Light Dominates Peripheral Circadian Oscillations in *Drosophila melanogaster* During Sensory Conflict

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Abstract

In *Drosophila*, as in other animals, the circadian clock is a singular entity in name and concept only. In reality, clock functions emerge from multiple processes and anatomical substrates. One distinction has conventionally been made between a *central clock* (in the brain) and *peripheral clocks* (e.g. in the gut and the eyes). Both types of clock generate robust circadian oscillations, which do not require external input. Furthermore, the phases of these oscillations remain exquisitely sensitive to specific environmental cues, such as the daily changes of light and temperature. When these cues conflict with one another, the central clock displays complex forms of sensory integration; how peripheral clocks respond to conflicting input is unclear. We therefore explored the effects of light and temperature misalignments on peripheral clocks. We show that under conflict, peripheral clocks preferentially synchronize to the light stimulus. This photic dominance requires the presence of the circadian photoreceptor, Cryptochrome.

Keywords

Circadian rhythms, *Drosophila*, peripheral clocks, *period*, *luciferase*, Cryptochrome, multisensory entrainment, sensory conflict, circadian entrainment, sensory integration

Introduction

The intrinsic periodicity of most earthly habitats has led to the evolution of circadian clocks across all phyla. These endogenous ~24-hour oscillators allow organisms to optimize their biology for an isochronal world. For instance, in the fruit fly, *Drosophila melanogaster*, locomotor activity shows daily periodicity with a steady increase towards the evening (Allada and Chung, 2010). In both insects and mammals, these circadian systems comprise 'molecular clocks' exhibiting transcription-translation feedback loops of specific clock genes.

To be robust, it is essential that such oscillations can be re-synchronized, or 'entrained', to external cues (Zeitgebers) (Dunlap et al., 2004). Research into entrainment has largely focused on cues from single modalities. In *Drosophila*, both light:dark cycles (LD) and temperature cycles (TC) entrain locomotor activity rhythms (Wheeler et al., 1993), via light- and temperature-entrainable molecular rhythms of the core clock proteins Period (PER) and Timeless (TIM) in the central clock network (Yoshii et al., 2005; Zerr et al., 1990). A major route for photic entrainment is the light-dependent degradation of TIM, mediated by the circadian photoreceptor, Cryptochrome (CRY) (Busza, 2004; Stanewsky et al., 1998). Less is known about the mechanisms of thermal entrainment.

Circadian systems have evolved in the presence of, and are subject to, multisensory challenges. Recent work investigating the coincidence of light and temperature shows that these cues act in a cooperative manner to entrain molecular rhythms in the brain of *Drosophila* (Yoshii et al., 2009). Conversely, misalignments between LD and TC lead to reduced-amplitude PER oscillations in these clock neurons, dissociation between different light- and temperature-sensitive cell groups, and an associated loss of evening anticipation behavior (Harper et al., 2016). Together, these findings highlight the exquisite sensitivity, and thus vulnerability, of the circadian system in *Drosophila* to environmental phase relationships; tolerating only certain degrees of Zeitgeber mismatch.

Along with neurons in the central clock network of the fly brain, the circadian system includes peripheral oscillators in tissues around the body, including the retina, antenna, proboscis, leg, wing, Malpighian tubules, gut, cuticle and reproductive organs (Giebultowicz et al., 2001; Giebultowicz and Hege, 1997; Ito et al., 2008; Krishnan et al., 1999; Plautz, 1997). These peripheral clocks are diverse, displaying varying levels of autonomy and involving different forms of molecular machinery (for review, see Ito and Tomioka, 2016).

The entrainment of peripheral clocks to single Zeitgebers has also been studied. For instance, many respond directly to both light and temperature, entraining PER rhythms to LD and TC when isolated *in vitro* (Glaser and Stanewsky, 2005; Ivanchenko et al., 2001; Plautz, 1997). As in the central clock, light sensitivity of peripheral clocks appears to act via CRY, which mediates the light-dependent degradation of TIM (Ivanchenko et al., 2001; Stanewsky et al., 1998). Unlike the central clock, however, peripheral CRY may serve a dual function as a core clock component (Collins et al., 2006; Krishnan et al., 1999; Levine et al., 2002b). This latter role of CRY resembles that which is observed in mammalian systems (Okamura, 1999; van der Horst et al., 1999).

While unimodal entrainment of peripheral clocks has been demonstrated, the responses of these

oscillators in multisensory environments remains unknown. Thus, building on previous work in the central clock, we asked how peripheral circadian networks respond to conflicts between light and temperature. For this, we used a well-established *period-luciferase* fusion gene (*XLG-luc*) reporting PER expression in peripheral clocks (Veleri et al., 2003). Furthermore, given that peripheral clocks form part of a wider circadian network throughout the fly, we investigated responses *in vivo* rather than purely in isolated tissues. We show that, unlike the central clock network, peripheral clock oscillations do not collapse under sensory conflict. Instead, light dominates peripheral clock entrainment and this light dominance depends on the circadian photoreceptor, CRY.

Materials and Methods

Fly Maintenance and Stocks

Flies were reared under 12 hr:12 hr (12:12) LD cycles on *Drosophila* medium (0.8% agar, 2.2% sugar-beet syrup, 8.0% malt extract, 1.8% yeast, 1.0% soy flour, 8.0% corn flour, and 0.3% hydroxybenzoic acid) at 25°C and approximately 60% humidity. The following fly stocks were used: *XLG-luc1-1* (Veleri et al., 2003), tim^{01}*;XLG-luc1-1* (Glaser and Stanewsky, 2005), and *XLG-luc1-1* cry^{b} double mutants generated by meiotic recombination between *XLG-luc1-1* and cry^{b} bearing chromosomes. Only male flies between 3 and 6 days old were used in experiments.

Activity Monitoring

Flies were individually placed into small glass recording tubes containing 5% sucrose and 2% agar medium, which occupied approximately one third of the tube. These tubes were then loaded into MB5 activity monitors (Trikinetics, Waltham, USA), with seventeen infrared beam detectors distributed across the length of each activity tube. An interruption of the infrared light beam by the movement of a fly produced a signal, which was then recorded by a microprocessor. The number of beam breaks was recorded for each fly in 5-minute time bins and summed into bin counts. Thus, 12 activity scans were obtained for each fly per hour. Monitors were placed in a light- and temperature-controllable incubator (Percival) for the duration of the experiments.

