Sci Nat (2016) 103: 74
DOI 10.1007/s00114-016-1399-3

ORIGINAL PAPER

Evolution of robust circadian clocks in *Drosophila melanogaster* populations reared in constant dark for over 330 generations

Radhika Shindey[1] · **Vishwanath Varma**[1] · **K. L. Nikhil**[1] · **Vijay Kumar Sharma**[1]

Received: 7 June 2016 / Revised: 9 August 2016 / Accepted: 19 August 2016 / Published online: 1 September 2016

Abstract Robustness is considered to be an important feature of biological systems which may evolve when the functionality of a trait is associated with higher fitness across multiple environmental conditions. Thus, the ability to maintain stable biological phenotypes across environments is thought to be of adaptive value. Previously, we have reported higher intrinsic activity levels (activity levels of free-running rhythm in constant darkness) and power of rhythm (as assessed by amplitude of the periodogram) in *Drosophila melanogaster* populations (stocks) reared in constant darkness (DD stocks) as compared to those reared in constant light (LL stocks) and 12:12-h light-dark cycles (LD stocks) for over 19 years (~330 generations). In the current study, we intended to examine whether the enhanced levels of activity observed in DD stocks persist under various environments such as photoperiods, ambient temperatures, non-24-h light-dark (LD) cycles, and semi-natural conditions (SN). We found that DD stocks largely retain their phenotype of enhanced activity levels across most of the above-mentioned environments suggesting the evolution of robust circadian clocks in DD stocks. Furthermore, we compared the peak activity levels of the three stocks across different environmental conditions relative to their peaks in constant darkness and found that the change in peak activity levels upon entrainment was not significantly different across the three stocks for any of the examined environmental conditions. This suggests that the enhancement of activity levels in DD stocks is not due to differential sensitivity to environment. Thus, these results suggest that rearing in constant darkness (DD) leads to evolution of robust circadian clocks suggesting a possible adaptive value of possessing such rhythms under constant dark environments.

Keywords Circadian rhythms · Evolution · Robustness · Activity-restrhythm

Communicated by: Volker Loeschcke

Electronic supplementary material The online version of this article (doi:10.1007/s00114-016-1399-3) contains supplementary material, which is available to authorized users.

✉ Vijay Kumar Sharma
vsharma@jncasr.ac.in; vksharmas@gmail.com; http://www.jncasr.ac.in/vsharma/

[1] Chronobiology Laboratory, Evolutionary and Organismal Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore, Karnataka 560064, India

Introduction

Robustness is considered to be an important feature which enables organisms to maintain its functions against internal or external perturbations (Kitano 2004). Thus, the ability to maintain biological functions under multiple environments is likely to provide some adaptive value and to have evolved as a consequence of natural selection. Circadian clocks are ubiquitous in the living world as evidenced by its presence in almost all organisms ranging from the single-celled cyanobacteria to complex multicellular systems. The presence of circadian clocks helps organisms to adapt to both daily as well as seasonal changes in the environment they inhabit, rather than passively respond to such changes (Sharma 2003). Hence, circadian clocks are considered to be an evolutionary adaptation to cyclic changes in the environmental factors (Vaze and Sharma 2013). Although the design principles associated with robustness of circadian clocks have been explored by several theoretical studies (Cheng et al. 2001; Gonze et al. 2002; Stelling et al. 2004; Akman et al. 2010; Thommen et al. 2010; Gurevich et al. 2015), studies on the evolution of robustness (Wagner 2005) and empirical evidence for

the same are relatively sparse (Kannan et al. 2012; Nikhil et al. 2015).

Activity-rest rhythm in fruit flies *Drosophila melanogaster* is known to be under the control of circadian clocks (Konopka and Benzer 1971; Young 1998) whereby activity is restricted mainly to the daytime and consists of the morning and evening peaks separated by an afternoon "siesta" (Hamblen-Coyle et al. 1992; Wheeler et al. 1993; Helfrich-Förster 2000). There is some evidence to suggest that desiccation stress due to high temperature and low humidity during the midday might have influenced the evolution of such bimodal activity patterns (Simunovic and Jaenike 2006). Furthermore, increases in temperature, day-length, and light-intensity (all of which mimic summer conditions) are known to cause a reduction in afternoon activity and a shift in the two activity peaks to early morning and late evening hours (Majercak et al. 1999; Rieger et al. 2003, 2007; Yoshii et al. 2009) which is believed to be mediated by the splicing of the clock gene *period* (*per*; Majercak et al. 2004). Additionally, it has been observed that polymorphisms in the *per* gene in wild-caught populations of *D. melanogaster* influence the splicing efficiency and are associated with temperature-induced shifting of activity (Low et al. 2012). Thus, it is apparent that there is natural variation for the regulation of activity-rest rhythm by circadian clocks which enables flies to modulate their activity in response to harsh environmental conditions, and thus can evolve when subjected to selection.

