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Supplemental Data

Natural Variation in the Splice Site Strength of a Clock Gene and Species-Specific Thermal Adaptation

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Expanded Experimental Procedures

Fly strains and general handling

The wildtype *D. melanogaster* data shown in this manuscript was obtained with the laboratory strain, Canton S. Similar results were observed with other standard strains of *D. melanogaster* (e.g., Oregon R, *y w*; data not shown). We show results from two different *D. yakuba* strains: In figure 1 we used descendents of a strain originally captured in 1955 in the Ivory Coast (Burla strain) and obtained from the Tucson *Drosophila* Stock Center (stock number; 14021-0261.00). In figure 8, we used the Tai18E2 strain, a gift from Dr. Coyne, University of Chicago. This line is derived from an isofemale line termed Tai18 collected in 1981 in the Tai rainforest on the border between Liberia and Ivory Coast (described in, (Coyne et al., 2004)) and subsequently laboratory inbreeding led to the subline Tai18E2. Similar results were obtained using other *D. yakuba* isofemale lines that we received from Dr. Coyne (e.g., Tai30, SJ2, D. yakuba 2 and D. yakuba 45; data not shown). *D. santomea* (isofemale line ST0.4) was a gift from Dr. Coyne and originally collected in 1998 by Lachaise and co-workers (Cariou et al., 2001; Lachaise et al., 2000), whereas *D. simulans* (sim4 strain; originally captured in New Caledonia, Scotland) was obtained from the Tucson *Drosophila* Stock Center (stock number; 14021-0251.216). The

generation of transgenic flies is described below. All flies were routinely reared at room temperature (22-25^{o}C) and maintained in vials or bottles containing standard agar-cornmeal-sugar-yeast-Tegosept-media.

Tissue culture constructs

We used the pUChsNeoAct5C vector (kindly provided by Dr. K. Irvine, Rutgers University, USA) as the backbone for generating constructs that express the *luciferase* (*luc*) open reading frame (ORF) fused to the dm*per* 3' UTR and flanking 3' genomic sequences. PCR was used in the presence of a previously described CaSpeR-4 based transformation vector containing a 13.2kb genomic dm*per* insert (termed CaSpeR13.2) (Cheng et al., 1998; Lee et al., 1998) to amplify dm*per* sequences from the stop codon to 90bp after the presumed poly(A) cleavage site (nucleotides 6869 to 7465, numbering according to (Citri et al., 1987). In addition, during the PCR we introduced a StuI restriction site just upstream of the dm*per* stop codon and a SalI site immediately after position 7465. This dm*per*-containing fragment was digested with StuI and SalI, then purified. In a second PCR we used the pGL3 plasmid (Promega, USA) as a template to amplify the *luc* ORF and introduce an EcoRI site just upstream of the start codon and a StuI site immediately before the *luc* stop codon. This *luc*-containing fragment was digested with EcoRI and StuI, then purified. Subsequently, a three-way ligation was performed with the two purified fragments and the backbone of pUChsNeoAct5C after digestion with EcoRI and SalI, resulting in a *luc*-dm*per* hybrid gene downstream of the pAct5C promoter (termed 8:8; Fig. 3). Finally, we used standard PCR-based techniques to introduce an XhoI site 9bp upstream of the dmpi8 5'ss, and a KpnI site 10bp downstream of the 3'ss, yielding 8:8kx. We also generated a derivative of this plasmid by performing the same general procedure but further introducing BamHI sites immediately 3'

to the XhoI site and 5' to the KpnI site. Digestion with BamHI followed by ligation generated a construct that still retains the XhoI and KpnI sites but now linked via a BamHI site eliminating the dmpi8 intron to yield Δ8:8kx. To simplify the swapping of intronic sequences we digested the Δ8:8kx plasmid with EcoRI and SalI and subcloned the released *luc-per* fragment into the smaller pGEMT-Easy vector (Promega, USA), resulting in the intermediate vector termed Luc-Δ8-TA. Intron-containing sequences were first subcloned into Luc-Δ8-TA at the XhoI and KpnI sites. Subsequently, the plasmid was digested with StuI and SalI and the released fragment subcloned into either the 8:8kx or Δ8:8kx backbones digested with the same restriction enzymes.

For the dyp3' plasmid (Figs. 3 and 4), the dyp3' intron with 9bp of 5' and 10bp of 3' flanking sequences were amplified with XhoI and KpnI sites using PCR and *D. yakuba* genomic DNA (using the Ivory Coast strain used in this study) as template, and subcloned into the Luc-Δ8-TA backbone followed by the steps described above. A similar strategy was used to generate the 3:3 plasmid (Fig. 4) using PCR in the presence of CaSpeR13.2 to amplify intron 3 from dm*per*. Oligonucleotides with overhanging XhoI and KpnI sites were used to generate the 8:3 and 3:8 constructs (Fig. 4), which are hybrids between dmpi8 and intron 3 of dm*per* fused at the putative branchpoint (both have the same sequence, CTAAC). We used PCR to generate the hybrids between the dmpi8 and dyp3' introns [i.e., dyp3':8, 8:dyp3' and 8:dyp3'(3'ss)] (Fig. 4). Finally, mutants of dmpi8 with altered 5' and 3'ss (i.e., M1, M2, M3, M2M1 and M3M1; Fig. 3) were generated using the Quick Change site-directed mutagenesis kit (Stratagene, CA, USA) and the 8:8kx vector as template. All final constructs used in this study (i.e., Figs. 3 and 4) were validated by DNA sequencing prior to further use.