Zeitgebers

Field studies show that temperature rises continuously (and almost linearly) throughout the day and falls in a similar fashion during the night (Vanin et al., 2012). However, apart from some recent exceptions (e.g. Yoshii et al., 2010), it has been traditional to use sharp, 'square-wave-like' transitions between cold and warm conditions to study temperature-dependent circadian entrainment. Our preliminary experiments showed that such square-wave-like temperature cycles gave rise to sharp peaks in bioluminescence readings at the transitions between cold and warm (see Fig. S1 A for examples). The fact that these peaks were also present in clock-null mutant flies (tim^{01}; Fig. S1 A, bottom trace) suggests they are extrinsic to the circadian oscillator, possibly reflecting an altered metabolic activity during sharp temperature transitions (Sehadova et al., 2009). At a conceptual level, we can draw a comparison with 'masking', which commonly describes direct stimulus-evoked (i.e. clock independent) changes in locomotor behavior at points of sharp environmental transition. In analogy, the clock-independent bioluminescence responses could be described as a form of 'molecular masking', which might well be linked to the behavioural responses. To mitigate these masking effects, we used more naturalistic, 'ramped' temperature cycles (Vanin et al., 2012), which made the transitional bioluminescence responses disappear (as is also the case with behavioral masking) (Fig. S1 A).

12:12 light-dark cycles were generated through square wave transition between ~2500 and 0 lux respectively. 12:12 temperature cycles were achieved through gradual transitions between 26°C and 16°C occurring over 9.5 hours (see Fig. S1 B,C). ZT0 denotes the beginning of an increase from 16°C to 26°C over 9.5 hours, and ZT12 denotes the start of a corresponding decrease from 26°C to 16°C.

Cue misalignment is quantified as the absolute distance, in hours (delta time, or Δt), between the onset/offset of two cyclic 12:12 signals. For example, $\Delta t_{L,T} = 3$ hr denotes that light onset/offset occurs 3 hr after the beginning of temperature rise/fall.

Environmental conditions were recorded with an environmental monitor placed inside the incubator. These were checked to validate scheduled conditions. The average activity of the population was plotted as histograms using the MATLAB Flytoolbox library (Levine et al., 2002a).

Bioluminescence Imaging

The images taken in Fig. 1 were acquired using an LV200 bioluminescence imaging system (Olympus). Flies were kept in 12:12 LD at 25°C (Fig. 1 A,B) or 12:12 naturalistic TC in DD (Fig. 1 C,D) for at least 3 days in vials containing a 1% agar and 5% sucrose food supplemented with 15mM luciferin. Individuals were then anaesthetized using ether and dry mounted under a UPLSAPO 20x Apochromat objective. Light microscopy was used to obtain a reference image of the fly. The lights were then turned off and a CCD camera (Hamamatsu ImageEM X2) was used to capture the bioluminescence signal over a 5 min exposure period. All images were processed using the same settings in FIJI (Schindelin et al., 2012)

Bioluminescence Rhythms Acquisition

Bioluminescence assays were performed as previously described (Glaser and Stanewsky, 2005). 3-6 day-old male flies were placed in alternate wells of a 96-microtiter plate (48 flies per plate). Each well contained 100μl luciferin medium (1% agar, 5% sucrose, and 15mM luciferin, (Biosynth, Switzerland)). Using a Packard TopCount Multiplate Scintillation Counter (Perkin-Elmer) that was placed in a light- and temperature-controllable incubator (Percival), the photon count per second (CPS) emitted from each well was measured every 60 minutes for 15 seconds per well, unless otherwise stated in the text. Flies were exposed to the experimental regime as specified at relevant points in Results.

If at any point in the experiment individual well emissions dropped to background levels (approximately 50-100 CPS), flies were regarded as dead, and their data were excluded from this point onwards.

Bioluminescence Data Analysis

A custom analysis toolbox was developed in MATLAB and R. Raw data were loaded into MATLAB and bioluminescence signals from living flies were selected. A mean trace of 20-40 *tim^{01} XLG-luc1-1* flies lacking a functional clock was subtracted from each individual in the *XLG-luc1-1* and *XLG-*

luc1-1 cry^{b} groups to remove components of the bioluminescence signal that were not clock-driven (Fig. S1 A; Fig. S2; Fig. S3). Individual traces were then detrended and normalized as described in (Levine et al., 2002a) to have a mean time course equal to 1, and to preserve the appearance of percentage changes for oscillations around the long-term trend line (Fig. S2).

Next, individual bioluminescence signals in the *XLG-luc1-1* and *XLG-luc1-1 cry*^{b} groups were quantified to produce an empirical distribution of values, from which the median and 95% confidence intervals were calculated in R. Non-parametric Mann-Whitney U tests were also used for statistical comparisons between genotypes and environmental conditions.

Peak phases were determined by first smoothing individual traces using a low-pass filter to remove noise (threshold = 12 hr). The mean peak phase was then measured across multiple days (see text) according to ZT_T (Fig. S2). The amplitude was measured from the smoothed trace as the mean difference between the peak and trough across multiple days (Fig. S2). Finally, autocorrelation analysis of the raw signal was used to calculate rhythm strength (RS) as the height of the third peak in the correlogram divided by the confidence interval (see Levine et al., 2002a). As the rhythm strength analysis is sensitive to the amount of data provided, exactly two days were used for all analyses of this type. Free running period was estimated as the location of the third peak in the correlogram divided by two (see Levine et al., 2002a). A mean average trace, with shaded region showing SEM, was then plotted in R for each genotype (Fig. S2).

Separate sections of the experimental regime (described in Results) were quantified as separate experimental regions of interest (ROIs). In this way, data loss resulting from fly death during the experiment only affected subsequent ROIs, leaving previous ROIs intact and thus improving the overall power of our analysis. Note that n-numbers within a genotype accordingly vary between different ROIs during a single experiment (reported in the text).

Results

Spatial Expression Pattern of the XLG-luc Transgene

We studied bioluminescence changes using the *XLG-luc* transgene. *XLG-luc* contains the endogenous *period* promoter, driving the expression of a *period-luciferase* fusion gene. The XLG-LUC protein is expressed in most, if not all, *per*-expressing cells (Veleri et al., 2003), including neurons in the fly brain (Veleri et al., 2003) as well as those in the periphery (Glaser and Stanewsky, 2005). However, the construct is widely used as a peripheral clock reporter, since signals from the central clock are expected to be overwhelmed by those from the peripheral *per*-expressing cells (Glaser and Stanewsky, 2005; Sehadova et al., 2009; Veleri et al., 2003). Evidence for this comes from the fact that rhythmic photon counts in *XLG-luc* flies are by a factor of ~250-times greater than those observed for an 8.0-*luc* transgenic line, in which luciferase expression is restricted to central clock cells (Fig. S3; Veleri et al., 2003). Nevertheless, exactly which *per*-expressing tissues predominantly contribute to the bioluminescence signal of *XLG-luc* flies is not known.

To visualize the source of the luciferase reporter signal measured in our time series assays, we performed whole-animal bioluminescence imaging on flies that had been reared in circadian light and temperature conditions (Fig. 1). *XLG-luc* transgenic flies in wild type and *cry* mutant background were imaged between ZT 19-23 and ZT 7-11 in LD and naturalistic TC, when PER expression is expected to be at peak and trough levels respectively (Glaser and Stanewsky, 2005) (Fig. S1 F,G). Bioluminescence levels varied markedly between time points for wild type flies in both LD and TC. However, a similar change in *cry* mutants was only observed during TC, consistent with a drastically weakened light input pathway in flies lacking functional CRY (Fig 1 A-D) (Glaser and Stanewsky, 2005; Stanewsky et al., 1998). This finding was confirmed in our own bioluminescence time series assays (Fig. S1 F,G).

