Parametric effects of light acting via multiple photoreceptor types contribute significantly to circadian entrainment in *Drosophila melanogaster*

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Abstract

Circadian rhythms in physiology and behavior have near 24-hour periodicities that must adjust to the exact 24-hour geophysical cycles on earth to ensure stable and adaptive daily timing, a phenomenon referred to as entrainment. Entrainment can either occur via instantaneous shifts in the phase of the clock, a process referred to as non-parametric entrainment, or by light acting continuously to alter the speed of the clock, a process referred to as parametric entrainment. Though typically studied in isolation, real-world entrainment involves both parametric and non-parametric processes. While *Drosophila melanogaster* has been a powerful model organism for understanding the molecular and neuronal underpinnings of entrainment, the vast majority of the work on entrainment in this species has focused on non-parametric light effects. Though parametric effects of light have been described for *D. melanogaster*, there have been few studies examining the mechanisms underlying such effects. This has been due, in large part, to the fact that the fly's circadian system does not free-run strongly under constant light conditions, which is required to experimentally address parametric effects of light. In this study, we demonstrate that light has robust parametric effects on the *Drosophila* circadian clock and that the timing of daily activity is strongly influenced by these effects, even under the standard step-function light/dark cycles employed by the field. We also show that parametric effects of light depend on external photoreceptors and, to a surprising extent, on the blue light photoreceptor *cryptochrome*. Furthermore, we introduce ramped light cycles as an entrainment paradigm that produces entrainment features that are similar to those seen in mammalian systems, in that they eliminate the confounding effects of the large startle responses displayed by flies under step function light/dark cycles and require multiple days for re-entrainment to shifted cycles. These features make possible a new experimental approach to understanding the mechanisms underlying entrainment in the fly.

Keywords: *parametric entrainment, continuous entrainment, tonic entrainment, Drosophila, circadian, locomotor activity, Aschoff's rule, free-running period, constant light, skeleton photoperiods*

Introduction

Circadian rhythms are ubiquitous endogenous rhythms in behavior, physiology and metabolism. They “free-run” in the absence of cyclic environments at periodicities close to but significantly different from 24-h. In order to support optimal daily timing on an ever-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 to optimal times of the day and anticipate changes in the environment [1–4]. Poor entrainment is at the heart of many health problems in modern societies, making entrainment a central problem to circadian biology and general well-being [4].

Mechanistically, 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 savings 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 modern 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 light induced CRYPTOCHROME (CRY; blue light photoreceptor) mediated TIMELESS (TIM; core component of the molecular circadian clock) degradation ([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 *cryptochrome* 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 of light entrainment. For this reason, we have sought to develop approaches that allow us to use the powerful fly model to understand the parametric effects of light.

Therefore, we set out to identify light regimes that better support an examination of parametric light effects and their contribution to circadian entrainment. We find that constant light regimes in which UV and blue wavelengths have been blocked (referred to as yellow light throughout the manuscript) support robust free-running rhythms with lengthened periods. We also show that external photoreceptors/phototransduction and the blue light circadian photoreceptor *cryptochrome* differentially govern the parametric effects of light under these conditions. Using various skeleton photoperiods (see Methods), we show that parametric effects of light shape circadian entrainment, even under the step-function/rectangular LD cycles commonly used in the field. Finally, we define light regimes that entrain behavioral rhythms in a manner that fails to

90 induce the confounding startle responses associated with LD12:12 and that, as in mammalian
91 systems, require multiple cycles for re-entrainment to shifted light cycles. These results reveal
92 important features of entrainment in *Drosophila* and establish a useful new approach to
93 understanding light resetting in this important model organism.

94

Results

Wildtype and background strains have longer free-running periods under constant yellow light

The most direct method of measuring parametric effects of light is the demonstration that free-running periods of a circadian rhythm differ between LL and DD conditions. Given the fact that the presence of the blue light circadian photoreceptor CRY renders the *Drosophila* clock arrhythmic under white LL conditions, we first asked if filtering blue and UV wavelengths from a constant light regime would support free-running rhythms under LL. We examined the free-running periods of a wildtype strain and two common genetic background strains under UV/blue filtered (yellow) LL conditions (see Methods for details on the spectral composition of light). We found that wildtype *Canton-S* (CS) flies showed strong endogenous circadian locomotor rhythms with significantly longer free-running periods under LL compared to DD, with an average difference of ~1-h (Figs. 1A and B).

For the white-eyed *w¹¹¹⁸* mutant, a commonly used background strain, the difference between LL and DD periods was ~3.5-h, remarkably higher than the difference displayed by CS flies (Fig. 1A and B). In case of the yellow-white mutant (*yw*), another common background strain, we found that a large percentage of flies were arrhythmic (Table 1), but among those that were rhythmic, free-running periods were also longer under LL than under DD (Figs. 1A and B). We also examined the strength of these rhythms under LL and DD (see Methods) and found that CS flies had higher rhythmic strength under LL whereas *w¹¹¹⁸* and *yw* flies had significantly higher strengths under DD (Fig. 1C). These results clearly indicate that parametric effects of light can induce period changes in the *D. melanogaster* clock. However, the differences displayed between

CS and *w¹¹¹⁸* flies prompted us to ask how this LL regime would affect mutants for photoreception and phototransduction.

