Loss of Timeless Underlies an Evolutionary Transition within the Circadian Clock

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Abstract

Most organisms possess time-keeping devices called circadian clocks. At the molecular level, circadian clocks consist of transcription—translation feedback loops (TTFLs). Although some components of the negative TTFL are conserved across the animals, important differences exist between typical models, such as mouse and the fruit fly. In Drosophila, the key components are PERIOD (PER) and TIMELESS (TIM-d) proteins, whereas the mammalian clock relies on PER and CRYPTOCHROME (CRY-m). Importantly, how the clock has maintained functionality during evolutionary transitions between different states remains elusive. Therefore, we systematically described the circadian clock gene setup in major bilaterian lineages and identified marked lineage-specific differences in their clock constitution. Then we performed a thorough functional analysis of the linden bug Pyrrhocoris apterus, an insect species comprising features characteristic of both the Drosophila and the mammalian clocks. Unexpectedly, the knockout of timeless-d, a gene essential for the clock ticking in Drosophila, did not compromise rhythmicity in P. apterus, it only accelerated its pace. Furthermore, silencing timeless-m, the ancestral timeless type ubiquitously present across animals, resulted in a mild gradual loss of rhythmicity, supporting its possible participation in the linden bug clock, which is consistent with timeless-m role suggested by research on mammalian models. The dispensability of timeless-d in P. apterus allows drawing a scenario in which the clock has remained functional at each step of transition from an ancestral state to the TIM-d-independent PER + CRY-m system operating in extant vertebrates, including humans.

Key words: circadian clock, reverse genetics, timeless, gene loss, Bilateria, Insecta.

Introduction

From the very beginning of life on our planet, nearly every organism has been exposed to periodic changes of day and night. Often, it is a great advantage for an organism to anticipate dawn, avoid mid-day heat, or target particular activity to a specific time of day. Accordingly, organisms have evolved circadian clocks, internal genetically determined time-measuring devices that “keep ticking” with a free-running period (~) close to 24 h, even when environmental cues are absent.

The key feature of circadian clocks is the transcription—translation feedback loop (TTFL) with positive and negative regulators. The positive regulators (transcription factors) drive the expression of mRNAs from which the negative regulators are translated, gradually accumulate, and once reach a threshold level, they suppress their own transcription by inhibiting the activity of the positive regulators. The similarities of the circadian clock shared by the fruit fly Drosophila melanogaster and mammals have facilitated elucidation of molecular mechanisms underlying rhythmicity (Allada et al. 2001; Zhang et al. 2011; Ozkaya and Rosato 2012). The core of the clock in animals is composed of the positive transcription factors, CLOCK (CLK) and BMAL/CRY (Darlington et al. 1998; Rutila et al. 1998; Huang et al. 2012; Menet et al. 2014), whose activity is suppressed by negative regulators. Within the negative feedback loop, we can see notable differences in the setup of the fruit fly and mammalian clocks. In the fruit fly, PERIOD and Drosophila-type TIMELESS (here referred to as TIM-d) are the key negative regulators essential for behavioral and molecular rhythmicity (Konopka and Benzer 1971; Bargiello et al. 1984; Zehring et al. 1984; Hardin et al. 1990; Sehgal et al. 1994). The chief light-cued synchronization pathway involves the interaction of TIM-d with Drosophila-type
CRYPTOCHROME (CRY-d) (Stanewsky et al. 1998; Ceriani et al. 1999; Emery et al. 2000). However, mammalian clocks lack both TIM-d and CRY-d; instead, their negative feedback relies on PER and mammalian CRY (hereafter CRY-m) (Kume et al. 1999). Thus, CRY-d serves in Drosophila clock neurons as a blue light photoreceptor that triggers light-mediated degradation of the negative regulators, whereas CRY-m in mammals is itself a potent repressor of CLOCK-BMAL. Phylogenetic analyses indicate that CRY-m is present in numerous insect species (Yuan et al. 2007), and in some of them, CRY-m was confirmed as a component essential for circadian rhythmicity (Ikeno, Katagiri, et al. 2011; Ikeno, Numata, et al. 2011; Zhang et al. 2017). The evolution of CRY might be shaped by its additional roles, including its involvement in seasonality, as was shown in the bean bug (Ikeno, Katagiri, et al. 2011; Ikeno, Numata, et al. 2011) and in the linden bug Pyrrhocoris apterus (Urbanova et al. 2016).