Previous studies have demonstrated the evolution of robust circadian clocks in *Drosophila* populations subjected to laboratory selection. For instance, *D. melanogaster* populations selected for narrow gate of adult emergence exhibited enhanced amplitude for adult emergence and activity-rest rhythms in a wide range of environmental conditions, suggesting the robust nature of the evolved circadian clocks (Kannan et al. 2012). Similarly, Nikhil et al. (2015) demonstrated that *D. melanogaster* populations selected for delayed phase of adult emergence (late emergence chronotypes) evolved higher accuracy of entrainment in both emergence and activity-rest rhythms, which was robust across multiple environmental conditions. These studies demonstrate that selection for different clock properties might be associated with the coevolution of robustness in other clock-controlled traits.

We had previously studied activity-rest rhythm of *D. melanogaster* populations that were reared under three different light regimes—periodic environment of 12:12-h light-dark cycles (LD stocks), constant light (LL stocks), and constant dark (DD stocks) conditions for over 330 generations, and reported that DD stocks have evolved higher activity levels and amplitude of free-running activity-rest rhythm (Shindey et al. 2016). We also observed that DD stocks have evolved greater power (as assessed by the amplitude of the periodogram) of free-running rhythms as compared to LD and LL stocks (Shindey et al. 2016). Here, we intended to study whether such high activity levels in DD stocks might be associated with a robust underlying circadian clock, and therefore, we examined whether high activity levels seen in DD stocks are restricted only to DD conditions, or if they persist across different environments. We assayed activity-rest rhythm of all three stocks under multiple environments comprising short (LD04:20—4-h light and 20-h darkness) and long (LD20:04—20-h light and 04-h darkness) photoperiods, LD12:12 (12-h light and 12-h darkness) at low and high ambient temperatures (18 and 28 °C), non-24 h LD cycles of LD09:09 (9-h light and 9-h darkness) and LD15:15 (15-h light and 15-h darkness), and semi-natural (SN) conditions. We observed that the enhanced activity levels of DD stocks in comparison to LL and LD stocks persist under most environmental conditions. It has been proposed that to retain the functionality of important traits, the underlying genetic architecture evolves mechanisms to buffer the phenotype against environmental perturbations (Waddington 1957). Therefore, we also compared the change in peak activity levels of the three stocks between the above-mentioned environmental conditions and the activity levels in constant darkness. We found that the change in peak activity levels upon entrainment was not significantly different across the three stocks for any of the environmental conditions examined. This suggests that the robustness of enhanced activity levels in DD stocks is not due to differential sensitivity to the environment but stems from the enhanced activity levels observed in constant darkness. Thus, our results demonstrate that long-term rearing of *D. melanogaster* populations in constant darkness results in the coevolution of robust circadian clocks that maintain higher activity levels across a wide variety of environmental conditions.

Materials and methods

Population maintenance and standardization

The current study was performed on three sets of *D. melanogaster* stocks (with four replicates/blocks each) maintained in the laboratory as large outbred populations under constant light (LL), 12:12-h light-dark (LD) cycles, and constant darkness (DD). These populations ($LL_{1–4}$, $LD_{1–4}$, $DD_{1–4}$) were derived from four baseline laboratory populations of *D. melanogaster* which are described in detail elsewhere (Sheeba et al. 1998). Briefly, five replicate populations were derived from an ancestral population established from wild-caught flies collected from South Amherst, Massachusetts (Ives 1970). Four out of these five replicate populations served as the baseline populations from which LL, LD, and DD stocks were later derived. These fly populations were maintained on a 21 day generation cycle on banana-jaggery medium in LL for over 600 generations before

being segregated into the LL, LD, and DD stocks. Thereafter, all the three sets of populations (stocks) were maintained under controlled laboratory conditions at constant temperature of 25 ± 1 °C and relative humidity of 65–75 %. The LL and DD stocks were maintained under LL (~100 lx) and DD, respectively, while LD stocks were maintained under 12:12-h LD cycles with light phase of ~100 lx. White compact fluorescent lamps (Philips, Genie, 8 W, Philips India Limited, Gurgaon, India) were used to illuminate the cubicle during the light phase for LD and LL, and the spectrum of the fluorescent lamp predominantly consists of three wavelengths around 450, 550, and 620 nm. All populations were maintained on a 21 day discrete (non-overlapping) generation cycle similar to their ancestors as described above. The maintenance protocol for all the three stocks has been described in detail in Shindey et al. (2016).

