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Robustness of Circadian Timing Systems Evolves in the Fruit Fly *Drosophila melanogaster* as a Correlated Response to Selection for Adult Emergence in a Narrow Window of Time

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Robustness is a fundamental property of biological timing systems that is likely to ensure their efficient functioning under a wide range of environmental conditions. Here we report the findings of our study aimed at examining robustness of circadian clocks in fruit fly *Drosophila melanogaster* populations selected to emerge as adults within a narrow window of time. Previously, we have reported that such flies display enhanced synchrony, accuracy, and precision in their adult emergence and activity/rest rhythms. Since it is expected that accurate and precise circadian clocks may confer enhanced stability in circadian time-keeping, we decided to examine robustness in circadian rhythms of flies from the selected populations by subjecting them to a variety of environmental conditions comprising of a range of photoperiods, light intensities, ambient temperatures, and constant darkness. The results revealed that adult emergence and activity/rest rhythms of flies from the selected stocks were more robust than controls, as they displayed enhanced stability under a wide variety of environmental conditions. These results suggest that selection for adult emergence within a narrow window of time results in the evolution of robustness in circadian timing systems of the fruit fly *D. melanogaster*. (Author correspondence: vsharma@jncasr.ac.in or vksharmas@gmail.com)

Keywords: Circadian, *Drosophila*, Light, Photoperiod, Precision, Robustness, Selection, Temperature

INTRODUCTION

Most organisms show precise daily rhythms in behavioral and metabolic processes that persist with near-24 h (circadian) period under constant conditions of the laboratory. Interestingly, rhythms with intrinsic period close to 24 h are often found to be more precise than those far removed from it (Daan & Beersma, 2002; Pittendrigh & Daan, 1976; Sharma & Chandrashekaran, 1999), which suggests that circadian rhythms are by-products of adaptation to daily cycles of nature (Dunlap et al., 2004; Pittendrigh, 1993; Sharma, 2003). Traits capable of maintaining biological functions stably and efficiently in the face of fluctuations in environmental conditions are more likely to be selected for than those that are weak and unstable (Kitano, 2004). Such traits responsible for relative homeostasis in biological functions when faced with environmental perturbations are said to be "robust"—the lesser the degree to which the function varies in different environments the higher is its robustness. It is concomitantly important to take note of the relative nature of a measure of robustness—a trait or function can only be defined to be more or less robust in comparison with some other trait or function. With such a definition, it is likely that organisms with more stable circadian time-keeping ability are likely to be favored by natural selection where robustness of circadian systems can be defined as the ability to maintain stable phase relationship with the changing environment. This is corroborated in two relatively recent studies that showed that features of light profiles from the real world that promote evolution of complexity in circadian clock networks also contribute to the evolution of its robustness (Merrow & Maas, 2009; Troein et al., 2009).

Robustness enhances the ability of circadian clocks to maintain stable phase relationship with environmental cycles with a range of light intensities (Aschoff et al., 1971), daylengths (Aschoff et al., 1972), and temperatures (Liu et al., 1997) due to changing seasons (Akman et al., 2010; Moore-Ede et al., 1982; Shafer et al., 2004).

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Seasonal changes in daylength modulate circadian clocks to an extent that it is reflected in some of the major clock-controlled behavioral outputs (Lankinen, 1993; Pittendrigh, 1954; Rieger et al., 2003; Stoleru et al., 2007). Previous studies in *D. melanogaster* have shown that the morning and evening activity peaks (Rieger et al., 2003, 2012; Shafer et al., 2004; Stoleru et al., 2007) as well as oscillations in the expression of some of the core clock genes (Majercak et al., 1999; Qiu & Hardin, 1996; Shafer et al., 2004) were altered when flies were subjected to light/dark (LD) cycles of different daylengths. In addition, interaction of circadian clocks with environmental conditions in nature brings about a characteristic time course and waveform in adult emergence (De et al., 2012) and activity/rest (Vanin et al., 2012) rhythms in *Drosophila* which is quite different from that observed under typical laboratory conditions. Moreover, the phase of circadian rhythms and its day-to-day precision was altered under varying photoperiods (Aschoff et al., 1972) and latitudes (Daan and Aschoff, 1975). Precision was maximum at equinoxes (Aschoff et al., 1972) and minimum at higher latitudes (Daan & Aschoff, 1975), which is consistent with the notion that cyclic environmental factors may have acted as selection pressure for the evolution of robustness and precision in circadian timing systems (Sharma, 2003).

Light intensity during the day is a key determinant of the strength of LD cycles in nature (Boulos et al., 2002; Moore-Ede et al., 1982). For example, a previous study on the effect of LD light intensity in *D. melanogaster* showed marked differences in activity/rest rhythm with changing light intensity (Rieger et al., 2007). Apart from dealing with different light conditions, robustness of circadian clocks is likely to ensure stability in circadian time-keeping when organisms are subjected to LD cycles at different ambient temperatures. Although considerable progress has been made to our understanding of circadian entrainment of activity/rest rhythm to temperature cycles (Boothroyd et al., 2007; Glaser & Stanewsky, 2005; Hastings et al., 1991; Tomioka et al., 1998; Wheeler et al., 1993), and to LD cycles with different ambient temperatures (Majercak et al., 1999), entrainment of adult emergence rhythm under such ambient temperature conditions remains largely unexplored.

The variety of environmental conditions likely to be experienced by an organism raises several questions regarding the maintenance of stable entrainment in circadian rhythms to varying environmental cues. More specifically, it is critical to understand the relationships between precision, stability, and robustness of circadian timing systems when an organism is faced with a wide range of environmental conditions. To this end, we used populations of *D. melanogaster* that were selected to emerge in a narrow window of time (Kannan et al., 2012). These flies displayed stable emergence and activity rhythms with enhanced synchrony, accuracy, and precision compared with controls. Using these populations in the current study, we aimed to elucidate whether circadian clocks are robust enough to withstand a wide range of environmental conditions. To assess this, we used the percentage emergence during the selection window, height of emergence peak, gate width of emergence, synchrony and accuracy in emergence rhythm, and waveform and morning activity peak in activity rhythm under a variety of environmental conditions.

We studied robustness of circadian clocks of the selected populations by comparing their emergence and activity rhythms under different environmental conditions with those of the controls. We considered the persistence of circadian rhythms with greater synchrony, accuracy, and precision under different light intensities, temperatures, daylengths, and constant darkness compared with controls as a measure of enhanced robustness of the circadian clocks of the selected fly populations. Our working definition of robustness, as expected, is comparative in nature—we contrast various parameters of adult emergence and activity rhythms of the selected populations and find the continued presence of stable circadian rhythms in comparison with the controls. Our study, reveals that robustness in circadian time-keeping evolves as a consequence of selection for emergence during a narrow window of time.

MATERIALS AND METHODS

Fly Population Maintenance and Selection Protocol

Four precision populations (PP_{1-4}) were derived from four ancestral baseline populations of *D. melanogaster* that have been maintained for several hundred generations in the laboratory as four independent populations under 12:12 h LD cycles at 25°C on banana-jaggery (BJ) medium (Sheeba et al., 1998). The PP_{1-4} populations were initiated from four baseline populations by selecting for flies that emerged during ZT01–02 (zeitgeber time 01–02; henceforth will be referred as the selection window) for four successive days (9–12 d after egg collection), where ZT00 is the time of lights-on and ZT12 of lights-off under 12:12 h LD cycles. Simultaneously, four control populations (CP_{1-4}) were also initiated from the four baseline populations that experienced all other conditions similar to the selected populations but were not subjected to any conscious selection pressure for timing of emergence (henceforth the sets of selected and control populations will be referred to as selected and control stocks). In case of *CP*, flies emerging throughout the day were used as breeding adults for the next generation. At every generation, a total of ~1200 breeding adults per population, with roughly equal number of males and females, were collected in Plexiglas cages with BJ medium in a Petri dish. Six days post emergence, yeast–acetic acid paste was applied on the BJ medium to induce greater egg output. Three days later, eggs laid during a 3 h window (ZT01–04) were collected on BJ medium and dispensed at a density of ~300 eggs per vial into long glass vials (18 cm height × 2.4 cm diameter) containing ~10 mL of BJ medium. Both selected and

control stocks were maintained under 12:12 h LD cycles at constant temperature (~25°C), light intensity (~100 lux), and relative humidity (~75%) on a 21 d nonoverlapping generation cycle. This selection experiment has been ongoing for over 80 generations (Kannan et al., 2012).

We chose the selection window for emergence between ZT01 and ZT02 for two reasons. Firstly, because significant masking of the *D. melanogaster* emergence rhythm by light has been seen previously—as a "startle" response to lights-on evidenced by sudden increase in emergence immediately after (within 10 min) lights-on (McNabb & Truman, 2008). We delayed the start of selection window by 1 h (at ZT01) with respect to lights-on (at ZT00) at first to ensure that we do not inadvertently select flies that emerge as a consequence of startle response to daily switching-on of lights. Secondly, because it would enable us to select flies that form the peak of emergence rhythm.

Standardization of Selected Stocks

To minimize nongenetic parental effects that could have inadvertently been caused by the imposition of different selection protocols, all selected and control stocks were subjected to a common rearing condition (like *CP*) for one generation prior to each assay. In this generation, selection pressure was relaxed and flies emerging throughout the day were collected to form breeding adults for the next generation. Approximately 300 eggs were collected per vial containing 10 mL BJ medium, with equal number of vials per population. On the 12th day after egg collection, freshly emerged adults from each population were collected into Plexiglass cages with BJ medium. Yeast–acetic acid paste was provided to flies for 2 d prior to egg collection. The progeny of these stocks will henceforth be referred to as standardized populations.