Constructs for transgenic flies

We first generated a construct that contains a hybrid between dm*per* cDNA and genomic sequences with a StuI site just 5' upstream of the dm*per* stop codon (termed 8:8-CRS/hs/cper). This was generated by amplifying genomic dm*per* sequences from positions 5903 (137bp upstream of the SfiI site in exon 5 of dm*per*) to 7529 (225bp downstream of the Bsu36I site in the 3' UTR of 13.2 dm*per* genomic sequence) using CaSpeR13.2 (referred to as per^{G} in (Cheng et al., 1998) as a template and introducing an AatII and SfiI sites at the 5' and 3' ends of the fragment, respectively. The amplified fragment was digested with AatII and EcoRI and subcloned in the shuttle vector, pSP72 (Promega, USA) to yield pSP72-per13.2-3'end. We then used the Quick Change site-directed mutagenesis kit (Stratagene, CA, USA) to introduce a StuI site immediately upstream of the stop codon (pSP72-per13.2-3'endStuI). Subsequently, the StuI-to-Bsu36I fragment spanning from the stop codon to 234bp downstream of the dmpi8 3'ss was replaced with variants from the tissue culture constructs (8:8kx, dyp3', M2M1). Finally, the resulting constructs were digested with SfiI and Bsu36I and subcloned into the previously described CRS/hs/cper transformation vector (Hao et al., 1999) to yield 8:8-CRS/hs/cper, dyp3'-CRS/hs/cper and M2M1-CRS/hs/cper. Transgenic flies were generated by Genetic Services, Inc. (Sudbury, MA, USA) in a w^{1118} background and subsequently crossed into a *w* per^{01} background with a double balancer line (*w* per^{01};*Sco*/*Cyo*;*MKRS*/*TM6B*), resulting in the transgenic lines termed P{dmper/8:8}, P{dmper/dyp3'} and P{dmper/M2M1}. At least three independent lines for each construct were obtained. The results shown in this manuscript were derived by pooling data from the following lines: P{dmper/8:8}, f9, f19, f46; P{dmper/dyp3'}, f6, f14, f22; P{dmper/M2M1}, f13, m17, m32.

Locomotor activity

Locomotor activity was continuously monitored and recorded in 15-min bins by placing individual adult male flies (three to seven day-old males) in glass tubes and using a Trikinetics (Waltham, MA, USA) system, as previously described (Rosato and Kyriacou, 2006). Briefly, throughout the testing period flies were maintained at the indicated temperature (18^{o}, 25^{o} or 29^{o}C) and subjected to 5 days at the indicated photoperiod [LD; where zeitgeber time 0 (ZT0) is defined as lights-on], and in some cases followed by 5-7 days of constant dark conditions. Cool white fluorescent light (~2000 lux) was used during LD and the temperature did not vary by more than 0.5^{o}C between the light and dark periods. Data analysis was done on a Macintosh computer with the FaasX software (kindly provided by M. Boudinot and F. Rouyer, CNRS, France), which is based on the Brandeis Rhythm Package (originally developed in the laboratories of J. Hall and M. Rosbash, Brandies University, MA, USA). The histograms (eductions) showing the distribution of locomotor activity through a 24 hr period (e.g., see Fig. 1) were obtained using the 'eduction' option of the FaasX software. The last 3 days worth of LD data were averaged for each fly, and data pooled to generate the group averages shown in 15-min or 30-min bins, as indicated in the figure legends. This included multiple independent experiments and for the transgenics, pooling results from at least two independent lines for each genotype. A correction applied to neutralize "startle response" (i.e., increased bout of fly activity following the light-to-dark and dark-to-light environmental transitions; essentially the activity counts in the bin right after the environmental transition is replaced by an average of the activity counts in the bins just before and after) (Wheeler et al., 1993). In figure 5, daily locomotor activity profiles were normalized such that the peak of evening activity was set to 1, facilitating visual comparison of the different transgenic genotypes.

Free-running periods and power (amplitude or strength of the rhythm) were obtained using the *Chi*-square periodogram module available within the FaasX program using activity data collected in 30 min bins during at least 5 consecutive days in DD. Flies with power ≥10, width ≥2, and periods between 20-30 hr were designated rhythmic. Values for individual flies were pooled to obtain an average value for each genotype. The timing of morning and evening peaks, 50% morning offset and 50% evening onset were determined on a Unix command line version of the Brandeis Rhythm Package (BRP) Phase module. The values were based on pooling data from multiple individual flies over the last three days of LD using data collected in 30 min bins. ANOVA and appropriate post-hoc analysis were performed using SPSS 16.0 (SPSS Inc., Chicago, USA). Similar results were obtained when we varied the onset and offset phase reference points from 25 to 75% of peak values (data not shown), and results with 50% are shown as they were the most reproducible.