In order to eliminate non-genetic parental effects and to unambiguously test the genetic differences between the stocks, all stocks were subjected to common rearing in 12:12-h LD cycles for one generation before the experiments (referred to as standardized stocks). The progeny of standardized stocks was used for all the experiments discussed below.

Activity-rest rhythm assay under different photoperiods, ambient temperatures, and *T* cycles

The activity-rest behavior of 32 virgin males (3–4 days old) from each standardized block was recorded in locomotor activity tubes (5-mm diameter × 65-mm length) containing corn food for 7–8 days each, under short/winter-type (LD04:20—4-h light and 20-h dark) and long/summer-type (LD20:04—20-h light and 4-h dark) photoperiods at ~25 °C, and LD12:12 (12-h light and 12-h dark) with ambient temperatures of 18 and 28 °C, using Trikinetics Drosophila Activity Monitors (DAM, Trikinetics, Waltham, MA). The activity-rest rhythm was also assayed under two different non-24-h LD cycles (LD09:09—9-h light and 9-h dark; LD15:15—15-h light and 15-h dark) for 12 days. For all environmental conditions discussed in this section, light intensity of 0.1 lx measured using LICOR light-meter L250 (Lincoln, NE, USA) was used during the photophase. This light intensity was chosen since maximum differences in activity levels between the three stocks are observed at this light intensity (*Radhika Shindey, personal observation*).

Activity-rest rhythm assay under semi-natural condition (SN)

For the experiments under semi-natural conditions (SN), DAM monitors were placed inside an enclosure (122 × 122 × 122 cm^3) with grids (6 × 6 cm^2) covered only on the top with a sloping translucent plastic sheet (described in De et al. 2013). The experiments were performed in the above-described enclosure at JNCASR, Bangalore (12°59′N 77°35′E). An environmental monitor (DEnM, Trikinetics, Waltham, MA) was used to record daily profiles of light, temperature, and humidity in 15-min bins alongside the activity recording. The activity-rest behavior of 32 virgin males (3–4 days old) from each standardized block was recorded for a minimum of 7 days in March (2016) when the maximum light intensity experienced by flies was ~340 lx and maximum temperature was ~32 °C.

Activity-rest rhythm assay in constant darkness in semi-natural condition (SN-DD)

Light-tight metal boxes were used for recording activity-rest rhythm under DD in otherwise SN conditions (SN-DD) of cycling temperature and humidity. An environmental monitor was placed inside the box to verify that there was no leakage of light, and that temperature and humidity closely matched the conditions outside the box. The activity-rest behavior of 32 virgin males (3–4 days old) from each standardized block was recorded for a minimum of 7 days inside the metal box simultaneously with the recording under SN.

Analysis of change in peak activity levels upon entrainment

Since we wished to test if enhanced activity levels observed in DD stocks under DD persist across multiple environments, we also estimated the magnitude by which the peak activity levels of all three stocks changed under different environmental regimes with respect to their activity levels seen under DD. This would help us assess whether the high peak activity levels in DD stocks (if) persisting across environments is due to intrinsic high peak activity levels, or is a response to light and/or temperature conditions. To do so, we calculated the difference between the peak activity level of a stock under a given environmental condition and that under DD. The activity data for DD was reanalyzed from Shindey et al. (2016). To calculate the change in activity level, the activity counts in the highest 1-h bin under DD were subtracted from the activity counts at the activity peak (calculated as the sum of activity counts in 2-h windows around the activity peak) in a given environmental regime (Nikhil et al. 2016b). The change in peak activity levels was calculated separately for every block and analyzed statistically as described below.

Statistical analyses

The raw activity profiles for all environmental conditions were plotted by binning activity counts in 1-h intervals. For the analysis of activity-rest rhythm under different environmental conditions, block means of raw activity counts binned in 1-h intervals were subjected to mixed model analysis of

variance (ANOVA) with stock and time-point as fixed factors and block as random factor. We further quantified the activity counts in 6-h intervals starting from lights-ON (Zeitgeber Time (ZT) 00) or "dawn" (06:00-h local time) for all the environmental conditions, and the data was subjected to ANOVA with stock and time interval as fixed factors, and block as random factor. Similarly, the data for change in peak activity levels was subjected to a mixed model ANOVA with stock as fixed and block as random factor.