CRY proteins seem to participate in magnetoreception as was reported for CRY-d in the monarch butterflies (Wan et al. 2021) and Drosophila (Yoshii et al. 2009; Fedele et al. 2014), and for CRY-m in cockroaches (Bazalova et al. 2016), P. apterus (Netusil et al. 2021), and birds (Xu et al. 2021).

Mammalian-type TIM (TIM-m), first identified in mice (Zylka et al. 1998) and subsequently in Drosophila (Benna et al. 2000), was first deemed as a nonclock protein. TIM-m is essential for development, which further complicated its functional analysis (Gotter et al. 2000; Benna et al. 2010). However, conditional knockdown experiments in the rat disrupted neuronal activity rhythms in the suprachiasmatic nucleus (Barnes et al. 2003), and mutation in human TIM-m causes familial advanced sleep phase syndrome (Kurien et al. 2019) belonging to a family of circadian rhythm sleep disorders.

The above-described idiosyncrasies between the circadian clocks within Drosophila and mice prompted us to elucidate the evolution of the circadian clock set up in Bilateria, that is animals with exception of Porifera, Ctenophora, and Cnidaria. Assuming that the clock must have remained functional throughout evolution, we asked what were the transitional steps leading to the fruit fly organization (PER/TIM-d) on the one hand and the mammalian one (PER/CRY-m) on the other?

Recent progress in transcriptome sequencing of Bilateria in general and insect lineages in particular (Misof et al. 2014; Johnson et al. 2018; Kawahara et al. 2019; McKenna et al. 2019; Wipfler et al. 2019) allowed us to perform a systematic search for and subsequent evolutionary analysis of the circadian clock genes in Bilateria. Then we functionally tested all major clock components in the linden bug, P. apterus, an insect where a combination of both mammalian and Drosophila clock components were identified. Although a majority of P. apterus clock genes are functionally conserved with the fruit fly or mouse orthologs, unexpectedly, the knockout of tim-d, a gene essential for the clock ticking in Drosophila, did not compromise rhythmicity in P. apterus, but only accelerated its pace.

Results

Complex Evolution of Bilaterian Clock Setup

First, we retrieved genes coding for the key circadian clock negative feedback loop proteins from organisms representing major lineages of animals. To classify the CRY types (Kume et al. 1999; Yuan et al. 2007), we identified all cryptochrome/photolyase family members and reconstructed their phylogeny. The analysis revealed five well-defined clades: CRY-m, 6-4 DNA PHOTOLYASE (6-4 PL), CRY-d, CYCLOBUTANE PYRIMIDINE DIMER PHOTOLYASE (CPD-PL), and Drosophila, Arabidopsis, Synecocystis, and Human (DASH)-type CRY (fig. 1A; and supplementary fig. 1, Supplementary Material online). Although our primary interest was a reliable detection of CRY-d and CRY-m types across Bilateria, obtained phylogeny and distribution of 6-4 PL, CPD-PL, and DASH might be useful in the design of experiments (see Discussion).