	<i>N</i>		% <i>Rhythmic</i>		<i>Period (h)</i>		<i>Rhythm power</i>		<i>Number of independent runs</i>	
	<i>DD</i>	<i>LL</i>	<i>DD</i>	<i>LL</i>	<i>DD</i>	<i>LL</i>	<i>DD</i>	<i>LL</i>	<i>DD</i>	<i>LL</i>
CS	120	128	80.46	86.32	23.42	24.67	229.12	306.26	4	4
<i>w¹¹¹⁸</i>	96	128	97.92	81.25	23.33	26.89	972.90	535.07	3	4
<i>yw</i>	113	126	96.46	25.47	24.08	25.17	270.76	57.16	4	4
<i>yw;;cry^{OUT}</i>	100	119	96.77	100.00	23.42	23.00	718.05	729.37	4	4
<i>w;;glass^[60j]</i>	119	128	81.04	18.15	24.54	26.39	171.05	23.40	4	4
<i>norpA^[7]</i>	62	62	98.08	100.00	24.67	28.25	884.40	400.73	2	2

Table 1: Details of experiments performed to assay free-running locomotor activity rhythms in various genotypes of flies under constant darkness and constant light (also see Figs. 1-2).

Parametric 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 *cryptochrome* (*cry*), and the other consisting of external photoreceptors, i.e, the eyes, ocelli and the HB eyelets [19]. The loss-of-function *cry^{OUT}* mutation lacks functional *cry* [20]. 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]. We also examined the effect of LL on the *norpA* mutant, which lacks the phospholipase-C signaling that mediates rhodopsin-based phototransduction. We found that loss-of-function *cry^{OUT}* mutants did not increase their free-running period under LL; rather, they showed a

significant *reduction* in free-running period under LL compared to DD, although the difference was quite small. This was surprising, as it indicates that *cryptochrome* is sensitive to long wavelength light under LL and that it contributes to parametric light effects under a broader range of wavelengths than expected from its action spectra [21] (Figs. 2A and B; Table 1).

We 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 ~1.75-h compared to DD (Figs. 2A and B; Table 1). In addition, we found that all *norpA* mutants were rhythmic and their free-running periods were longer in LL by ~3.5-h (Figs. 2A; Table 1). Thus, in contrast to *cry*^{OUT} mutants, which showed a very small reduction in period under LL, flies lacking external photoreceptors were characterized by remarkably large parametric increases in period. When we compared the rhythmic strengths of these mutants under LL and DD, we found that while *cry*^{OUT} displayed comparable rhythmic strength under LL and DD (Fig. 2C; Table 1), both *glass*^[60j] and *norpA* both displayed significantly weaker rhythms under LL (Figs. 2C; Table 1). Thus, the loss of *cry* function drastically decreases the parametric effects of light, while the loss of external photoreceptor function increases them and weakens rhythms under LL conditions.

These results suggest that parametric effects of light modulate clock speed in *Drosophila* and suggest that these are likely to contribute to light entrainment. Surprisingly, *cryptochrome* appears to be required for such lengthening of free-running periods, even under light conditions lacking blue and UV light. Furthermore, the increased extent of period lengthening by LL in *norpA* mutants compared to wildtype *CS* flies suggest that phospholipase-C dependent phototransduction normally acts in opposition to *cry* with regard to the parametric effects of light, a result that

supports previous work supporting antagonistic roles for *cry* and external photoreceptors in the adjustment of the evening peak of activity under step function LD cycles [22], and suggesting that parametric light effects make significant contributions to the timing of the entrained evening peak of activity.

Non-parametric effects of light do not account for entrainment under standard LD12:12 conditions

One major prediction from the non-parametric (a.k.a., 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 superfluous [5,6]. If this were true of *D. melanogaster* locomotor rhythms, we would expect the locomotor activity waveform of flies to be identical under LD12:12 and symmetric skeleton photoperiods (SPP), in which only two 30-min light pulses are provided to flies in an otherwise dark environment (see Methods; Fig. 3A). All wild-type and genetic background control strains we examined readily entrained to SPP displaying no differences from LD12:12 flies (Fig. 3B). Interestingly, two of the mutant strains examined here displayed a significantly reduced number of entrained flies under SPP compared to LD12:12: in case of *cry*^{OUT} and *glass*^[60j] flies (Fig. 3B), but such reduction in entrainment was seen in *norpa* mutants (Fig. 3B).

Averaged locomotor activity profiles of *CS*, *w*¹¹¹⁸ and *yw* flies indicate that there were distinct differences in waveforms between LD12:12 and SPP (Fig. 3C). We quantified the phases of evening activity peaks from smoothed data (see Methods) and found that median phases in all three genotypes were significantly advanced under SPP compared to LD12:12 (See Methods for

details of statistical analysis; Fig. 3D). These results clearly show that skeleton photoperiods do not recapitulate entrainment under LD12:12. Furthermore, we found that the phase of evening peak of activity was advanced even in *cry^{OUT}* and *glass^[60j]* mutants (Fig. 3D), whereas there was no significant difference in phases of evening activity between SPP and LD 12:12 *norpA* mutants (Fig. 3D).