For all the above-described experiments, ANOVA was followed by post hoc multiple comparisons using Tukey's HSD test at a significance level $\alpha = 0.05$. All statistical analyses were executed on Statistica software for Windows Release 5.0B (STATISTICA™ 1995).

Results

Activity-rest rhythm under two different photoperiods

Raw activity profiles for LL, LD, and DD stocks under LD04:20 and LD20:04 are shown in Fig. 1a. ANOVA on activity data revealed a statistically significant effect of stock for LD04:20 ($F_{2,6} = 6.38$, $p < 0.05$), while the effect of stock was not statistically significant for LD20:04 (LD20:04: $F_{2,6} = 3.35$, $p > 0.05$). The effect of time-point was statistically significant for both the conditions (LD04:20: $F_{23,69} = 17.96$, $p < 0.05$; L20:04: $F_{23,69} = 87.87$, $p < 0.05$). The stock × time-point interaction was also statistically significant for LD04:20 ($F_{46,138} = 1.50$, $p < 0.05$) and LD20:04 ($F_{46,138} = 1.51$, $p < 0.05$).

ANOVA performed on 6-h activity counts revealed statistically significant effect of stock for LD04:20 ($F_{2,6} = 60.29$, $p < 0.05$) with DD stocks showing higher activity, while the effect of stock was not statistically significant for LD20:04 ($F_{2,6} = 3.35$, $p > 0.05$). The effect of time interval was statistically significant for both the photoperiods (LD04:20: $F_{3,9} = 22.49$, $p < 0.05$; LD20:04: $F_{3,9} = 104.01$, $p < 0.05$). The stock × time interval interaction was statistically significant for LD04:20 ($F_{6,18} = 2.92$, $p < 0.05$) but not for LD20:04 ($F_{6,18} = 1.87$, $p > 0.05$). Post hoc comparisons revealed significantly higher activity counts for DD stocks as compared to LL stocks in ZT18-00 and ZT00-06 time intervals under LD04:20 and LD12:12 ($p < 0.05$; Fig. 1b).

We also compared the change in peak activity levels of the three stocks under short photoperiod relative to their respective values under DD (as described in "Materials and methods"). ANOVA revealed that the effect of stock on the change in peak activity levels was not statistically significant ($F_{2,6} = 4.32$, $p > 0.05$; Fig. 1c; Online resource 1) suggesting that all three stocks undergo similar changes in peak activity levels from DD to short photoperiod conditions.

Similarly, under LD20:04, trends for higher activity levels for DD stocks were observed as compared to LL and LD stocks, though the differences were not statistically significant. Interestingly, under these long-day conditions, LD stocks appeared to show lowest activity levels as opposed to LL stocks which show lowest activity under LD12:12 and LD04:20 (Fig. 1a, b). ANOVA on the change in peak activity levels between DD and LD20:04 conditions for the three stocks revealed that the effect of stock was not statistically significant ($F_{2,6} = 0.73$, $p > 0.05$; Fig. 1c; Online resource 1).

Thus, DD stocks show significantly higher activity levels as compared to LD and LL stocks under short photoperiod and a trend of higher activity under long photoperiod, suggesting that enhanced activity in DD stocks is robust across multiple photoperiods. Furthermore, higher activity levels in DD stocks under these photoperiods are not due to significant change in peak activity levels of DD stocks upon entrainment to both photoperiods relative to the other two stocks.

Activity-rest rhythm under 12:12-h light-dark (LD) cycles at different ambient temperatures

Raw activity counts in 1-h bins under different ambient temperatures are shown in Fig. 2a. ANOVA on activity data under different ambient temperatures revealed no statistically significant effect of stock at 18 °C ($F_{2,6} = 3.89$, $p > 0.05$) and 28 °C ($F_{2,6} = 3.67$, $p > 0.05$). However, a statistically significant effect of time-point was seen for both the temperatures (18 °C: $F_{23,69} = 73.8$, <0.05; 28 °C: $F_{23,69} = 173.3$, $p < 0.05$). The effect of stock × time-point interaction was statistically significant for both 18 °C ($F_{46,138} = 2.13$, $p < 0.05$) and 28 °C ($F_{46,138} = 2.82$, $p < 0.05$). Post hoc multiple comparisons on 1-h activity counts under 18 and 28 °C revealed significantly higher activity at multiple time-points (ZT10 and ZT11 for 18 °C and ZT00, ZT10, ZT11, ZT22, and ZT23 for 28 °C; $p < 0.05$) for DD stocks as compared to LL stocks.

