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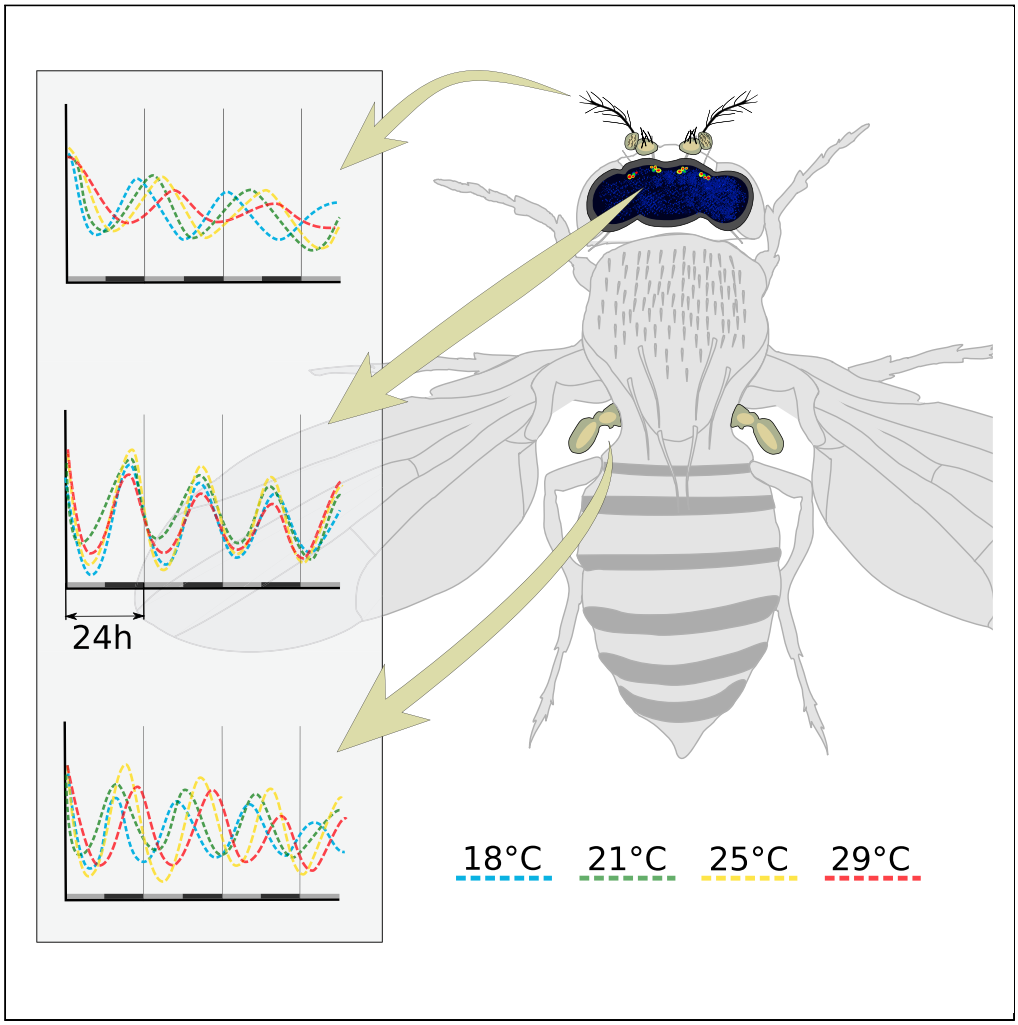

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HIGHLIGHTS

Drosophila halteres contain a robust circadian oscillator

Circadian clocks of halteres and antennae are over-compensated

The *Drosophila* central brain clock is temperature compensated

The neuropeptide PDF is not required for temperature compensation of clock neurons

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A Robust and Self-Sustained Peripheral Circadian Oscillator Reveals Differences in Temperature Compensation Properties with Central Brain Clocks

Marijke Versteven,[1] Karla-Marlen Ernst,[1] and Ralf Stanewsky[1,2,*]

SUMMARY

Circadian clocks are characterized by three properties: they run in constant conditions with a period of ~24 h, synchronize to the environmental cycles of light and temperature, and are temperature compensated, meaning they do not speed up with temperature. Central brain clocks regulate daily activity rhythms, whereas peripheral clocks are dispersed throughout the body of insects and vertebrates. Using a set of luciferase reporter genes, we show that *Drosophila* peripheral clocks are self-sustained but over-compensated, i.e., they slow down with increasing temperature. In contrast, central clock neurons in the fly brain, both in intact flies and in cultured brains, show accurate temperature compensation. Although this suggests that neural network properties contribute to temperature compensation, the circadian neuropeptide Pigment Dispersing Factor (PDF) is not required for temperature-compensated oscillations in brain clock neurons. Our findings reveal a fundamental difference between central and peripheral clocks, which likely also applies for vertebrate clocks.

INTRODUCTION

Circadian clocks temporally organize physiology and behavior of all organisms exposed to the daily changes of light intensities and temperature. There are growing numbers of examples that circadian clock function positively contributes to an organism's fitness and to human health, highlighting the importance of understanding their underlying molecular mechanisms. In order to function as reliable time keepers, one hallmark of circadian clocks is their temperature independence (e.g., Hall, 1997; Pittendrigh, 1954). Particularly in poikilothermic animals, a circadian clock that would change its pace with temperature would be of little use to accurately measure time. As a consequence, circadian clocks are "temperature compensated," meaning they keep ticking with a period close to 24 h over a large variation of ambient temperatures within the organism's physiological range (Hall, 1997). This ability to keep a temperature-compensated 24-h rhythm therefore is also one of the three characteristics that define a circadian rhythm, the other two being the self-sustained nature and the ability to be entrained by environmental and other time cues (Zeitgeber).

In *Drosophila*, the free-running period length (Tau, τ) for both rhythmic eclosion of pharate adults from the pupal case, as well as adult locomotor activity, remains almost exactly 24 h between 16°C and 28°C, revealing a remarkable degree and precision of temperature compensation (Konopka et al., 1989; Pittendrigh, 1954; Singh et al., 2019; Zimmerman et al., 1968). Both eclosion and locomotor activity rhythms are controlled by specialized clock neurons in the pupal or adult brain (Ewer et al., 1992; Myers et al., 2003). These clock neurons are defined through their expression of clock genes like *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), and *cycle* (*cyc*), which are all required to maintain the rhythmic behaviors of the fly (Peschel and Helfrich-Förster, 2011). These clock genes form the molecular bases of the circadian clock by generating self-sustained 24-h oscillations of clock gene transcription and protein abundance. Transcription is activated by binding of the transcription factors CLK and CYC to *per* and *tim* promoter elements. PER and TIM proteins slowly accumulate in the cytoplasm before entering the nucleus, where they shut down their own transcription by binding to CLK and CYC. Only after PER and TIM degradation this 24-h lasting cycle can start again. The period length of this molecular cycle is controlled by the stability and subcellular localization of the PER and TIM repressor proteins, which are both determined by several kinases and phosphatases targeting PER and/or TIM (Tataroglu and Emery, 2015). Considering the molecular and biochemical nature of this feedback loop underlying circadian clocks, their ability to drive temperature-compensated biological rhythms seems surprising. Current models

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predict that counter balancing biochemical reactions, some that speed up and others that slow down with increasing temperature, result in overall temperature-independent circadian oscillations (e.g., Shinohara et al., 2017).

Based on the temperature-independent τ of clock-controlled behaviors, it seems required that the molecular oscillations and activity of the central clock neurons driving these output rhythms must somehow be temperature compensated. This is less clear for peripheral clocks for several reasons. Peripheral clocks are widely distributed within the fly, for example, in eyes, wings, legs, antennae, proboscis, Malpighian tubules, testes, and even the cuticle (Beaver et al., 2002; Chatterjee et al., 2010; Ito et al., 2008; Ivanchenko et al., 2001; Levine et al., 2002; Plautz et al., 1997). Yet, only for the antennal, proboscis, and cuticle peripheral clocks it is known which biological output rhythm they control, but it is not known if these rhythms are temperature compensated (Chatterjee et al., 2010; Ito et al., 2008; Krishnan et al., 1999). In addition, the molecular machinery in peripheral clocks differs from that in central clock neurons. The main difference concerns the function of *cryptochrome* (*cry*), which encodes a blue-light photoreceptor for the synchronization of both central clock neurons and peripheral clocks (Ito et al., 2008; Ivanchenko et al., 2001; Stanewsky et al., 1998). In addition, CRY appears to be required for clock function in most peripheral tissues, for example, in the eye and the antennae (Krishnan et al., 2001; Levine et al., 2002; Stanewsky et al., 1998), presumably by acting as repressor for *per* and *tim* expression, similar to the mammalian CRY proteins (Collins et al., 2006). Although peripheral clocks can be entrained to light:dark (LD) and temperature cycles (Glaser and Stanewsky, 2005; Harper et al., 2017; Ito et al., 2011; Ivanchenko et al., 2001; Levine et al., 2002), it surprisingly is still unclear if they meet the remaining two criteria, which would qualify them as genuine circadian clocks: self-sustained rhythmicity and temperature compensation. It is difficult to determine if peripheral clocks are temperature compensated, because their molecular oscillations dampen out very quickly in constant conditions, mitigating against reliable estimation of τ (e.g., Harper et al., 2017; Kidd et al., 2015; Levine et al., 2002; Veleri et al., 2003). Furthermore, it is not clear if this dampening is due to the lack of a self-sustained molecular oscillator or caused by rapid desynchronization between individual peripheral oscillator cells. To address the properties of peripheral clocks in more detail, we applied an established luciferase reporter system, allowing the measurement of clock gene expression in real time in live tissues (e.g., Glaser and Stanewsky, 2005). We identified a robust, self-sustained 24-h oscillator in the fly's haltere, an organ important for proprioceptive feedback during flight and in some insects also walking (Yarger and Fox, 2016). Surprisingly, the haltere clock is over-compensated against temperature changes, i.e., it slows down with increasing temperature rather than speeding up. We show that the same applies for the antennal peripheral clock, indicating that this a general feature of peripheral fly oscillators. Using the same assay, we show that molecular oscillations in the central brain clock are perfectly temperature compensated, neither slowing down nor speeding up with temperature. Although this difference indicates the importance of neuronal network properties, we show that the neuronal synchronizing peptide Pigment Dispersing Factor (PDF) is not required for accurate temperature compensation.