Parametric effects of light during late day are necessary for recapitulating entrainment to rectangular LD cycles

If parametric effects contribute to entrainment under step function LD cycles, we would expect increased durations of skeleton light exposure to have measurable effects on the phase of entrainment in a manner that depends on the timing of the increased light (Swade, 1969; Vaze et al., 2012). The parametric model of entrainment posits that long durations of light delivered at dawn will accelerate the clock, whereas light delivered at dusk will have a decelerating effect on the clock [7,23]. The prediction, therefore, is that long durations of light around dawn would lead to reduced free-running period, thereby leading to an advanced phase of entrainment and that long durations of light during dusk would lengthen free-running period and consequently delay the phase of entrainment [24]. In contrast, if non-parametric effects dominate entrainment at transitions, activity profiles under skeleton light cycles should be largely unaffected by the length of the skeleton light periods. In order to test the extent to which the fly is entrained to full length LD cycles via parametric or non-parametric effects, we subjected our flies to two asymmetric skeleton photoperiods (ASPP), one with 6-h of light during the first half of the day and a brief pulse at dusk (30-min; ASPP-1; Fig. 4), and one with a brief dawn pulse (30-min) and 6-h of light during the second half of the day (ASPP-2; Fig. 5). As for SPP, we first sought to determine the

extent to which wild-type, genetic background, and mutant 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 (Fig. 4 and 5; Table 2).

	% <i>Entrained</i> (N)			
	<i>LD12:12</i>	<i>SPP</i>	<i>ASPP-1</i>	<i>ASPP-2</i>
<i>CS</i>	100 (61)	100 (62)	100 (62)	100 (64)
<i>w¹¹¹⁸</i>	100 (60)	100 (63)	100 (62)	100 (58)
<i>yw</i>	100 (62)	98.38 (62)	100 (64)	100 (62)
<i>yw;;cry^{OUT}</i>	100 (60)	82.54 (63)	98.36 (61)	100 (49)
<i>w;;glass^[60j]</i>	98.31 (59)	77.59 (58)	98.44 (64)	93.55 (62)
<i>+,;norpA^[7]</i>	100 (47)	100 (45)	100 (63)	98.31 (59)

Table 2: Percentage of flies entraining to each of the four environmental condition and the total number of flies based on which these percentages were computed. Only values in red are significantly lower than the values under LD12:12.

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 wildtype *CS* flies, there was a significant advance in the timing of evening activity when the long-duration of light was delivered around dawn (ASPP-1; Figs. 4C and D). 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; Figs. 5D). Similar effects of long-duration light around dawn were observed for *w¹¹¹⁸* and *yw* flies (ASPP-1; Fig. 4) and the evening peak was significantly delayed in these strains when long duration light was delivered around dusk (ASPP-2; Fig. 5). In case of both *cry^{OUT}* and *glass^[60j]* mutants there was a significant phase-advance when long-duration light was delivered at dawn (ASPP-1; Fig. 4). While there was no difference in the phases of evening peak between LD12:12 and long duration evening light for *glass^[60j]* mutants, there was a significant delay in the phase of evening peak for *cry^{OUT}* mutants when long duration light was

delivered at dusk compared to LD12:12 (ASSP-2; Fig. 5). In the case of *norpA* mutants, there was no significant difference in phase under either asymmetric skeleton photoperiod compared to LD12:12 (Figs. 4 and 5).

Taken together, these results reveal that long durations of light at the end of the day are sufficient for flies to achieve the waveform displayed under LD12:12. Therefore, entrained waveforms of locomotor activity under standard laboratory LD conditions are strongly shaped by parametric effects of light.

Ramped yellow light cycles can only entrain wildtype and background strains

Although our skeleton photoperiod experiments revealed that entrained waveforms of locomotor activity under LD12:12 are significantly shaped by parametric effects of light, there are large startle responses to the discrete transitions between darkness and light which can be seen clearly in the activity profiles (Figs. 3-5). Such startles 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 like wildtype flies under step function LD cycles [13,25]. In order to most accurately characterize the timing of entrained circadian outputs there is need for a light entrainment paradigm to which wildtype flies readily entrain but that do not produce strong diurnal rhythms in loss-of-function clock mutants and that reveal entrainment deficits in flies lacking normal light input. Experiments conducted under such an environmental cycle, although perhaps no less natural than step-function LD cycles, would be more likely to reveal the true neuronal and molecular underpinnings of circadian entrainment to light.

To this end, we asked if a ramped light cycle in which light intensity gradually changes with time of day from a low but non-zero value to a high value might be experimentally useful. 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 cause for large startle responses, (ii) ramping of the intensity is a more challenging condition for entrainment, 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, therefore offering a form of entrainment biased toward the parametric effects of light, making it a useful addition to current approaches. Light ramps might, therefore, make possible new approaches to understanding the mechanisms underlying this important form of circadian entrainment.