In both genotypes, we saw an overwhelming majority of signal emanating from the abdomen and eyes, consistent with that observed in other *per-luc* transgenics (Fig 1 A, C) (Stanewsky et al., 1997). We thus conclude that *in vivo* assays monitoring bioluminescence changes in *XLG-luc* reporter lines will be dominated by signals from these peripheral clock components.

Sensory Conflict Generates P-like Behavior under Naturalistic Temperature Cycles

Recent work has shown that a 6 hr misalignment between LD and (square wave-like) TC can disrupt normal circadian locomotor patterns in Canton S wild type flies, characterized by a loss of evening anticipation. In place of this, flies display 'Plateau' (P) behavior, which features a period of sustained high activity, bordered by relative inactivity (Harper et al., 2016) (Fig. 2B). This P behavior is also associated with a breakdown of molecular oscillations in central clock neurons.

We first examined whether P behavior is present in *XLG-luc* transgenic flies during misaligned LD:TC for temperature cycles that are naturalistic. We administered an environmental regime

comprising aligned LD:TC ($\Delta t_{L,T} = 0$) for 3 days, followed by incremental 2 hr delays of LD ($\Delta t_{L,T} = 2$, $\Delta t_{L,T} = 4$) to generate what is ultimately a 6 hr misalignment ($\Delta t_{L,T} = 6$) for 3 days. Here, Δt is the absolute distance, in hours between the onset/offset of two cyclic 12:12 signals (see Methods). During aligned LD:TC, *XLG-luc* flies displayed a characteristic bimodal profile of activity, with evening anticipation and a peak coinciding with the end of photo/thermo-phase (Fig. 2C). During, 6 hr misaligned LD:TC, *XLG-luc* locomotor activity displayed P behavior as observed previously (Harper et al., 2016; Fig. 2B and 2D) – forming a broad, flat bout of activity between lights off and the beginning of falling temperature.

As expected, there were some differences in the behavioral pattern between misaligned LD:TC using naturalistic TC, compared to that using rectangular TC. *XLG-luc* flies did not exhibit the sharp activity increases at the beginning of the warm phase, nor did they show a rapid drop in activity after lights-off (compare Fig. 2 B,D). Instead they showed a smoother activity increase during the rising temperature phase, punctuated by the lights-on transition, and a similarly smooth activity decrease during the falling temperature phase after lights-off (Fig. 2D). We attribute these minor differences to the fact that we applied naturalistic temperature cycles (cf. Yoshii et al 2009). Importantly however, we did observe P behavior during misaligned naturalistic TC and LD conditions, demonstrating that the clock's behavioural output during sensory conflict is broadly similar between rectangular and naturalistic entrainment conditions.

Light Dominates Peripheral Clock Entrainment in Wild Type Flies

We next sought to investigate the response of the peripheral clock system during the P behavior induced under our conflicting 6 hr phase-shifted LD:TC. We first assessed the stability of peripheral endogenous rhythms in free running conditions (DD:26°C) after entrainment to aligned and misaligned LD:TC (Fig. 3 A,B). To investigate potentially richer forms of conflict, we conducted further experiments in which the period of free running conditions was then followed by either aligned or misaligned LD:TC (Fig. 3C; Fig. S1 D,E). These experiments were performed in *XLG-luc* flies in both wild type and cry^b mutant genetic backgrounds.

During aligned conditions, both wild type and cry^b flies displayed rhythms of bioluminescence, peaking during the night (as defined by both light and temperature) (Fig. 3 A,C; Fig. 3D left; Fig. S1 D,E). This observation agrees with previously reported PER protein oscillations in wild type flies during unimodal LD and TC entrainment (Glaser and Stanewsky, 2005), and also agrees with our own observations (Fig. S1 F,G). During 6 hr delay of LD relative to TC, peak bioluminescence in wild type flies was also delayed by approximately 6 hours (Fig. 3 B,C,D left; Fig. S1 D,E). However, a similar shift was not observed in the cry^b background, which instead showed no change in peak phase (Fig. 3 B,C,D left; Fig. S1 D,E). Together, these results indicate that peripheral PER rhythms in wild type flies entrain preferentially to light during conflicting LD:TC, whereas cry^b flies entrain preferentially to temperature. This directly contrasts results obtained in the central clock of the fly brain.

Strikingly, no significant effect of misaligned LD:TC was observed on the amplitude or rhythmicity in either wild type or *cry* mutant flies when pooling across experiments (Fig. 3D middle, right). This again contrasts findings in the central clock neurons of wild type flies under similar environmental

conflicts, in which the amplitude of PER oscillations was severely dampened during misaligned conditions (Harper et al., 2016). In fact, the amplitude of peripheral clock bioluminescence rhythms was consistently larger in wild type flies compared to cry^b mutants (Fig. 3D middle), contrasting observations made during conflict in the central clock neurons. Because we did observe a comparable degree of P behavior, suggesting a similar breakdown of PER oscillations in the central clock neurons as described previously (Fig. 2 B,D; Harper et al., 2016) we conclude that peripheral molecular clocks do not collapse during 6 hr misalignment of LD and TC, but rather synchronize to the light cue. In contrast, removal of CRY has similar consequences on both central and peripheral oscillators, rendering them preferentially sensitive to temperature cycles.

During free-running conditions, *cry*-negative flies became arrhythmic, consistent with the reported role of cryptochrome in the core clock machinery of peripheral clocks (Collins et al., 2006). *cry*-positive flies, in contrast, continued to show bioluminescence rhythms after both aligned and misaligned conditions. These free running rhythms then gradually dampened with time (Fig. 3 A-C; Fig S1 D,E), consistent with previous studies in *XLG-luc* (Veleri et al., 2003). Median free running period of wild type flies after aligned and misaligned LD:TC was 21.5 hr and 22.0 hr, respectively, with no significant difference observed between entrainment conditions. This suggests no lasting effect of sensory conflict on peripheral clock rhythms. An equivalent analysis for *cry* mutant flies was made impossible by the complete lack of free running rhythmicity.

Discussion

Sensory entrainment of circadian systems is a multimodal problem. We used a *per-luciferase* transgenic reporter to study the combination of light and temperature Zeitgebers in peripheral clocks of *Drosophila*. We showed that the responses of peripheral clocks during conflicting entrainment conditions differ markedly from those of the central clock, further highlighting the diversity within the wider circadian system.

Disruptions of locomotor behavior in wild type flies (leading to P behavior) have been shown to result from a 6 hr delay of LD relative to square-wave-like TC (Harper et al., 2016). We here report that evening locomotor behavior is also altered during equivalent misalignments under more naturalistic temperature fluctuations. Indeed, the activity profile that results from a more naturalistic form of sensory conflict (using ramped TC) closely mimics the previously reported P behavior, displaying a similar breakdown of evening anticipation, yet without the abrupt changes in activity observed previously at the end of thermo-phase and photo-phase. While conflict between light and temperature caused behavioral disruptions in *XLG-luc* control flies, we did not see an associated disruption of molecular cycling in the peripheral clocks using an *XLG-luc* reporter assay: a 6 hr misaligned LD:TC results in an equivalent phase shift of PER rhythms (i.e. peripheral clock rhythms remain synchronized to the light stimulus).

