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Parametric effects of light acting via multiple photoreceptors contribute to circadian entrainment in *Drosophila melanogaster*

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Circadian rhythms in physiology and behaviour have near 24 h periodicities that must adjust to the exact 24 h geophysical cycles on earth to ensure adaptive daily timing. Such adjustment is called entrainment. One major mode of entrainment is via the continuous modulation of circadian period by the prolonged presence of light. Although *Drosophila melanogaster* is a prominent insect model of chronobiology, there is little evidence for such continuous effects of light in the species. In this study, we demonstrate that prolonged light exposure at specific times of the day shapes the daily timing of activity in flies. We also establish that continuous UV- and blue-blocked light lengthens the circadian period of *Drosophila* and provide evidence that this is produced by the combined action of multiple photoreceptors which, includes the cell-autonomous photoreceptor *cryptochrome*. Finally, we introduce ramped light cycles as an entrainment paradigm that produces light entrainment that lacks the large light-driven startle responses typically displayed by flies and requires multiple days for entrainment to shifted cycles. These features are reminiscent of entrainment in mammalian models systems and make possible new experimental approaches to understanding the mechanisms underlying entrainment in the fly.

1. Introduction

Circadian rhythms are ubiquitous endogenous rhythms in behaviour, physiology and metabolism. Such rhythms 'free-run' in the absence of cyclic environments at periodicities close to but significantly different from 24 h. To achieve optimal daily timing on a rotating planet, these rhythms must be coordinated with the exact 24 h rhythms of the earth's geophysical cycles, which include daily changes in light and temperature. Such coordination, called entrainment, enables organisms to restrict their daily activities and other biological processes to adaptive times of the day and to anticipate predictable daily changes in the environment [1–4]. Poor entrainment contributes significantly to many health problems in modern societies, making entrainment a central problem to circadian biology and general well-being [4].

Organisms are thought to entrain to cyclic environments using two distinct processes [1,5]. In the first, referred to as non-parametric, environmental transitions instantaneously reset the phase of the circadian clock, much like watches being reset upon the switch to daylight saving time [1,6]. In the second, referred to as parametric, the continuous action of light serves to speed-up or slow-down the angular velocity of internal clocks in a time-of-day-dependent manner [1,5,7]. Real-world entrainment likely involves both parametric and non-parametric processes [5] and understanding both processes

is critical if we are to understand the entrainment of the circadian system and how clocks operate under both natural and artificial light environments.

Both parametric and non-parametric effects of light have been incorporated into unified models of circadian entrainment, including for *Drosophila*, an organism that offers a powerful model system for understanding the neuronal and molecular basis of light entrainment [8–10]. However, the fly's circadian system does not maintain robust endogenous circadian rhythms under constant light (LL), making the mechanisms of parametric entrainment difficult to address. This is because of CRYPTOCHROME (CRY; blue light photoreceptor) mediated degradation of TIMELESS (TIM), a core component of the molecular circadian clock [11] (reviewed in [12]). Indeed, very few studies have examined the parametric effects of light on the fly clock. Free-running rhythms have been observed for *Drosophila*, both in *cry* loss of function mutations, which maintain rhythms in LL [13–16] and in small numbers of wild-type flies [17,18]. These studies suggest that LL conditions lengthen the clock's endogenous period relative to constant darkness (DD). However, the sensitivity of the fly clock to light means that LL conditions most often cause arrhythmicity or internal desynchronization in wild-type strains [15] and this has limited the utility of the fly as a model for understanding parametric light entrainment. For this reason, we have sought to develop approaches that allow us to use the powerful fly model to probe the parametric effects of light.

Using various light regimes (see Methods), we show that the timing of locomotor activity under standard laboratory conditions is strongly shaped by long durations of light at specific times of the day. This suggested that speed of the clock regulating these rhythms must be modulated by time-of-day-dependent continuous effects of light. In our search for LL conditions that support robust free-running rhythms in the fly, we find that LL regimes in which UV and blue wavelengths have been blocked perform remarkably well and produce lengthened circadian periods (slower running clocks). We also show that external photoreceptors and the blue light circadian photoreceptor *cry* differentially govern the parametric effects of light under these conditions. Finally, we define light regimes that entrain behavioural rhythms in a manner that fails to induce the confounding startle responses associated with widely used LD12 : 12 cycles (12 h of light and 12 h of darkness) and that, as in mammalian systems, such regimes require multiple cycles for re-entrainment to shifted light cycles. These results reveal important features of entrainment in *Drosophila* and establish a useful new approach to understanding light resetting in this important model organism.

2. Methods

(a) Fly stocks

The following fly lines were used in this study: *Canton-S* (*CS*; Bloomington Drosophila Stock Center (BDSC) stock number: 64349), white-eye (w^{1118}) [19], yellow-white (*yw*; BDSC stock number: 1495), $yw;;cry^{OUT}$ [20], $w;;glass^{[60j]}$ [13],$+;;norpA^{[7]}$ (BDSC stock number: 5685),$+;;per^{01}$ (BDSC stock number: 80928) and $+;;clk^{JRK}$ (BDSC stock number: 80927). All these genotypes were maintained in bottles with corn syrup soy media (Archon Scientific; Durham, NC). Three- to five-day-old male flies were collected from bottles for loading into *Drosophila* Activity Monitors (DAM; Trikinetics, Waltham, MA, USA). Sample sizes and replication for each experiment and the environmental conditions experienced before behavioural experiments began are described separately for each experiment below.

(b) Entrainment of locomotor activity rhythms under skeleton photoperiods

(i) Fly lines, recording conditions and light conditions

CS, w^{1118} and *yw* males were used in these experiments. Flies were maintained under white light LD12 : 12 before being loaded into locomotor tubes for one cycle after being loaded into the DAM systems before being transferred to the different skeleton photoperiod conditions. Approximately 32 flies of each genotype were collected under CO_2 anaesthesia and gently placed into 5 mm glass capillary tubes containing sucrose-agar media, which was sealed with paraffin wax at the media containing end and plugged with a short length of white yarn. Fly-containing tubes were then loaded into DAMs and secured with rubber bands woven between tubes. Infrared beam crossings were recorded every minute for the duration of the experiment. Our symmetric skeleton photoperiod (SPP; figure 1*a*), consisted of 30 min pulses starting and ending at dawn (Zeitgeber Time (ZT) 00) and dusk (ZT 12) of the LD cycle used during rearing, respectively. Additionally, we used two asymmetric skeleton photoperiods (aSPP), the first of which consisted of a dawn light pulse lasting 6 h with a 30 min pulse ending at dusk (aSPP-1; figure 1*a*), and the second of which consisted of a dusk light pulse lasting 6 h and a 30 min pulse starting at dawn (aSSP-2; figure 1*a*). All these conditions employed the standard broad spectrum white light produced by the incubators without the use of any wavelength filters. These incubators were programmed to run at a constant 25°C. The light intensity for all these experiments was 400–500 lux, which approximates to approximately 228–285 µW cm^{-2}.

(ii) Statistical analyses

The first step in the analysis of these data was to identify flies that were entrained versus flies that were free-running. Owing to the strong startle responses associated with step-function cycles, especially under the skeleton photoperiod conditions, it was not possible to use raw data to perform periodogram analyses and estimate period values. This is because startles to transitions (figure 1*a*) cause a spike in activity at recurring 24 h intervals which produce strong 24 h signals in standard periodogram analyses, even when the non-startled component of activity appear to be free-running. Therefore, we used the Savitzky–Golay (SG) filter with a filter order of 2 and filter frame length of 251 min to smooth the raw data, to minimize the contributions of startle to the periodogram results. We then used the χ^2 periodogram on the filtered data and estimated period values. Flies that had period values between 23.67 h and 24.33 h were considered entrained, and the ones outside this range were considered free-running. These values were chosen because they're the two closest points to 24 h (on either side) in our χ^2 periodogram analysis; this enabled us to include flies that entrain but have periodicities that may be slightly wavering around 24 h. We note that only approximately 10% of flies under DD display exactly 24 h periodicities. Thus, the likelihood of finding exactly 24 h free-running flies under these light conditions is very small. Though we must acknowledge that some small proportion of flies that we considered entrained may have been free-running, a period of 24 h with reproducible phase-relationships with the zeitgeber across experiments amounts to strong evidence of entrainment.