We therefore placed our wild-type, genetic background, and mutant strains under ramping light cycles, filtered for UV and blue wavelengths (see Methods), and characterized individual flies as either (i) entrained, (ii) displaying relative coordination (transient or unstable entrainment), (iii) free-running, or (iv) arrhythmic (Fig. 6). We found that while close to 80% of wildtype *CS* flies entrained to yellow ramps, only ~50% of *w¹¹¹⁸* and *yw* flies entrain. Interestingly, ~80% of *cry^{OUT}* and ~97% of *norpA* mutants displayed free-running rhythms under these conditions (Fig. 6; Table 3) indicating the absence of entrainment of a strong circadian oscillation. In case of *glass^{l60j}* mutants, ~50% of the flies entrained and ~40% of flies are arrhythmic under these ramping light cycles (Fig. 6; Table 3).

	<i>Entrained</i>	<i>Relative coordination</i>	<i>Free- running</i>	<i>Arrhythmic</i>	<i>N</i>
<i>CS</i>	79.31	5.17	8.62	6.90	58
<i>w¹¹¹⁸</i>	51.61	27.42	14.52	6.45	62
<i>yw</i>	49.21	4.76	7.94	38.10	63
<i>yw;;cry^{OUT}</i>	16.33	2.04	79.59	2.04	49
<i>w;;glass^[60j]</i>	49.18	0	9.84	40.98	61
<i>+;;norpA^[7]</i>	0	0	96.77	3.23	31

Table 3: Percentage of flies that belong to one of the four categories under ramped yellow light cycles, along with the total number of flies based on which these percentages were calculated.

We examined the locomotor activity profiles of entrained wildtype *CS*, *w¹¹¹⁸* and *yw* flies under these conditions, and found that there is one major peak of activity roughly centered on the declining phase of light intensity (Fig. 7A). There is also a smaller peak of activity especially visible in *w¹¹¹⁸* flies, which commences at the beginning of the inclining phase of light intensity (Fig. 7A). Interestingly, while the median phase of the dominant peak of activity for *CS* and *w¹¹¹⁸* flies was ~ZT19 (the start of the inclining light phase, in this case, was defined as ZT00), the median phase of the dominant peak in *yw* flies was ~ZT17 (Fig. 7B), a difference of ~2-h in the phase of entrained activity peaks that is not apparent under standard LD12:12 entrainment conditions (e.g., Fig. 3C and D). This surprisingly large difference between strains under light ramps but not under LD12:12 lends strong support to our notion that such light ramp cycles will allow the experimentalist to detect the effects of genetic and neuronal manipulations on entrainment that would not be apparent using standard light regimes, thanks to the absence of startle/arousal mediated effects on the timing of behavior and to the biasing of entrainment toward parametric effects of light.

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 the same phase of 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. 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 (Fig. 7B). We found similar significant clustering in case of the *w¹¹¹⁸* (Fig. 7B) and *yw* strains (Fig. 7B), thereby indicating that these flies had entrained to the previous light ramps rather than being driven directly by changes in environmental light.

To further establish the extent to which our light ramp conditions provide experimental conditions that reduce the masking effects of light on behavior, we examined loss-of-function clock mutants under these conditions. We found that only of ~35% of *per⁰¹* loss-of-function mutants displayed some form of rhythmicity, and the majority of flies were arrhythmic (~65%; Fig. 7C; Table 4). We note that the residual rhythmicity was much weaker than what is observed under LD12:12, a condition in which the majority of *per⁰¹* mutant display rhythms. In the *per⁰¹* flies that were rhythmic, there was a single peak of activity very close to ZT00 or the time of the day when light intensity was at a minimum (Fig. 7D). We quantified their phases and found that the median phase of activity was ~ZT22.29. We think that such residual rhythmicity in *per⁰¹* flies is some form of bright light avoidance behavior/masking. We found that this phase of peak is significantly delayed compared to the phase of their background control CS flies (Wilcoxon's test: $W = 36$, $p = 9.3 \times 10^{-9}$), which is not typically the case under standard LD12:12 conditions, 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 (Fig. 7C), which is in stark contrast to the relatively

nocturnal activity rhythms displayed by this mutant under LD12:12 [26]. These results suggest that light ramp cycles will offer advantageous conditions for the examination of light entrainment in *Drosophila* that are significantly less subject to the masking effects of light.

	<i>Rhythmic</i>	<i>Arrhythmic</i>	<i>N</i>
<i>+,;per⁰¹</i>	34.92	65.08	63
<i>+,;clk^{JRK}</i>	0	100	60

Table 4: Percentage of flies that are rhythmic and arrhythmic for loss-of-function clock mutants under ramped yellow light cycles, along with the total number of flies based on which these percentages were calculated.

Discussion

Real world entrainment is mediated by a combined contribution of parametric and non-parametric effects of light. Such effects of light have been modeled 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 [5,23]. In fact, the non-parametric model alone also has limited utility in predicting entrainment in humans (see [8] and references therein, and [27]). 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.