RESULTS

The *Drosophila* Haltere Contains a Robust and Self-Sustained Peripheral Circadian Oscillator

In contrast to circadian pacemaker neurons in the fly brain, peripheral circadian clocks of *Drosophila* are generally considered as weak or dampened circadian oscillators, owing to their inability of maintaining molecular oscillations in constant conditions (e.g., Levine et al., 2002; Stanewsky et al., 1997; Veleri et al., 2003). We were therefore surprised to observe sustained and non-dampening oscillations during constant conditions in cultured halteres of transgenic *ptim-TIM-luc* flies, a reporter for TIM protein expression (Lamba et al., 2018, Figures 1A, 1B, and S1). After initial synchronization to LD cycles, bioluminescence signals from individual pairs of halteres were measured in constant darkness and temperature (DD, 25°C) with 30 min or 1 h time resolution. In order to determine the period-length of the bioluminescence oscillations, data were detrended and subjected to curve fitting (Figure 1C, Klemz et al., 2017). At 25°C, *ptim-TIM-luc* oscillations have a τ of 24.0 ± 0.1 h demonstrating that they are indeed circadian (Figure 1D, Table 1). In addition, we analyzed *ptim-TIM-luc* expression in halteres of clock-less per^{01} flies, which led to arrhythmic reporter gene expression as expected (Figure S2).

Clock Protein Oscillations in the Haltere Slow Down with Increasing Temperature

We next asked if these oscillations are also temperature compensated, as this feature is a defining criterion for circadian clocks (Pittendrigh, 1954). To our surprise, although robust oscillations were also observed at 18°C, 21°C, and 29°C, τ increased quite dramatically by 4.2 h from the lowest (18°C) to the highest (29°C)

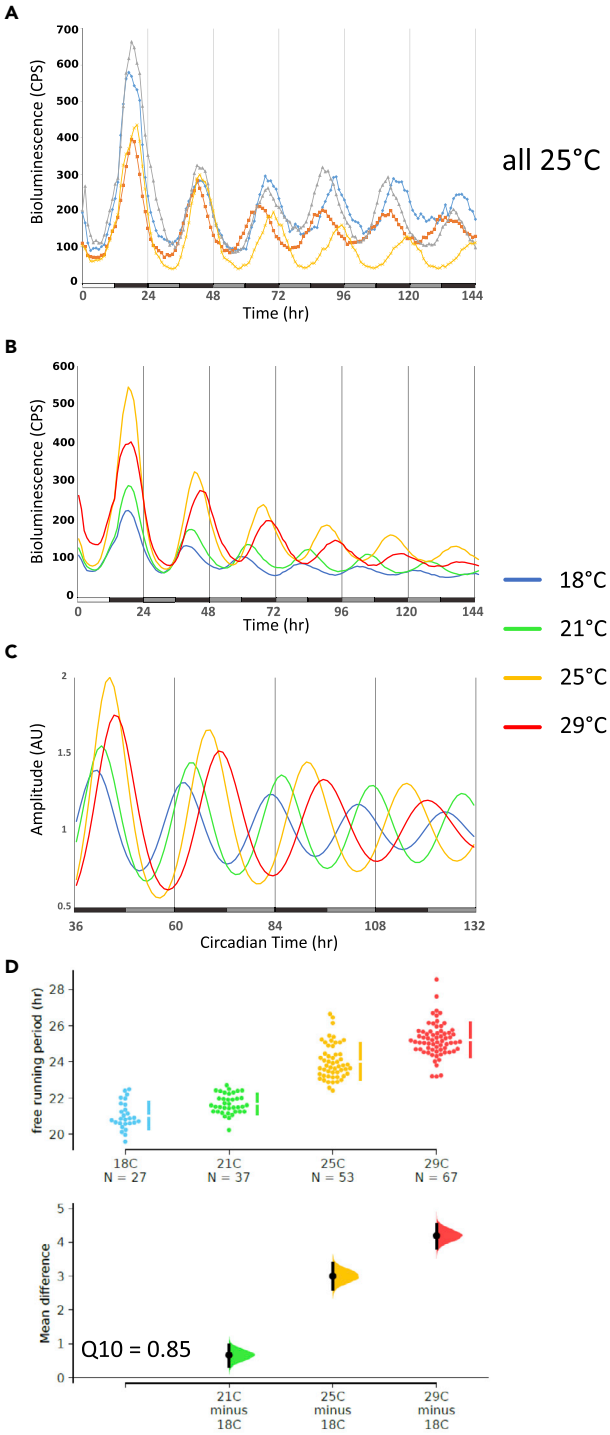

Figure 1. The *Drosophila* Haltere Contains a Non-dampened Circadian Oscillator

Bioluminescence recordings of halteres dissected from *tim-TIM-luc* flies. Tissues were exposed to two LD cycles (data of the first LD cycle were discarded) followed by DD at different constant temperatures.

(A) Raw data traces of four pairs of halteres at 25°C. White portion of x axis bar indicates "lights on," black portion "lights off," and gray portions subjective day (= lights off, but the time lights would have been on if the cultures were still in LD). CPS, counts per second.

(B) Average traces at 18°C (n = 27), 21°C (n = 12), 25°C (n = 53), 29°C (n = 67), same conditions as in (A).

(C) Curve-fitted oscillations after detrending the DD data in (B).

Figure 1. *Continued*

(D) Period estimates for individual haltere pairs obtained from curve-fitted data (see Transparent Methods). To precisely visualize potential period differences between temperatures we applied estimation statistics (ES) rather than significance tests (Ho et al., 2019). Because ES focusses on the magnitude of the effect size (i.e., mean difference) and its precision, rather than acceptance or rejection of the null hypothesis, ES gives a more informative way to analyze and interpret results (Ho et al., 2019). We generated shared-control plots, which are analogous to an ANOVA with multiple comparisons, whereby the 18°C data served as shared-control. The top part shows all the period and expression level data points observed, with the mean and standard error plotted as a discontinuous line to the right (the gap indicates the observed mean). In the bottom part a difference axis displays the effect size (mean difference, the black dots). The colored filled curves indicate the resampled distribution, and the black vertical line indicates the 95% confidence interval (CI). The size of the CI shows the precision of the mean difference: if it crosses the reference line (x axis) both datasets may originate from the same distribution ($p > 0.05$), if not, they are different ($p < 0.05$) (see Transparent Methods and Ho et al., 2019 for more details). See also Table 1 for numeric τ and expression level values.

temperature (Q_{10} = 0.85) (Figures 1B–1D, Table 1). These results indicate that the haltere circadian oscillator is temperature compensated, because it does not speed up with increasing temperature. Nevertheless, this temperature compensation is not normal, since the oscillations slow down with temperature, therefore suggesting an "over-compensation" against temperature changes. In order to investigate if this over-compensation is specific for the TIM protein, we next performed the same experiments with *XLG-luc* transgenic flies, expressing a reporter gene that reflects spatial and temporal PER expression (Veleri et al., 2003) (Figure S1). Comparable with the *ptim-TIM-luc* reporter, *XLG-luc* halteres showed robust oscillations at all four temperatures (Figures 2A and S3A). Similar to what we observed for *ptim-TIM-luc*, the τ associated with the *XLG-luc* oscillations increased by about 3.5 h with increasing temperature (Q_{10} = 0.86), suggesting that the oscillations of clock proteins in halteres are over-compensated against temperature changes (Figures 2A and S3A, Table 1).

To investigate if the temperature compensation phenotype in halteres extends to clock gene transcription, we employed transcriptional reporters for *per* (*plo*) (Brandes et al., 1996) and *tim* (*tim-luc*) (Stanewsky et al., 2002) (Figure S1) and performed the same experiments as described above for the protein reporters. Halteres expressing *tim-luc* were robustly rhythmic at all four temperatures (Figures 2B and S3A). Interestingly, τ increased only by 2.7 h from 18°C to 29°C (Q_{10} = 0.90), indicating less over-compensation of *tim* transcription compared with PER and TIM proteins (Figures 2B and S3A, Table 1). Similar results were obtained for *plo* halteres, which exhibited high-amplitude oscillations at all four temperatures with a temperature-dependent τ increase of only 1.6 h (Q_{10} = 0.94) (Figures 2C and S3, Table 1). In summary, the results show that PER and TIM oscillations in halteres are over-compensated, largely caused by posttranscriptional mechanisms leading to period lengthening with increasing temperatures.

In addition to temperature-dependent changes in τ, we also observed differences in the expression levels of the same transgene at different temperatures. Consistent with previous observations (Kidd et al., 2015; Majercak et al., 1999), both PER and TIM protein reporters show higher expression at 25°C and 29°C compared with the lower temperatures (Figure S3, Table 1). From these results it could be expected that, owing to the repressor activity of PER and TIM, transcription rates of *per* and *tim* would be lower at the warm temperatures compared with the cooler ones. Indeed, in the case of *per*, low protein (*XLG-luc*) expression levels at 18°C and 21°C compared with 25°C and 29°C coincide with elevated levels of *per* transcription (*plo*) at 18°C and 21°C compared with 29°C (Figure S3, Table 1). In contrast, expression levels of both *tim* constructs were significantly reduced at 18°C compared with the higher temperatures, whereas there was no difference (*tim-luc*) or a slight increase (*tim-TIM-luc*) between 25°C and 29°C (Figure S3; Table 1). These results are not readily compatible with direct negative feedback regulation through PER and TIM repression, where one would expect higher protein levels to be correlated with a lower rate of *tim* transcription.