In further contrast to the central clock, the amplitude of PER oscillations is not significantly changed between aligned and misaligned conditions. Taken together, this strongly suggests that, during sensory conflict, peripheral clocks in flies entrain preferentially, perhaps exclusively, to light. Peripheral clocks thus exhibit a separate, and distinct, response to sensory conflict compared to that observed in the central clock neurons. Future work would benefit from an investigation into how this response is affected by varying environmental phase relationships.

One outstanding question, for example, is whether the peripheral clock has no effect on locomotor behavior at all. Indeed, our own finding that peripheral PER rhythms in control flies display no other change during sensory conflict than shifting their phase with the light cue, might indicate that the circadian anomalies observed during sensory conflict purely result from disruptions of the central clock. However, an alternative explanation could be that the P-like behavior we observe emerges from a discrepancy between the peripheral and central clock networks. If peripheral clocks do contribute to locomotor behavior, then activity under sensory conflict will necessarily be driven by two out-of-sync circadian networks. A potential route for future research would be to use the kinases Doubletime and Shaggy to generate period discrepancies between peripheral and central clocks, as was used previously to assess autonomy between neuronal subgroups within the central clock network (Yao and Shafer, 2014). Another option would be to simultaneously measure peripheral clock bioluminescence and activity in individual flies and investigate any subtle correlations therein (Guo et al., 2016; Khabirova et al., 2016).

Our bioluminescence imaging data shows *XLG-luc* – a previously used transgenic reporter line – is expressed primarily in the eyes and abdomen. Thus, our findings predominantly relate to peripheral

clocks located in these body parts. The peripheral circadian system, however, exhibits much heterogeneity, specifically with regard to the degree of independence from the central clock (Ito and Tomioka, 2016). It is not yet clear how our findings translate to other areas in the wider peripheral network. A similar rationale could be applied to the role of cryptochrome in peripheral clocks. In this study, we show that light dominance in the periphery depends on *cry* expression. Whether the *cry* dependence of this light dominance reflects a *cry* dependence within the peripheral clock itself or elsewhere in the circadian system, however, remains unclear.

The question of *why* the peripheral clock network might respond differently to sensory conflict, when compared to the central clock, remains unclear. The central clock is a highly interconnected network with strong coupling through the action of PDF (e.g. Lin et al., 2004). Thus, a dissociation between oscillatory components, resulting from sensory conflict, is a potential cause for the disruptions observed. Less is known about connectivity in the peripheral clock system. The resilience of these oscillators to sensory conflict may therefore hint at a more independent network architecture, with less coupling between subparts. This theory lends itself to modelling approaches; weakly coupled oscillator theory, for example, might provide a useful framework to infer coupling strengths and guide experimentation. Equally, from a more Bayesian perspective, we might ask why the central clock does not appear to coordinate peripheral gut and eye clocks during conflict. Perhaps an uncertainty in central clock oscillations, reflected in their reduced amplitude, is projected to the periphery in the form of a low precision signal, thus leading to more autonomous behavior in these peripheral clocks. Such hypotheses could explain aspects of the heterogeneity observed throughout the wider clock network. We anticipate the benefits to come from embracing these mathematical viewpoints, alongside more holistic experimental studies of circadian systems in multisensory environments.

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Author Contributions

REFH and RS conducted the bioluminescence assays. REFH conducted the behavioral assays, analyzed the data and developed the bioluminescence data analysis toolbox. MO performed the bioluminescence imaging. REFH, RS, PD and JTA designed the experiments and prepared the manuscript.

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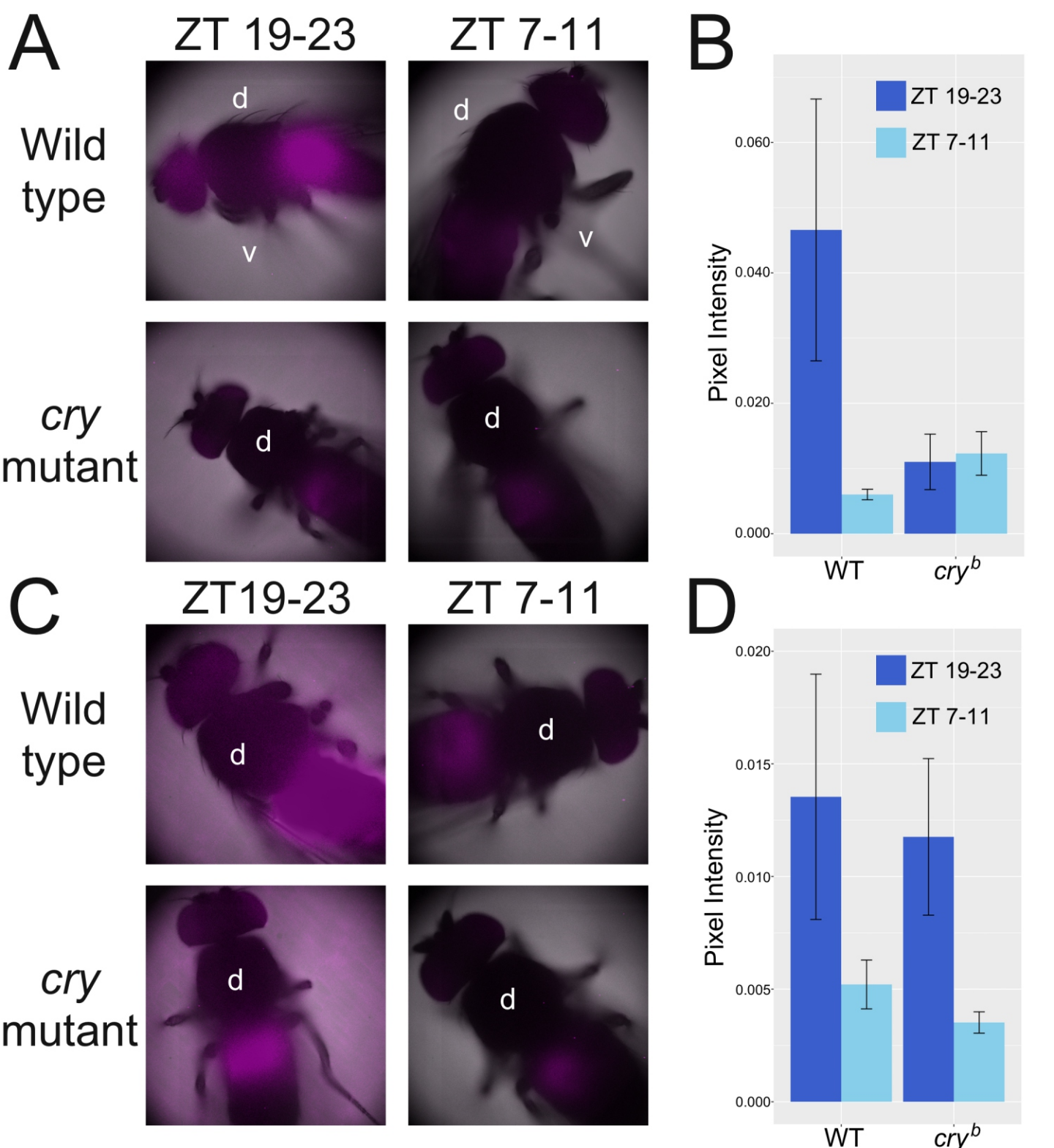
Figure Legends