The stability of CRY proteins is regulated, among others, by three proteins from the FBXL subfamily: JETLAG (JET), a protein essential for the interaction between CRY-d and TIM-d in Drosophila (Koh et al. 2006; Peschel et al. 2006, 2009), and two mammalian clock components interacting with CRY-m, FBXL3, and FBXL21, known also as over-time and after-hours, respectively (Godinho et al. 2007; Siepka et al. 2007; Hirano et al. 2013). Our phylogenetic reconstruction revealed a clear separation of JET and its deuterostomian homolog JET-like (also known as FBXL15) from FBXL3 and FBXL21 (fig. 1B; supplementary fig. 2, Supplementary Material online). FBXL3 and FBXL21 share one common ancestor present in basal Deuterostomia, whereas FBXL21 is only present in Gnathostomata (fishes, amphibians, reptiles, birds, and mammals). During our search for FBXL3, FBXL21, and JET proteins in bilaterian lineages, we retrieved all homologs, including the distantly related proteins. Therefore, the absence of target proteins reflects gene loss rather than the technical inability to identify a protein in prospected genomes and transcriptomes.

Then, we reconstructed PER and TIM phylogeny. Three paralogs (PER1–3) found in the majority of vertebrates, albeit some lineage-specific losses were identified in reptiles and birds, are separated from a single PER identified in Protostomia (fig. 1C; supplementary fig. 3, Supplementary Material online). Phylogeny of TIMELESS proteins confirmed clear separation of TIM-d from the ubiquitously present mammalian-type TIM-m (fig. 1D; supplementary fig. 4, Supplementary Material online). Furthermore, the presence of TIM-d in echinoderms and hemichordates confirms that the duplication leading to TIM-d and TIM-m arose in the ancestor of deuterostomes and protostomes.

Having well-defined TIM, CRY, and PER data sets, we mapped their presence on the bilaterian phylogeny, with a particular focus on gene losses in entire lineages. Full analysis depicted in figure 2 (see supplementary tables 1 and 2, Supplementary Material online) revealed a complex evolution of the circadian clock setup. TIM-m is, consistently with its essential role in development (Gotter et al. 2000; Benna et al. 2010), the only protein identified in all organisms.
Presented trees were inferred using RAxML maximum likelihood GAMMA-based model. For detailed trees see supplementary figures 1–4, Supplementary Material online.

The Linden Bug Is a Model with Combination of Both Mammalian and Drosophila Components

Surprisingly, the canonical clock component PER has been lost in two basal deuterostomian lineages, echinoderms, and hemichordates.

Notably, TIM-d has been lost in the entire bilaterian lineage only 3 times: in vertebrates, nearly all termites, and bees/wasps/ants (Hymenoptera; Rubin et al. 2006). In all three cases, TIM-d loss coincides with the absence of CRY-d, which seems to have been lost either simultaneously with TIM-d or even earlier, as seen in termites. Furthermore, CRY-d is absent in three additional lineages (supplementary tables 1 and 2, Supplementary Material online). In contrast, CRY-m was lost only in cyclorrhaphan Diptera, indicating the uniqueness of the Drosophila model. JET was lost in several lineages including those where either CRY-d or TIM-d is absent, such as a subset of Coleoptera, Hymenoptera, and Pentatomomorpha + Cimicomorpha. FBXL3 and FBXL21 were found in Gnathostomata, their single ancestor FBXL3 was found in all analyzed basal Deuterostomia. However, FBXL3 was lost in the majority of Protostomia and has remained only in the horseshoe crab Limulus, and four insect lineages: Ephemeroptera, Blattodea, Thysanoptera, and Hymenoptera (supplementary fig. 2, Supplementary Material online). Remarkably, all three TIM-d losses observed in the entire Bilateria coincide with the presence of FBXL3/21 and CRY-m.

The Linden Bug Is a Model with Combination of Both Mammalian and Drosophila Components

Clearly, the evolution of the circadian clock architecture was more complex than previously appreciated. To understand the functionality of the clock during its evolutionary transitions, we conducted a systematic molecular-genetic analysis of the clock components in the linden bug, P. apterus, a species comprising features of both mammalian and Drosophila clock types (fig. 2; supplementary tables 1 and 3, Supplementary Material online). Paradoxically, P. apterus possesses CRY-m and TIM-d but lacks CRY-d (Bajgar et al. 2013).