Peripheral Clocks in the Antennae Slow Down with Increasing Temperatures

To investigate if overcompensation against temperature changes is a general feature of *Drosophila* peripheral clocks, we next performed the same analysis of clock gene expression in dissected antennae. This sensory organ contains a circadian clock controlling the daily sensitivity changes of the olfactory system (Krishnan et al., 1999). Consistent with published reports (Krishnan et al., 2001; Levine et al., 2002), rhythmic gene expression was readily detectable in DD at 25°C after initial synchronization to LD cycles in antennal pairs prepared from the four different luciferase transgenic strains (Figures 3 and S4A, Table S1). In contrast

Genotype	°C	N	Tau ± SEM (h)	Error ± SEM	Mean ± SEM (CPS)
Halteres					
plo	18	23	22.8 ± 0.2	0.15 ± 0.01	377.9 ± 31.3
	21	30	22.9 ± 0.1	0.04 ± 0.01	302.4 ± 18.9
	25	28	24.2 ± 0.1	0.04 ± 0.01	385.2 ± 35.7
	29	11	24.4 ± 0.2	0.05 ± 0.01	190.2 ± 23.1
			Q_{10} = 0.94		
tim-luc	18	25	22.1 ± 0.2	0.32 ± 0.03	505.6 ± 33.7
	21	23	23.0 ± 0.1	0.07 ± 0.01	705.7 ± 53.4
	25	33	24.6 ± 0.1	0.03 ± 0.01	1361.8 ± 85.8
	29	25	24.8 ± 0.1	0.03 ± 0.01	1340.3 ± 85.5
			Q_{10} = 0.90		
XLG-luc	18	22	19.9 ± 0.4	0.25 ± 0.03	115.3 ± 6.1
	21	12	20.2 ± 0.3	0.26 ± 0.03	117.4 ± 9.7
	25	28	21.2 ± 0.1	0.07 ± 0.01	157.2 ± 7.2
	29	14	23.4 ± 0.1	0.07 ± 0.01	149.1 ± 8.6
			Q_{10} = 0.86		
ptim-TIM-luc	18	27	21.0 ± 0.1	0.17 ± 0.02	77.7 ± 4.2
	21	37	21.7 ± 0.1	0.09 ± 0.01	99.2 ± 5.8
	25	53	24.0 ± 0.1	0.10 ± 0.01	112.2 ± 8.6
	29	67	25.2 ± 0.1	0.04 ± 0.01	134.4 ± 6.9
			Q_{10} = 0.85		
Brains					
ptim-TIM-luc	18	14	28.6 ± 0.4	0.33 ± 0.02	184.3 ± 16.6
TopCount	21	13	30.8 ± 0.6	0.20 ± 0.02	167.7 ± 18.2
	25	14	30.0 ± 0.8	0.30 ± 0.03	187.7 ± 8.1
	29	5	30.0 ± 1.2	0.32 ± 0.06	324.3 ± 80.1
			Q_{10} = 0.96		
LV200	21	1	31.7	0.18	NA
	21	1	31.4	0.20	NA
Flies					
8.0-luc	18	38	24.8 ± 0.3	0.42 ± 0.03	85.1 ± 5.2
Cut off < 0.7	21	10	23.9 ± 0.8	0.59 ± 0.04	89.9 ± 10.4
	25	31	24.5 ± 0.2	0.43 ± 0.03	71.5 ± 5.8
	29	17	23.2 ± 0.3	0.17 ± 0.02	40.3 ± 2.9
			Q_{10} = 1.06		
8.0-luc; Pdf^{01}	18	41	21.7 ± 0.2	0.27 ± 0.03	23.1 ± 0.9

Table 1. Free Running Period (τ) and Expression Levels of Clock Gene Expression in Halteres, and Clock Neurons

(Continued on next page)

Genotype	°C	N	Tau ± SEM (h)	Error ± SEM	Mean ± SEM (CPS)
Cut off < 0.7	21	18	22.7 ± 0.1	0.54 ± 0.03	17.7 ± 0.7
	25	30	21.7 ± 0.1	0.27 ± 0.03	20.4 ± 1.0
	29	15	22.5 ± 0.1	0.26 ± 0.03	15.2 ± 0.8
			Q_{10} = 0.97		

Table 1. Continued

τ values were calculated in Chronostar (see Transparent Methods). The "Error" value depicts the correlation between the curve fit and the detrended data (one minus correlation coefficient, so the lower the error value the more trustable the period value). All individual τ values with an error <0.5 or <0.7 were used for calculating the average τ in the tissue culture and adult fly experiments, respectively. Q_{10} values were calculated using the τ values for 18°C and 29°C. The "mean" reflects the average expression level (CPS) during the entire time series. The brain *tim-TIM-luc* LV200 values refer to the two DN1 groups recorded at 21°C with the LV200 CCD camera system (Figures 4C–4F and Video S1).

to halteres, robust rhythmic reporter gene expression was not observed at all temperatures (in particular at 29°C), indicating that the antennal oscillator is less robust at least in warmer temperatures (Figures 3 and S4A, Table S1). We therefore decided to determine temperature compensation properties of the antennal oscillator only for 18°C, 21°C, and 25°C, where we obtained reliable period estimates (Figures 3 and S4A, Table S1). Comparing *ptim-TIM-luc* period values at the three different temperatures revealed a severe over-compensation phenotype, with a lengthening of τ by > 4 h (Q_{10} = 0.79) (Figures 3 and S4A, Table S1). In contrast, *XLG-luc* antennae, which were arrhythmic at 29°C, lengthened their period only by > 1 h between 18°C and 25°C (Q_{10} = 0.92) (Figures 3 and S4A, Table S1), indicating less over-compensation for this reporter. Nevertheless, the fact that τ increases significantly at 25°C compared with 18°C and 21°C and expression becomes arrhythmic at 29°C may indicate a strong over-compensation phenotype of PER expression in antennae, resulting in arrhythmicity instead of severe period lengthening. Strikingly, and in contrast to the situation in halteres, gene expression rhythms of the two transcriptional reporters for *per* and *tim* also showed severe over-compensation with a dramatic τ lengthening > 5–6 h between 18°C and 25°C (Q_{10} = 0.77 and 0.69, respectively) (Figures 3 and S4A, Table S1).

Mean expression levels of all four reporters changed with temperature in a very similar manner, with low expression levels at the extremes (18°C and 29°C) and higher expression levels at 21°C and 25°C (Figure S4, Table S1), precluding any meaningful correlation between protein levels and transcription rates as was the case for the halteres. It has previously been reported that luciferase activity is temperature dependent, either increasing or decreasing with temperature (e.g., Kurosawa et al., 2017; McElroy and Seliger, 1961). Although we cannot rule out direct effects of temperature on luciferase activity, we do not observe consistent changes of luciferase activity with temperature. For example, *tim-luc* bioluminescence signals are lowest at 29°C in antennae but at peak levels in halteres at the same temperature (Figures S3 and S4). Because the *tim-luc* reporter does not encode any TIM protein sequences, we are confident that the bioluminescence levels report endogenous gene expression, and not temperature effects on luciferase activity. In summary, the antennal results confirm the over-compensation phenotypes observed in halteres, thereby strongly suggesting that this is a general inadequacy of peripheral circadian clocks. Moreover, the more severe τ-lengthening defects in antennae point to a correlation of temperature compensation with oscillator strength (see Discussion).

PER Oscillations in Brain Clock Neurons Are Accurately Temperature Compensated

To investigate if the over-compensation of peripheral clocks is also a feature of the neuronal clocks in the fly brain we turned to the *8.0-luc* transgene, where PER expression is restricted to subsets of the dorsal clock neurons (DN and LNd) and excluded from the periphery (Veleri et al., 2003; Yoshii et al., 2009) (Figure S1). Since fruit fly behavioral rhythms are well temperature compensated (Konopka et al., 1989; Singh et al., 2019) and controlled by brain clock neurons (Ewer et al., 1992), we expected *8.0-luc* oscillations to be temperature compensated as well. Indeed, when we recorded *8.0-luc* oscillations in DD, periods were fairly constant between 18°C, 21°C, 25°C, and 29°C with no sign of τ lengthening with increasing temperatures (Q_{10} = 1.06) (Figure 4A, Table 1). Because both *XLG-luc* and *8.0-luc* encode full-length PER-LUC fusion proteins, it seems clear that the observed period lengthening in peripheral clocks is indeed caused by an impairment of temperature compensation and not by intrinsic properties of PER-LUC fusion proteins.

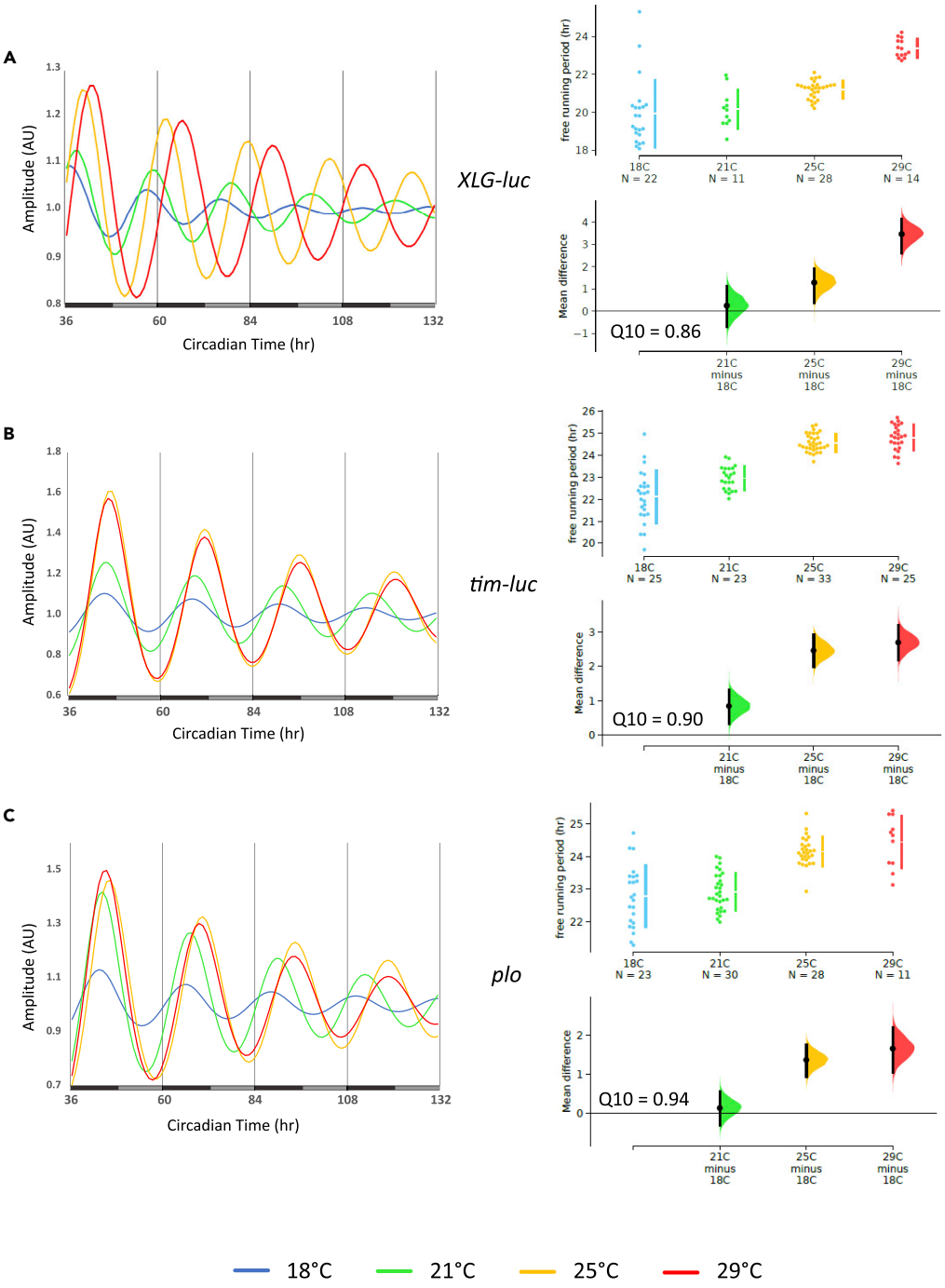

Figure 2. Clock Protein Rhythms in the Haltere Slow Down with Increasing Temperature

Bioluminescence recordings of halteres dissected from (A) *XLG-luc*, (B) *tim-luc*, and (C) *plo* at the indicated constant temperatures in DD. The plots on the left show curve-fitted data for the DD portion of the experiment. To the right, ES shared control plots comparing the distribution of period values at the different temperatures (see legend to Figure 1D and Transparent Methods for details).