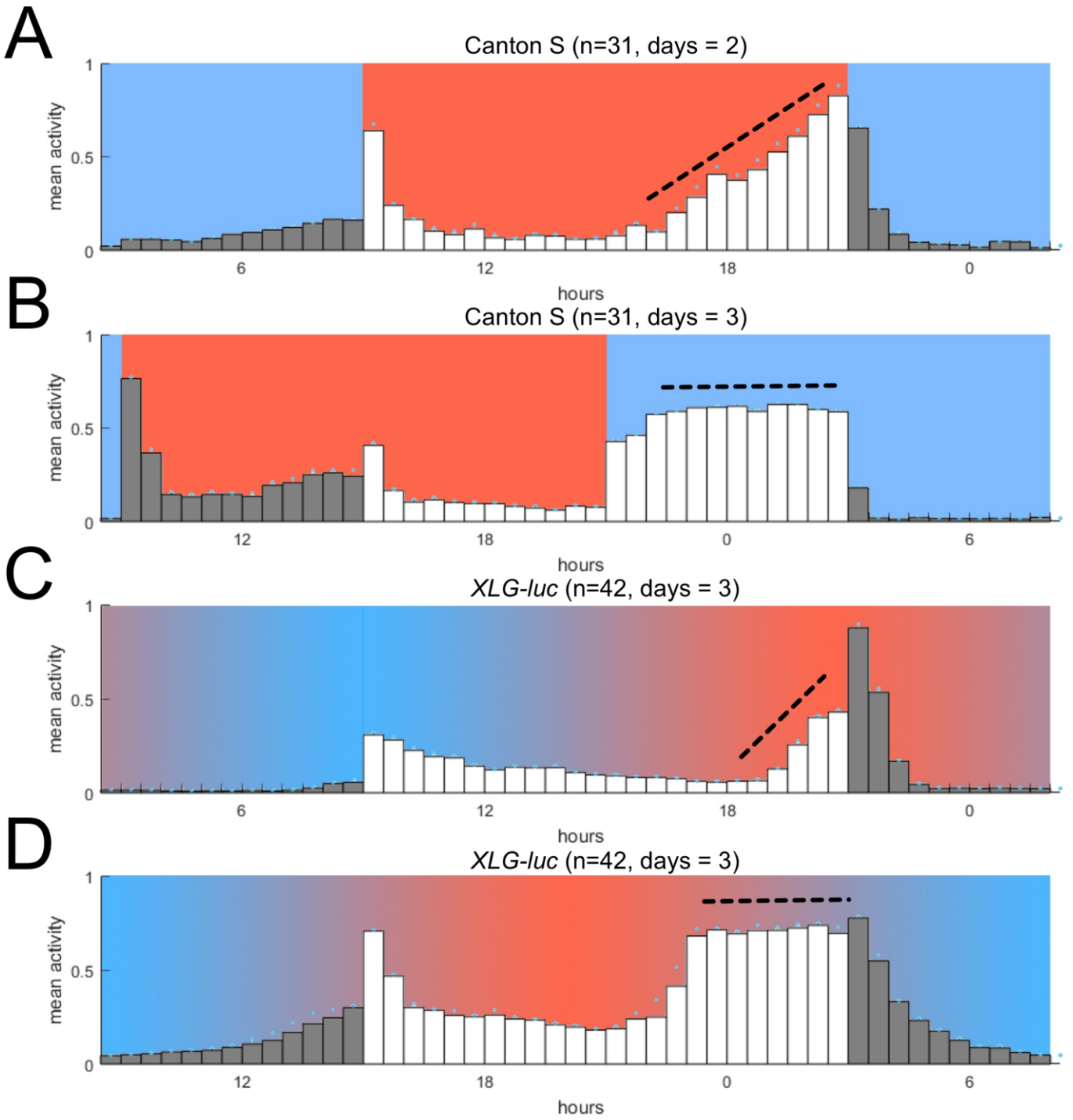
Figure 1: Bioluminescence imaging of *XLG-luc* transgenic flies. Pseudo-colour image of *XLG-luc* bioluminescence superimposed on a bright-field (black and white) reference image. Male *XLG-luc* and *XLG-luc cry^b* flies were fed on luciferin fortified food (15 mM) for 3 days in LD (A,B) or 16:26°C ramping TC (C,D) conditions, and imaged for 10min with the LV200 Bioluminescence imaging system (Olympus). Images were taken between ZT 19 to ZT 23 and between ZT 7 to ZT 11, reflecting PER peak and trough expression levels, respectively. Panels B and D show quantifications of abdominal bioluminescence intensities for both LD (B) and TC (D) entrainment regime at the different circadian times indicated. The orientation of the fly is specified by the label 'd' (dorsal) and 'v' (ventral). Note the main signal sources in the abdomen and eyes. B) /$n = 8$, and $n = 4$/ $n = 10$, for peak/trough expression in wild type and *cry* mutant flies respectively. D) $n = 10$/$n = 8$, and $n = 7$/ $n = 5$, for peak/trough expression in wild type and *cry* mutant flies respectively.