Adult Emergence Rhythm Assay

The emergence rhythm of flies was assayed under LD with (i) different photoperiods, (ii) different light intensities during the light phase of LD, and (iii) different ambient temperatures. To study the rhythm under different photoperiods, 10 vials of each replicate population, with ~300 eggs per vial, were introduced to LD08:16, LD12:12, and LD16:08 conditions created at a constant temperature of ~25°C and light intensity of ~100 lux during the light phase of LD. In separate experiments, emergence rhythm was assayed under LD with different light intensities (1, 10, 100, and 1000 lux) and constant (~25°C) ambient temperature, or under LD with different ambient temperatures (18°C, 25°C, and 29°C) and ~100 lux intensity during the light phase. Light intensity was measured by LI-COR light meter L250 (Lincoln, NE, USA). Assays under different environmental conditions were carried out with 10 vials for each of the four replicate populations taking ~300 eggs per vial. Vials kept under different environmental conditions were closely monitored for emergence of the first fly and thereafter regularly at every 2-h interval for five successive days. To have a better resolution of the emergence profile, particularly during lights-on and around the selection window, we assayed the number of adults emerging every 1 h in the duration ZT23–04. To estimate the percentage of flies emerging in every reading interval of 1 or 2 h, the number of adults that emerged during those reading intervals was divided by the total number of flies that emerged from that particular vial in one complete day. The average values thus obtained for five successive days were plotted as a function of time, to obtain the daily emergence waveform. The gate width of emergence was estimated as the time interval between the start and end of emergence on each day, with start and end defined as the time when emergence was greater than and less than an arbitrary cutoff of 5% of the total daily emergence. The phase of the emergence peak was estimated as the mean time point at which maximum emergence occurred over five successive days.

Locomotor Activity Rhythm Assay

Locomotor activity rhythm of flies was assayed individually under different environmental conditions as described above. For this assay, eggs collected from the standardized stocks were dispensed into vials containing ~10 mL of BJ medium at a density of ~300 eggs per vial. Freshly emerged (2 d old) virgin males were introduced individually into glass tubes and their locomotor activity behavior was monitored for a minimum of 10 d under different environmental conditions, using Drosophila Activity Monitors from Trikinetics (Waltham, Massachusetts, USA). The waveform of activity rhythm was obtained by dividing hourly pooled activity data by the total amount of activity during one complete day. The phases of morning and evening activity peaks were estimated as the average time during morning and evening when the activity in 1 h bin was at its maximum. Daytime activity was estimated as percentage of activity of an individual fly during ZT01–12 and nighttime activity as percentage of activity during ZT13–00. Circadian period (τ) of activity rhythm was estimated by treating activity data collected in DD for a minimum of 10 d through CLOCKLAB software from Actimetrics (Wilmette, IL, USA). For the estimation of precision of free-running rhythm in DD, cycle-to-cycle period was estimated as the time interval between two successive activity peaks.

We estimated synchrony and accuracy as the measures of inter and intraindividual (or inter and intravial for emergence rhythm) variance, respectively, for the rhythms (Daan & Beersma, 2002; Kannan et al., 2012). Synchrony was estimated as the inverse of standard deviation (SD) of the phase relationship (ψ) between emergence or activity rhythm and lights-on in LD. To estimate synchrony in emergence rhythm of each replicate population, mean phase of the emergence peak for each of the 10 vials was obtained by averaging across

five cycles. The SD across 10 such average ψ values was used as a measure of intervial variance in emergence rhythm of each replicate population. Similarly, mean ψ of morning activity peak for each individual fly was obtained by averaging across 10 cycles. The SD across 32 such average ψ values was used as a measure of inter-individual variance in activity rhythm of each replicate population. Accuracy was estimated as the reciprocal of SD of the day-to-day ψ for emergence or activity rhythm. To estimate accuracy of emergence rhythm of each replicate population, we first calculated the inverse of SD of daily ψ of emergence peak across 5 d for each vial and then averaged it across all 10 vials. Similarly for activity rhythm, we estimated reciprocal of SD of daily ψ of morning activity peak across 10 d for each individual and then averaged it across all flies. Homogeneity of τ in each replicate population was estimated as the inverse of interindividual SD of τ (n = 32). To calculate precision, variance in phase of morning activity peak across 10 cycles of each individual was first calculated. Precision of activity rhythm was estimated as the reciprocal of SD of cycle-to-cycle τ in individual flies. Since all the four measures are estimated as 1/SD, they have a unit of h^{-1}.

Statistical Analyses

The emergence in 1 or 2 h bins, percentage emergence during selection window, gate width of emergence, height of emergence peak, activity in 1 h bins, morning and evening activity peaks, daytime and nighttime activity levels, and measures of synchrony, accuracy, homogeneity, and precision of emergence and activity rhythms under various environmental conditions were analyzed separately using mixed-model analysis of variance (ANOVA) treating replicate population (block; B) as a random factor, whereas photoperiod (P), light intensity (I), temperature (TP), stock (S), and time of day (T) were treated as fixed factors crossed with block. ANOVA was followed by post hoc multiple comparisons using Tukey's test. Ninety-five percent confidence interval (calculated using minimum significant difference in Tukey's test; Sokal & Rohlf, 1995) were used in the figures as error bars to facilitate visual hypothesis testing (Gabriel, 1978). Thus, overlapping error bars would imply that the means are not significantly different. All analyses were implemented on STATISTICA for Windows Release 5.0 B (StatSoft, 1995).

The experiments and procedures described in this paper conform to international ethical standards (Portaluppi et al., 2010).

RESULTS

Selected Stocks Display Robust Emergence Rhythm Under a Wide Range of Photoperiods

Under LD12:12, the emergence waveform of *PP* had a more prominent peak and a higher percentage of flies emerged during the selection window compared with *CP* (Figure 1a–c). Under LD08:16 and LD16:08, this trend of emergence pattern persisted in *PP*, with higher percentage of flies emerging during the selection window, which resulted in enhanced morning peak compared with *CP* (Figure 1a, b). ANOVA showed statistically significant effects of T (LD08:16, $F_{11,33} = 196.8$, $p < .0001$; LD12:12, $F_{11,33} = 350.32$, $p < .0001$; LD16:08, $F_{11,33} = 38.6$, $p < .0001$) and S × T interaction (LD08:16, $F_{11,33} = 3.8$, $p < .001$; LD12:12, $F_{11,33} = 8.19$, $p < .0001$; LD16:08, $F_{11,33} = 3.8$, $p < .001$), whereas the effect of S was statistically not significant (LD08:16, $F_{1,3} = .27$, $p = .6$; LD12:12, $F_{1,3} = .74$, $p = .4$; LD16:08, $F_{1,3} = 5.13$, $p = .1$; Table 1). Post hoc multiple comparisons using Tukey's test revealed that emergence during ZT01–02 (selection window) was significantly higher in *PP* compared with *CP* under all three photoperiods (Figure 1b). This suggests that under a wide range of photoperiods, selected flies emerge in greater number during the selection window compared with controls.

Under all three photoperiods, *PP* had a narrower gate width of emergence than *CP* (Figure 1d). ANOVA on the gate width data showed statistically significant effects of P ($F_{2,6} = 139.3$, $p < .0001$) and S ($F_{1,3} = 477.4$, $p < .0002$), whereas the effect of S × P interaction was statistically not significant ($F_{2,6} = .27$, $p = .7$; Table 1). Post hoc multiple comparisons using Tukey's test revealed that although gate widths of both populations were significantly broader under LD16:08 compared with LD08:16 and LD12:12, gate width of *PP* was significantly narrower than *CP* in all three photoperiods (Figure 1d). These results suggest that selected stocks continue to show tighter emergence rhythm than controls even under photoperiods different from that imposed as a part of the selection regime.

In addition to a tighter emergence profile, under a wide range of photoperiods the selected stocks displayed enhanced synchrony (Figure 1e) and accuracy (Figure 1f) in emergence rhythm compared with controls. ANOVA on the synchrony and accuracy data showed statistically significant effects of P (synchrony, $F_{2,6} = 16.1$, $p < .03$; accuracy, $F_{2,6} = 8.6$, $p < .01$) and S (synchrony, $F_{1,3} = 46.9$, $p < .006$; accuracy, $F_{1,3} = 103.2$, $p < .002$), whereas the effect of S × P interaction was statistically not significant (synchrony, $F_{2,6} = 1.75$, $p = .2$; accuracy, $F_{2,6} = 3.1$, $p = .1$; Table 1). Post hoc multiple comparisons using Tukey's test revealed that under all three photoperiods, *PP* exhibited higher synchrony and accuracy in their emergence rhythm compared with *CP*.

Taken together, the above results suggest that more consolidated emergence rhythm in the selected stocks as compared with controls persists under a wide variety of photoperiods, which indicates enhanced robustness of their underlying circadian timing systems.

Selected Stocks Exhibit Enhanced Morning Activity Peak Under Different Photoperiods

Under LD12:12, *PP* had higher morning activity compared with *CP* (Figure 2a). Although under LD08:16 and LD16:08 morning activity peak of both stocks occurred prior to and after lights-on, respectively,