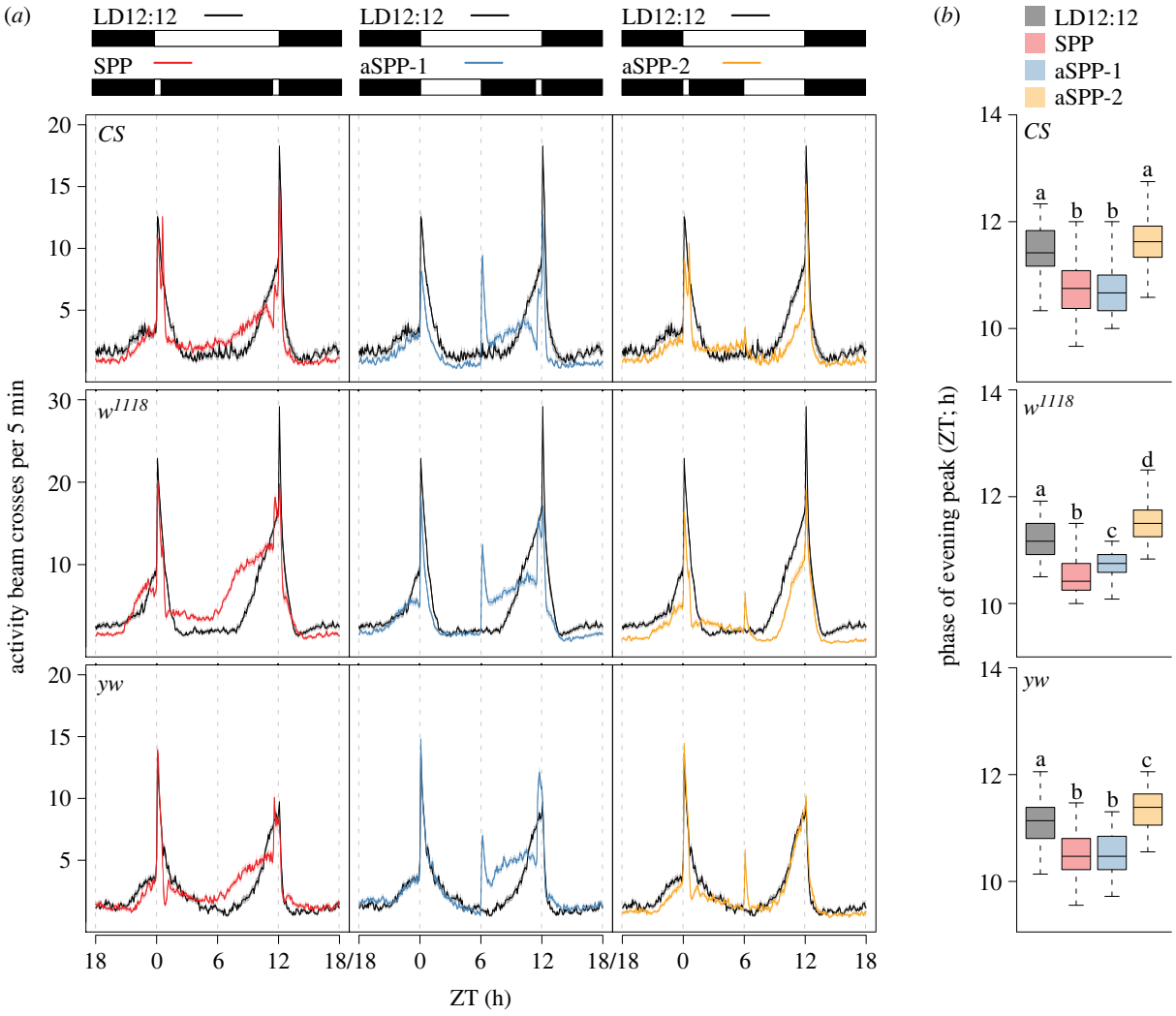

Figure 1. Entrainment to white light skeleton photoperiods. (*a*) Mean (±s.e.m.) locomotor activity profiles of *CS* (top), w^{1118} (middle) and *yw* (bottom) flies under symmetric skeleton photoperiods (left) and two asymmetric skeleton photoperiods (middle and right). The photoperiodic regimes are shown on top of the profile plots. Black shaded regions indicate darkness and white regions indicate light phases (400–500 lux) of the 24 h cycle. Note that the profiles under LD12 : 12 are replotted across panels to facilitate pair-wise comparisons. (*b*) Phases of the evening peak of activity for the three strains across the different photoperiodic conditions are shown. Boxplots that share the same letter are not statistically significantly different from each other. Statistical comparison of phases across all four photoperiodic conditions were done using Kruskal–Wallis tests. *CS*: $\chi^2_3 = 95.05$, $p = 0$; w^{1118}: $\chi^2_3 = 123.47$, $p = 0$; *yw*: $\chi^2_3 = 113.58$, $p = 0$. Profiles and phases of evening peak of activity are pooled from two independent replicate runs for each genotype.

Profiles of activity were averaged across only the flies that were categorized as entrained. Phases of evening peaks of activity were estimated by applying the SG filter on raw data of entrained flies only. For this purpose, the filter order was kept at two, but the filter frame length was reduced to 81 min so that small nuances of peaks could be identified. For each genotype, the effect of light environment on phases of evening peaks of activity were assessed by Kruskal–Wallis tests followed by Bonferroni corrections to account for multiple comparisons from the *agricolae* package for R [21]. All the analyses were carried out using custom R scripts.

(c) Assessment of free-running period under constant light

(i) Fly lines, recording conditions and the constant light paradigm

CS, w^{1118}, *yw*, cry^{OUT}, $glass^{[60j]}$ and $norpA^{[7]}$ males were used in these experiments. Approximately 32 flies of each genotype were collected and loaded into locomotor tubes as described above. Infrared beam crossings were recorded every minute for at least 10 days under DD or LL in Percival incubators (Model: LED-30HL1, Geneva Scientific, Fontana, WI, USA) at 25°C. Data from at least two independent runs are reported. See electronic supplementary material, table S1 for sample sizes and number of replicate runs.

Given that wild-type *Drosophila* are arrhythmic under white LL, we sought to establish light conditions under which wild-type flies maintain robust free-running rhythms. The arrhythmicity of flies under white LL is driven by the CRY mediated TIM degradation [22,23]. CRY is maximally sensitive to blue and UV wavelengths of light [24]. We therefore used multiple light filters which had varying degrees of overlap with the CRY action spectrum (electronic supplementary material, figure S1A). We used filters to produce constant green (Filter number: 139), orange (Filter number: 105), true yellow (Filter number: 101) and UV- and blue-blocked (Filter number: 821) light (Lee Filters, UK; http://www.leefilters.com/). We created red LL using the in-built red LED in our Percival incubators (electronic supplementary material, figure S1A). Light intensities between 6 and 12 µmol m^{-2} s^{-1} were used for this colour series. For examining the dose-dependent effect of UV- and blue-blocked light, we used three intensities: 14–18 (approx. 16) µW cm^{-2}, 28–32 (approx. 30) µW cm^{-2} and 47–53 (approx. 50) µW cm^{-2} at 600 nm. We used the S120C sensor and the PM100D console from Thorlabs (Newton, NJ; https://www.