First, we employed systemic RNA interference (RNAi) to functionally test the circadian clock candidates in P. apterus. To identify possible off-target effects in RNAi experiments, each gene was separately knocked down with two nonoverlapping double-stranded RNA (dsRNA) fragments. The knockdown experiments confirmed a conserved role for the majority of P. apterus circadian clock genes (fig. 3; supplementary fig. 5, Supplementary Material online); specifically, knockdown of Clock, cycle/Bmal, or Par domain protein 1 (Pdp1) resulted in arrhythmicity, whereas clockwork orange (cwo) silencing slowed down the clock, consistent with the roles of the Drosophila homologs (Allada et al. 1998; Rutila et al. 1998; Cyran et al. 2003; Matsumoto et al. 2007). Weaker phenotypes were observed in vrille-silenced bugs, with nonsignificantly decreased τ. Consistently with the role of per and cwo in established models, their knockdown in P. apterus resulted in severely reduced rhythmicity (fig. 3).

Silencing of posttranslational components essential for the clock, such as the casein kinase 1 doubletime (dtt; Price et al. 1998) and F-box protein slimb (Grima et al. 2002), remarkably extended τ (fig. 3; supplementary fig. 6, Supplementary Material online). In nemo (nmo)-silenced bugs, each fragment resulted in a faster clock, although only fragment’s #1 phenotype was statistically significant. Nevertheless, this observation is consistent with the role of nmo in Drosophila (Chiou et al. 2011; Yu et al. 2011).

Since RNAi is performed in adults, we were able to test the role of TIM-m on the τ without interfering with its developmental role reported for both insects and mammals (Benna et al. 2010; Kurien et al. 2019). Although tim-m silencing did not significantly impact τ, a substantial number of dsRNA-injected bugs displayed a gradual loss of rhythmicity in
When the activity from the first 5 days of constant darkness was analyzed, only 3.4% \((tim-m\#1)\) and 2.5% \((tim-m\#2)\) of bugs were arrhythmic. However, when the rhythmicity was analyzed for Days 6–10 of constant darkness, arrhythmicity was recorded in 34.5% \((tim-m\#1)\) and 22.5% \((tim-m\#2)\) of dsRNA-injected bugs (fig. 3; supplementary fig. 7, Supplementary Material online).

Surprisingly, \(tim-d\)-silenced linden bugs displayed robust rhythmicity (>90%) with \(\tau\) shortened by >1 h \((P < 0.0001)\). This observation strongly contrasts with the key role of \(tim-d\) in \(Drosophila\), where its depletion results in complete arrhythmicity (Sehgal et al. 1994). Notably, silencing \(shaggy\), a kinase

**Fig. 2.** Circadian clock gene losses mapped on the bilaterian phylogeny. Representative insect species are shown at the terminal nodes with indicated gene presence (full circle) or absence (empty circle) where the lineage-specific losses are highlighted with red rectangles (see details in supplementary tables 1 and 2, Supplementary Material online). The phylogenetic tree corresponds to a consensus of recent phylogenomic studies (Misof et al. 2014; Johnson et al. 2018; Kawahara et al. 2019; McKenna et al. 2019; Wipfler et al. 2019). Filled circles indicate the presence of PER (black), TIM-m (brown), TIM-d (bright magenta), JET (dark blue), FBXL3/21 (light purple), CRY-m (grey), CRY-d (blue), 6-4 PL (deep purple), CPD-PL (yellow), and DASH-type cryptochrome (green). For phylogenetic relationship see supplementary figures 1–4, Supplementary Material online. Numbers indicate the presence of multiple paralogs in one taxon. The question mark indicates a suspicious occurrence of DASH in \(Bemisia\). Supplementary figure 16, Supplementary Material online, illustrates clear relatedness of this sequence with DASH from plants and fungi which can either be explained as contamination or as a horizontal gene transfer (HGT) from plant to insect. The latter would be consistent with a recent HGT of a plant detoxification component to \(Bemisia\) (Xia et al. 2021).