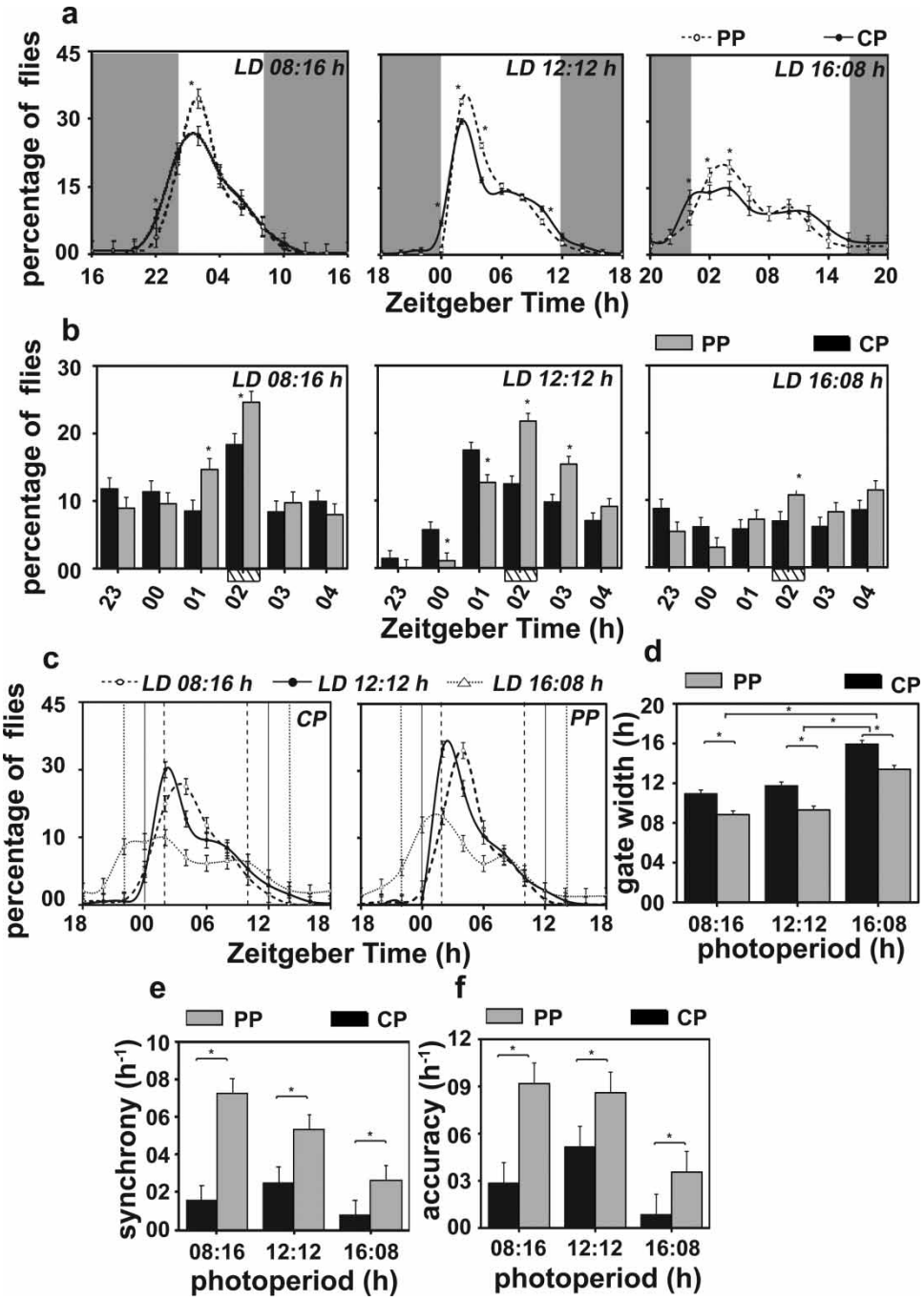

FIGURE 1. Enhanced emergence peak in selected (*PP*) populations compared with controls (*CP*) in three different photoperiods. Emergence rhythm in selected populations displayed increased stability as evidenced by increased emergence peak, tightened gate, and more consolidated emergence waveform under widely different photoperiods. (a) Waveforms of adult emergence rhythm in selected and control flies assayed under LD08:16, LD12:12, and LD16:08. Percentage of flies that emerged at every 2 h interval is plotted across zeitgeber time. Zeitgeber time 00 (ZT00) is taken as the time of lights-on under LD cycles. Shaded areas represent the duration of darkness under LD. (b) Percentage of flies emerging during ZT23–04, when assayed under LD08:16, LD12:12, and LD16:08. Percentage of flies that emerged in 1 h intervals is plotted across zeitgeber time. Hatched boxes on the *x*-axes denote the selection windows. (c) Emergence rhythms of selected and control populations assayed under LD08:16, LD12:12, and LD16:08. Vertical dashed, solid, and dotted lines represent the lights-on and lights-off under LD08:16, LD12:12, and LD16:08, respectively. All assays were performed under ~100 lux light intensity. The *x*-axis is labeled according to the schedule of LD12:12—ZT00 is therefore lights-on under LD12:12. (d) Gate widths of adult emergence rhythm under LD08:16, LD12:12, and LD16:08. Under all three photoperiods, gate widths of emergence rhythm in selected populations were smaller compared with controls. Error bars indicate 95% confidence intervals (95% CIs) around the means for visual hypothesis testing. (e, f) Synchrony and accuracy of adult emergence rhythms under LD08:16, LD12:12, and LD16:08. *PP* exhibit enhanced synchrony and accuracy in emergence rhythms across all three photoperiods compared with controls. A total of 10 vials with ~300 eggs were used for each population of both *PP* and *CP* in this assay. Statistically significant levels of difference between the selected and control stocks are indicated by asterisks.

TABLE 1. Adult emergence rhythm of populations selected for emergence in a narrow window of time under wide range of environmental conditions.

		% emergence ZT01–02	Gate width (h)	Synchrony (h^{-1})	Accuracy (h^{-1})
Control populations *(CP)*					
Photoperiod (h)	08:16	18.3 ± 1.6	10.9 ± .7	1.5 ± .8	2.8 ± 1.3
	12:12	12.1 ± 1.5	11.7 ± .7	2.5 ± .8	5.1 ± 1.3
	16:08	6.8 ± 1.3	15.9 ± .7	.8 ± .8	.8 ± 1.3
Light intensity (lux)	1	11. ± 1.4	13.0 ± .6	.9 ± 1.2	2.9 ± .7
	10	12.0 ± .8	11.8 ± .6	.8 ± 1.2	1.8 ± .7
	100	12.4 ± 1.1	11.7 ± .6	2.5 ± 1.2	5.1 ± 0.7
	1000	13.4 ± 1.6	12.2 ± .6	1.6 ± 1.2	5.4 ± .7
Temperature (°C)	18	14.8 ± 2.0	9.5 ± .4	1.0 ± .6	3.0 ± 1.2
	25	12.1 ± 1.5	11.9 ± .4	2.5 ± .6	5.2 ± 1.2
	29	8.3 ± 1.4	12.1 ± .4	1.9 ± .6	3.5 ± 1.2
Precision populations (*PP*)					
Photoperiod (h)	08:16	24.6 ± 1.6	10.15 ± .7	7.2 ± .8	9.1 ± 1.3
	12:12	22.9 ± 1.5	9.3 ± .7	5.3 ± .8	8.6 ± 1.3
	16:08	10.7 ± 1.3	13.4 ± .7	2.6 ± .8	3.6 ± 1.3
Light intensity (lux)	1	11.3 ± 1.4	11.1 ± .6	3.3 ± 1.2	6.9 ± .7
	10	16.8 ± .8	9.6 ± .6	3.8 ± 1.2	4.4 ± .7
	100	21.8 ± 1.1	9.3 ± .6	5.3 ± 1.2	8.6 ± .7
	1000	23.0 ± 1.3	10.7 ± .6	4.1 ± 1.2	8.3 ± .7
Temperature (°C)	18	8.6 ± 2.0	14.8 ± 0.4	1.5 ± .6	4.6 ± 1.2
	25	22.9 ± 1.5	9.2 ± .4	5.3 ± .6	8.6 ± 1.2
	29	11.4 ± 1.4	10.3 ± .4	2.6 ± .6	8.0 ± 1.2

Selected flies emerged in a narrow gate width of adult emergence rhythm under 25°C and 29°C ambient temperatures (Mukherjee et al., 2012). All values are mean ± 95% confidence interval (CI).

in LD08:16 *PP* flies were more active before lights-on than *CP* (Figure 2a, b). Although the evening activity peak of both stocks occurred after lights-off under LD08:16 and before lights-off in LD16:08, there was no difference in their amplitudes (Figure 2a, b). ANOVA on hourly binned activity data revealed statistically significant effects of S (LD08:16, $F_{1,3} = 20.9$, $p < .01$), T (LD08:16, $F_{23,69} = 323.2$, $p < .0001$; LD12:12, $F_{23,69} = 166.9$, $p < .0001$; LD16:08, $F_{1,3} = 289.54$, $p < .0001$), and S × T interaction (LD08:16, $F_{23,69} = 1.8$, $p < .03$; LD12:12, $F_{23,69} = 2.47$, $p < .002$). However, the effects of S (LD12:12, $F_{1,3} = .1$, $p = .4$; LD16:08, $F_{1,3} = .1$, $p = .08$) and S × T interaction (LD16:08, $F_{23,69} = 1.16$, $p = .3$; Table 2) were statistically not significant. Post hoc multiple comparisons using Tukey's test revealed that under LD08:16, activity during ZT21–23 was significantly greater in *PP* compared with *CP*. Furthermore, under LD12:12, activity levels during ZT21–22 and ZT00–02 were significantly greater in *PP* compared with *CP*. Although synchrony and accuracy in phase of *PP* were greater than *CP*, the differences did not reach statistical levels of significance (Figure 2c, d). In essence, persistence of enhanced morning peak of activity under a wide range of photoperiods in *PP* as compared with *CP* suggests that flies selected for emergence during a narrow window of time have more robust activity pattern.

Selected Stocks Display Stable and Robust Emergence Rhythm Under a Wide Range of Light Intensities

To study if emergence rhythm of selected stocks was robust enough to cope with variations in zeitgeber strength, we assayed this rhythm under LD12:12 with four different light intensities (1, 10, 100, and 1000 lux). Under all four light intensities, amplitude of emergence peak in *PP* was greater and emergence waveform was more stable than *CP* (Figure 3a, b). ANOVA on the emergence data revealed statistically significant effects of S ($F_{1,3} = 197.4$, $p < .0007$), I ($F_{3,9} = 32.6$, $p < .0001$), T ($F_{11,33} = 283.6$, $p < .0001$), and S × I ($F_{23,69} = 6$, $p < .0001$), S × T ($F_{11,33} = 49.9$, $p < .0001$), I × T ($F_{33,99} = 25.1$, $p < .0001$), and S × I × T ($F_{33,99} = 4.65$, $p < .0001$; Table 1) interactions. Post hoc multiple comparisons using Tukey's test revealed that under LD with intensity greater than 1 lux, emergence was significantly higher during ZT01–-02 (selection window) in *PP* compared with *CP*. The most striking difference between the emergence profiles of selected and control stocks was seen under LD with 1 lux intensity; emergence rhythm in control stocks damped considerably, whereas that in selected stocks persisted robustly albeit with a phase delay of 2 h (Figure 3a, b).