Tissue culture transfection and collection

The S2 cells and DES expression medium were purchased from Invitrogen and all procedures were performed according to manufacturer's instructions. To generate stable transformants, the Calcium Phosphate Transfection Kit (Invitrogen, USA) was used according to the manufacturer's instructions. Transient transfections were performed using Effectene (Qiagen, USA) according to manufacturer's instruction. Briefly, 0.5 mg of plasmid were mixed with 4 ml of Enhancer and 5 ml of Effectene and incubated with 3.0×10^6 cells for 12 to 16 hr. Subsequently, cells were transferred to the indicated temperatures for overnight incubation before collection. During collection, cells were resuspended and washed twice with PBS on ice. Cell pellets were subjected to RNA

extraction and further analysis as described below. The results shown in figures 3 and 4 were based on pooling data from at least two independent stable transformants for the stable cell lines and at least three independent experiments for the data obtained using transient transfections.

Splicing assay

For RNA analysis in flies, vials containing ~100 young (2- to 6-day-old) adult flies were placed in controlled environmental chambers (Percival, USA) at the indicated temperature and exposed to at least five 24-h photoperiods of alternating LD cycles as described above for recording locomotor activity. At selected times during LD, flies were collected by freezing and heads isolated.

Total RNA was extracted and the relative levels of dmpi8 spliced and unspliced *per* RNA variants in fly heads and S2 cells were measured using a semi-quantitative reverse transcriptase-PCR (RT-PCR) assay as previously described (Majercak et al., 2004; Majercak et al., 1999). Briefly, RNA was collected from isolated fly heads and S2 cells using Tri-reagent (Sigma). Approximately 2μg of total RNA was reversed transcribed using oligo$(dT)_{20}$ and Thermoscript RT enzyme (Invitrogen) in a 20μl reaction. Gene specific primers flanking the 3' UTR intron of the different dm*per* variants were used to amplify both the spliced and unspliced forms in a 50μl reaction using 2μl of RT product as template. The following primers were used to amplify the target regions: for *D. melanogaster* (Canton S) flies and S2 cells, sense primer P6869 (5' TAGTAGCCACACCCGCAGT 3') and antisense primer P7197 (5' TCTACATTATCCTCGGCTTGC 3'), as previously described (Majercak et al., 2004); for *D. simulans*, sense primer P6890 (5' CTGCTGACCGACGTACACAAC 3') and antisense primer P7184 (5' GGCTTGAGATCTACATTATCCTC 3'); for *D. yakuba* and

D. santomea, sense primer yakF1 (5’ AGCACGGCGATGGGTAGTAG 3’) and antisense primer yakR1 (5’ CCTTAGGGCTGAGCCACTCTAG 3’); for transgenic flies, we used sense primers P6851 (5’ ACACAGCACGGGGATGGGTAGT 3’) and P6851-StuI (5’ ACACAGCACGGGGATGGGAGGC 3’) to differentiate between the endogenous per^{01} mRNA transcripts and the dm*per* transgene derived RNA, respectively. The latter primer will only amplify transgenic dm*per* RNA that contains the engineered StuI site upstream of the stop codon. All RT-PCRs included gene specific primers targeting the non-cycling Cap Binding Protein 20 (CBP20) gene as an internal control (Majercak et al., 2004). Species-specific primer sets were used to amplify CBP20 from *D. melanogaster, D. simulans*, *D. yakuba* (for both Tai18E2 and Ivory Coast, Burla strain) and *D. santomea*, as follows: for *D. melanogaster* (Canton S) flies and S2 cells, sense primer CBP540F (5’ GTCTGATTCGTGTGGACTGG 3’) and antisense primer CBP673R (5’ CAACAGTTTGCCATAACCCC 3’); for *D. simulans* (sim4), sense primer CBP540F (5’ GTCTGATTCGTGTGGACTGG 3’) and antisense primer CBP500R (5’ TGTGACAACAGTTTGCCATAACC 3’); for *D. yakuba* and *D. santomea*, yakCBP2066 (5’ ACTGATTCGCGTGGACTGG 3’) and yakCBP2207 (5’ CTTCTGCGACAACAGTTTGC 3’). PCR products were separated and visualized by electrophoresis on 2% agarose gels containing Gelstar (Cambrex Co., USA), and the bands were quantified using a Typhoon 9400 Imager. The values of *per*-containing amplified products were normalized relative to CBP20 and expressed as either total RNA or the proportion with the 3’-terminal intron removed. Total RNA was calculated by adding the values for the two RT-PCR products; i.e., with and without the dmpi8 intron. We routinely collected samples after different cycle lengths to ensure that the PCR products were in the linear range.