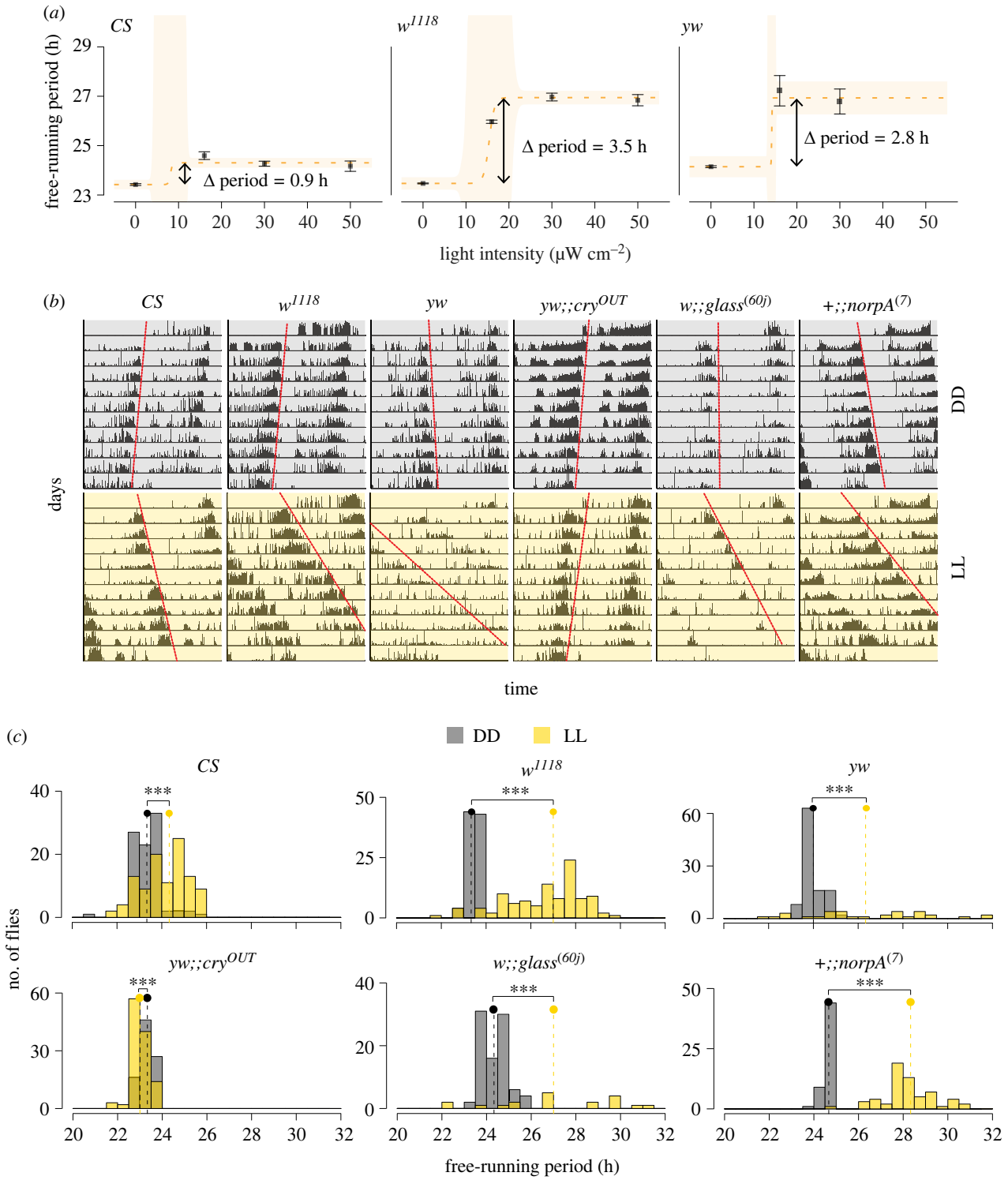

Figure 2. Free-running locomotor activity rhythms of flies under constant UV- and blue-blocked light. (*a*) Dose-dependent effect of constant light on the free-running period of *CS* (left), w^{1118} (middle) and *yw* (right) flies. The lack of data point for highest intensity of light in *yw* flies is because flies were arrhythmic under this condition. The yellow dashed line is dose response curve fitted through a logistic model (see Methods). ANOVAs on the fitted logistic model revealed a statistically significant effect of intensity on the free-running period for all three genotypes (*CS*: $p < 2 \times 10^{-16}$; w^{1118}: $p < 2 \times 10^{-16}$; *yw*: $p < 2 \times 10^{-16}$). (*b*) Representative actograms of *Canton-S* (*CS*), w^{1118}, *yw*, *yw;;cry*^{OUT}, *w;;glass*$^{[60j]}$ and *+;;norpA*$^{[7]}$ flies under constant darkness (top) and constant UV- and blue-blocked light (bottom) of 30-45 μW cm^{-2}. (*c*) Frequency distributions of free-running periods of all genotypes under constant dark and constant light conditions. The dots and dashed vertical lines represent the median values of free-running periods under each regime. Statistical comparisons are based on Wilcoxon's tests (*CS*: $W = 2676.5$, $p = 6.036 \times 10^{-08}$; w^{1118}: $W = 502$, $p = 2.2 \times 10^{-16}$; *yw*: $W = 618.5$, $p = 1.583 \times 10^{-08}$; *yw;;cry*^{OUT}: $W = 7391$, $p = 2.233 \times 10^{-08}$; *w;;glass*$^{[60j]}$: $W = 305$, $p = 1.092 \times 10^{-05}$; *+;;norpA*$^{[7]}$: $W = 10$, $p = 2.2 \times 10^{-16}$). Values are pooled from at least two independent runs (see electronic supplementary material, table S1).

thorlabs.com/) to measure single-wavelength light intensities. Comparisons of period for various genotypes reported in figure 2*b* and *c* (and electronic supplementary material, figure S2) under UV-and blue-blocked light were done after assaying flies under intensities of 30–45 μW cm^{-2} at 600 nm.

(ii) Statistical analyses

Free-running periods and corresponding rhythmic power values were estimated using the χ^2 periodogram. Period and power values were pooled from all replicate runs and were compared using Wilcoxon's tests for two independent samples.

Dose–response analyses were carried out using functions from the *drda* package [25] on R using logistic models. The logistic model is a form of nonlinear (sigmoidal) growth curve that was fit with our observed data using the default four-parameter function. An ANOVA on the fitted model then tests the model against a null hypothesis of a flat line representing the absence of light intensity-dependent changes in the free-running period. All analyses were carried out on R.

(d) Entrainment to ramped light cycles

(i) Fly lines, recording conditions and light conditions

CS, w^{1118}, *yw*, cry^{OUT}, $glass^{l60j1}$, $norpA^{[7]}$, per^{01} and clk^{JRK} males were used in these experiments. Flies were reared under ramped UV- and blue-blocked light cycles in Percival incubators (Model: LED-30HL1, Geneva Scientific, Fontana, WI, USA) at a constant temperature of 25°C. Approximately 32 males of each genotype were collected for each run and loaded into locomotor tubes. Ramping light cycles were generated such that there was no darkness at any point in the cycle. We programmed gradual light increases from approximately 10 µW cm^{-2} to 40–50 µW cm^{-2} (measured at 600 nm) (ZT00-ZT12) followed by gradual light decreases back to approximately 10 µW cm^{-2} during the next 12 h to complete the cycle. We settled on these light cycles based on our desire to avoid distinct jumps in light intensity from complete darkness, which produce large startle responses, and to reduce the possibility of any stark transition in light intensity that could induce non-parametric clock resetting. We recorded beam crossings, as described above, under the ramped light cycles for 9 consecutive days, at the end of which flies were kept under constant light starting from the nadir of the ramped cycle (approx. 10 µW cm^{-2} intensity). If entrained, flies must have a similar phase of locomotor activity on the first day under constant conditions as they did during entrainment—a phenomenon referred to as phase-control [2].

(ii) Statistical analyses

Owing to the novelty of this light regime, it was necessary to assess if flies could entrain to it at all. For each fly, we plotted an actogram over nine ramp cycles, computed a wavelet power spectrum to get daily estimates of period and plotted the standard χ^2 periodogram. The wavelet power spectrum was computed using the *WaveletComp* package on R [26] and is appropriate for use with circadian time series [27,28]. Individual flies were considered 'entrained' only if visual inspection of the actogram showed that timing of activity had stabilized by the sixth ramping cycle, if the wavelet power spectrum showed stable period values between 23.5 and 24.5 h post the sixth cycle, and/or if the χ^2 periodogram showed peaks in the same range. We used a slightly wider range of periods for the wavelets owing to the increased sensitivity of the method and consequently larger variation in detected periodicities. A fly was categorized as 'transiently entrained' or 'relatively coordinated' if there were obvious period changes during the time-course (for instance, a period that shifted from period of less than 24 h to greater than 24 h) in the actograms, if there was no period stability established by the sixth cycle based on the wavelet power spectrum, if there were multiple peaks in the χ^2 periodogram, or if flies appeared entrained for the first few cycles and then appeared to free-run. Flies were deemed 'free-running' if the actograms showed activity bouts drifting in a single direction and if the χ^2 periodogram showed peaks at period values less than 23.67 or greater than 24.33 h. Flies were considered 'arrhythmic' if there was no significant peak in the χ^2 periodogram or if there was no detectable period in the wavelet power spectrum. For loss-of-function clock mutants, flies were considered rhythmic if there was a significant peak in the χ^2 periodogram or if there was a detectable period in the wavelet power spectrum. All these analyses were carried out using custom R scripts.

Only the flies categorized as entrained (or rhythmic, in the case of loss-of-function clock mutants) were used to construct the average profiles of activity and to assess phase control under constant dim light. We estimated phases of activity peaks using the SG filter for the entrained flies under cyclic conditions and on the first day of constant dim light. We used a filter order of one and filter frame length of 41 on activity data binned in 15 min intervals to smooth the data before identifying peaks. Phase control was assessed by *V*-tests implemented through the *CircStats* package for R [29]. *V*-tests are Rayleigh's tests of uniformity on circular data that have an alternate hypothesis of a known mean angle [30]. Difference in phases of peaks between *CS* and per^{01} flies was tested using a Wilcoxon's test, also implemented on R.

3. Results

(a) Pulses of light at dawn and dusk are insufficient to mirror the entrained waveform of locomotor activity under standard LD12 : 12 conditions

One major prediction of the non-parametric (aka instantaneous) model of entrainment is that light presented at dawn and dusk are the most critical signals for entrainment, and that light during the rest of the day is largely superfluous [5,6]. If this were true of *D. melanogaster* locomotor rhythms, we would expect the locomotor activity waveform of flies be highly similar under LD12 : 12 and SPP, in which only two 30 min light pulses are provided to flies in an otherwise dark environment (figure 1*a*). Although such experiments have been done on *Drosophila* eclosion rhythms [31,32], ours is the first, to our knowledge, to report results of such experiments on *Drosophila* locomotor activity rhythms. All wild-type and genetic background control strains we examined readily entrained to SPP displaying no differences from LD12 : 12 flies in the percentage of entrained flies (electronic supplementary material, table S2).