In addition, under LD with relatively dim light intensities (≤100 lux), *PP* had a narrower gate width of emergence compared with *CP* (Figure 3c). ANOVA on the gate width data revealed statistically significant effects of S ($F_{1,3} = 245.7$, $p < .0005$), I ($F_{3,9} = 9.4$, $p < .003$), and S × I interaction ($F_{3,9} = 12.6$, $p < .001$; Table 1). Post hoc multiple comparisons using Tukey's test revealed that selected stocks have much tighter emergence rhythm than controls under a wide range of light intensities (≤100 lux; Figure 3a, c).

Persistence of enhanced peak and tighter emergence waveform were associated with improved synchrony (Figure 3d) and accuracy (Figure 3e) in *PP* under a wide range of light intensities. ANOVA revealed

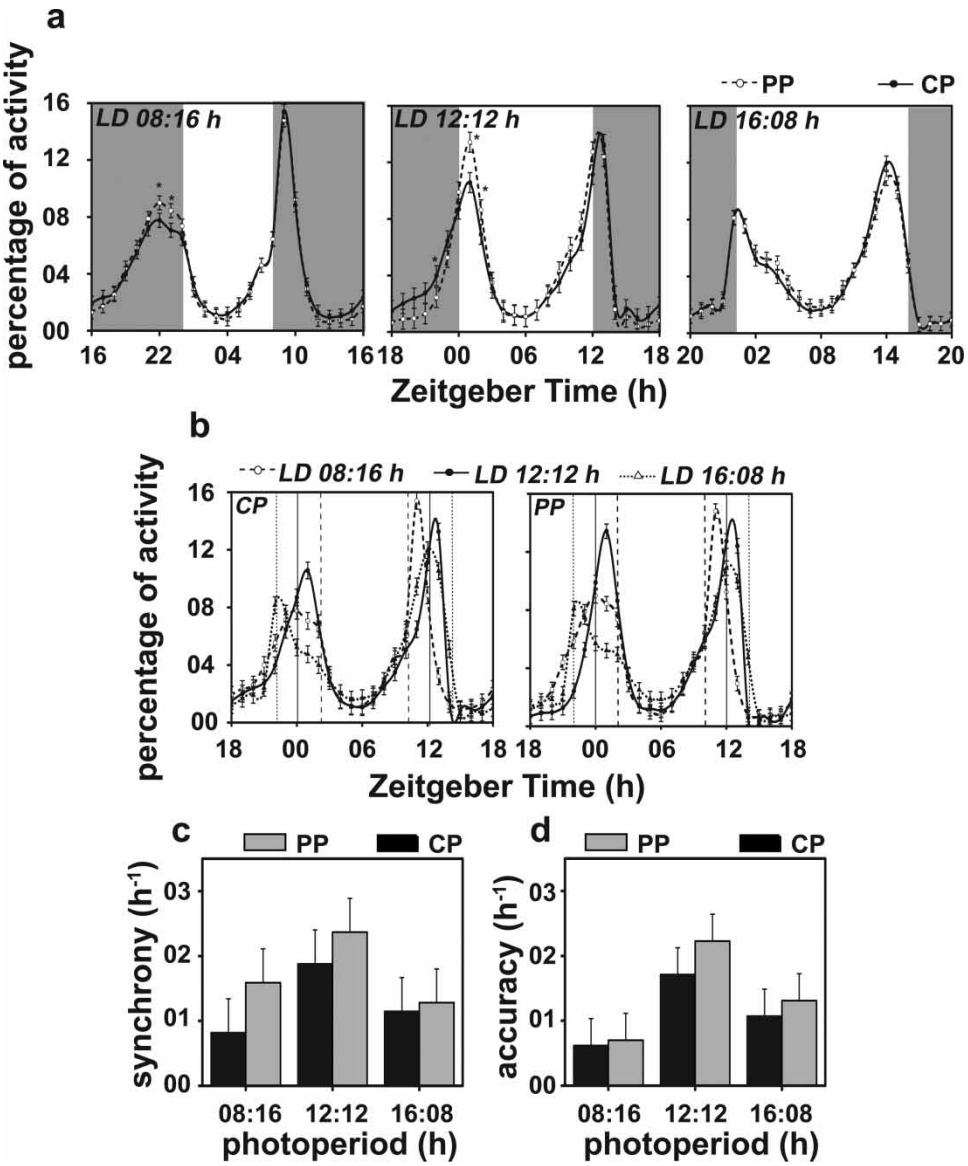

FIGURE 2. Morning peak of activity is enhanced in selected (*PP*) flies compared with controls (*CP*) under widely different photoperiods. (a) Waveforms of activity rhythm in selected and control flies under LD08:16, LD12:12, and LD16:08. Percentage activity in every 1 h interval is plotted across zeitgeber time. Under LD08:16, morning peak of activity is phase-advanced in both control and selected populations; however, flies from selected populations show enhanced morning peak compared with controls. (b) Waveforms of activity rhythm under LD08:16, LD12:12, and LD16:08. Percentage activity in every 1 h interval is plotted along the *y*-axis and zeitgeber time along the *x*-axis. Dashed, solid, and dotted lines represent the lights-on and lights-off under LD08:16, LD12:12, and LD16:08, respectively. (c, d) Synchrony and accuracy of phase under various photoperiods. Light intensity during the light phase of LD was ~100 lux. Error bars indicate 95% confidence intervals (95% CIs) around the means for visual hypothesis testing. In this assay, a total of 32 flies were used for each of the selected and control populations. All other details are the same as in Figure 1.

statistically significant effects of I (synchrony, $F_{3,9} = 7.8$, $p < .01$; accuracy, $F_{3,9} = 12.4$, $p < .001$) and S (synchrony, $F_{1,3} = 102.2$, $p < .002$; accuracy, $F_{1,3} = 482.4$, $p < .0002$), whereas the effect of S × I interaction was statistically not significant (synchrony, $F_{3,9} = .06$, $p = .9$; accuracy, $F_{3,9} = .57$, $p = .6$; Table 1). Post hoc multiple comparisons using Tukey's test revealed that, under a wide range of light intensities, selected stocks display higher synchrony and accuracy in their emergence rhythm compared with controls. With the selection being imposed on the emergence phenotype, enhanced stability of emergence profiles in selected populations compared with controls, across different zeitgeber strengths (in the form of various light intensities), offers a clear evidence for enhanced robustness of their circadian timing systems in the face of changing environments.

Selected Flies Display Robust Activity Rhythm Under a Wide Range of Light Intensities

Under a wide range of light intensities, *PP* flies were more active in the morning and their morning activity peak (ZT21–03) was of greater amplitude than *CP*

TABLE 2. Activity/rest rhythm of flies from populations selected for emergence in a narrow window of time under wide range of environmental conditions.

		% activity ZT21–03	synchrony (h^{-1})	accuracy (h^{-1})
Control populations *(CP)*				
Photoperiod (h)	08:16	36.5 ± 2.8	.8 ± .5	.6 ± .4
	12:12	38.4 ± 3.3	1.9 ± .5	1.7 ± .4
	16:08	34.6 ± 4.7	1.1 ± .5	1.1 ± .4
Light intensity (lux)	1	34.9 ± 1.5	2.1 ± .2	1.5 ± .3
	10	36.4 ± 3.3	2.2 ± .2	1.7 ± .3
	100	38.4 ± 3.3	1.9 ± .2	1.7 ± .3
	1000	39.9 ± 3.5	2.2 ± .2	1.8 ± .3
Temperature (°C)	18	33.7 ± 2.1	2.2 ± .5	1.4 ± .1
	25	38.4 ± 3.3	1.9 ± .5	1.7 ± .1
	29	41.2 ± 3.7	2.0 ± .5	1.2 ± .1
Precision populations (*PP*)				
Photoperiod (h)	08:16	39.0 ± 2.8	1.6 ± .8	.7 ± .4
	12:12	44.6 ± 3.3	2.4 ± .8	2.3 ± .4
	16:08	37.5 ± 4.7	1.3 ± .8	1.3 ± .4
Light intensity (lux)	1	37.2 ± 1.5	2.4 ± .2	1.7 ± .3
	10	39.5 ± 3.3	2.4 ± .2	2.2 ± .3
	100	44.6 ± 3.3	2.4 ± .2	2.3 ± .3
	1000	43.8 ± 3.5	2.1 ± .2	2.5 ± .3
Temperature (°C)	18	35.0 ± 2.1	2.3 ± .5	1.4 ± .1
	25	44.6 ± 3.3	2.4 ± .5	2.3 ± .1
	29	41.0 ± 3.7	2.4 ± .5	1.2 ± .1

Activity between ZT21 and ZT03 (3 h before lights-on to 3 h after lights-on) is considered as the morning peak of activity. All values are mean ± 95% confidence interval (CI).

(Figure 4a). ANOVA revealed statistically significant effects of T ($F_{23,69} = 726$, $p < .0001$) and S × T ($F_{23,69} = 6.91$, $p < .0001$), I × T ($F_{46,138} = 8.09$, $p < .0001$), and S × I × T ($F_{46,138} = 4.31$, $p < .0001$) interactions, whereas the effects of I ($F_{2,6} = 2.8$, $p = .4$) and S ($F_{1,3} = 3.3$, $p = .4$; Table 2) were statistically not significant. Post hoc multiple comparisons using Tukey's test revealed that under four different light intensities, selected flies displayed significantly enhanced morning activity (ZT00–01) compared with controls (Figure 4a). Additionally, *PP* flies also exhibited increased activity during the selection window (ZT01–02) compared with controls at light intensities greater than 1 lux. This was associated with enhanced accuracy in phase of *PP* flies. Although *PP* displayed enhanced synchrony only at 100 lux, they showed higher accuracy under light intensities higher than 10 lux (Figure 4b, c). At 1 lux, the activity profiles of the selected and control stocks were most strikingly different—the morning activity peak and evening anticipatory activity of selected flies were significantly greater than controls, which exhibited enhanced nighttime activity. The robustness of activity profiles of the selected flies under various zeitgeber strengths taken together with similar observations made for the emergence profiles offers clear evidence for the evolution of robustness in circadian timing systems as a correlated response to selection for emergence in a narrow window of time.