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 the circadian clock by driving the rapid degradation of TIM upon exposure to light, thereby causing phase dependent advances or delays in the circadian oscillator (e.g., [11]). However, the construction of light phase response curves for the *cry^{baby}* mutant, revealed that parametric light effects were still present, though reduced, in the absence of CRY function [28]. The presence of CRY is also thought to explain why dipteran clocks cannot maintain their rhythms under LL conditions: CRY action under LL prevents sufficient build-up of TIM for the molecular clock to run [29–31]. Given that CRY has long been thought to be sensitive specifically to UV/blue wavelengths, we assumed that our yellow LL regime would “work-around” CRY. Indeed, the strong endogenous timekeeping displayed by wild-type flies under yellow LL was reminiscent of the rhythms displayed by loss of function *cry* mutants under the

white LL conditions that render normal flies arrhythmic [29]. This suggested that CRY was not engaged under yellow LL. However, the lack of parametric effects of yellow LL in *cry^{OUT}* flies (Fig. 2) 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 yellow LL in loss-of-function *cryptochrome* mutants, we hypothesize that CRY activation and CRY mediated TIM degradation may be at the heart of period lengthening in yellow LL. This is because the action spectrum of CRY suggests that it is likely to be weakly sensitive to wavelengths of light longer than 500nm [21]. Furthermore, flies display clear behavioral preferences for specific wavelengths of light throughout the circadian cycle, avoiding blue light and seeking out green and red shifted wavelengths during the day [32]. We suspect that, in real world settings, flies are likely to be exposed to long durations of light that are 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 lab. This would allow CRY to mediate the parametric effect of light by controlling TIM turnover in a manner that depends on light intensity and duration, thereby adjusting PERIOD accumulation and nuclear entry to modulate the clock's free-running period. This, we argue, is mechanistically similar to the effects of a hypomorphic *cry* mutation under bright white LL, which produces increased free-running periods [33].

Antagonistic effects of CRY and norpA in parametric light entrainment

Our results show that wildtype flies have longer free-running periods under LL. The amount of this lengthening appears to be dependent strongly on both *cry* and *norpA* mediated phototransduction. Not only does absence of CRY prevent constant light from lengthening free-running period, it appears to modestly reduce it. On the other hand, absence of *norpA* function lengthens period by a larger than wildtype amount (see Figures 1-2 and Table 1). These clearly indicate an antagonistic interaction between *cryptochrome* and *norpA*, at least under the presence of 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 [22].

Such an antagonistic relationship can be hypothesized to be of adaptive value in the context of parametric entrainment, wherein organisms have to modulate the speed of their clocks in a time-of-day dependent manner. Such antagonism can explain the entrainment of locomotor rhythms to skeleton photoperiods. *CS* flies displayed a significantly advanced phase under the symmetrical skeleton photoperiod (SPP) relative to LD12:12, indicating that absence of a full 12-h photoperiod leads to a faster running clock and advanced evening peak phase under SPP. Similar results in the *w¹¹¹⁸* and *yw* background strains also support this idea. Remarkably, loss-of-function *cryptochrome* mutants show twice as much advance in the phase of their evening peaks of activity under SPP compared to their background controls. This is also consistent with the idea that *cryptochrome* tends to lengthen period under long durations of light.

Our results that wildtype and background strains display a significant advance of evening peak phase under SPP indicates that the two brief light pulses do not comprise the minimum required

light schedule to recapitulate LD12:12 behavior. Therefore, we extended our analyses to asymmetric SPPs to ask if long durations of light are necessary for entrainment of waveforms in a manner similar to that under standard LD12:12 as a means of testing the prediction that long durations of light contiguous with dawn would have a net accelerating effect on the clock while long durations of light contiguous with dusk would have a net decelerating effect on the clock. Indeed, this is exactly what we found: wild-type and genetic background flies had significantly advanced phase under long dawn skeletons and a tendency towards increased similarity to LD12:12 under long dusk skeletons. To the best of our knowledge, this is the first study to demonstrate that long durations of light at specific times of the day are required for entrainment phases largely similar to those in LD12:12 for the locomotor activity rhythm in flies. Similar experiments have been performed to examine the light requirement schedules of the eclosion rhythm for flies that were artificially selected for morning and evening adult emergence [34]. The authors found that for genotypes that had shorter than 24-h periods under DD 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 behavior under LD12:12 [34], providing further support for time-of-day specific parametric light effects.

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, one aspect of light entrainment in flies 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 12:12 LD cycles almost instantaneously upon encountering a shifted light transition (reviewed in [19]), and even flies lacking major components of light input pathway re-entrain fairly rapidly (reviewed in [19]). For these reasons, new experimental light regimes are necessary if *Drosophila* is to be used to address entrainment in a manner with the greatest possible relevance to animals generally.

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. It is clear that standard step-function LD cycles (and our various skeleton photoperiods – Figs. 3-5) 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 therefore sought a light regime that would produce entrainment in flies more akin to that seen in mammalian systems, which are characterized by several cycles of transient phases before establishing stable re-entrainment to the new LD cycles phase (examples in [2]).

We propose the yellow light ramped cycles used here as one such regime, as it fails to produce the large startles associated with step-function LD cycles and require multiple cycles for entrainment (i.e., transients; Fig. 6). Furthermore, unlike standard LD12:12 conditions, yellow light ramps reveal significant entrainment defects in flies lacking light input pathways and largely fail to drive 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 relatively under-studied.