\(TIM-d\) Knockdown and Knockout Linden Bugs Remain Robustly Rhythmic

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essential for TIM-d-dependent phosphorylation in Drosophila (Martinek et al. 2001), had no impact on the
s
in the linden
bug (fig. 3). Therefore, to fully explore and clarify this unex-
p	tim-d
pted phenotype, we applied the CRISPR/Cas9 tech-

nique (Kotwica-Rolinska et al. 2019) and produced well-
defined genetic mutants in
	tim-d
	and two additional compo-
nents of the negative feedback loop, cry-m and per, in
P. apterus (fig. 4A; supplementary figs. 8–11, Supplementary
Material online).

Not only did the P. apterus tim-d03 homozygous mutants
display robust rhythmicity (98%, fig. 4B and C), but the
strength of their rhythm, plotted as PN (power of the perio-
dogram), was comparable to values observed in the wild-type
(wt; supplementary fig. 12, Supplementary Material online).
The
	tim-d03
mutation causes a frameshift followed by a pre-
mature stop codon (fig. 4A), therefore, tim-d03 encodes only
the initial one-third of the P. apterus TIM-d protein lacking
the conserved PER-interaction regions, nuclear localization
signal, and several key amino acid residues downstream
(fig. 4A; supplementary figs. 13 and 14, Supplementary
Material online). These features, essential for its proper func-
tion in Drosophila (Saez and Young 1996; Rothenfluh et al.
2000; Hara et al. 2011), are remarkably conserved in
P. apterus TIM-d. Importantly, tim-d03 resulted in
s
that was 0.6 h
shorter than that of the wt (P < 0.0001), which corroborates
the shorter
s
observed after
	tim-d
knockdown (fig. 3).

Three different mutations in cry-m were analyzed in detail
(supplementary fig. 9, Supplementary Material online). The
absence of CRY-m (cry-m04 and cry-m05) or a n-frame inser-
tion (cry-m9in; supplementary fig. 10, Supplementary
Material online) virtually destroyed rhythmic behavior
(fig. 4B and supplementary fig. 12A–C, Supplementary
Material online). However, a substantial percentage of homo-
yzous mutants displayed aberrant complex rhythmicity,
where either multiple periodic components were identified
or the
s
changed during the 10-day recording (fig. 4B;
supplementary fig. 15, Supplementary Material online).
Nevertheless, these rhythmic linden bugs, observed both in
cry-m mutant homozygotes (fig. 4B) and in cry-m knock-
down males (fig. 3A), displayed non-24-h rhythmicity
Fig. 4. Either cry-m or per depletion completely abolishes circadian rhythmicity in P. apterus, whereas tim-d mutants demonstrate robust rhythmicity with significantly shorter \( \tau \). (A) Schematic representation of tim-d mutants demonstrate robust rhythmicity with significantly shorter \( \tau \). (A) Schematic representation of tim-d gene structure with coding regions, alternative splicing, and engineered mutation (tim\(^{03}\)). Corresponding wt and mutant proteins are shown with major functional domains highlighted (for details, see supplementary figs. 13 and 14, Supplementary Material online). Alternative splicing of tim-d was detected in exons 9, 17, and 18. (B) Summary indicating the number and rhythmicity of the tested mutant and heterozygous animals compared with corresponding control siblings. (C) Individual \( \tau \) values are plotted as a dot for each male; red bars depict means \pm SEMs (calculated only if >10% individuals were rhythmic). Statistical differences from the controls is shown as \( P \)-value (Kruskal–Wallis test with Dunn’s post hoc). Double-plotted actogram of (D) wt and tim\(^{03}\) P. apterus compared with (E) D. melanogaster wt (Canton S) and tim\(^{01}\) mutant (arrow indicates the beginning of constant darkness).