Selected Stocks Display Robust Emergence Rhythm Under Light/Dark Cycles at Different Ambient Temperatures

Under 25°C and 29°C, *PP* had higher and more consolidated emergence rhythm compared with *CP* (Figure 5a–c). ANOVA on the emergence data revealed statistically significant effects of TP ($F_{11,11} = 26.7$, $p < .0001$) and S × TP ($F_{11,11} = 9.5$, $p < .0004$), T × TP ($F_{22,22} = 4.6$, $p < .0003$), and S × TP × T ($F_{22,22} = 8.9$, $p < .0001$) interactions, whereas the effect of S was statistically not significant ($F_{1,11} = 1$, $p = .5$; Table 1). Post hoc multiple comparisons using Tukey's test revealed that at 25°C and 29°C, percentage emergence during ZT01–02 (selection window) in *PP* was significantly higher compared with *CP*. Furthermore, in an earlier study we have shown that at 29°C selected stocks had a narrower gate width than controls ($p < .02$), and the difference in gate width was reduced considerably at 18°C (Mukherjee et al., 2012). Also at lower temperature, emergence waveform of selected stocks was phase-delayed compared with controls (Figure 5a–c).

At 25°C, *PP* stocks had higher synchrony and accuracy in emergence rhythm compared with *CP* (Figure 5d, e), whereas at 29°C *PP* stocks display higher accuracy and at 18°C synchrony and accuracy of both stocks did not differ. ANOVA revealed statistically significant effects of TP (synchrony, $F_{2,2} = 74.7$, $p < .01$; accuracy, $F_{2,2} = 163.8$, $p < .006$) and S (accuracy, $F_{1,1} = 5.69$, $p < .02$), whereas the effects of S (synchrony, $F_{1,1} = 24.6$, $p = .1$) and S × P interaction (synchrony, $F_{2,2} = .1$, $p = .8$; accuracy, $F_{2,2} = .89$, $p = .5$; Table 1) were statistically not significant. Post hoc multiple comparisons using Tukey's test revealed a significantly higher accuracy in *PP* compared with *CP* under higher temperature. These results suggest that selected stocks display more consolidated and stable emergence rhythm compared with controls particularly at higher temperatures.

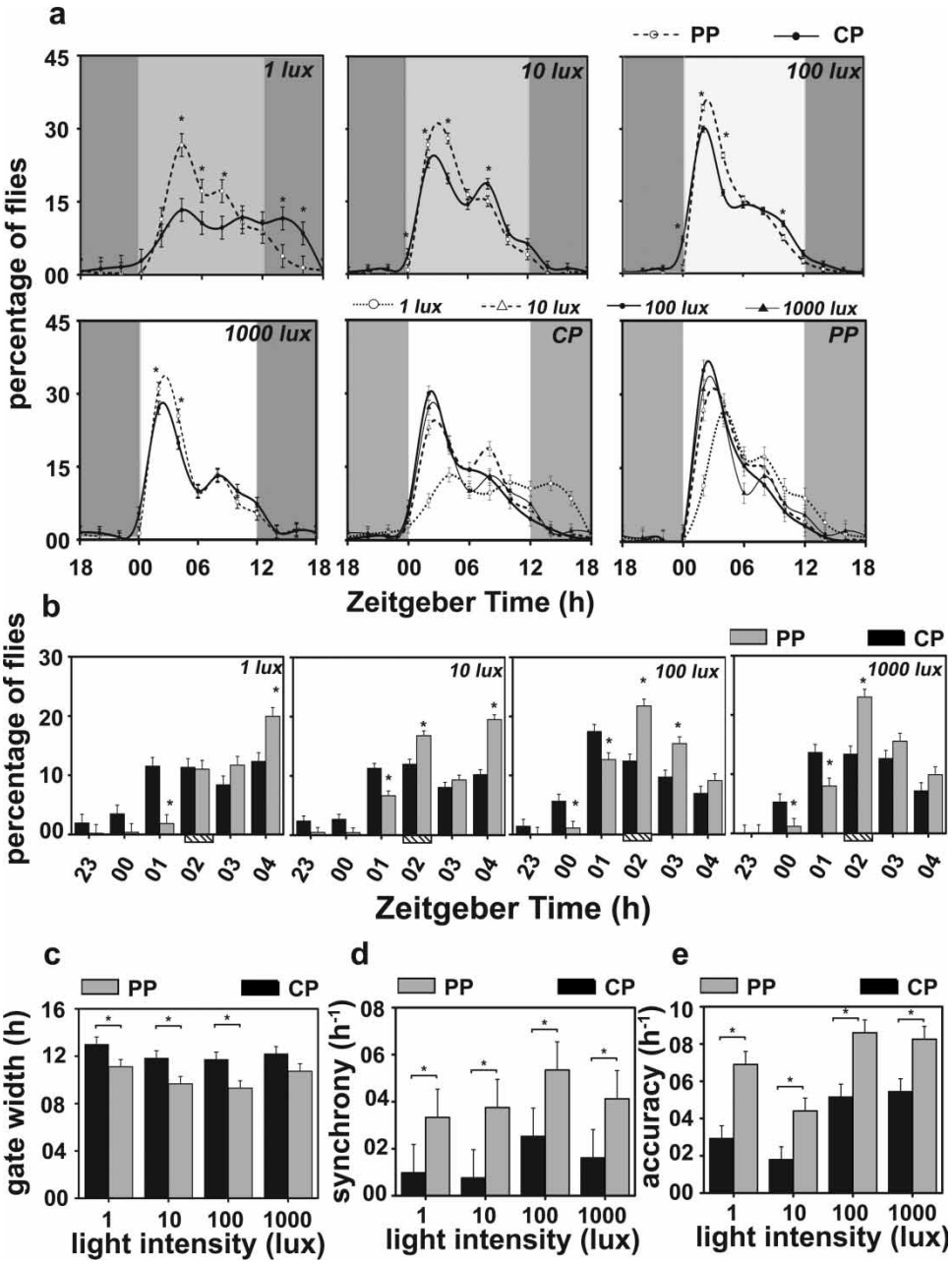

FIGURE 3. Selected (*PP*) populations display enhanced peak of emergence compared with controls (*CP*) under various light intensities. (a) Waveforms of emergence rhythm in selected and control populations under 12:12 h light/dark (LD) cycles at ~25°C, with light intensities of 1, 10, 100, and 1000 lux. With decreasing light intensity, the emergence rhythm weakened in control populations, whereas in selected populations it persisted stably. (b) Percentage of flies emerged during ZT23–04 when assayed under 1, 10, 100, and 1000 lux. Although light intensity modified emergence waveforms of both populations, emergence during the selection window (ZT01–02) continued to be enhanced in selected populations compared with controls. Clear emergence peak was seen in selected populations even under low light (1 lux) albeit phase-delayed by 2 h, in control flies it disappeared and flies emerged throughout the day. (c) Gate widths of emergence rhythm under LD of 1, 10, 100, and 1000 lux intensities. Selected flies continued to have narrower gate of emergence under LD of light intensity less than 1000 lux, whereas at higher light intensity (1000 lux) the gate widths of selected and control populations did not differ. (d, e) Synchrony and accuracy of adult emergence rhythms under various light intensities. The selected flies exhibited enhanced synchrony and accuracy across a range of light intensities. In this assay, a total of 10 vials with ~300 eggs were used for each of the selected and control populations. All other details are the same as in Figure 1.

Selected Stocks Display Altered Activity Rhythm to Suit Different Ambient Temperatures Under Light/Dark Cycles

The selected flies exhibited better ability to adjust to LD at different ambient temperatures compared with *CP*. At 25°C, morning activity peak of *PP* was higher than *CP*, at 18°C *PP* flies became more active in the morning and less active by evening, and at 29°C they were less active during the day and more active at night (Figure 6a–c). Although *PP* flies exhibited enhanced phase synchrony at 25°C and 29°C, these differences did not reach statistical levels of significance (Figure 6d). *PP* flies exhibited enhanced accuracy at 25°C; however, at lower as well as higher temperatures the