Averaged locomotor activity profiles of *CS*, w^{1118} and *yw* flies indicate that there were distinct differences in waveforms between LD12 : 12 and SPP (figure 1*a*). We quantified the phases of evening activity peaks from smoothed data and found that median phases in all three genotypes were significantly advanced under SPP compared to LD12 : 12 (figure 1*b*). These results indicate that skeleton photoperiods do not recapitulate entrainment under LD12 : 12 and that light in the middle of the day has significant effects on the phase of evening activity.

(b) The timing and shape of the locomotor activity waveform is driven by time-of-day-dependent long durations of light

If parametric effects contribute to entrainment under step function LD cycles, it is expected that increased durations of skeleton light exposure will have measurable effects on the phase of entrainment in a manner that depends on the timing of the increased light [7,32]. The parametric model of entrainment posits that long durations of light at dawn will accelerate the clock, whereas light at dusk will decelerate it [7,33]. The prediction, therefore, is that long durations of dawn light would lead to reduced free-running period and

lead to an advanced phase of entrainment and that long durations of dusk light would lengthen free-running period and delay the phase of entrainment [34]. We subjected our flies to two aSPP, one with 6 h of light during the first half of the day and a 30 min pulse at dusk (aSPP-1; figure 1*a*), and one with a 30 min dawn pulse and 6 h of light during the second half of the day (aSPP-2; figure 1*a*). As for SPP, we first sought to determine the extent to which wild-type and genetic background strains entrain to aSPP regimes. We found that there was no significant reduction in the number of flies entraining to either of the asymmetric skeleton photoperiods for any genotypes (electronic supplementary material, table S2).

We next compared the phases of the evening peak of activity under our two aSPP conditions to those seen under LD12 : 12 and found that, for wild-type *CS* flies, there was a significant advance in the timing of evening activity when the long-duration of light was delivered at dawn (aSSP-1; figure 1*a* and *b*). Though the evening peak appeared to be delayed when long duration light was delivered around dusk, the differences in the median phases of individual flies did not reach significance (aSPP-2; figure 1*a* and *b*). Similar effects of long-duration light around dawn were observed for w^{1118} and *yw* flies (aSPP-1; figure 1) and the evening peak was significantly delayed in these strains when long duration light was delivered around dusk (aSSP-2; figure 1). The difference in phases of evening peak of *yw* flies under LD12 : 12 and aSPP-2, although significant was only approximately 15 min and is therefore barely apparent in the profiles (figure 1). Importantly, in all three genotypes, there appears to be delaying effect of light on the overall waveform of the locomotor activity rhythm under aSPP-2 (figure 1).

Taken together, these results suggest that long durations of light at the start of the day advance the clock and at the end of the day delay the clock. However, longer durations of light during the second half of the day appear to mimic the waveform under LD12 : 12 more closely. Therefore, entrained waveforms of locomotor activity under standard laboratory LD conditions are strongly shaped by continuous effects of light in a time-of-day-dependent manner.

(c) Wild-type and background strains have longer free-running periods under constant UV- and blue-blocked light

While the number of discrete transitions between light and dark are the same across the different skeleton photoperiod regimes, the timings of these transitions are different (figure 1*a*). This may therefore appear as a confound in our experimental design. However, instantaneous effects of light on the clock around mid-day (which is where our transitions occur) are almost non-existent [2,18,35,36]. Our results are therefore consistent with the speed of internal clocks being modulated by long durations, and continuous effects, of light.

The most direct method available for measuring such effects is the demonstration that free-running periods differ between LL and DD. Because the presence of the blue light circadian photoreceptor CRY renders the *Drosophila* clock arrhythmic under white LL, we assayed locomotor activity in flies under various LL conditions with differing degrees of overlap with the CRY action spectrum (electronic supplementary material, figure S1A). We assayed *CS* flies under green, orange, yellow, UV- and blue-blocked and red LL and found that flies were arrhythmic under green and yellow LL (see example actograms in electronic supplementary material, figure S1B). While many flies were arrhythmic under orange light, some flies displayed complex rhythms (electronic supplementary material, figure S1B) in which spontaneous changes in free-running period are apparent. Under red LL, flies were rhythmic and had periodicities similar to those measured under constant darkness (a little less than 24 h; electronic supplementary material, figure S1B). By contrast, we found that when the UV- and blue-components of white light are blocked, flies are robustly rhythmic with periods longer than those measured under constant darkness (electronic supplementary material, figures S1B and S2A; tables S1 and S3). Thus, the presence and speed of endogenous rhythms appear to depend on the extent of overlap between the spectrum of LL used and the action spectrum of CRY. The large overlap in the case of green and yellow LL (electronic supplementary material, figure S1A) rendered flies arrhythmic. In the case of orange LL, there is much lower overlap, and this was associated with complex rhythms in a subset of flies. The complete lack of overlap in case of red light does not seem to affect the presence or speed of the rhythm. However, the UV- and blue-blocked light, which has low but non-zero overlap with the CRY absorption spectrum and therefore likely engages CRY at a much lower rate [24], supports robust but slower rhythms. Taken together, these results suggest that CRY may play a role in the regulation of clock speed during prolonged illumination when light conditions do not drive the complete CRY-mediated loss of circadian timekeeping (see Discussion).

Before examining the role of CRY on rhythm speed, we performed dose–response analyses to examine effects of light intensity on the free-running period of the clock driving rhythms in locomotor activity. We used *CS*, w^{1118} and *yw* under three intensities of constant UV- and blue-blocked light. Fitting the logistic model indicated that there was significant light intensity-dependent change in speed of the locomotor activity rhythm in all three fly strains (figure 2*a*). However, the extent of change in rhythm speed depended on the strain used (figure 2*a–c*). While *CS* flies displayed a period lengthening of approximately 1 h, the w^{1118} and *yw* strains displayed lengthening of approximately 3–3.5 h (figure 2*a–c*). We also examined the strength of these rhythms under LL and DD and found that w^{1118} and *yw* flies had significantly lower rhythm strengths under LL (electronic supplementary material, figure S2A and table S1). w^{1118} and *yw* strains lack normal screening pigment in the eyes due to a loss of function mutation in the *white* gene. Our results therefore suggest that external photoreceptors may play a role in light-dependent changes in rhythm strength and speed, owing to the *white* gene's role in photoreception (e.g. [37]). Additionally, it is also likely that the absence of screening pigment in white-eyed flies, which ensures that the light entering through the lens stays within the ommatidium (discussed in [38,39]), is associated with an increase in the amount of light reaching CRY in the clock neurons within the brain, thereby affecting period lengthening under LL.

(d) Continuous effects of light are dependent on both external photoreceptors and *cryptochrome*

The *Drosophila* circadian system has two major photoreceptive systems, one mediated by the deep brain, cell-autonomous

blue-light photoreceptor *cry*, and the other consisting of external photoreceptors, i.e. the eyes, ocelli and the HB eyelets [40]. Previous work by others has established that external photoreceptors and CRY cooperate to entrain the fly's circadian clock to LD12 : 12 (e.g. [41,42]). Our results also suggest that both CRY and external photoreceptors likely contribute to light-dependent changes in endogenous period. We therefore expanded our analyses to fly strains that have mutations in these light input pathways. The loss-of-function cry^{OUT} mutation lacks functional *cry* [20] and the $glass^{[60j]}$ mutant lacks all external photoreceptors due to the loss of a transcription factor required for their development [13]. Previous work has shown that *cry*/*glass* double mutants are circadian blind to environmental light, suggesting that, taken together, *cry* and external photoreceptors account for most if not all light input to the circadian clock [13]. Furthermore, studies have also shown that *cry* may have the ability to integrate dim light information over long durations and that *cry* mutants continue to display weak resetting responses to light [35,43].

To further examine the extent to which external photoreceptors and CRY contribute to the parametric effects of light, we examined the effects of LL on mutants lacking normal photoreception/transduction. We found that loss-of-function cry^{OUT} mutants do not increase their free-running period under continuous light; rather, they showed a significant *reduction* in free-running period compared to their values under constant darkness, although this difference was quite small. This indicates that CRY is sensitive to longer wavelengths of light under prolonged illumination, and that it contributes to parametric light effects under a broader range of wavelengths than might have been expected from its action spectra [24] (figure 2*b* and *c*; electronic supplementary material, table S1). Remarkably, loss-of-function *cry* mutants show twice as much advance in the phase of their evening peaks of activity under SPP compared to their background controls (compare figure 1; electronic supplementary material, figure S3). This is also consistent with the idea that *cry* acts to lengthen period under long durations of light lacking strong UV and blue components.