In contrast to peripheral clocks, the central brain clock in fruit flies is organized in a neuronal network, and these network properties may contribute to accurate temperature compensation. The neuropeptide PDF acts as a synchronizer of this network and differentially influences the period and amplitude of clock protein expression, depending on the particular clock neuronal group (Yoshii et al., 2009). For example, at 25°C

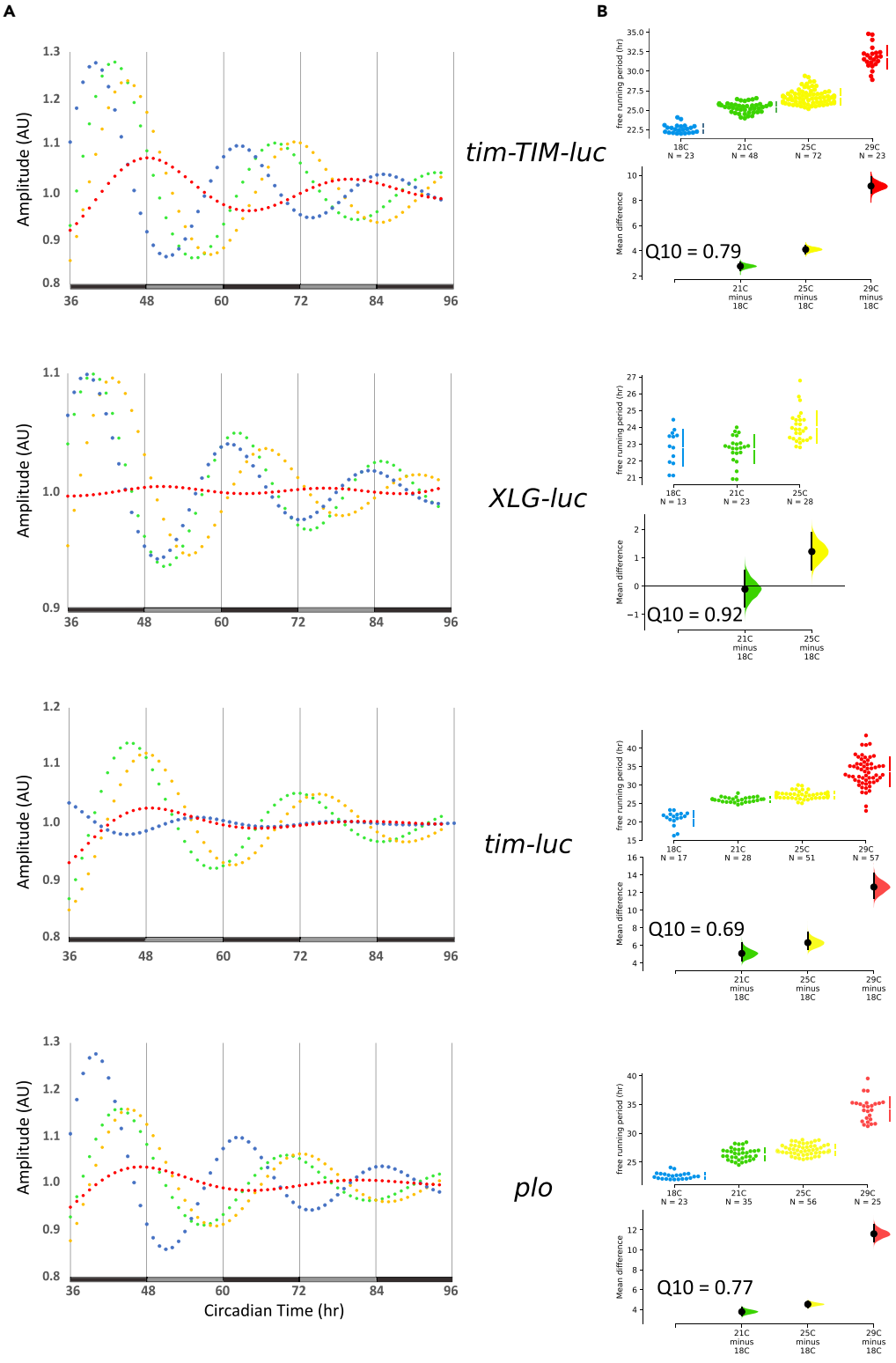
A
B
Amplitude (AU)
Circadian Time (hr)
tim-TIM-luc
XLG-luc
tim-luc
plo
free running period (hr)
Mean difference
Q10 = 0.79
Q10 = 0.92
Q10 = 0.69
Q10 = 0.77
18C
21C
25C
29C
18°C
21°C
25°C
29°C

Figure 3. The Antennal Clock Slows Down with Increasing Temperature

(A) Curve fits of bioluminescence recordings of antennae dissected from *tim-TIM-luc*, *XLG-luc*, *tim-luc*, and *plo* flies at the indicated constant temperatures in DD. Black bars indicate subjective night, gray bars subjective day.

(B) Period estimates for antennal pairs obtained from curve-fitted data in (A).

and in the absence of PDF, *8.0-luc* oscillations have a short period (22.7 h) and increased amplitude, most likely reflecting the enhanced PER oscillations in the CRY-negative LNd (Yoshii et al., 2009). To test if PDF is required for temperature compensation, we analyzed bioluminescence rhythms of *8.0-luc;Pdf*^{01} flies at four different temperatures. As expected, at 25°C we observed robust short-period bioluminescence rhythms, and similar results were obtained for the other three temperatures (Q_{10} = 0.97) (Figure 4B, Table 1). Therefore, PDF is not required for temperature compensation, at least with regard to clock protein expression in the *8.0-luc* expressing neurons.

Isolated Brains Maintain Slow Running but Temperature-Compensated Clock Protein Oscillations

Brain clock protein oscillations were measured in intact *8.0-luc* flies and not in isolation as was done for the halteres. To determine if isolated brains also maintain temperature-compensated oscillations in DD, we measured bioluminescence rhythms from dissected *tim-TIM-luc* brains in the same culture conditions as described above for the halteres. Surprisingly, oscillations were not as robust as observed in the halteres and the period length was drastically increased to about 30 h at all four temperatures tested (Q_{10} = 0.96) (Figures 5A and 5B, Table 1). Although this indicates that neuronal clocks require some kind of peripheral input in order to run with a 24-h period, clock protein oscillations in isolated brains are nevertheless properly temperature compensated.

The bioluminescence signals recorded from isolated *tim-TIM-luc* brains are expected to be composed of clock glia cells in addition to the clock neurons (Ewer et al., 1992). Since the TopCount plate reader measures overall light output from individual wells, it does not allow for any spatial resolution of gene expression. To confirm if clock neurons in culture indeed oscillate with long ~30-h periods, we therefore applied a bioluminescence imaging system (LV200, Olympus) to measure clock gene oscillations in a subset of the clock neurons. For this, *tim-TIM-luc* flies were combined with a red-fluorescent reporter, to label the nuclei of all clock neurons (Mezan et al., 2016). Brains of *tim-TIM-TOMATO*; *tim-TIM-luc* flies were dissected, and using the fluorescence mode of the microscope, we focused on a subset of the DN1 clock neurons (Figures 5C and 5E). In the absence of fluorescence light (Figure 5D), photons were then captured automatically once per hour for 4 days using a cooled charge-coupled device (CCD) camera (see Transparent Methods). Within two DN1 clusters bioluminescence oscillated with a period around 31 h (Figure 5F, Video S1, Table 1). Combined, the brain culture results indicate that clock neurons in isolated brains oscillate with a period $\geq$30 h and that these oscillations are temperature compensated.

DISCUSSION

We report here important characteristics of peripheral clocks in the insect *Drosophila melanogaster*. Although previous work showed that these peripheral clocks can be synchronized tissue-autonomously to LD and temperature cycles, it was not clear if they indeed constitute self-sustained and temperature-compensated oscillators. Hence, up to now it was questionable if peripheral fly clocks qualify as true circadian clocks. Our work shows that at least the haltere peripheral clock maintains robust and self-sustained 24-h oscillations and that the period of PER and TIM protein expression rhythms increases with temperature (over-compensation). Transcriptional rhythms for both genes are less over-compensated, with Q_{10} values close to 1 (0.90 for *tim-luc* and 0.94 for *plo*). Although this may indicate better temperature compensation for transcription, in the antennae we observed more severe over-compensation with the transcriptional reporters compared with the protein reporters (Figure 3, Table S1). In summary, our results suggest that over-compensation in the face of increasing ambient temperatures is a general feature of peripheral clocks in fruit flies, whereas the molecular mechanisms of temperature compensation may differ between different tissues.