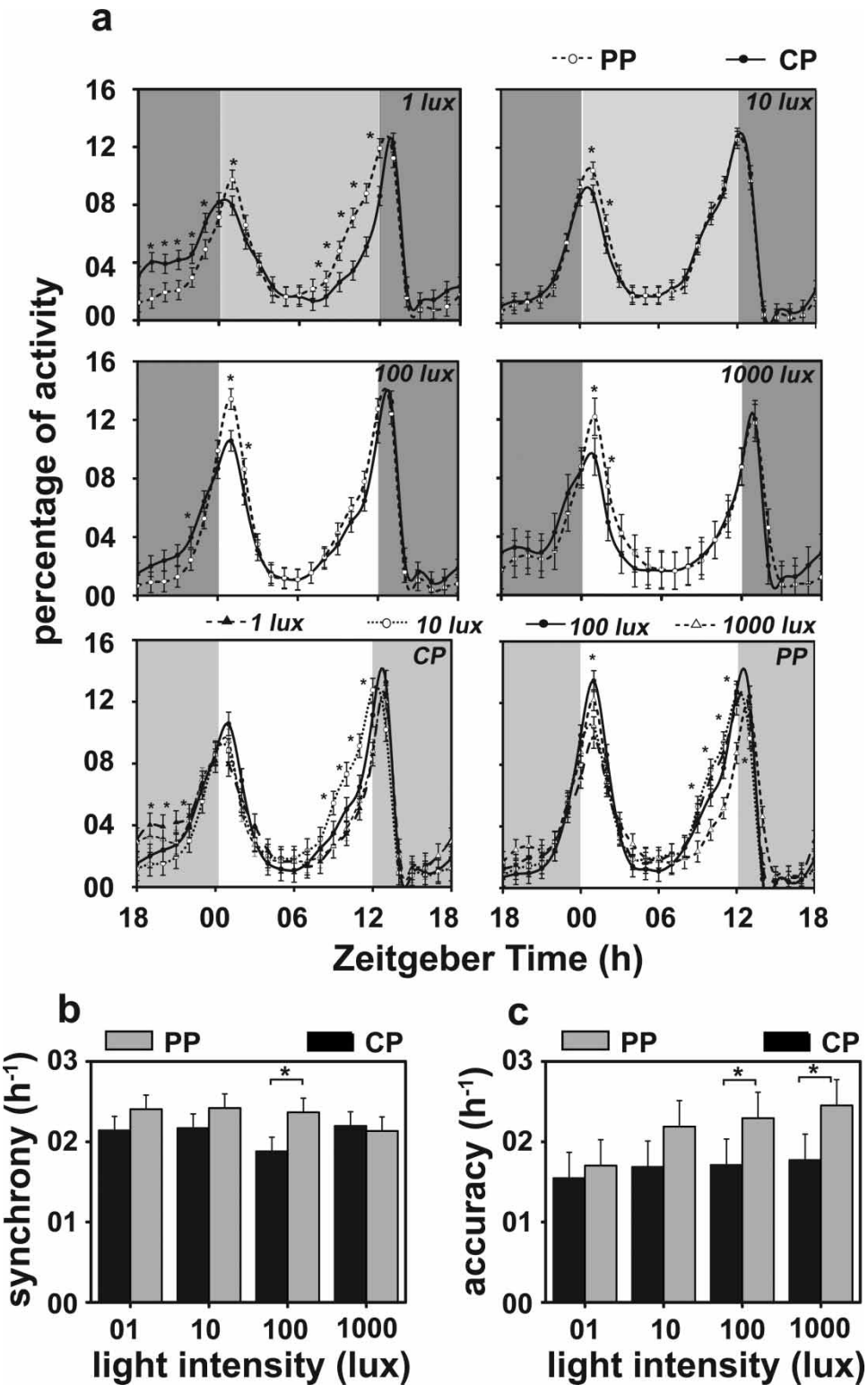

FIGURE 4. Enhanced morning activity peak in selected (*PP*) flies compared with controls (*CP*) under a wide range of light intensities. (a) Waveforms of activity rhythm of selected and control flies under 12:12 h light/dark (LD) cycles at ~25°C with light intensities of 1, 10, 100, and 1000 lux during light phase. Percentage of activity in 1-h intervals is plotted along the *y*-axis and zeitgeber time in h along the *x*-axis. Under 1 lux, selected flies displayed phase delay in morning activity peak compared with controls that is comparable to phase delay in emergence rhythm observed under the same environmental condition. Moreover, selected flies displayed enhanced morning peak of activity in all light intensities. (b, c) Synchrony and accuracy of phase under wide range of light intensities. The selected flies exhibit more accuracy at light intensities above 10 lux compared with controls. In this assay, a total of 32 flies were used for each of the selected and control populations. All other details are same as in Figure 1.

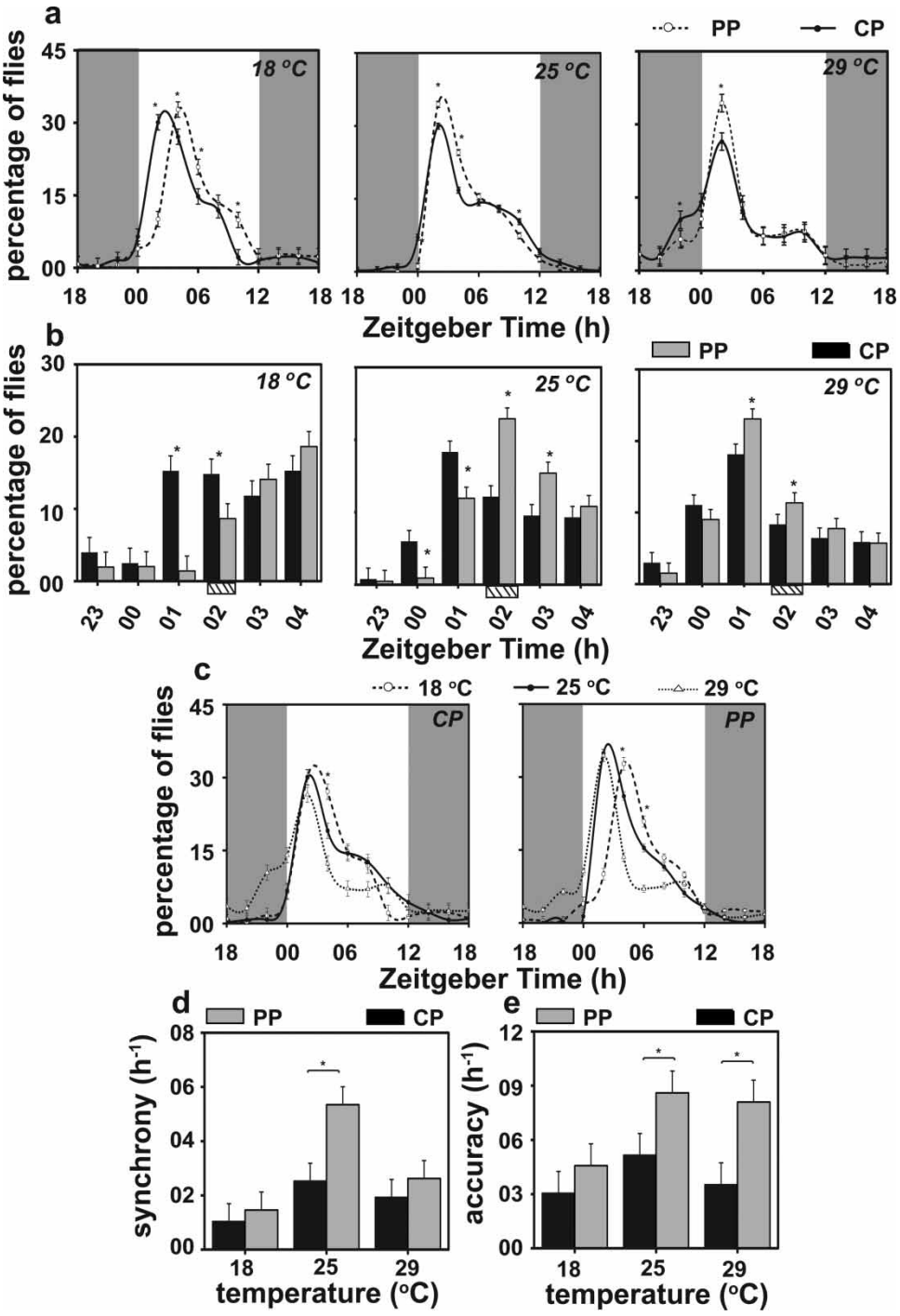

FIGURE 5. Robust emergence rhythms of selected (*PP*) and control (*CP*) stocks under 12:12 h light/dark cycles at different temperatures. (a) Waveforms of emergence of selected and control stocks were assayed under 12:12 h light/dark (LD) cycles at 18°C, 25°C, and 29°C. Emergence peak of selected stocks exhibited phase delay of 2 h under lower temperature, whereas under higher temperature they emerged with a narrower emergence gate and sharper emergence peak compared with controls. (b) Percentage emergence during ZT23–04 under 12:12 h LD at 18°C, 25°C, and 29°C. The selected flies exhibited increase in percentage of emergence during selection window (ZT01–02) at 25°C and 29°C. Percentage emergence is plotted with 2 h resolution in a and 1 h resolution in b. Hence the difference between selected and control flies at ZT04 is statistically significant in a, whereas this difference is diminished in b due to the higher resolution. (c) Adult emergence rhythms of selected and control stocks assayed under 12:12 h LD cycles at 18°C, 25°C, and 29°C. (d, e) Synchrony and accuracy of adult emergence rhythms under different temperatures. The selected flies exhibited enhanced accuracy under higher temperature. In this assay, a total of 10 vials with ~300 eggs were used for each of the selected and control flies. All other details are the same as in Figure 1.

differences between *PP* and *CP* did not reach statistical levels of significance (Figure 6e). ANOVA on activity data revealed statistically significant effects of TP ($F_{23,69} = 235$, $p < .0001$) and S × T ($F_{23,69} = 4.2$, $p < .0001$), TP × T ($F_{46,138} = 19.18$, $p < .0001$), and S × TP × T ($F_{46,138} = 2.4$, $p < .0001$) interactions; however, the effects of T ($F_{2,6} = 1$, $p = .4$) and