In summary, we have established light regimes that demonstrate significant parametric effects of light on the *Drosophila* circadian clock and reveal 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. Our results also show clearly that these parametric effects have a strong influence on entrainment even 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. 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 light entrainment.

Methods

Fly stocks

The following fly lines were used in this study: *Canton-S* (CS; BDSC stock number: 64349), white-eye (*w¹¹¹⁸*) [35], yellow-white (*yw*; BDSC stock number: 1495), *yw;;cry^{OUT}* [20], *w;;glass^[60j]* [13], *+;;norpA^[7]* (BDSC stock number: 5685), *+;;per⁰¹* (BDSC stock number: 80928) and *+;;clk^{IRK}* (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). Sample sizes and replication for each experiment and environmental conditions experienced before behavioral experiments began are described separately for each experiment below.

Assessment of free-running period under constant light

Fly lines, recording conditions and the constant light paradigm:

CS, *w¹¹¹⁸*, *yw*, *cry^{OUT}*, *glass^[60j]* and *norpA^[7]* males were used in these experiments. About 32 flies of each genotype were collected under CO₂ anesthesia 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 DAM monitors. Infrared beam crossings were recorded every minute for at least 10 days under constant darkness (DD) or constant light (LL) in Percival incubators (Model: LED-30HL1, Geneva Scientific, Fontana, WI) at 25 °C. Data from at least two independent runs are reported. See Table 1 for sample sizes and number of replicate runs.

Given that wildtype *Drosophila* is arrhythmic under constant white light, we sought to establish light conditions under which wild-type flies maintain robust free-running rhythms. It is well established that the arrhythmicity of flies under white LL is driven by the Cryptochrome (CRY) mediated TIMELESS (TIM) degradation [36,37]. Furthermore, CRY is maximally sensitive to blue and UV wavelengths of light [21]. We therefore used a light filter which blocks all blue and UV components of white light (Filter number: 821; Lee Filters, UK; <http://www.leefilters.com/>). We refer to this light as yellow light throughout the manuscript owing to the color of the light to the human eye, but it is important to note that this light is not true yellow; it only has the blue and UV components of white light blocked. The intensity of yellow LL for all these experiments were 30-45 $\mu\text{W}/\text{cm}^2$ at 600nm. We used the S120C sensor and the PM100D console from Thorlabs (Newton, NJ; <https://www.thorlabs.com/>) to measure the single wavelength light intensities.

Statistical analyses:

Free-running period values and corresponding rhythmic power were estimated using the χ^2 periodogram (implemented using in-house custom R scripts; codes available upon request). Period and power values were pooled from all independent replicate runs and were compared using Wilcoxon's tests for two independent samples (also implemented using custom R scripts).

Entrainment of locomotor activity rhythms under skeleton photoperiods

Fly lines, recording conditions and light conditions:

CS, *w¹¹¹⁸*, *yw*, *cry^{OUT}*, *glass^[60j]* and *norpA^[7]* males were used in these experiments. Flies were maintained under white light LD12:12 before being loaded into locomotor tubes and again 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 and loaded into locomotor tubes as described above. Our symmetric skeleton photoperiod (SPP; Fig. 3A), consisted of 30-min pulses starting and ending at dawn and dusk 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 that was 6-h with the standard 30-min pulse at dusk (ASPP-1; Fig. 4A), and the second of which consisted of a 6-h dusk light pulse and 30-min dawn pulse (ASPP-2; Fig. 5A). All these conditions employed the standard white light produced by the incubators without the use of any wavelength filters. These conditions were programmed to run at a constant 25 °C. The light intensity for all these experiments were 400-500 lux.

Statistical analyses:

The first step to analyzing this 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. 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. For each genotype, we used the Fisher's exact test to ask if the

number of entrained versus free-running flies were significantly different under SPP, ASPP-1, and ASPP-2, compared to standard LD12:12 conditions.

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. All the analyses were carried out using custom R scripts.

Entrainment to ramped light cycles

Fly lines, recording conditions and light conditions:

CS, *w¹¹¹⁸*, *yw*, *cry^{OUT}*, *glass^[60j]*, *norpA^[7]*, *per⁰¹* and *clk^{JRK}* males were used in these experiments. Flies were reared under ramped yellow light cycles in Percival incubators (Model: LED-30HL1, Geneva Scientific, Fontana, WI) at a constant temperature of 25 °C. Approximately 32 males of each genotype were collected for each run and loaded into locomotor tubes as described above. Ramping light cycles were generated such that there was no darkness at any point in the cycle. We programmed gradual light increases from ~10 $\mu\text{W}/\text{cm}^2$ to 40-50 $\mu\text{W}/\text{cm}^2$ (measured at 600nm) (ZT00-ZT12) followed by gradual light decreases back to ~10 $\mu\text{W}/\text{cm}^2$ 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 that could induce non-parametric clock resetting. We recorded beam crossings, as described above, under the ramped light cycles for nine consecutive days, at the end of which flies were kept under constant light from the nadir of the ramped cycle ($\sim 10 \mu\text{W}/\text{cm}^2$ intensity) to assess phase control.