ANOVA on activity counts in 6-h intervals revealed no statistically significant effect of stock for any of the temperatures (18 °C: $F_{2,6} = 3.89$, $p > 0.05$; 28 °C: $F_{2,6} = 3.67$, $p > 0.05$), while the effect of time interval was statistically significant for both the temperatures (18 °C: $F_{3,9} = 91.13$, $p < 0.05$; 28 °C: $F_{3,9} = 174.55$, $p < 0.05$). The stock × time interval interaction was statistically significant for 28 °C ($F_{6,18} = 4.11$, $p < 0.05$) but not 18 °C ($F_{6,18} = 0.54$, $p > 0.05$). Post hoc comparisons for activity counts under 28 °C revealed that DD stocks show significantly higher activity counts during late-night (ZT18-00) and daytime (ZT00-06 and ZT06-12) as compared to LD stocks which showed the lowest activity counts during the daytime ($p < 0.05$; Fig. 2b).

ANOVA on the change in peak activity level for the three stocks revealed that the effect of stock was not statistically significant either at 18 °C ($F_{2,6} = 3.19$, $p > 0.05$; Fig. 2c) or

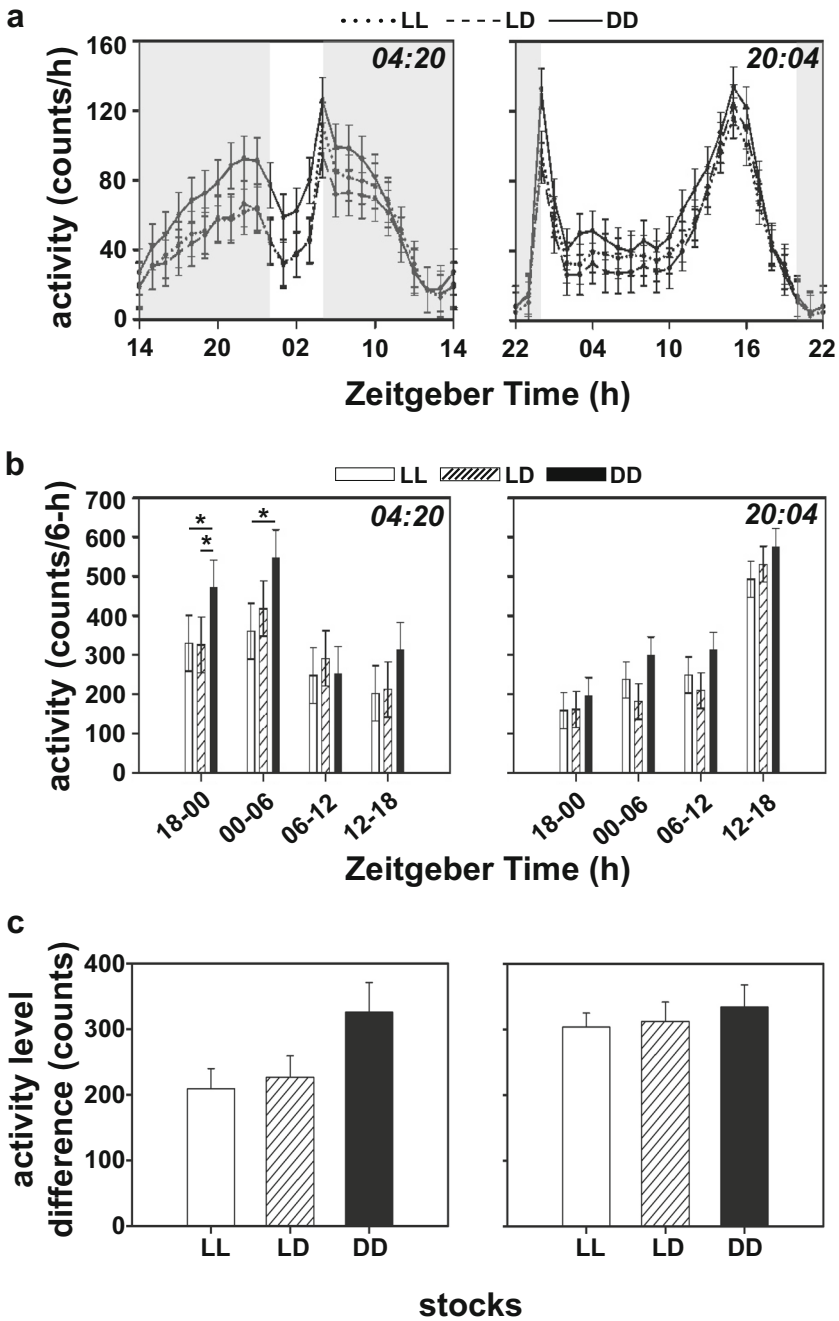

Fig. 1 **a** The activity-rest profile of LL, LD, and DD stocks under LD04:20 (*left*) and LD20:04 (*right*). The raw activity counts averaged over seven cycles are plotted in 1-h bins along the *y*-axis and Zeitgeber time (ZT in h) along the *x*-axis. ZT00 represents lights-ON and ZT04 and ZT20 represent lights-OFF in LD04:20 and LD20:04, respectively. The *gray shaded and unshaded areas* represent dark and light phases, respectively. Light intensity of 0.1 lx was used in the light phase of LD cycles. **b** Comparison of 6-h activity counts for the three stocks in LD04:20 (*left*) and LD20:04 (*right*). **c** Peak activity level differences between DD and LD04:20 (*left*) and between DD and LD20:04 (*right*) for all three stocks. *Error bars* in panels **a** and **b** are 95 % confidence interval (95 %CI), and those for panel **c** are standard error of mean (SEM). Statistically significant differences between the stocks are indicated by *asterisks*

at 28 °C ($F_{2,6}$ = 3.79, $p > 0.05$; Fig. 2c; Online resource 1). Hence, similar to that observed for photoperiods, higher activity levels seen in DD stocks are not due to significant change in activity levels of DD stocks upon entrainment to LD12:12 cycles of high or low temperatures.

Activity-rest rhythm under non-24-h light-dark (LD) cycles

We have plotted average activity profiles of flies exhibiting entrained activity-rest rhythm under 18-h and 30-h LD cycles

