.72*	−0.55*
sod	−0.51*	+0.68*
est	+0.70*	−0.59*
tf	−0.77*	+0.74*

*Significant correlation at $P < 0.01$ (Spearman rank-order correlations).

Table S2. Primers used for qRT-PCR analyses

Gene	Forward primer	Reverse primer
cry2	GCGGTCGCCTGTTTTTTGACTCG	ACATCCACATACCAGCATTTACA
Pdp1$_{iso1}$	AGATCCAGAATTAGAAGCAGTCAC	GGTCAGTGTCGTAAGGGAGCGTC
Pdp1$_{iso2}$	CGGCCAGGTGGTCGCAGTA	GGTCAGTGTCGTAAGGGAGCGTC
rp49	CCGATATGTAAAACTGAGGAGAAAC	GGAGCATGTGCCTGGTCTTTT
Met	TTCTGATGATGGTGAAAAGATG	TATCGCCCCTGACTACTTGG
cyc	TGCCTTGTAGCCATGGGTAGAG	ATTATTTTGTTTCCATAGTATTCGTAAG
Clk	CCAACTTCATCACTTGTTCCAG	CCCTTCGTAAGAAATCTATAGTAG
cwo	CCAACTTCATCACTTGTTCCAG	TAACGGAGGTGGCCTGAAG
tgo	TCCCAAGCTCCTTATCACAAC	AAAGTTCGTGAGGTTGGTTGG
lip	CCTTTGCAACAACGCTCTCTAC	CATTCTGAGGCCTTGCTATGTA
def	CACAGTAGTTGTAGCAATGGC	CATGCGGAGTGGTTTGGAG
est	GGGCCGATGTATGCTTACATATT	CTTACATTCTTGTCCTCTTGACG
sod	ACCCCATCACGGAGGACC	TCGTCCGAAGTCGTCCTTG
tf	TTCGACTGTAGAGCAATCCATG	CTTGGAGGAATAGTGATTAGACC
Kr-h1	GAACGTCTTGTTACACACACC	CCCTACCAGTGTAACTTTTGC

Table S3. PCR primers for cloning of dsRNA templates and for the *cry2* hybridization probe

Gene	Forward primer	Reverse primer
cry2	TTGAAGAAGACCCAGAACCATTT	CAAAAACTTTCATTCCTTCTTC
Pdp1$_{iso1}$	AGTCTACCGCCGGATGGCAAGGTG	TCCTTCACAATCAGGGTCATGGTG
Pdp1$_{iso2}$	GAGGGGATGGTGTTAGGAGTG	TCCATTGATAAACATGTGATGTTG
cyc	CAGGAAAATGGATAAGTCTACGG	CCCTTCGTAAGAAATCTATAGTAG
Clk	TGCCTTGTAGCCATGGGTAGAG	ATTATTTTGTTTCCATAGTATTCGTAAG
cwo	ATGATGGAGCACACCTGTTGG	GGCCGGGTATTGAGTTCTC
tgo	ATGGCCGATGTTTATATGATAAGGTC	CGGAGGCGTATAACCAAGG
tai	GCCTCCTTCACCGACATGAGCAG	ACTGCACTCTCACATTGCATGAGCG