Differences between Central and Peripheral Oscillators

Why are peripheral clocks not able to maintain stable period lengths at different temperatures? One possible answer is the different make-up of the molecular oscillator in peripheral clocks, which requires CRY function in most studied cases (Ivanchenko et al., 2001; Krishnan et al., 2001; Levine et al., 2002). One exception is the

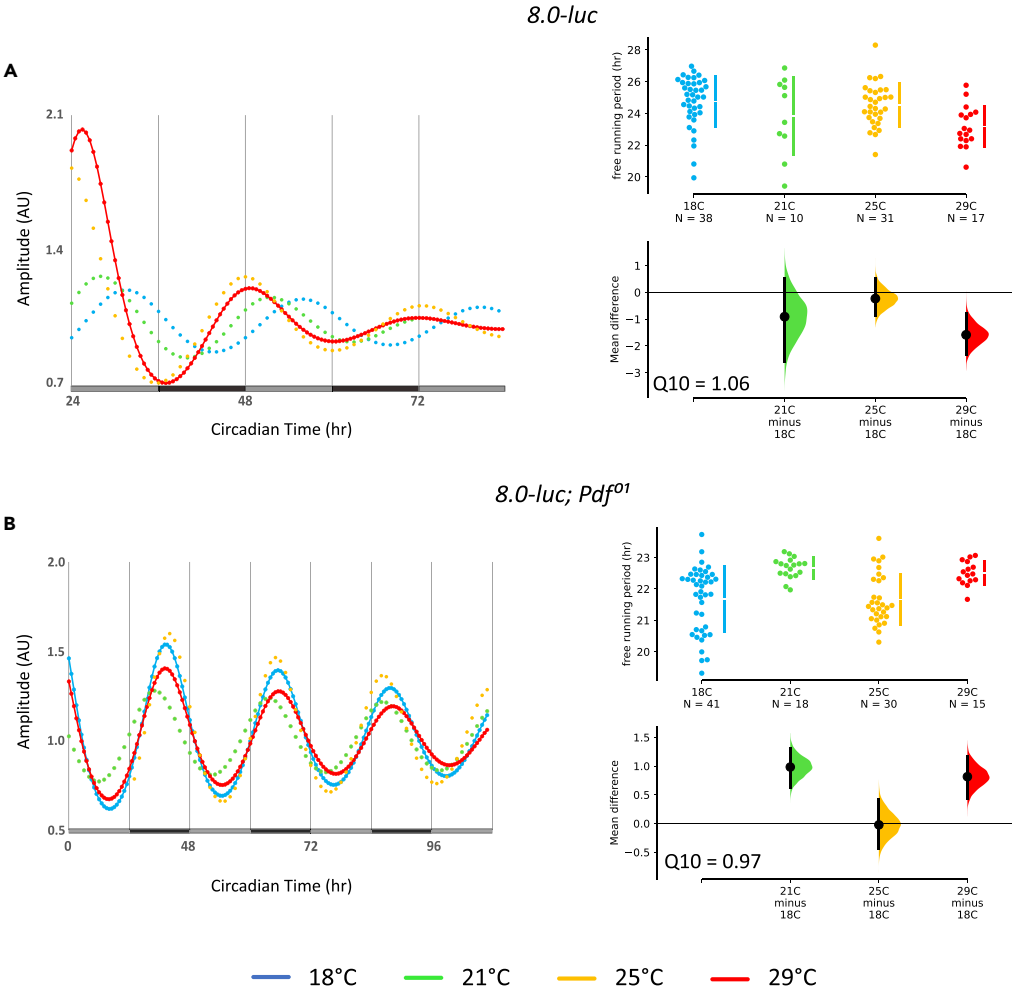

Figure 4. Clock Gene Expression in Central Brain Clock Neurons Is Temperature Compensated and Does Not Require PDF

Bioluminescence recordings of *8.0-luc* flies recorded at different constant temperatures in DD.

(A) Curve fits of DD data of *8.0-luc* flies (left), and ES comparing individual τ values at different temperatures (right).

(B) Same as in (A) for *8.0-luc; Pdf*^{01} flies.

cuticle deposition rhythm, but it is not known if this rhythm is temperature compensated (Ito et al., 2008). Another possibility is related to the cell-type harboring central versus peripheral clocks. In addition to rhythms of clock gene expression, central clock neurons also display clock-regulated daily rhythms of neuronal activity, which is thought to feedback and enhance clock gene expression rhythms (reviewed in Allen et al., 2017). Considering the non-neuronal nature of a subset of the peripheral clock cells (e.g., MT, testes, epidermal cells that give rise to the cuticle deposition rhythm) it is possible that neuronal network properties of the circadian clock neurons contribute to temperature compensation. For example, in the mammalian suprachiasmatic nuclei (SCN), coupling between the individual clock neurons prevents their synchronization to temperature cycles, suggesting that network properties contribute to resilience against temperature-induced changes to the molecular clock (Abraham et al., 2010; Buhr et al., 2010). Our finding that removal of the circadian neuropeptide PDF, a potent coupling signal between different circadian clock neurons, did not impair temperature compensation of clock neurons, argues against an important role of network communication in temperature compensation. Nevertheless, our results do not rule out that PDF-independent neuronal network properties contribute to temperature compensation of the fruit fly brain clock.

Alternatively, the temperature dependence of peripheral clock oscillations may contribute to the temperature independence of the brain oscillators. Perhaps the brain clocks are connected to the peripheral

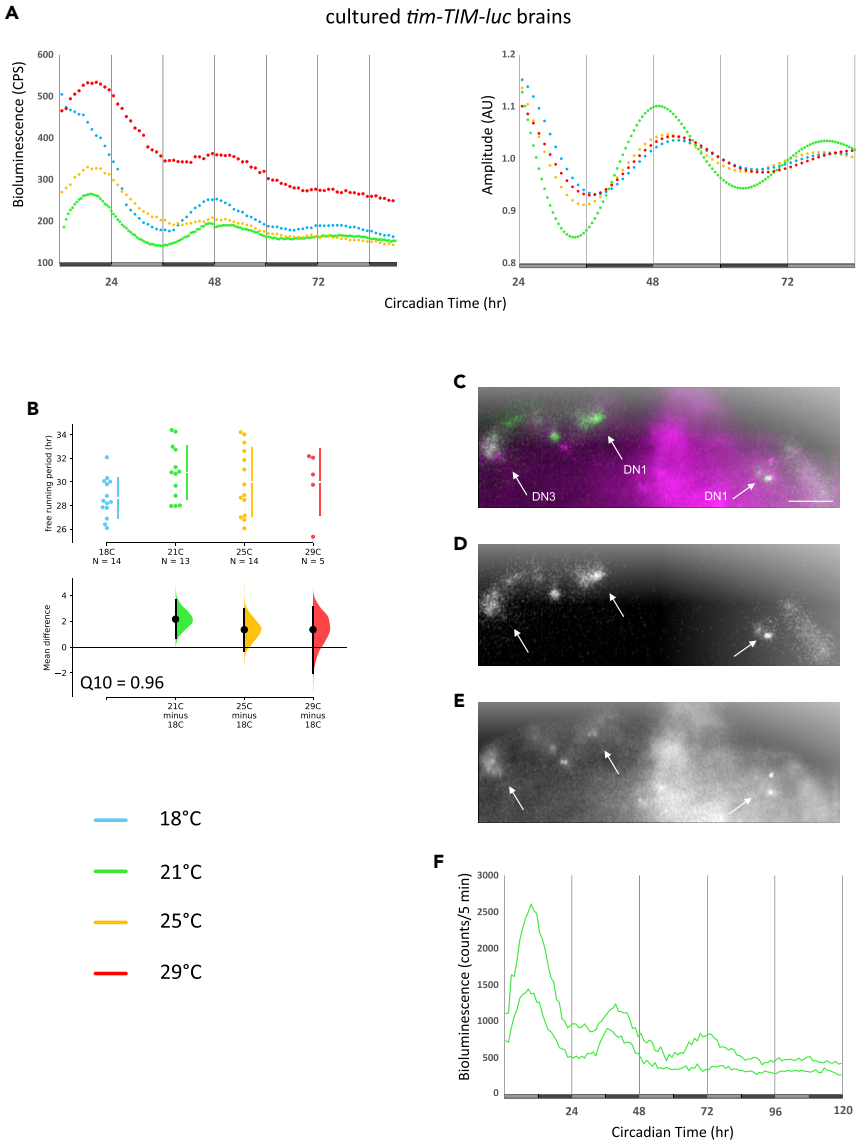

Figure 5. Long-Period Clock Gene Expression in Cultured Brain Neurons Is Temperature Compensated

Brains of *tim-TIM-luc* flies were dissected and transferred to cell culture medium followed by bioluminescence recordings in a TopCount plate reader (A) or using a CCD camera (C–F). (A) Average raw bioluminescence of brains at the indicated temperature during DD (left), and curve fitted data (right) as described in the legend to Figure 1. N's are indicated in (B) and Table 1. (B) ES of individual τ values as described in the legend to Figure 4. (C–E) Merged (C), bioluminescence (D, green in merge), and fluorescence images (E, magenta in merge) of *tim-TIM-TOMATO*; *tim-TIM-luc* brains. Scale bar, 50 μm. (F) CCD camera bioluminescence recordings of the two groups of DN1 neurons shown in (C)–(E) at 21°C in DD (see Transparent Methods). For τ see Table 1.

clocks and somehow receive temperature information encoded by the period changes in the peripheral oscillators. This way, the central oscillators could be instructed to speed up or slow down their molecular oscillations, in order to maintain a stable τ at different temperatures. In this scenario, the "outside" location of the peripheral oscillators (antennae, haltere, legs) would help them to react quickly to temperature changes. Although this is an interesting speculation, the fact that we observed temperature-compensated rhythms of $\geq$30 h in isolated brains shows that peripheral clocks are not required for temperature compensation of the brain oscillator. We have no explanation for why clock gene oscillations in isolated brains show long periods, but the reproducible results, obtained with different recording methods, indicate that they are genuinely long. In fact, long periods of *tim-luc* expression in subsets of the clock neurons of isolated

brains, recorded with a similar CCD camera system, were reported previously (Sellix et al., 2010). One reason for the long periods could be that brains in culture change their neuronal firing properties. For example, it has been shown that the bursting firing mode of the l-LNv decays rapidly within the first 30 min after dissection and also depends on input from the visual system (Muraro and Ceriani, 2015). It is therefore likely that the firing properties of the clock neurons after several days in culture are quite different compared with an intact brain. Because neuronal firing feeds back on molecular oscillations, changed neuronal activity may influence (slow down) the free running molecular oscillations independent of temperature. Future work will reveal if the long periods are linked to specific subsets of clock neurons or a general feature observed in isolated brains in culture. The latter would indicate that the period of central clock neurons is normally accelerated by an unknown input from outside the brain, as for example, the visual system or other peripheral clock-containing tissues and sensory organs.

Robustness of Peripheral and Central Oscillators

Our comparison between peripheral oscillators revealed that the haltere oscillator is more robust and also less over-compensated compared with the antennal oscillator. It is possible that this correlation reflects a causal relationship between oscillator strength and temperature compensation. On the other hand, although the haltere oscillator is very robust and basically non-dampening (Figure 1A), it does show significant over-compensation compared with the brain oscillator, ruling out that oscillator strength is the unique determinant for fully functional temperature compensation.

Why a Peripheral Clock in the Haltere?