We also found that a large proportion of $glass^{[60j]}$ mutants were arrhythmic under LL, but the free-running period of rhythmic flies was longer under LL by approximately 1.75 h (figure 2*b*,*c*; electronic supplementary material, table S1). In addition, we found that all *norpA* mutants, which lack the phopholipase-C signalling that mediates rhodopsin-based phototransduction, were rhythmic and their free-running periods were longer under LL by approximately 3.5 h (figures 2*a*,*c*; electronic supplementary material, table S1). Thus, in contrast to cry^{OUT} mutants, which showed a very small reduction in period under LL, flies lacking external photoreceptor signalling were characterized by large parametric increases in period.

When we compared the rhythmic strength of these mutants under LL and DD, we found that while cry^{OUT} displayed comparable rhythmic strength under the two regimes (electronic supplementary material, figure S2B; table S1), both $glass^{[60j]}$ and *norpA* flies displayed significantly weaker rhythms under LL (electronic supplementary material, figure S2B; table S1). Thus, the loss of *cry* function drastically decreases the parametric effects of light, while the loss of external photoreceptor function increases them and weakens endogenous rhythms under LL. Finally, to further tease apart the role of phototransduction mechanisms in the lengthening of period under LL versus lengthening caused by the screening pigment acting as a gatekeeper for the amount of light reaching the brain, we examined free-running periods of white-eyed and red-eyed *cry* mutants. We found no significant difference between the free-running period of the two *cry* mutants (electronic supplementary material, figure S4), suggesting little effect of the screening pigment's action on light-mediated changes in the brain in the absence of CRY.

These results suggest that parametric effects of light modulate clock speed in *Drosophila* and that these are likely to contribute to light entrainment. Surprisingly, *cry* appears to be required for such lengthening of free-running periods, even under light conditions lacking UV and blue light. Furthermore, the increased extent of period lengthening by LL in *norpA* mutants compared to wild-type *CS* flies suggests that phospholipase-C dependent phototransduction normally acts in opposition to *cry* with regard to the parametric effects of light, [41] and that parametric light effects make significant contributions to the timing of the entrained evening peak of activity.

(e) Ramped UV- and blue-blocked light cycles have significant experimental utility for understanding entrainment

Although our skeleton photoperiod experiments revealed that entrained waveforms of locomotor activity under LD12 : 12 are significantly shaped by parametric effects of light, the large startle responses to the discrete transitions between darkness and light which can be seen clearly in the activity profiles (figure 1), may contribute to the daily timing of activity in ways that may obscure the effects of light on the clock. For example, arousal signals caused by the masking effects of light likely contribute significantly to the timing of major peaks of activity. Thus, such startle responses pose a challenge to understanding the true contribution of the entrained circadian system to these waveforms. Moreover, waveforms of loss-of-function clock and light input mutants look remarkably normal under step-function LD cycles in that they reliably produce two daily peaks of activity at dawn and dusk [13,44]. To characterize the timing of entrained circadian outputs most accurately there is, therefore, a need for a light entrainment paradigm to which wild-type flies readily entrain but that does not induce strong diurnal rhythms in loss-of-function clock mutants. Furthermore, light regimes that better reveal entrainment deficits in flies lacking normal light input pathways would be of great utility. Experiments conducted under such light regimes, although structurally very different than widely used step-function LD cycles, could reveal neuronal and molecular underpinnings of circadian entrainment that step-function LD cycles will not.

To this end, we asked if ramping light cycles in which light intensity gradually changes from a low but non-zero value to a high value and then returns to the low value over 24 h, might provide novel experimental utility. We argue that such a regime would be useful because (i) there are no discrete jumps between states of darkness and light and therefore no large startle responses, (ii) ramping of the intensity represents a much more challenging light condition for entrainment than step-function LD cycles, as it requires the circadian system to constantly monitor a slowly changing

environment, and (iii) there is no opportunity under such a light cycle for discrete phase-shifts of the clock. If so, ramping light cycles would offer a unique regime of entrainment biased toward the parametric effects of light, making it a useful complement to the widely employed LD12 : 12 cycle.

To examine the potential utility of ramping light cycles, we placed our wild-type, genetic background, and several mutant strains under ramping light cycles that were filtered for UV and blue wavelengths and characterized individual flies as either (i) entrained, (ii) displaying relative coordination (transient or unstable entrainment), (iii) free-running or (iv) arrhythmic (electronic supplementary material, figure S5). We found that while close to 80% of wild-type *CS* flies entrained to light ramps, only approximately 50% of w^{1118} and *yw* flies entrain. Interestingly, approximately 80% of cry^{OUT} and approximately 97% of *norpA* mutants displayed free-running rhythms under these conditions (electronic supplementary material, figure S5 and table S4) indicating the presence of a strong circadian oscillation that had failed to entrain to the light cycle. In the case of $glass^{[60j]}$ mutants, approximately 50% of the flies entrained and approximately 40% of flies are arrhythmic under these ramping light cycles (electronic supplementary material, figure S5; table S4). Similar percentages of entrained flies were also observed when wild-type flies were reared under LD12 : 12 before transfer to light ramps, indicating that the ability to entrain to ramped regimes is not strongly dependent on light environment during rearing (electronic supplementary material, table S5). In the case of $glass^{[60j]}$ mutants, there was a small reduction in the percentage of flies that entrained to ramps after rearing in LD12 : 12, suggesting that light environment during rearing may have slightly stronger effects on this mutant (electronic supplementary material, table S5).

We examined the locomotor activity profiles of entrained wild-type *CS*, w^{1118} and *yw* flies under these conditions, and found one major peak of activity that was approximately centred around the second half of the declining phase of light intensity (figure 3*a*). There is also a smaller peak of activity especially visible in w^{1118} flies, which commences at the beginning of the inclining phase of light intensity (figure 3*a*). Interestingly, while the median phase of the dominant peak of activity for *CS* and w^{1118} flies was ~ZT19, the median phase of the dominant peak in *yw* flies was ~ZT17 (figure 3*b*), a difference of approximately 2 h in the phase of entrained activity peaks that is not apparent under standard LD12 : 12 entrainment conditions (e.g. figure 1). This surprisingly large difference between strains under light ramps but not under LD12 : 12 supports our notion that such ramped cycles will allow the experimentalist to detect the effects of genetic and neuronal manipulations on entrainment that may not be apparent using standard LD12 : 12 cycles, thanks, most likely, to the absence of startle/arousal mediated effects on behaviour.

A critical property of entrainment is phase-control, which means that once allowed to free-run following entrainment to a cyclic environment, organisms must show similar phases of the rhythm on the first day of constant conditions as it did during stable entrainment [2]. In the absence of phase control, one cannot conclude that the organism was really entrained during their exposure to any particular environmental cycle [2]. We estimated phase control in the wild-type and background control strains and found that phases of the dominant peak of *CS* flies on the first day under constant conditions is significantly clustered around the median phase of the same flies under entrainment (figure 3*b*). We found similar significant clustering in case of the w^{1118} (figure 3*b*) and *yw* strains (figure 3*b*), thereby indicating that these flies had truly entrained to the light ramps rather than being driven directly by changes in environmental light.

To further establish the extent to which our ramped light cycles provide experimental conditions that reduce the masking effects of light on behaviour, we examined loss-of-function clock mutants under these conditions. We found that only of approximately 35% of per^{01} loss-of-function mutants displayed some form of rhythmicity, with most flies displaying arrhythmicity (approx. 65%; figure 3*c*; electronic supplementary material, table S6). We note that the residual rhythmicity was much weaker than what is observed under LD12 : 12, a condition in which the majority of per^{01} mutant display rhythms. In the per^{01} flies that were rhythmic under ramps, there was a single peak of activity very close to ZT00 or the time of the day when light intensity was at a minimum (figure 3*d*). We quantified behavioural phases and found that the median phase of activity was ~ZT22.29. We think that such residual rhythmicity in per^{01} flies likely represents bright light avoidance behaviour/masking. This is an extremely mild masking effect compared to those observed under LD12 : 12. We found the phase of per^{01} mutants is significantly delayed compared to the phase of their background control *CS* flies (Wilcoxon's test: $W = 36$, $p = 9.3 \times 10^{-09}$), which is not typically the case under LD12 : 12, wherein evening peaks coincide with dusk. For clk^{JRK} mutants, which bear a loss-of-function mutation of the *clock* gene, all flies were arrhythmic under light ramps (figure 3*c*), which is in stark contrast to the relatively robust nocturnal activity rhythms displayed by this mutant under LD12 : 12 [45]. These results further suggest that light ramp cycles will offer significantly increased experimental utility for the examination of light entrainment in *Drosophila*.

4. Discussion

Real-world entrainment is mediated by a combined contribution of parametric and non-parametric effects of light. Parametric effects of light have been modelled in the past and have successfully explained entrainment under various photoperiodic conditions in *Neurospora*, *Drosophila* and mammals [8–10], unlike the non-parametric model which has limited ability to predict entrainment under different photoperiods [5,33]. The non-parametric model alone also has limited utility in predicting entrainment in humans (see [8] and references therein, and [46]). Therefore, a holistic study of entrainment requires the understanding of both parametric and non-parametric processes, and our study is a significant first step in that direction for *Drosophila*.