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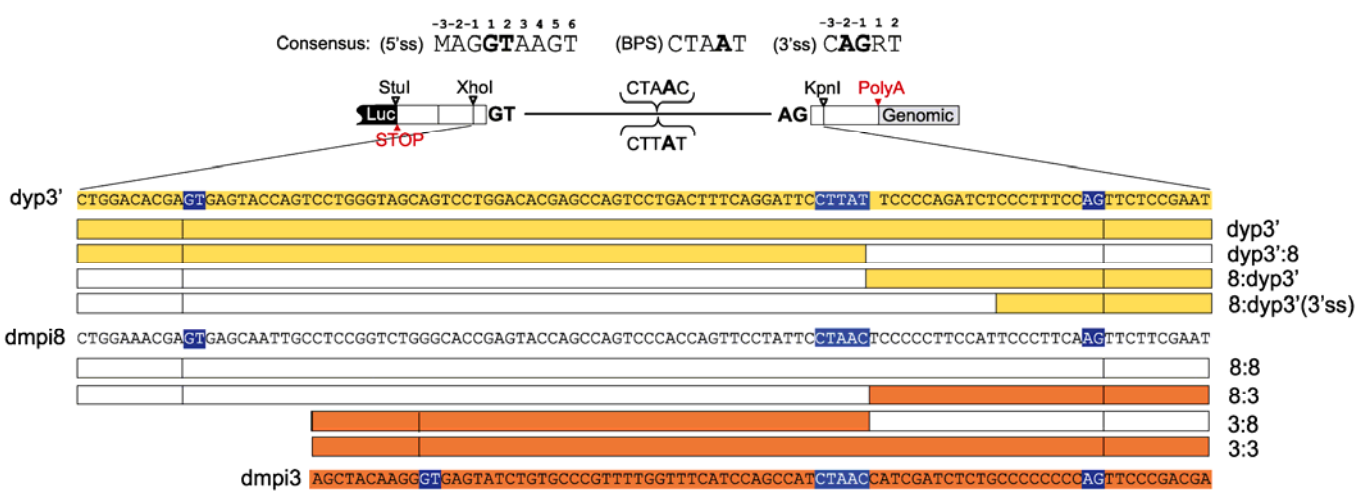
Supplemental figure.

Figure S1. Sequences and splice site strengths of *per* 3'-terminal introns and hybrids used in this study.

(A) Shown at top are the *Drosophila* consensus sequences for the 5'ss and 3'ss, as explained in figure 3. The schematic of the *luc*-dm*per* construct is identical to that shown in figure 3, except that here we also indicate the two branch point sites found in the different introns (CTA**A**C or CTT**A**T; where the presumptive branch point A is indicated in bold) used to generate the hybrid introns used in this study and indicated below as follows: Shown are the sequences of the different introns including 9bp and 10bp of flanking 5' and 3', respectively; yellow, dyp3'; gray, dmpi8; red, dm*per* intron 3. High-lighted in blue are the 5' and 3'ss and the presumptive branch point region for each construct. **(B)** Intronic and flanking sequences of the *per* 3'-terminal introns from *D. simulans* (sim4), *D. santomea* (ST0.4) and *D. yakuba* (Tai18E2) used in this study. **(C)** Predicted 5' and 3'ss strengths.

A



B

dmpi8	CTGGAAACGAGTGAGCAATTGCCTCCGGTCTGGGCACCGAGCACCAGCCAGTCCCACCAGTTCCCCCTATTCCTAACTCCCCCTTCCATTCCCTTCAAGTTCTTCGAATCAACGCGAAC
D. simulans (sim4)	CTGGAAACGAGTGAGCAGTTGCCTCCGGTCTGG---TCGAGCACCAGCCAGTCCCACCAGTTCCCCCCATTCCTAACTCCCCCTTCCATTCCCTTCAAGTTCTCCGAATCAACGCAAAC
D. yakuba (Tai18E2)	CTGGACACGAGTGAGTACCAGTCCTGGGTAGCAGTCCTGGACACGAGCCAGTCCTGACTTTCAGGATTCCTTATTCCCCAGATCTCCCTTTCCAGTTCTCCGAATCAGCACGAAC
D. santomea (ST0.4)	CTGGACACGAGTGAGTATCAGTCCTGG----------ACACGAGCCAGTCCTGACTTCCAGGATTCCTTATTCCCCAGATCTCCCTTTCCAGTTCTCCGAATCAGCACGAAC

C

Genotype	Splice site prediction score	
	5'ss	3'ss
dmpi8	0.45	0.21
D. simulans (sim4)	0.39	0.44
D. yakuba (Tai18E2)	0.79	0.78
D. santomea (ST0.4)	0.80	0.78

Supplemental tables.

Table S1. Timing of daily activity in *D. melanogaster* and *D. yakuba* at different temperatures and photoperiods