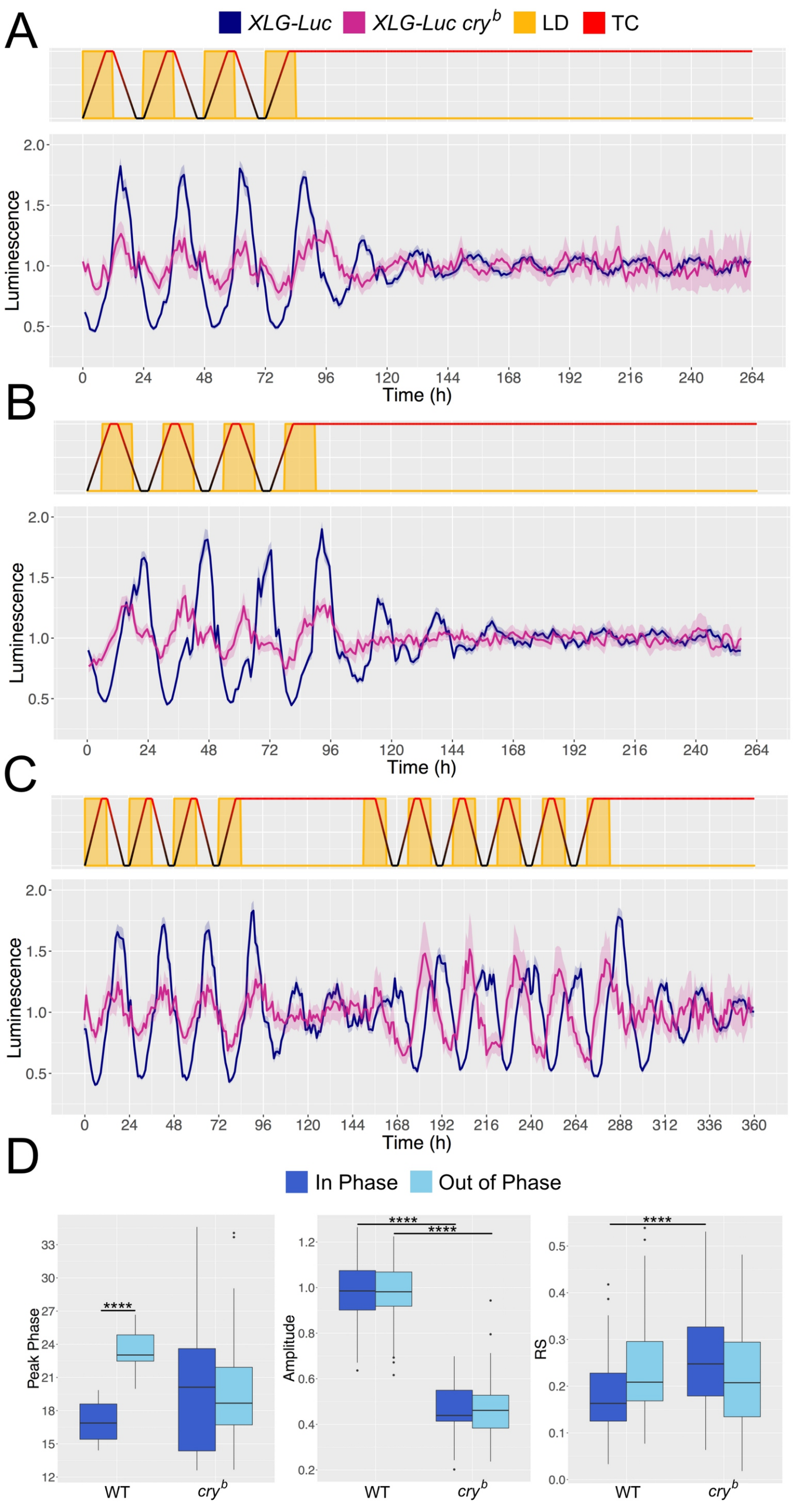
Figure 2: Locomotor behavior during sensory conflict using square-wave and naturalistic TC. A) Average locomotor activity in aligned LD:TC (lights-on coincides with temperature-on, $n = 31$). B) Average locomotor activity in 6 hr misaligned LD:TC (lights-on delayed by 6 hours relative to temperature-on, $n = 31$). C,D) New data in which a naturalistic temperature regime was used to generate sensory conflict. C) Average locomotor activity in aligned LD:TC (lights-on coincides with the start of temperature-rise, $n = 42$). D) Average locomotor activity in 6 hr misaligned LD:TC (LD delayed by 6 hours relative to temperature-rise, $n = 42$). Locomotor data for each plot was rescaled in the range of 0 and 1 to facilitate profile comparisons. Mean raw activity across days (total beam breaks/5min/fly) was 13.3 (A), 11.8 (B), 10.4 (C), and 17.9 (D). Black dashed lines highlight presence/absence of evening anticipation. (Data in A,B adapted from Harper et al. 2016 in which a square-wave temperature regime was used to generate sensory conflict).

Figure 3: Bioluminescence recordings. Dark blue and magenta lines show mean average of *XLG-luc* and *XLG-luc, cry^b* flies respectively. Shaded regions show SEM. A) Experimental regime in which environmental conditions followed 4 days of LD:TC in phase, and 7 days of free run in DD at 26°C. ($n = 43 - 46$ and $n = 15 - 30$ for wild type and *cry* mutants respectively). B) Experimental regime in which environmental conditions followed 4 days of misaligned LD:TC, and 7 days of free run in DD at 26°C. ($n = 47 - 48$ and $n = 28 - 35$). C) Experimental regime in which environmental conditions followed 4 days of LD:TC in phase, 2 days of free run in DD at 26°C, 6 days of misaligned LD:TC via 6h delay of LD, and finally 3 days of free run at DD and 26°C. ($n = 37 - 44$ and $n = 7 - 14$ for wild type and *cry* mutants respectively). All bioluminescence readings recorded at a resolution of 1 hr. D) Quantification of bioluminescence signal peak phase (Left), amplitude (Middle) and rhythmicity (Right) during aligned and misaligned LD:TC. Analysis of the aligned condition used adult flies taken from the last full two days of aligned LD:TC (Fig. 3A and 3C) ($n = 80$ and $n = 22$ for wild type and *cry* mutants respectively). Analysis of the misaligned condition used flies taken

from the last full two days of misaligned LD:TC (Fig. 3B and Fig. S1D) ($n = 91$ and $n = 47$ for wild type and *cry* mutants respectively). Box plots show 1st, 2nd and 3rd quartiles of the data, with the upper and lower whiskers extending to $\pm$IQR from the 3rd and 1st quartiles respectively. Data points beyond the whiskers are plotted as outliers. Mann-Whitney U test used to compare between condition and genotype ($p < 0.00001$ shown by ****).


A
ZT 19-23
ZT 7-11
Wild type
cry mutant
d
v
B
ZT 19-23
ZT 7-11
Pixel Intensity
0.060
0.040
0.020
0.000
WT
cry^b
C
ZT19-23
ZT 7-11
Wild type
cry mutant
D
ZT 19-23
ZT 7-11
Pixel Intensity
0.020
0.015
0.010
0.005
0.000
WT
cry^b


A
Canton S (n=31, days = 2)
mean activity
1
0.5
0
6
12
18
0
hours
B
Canton S (n=31, days = 3)
mean activity
1
0.5
0
12
18
0
6
hours
C
XLG-luc (n=42, days = 3)
mean activity
1
0.5
0
6
12
18
0
hours
D
XLG-luc (n=42, days = 3)
mean activity
1
0.5
0
12
18
0
6
hours


A
XLG-Luc
XLG-Luc cry^b
LD
TC
Luminescence
Time (h)
B
Luminescence
Time (h)
C
Luminescence
Time (h)
D
In Phase
Out of Phase
Peak Phase
Amplitude
RS
****
WT
cry^b

Light Dominates Peripheral Circadian Oscillations in *Drosophila melanogaster* During Sensory Conflict