Statistical analyses:

Owing to the novelty of this light regime, it was necessary to assess if flies could entrain to such a regime at all. For each fly, we plotted the 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 and has been suggested for use with circadian time series (Tanya Leise's papers). All three of these assessments were used for every fly. Individual flies were considered entrained only if visual inspection of the actogram showed that timing of activity had stabilized by the sixth ramping cycle, and if the wavelet power spectrum showed stable period values between 23.5-24.5-h post the sixth cycle, and/or if the χ^2 periodogram showed peaks in the same range. A fly was categorized as transiently entrained or relatively coordinated if there were obvious period changes (for instance, a period that shifted from period of $< 24\text{-h}$ to $> 24\text{-h}$) 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 or if the χ^2 periodogram showed peaks at period values less than 23.5 or greater than 24.5-h. Flies were considered arrhythmic if there was there was no significant peak in the χ^2 periodogram or if 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 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. Phase control is the phenomenon wherein the timing of behavior during entrainment and the first cycle of constant conditions are the same. 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. *V*-tests are Rayleigh's tests of uniformity on circular data that have an alternate hypothesis of a known mean angle [38]. Difference in phases of peaks between *CS* and *per⁰¹* flies was tested using a Wilcoxon's test, also implemented on R.

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Figure Legends

Figure 1: Free-running locomotor activity rhythms of wildtype and common background strains under constant darkness and light. (A) Representative actograms of *Canton-S* (CS; top), *w¹¹¹⁸* (middle) and *yw* (bottom) under constant darkness (DD; left) and constant yellow light (LL; right) of 30-45 $\mu\text{W}/\text{cm}^2$. (B) Frequency distributions of free-running periods of CS (top), *w¹¹¹⁸* (middle) and *yw* (bottom) under DD and LL. 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: $W = 2676.5$, $p = 6.036\text{e-}08$; *w¹¹¹⁸*: $W = 502$, $p = 2.2\text{e-}16$; *yw*: $W = 618.5$, $p = 1.583\text{e-}08$). (C) Power of the rhythm under DD and LL for CS (left), *w¹¹¹⁸* (middle) and *yw* (right). Statistical comparisons are based on Wilcoxon's tests (CS: $W = 3749$, $p = 0.0071$; *w¹¹¹⁸*: $W = 7579$, $p = 1.525\text{e-}12$; *yw*: $W = 3196$, $p = 4.799\text{e-}12$). Values of free-running period and rhythms power are pooled from at least three independent runs (see Methods and Table 1). * < 0.05 , ** < 0.01 , *** < 0.001 , and NS (Not Significant).

Figure 2: Free-running locomotor activity rhythms of fly mutants for photoreception and phototransduction under constant darkness and light. (A) Representative actograms of *yw;;cry^{OUT}* (top), *w;;glass^[60j]* (middle) and *+;;norpA^[7]* (bottom) under constant darkness (DD; left) and constant yellow light (LL; right) of 30-45 $\mu\text{W}/\text{cm}^2$. (B) Frequency distributions of free-running periods of all three genotypes under DD and LL. 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 (*yw;;cry^{OUT}*: $W = 7391$, $p = 2.233\text{e-}08$; *w;;glass^[60j]*: $W = 305$, $p = 1.092\text{e-}05$; *+;;norpA^[7]*: $W = 10$, $p = 2.2\text{e-}16$). (C) Power of the rhythm under DD and LL for the three genotypes. Statistical comparisons are based on Wilcoxon's tests (*yw;;cry^{OUT}*: $W = 5289$, $p = 0.76$; *w;;glass^[60j]*: $W = 1315$, $p < 0.001$; *+;;norpA^[7]*: $W = 2365$, $p < 0.001$). Values of free-running

period and rhythms power are pooled from at least two independent runs (see Methods and Table 1). * < 0.05, ** < 0.01, *** < 0.001, and NS (Not Significant).

Figure 3: Entrainment to white light symmetric skeleton photoperiod. (A) Schematic representations of the two different photoperiods under which the locomotor activity of the following six genotypes were assayed: *CS*, *w¹¹¹⁸*, *yw*, *yw;;cry^{OUT}*, *w;;glass^[60j]* and *++;norpA^[7]*. The black shaded regions indicate the durations of darkness over one 24-h cycle, and the white regions indicate the durations of white light (400-500 lux) within the 24-h cycle (see Methods for more details). (B) Mosaic plots showing the relative proportions of entrained and free-running flies in each photoperiod condition. Widths of the vertical bars are relative indicators of the number of flies in each photoperiod (see Table 2). Statistically significant effects of skeleton photoperiod on the number of flies that entrain were tested using Fisher's exact tests (only significant effects are reported here – *yw;;cry^{OUT}*: $p < 0.001$; *w;;glass^[60j]*: $p < 0.001$). (C) Mean ($\pm$ SEM) locomotor activity profiles of the wildtype and background control flies (top), and the photoreceptor and phototransduction mutants (bottom) under LD12:12 compared with the symmetric skeleton photoperiod. (D) Phases of the evening peak of activity across the two regimes for all genotypes. Boxplots that share the same alphabet are not statistically significantly different from each other (also, see Table 2). Profiles and phases of evening peak of activity are pooled from two independent replicate runs for each genotype. Statistical comparison of phases across all four photoperiodic conditions (see Methods) were done using Kruskal-Wallis tests. Test results reported here are from the Kruskal-Wallis tests and are the same for this figure and Figs. 4 and 5.