Genotype[a]	Photoperiod[b] (L:D)	Temperature (°C)	n[c]	Morning peak (hr ± sem)[d]	Morning offset[e] (hr ± sem)[d]	Evening peak (hr ± sem)[d]	Evening onset[f] (hr ± sem)[d]	Siesta[g] (hr ± sem)
D. melanogaster	12:12	18	36	0.6 ± 0.1	3.1 ± 0.2	10.0 ± 0.1	7.1 ± 0.2	4.1 ± 0.3
		25	61	0.6 ± 0.1	2.6 ± 0.1	11.0 ± 0.1	9.2 ± 0.3	6.5 ± 0.3
		29	64	22.6 ± 0.1	0.7 ± 0.1	12.1 ± 0.0	10.6 ± 0.1	10.0 ± 0.1
D. yakuba		18	62	1.0 ± 0.3	2.7 ± 0.2	11.1 ± 0.2	9.3 ± 0.2	6.7 ± 0.3
		25	139	0.3 ± 0.1	2.0 ± 0.1	10.9 ± 0.1	9.0 ± 0.1	7.1 ± 0.2
		29	222	0.2 ± 0.1	2.1 ± 0.1	10.5 ± 0.1	8.7 ± 0.1	6.6 ± 0.1
D. melanogaster	9:15	18	35	23.7 ± 0.2	2.5 ± 0.3	7.3 ± 0.1	4.4 ± 0.2	2.3 ± 0.2
		25	59	23.2 ± 0.1	1.0 ± 0.2	8.9 ± 0.1	7.4 ± 0.1	6.4 ± 0.2
		29	62	21.4 ± 0.1	23.3 ± 0.1	10.1 ± 0.0	8.6 ± 0.0	9.2 ± 0.1
D. yakuba		18	25	23.5 ± 0.3	1.3 ± 0.4	8.4 ± 0.1	6.8 ± 0.2	5.7 ± 0.4
		25	36	23.5 ± 0.2	1.2 ± 0.3	8.6 ± 0.2	6.8 ± 0.3	5.7 ± 0.4
		29	43	23.0 ± 0.1	0.9 ± 0.2	8.6 ± 0.3	6.8 ± 0.2	6.0 ± 0.3

[a]Young male flies were maintained at the indicated temperature and photoperiod for five days. The last three days worth of activity data was pooled for each individual fly and then a group average was determined. *D. melanogaster* is Canton S and *D. yakuba* is Ivory Coast, Burla strain.
[b]The light/dark cycles were either 12hr light followed by 12 hr dark (12:12) or 9 hr light followed by 15 hr dark (9:15).
[c]n, number of flies that gave significant values for both morning peak, morning offset, evening peak, and evening onset and that survived throughout the entire testing period.
[d]Values denote zeitgeber time, with ZT0 defined as the start of lights-on.
[e]Morning offset is defined as the time when 50% of peak morning activity was attained following the morning peak of activity.
[f]Evening onset is defined as the time when 50% of peak evening activity was attained prior to the evening peak of activity.
[g]Siesta time is defined as the length of time between 50% of morning offset and 50% of evening onset.

Table S2. P-values for ANOVA analysis of results shown in Table S1

Comparison	Effect	Siesta[a]			P-value[c]			
		Statistic[b]		P-value[c]	Morning peak	Morning offset[a]	Evening peak	Evening onset[a]
D. melanogaster and D. yakuba; 18, 25 and 29ºC; LD of 9:15, 12:12	Temperature	$F_{2,568}$	= 169.9	0.0000	0.0000	0.0000	0.0000	0.0000
	Photoperiod	$F_{1,568}$	= 38.1	0.0000	0.0000	0.0000	0.0000	0.0000
	Genotype x Temperature	$F_{2,568}$	= 170.0	0.0000	0.0000	0.0000	0.0000	0.0000
	Genotype x Photoperiod	$F_{1,568}$	= 2.1	0.1451	0.0060	0.0060	0.3110	0.3184
D. melanogaster; 18, 25 and 29ºC; LD of 9:15, 12:12	Temperature	$F_{2,234}$	= 741.20	0.0000	0.0000	0.0000	0.0000	0.0000
	Photoperiod	$F_{1,234}$	= 24.70	0.0000	0.0000	0.0000	0.0000	0.0000
D. yakuba; 18, 25 and 29ºC; LD of 9:15, 12:12	Temperature	$F_{2,234}$	= 0.25	0.7811	0.0260	0.1160	0.2470	0.1327
	Photoperiod	$F_{1,234}$	= 14.98	0.0001	0.0000	0.0010	0.0000	0.0000

[a]Morning offset, evening onset and siesta are as defined in Table S1.
[b]F ratio from ANOVA analysis with degree of freedom shown in subscript.
[c]P-values of significance test from ANOVA analysis.

Table S3. Post-hoc Tukey HSD multiple comparisons test for data shown in Table S1[a]

Genotype	Comparison	P-value[c]				
		Siesta[b]	Morning peak	Morning offset[b]	Evening peak	Evening onset[b]
D. melanogaster	18 °C VS. 25 °C	0.0000	0.0000	0.0000	0.0000	0.0000
	18 °C VS. 29 °C	0.0000	0.0000	0.0000	0.0000	0.0000
	25 °C VS. 29 °C	0.0000	0.0000	0.0000	0.0000	0.0000
D. yakuba	18 °C VS. 25 °C	0.8351	0.0770	0.1670	0.9970	0.5946
	18 °C VS. 29 °C	0.9972	0.0120	0.0740	0.2330	0.0556
	25 °C VS. 29 °C	0.7475	0.7840	0.9420	0.2060	0.3331

[a]Multiple comparisons test was done separately within each genotype with data from different temperatures and photoperiods.
[b]Morning offset, evening onset and siesta are as defined in Table S1.
[c]P-values of significance test from Tukey HSD test.