(supplementary fig. 15B, Supplementary Material online). Similar to cry-m mutants, about one-third of P. apterus per mutants were arrhythmic, whereas the remaining bugs displayed rhythmicity with a short \( \tau \) of \(~19\text{ h}\) (fig. 4B and C).

Discussion

Phylogenetic analyses were primarily focused on discerning clock setup and its evolution in Bilateria. However, clustering within a specific group reflects the evolutionary origin of the proteins, though, in some of them, mutations affecting biochemical properties might have occurred. It is conceivable that especially closely related groups, such as CRY-m and 6-4 Pi, might relatively easily accumulate changes affecting their function. The combination and absence of particular CRY-type proteins in a given species might also be considered in experiments when antibodies recognizing conserved protein motifs are used, and vice versa, the design of either specific or universal antibodies should benefit from the available data sets.

The specific changes in the circadian clock setup, such as the absence of CRY-d and JET, pointed our attention to P. apterus. Altogether, the thorough RNAi and focused CRISPR/Cas9 analyses indicate nine P. apterus clock components (Clk, cyc/Bmal, Pdp1, vri, cwo, dbt, slimb, per, and nmo) work comparably to D. melanogaster orthologs. RNAi silencing of tim-m supports its role in the clock, which is consistent with its oscillatory role in the rat neuronal cells (Barnes et al. 2003). Nevertheless, relatively low P. apterus activity levels and their broad peaks (see Kaniewska et al. 2020) make it difficult to determine whether alterations of clock gene expression cause phase shifts in behavioral outputs. Thus, the role of TIM-m in circadian photoreception, proposed by the fruit fly experiments (Benna et al. 2010) and suggested by the altered activity phase in mammals (Kurien et al. 2019), could only be addressed in P. apterus with great difficulties.

Pyrrhocoris apterus possesses CRY-m, an ortholog of a key mammalian negative feedback loop protein, which functions consistent with its role in the monarch butterfly (Zhang et al. 2017), the bean bug (Ikeno, Katagiri, et al. 2011; Ikeno, Numata, et al. 2011), or the mouse (Kume et al. 1999). A substantial proportion of homozygous cry-m mutants (\(~40–80%\)) displayed aberrant complex rhythmicity. Comparable aberrant residual rhythmicity has been reported for per\(^{01}\) Drosophila mutants, where \(~30\%) of flies were shown to display a non-24-h \( \tau \) (Helfrich-Forster 2001). An interesting recent study on CRY-deficient mice describes their locomotor activity rhythms, which have shorter and variable \( \tau \) when compared with wt controls (Putker et al. 2021).

The major difference between mammals and P. apterus is that linden bugs possess TIM-d. However, unlike in Drosophila where tim-null flies are completely arrhythmic, TIM-d only modulates the circadian period in P. apterus. This result is in agreement with earlier RNAi experiments in crickets and firebrats, which also reported shorter \( \tau \) upon tim-d silencing (Danbara et al. 2010; Kamae and Tomioka 2012), dramatically contrasting with the key role of tim-d in Drosophila (Sehgal et al. 1994; fig. 4D and E).

The dispensability of TIM-d in P. apterus suggests a scenario of transition between clock architectures relying on...
Evolutionary Transitions in Animal Clock

distinct components of their negative feedback loops. The proposed model implies that the clock would be functional in each step of the transition from the ancestral state to the PER + CRY-m system known today in vertebrates (fig. 5). A similar clock gene combination in Hymenoptera indicates a convergent evolution of the circadian system, although functional evidence from this insect group is not yet available. The circadian clock observed in P. apterus could then correspond to an early clock setup that facilitates tim-d loss without a complete collapse of the circadian cycling. However, the timing and causality of the proposed events might have been lineage specific, where either the loss of cry-d triggered the transition of TIM-d to its modulatory role, or alternatively, the loss of JET or change in TIM-d properties compromised its interaction with CRY-d, which in turn was subsequently lost.