Although it is formally possible that the haltere clock supports temperature synchronization of the central brain clock (see above), it seems more likely that it fulfills a more direct physiological function in this proprioceptive sensory organ. Clock gene expression in the *Drosophila* haltere was observed in cells that most likely represent campaniform sensilla, the principle haltere mechanosensory neurons (Sehadova et al., 2009; Yarger and Fox, 2016). In contrast, no clock gene expression in the other haltere mechanosensory cell type, the chordotonal organ neuron, could be detected (Sehadova et al., 2009). We therefore conclude that the rhythmic haltere gene expression we report here emanates from the campaniform sensilla. At present, it is unknown which biological rhythm(s) the haltere clock regulates. Compared with other peripheral clocks the haltere rhythms are strikingly more robust (e.g., compare Figures 1, 2, and S3 with Figures 3 and S4), suggesting that the haltere clock does have an important physiological function.

Conclusions

In summary, our work reveals surprising features of peripheral clocks, based on the discovery of a robust and non-dampening 24-h oscillator in the fly haltere. Unlike the central clock neurons in the brain, the haltere oscillator is over-compensated against temperature increases. Our findings extend beyond poikilothermic invertebrates, because it has been reported that peripheral clocks in both ectothermic and endothermic vertebrates are also over-compensated (Barrett and Takahashi, 1995; Izumo et al., 2003; Kaneko et al., 2006; Lahiri et al., 2005; Reyes et al., 2008; Tsuchiya et al., 2003). Careful comparison of our invertebrate data with the vertebrate studies reveals that in embryonic zebrafish and mammalian fibroblast cell lines, as well as in dispersed chick pineal cells, clock gene oscillations slow down with increasing temperature, with similar Q_{10} values as reported here ($Q_{10} \leq 0.9$) (Izumo et al., 2003; Lahiri et al., 2005; Tsuchiya et al., 2003). Circadian clocks of various cultured adult zebrafish tissues have Q_{10} values between 0.9 and 1.0 (Kaneko et al., 2006), whereas in cultured tissues of *mPer2-luc* mice the only tissue with a $Q_{10} \geq 1.0$ was the SCN, and all peripheral clocks slowed down with temperature. Moreover, the liver oscillator did not show self-sustained oscillations below 37°C (Reyes et al., 2008) similar to what we show here for the antennae at 29°C. Combined, the available data from various circadian model organisms point to a more prominent difference between the makeup of peripheral and central circadian oscillators than previously thought. This is also supported by the lack of a PRC dead zone in mammalian peripheral clocks (Balsalobre et al., 2000). Our results indicate that in *Drosophila* the differences between central ($Q_{10} = 1$) and peripheral clocks are particularly prominent, as Q_{10} values for PER and TIM protein rhythms in the haltere and transcriptional and protein rhythms in the antennae are at the lower end, or even lower, compared with what has been reported for most vertebrate peripheral tissues (Tables 1 and S1). The question which of the differences between peripheral and central oscillators (e.g., molecular and/or neuronal network properties) is responsible for the different temperature compensation properties cannot be finally answered and requires more detailed analysis and comparisons between different organisms, tissues, and organs.

Limitations of the Study

We were surprised that there was no clear correlation between protein levels reported by *XLG-luc* and *tim-TIM-luc* and transcriptional levels reported by *plo* and *tim-luc*. As described in Results, luciferase activity was not a simple function of temperature, either increasing or decreasing depending on the reporter gene and tissue. Although this suggests that luciferase activity—apart from accurately reporting clock gene oscillations—is also a faithful reporter of gene expression levels, we cannot rule out that other endogenous factors affect this enzyme.

Resource Availability

Lead Contact

Further information and requests for resources, reagents, and raw datasets should be directed to and will be fulfilled by the Lead Contact, Ralf Stanewsky (stanewsky@uni-muenster.de).

Materials Availability

All reagents (fly stocks) used in this study are available from the Lead Contact.

Data and Code Availability

This study did not generate large datasets or code.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101388.

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AUTHOR CONTRIBUTIONS

M.V. performed all experiments, analyzed data, and wrote parts of the Methods. K.-M.E. performed adult fly recordings. R.S. designed the study, analyzed data, and wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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iScience, Volume *23*

Supplemental Information

A Robust and Self-Sustained Peripheral Circadian Oscillator Reveals Differences in Temperature Compensation Properties with Central Brain Clocks

Marijke Versteven, Karla-Marlen Ernst, and Ralf Stanewsky

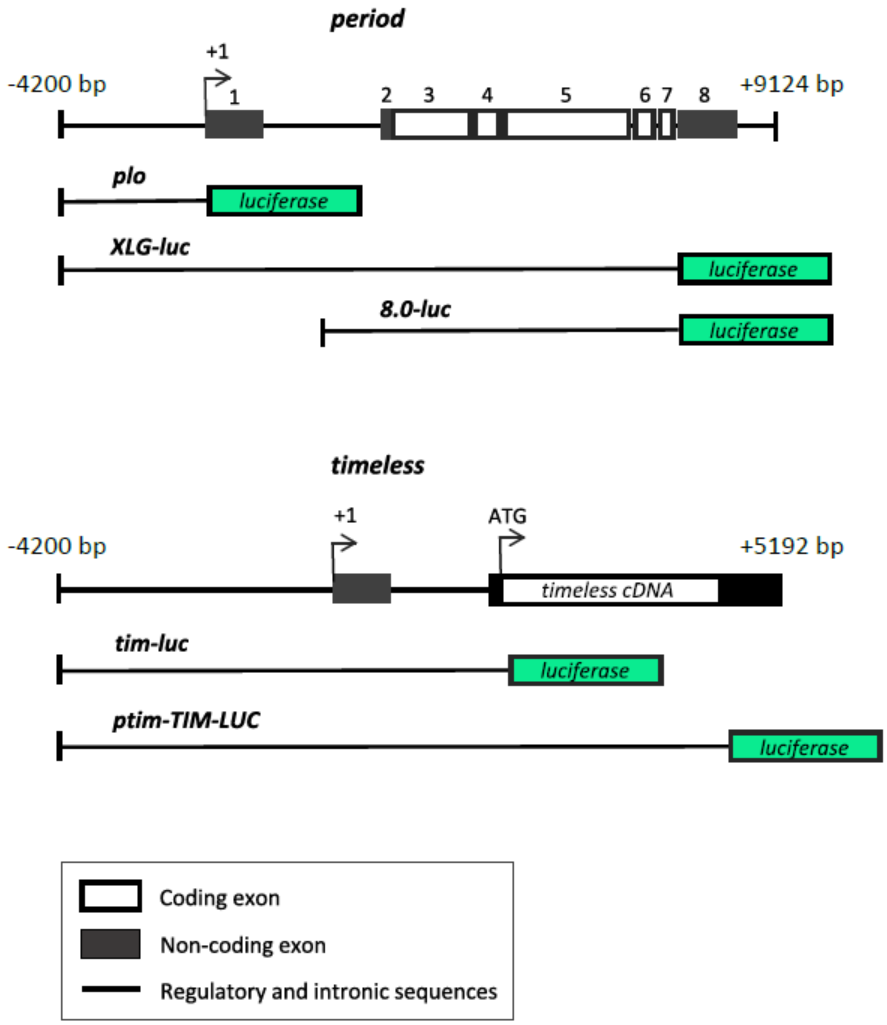

Figure S1: Map of the *period* and *timeless* luciferase constructs, Related to Figures 1-5. Top: genomic organization of the *per* locus and *per* content of the 3 *per-luciferase* constructs applied here. *plo* contains only 5' *per*-regulatory sequences, reporting *per* transcription (Brandes et al., 1996; Stanewsky et al., 1997), while *XLG-luc* and *8.0-luc* report PER protein expression (Veleri et al., 2003). Due to the lack of regulatory *per* promoter sequences, *8.0-luc* expression is restricted to subsets of the clock neurons and excluded from the peripheral clocks (Gentile et al., 2013; Veleri et al., 2003; Yoshii et al., 2009). Bottom: map of a *timeless* construct containing ~6.3 kb 5'-regulatory sequences fused to the *tim* cDNA promoter (Rutila et al., 1998) and the extend of *tim* sequences contained in the *tim-luc* and *tim-TIM-luc*, reporting *tim* transcription and TIM protein expression, respectively (Lamba et al., 2018; Stanewsky et al., 2002).

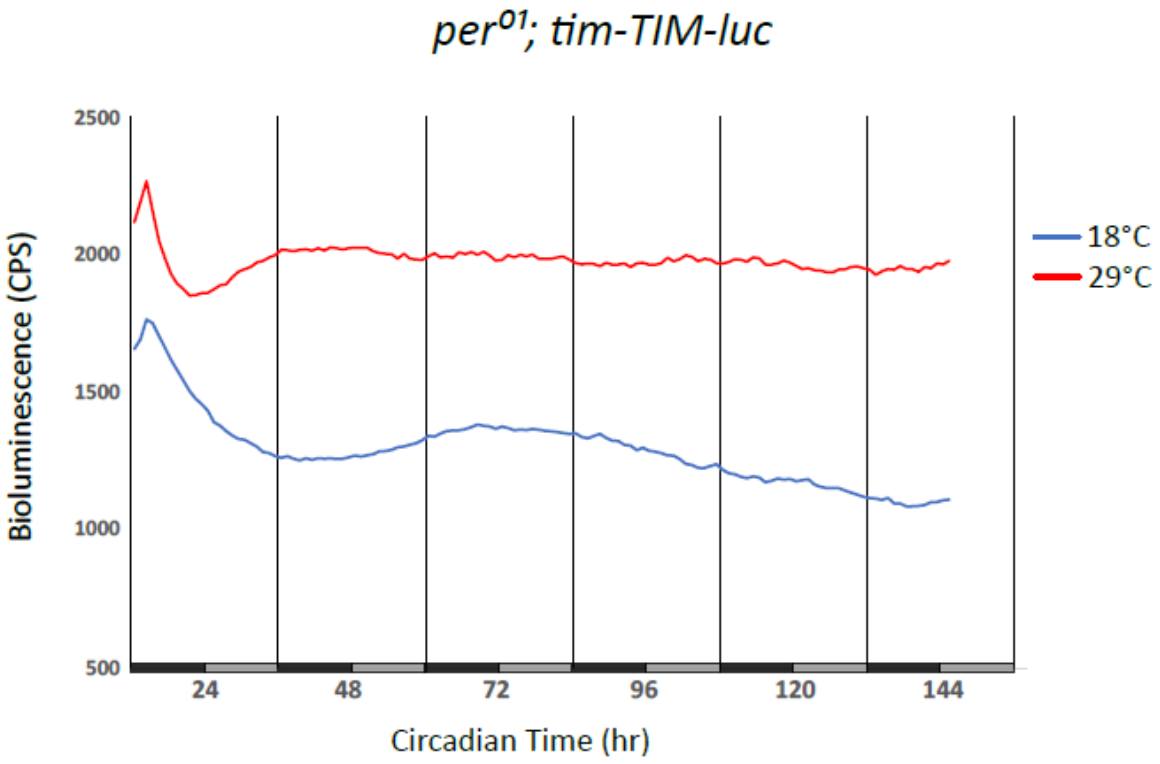

Figure S2: Rhythmic *tim-TIM-luc* expression in the haltere depends on a functional clock, Related to Figure 1. Bioluminescence recordings of halteres dissected from *per^{01}; tim-TIM-luc* flies during DD at 18°C (n=48) and 29°C (n=46). Note the arrhythmic, but drastically increased (> 10 fold) expression levels compare to *per^{+}* due the lack of PER repressor activity (compare to Figure 1).