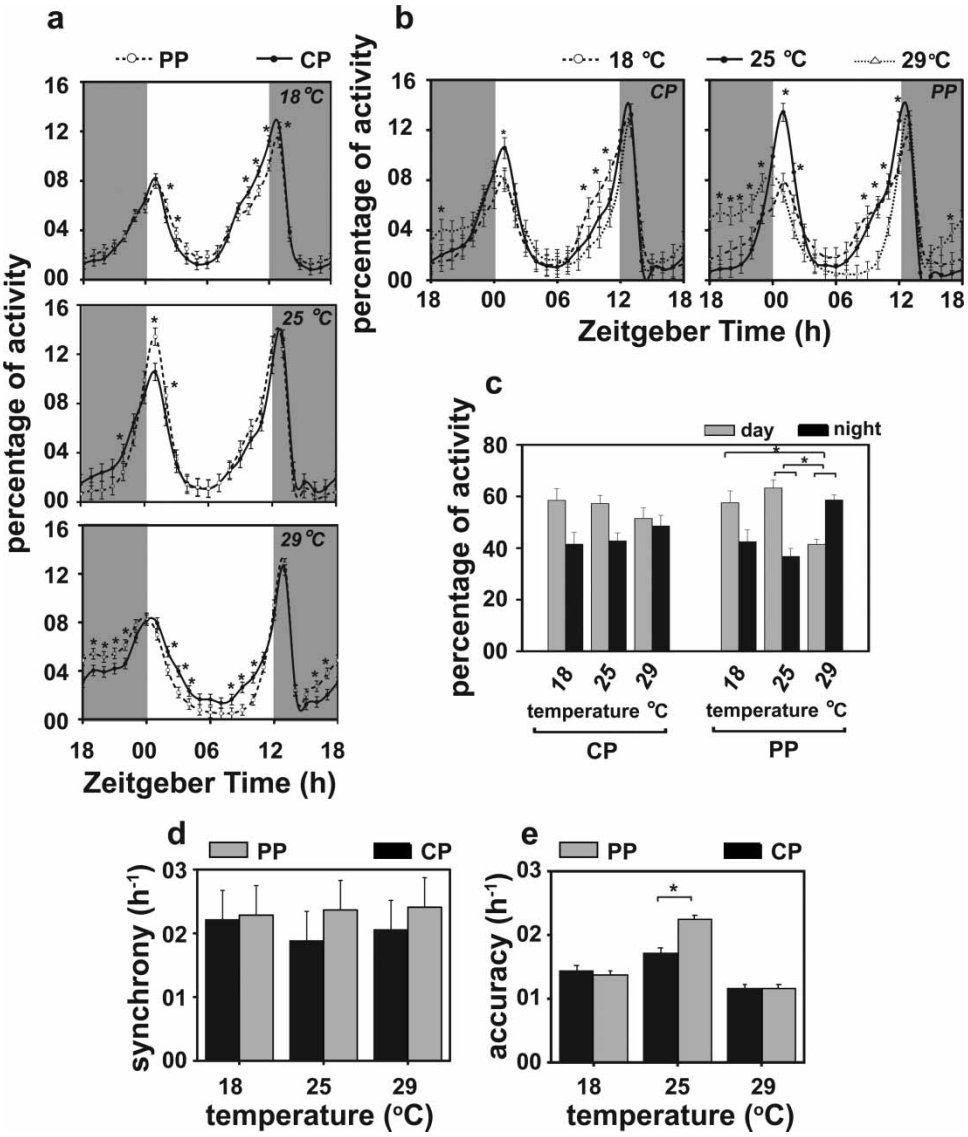

FIGURE 6. Activity rhythms of selected (*PP*) and control (*CP*) populations assayed at 18°C, 25°C, and 29°C under 12:12 h light/dark (LD) cycles. (a) Waveforms of activity rhythm of selected and control flies under 12:12 h LD cycles at 18°C, 25°C, and 29°C. At 18°C, selected flies exhibited enhanced activity in the morning (ZT01–03) and delayed build-up of evening activity peak. At 29°C, these flies exhibited enhanced nighttime and reduced daytime activity. (b) Waveforms of activity rhythm of selected and control flies assayed under 12:12 h LD cycle at 18°C, 25°C, and 29°C. (c) Percentage activity of selected and control flies during daytime (ZT01–12) and nighttime (ZT13–00) under 12:12 h LD cycles at 18°C, 25°C, and 29°C. At 18°C and 25°C, selected flies exhibited enhanced activity during daytime and reduced activity during nighttime, and vice versa under high temperature (29°C). (d, e) Synchrony and accuracy of phase under various temperatures. In this assay, a total of 32 flies were used for each of the selected and control populations. All other details are the same as in Figure 1.

S ($F_{1,3} = 1$, $p = .4$; Table 2) were not statistically significant. Post hoc multiple comparisons using Tukey's test revealed that at lower temperature *PP* flies display enhanced activity compared with *CP* during morning hours (ZT01–03), whereas the activity decreases compared with *CP* during evening hours (ZT10–12; Figure 6a, b). At higher temperature, *PP* showed reduced daytime (ZT02–04, 06, 08–10) activity and increased nighttime activity (ZT17–23; Figure 6a, b). These results suggest that flies selected for emergence during a narrow window of time are better at adapting to harsh environmental conditions by increasing the nocturnal activity at higher temperature compared with controls.

Selected Populations Have Enhanced Homogeneity and Precision of Circadian Clocks

In order to study how precise the endogenous clocks of *PP* flies are, we assayed activity rhythm under DD at 25°C, after 90 generations of selection. During the first subjective day, *PP* maintained higher amplitude of activity with prominent morning peak compared with *CP* (Figure 7a). In *PP*, a significantly greater proportion of flies displayed circadian rhythmicity with τ significantly shorter than *CP* (Figure 7b) and have evolved faster running circadian clocks compared with *CP* (Figure 7c). Besides, *PP* flies were more homogenous in terms of their τ (Figure 7d) and evolved enhanced precision in

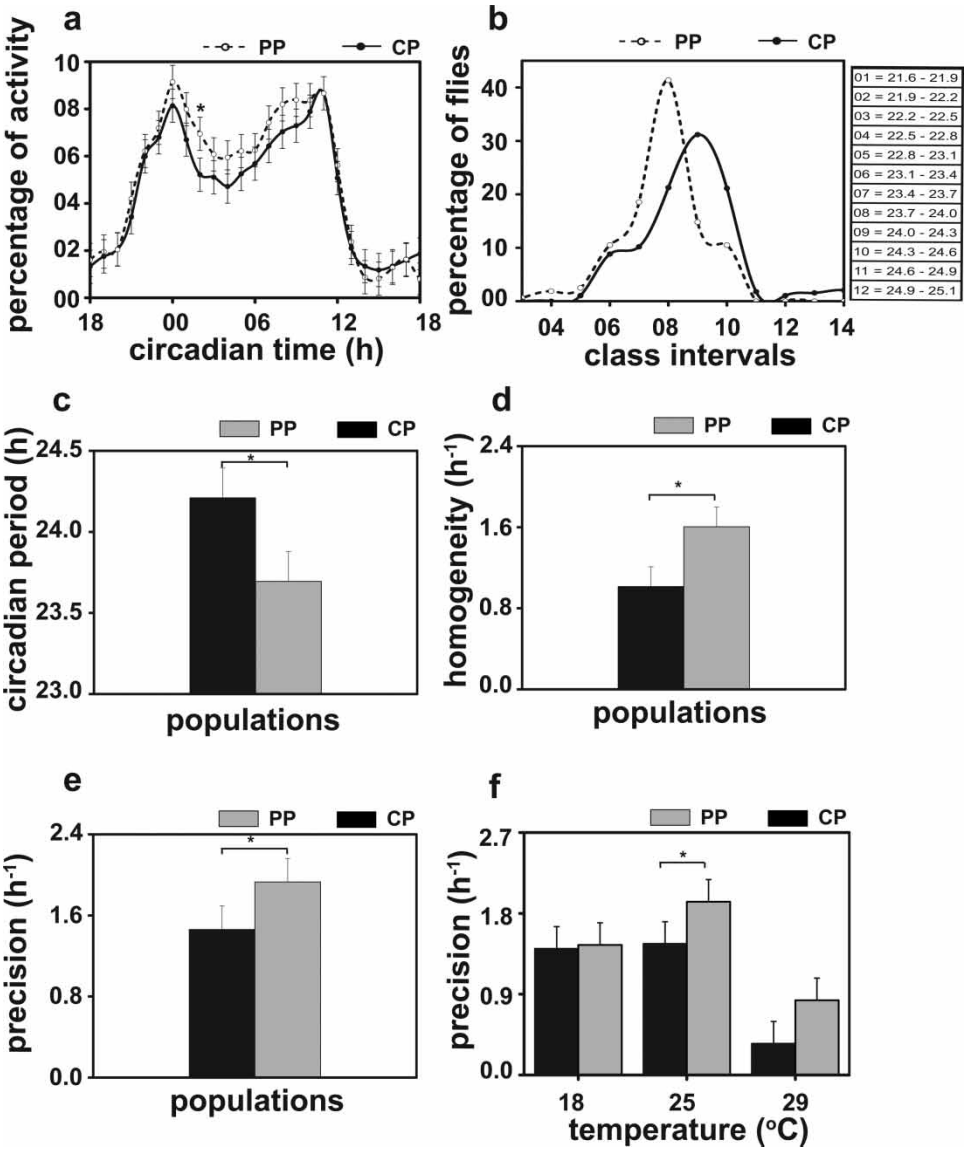

FIGURE 7. Stability of circadian timing system of flies selected for emergence in a narrow window of time. (a) Amplitude of activity rhythm during the first cycle under constant darkness (DD). Circadian time (h) is plotted along the *x*-axis and percentage of activity along the *y*-axis. The selected (*PP*) flies maintained higher amplitude in activity rhythm during first subjective day compared with controls (*CP*). (b) Frequency distributions of τ in *PP* and *CP* populations. Class interval of time (h) is plotted along *x*-axis and percentage of flies along the *y*-axis. The class intervals are defined in the table to the right of the figure. *PP* flies had shorter τ with a narrower distribution between 23.7 and 24 h. (c) Circadian period (τ) of activity/rest rhythm. *PP* flies had faster running circadian clocks compared with controls. Homogeneity (d) in τ and clock precision (e) were enhanced in the selected flies compared with *CP* controls. (f) Precision of activity/rest rhythms under 18°C, 25°C, and 29°C. In this assay, a total of 32 flies were used for each of the selected and control populations. All other details are the same as in Figure 1.

their free-running circadian rhythm (Figure 7e). ANOVA on τ, homogeneity, and precision data revealed a statistically significant effect of S (circadian period, $F_{1,3} = 38.01$, $p < .008$; homogeneity, $F_{1,3} = 16.6$, $p < .02$; precision, $F_{1,3} = 19.7$, $p < .02$; Table 2). Post hoc multiple comparisons using Tukey's test revealed that *PP* flies had shorter τ and increased homogeneity and precision compared with controls. The Kolmogorov-Smirnov two-sample test also revealed that the distribution of τ ($p < .01$) was significantly different in *PP* relative to *CP*. These results suggest that selection for emergence in a narrow window of time led to the evolution of increased robustness of circadian clocks. Further support for this result is obtained from the increased precision in emergence rhythm as measured by the inverse of cycle-to-cycle variance in emergence peaks.