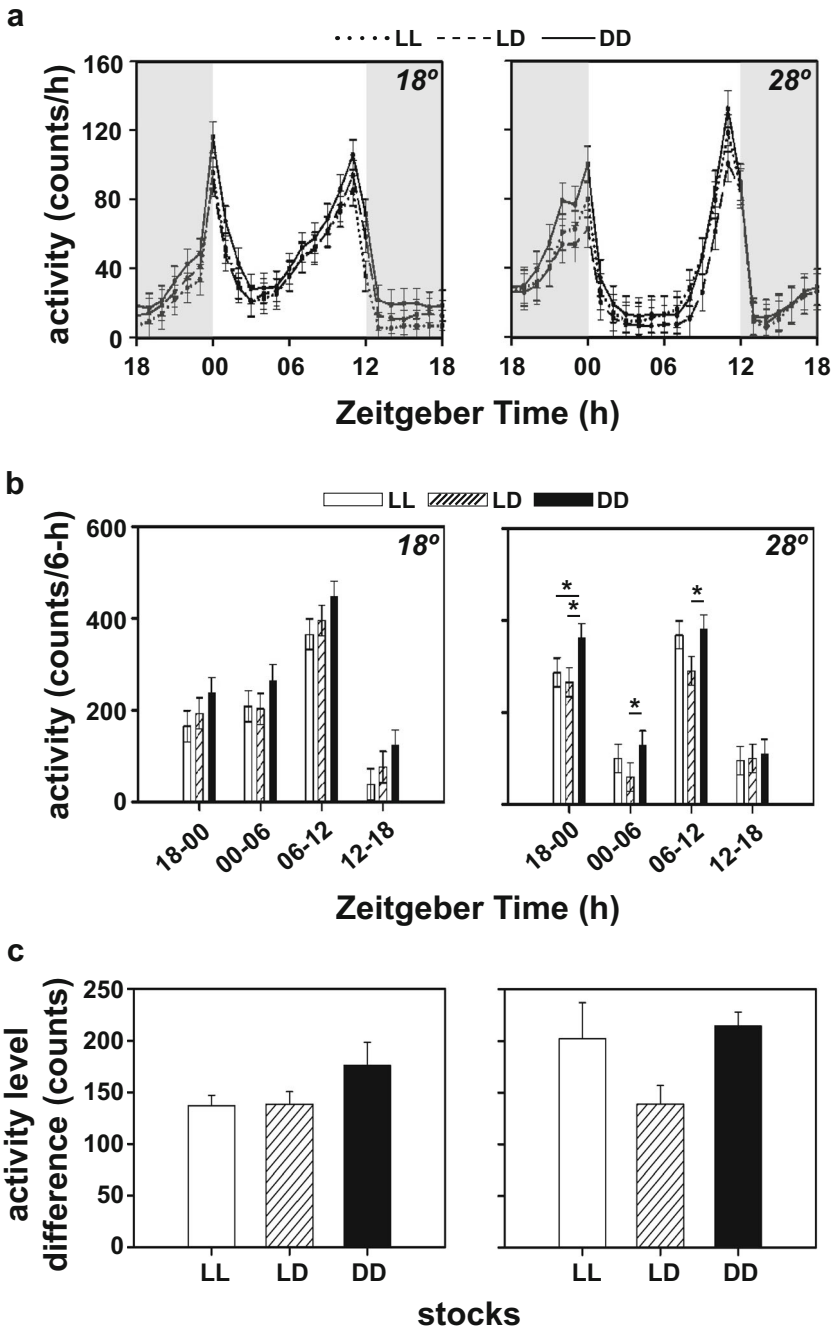

Fig. 2 **a** The activity-rest profile of LL, LD, and DD stocks under light-dark cycles at ambient temperature of 18 °C (*left*) and 28 °C (*right*). The raw activity counts in 1-h bins averaged over seven cycles are plotted along the *y*-axis and Zeitgeber time (ZT in h) along the *x*-axis. ZT00 and ZT12 represent lights-ON and OFF, respectively. **b** Comparison of 6-h activity counts for the three stocks at 18 °C (*left*) and 28 °C (*right*) ambient temperatures. **c** Peak activity level differences between DD (25 °C) and LD at 18 °C (*left*) and between DD (25 °C) and LD at 28 °C (*right*) for all three stocks. All other details are the same as in Fig. 1

(Fig. 3a). We verified entrainment of individual flies by examining their actograms and choosing only those individuals for analysis which exhibited phase-locking of onset and offset of activity with lights-ON and lights-OFF, respectively. ANOVA on raw activity counts under 18-h LD cycles (LD09:09) revealed statistically significant effect of time-point ($F_{17,51} = 71.3$, $p < 0.05$) and stock × time-point interaction ($F_{34,102} = 2.19$, $p < 0.05$), but not of stock ($F_{2,6} = 2.93$, $p > 0.05$). Post hoc comparisons revealed that DD stocks showed significantly higher activity levels as compared to LL stocks at multiple time-points (ZT00-03, $p < 0.05$; Fig. 3a). Further, ANOVA on activity counts in 6-h intervals