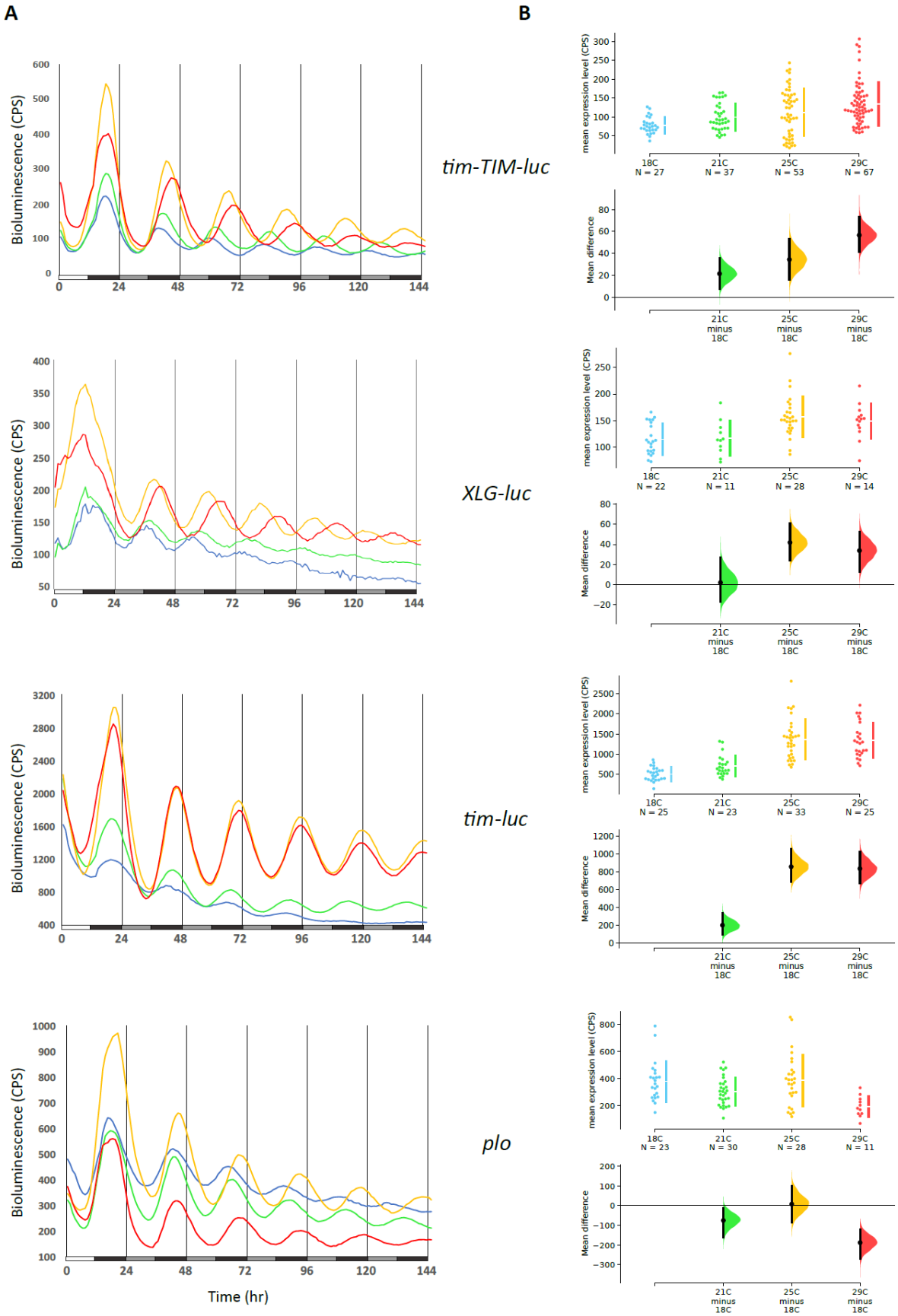
A
B
Bioluminescence (CPS)
tim-TIM-luc
XLG-luc
tim-luc
plo
Time (hr)
mean expression level (CPS)
Mean difference
18C
N = 27
21C
N = 37
25C
N = 53
29C
N = 67
21C minus 18C
25C minus 18C
29C minus 18C
N = 22
N = 11
N = 28
N = 14
N = 25
N = 23
N = 33
N = 30
N = 11

Figure S3: Raw levels of reporter gene expression in halteres at different temperatures, Related to Figures 1, 2, Table 1. (A) Bioluminescence recordings of halteres dissected from *tim-TIM-luc* (replotted from Figure 1B), *XLG-luc*, *tim-luc*, and *plo* flies at the indicated constant temperatures. The plots show raw-data averages from the last day in LD followed by 5 days in DD. White bars on the X-axis indicate lights-on, black bars lights-off, and grey bars, subjective day. **(B)** Gene expression levels reflecting the mean CPS of a single haltere pair during the entire recording. To precisely visualize potential gene expression differences between temperatures we applied ES (see legend to Figure 1D and Transparent Methods for details).

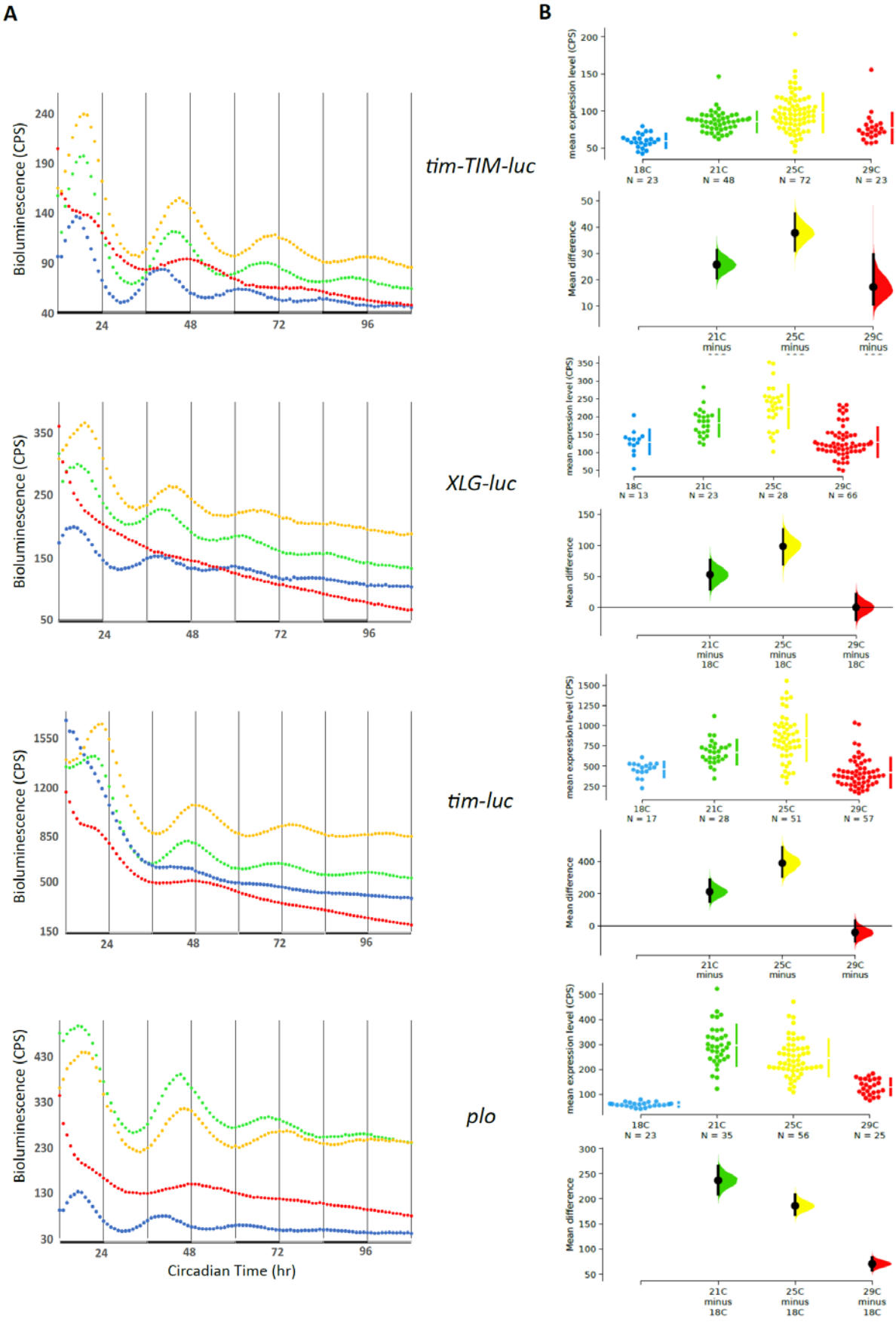
A
B
Bioluminescence (CPS)
Circadian Time (hr)
tim-TIM-luc
XLG-luc
tim-luc
plo
mean expression level (CPS)
Mean difference
18C
21C
25C
29C
N = 23
N = 48
N = 72
N = 23
N = 13
N = 23
N = 28
N = 66
N = 17
N = 28
N = 51
N = 57
N = 23
N = 35
N = 56
N = 25
21C minus
25C minus
29C minus
21C minus 18C
25C minus 18C
29C minus 18C

Figure S4: Raw levels of reporter gene expression in antennae at different temperatures, Related to Figure 3 and Table S1. (A) Bioluminescence recordings of antennae dissected from *tim-TIM-luc, XLG-luc*, *tim-luc*, and *plo* flies at the indicated constant temperatures. The plots on the left show raw-data averages from the 4 days in DD immediately following LD entrainment. Black bars indicate subjective night; grey bars subjective day. **(B)** Gene expression levels reflecting the mean CPS of a single antennal pair during the entire recording. To precisely visualize potential gene expression differences between temperatures we applied ES (see legend to Figure 1D and Transparent Methods for details).