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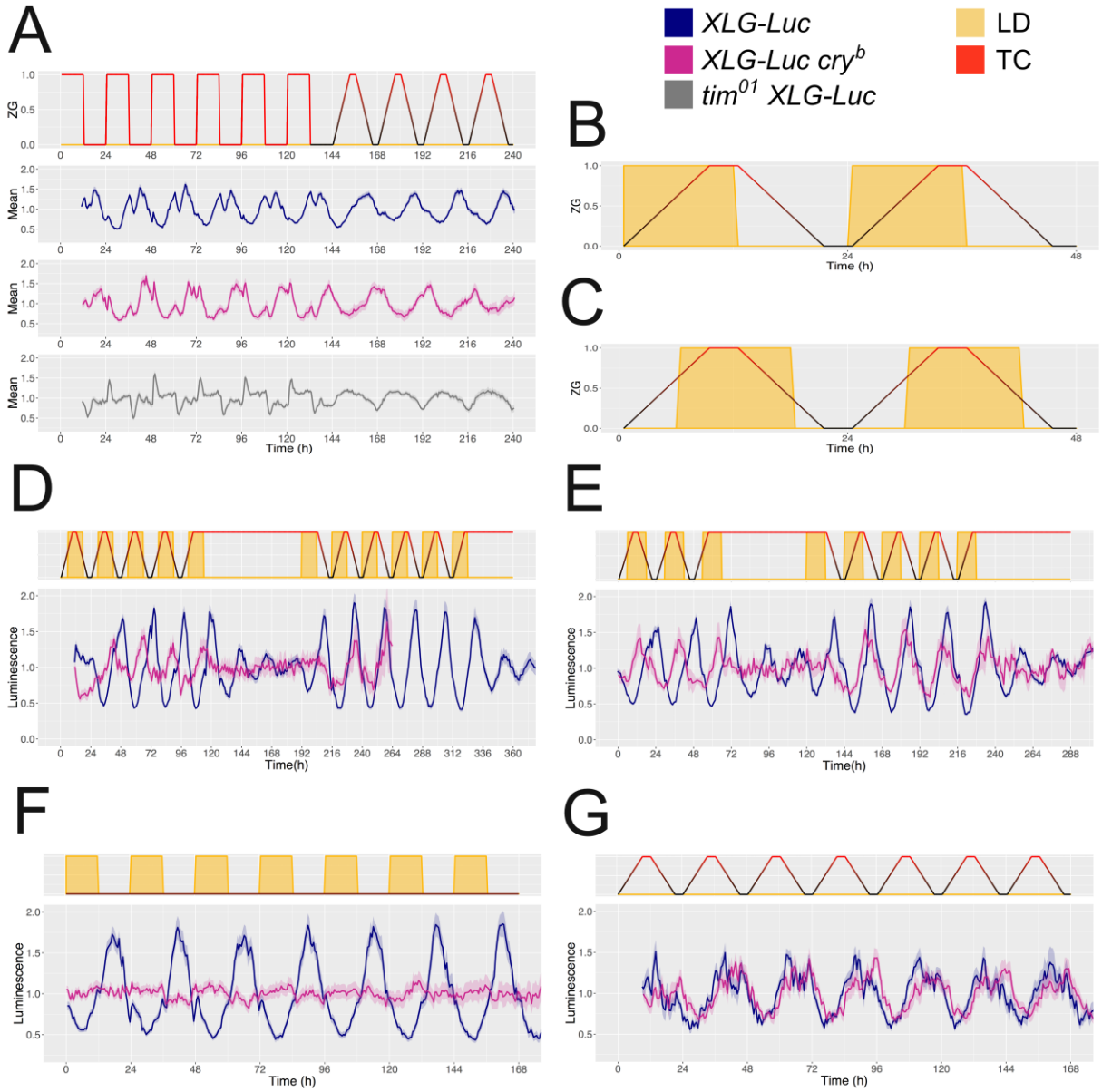
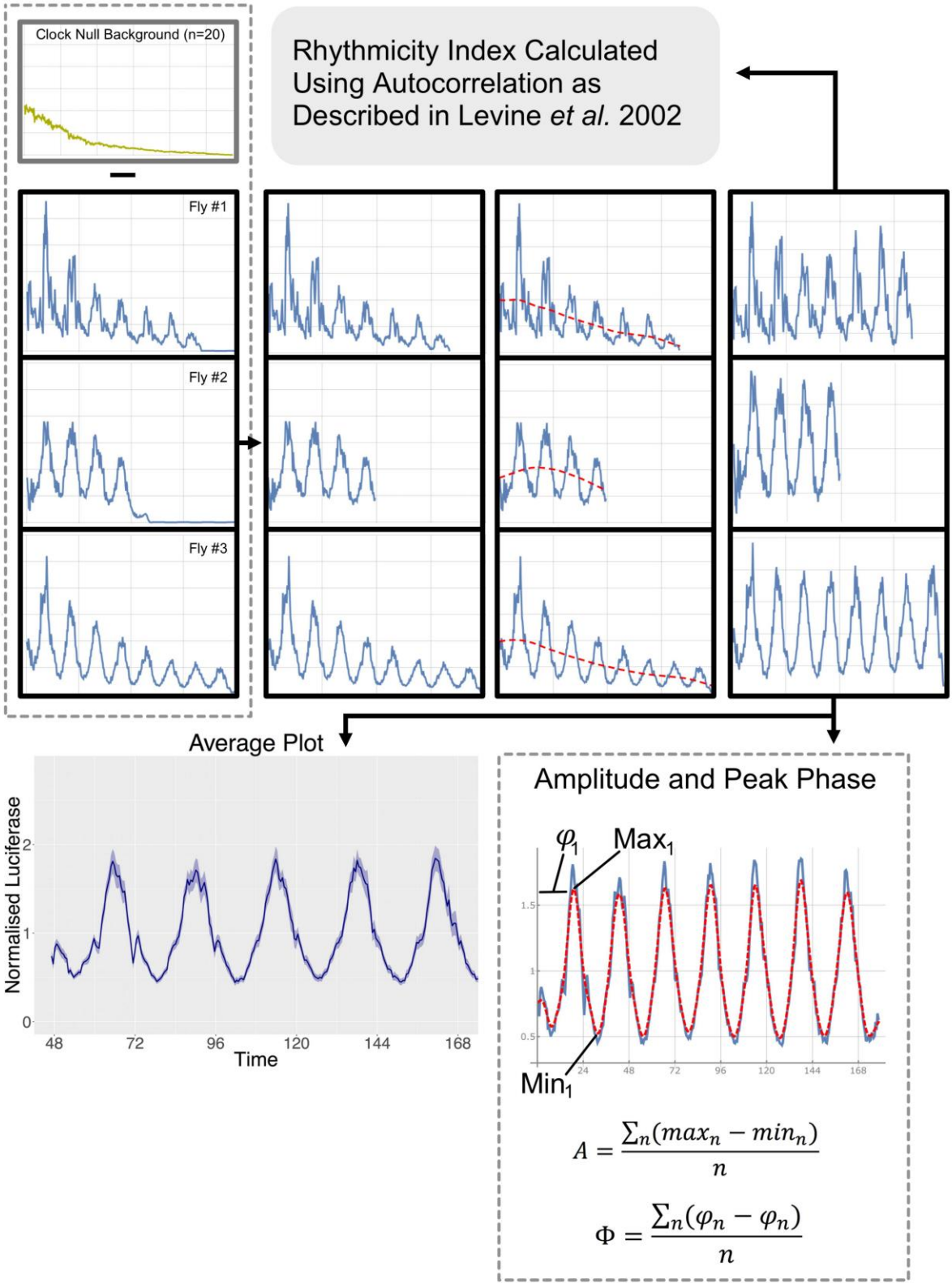
Supplementary Online Material