(a) Antagonistic effects of CRY and norpA in parametric light entrainment

Although few previous studies have suggested that the *Drosophila* clock lengthens its period under LL [17,47], our results show that wild-type flies have robust circadian rhythms with longer free-running periods under LL. The

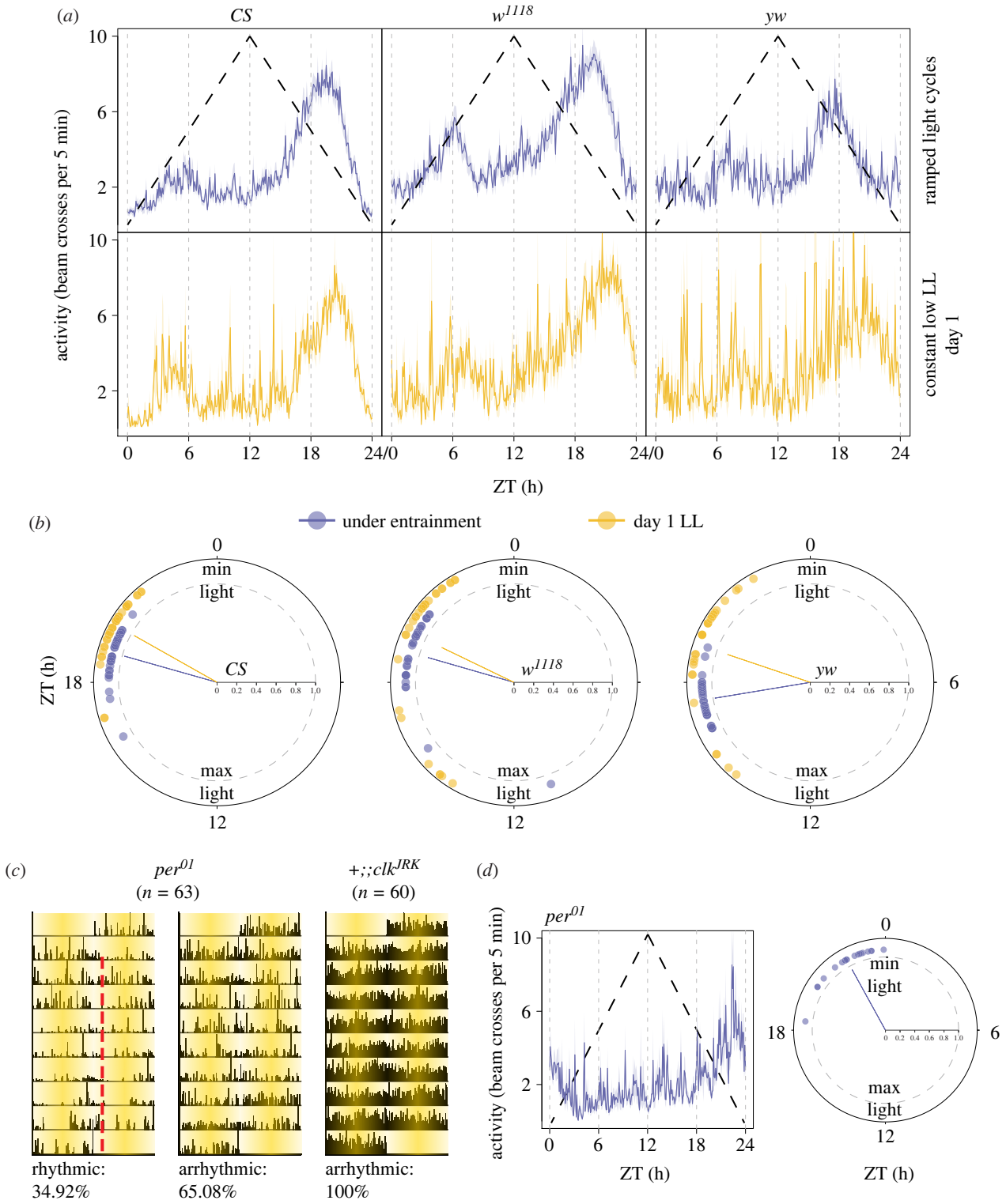

Figure 3. Entrainment to ramped UV- and blue-blocked light cycles. (*a*) Mean (±s.e.m.) locomotor activity profiles of entrained *CS* (left), w^{1118} (middle) and *yw* (right) flies under ramped light cycles (top) and on the first day under constant low intensity light (bottom). (*b*) Polar plots showing the phases of the predominant peak of locomotor activity for the three genotypes (*CS*, left; w^{1118}, middle; *yw*, right) under entrainment and on the first day post entrainment under constant conditions. Phase control was statistically examined using V-tests (see Methods). *CS*: $p = 7.67 \times 10^{-17}$; w^{1118}: $p = 1.45 \times 10^{-10}$; *yw*: $p = 7.38 \times 10^{-10}$. (*c*) Representative actograms showing rhythmic and arrhythmic flies under ramped cycles for loss-of-function clock mutants. The red dashed line indicates the offset of locomotor activity. The shading on the actograms represents the ramping up and down of light, starting at Zeitgeber Time (ZT) 00. (*d*) Mean (±s.e.m.) locomotor activity profile (left) and polar plot depicting phases of the peak of activity of per^{01} flies under ramped light cycles. The black dashed line in the top row of (*a*) and (*d*-left) is a schematic of light ramping from a lower minimum value (approx. 10 µW cm^{-2}) at ZT00 to the maximum value (40–50 μW cm^{-2}) at ZT12 and back to the minimum value by ZT24. In all the polar plots, individual dots represent a single fly and the line from the centre points to the mean phase across all flies. The distance of these lines from the centre is an estimate of the across-fly variation in phase. Values close to 1 (grey dashed circle) have lower variances.

amount of this lengthening appears to be strongly dependent on both *cry* and *norpA*-mediated phototransduction. Not only does the loss of CRY prevent LL from lengthening free-running period, but it also appears to modestly reduce it. On the other hand, the loss of *norpA* function results in larger LL-induced increases in period compared to

wild-type controls (figure 2; electronic supplementary material, table S1). These indicate an antagonistic interaction between *cry* and *norpA* under constant light. The idea of such an antagonism is also supported by studies in flies showing opposite effects of *cry* and visual photoreception on the evening peak of locomotor activity and their effects on clock oscillations under long days [41,42]. It is important to note here that Kistenpfennig *et al*. [41] report delayed evening peak of activity under long days in *cry* mutants and advanced evening peak in case of the absence of eyes. The opposing directionality of this antagonism as compared to what we observe here could be attributed to the presence of strong UV and blue components of white light used by the authors under long day conditions. However, it is interesting that the apparent directionality of antagonism between CRY and external photoreceptors may be context specific and may have adaptive value in the context of parametric entrainment under different light schedules.

The significant evening peak advances displayed by wild-type and background strains under SPP indicates that the two brief light pulses do not comprise the minimum required light schedule to recapitulate LD12 : 12 behaviour. To the best of our knowledge, this is the first study to use asymmetric skeleton photoperiod regimes to demonstrate that long durations of light at specific times of the day are required for entrainment phases like those observed under LD12 : 12 in the locomotor activity rhythm of flies. Similar experiments have been performed to examine the light requirement schedules of the eclosion rhythm for *D. melanogaster* flies that were artificially selected for morning and evening adult emergence [32]. This study found that for genotypes that had shorter than 24 h periods under constant darkness and a larger delay/advance ratio of their phase response curves, regimes that had long durations of light contiguous with dusk comprised the minimum light requirement schedule to mirror behaviour under LD12 : 12 [32], providing further support for time-of-day specific parametric light effects.

(b) *Cryptochrome* mediates the parametric effects of light in *D. melanogaster*

The most direct effect of light on the molecular circadian clock in *D. melanogaster* is through the deep brain blue light photoreceptor CRY, which has long been thought to mediate the non-parametric effects of light on the circadian clock by driving the rapid degradation of TIM upon exposure to light, thereby causing phase dependent advances or delays in the molecular circadian oscillator (e.g. [11]). However, the construction of light phase response curves (plots of time-of-day-dependent phase-shifts in response to brief pulses of light) for the cry^{baby} mutant, revealed that light effects were still present, though reduced, in the absence of CRY function [35]. The presence of CRY is also thought to explain why dipteran clocks cannot maintain their rhythms under constant light conditions: CRY action under continuous presence of light prevents sufficient build-up of TIM for the molecular clock to run [48–50]. Given that CRY has long been thought to be sensitive specifically to UV/blue wavelengths, we assumed that our UV- and blue-blocked LL regime would 'work-around' CRY. Indeed, the strong endogenous time-keeping displayed by wild-type flies under this regime was reminiscent of the rhythms displayed by loss of function *cry* mutants under the continuous white light conditions that render normal flies arrhythmic [48]. This suggested that CRY was not engaged under UV- and blue-blocked light. However, the lack of parametric effects in cry^{OUT} flies (figure 2; electronic supplementary material, figure S4) revealed, surprisingly, that non-UV/blue wavelengths likely do engage CRY and contribute to the parametric effects of light.