Table S4. Little effect of temperature on period length in *D. melanogaster* and *D. yakuba* flies

Genotype[a]	Temperature (°C)	n	Rhythmicity[b] (%)	Period (hr ± sem)	Power[c] (± sem)
D. melanogaster	18	32	84	23.7 ± 0.1	92.6 ± 6.9
	25	32	100	23.9 ± 0.1	91.1 ± 5.5
	29	32	91	23.7 ± 0.1	93.1 ± 6.6
D. yakuba	18	23	87	24.2 ± 0.2	46.0 ± 5.0
	25	31	81	24.0 ± 0.2	52.1 ± 6.0
	29	20	45	24.2 ± 0.5	17.1 ± 3.5

[a]Young male flies were entrained for five 12:12LD cycles followed by five days in constant dark conditions at the indicated temperature. The results are a subset of the same experiments used to calculate the data shown in Table S1.

[b]Flies with a power value of greater than 10 and period $\geq$ 20 and $\leq$ 30, were defined as rhythmic.

[c]Power is a measure of the strength or amplitude of the rhythm.

Table S5. P-values for ANOVA analysis of results shown in Table S4

Comparison	Effect	Statistic[a]	P-value[b]
D. melanogaster and D. yakuba; 18, 25 and 29ºC	Temperature	$F_{2,141} = 0.007$	0.9930
	Genotype	$F_{1,141} = 6.7$	0.0106
	Genotype x Temperature	$F_{2,141} = 1.1$	0.3477

[a]F ratio from ANOVA analysis with degree of freedom shown in subscript.

[b]P-values of significance test from ANOVA analysis.

Table S6. Timing of daily activity in P{dmper/8:8}, P{dmper/dyp3'} and P{dmper/M2M1} transgenic flies at different temperatures and photoperiods

Genotype[a]	Photoperiod (L:D)	Temperature (°C)	n[b]	Morning peak (hr ± sem)[c]	Morning offset[d] (hr ± sem)[c]	Evening peak (hr ± sem)[c]	Evening onset[e] (hr ± sem)[c]	Siesta[f] (hr ± sem)
P{dmper/8:8}	11:13	18	51	0.2 ± 0.1	1.3 ± 0.1	9.9 ± 0.1	6.9 ± 0.2	5.6 ± 0.2
P{dmper/dyp3'}			39	23.9 ± 0.1	1.0 ± 0.3	9.3 ± 0.1	5.8 ± 0.2	4.8 ± 0.3
P{dmper/M2M1}			65	0.2 ± 0.1	1.6 ± 0.1	9.4 ± 0.1	6.3 ± 0.1	4.7 ± 0.2
P{dmper/8:8}	11:13	25	76	22.9 ± 0.1	1.1 ± 0.1	10.1 ± 0.1	8.2 ± 0.1	7.1 ± 0.1
P{dmper/dyp3'}			62	22.6 ± 0.2	1.6 ± 0.1	9.2 ± 0.1	6.4 ± 0.1	4.8 ± 0.2
P{dmper/M2M1}			91	23.5 ± 0.1	2.3 ± 0.2	9.4 ± 0.1	6.4 ± 0.2	4.1 ± 0.3
P{dmper/8:8}	11:13	29	79	22.3 ± 0.1	0.5 ± 0.1	10.1 ± 0.1	8.3 ± 0.2	7.8 ± 0.2
P{dmper/dyp3'}			91	21.6 ± 0.1	0.3 ± 0.1	9.9 ± 0.1	7.8 ± 0.1	7.5 ± 0.2
P{dmper/M2M1}			127	22.6 ± 0.2	2.1 ± 0.2	10.4 ± 0.1	7.7 ± 0.1	5.6 ± 0.2
P{dmper/8:8}	12:12	18	15	23.1 ± 0.4	1.7 ± 0.4	11.3 ± 0.1	9.3 ± 0.2	7.5 ± 0.4
P{dmper/dyp3'}			31	23.1 ± 0.3	1.7 ± 0.2	10.7 ± 0.2	7.5 ± 0.2	5.7 ± 0.3
P{dmper/M2M1}			45	0.6 ± 0.2	2.8 ± 0.3	10.8 ± 0.1	8.0 ± 0.1	5.3 ± 0.3
P{dmper/8:8}	12:12	25	77	22.7 ± 0.1	1.0 ± 0.1	11.2 ± 0.1	9.3 ± 0.1	8.3 ± 0.2
P{dmper/dyp3'}			62	22.8 ± 0.1	1.5 ± 0.1	10.4 ± 0.0	7.7 ± 0.1	6.2 ± 0.2
P{dmper/M2M1}			94	23..9 ± 0.1	2.4 ± 0.1	10.5 ± 0.1	8.0 ± 0.1	5.6 ± 0.2
P{dmper/8:8}	12:12	29	16	22.1 ± 0.2	0.7 ± 0.3	11.4 ± 0.1	9.9 ± 0.1	9.2 ± 0.3
P{dmper/dyp3'}			32	21.9 ± 0.2	0.3 ± 0.2	10.8 ± 0.1	9.1 ± 0.1	8.8 ± 0.3
P{dmper/M2M1}			46	23.0 ± 0.2	2.0 ± 0.2	11.6 ± 0.2	9.8 ± 0.2	7.8 ± 0.3
P{dmper/8:8}	13:11	18	44	0.6 ± 0.2	2.0 ± 0.2	11.4 ± 0.1	8.4 ± 0.1	6.4 ± 0.2
P{dmper/dyp3'}			25	0.8 ± 0.3	2.9 ± 0.3	11.3 ± 0.1	7.6 ± 0.3	4.6 ± 0.3
P{dmper/M2M1}			44	2.1 ± 0.2	4.2 ± 0.3	10.7 ± 0.1	8.0 ± 0.2	3.9 ± 0.3
P{dmper/8:8}	14:10	25	63	23.6 ± 0.2	2.0 ± 0.2	12.8 ± 0.0	10.9 ± 0.1	8.9 ± 0.2
P{dmper/dyp3'}			126	0.2 ± 0.1	2.4 ± 0.1	12.4 ± 0.0	10.0 ± 0.1	7.6 ± 0.1
P{dmper/M2M1}			91	0.1 ± 0.1	2.5 ± 0.1	12.7 ± 0.1	10.4 ± 0.1	8.0 ± 0.2
P{dmper/8:8}	13:11	29	186	23.2 ± 0.1	1.1 ± 0.1	11.9 ± 0.0	10.0 ± 0.1	8.8 ± 0.1
P{dmper/dyp3'}			152	22.7 ± 0.1	0.9 ± 0.1	11.6 ± 0.1	9.6 ± 0.1	8.6 ± 0.1
P{dmper/M2M1}			148	23.1 ± 0.1	2.0 ± 0.1	11.8 ± 0.1	9.6 ± 0.1	7.5 ± 0.2