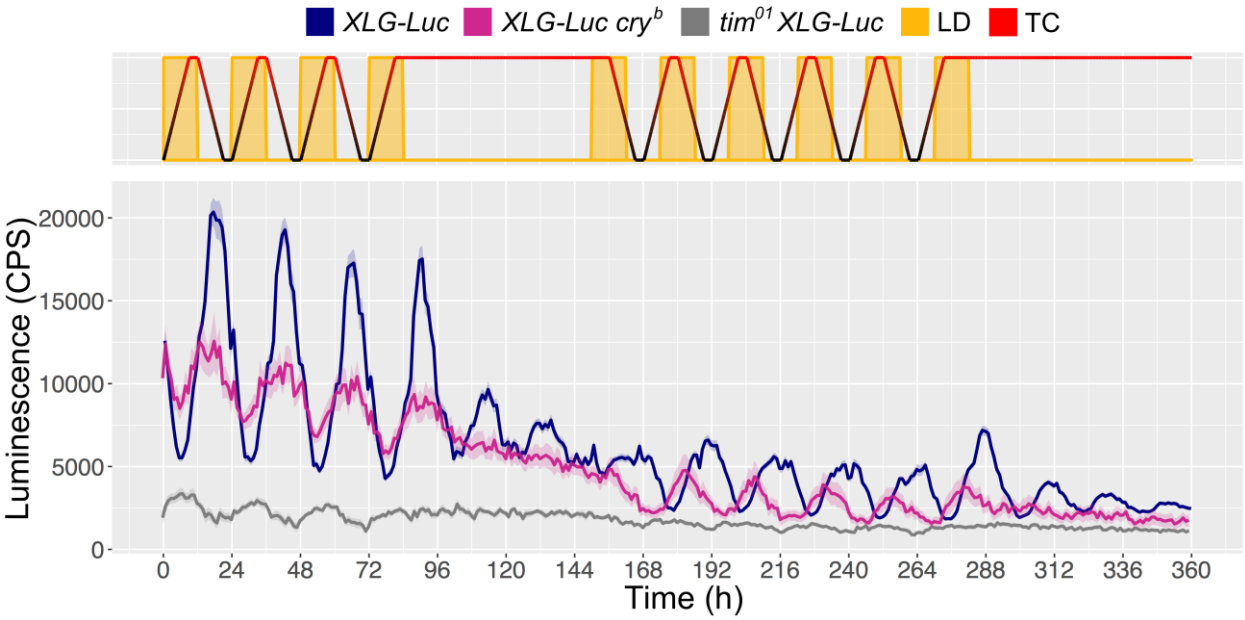
-Supplementary Figures-



Supplementary Figure 1: Bioluminescence data analysis workflow. Raw data from individual wells (flies) were extracted in MATLAB. A mean average trace of a clock null mutant (*tim*^{01} *XLG-luc1-1*) was obtained, and this was subtracted from each individual in the test genotypes (*XLG-luc* and *XLG-luc cry*^{b}). Where emissions drop to background levels (50-100 CPS) flies were deemed to have died and data from this point onwards were excluded from subsequent analysis. Each individual signal was then detrended and normalized as described in Levine et al. 2002a by applying a low-pass filter to extract the long-term trend and dividing each point in the raw trace by this baseline. Peak phase, amplitude, and rhythmicity was then measured from these normalized signals as shown, and a mean average was plotted in R with shaded region showing SEM.


Clock Null Background (n=20)
Rhythmicity Index Calculated Using Autocorrelation as Described in Levine *et al.* 2002
Fly #1
Fly #2
Fly #3
Average Plot
Normalised Luciferase
Time
Amplitude and Peak Phase
φ_1
Max_1
Min_1
$A = \frac{\sum_n (max_n - min_n)}{n}$
$\Phi = \frac{\sum_n (\varphi_n - \varphi_n)}{n}$

Supplementary Figure 2: Bioluminescence recordings. Dark blue, magenta and grey lines show mean average of *XLG-luc*, *XLG-luc cry^{b}* and *XLG-luc tim^{01}* flies, respectively. Shaded regions show SEM. A) Experimental regime in which 5 days of square-wave-like TC were followed by 4 days of ramped TC (16:26°C and DD in both cases, see upper panel). ($n = 30$, $n = 16$ and $n = 24$ for wild type, *cry* mutant, and *tim* null flies respectively). Note the marked changes in bioluminescence responses between the two entrainment regimes, which can be seen across all three experimental lines. B) Experimental conditions showing aligned LD and TC. C) Experimental conditions showing misaligned LD and TC. D) Experimental regime in which environmental conditions followed 5 days of misaligned LD:TC via 6h delay of LD, 2 days of free run in DD at 26°C, 6 days of aligned LD:TC, and finally 2 days of free run at DD and 26°C. ($n = 41 - 44$ and $n = 8 - 19$ for wild type and *cry* mutants respectively). *XLG-luc cry^{b}* flies died during the experiment, leading to incomplete data for this genotype. E) A repeat of (D) where fewer days out-of-phase condition were used to improve chances of survival for the duration of the experiment ($n = 43 - 48$ and $n = 7 - 10$ for wild type and *cry* mutants respectively). F) Experimental regime in which environmental conditions followed 7 days of LD at 26°C. ($n = 18$ and $n = 22$ for wild type and *cry* mutant flies respectively). Median peak phase of *XLG-luc* flies was at ZT 18.21; no discernible peak phase for *XLG-luc cry^{b}*. G) Experimental regime in which environmental conditions followed 7 days of ramped TC (16:26°C) in DD ($n = 17$ and $n = 22$ for wild type and *cry* mutants respectively). Median peak phase of *XLG-luc* and *XLG-luc cry^{b}* was at ZT 16.65 and ZT 18.83 respectively. A,F,G) Bioluminescence readings acquired at 30 min resolution.



Supplementary Figure 3: Raw bioluminescence recordings of *XLG-luc*, *XLG-luc cry^b* and tim^{01} XLG-*luc* flies.

Raw bioluminescence signals recorded in Fig. 3C prior to processing described in Fig. S1. Dark blue, magenta and grey lines show mean average of *XLG-luc* ($n = 43 - 44$), *XLG-luc cry^b* ($n = 8 - 16$) and *XLG-luc tim^{01}* ($n = 12 - 13$) flies, respectively. Note the comparably weak signal in *XLG-luc tim^{01}*, as well as the minor fluctuations in response to the changing temperature conditions (see Methods for discussion).