Owing to the clear absence of period lengthening under UV- and blue-blocked light in loss-of-function *cry* mutants, we hypothesize that CRY activation and CRY mediated TIM degradation may be at the heart of period lengthening in this regime. This is because the action spectrum of CRY suggests that it is likely to be weakly sensitive to wavelengths of light longer than 500 nm (electronic supplementary material, figure S1A) [24]. Remarkably, flies display stark behavioural preferences for specific wavelengths of light throughout the circadian cycle, avoiding blue light and seeking out green and red-shifted wavelengths during the day [51]. We suspect that, in real-world settings, flies are likely to be exposed, for significant durations, to light that is significantly more yellow/red shifted than the white light used in the laboratory. Therefore, under natural and freely behaving conditions, CRY-mediated TIM degradation is expected to be significantly lower than that seen in the laboratory. This would allow CRY to mediate the parametric effect of light by controlling TIM levels in a manner that depends on light intensity and duration, thereby adjusting PERIOD accumulation and nuclear entry to modulate the clock's speed. This, we argue, is mechanistically similar to the effects of a hypomorphic *cry* mutation under bright white LL, which produces lengthened free-running periods [52]. Future work examining molecular clock cycling under a range of light conditions will be necessary to test this hypothesis.

(c) On the utility of light ramps to study entrainment

The vastly simpler nervous system and highly conserved molecular clock in *Drosophila* has made it a superlative model system to understand mechanisms of entrainment that are relevant to entrainment in animals generally. However, there is an aspect of light entrainment in flies that has limited its relevance to light resetting in mammals: whereas mammals including humans require several cycles to entrain to a shifted (i.e. jet lagged) LD cycle (reviewed in [2]), flies adjust to even large shifts in LD12 : 12 almost instantaneously upon encountering a shifted light transition (reviewed in [40]), and even flies lacking major components of light input pathway re-entrain fairly rapidly (reviewed in [40]).

We wonder if this apparent instantaneous re-entrainment to shifted LD cycles is the result of the large startle responses produced by the light/dark cycles under which flies are typically studied. Standard step-function LD cycles (and our various skeleton photoperiods; figure 1; electronic supplementary material, figure S3) promote significant startle responses at light transitions, and these, rather than light's effect on the clock, may be driving the rapid re-entrainment seen in flies. We propose the UV- and blue-blocked ramping light cycles as an entrainment regime that fails to produce the large startles associated with step-function LD cycles and that requires multiple cycles for entrainment (i.e. they produce transients; electronic supplementary material, figure S5). Given that w^{1118} and *yw* flies show approximately 50% entrainment under these conditions, it is critical to use

these conditions cautiously in variants in these genetic backgrounds with all the appropriate parental and background controls when assessing entrainment to such regimes. Furthermore, unlike LD12 : 12 conditions, UV- and blue-blocked light ramps reveal significant entrainment defects in flies lacking light input pathways and fail to drive strong daily rhythms in loss-of-function clock mutants. This regime, therefore, allows us to explore aspects of entrainment in flies that are highly relevant but difficult to study using step-function LD cycles.

5. Conclusion

In summary, we have demonstrated that parametric effects have a strong influence on entrainment under the long-employed step-function LD12 : 12 and that, as predicted by parametric models of entrainment, there are clear time-of-day-dependent parametric effects of light. We have also established light regimes that reveal the effects of LL on the *Drosophila* circadian clock and an unexpected role for CRY in the mediation of these effects, at wavelengths beyond those associated with its established role as a blue light circadian photoreceptor. Interestingly, the apparent antagonism of CRY and phototransduction from external photoreceptors explains the patterns of entrainment displayed under various skeleton photoperiod regimes. Finally, our work establishes experimental light conditions that will offer great utility for future research aimed at understanding the parametric effects of light on the clock and the ways in which specific genes and neural pathways contribute to circadian photoentrainment.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. All data are available at Dryad Digital Repository [53].

Supplementary material is available online [54].

Authors' contributions. L.A.: conceptualization, data curation, formal analysis, investigation, methodology, software, validation, visualization, writing—original draft, writing—review and editing; O.T.S.: conceptualization, funding acquisition, project administration, resources, supervision, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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References

1. Pittendrigh CS. 1981 Circadian systems: entrainment. In *Biological rhythms* (ed. J Aschoff), pp. 95–124. Boston, MA: Springer.
2. Moore-Ede MC, Sulzman FM, Fuller CA, Charles A. 1982 *The clocks that time US: physiology of the circadian timing system*. Cambridge, MA: Harvard University Press.
3. Vaze KM, Sharma VK. 2013 On the adaptive significance of circadian clocks for their owners. *Chronobiol. Int.* **30**, 413–433. (doi:10.3109/07420528.2012.754457)
4. Roenneberg T, Merrow M. 2016 The circadian clock and human health. *Curr. Biol.* **26**, R432–R443. (doi:10.1016/j.cub.2016.04.011)
5. Daan S. 2000 Colin Pittendrigh, Jürgen Aschoff, and the natural entrainment of circadian systems. *J. Biol. Rhythms* **15**, 195–207. (doi:10.1177/074873040001500301)
6. Pittendrigh CS, Daan S. 1976 A functional analysis of circadian pacemakers in nocturnal rodents. IV. Entrainment: pacemaker as clock. *J. Comp. Physiol.* **106**, 291–331. (doi:10.1007/BF01417859)
7. Swade RH. 1969 Circadian rhythms in fluctuating light cycles: toward a new model of entrainment. *J. Theor. Biol.* **24**, 227–239. (doi:10.1016/S0022-5193(69)80048-2)
8. Rémi J, Merrow M, Roenneberg T. 2010 A circadian surface of entrainment: varying T, τ, and photoperiod in *Neurospora crassa*. *J. Biol. Rhythms* **25**, 318–328. (doi:10.1177/0748730410379081)
9. Beersma DGM, Daan S, Hut RA. 1999 Accuracy of circadian entrainment under fluctuating light conditions: contributions of phase and period responses. *J. Biol. Rhythms* **14**, 320–329. (doi:10.1177/074873099129000740)
10. Abhilash L, Sharma VK. 2020 Mechanisms of photic entrainment of activity/rest rhythms in populations of *Drosophila* selected for divergent timing of eclosion. *Chronobiol. Int.* **37**, 469–484. (doi:10.1080/07420528.2020.1727917)
11. Hunter-Ensor M, Ousley A, Sehgal A. 1996 Regulation of the *Drosophila* protein timeless suggests a mechanism for resetting the circadian clock by light. *Cell* **84**, 677–685. (doi:10.1016/S0092-8674(00)81046-6)
12. Hardin PE. 2011 Molecular genetic analysis of circadian timekeeping in *Drosophila*. *Adv. Genet.* **74**, 141–173. (doi:10.1016/B978-0-12-387690-4.00005-2)
13. Helfrich-Förster C, Winter C, Hofbauer A, Hall JC, Stanewsky R. 2001 The circadian clock of fruit flies is blind after elimination of all known photoreceptors. *Neuron* **30**, 249–261. (doi:10.1016/S0896-6273(01)00277-X)
14. Yoshii T, Funada Y, Ibuki-Ishibashi T, Matsumoto A, Tanimura T, Tomioka K. 2004 *Drosophila* cryb mutation reveals two circadian clocks that drive locomotor rhythm and have different responsiveness to light. *J. Insect. Physiol.* **50**, 479–488. (doi:10.1016/j.jinsphys.2004.02.011)
15. Rieger D, Shafer OT, Tomioka K, Helfrich-Förster C. 2006 Functional analysis of circadian pacemaker neurons in *Drosophila melanogaster*. *J. Neurosci.* **26**, 2531–2543. (doi:10.1523/JNEUROSCI.1234-05.2006)
16. Dolezelova E, Dolezel D, Hall JC. 2007 Rhythm defects caused by newly engineered null mutations in *Drosophila*'s cryptochrome gene. *Genetics* **177**, 329–345. (doi:10.1534/GENETICS.107.076513)
17. Konopka RJ, Pittendrigh C, Orr D. 2007 Reciprocal behaviour associated with altered homeostasis and photosensitivity of *Drosophila* clock mutants. *J. Neurogenet.* **21**, 243–252. (doi:10.1080/01677060701695391)
18. Nikhil KL, Vaze KM, Karatgi R, Sharma VK. 2016 Circadian clock properties of fruit flies *Drosophila* melanogaster exhibiting early and late emergence chronotypes. *Chronobiol. Int.* **33**, 22–38. (doi:10.3109/07420528.2015.1108981)
19. Barber AF, Erion R, Holmes TC, Sehgal A. 2016 Circadian and feeding cues integrate to drive rhythms of physiology in *Drosophila* insulin-producing cells. *Genes Dev.* **30**. 2596–2606. (doi:10.1101/gad.288258.116)
20. Yoshii T, Todo T, Wülbeck C, Stanewsky R, Helfrich-Förster C. 2008 Cryptochrome is present in the compound eyes and a subset of *Drosophila*'s clock neurons. *J. Comp. Neurol.* **508**. 952–966. (doi:10.1002/cne.21702)
21. de Mendiburu F. 2021 agricolae: statistical procedures for agricultural research. See https://cran.r-project.org/web/packages/agricolae/agricolae.pdf.
22. Stanewsky R, Kaneko M, Emery P, Beretta B, Wager-Smith K, Kay SA, Rosbash M, Hall JC. 1998 The cryb mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. *Cell* **95**, 681–692. (doi:10.1016/S0092-8674(00)81638-4)
23. Emery P, Stanewsky R, Hall JC, Rosbash M. 2000 A unique circadian-rhythm photoreceptor. *Nature* **404**, 456–457. (doi:10.1038/35006558)