[a]Young male flies were maintained at the indicated temperature and photoperiod for five days. The last three days worth of activity data was averaged for each individual fly and then a group average was determined. For each genotype, data from at least three independent lines was pooled.
[b]n, number of flies that gave significant values for both morning peak, morning offset, evening peak, and evening onset and that survived throughout the entire testing period.
[c]Values denote zeitgeber time, with ZT0 defined as the start of lights-on.
[d]Morning offset is defined as the time when 50% of peak morning activity was attained following the morning peak of activity.
[e]Evening onset is defined as the time when 50% of peak evening activity was attained prior to the evening peak of activity.
[f]Siesta time is defined as the length of time between 50% of morning offset and 50% of evening onset.

Table S7. P-values for ANOVA analysis of results shown in Table S6

Comparison[a]	Effect	Siesta[b]		P-value[d]				
		Statistic[c]			Morning peak	Morning offset[b]	Evening peak	Evening onset[b]
P{dmper/8:8}, P{dmper/dyp3'} and P{dmper/M2M1}; 18, 25 and 29ºC; LD of 11:13, 12:12, 13:11	Genotype	$F_{2,2522}$	= 136.3	0.0000	0.0000	0.0000	0.0000	0.0000
	Photoperiod	$F_{2,2522}$	= 99.2	0.0000	0.0000	0.0000	0.0000	0.0000
	Temperature	$F_{2,2522}$	= 200.4	0.0000	0.0000	0.0000	0.0000	0.0000
	Photoperiod x Genotype	$F_{4,2522}$	= 1.8	0.1200	0.0250	0.1350	0.0440	0.0020
	Temperature x Genotype	$F_{4,2522}$	= 12.0	0.0000	0.0000	0.0000	0.0000	0.0000
	Photoperiod x Temperature	$F_{4,2522}$	= 35.7	0.0000	0.0000	0.0000	0.0000	0.0000
	Temperature x Genotype x Photoperiod	$F_{8,2522}$	= 6.4	0.0000	0.0000	0.0000	0.0000	0.0000
P{dmper/8:8}, P{dmper/dyp3'} and P{dmper/M2M1}								
Photoperiod (L:D)	Temperature (ºC)							
11:13	18	Genotype	$F_{2,154}$	= 4.7	0.0110	0.1530	0.0170	0.0000	0.0000
11:13	25	Genotype	$F_{2,228}$	= 56.9	0.0000	0.0000	0.0000	0.0000
11:13	29	Genotype	$F_{2,296}$	= 36	0.0000	0.0000	0.0010	0.0100
12:12	18	Genotype	$F_{2,90}$	= 8.4	0.0000	0.0000	0.0080	0.0320	0.0000
12:12	25	Genotype	$F_{2,545}$	= 26.6	0.0000	0.0010	0.0000	0.0000	0.0000
12:12	29	Genotype	$F_{2,325}$	= 5.9	0.0030	0.0050	0.0000	0.0000	0.0000
13:11	18	Genotype	$F_{2,112}$	= 30.7	0.0000	0.0000	0.0000	0.0000	0.0300
14:10[e]	25	Genotype	$F_{2,279}$	= 19.6	0.0000	0.0020	0.0120	0.0000	0.0000
13:11	29	Genotype	$F_{2,485}$	= 27.5	0.0000	0.0010	0.0000	0.0000	0.0000

[a]ANOVA analysis was done with dataset specified.
[b]Morning offset, evening onset and siesta are as defined in Table S6.
[c]F ratio from ANOVA analysis with degree of freedom shown in subscript.