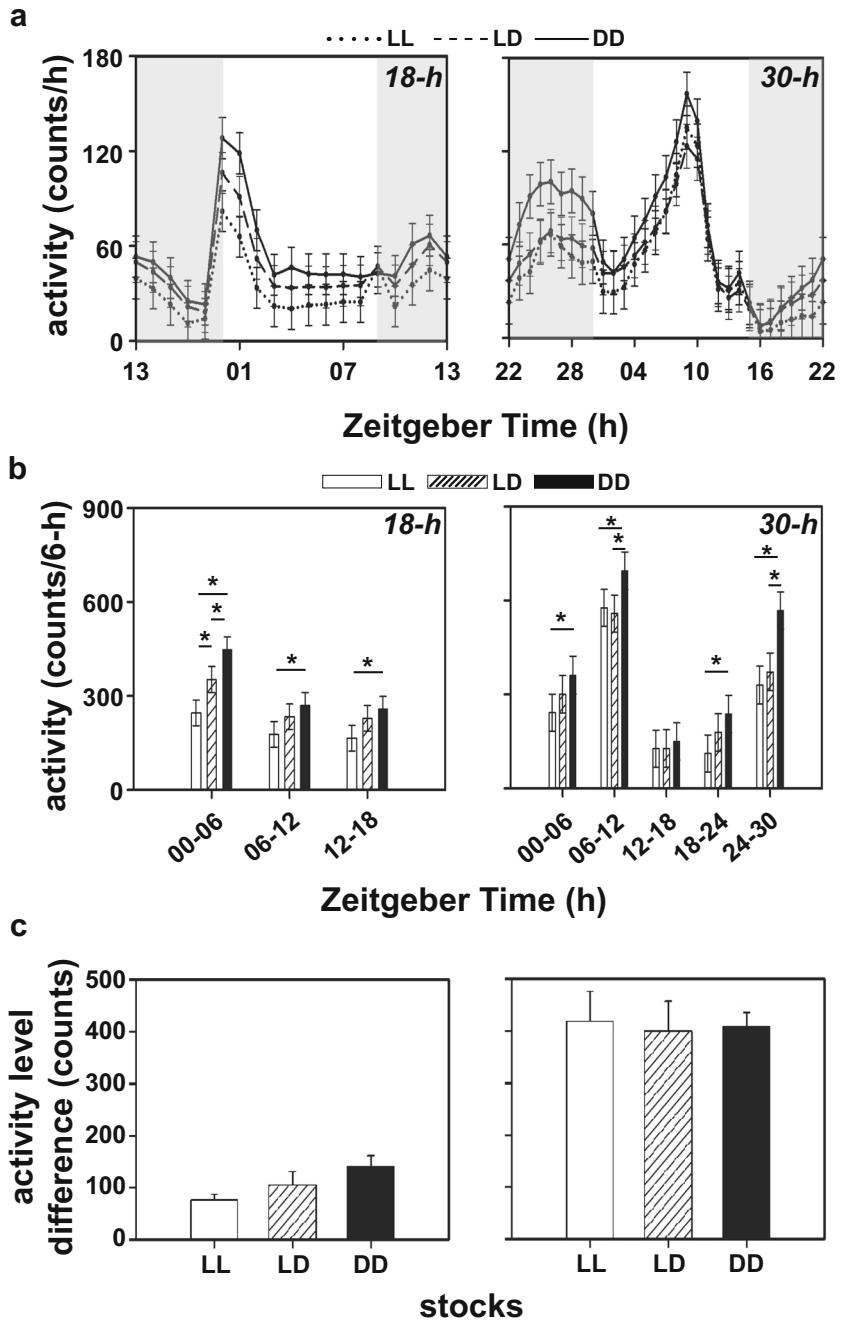

Fig. 3 **a** The activity-rest profile of LL, LD, and DD stocks under LD09:09 (*left*) and LD15:15 (*right*). The raw activity counts averaged over seven cycles are plotted in 1-h bins along the *y*-axis and Zeitgeber time (ZT in h) along the *x*-axis. ZT00 represent lights-ON while ZT09 and ZT15 represent lights-OFF in 18 and 30-h LD cycles, respectively. **b** Comparison of 6-h activity counts for the three stocks in LD09:09 and LD15:15 cycles. **c** Peak activity level differences between DD and LD09:09 (*left*) and between DD and LD15:15 (*right*) for all the three stocks. All other details are the same as in Fig. 1

revealed a statistically significant effect of time interval ($F_{2,6} = 113.34$, $p < 0.05$) and stock × time interval interaction ($F_{4,12} = 4.08$, $p < 0.05$) whereas the effect of stock was statistically not significant ($F_{2,6} = 2.93$, $p > 0.05$). Post hoc comparisons revealed significantly higher activity counts for DD stocks as compared to LL stocks for all time intervals ($p < 0.05$; Fig. 3b). Additionally, DD stocks showed higher activity counts than LD stocks in the ZT00-06 interval ($p < 0.05$; Fig. 3b).

For activity-rest rhythm under 30-h LD cycles (LD15:15), ANOVA on the raw activity counts revealed a statistically significant effect of stock ($F_{2,6} = 8.84$, $p < 0.05$), time-point ($F_{29,87} = 58.422$, $p < 0.05$), and stock × time-point interaction ($F_{58,174} = 2.85$, $p < 0.05$). The DD stocks demonstrated