Table S1: Free running period (τ) and expression levels of clock gene expression in antennae, Related to Figure 3 and S4.

Genotype	°C	N	Tau ± S.E.M (h)	Error ± S.E.M	Mean ± S.E.M (CPS)
antennae					
plo	18	23	22.6 ± 0.1	0.10 ± 0.01	59.7 ± 1.9
	21	35	26.4 ± 0.2	0.09 ± 0.01	296.3 ± 13.8
	25	56	27.2 ± 0.1	0.10 ± 0.01	245.8 ± 9.9
	29	90 (25)	33.9 ± 0.5	0.39 ± 0.02	102.3 ± 3.6
			Q_{10} = 0.69		
tim-luc	18	47 (17)	21.0 ± 0.5	0.43 ± 0.02	482.1 ± 17.0
	21	28	26.1 ± 0.1	0.07 ± 0.01	673.3 ± 28.7
	25	51	27.3 ± 0.1	0.06 ± 0.01	851.4 ± 40.3
	29	83 (58)	33.7 ± 0.1	0.29 ± 0.02	395.7 ± 20.2
			Q_{10} = 0.65		
XLG-luc	18	13	22.8 ± 0.3	0.25 ± 0.02	129.2 ± 9.7
	21	23	22.7 ± 0.2	0.19 ± 0.02	182.3 ± 7.9
	25	36	24.1 ± 0.2	0.16 ± 0.01	232.2 ± 9.3
	29	66	AR	N/A	129.5 ± 5.1
			Q_{10} = 0.92		
ptim-TIM-luc	18	23	22.6 ± 0.1	0.10 ± 0.01	59.7 ± 1.9
	21	48	25.4 ± 0.1	0.06 ± 0.01	85.6 ± 2.0
	25	72	26.7 ± 0.1	0.09 ± 0.01	97.6 ± 3.0
	29	57 (23)	31.8 ± 0.1	0.35 ± 0.02	67.0 ± 2.3
			Q_{10} = 0.73		

See Table 1 legend for details. Numbers in parentheses indicate N used for calculation of τ in cases where not all samples were rhythmic (cut-off error < 0.5). Q_{10} values were calculated using the τ values for 18°C and 29°C except for *XLG-luc* (18°C and 25°C).

Transparent Methods

Flies

Flies were kept at 25°C or 18°C on common cornmeal-yeast-sucrose food under light:dark cycles and ~60% humidity. *plo*, *tim-luc, XLG-luc, tim-TIM-luc* are *per* or *tim* fusions with the firefly *luciferase* cDNA that were all described previously and shown to report transcriptional or protein rhythms of the two clock genes in peripheral tissues (Brandes et al., 1996; Lamba et al., 2018; Stanewsky et al., 2002, 1997; Veleri et al., 2003). The transgenic *8.0-luc* line was described previously and encodes the entire PER protein fused to the firefly Luciferase cDNA (Veleri et al., 2003). Due to the absence of the *per* promoter, the 1st non-coding exon, and large parts of the 1st intron, reporter expression is limited to subsets of the DN1-3 and LNd both during TC and LD cycles (Gentile et al., 2013; Veleri et al., 2003; Yoshii et al., 2009). Pdf^{01} (Renn et al., 1999) and per^{01} (Konopka and Benzer, 1971) loss-of-function mutants were combined with the respective reporter constructs using standard crosses. The *tim-TIM-TOMATO* (chromosome *2)* flies express a destabilized red fluorescence protein in all clock cells (Mezan et al., 2016) and were combined with *tim-TIM-luc* (chromosome *3)* using standard crosses.

Bioluminescence measurements of adult flies

Luciferase expression of individual flies carrying the *8.0-luc period-luciferase* reporter gene was measured as described in (Veleri et al., 2003). 3-4 day old males were loaded in 96-well microtiter plates containing 100ul of 5% sucrose, 1% agar and 15mM luciferin. Bioluminescence was detected with a TopCount Multiplate Reader (Perkin Elmer) for serval days during conditions of the designated constant temperature and DD after the flies were entrained to LD at the same temperature for 2 days. Bioluminescence was measured once or twice per hour and data were analyzed using ChronoStar software (Klemz et al., 2017).

Culturing and plate reader bioluminescence measurements of *Drosophila* peripheral tissues and brains

Halteres and antennae of two- to seven-day old transgenic *plo*, *XLG-luc*, *tim-luc* or *ptim-TIM-luc* males kept under 12 h :12 h LD at 25°C were bilaterally dry dissected. Each pair was transferred into every other well of a 96 well plate (Topcount, Perkin Elmer) filled with culture medium containing 80% Schneider's medium (Sigma-Aldrich), 20% inactivated Fetal Bovine Serum (Capricorn Scientific, Ebsdorfergrund, Germany) and 1% PenStrep (Sigma-Aldrich) and fortified with Luciferin (Biosynth AG, Switzerland) to a final concentration of 226 µM. Plates were sealed with clear adhesive covers and transferred to a TopCount plate reader (Perkin Elmer). Bioluminescence emanating from each well was measured hourly or half-hourly in LD for two days, followed by 5 days of constant darkness (DD) at 18°C, 21°C, 25°C or 29°C, respectively, as described above for intact *8.0-luc* flies. For brain cultures, dissections of *ptim-TIM-LUC* male brains were done in Ca^{2+}-free Ringer's solution after briefly being rinsed in a 96% EtOH solution. Single brains were placed into a well containing the same medium as described above, but supplemented with 1 mM Luciferin.

Bioluminescence imaging of adult brains using the LV200 system

ptim-TIM-TOMATO; ptim-TIM-luc flies were synchronized to LD cycles at 21°C for at least 3 days. Thereafter, brains were dissected and collected in Ca^{2+} free Ringer's solution within 1 hour and subsequently placed on a Cellview glass bottom imaging dish (35x10mm; Greiner Bio One) treated with heptane glue. Brains were placed into a droplet of Ca^{2+} free Ringer's solution that was placed on top of the glue. The solution was removed leaving the brains glued to the dish and immediately replaced by a drop of culture medium (see above) containing 1 mM Luciferin where after additional culture medium was carefully added. Culture dishes were then placed into a sterile transparent glass container and entrained for two days in LD at 21°C, replacing two thirds of culture medium with fresh one each day. At day 3, culture dishes were transferred to a LV200 (Olympus) microscope placed in a temperature controlled dark room. Positioning of the brains was done by means of a mobile stage equipped with a Stage Top Incubator (Tokai Hit, Japan) and bright-field illumination. Fluorescence and use of the *ptim-TIM-TOMATO* construct allowed focussing on individual DN1 clock neuron cell bodies based on anatomical position using 40X magnification (Figure 4C-E). Bioluminescence was then measured during 5 min exposure periods with an EM gain of 400 (0.688 MHz EM-CCD camera CAM-ImageEM X2, Hamamatsu, Japan) followed by 5 s exposure to fluorescence light (to control focus) with an EM gain of 4. Exposures were performed once every hour from ZT12 onwards in DD for 5 days generating 512x512 pixel movies (see Movie S1). Images were processed with the CellSense software (Olympus, Japan). Average luminescence intensity of closely grouped DN1 cell bodies was measured within manually defined regions of interest (ROIs) over time, based on *ptim-TIM-TOMATO* signals. Dynamic ROIs were determined by re-positioning ROIs frame to frame to account for flattening of the cultured brains and to exclude cosmic rays as described previously (Roberts et al., 2015). Background correction was performed by subtracting the intensities of equally sized $ROI_{background}$ from $ROI_{cell\ bodies}$. Raw bioluminescence data were further processed with ChronoStar software to calculate period length as described above. Single frame images for Figure 6 C-E were processed in Fiji using a 1:14 gamma correction for the fluorescence channel.

Period and expression level calculation of bioluminescence data using ChronoStar software

Using ChronoStar software (Klemz et al., 2017) raw data from individual wells (tissues or flies) were first detrendend, using a running average with a 24 h window. After subtracting the resulting trend line from the raw data, the DD part of the data are subjected to a sinus fit operation. The parameters of the resulting curve include a period estimate (Tau in hr), along with a dimension less 'Error' value, depicting the correlation between the curve fit and the detrended data (1 minus correlation coefficient, so the lower the error value the more trustable the period value). For this study, we applied a cut-off of <0.5 for the error value for all tissue culture experiments and <0.7 for the experiments with adult flies and only samples meeting these criteria were included in the average calculations for 'Tau' and 'Error' plotted in Table 1. In addition, ChronoStar determined the mean expression level for the entire time series of each well, and the average 'Mean' is also tabulated in Table 1. Furthermore, the individual values for 'Tau' and 'Mean' were used for the estimation graphics and statistical tests (see below). To generate line and scatter graphs raw and curve fitted averaged time series were plotted in Excel and figures generated in Inkscape.

Estimation graphics and statistical tests

For the statistical analysis of the data, estimation statistics has been used. This approach gives a more informative way to analyze and interpret results (Claridge-Chang and Assam, 2016). It focuses on the effect size of one's experiment, as opposed to significance testing. While significance testing (p-values) focus on the acceptance or rejection of the null hypothesis, estimation stats focus on the magnitude of the effect size (i.e. mean difference) and its precision (Ho et al., 2019).

Data were analysed using DABEST (Ho et al., 2019), using the website available under https://www.estimationstats.com/#/. In order to compare the different groups of data at the same time, providing an overall view, a shared-control plot was used, which is analogous to an ANOVA with multiple comparisons (Ho et al., 2019). This plot is divided in two parts. First, the top part shows all the observed data points, showing the underlying distribution, right to every data set the mean and standard error are plotted as a discontinuous line, the gap indicates the observed mean. The bottom part shows the effect size (mean difference) as a bootstrap 95% confidence interval (CI) with BCa correction (Efron, 1979). Here the magnitude of the mean differences between groups can be easily compared with the reference. For all our plots the most left data set (18°C) is considered the reference and all mean differences are plotted relative to it. The size of the CI shows the precision of the mean difference. The smaller it is the more confident is the measure. The further apart the mean difference (black dot inside CI) is from the reference line, the more difference exists between the datasets (p value < 0.05). If the CI is cutting the reference line then, both datasets may originate from the same distribution. Therefore, they are not truly different (p value > 0.05).

Supplemental References

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