[d]P-values of significance test from ANOVA analysis.
[e]For simplicity, L:D of 14:10 was treated as 13:11 in the statistical analysis.

Table S8. Post-hoc Tukey HSD multiple comparisons test for data shown in Table S6[a]

Photoperiod (L:D)	Temperature (ºC)	Comparison		P-value[c]				
		Genotype 1	Genotype 2	Siesta[b]	Morning peak	Morning offset[b]	Evening peak	Evening onset[b]
11:13	18	P{dmper/8:8}	P{dmper/dyp3'}	0.0810	0.1490	0.2720	0.0000	0.0000
	18	P{dmper/8:8}	P{dmper/M2M1}	0.0110	0.8850	0.3570	0.0000	0.0300
	18	P{dmper/dyp3'}	P{dmper/M2M1}	0.8960	0.2770	0.0120	0.9970	0.1350
	25	P{dmper/8:8}	P{dmper/dyp3'}	0.0000	0.3420	0.0150	0.0000	0.0000
	25	P{dmper/8:8}	P{dmper/M2M1}	0.0000	0.0020	0.0000	0.0000	0.0000
	25	P{dmper/dyp3'}	P{dmper/M2M1}	0.0620	0.0000	0.0010	0.1950	0.9990
	29	P{dmper/8:8}	P{dmper/dyp3'}	0.5840	0.0120	0.7910	0.3010	0.0620
	29	P{dmper/8:8}	P{dmper/M2M1}	0.0000	0.2730	0.0000	0.0860	0.0090
	29	P{dmper/dyp3'}	P{dmper/M2M1}	0.0000	0.0000	0.0000	0.0000	0.8320
12:12	18	P{dmper/8:8}	P{dmper/dyp3'}	0.0080	0.9990	0.9990	0.0280	0.0000
	18	P{dmper/8:8}	P{dmper/M2M1}	0.0000	0.0020	0.0760	0.0710	0.0000
	18	P{dmper/dyp3'}	P{dmper/M2M1}	0.5340	0.0000	0.0130	0.7770	0.0160
	25	P{dmper/8:8}	P{dmper/dyp3'}	0.0000	0.5100	0.7540	0.0000	0.0000
	25	P{dmper/8:8}	P{dmper/M2M1}	0.0000	0.0740	0.0000	0.0000	0.0000
	25	P{dmper/dyp3'}	P{dmper/M2M1}	0.1140	0.0010	0.0000	0.2480	0.7010
	29	P{dmper/8:8}	P{dmper/dyp3'}	0.4140	0.1290	0.9040	0.0150	0.0080
	29	P{dmper/8:8}	P{dmper/M2M1}	0.0020	0.5130	0.0000	0.0000	0.1630
	29	P{dmper/dyp3'}	P{dmper/M2M1}	0.1130	0.0030	0.0000	0.0000	0.0000
13:11	18	P{dmper/8:8}	P{dmper/dyp3'}	0.0000	0.7870	0.0440	0.4190	0.0230
	18	P{dmper/8:8}	P{dmper/M2M1}	0.0000	0.0000	0.0000	0.0000	0.3980
	18	P{dmper/dyp3'}	P{dmper/M2M1}	0.1130	0.0020	0.0060	0.0020	0.2610
14:10	25	P{dmper/8:8}	P{dmper/dyp3'}	0.0000	0.0020	0.0260	0.0000	0.0000
	25	P{dmper/8:8}	P{dmper/M2M1}	0.0000	0.0050	0.0160	0.5220	0.0000
	25	P{dmper/dyp3'}	P{dmper/M2M1}	0.1100	0.9980	0.9240	0.0000	0.0000
13:11	29	P{dmper/8:8}	P{dmper/dyp3'}	0.5080	0.0010	0.3670	0.0000	0.0000
	29	P{dmper/8:8}	P{dmper/M2M1}	0.0000	0.8580	0.0000	0.8290	0.0000
	29	P{dmper/dyp3'}	P{dmper/M2M1}	0.0000	0.0080	0.0000	0.0010	0.9890

[a]Multiple comparisons tests were done comparing two genotypes at the indicated temperatures and photoperiods.

[b]Morning offset, evening onset and siesta are as defined in Table S6.
[c]P-values of significance test from Tukey HSD test.

Table S9. Similar period lengths in P{dmper/8:8}, P{dmper/dyp3'} and P{dmper/M2M1} flies

Genotype[a]	n	Rhythmicity[b] (%)	Period (hr ± sem)	Power[c] (± sem)
P{dmper/8:8}	140	89	23.4 ± 0.1	82.6 ± 3.1
P{dmper/dyp3'}	186	97	23.2 ± 0.0	96.5 ± 2.5
P{dmper/M2M1}	226	75	23.5 ± 0.1	62.6 ± 2.8

[a]Young male flies were kept at 25^{o}C for five 12:12LD cycles followed by five days in constant dark conditions. The results are a subset of the same experiments used to calculate the data shown in Table S6.

[b]Flies with a power value of greater than 10 and period $\geq$ 20 and $\leq$ 30, were defined as rhythmic.
[c]Power is a measure of the strength or amplitude of the rhythm.