Materials and Methods

Data Sets and Phylogenetic Analyses
To reconstruct the evolution of circadian clock genes in animals, we applied a similar approach as in Smykal et al. (2020). First, we compared data sets from representative organisms (mouse, zebrafish, Platynereis, linden bug, pea aphid, monarch butterfly, honeybee, and fruit fly). Then we used BLAST to systematically explore all major bilaterian lineages at NCBI for CRYs, TIMs, PER, and FBXL/JET in taxon-specific searches. To ensure that all homologs were retrieved, reciprocal searches were performed for each species separately. The absence of a given gene/protein was then tested and confirmed in the corresponding lineage (order, suborder, and in some cases even family). Retrieved protein sequences were aligned using the ClustalW algorithm in Geneious Prime (www.geneious.com). Initial trees were inferred with the Fast tree, redundant sequences identified and removed from further analysis. Presented trees were constructed using RAxML maximum likelihood GAMMA-based model, although the same conclusions were obtained from trees inferred using Phylm.

Pyrhocoris apterus circadian clock genes were retrieved from in-house transcriptomic and genomic databases, verified, and completed by Sanger sequencing and PCR using primers specific to each gene. The gene models of per, tim-d, and cry-m were reconstructed in P. apterus from draft genome assemblies based on Oxford Nanopore sequencing of genomic DNA. All newly generated sequences were uploaded to GenBank (see supplementary table 3, Supplementary Material online).

Gene Knockdowns and Gene Editing
Systemic RNAi is a well-established reverse genetic tool in P. apterus (Bajgar et al. 2013; Kotwica-Rolinska et al. 2017). Briefly, two non-overlapping cDNA fragments for each gene were designed to reduce RNAi off-targeting (see supplementary table 4, Supplementary Material online for primer’s sequences), products were cloned into plasmids from which the insert was amplified with primers introducing T7 promoter to both ends of the product (Smykal et al. 2014). Gene-specific dsRNAs were synthesized in vitro, purified by ethanol precipitation, and 8 μg injected into 2-day-old adult males. For details of gene editing in P. apterus see the Supplementary material and (Kotwica-Rolinska et al. 2019). Importantly, all mutant lines were outcrossed to the wt strain for seven to nine generations to remove possible off-target modifications.

Animal Rearing Conditions and Locomotor Activity Recordings
The majority of experiments were performed in Roana strain, whereas Oldrichovec strain was only used for cry-m gene editing. See Pivarciova et al. (2016) for details of the strain’s origin. In all experiments, adult males were used for locomotor activity analysis. Bugs were individually housed in test tubes (2.5 cm diameter, 15 cm in length) supplemented with dry linden seeds and water which the bugs were allowed to consume.
to consume ad libitum and placed in the Locomotor Activity Monitors (LAM 25, TriKinetics Inc, Waltham, MA, USA). Bugs were synchronized for 5 days in LD conditions (18 h light, 6 h darkness) at 25 °C, followed by at least 10 days in constant darkness at 25 °C. To determine rhythmicity and τ in the constant darkness, the Lomb–Scargle periodogram in Actogram plugin of ImageJ (Schmid et al. 2011) was employed. See Pivarciova et al. (2016) and Supplementary material for details.

**Supplementary Material**

Supplementary data are available at *Molecular Biology and Evolution* online.

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**Author Contributions**

Conceptualization: J.K.-R. and D.D.; investigation: J.K.-R., L.C., V.S., M.D., D.C., D.D., J.P., M.H., and B.C.-H.W.; formal analysis: J.K.-R., L.C., V.S., and D.D.; visualization: V.S. and D.D.; supervision: D.D.; and writing: D.D. with input from all coauthors. Authors declare that they have no competing interests.

**Data Availability**

cDNA and genome contigs connected to the project are deposited in the GenBank (see supplementary table 3, Supplementary Material online). All data are incorporated into the article and its online Supplementary material.

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