24. VanVickle-Chavez SJ, Van Gelder RN. 2007 Action spectrum of *Drosophila* cryptochrome. *J. Biol. Chem.* **282**, 10 561–10 566. (doi:10.1074/JBC.M609314200)
25. Malyutina A, Tang J. 2021 Pessia A. drda: an R package for dose-response data analysis. *BioRxiv*.
26. Roesch A, Schmidbauer H. 2018 WaveletComp: computational wavelet analysis. See https://cran.r-project.org/web//packages//WaveletComp/WaveletComp.pdf.
27. Leise TL. 2017 Analysis of nonstationary time series for biological rhythms research. *J. Biol. Rhythms* **32**, 187–194. (doi:10.1177/0748730417709105)
28. Leise TL. 2013 Wavelet analysis of circadian and ultradian behavioral rhythms. *J. Circ. Rhythms* **11**, 1–9. (doi:10.1186/1740-3391-11-5)
29. Lund U, Agostinelli C. 2018 CircStats: circular statistics, from 'Topics in circular statistics' (2001). See https://cran.r-project.org/web//packages//CircStats/CircStats.pdf.
30. Zar JH. 1999 *Biostatistical analysis*. Englewood Cliffs, NJ: Prentice Hall.
31. Pittendrigh CS, Minis DH. 1964 The entrainment of circadian oscillations by light and their role as photoperiodic clocks. *Am. Nat.* **98**, 261–294. (doi:10.1086/282327)
32. Vaze KM, Nikhil KL, Abhilash L, Sharma VK. 2012 Early-and late-emerging *Drosophila* melanogaster fruit flies differ in their sensitivity to light during morning and evening. *Chronobiol. Int.* **29**, 674–682. (doi:10.3109/07420528.2012.680557)
33. Roenneberg T, Hut R, Daan S, Merrow M. 2010 Entrainment concepts revisited. *J. Biol. Rhythms* **25**, 329–339. (doi:10.1177/0748730410379082)
34. Roenneberg T, Dragovic Z, Merrow M. 2005 Demasking biological oscillators: properties and principles of entrainment exemplified by the Neurospora circadian clock. *Proc. Natl Acad. Sci. USA* **102**, 7742–7747. (doi:10.1073/pnas.0501884102)
35. Kistenpfennig C, Hirsh J, Yoshii T, Helfrich-Förster C. 2012 Phase-shifting the fruit fly clock without cryptochrome. *J. Biol. Rhythms* **27**, 117–125. (doi:10.1177/0748730411434390)
36. Dollish HK, Kaladchibachi S, Negelspach DC, Fernandez FX. 2022 The *Drosophila* circadian phase response curve to light: conservation across seasonally relevant photoperiods and anchorage to sunset. *Physiol. Behav.* **245**, 113691. (doi:10.1016/j.physbeh.2021.113691)
37. Lee SJ, Montell C. 2004 Suppression of constant-light-induced blindness but not retinal degeneration by inhibition of the rhodopsin degradation pathway. *Curr. Biol.* **14**, 2076–2085. (doi:10.1016/j.cub.2004.11.054)
38. Tomlinson A. 2012 The origin of the *Drosophila* subretinal pigment layer. *J. Comp. Neurol.* **520**, 2676–2682. (doi:10.1002/cne.23063)
39. Stavenga DG, Wehling MF, Belušič G. 2017 Functional interplay of visual, sensitizing and screening pigments in the eyes of *Drosophila* and other red-eyed dipteran flies. *J. Physiol.* **595**, 5481–5494. (doi:10.1113/JP273674)
40. Helfrich-Förster C. 2017 The *Drosophila* clock system. In *Biological timekeeping: clocks, rhythms and behaviour* (ed. V Kumar), pp. 133–176. New Delhi, India: Springer. (doi:10.1007/978-81-322-3688-7_6)
41. Kistenpfennig C, Nakayama M, Nihara R, Tomioka K, Helfrich-Förster C, Yoshii T. 2018 A tug-of-war between cryptochrome and the visual system allows the adaptation of evening activity to long photoperiods in *Drosophila melanogaster*. *J. Biol. Rhythms* **33**, 24–34. (doi:10.1177/0748730417738612)
42. Schlichting M, Weidner P, Diaz M, Menegazzi P, Dalla Benetta E, Helfrich-Förster C, Rosbash M. 2019 Light-mediated circuit switching in the *Drosophila* neuronal clock network. *Curr. Biol.* **29**, 3266–3276.e3. doi:10.1016/J.CUB.2019.08.033.
43. Vinayak P, Coupar J, Hughes SE, Fozdar P, Kilby J, Garren E, Yoshii T, Hirsh J. 2013 Exquisite light sensitivity of *Drosophila melanogaster* cryptochrome. *PLoS Genet.* **9**, e1003615. (doi:10.1371/journal.pgen.1003615)
44. Wheeler DA, Hamblen-Coyle MJ, Dushay MS, Hall JC. 1993 Behavior in light-dark cycles of *Drosophila* mutants that are arrhythmic, blind, or both. *J. Biol. Rhythms* **8**, 67–94. (doi:10.1177/074873049300800106)
45. Kim EY, Bae K, Ng FS, Glossop NRJ, Hardin PE, Edery I. 2002 *Drosophila* clock protein is under posttranscriptional control and influences light-induced activity. *Neuron* **34**, 69–81. (doi:10.1016/S0896-6273(02)00639-6)
46. Roenneberg T, Kuehnle T, Juda M, Kantermann T, Allebrandt KV, Gordijn MCM, Merrow M. 2007 Epidemiology of the human circadian clock. *Sleep Med. Rev.* **11**, 429–438. (doi:10.1016/j.smrv.2007.07.005)
47. Yuan Q, Lin F, Zheng X, Sehgal A. 2005 Serotonin modulates circadian entrainment in *Drosophila*. *Neuron* **47**, 115–127. (doi:10.1016/j.neuron.2005.05.027)
48. Emery P, Stanewsky R, Helfrich-Förster C, Emery-Le M, Hall JC, Rosbash M. 2000 *Drosophila* CRY is a deep brain circadian photoreceptor. *Neuron* **26**, 493–504. (doi:10.1016/S0896-6273(00)81181-2)
49. Ingram KK, Kutowoi A, Wurm Y, Shoemaker DW, Meier R, Bloch G. 2012 The molecular clockwork of the fire ant *Solenopsis invicta*. *PLoS ONE* **7**, e45715. (doi:10.1371/journal.pone.0045715)
50. Rubin EB, Shemesh Y, Cohen M, Elgavish S, Robertson HM, Bloch G. 2006 Molecular and phylogenetic analyses reveal mammalian-like clockwork in the honey bee (*Apis mellifera*) and shed new light on the molecular evolution of the circadian clock. *Genome Res.* **16**, 1352–1365. (doi:10.1101/gr.5094806)
51. Lazopulo S, Lazopulo A, Baker JD, Syed S. 2019 Daytime colour preference in *Drosophila* depends on the circadian clock and TRP channels. *Nature* **574**. (doi:10.1038/s41586-019-1571-y)
52. Busza A, Emery-Le M, Rosbash M, Emery P. 2004 Roles of the two Drosophila CRYPTOCHROME structural domains in circadian photoreception. *Science (1979)* **304**, 1503–1506. (doi:10.1126/science.1096973)
53. Abhilash L, Shafer OT. 2023 Data from: Parametric effects of light acting via multiple photoreceptors contribute to circadian entrainment in *Drosophila melanogaster*. Dryad Digital Repository. (doi:10.5061/dryad.t4b8gtj5w)
54. Abhilash L, Shafer OT. 2023 Parametric effects of light acting via multiple photoreceptors contribute to circadian entrainment in *Drosophila melanogaster*. Figshare. (doi:10.6084/m9.figshare.c.6823042)