CS: $\chi^2_3 = 95.05$, $p = 0$; *w¹¹¹⁸*: $\chi^2_3 = 123.47$, $p = 0$; *yw*: $\chi^2_3 = 113.58$, $p = 0$; *yw;;cry^{OUT}*: $\chi^2_3 = 63.46$, $p = 1.07\text{e-}13$; *w;;glass^[60j]*: $\chi^2_3 = 50.50$, $p = 6.24\text{e-}11$; *++;norpA^[7]*: $\chi^2_3 = 7.02$, $p = 0.07$.

Figure 4: Entrainment to white light long dawn skeleton photoperiod (ASPP-1). (A) Schematic representations of the two different photoperiods under which the locomotor activity of the following six genotypes were assayed: *CS*, *w¹¹¹⁸*, *yw*, *yw;;cry^{OUT}*, *w;;glass^[60j]* and *++;norpA^[7]*. The black shaded regions indicate the durations of darkness over one 24-h cycle, and the white regions indicate the durations of white light (400-500 lux) within the 24-h cycle (see Methods for more details). (B) Mosaic plots showing the relative proportions of entrained and free-running flies in each photoperiod condition. Widths of the vertical bars are relative indicators of the number of flies in each photoperiod (see Table 2). (C) Mean ($\pm$ SEM) locomotor activity profiles of the wildtype and background control flies (top), and the photoreceptor and phototransduction mutants (bottom) under LD12:12 compared with the asymmetric skeleton photoperiod. (D) Phases of the evening peak of activity across the two regimes for all genotypes. Boxplots that share the same alphabet are not statistically significantly different from each other (also, see Table 2). Profiles and phases of evening peak of activity are pooled from two independent replicate runs for each genotype.

Figure 5: Entrainment to white light long dusk skeleton photoperiod (ASPP-2). (A) Schematic representations of the two different photoperiods under which the locomotor activity of the following six genotypes were assayed: *CS*, *w¹¹¹⁸*, *yw*, *yw;;cry^{OUT}*, *w;;glass^[60j]* and *++;norpA^[7]*. The black shaded regions indicate the durations of darkness over one 24-h cycle, and the white regions indicate the durations of white light (400-500 lux) within the 24-h cycle (see Methods for more details). (B) Mosaic plots showing the relative proportions of entrained and free-running flies in each photoperiod condition. Widths of the vertical bars are relative indicators of the number of flies in each photoperiod (see Table 2). (C) Mean ($\pm$ SEM) locomotor activity profiles of the wildtype and background control flies (top), and the photoreceptor and phototransduction

mutants (bottom) under LD12:12 compared with the asymmetric skeleton photoperiod. (D) Phases of the evening peak of activity across the two regimes for all genotypes. Boxplots that share the same alphabet are not statistically significantly different from each other (also, see Table 2). Profiles and phases of evening peak of activity are pooled from two independent replicate runs for each genotype. Note that the profiles and phase values for LD12:12 are the same as those reported in Figs. 3 and 4, and have been shown multiple times only to facilitate pairwise visual comparisons. Statistical analyses were done only once comparing each genotype across all four regimes (see Methods for details and legend for Fig. 3).

Figure 6: Example actograms from all six genotypes illustrating entrainment, relative coordination, free-running and/or arrhythmicity. The red dashed lines are drawn to trace the offset of locomotor activity in the top three rows. The percentage values on top of each actogram are the percentage of all flies showing that behavior. The shading on the actograms represents the ramping up and down of light, starting at Zeitgeber Time-00.

Figure 7: Entrainment to ramped yellow light cycles. (A) Mean ($\pm$ SEM) locomotor activity profiles of entrained *CS* (left), *w¹¹¹⁸* (middle) and *yw* (right) flies under ramped yellow light cycles (top) and on the first day under constant low yellow LL (bottom). (B) Polar plots showing the phases of the predominant peak of locomotor activity for the three genotypes (*CS* – left; *w¹¹¹⁸* – 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*: $p = 7.67\text{e-}17$; *w¹¹¹⁸*: $p = 1.45\text{e-}10$; *yw*: $7.38\text{e-}10$. (C) Representative actograms showing rhythmic and arrhythmic flies under ramped yellow 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 ($\pm$ SEM)

791 locomotor activity profile (left) and polar plots depicting phases of the peak of activity of *per⁰¹*
792 flies under ramped yellow light cycles. The black dashed line in the top row of panel (A) and
793 (D-left) is a schematic representation of light ramping from a lower minimum value (~10
794 $\mu W/cm^2$) at ZT00 to the maximum value (40-50 $\mu W/cm^2$) at ZT12 and back to the minimum
795 value by ZT24. In all the polar plots individual dots represent a single fly and the line from the
796 center points to the mean phase across all flies. The distance of these lines from the center is an
797 estimate of the across fly variation in phase. Values close to 1 (gray dashed circle) have lower
798 variances.
799

800

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

A

B

C

D

Figure 7