Precision of Circadian Clocks Under Various Ambient Temperatures

To study precision of circadian clocks of *PP* flies under DD at various ambient temperatures, we assayed activity rhythm at 18°C, 25°C, and 29°C. At 25°C, *PP* flies exhibited enhanced precision but at lower temperature it was not different from *CP* controls. At 29°C, precision was

reduced in both *PP* and *CP* but *PP* flies still showed a trend of enhanced precision compared with *CP* (Figure 7f). ANOVA on the precision data revealed statistically significant effects of TP ($F_{2,6} = 118.6$, $p < .0001$) and S ($F_{1,3} = 3.09$, $p < .01$), whereas the effect of TP × S interaction was statistically not significant ($F_{2,6} = 3.1$, $p = .1$). Post hoc multiple comparisons using Tukey's test revealed that circadian clocks of *PP* flies were more precise compared with controls at 25°C. These results suggest that circadian clocks of *PP* flies were more precise than *CP* controls and ambient temperature affects precision of circadian clocks.

DISCUSSION

The results of our study revealed that circadian clocks of fruit fly *D. melanogaster* populations selected for adult emergence in a narrow window of time evolved enhanced robustness in adult emergence and activity/rest rhythms. In our attempt to assess robustness, we discovered that emergence rhythms of selected and control stocks undergo photoperiod-dependent modulation, with more consolidated waveform under short photoperiod and less consolidated behaviors under longer photoperiod; however, in all three photoperiods selected stocks displayed persistence of higher amplitude and synchronous, accurate, and consolidated emergence rhythm compared with controls (Figure 1). This suggests that selection for emergence in a narrow window of time results in the evolution of robust adult emergence rhythm in the selected stocks, which can withstand a wide range of photoperiods.

Animals adjust their activity to seasonal changes in daylength by consistent phase modulation of morning and evening activity peaks (Majercak et al., 1999; Rieger et al., 2003; Shafer et al., 2004). The amplitude of the evening activity peaks of selected and control flies did not differ under any of the three photoperiods (Figure 2a); additionally, the evening peaks occurred prior to lights-off during long days and after lights-off on short days in both the populations. Unlike the similarity in the evening peak of activity, selected flies exhibited greater stability in their morning peak of activity compared with controls under different photoperiods, implying enhanced robustness in the underlying circadian clocks. Enhanced morning peak of activity indicates the possibility of enhanced accuracy of the underlying circadian timing systems. Accuracy of *PP* flies was indeed higher under LD12:12; however, this difference diminished under short- and long-day conditions (data not shown). In a previous study, morning peak of activity in *Drosophila* was reported to weaken with increasing photoperiods (Rieger et al., 2012). Although the morning activity peaks of both the stocks were reduced under longer photoperiod, under shorter period, activity rhythm of selected stocks continued to display greater stability compared with controls, although both showed lower amplitude morning peak. The ability of selected flies to adapt to short or long photoperiods is likely to be due to the altered genetic architecture of their core molecular clockwork. The core clock genes in *Drosophila timeless* (*tim*) occurs in two allelic forms, *ls-tim* and *s-tim* (Rosato et al., 1997), and *tim* polymorphism is thought to be involved in the seasonal adaptation of circadian rhythms (Kyriacou et al., 2008; Tauber et al., 2007). We therefore speculate that *tim* polymorphism may be involved in addition to other molecular mechanisms in maintaining the robustness of circadian timing systems in flies selected for emergence within a narrow window of time.

Apart from daylength, levels of light also modulate circadian rhythms. Under lower light intensity (1 lux), emergence rhythm of control flies became considerably weaker, whereas that of selected flies continued unabated, with a sharp and consolidated emergence peak with enhanced synchrony and accuracy (Figure 3). Since emergence in the selected populations is directly subjected to selection pressure, increased stability in the emergence rhythms of selected populations in comparison with controls across a variety of zeitgeber strengths suggests that these flies have evolved enhanced robustness in their underlying circadian clocks. To examine this further, we studied the activity rhythm of these flies under LD with different light intensities. Interestingly, selected flies continued to display consolidated bimodal activity rhythm over a wide range of light intensities; exhibiting enhanced morning peak compared with controls even under apparently weak LD cycles, confirming the robustness of their circadian clocks. Although control flies showed extremely feeble emergence rhythm at low (1 lux) light intensity (Figure 3a), clear morning and evening peaks could be seen in their activity profiles, though far less robust compared with selected flies (Figure 4). This result suggests that the threshold of light that can affect the consolidated nature of emergence and activity rhythms are different between selected and control flies. Following this logic, mechanisms underlying activity rhythm seem to be less sensitive to light than that governing emergence rhythm. This was confirmed when we studied the light pulse phase response curves (PRCs) of these flies. Although the selected flies differed from controls in terms of advance phase shifts during late subjective night, at most phases, the PRCs did not differ statistically (data not shown). This further confirms that the differential responsiveness of activity and emergence rhythms to light is not as a result of altered sensitivity at the level of circadian clocks. Since light for circadian photoreception in *Drosophila* acts through rhodopsin-containing photoreceptors and cryptochrome (Emery et al., 1998, 2000; Stanewsky et al., 1998), it would be interesting to examine if selection for emergence in a narrow window of time has altered elements of input pathways resulting in enhanced light sensitivity in the selected flies. However, given that selection in our study was imposed during the early morning hours, we cannot completely

rule out the contribution of startle effects of lights-on in altered light sensitivity of the selected flies. However, under DD, selected flies maintained marginally higher amplitude in their activity rhythm during the first subjective day and showed enhanced homogeneity and precision, which suggests that the selection process worked at the level of the clock system. Similarly, under DD emergence profile of the selected populations was found to display higher amplitude compared with controls, which confirmed the notion that response to selection for emergence in a narrow window of time is primarily mediated through changes in endogenous circadian clocks and not due to startle effect of emergence to lights-on (Kannan et al., 2012).

Temperature is a key environmental factor capable of serving as a zeitgeber for the circadian clocks of a wide variety of organisms (Glaser and Stanewsky, 2007; Sweeney and Hastings, 1960). Changes in ambient temperature altered the phase of emergence rhythm in the selected flies, with a phase delay of 2 h seen at lower (18°C) temperature and phase advance of 1 h at higher (29°C) temperature (Figure 5a). Furthermore, changes in ambient temperature altered the phase of evening activity peak; it was less phase advanced at lower (18°C) and higher (29°C) temperatures compared with controls (Figure 6a). At 29°C, selected flies had an extended siesta, probably to avoid the warm light phase of LD cycles and were active mostly during nights (Figure 6a, c) and displayed lower daytime and higher nighttime activity levels compared with controls. Altered phase of emergence and of activity rhythms in selected flies under different ambient temperatures suggests that these flies have also evolved greater thermal sensitivity compared with controls. From studies on splicing of clock genes we know that colder temperatures lead to more rapid daily increases in *period* (*per)* transcript levels, which in turn results in earlier evening activity peak, and higher temperatures cause low level of splicing during the day and enhanced nighttime activity (Majercak et al., 1999). Since selected populations displayed enhanced nighttime activity compared with controls, it would be interesting to examine if such changes in activity patterns are due to altered *per* splicing. Persistence of enhanced emergence peak with increase in accuracy in the selected flies suggests that their underlying circadian clocks are robust even under high temperature. However, robustness observed in population-level rhythm in the selected flies under LD with different ambient temperatures was manifested differently at the individual-level activity rhythm, which may be because 80 generations of selection may be too early for the selection effects to percolate down to individual-level rhythms. Alternatively, it is possible that any correlated response to selection in the direction of enhanced robustness of activity rhythm is not expressed under different temperatures due to an overriding effect of temperature on clock output involved in the regulation of activity rhythm. Thus, it is plausible that the underlying neuronal and endocrinal output pathways that govern emergence and activity would have been altered in different ways by selection for emergence in a narrow window of time.

In our experiments, we observed enhanced robustness in emergence and activity rhythms of selected flies compared with controls, under a wide variety of environmental conditions. This may be because with the selection window being relatively close to lights-on, it is possible that selected flies are better able to time their emergence and activity rhythms with reference to LD cycles. However, we were not able to clearly detect such robustness in the emergence and activity phenotypes of the selected flies, especially under certain conditions. Such flexibility to temperature changes seen in the face of robustness to changes in the light conditions could be possible, since the selection protocol maintains a constant ambient temperature throughout (~25°C). Additionally, in a biological system that is constantly exposed to varying degrees of fluctuations in environmental conditions, absolute lack of flexibility in the form of extreme robustness to any sort of environmental challenge may be a useful strategy. Biological systems controlled by a complex genetic network, such as circadian clocks, are generally expected to display robustness to relatively small environmental perturbations in combination with flexibility in the face of large and drastic environmental changes. Therefore, it is possible that temperature changes in our experiments represent relatively large environmental perturbations compared with those in light conditions and therefore induce a flexible response from the flies. Additionally, recent theoretical studies with the fungal circadian clocks have offered some indications that enhanced flexibility and robustness in circadian timing systems can go hand in hand (Akman et al., 2010).

From our observations, we can decipher very similar trends in the responses shown by the selected flies under a variety of environmental conditions. For instance, in selected flies, we observe phase delay in the onset of emergence under short photoperiod (Figure 1a) and in the morning peak of emergence under low light intensity (1 lux; Figure 3a) and in LD cycles with lower ambient temperature (Figure 5a), conditions that generally mimic winter conditions. Similarly, these flies emerged for extended time under long photoperiod (Figure 1a), high light intensity (Figure 3a), and in LD cycles with higher ambient temperature (Figure 5a), i.e., in summer-like conditions. All our experiments were performed under rectangular laboratory LD cycles where the levels of light changed abruptly. These artificial conditions, however, differ markedly from natural conditions, in terms of light intensity, light quality, twilight duration, photoperiod, and temperature, all of which undergo gradual daily and seasonal changes (De et al., 2012; Vanin et al., 2012). Assessment of robustness of circadian timing systems under natural conditions extended over different seasons would provide a more realistic idea

of how stable the circadian clocks of selected flies are (since similar trends are observed under artificial conditions mimicking summer and winter).

In summary, the results of our study suggest that robustness of circadian clocks, in terms of persistent stability of emergence and activity rhythms under a wide variety of environmental conditions, evolves in fruit fly *D. melanogaster* populations, as a by-product of selection for emergence in a narrow window of time.

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