significantly higher activity levels as compared to LD and LL stocks across several time-points (ZT22-29, $p < 0.05$; Fig. 3a). Further, ANOVA performed on activity counts in 6-h intervals showed statistically significant effect of stock ($F_{2,6} = 8.84$, $p < 0.05$), time interval ($F_{4,12} = 79.31$, $p < 0.05$), and stock × time interval interaction ($F_{8,24} = 3.93$, $p < 0.05$). Post hoc comparisons revealed that DD stocks exhibit significantly higher activity counts as compared to LL stocks in all time intervals ($p < 0.05$) except ZT12-18 (Fig. 3b), and that compared to LD stocks in ZT06-12 and ZT24-30 intervals ($p < 0.05$; Fig. 3b).

ANOVA on the change in peak activity levels between DD and LD09:09 for the three stocks revealed that the effect of stock was not statistically significant ($F_{2,6} = 2.03$, $p > 0.05$; Fig. 3c, Online resource 2), and the same was observed for change in peak activity levels between DD and LD15:15 ($F_{2,6} = 0.04$, $p > 0.05$; Fig. 3c; Online resource 2).

Thus, DD stocks consistently exhibit higher activity levels through most of the day even under non-24-h LD cycles, and this did not stem from a differential change in activity level upon entrainment to the two regimes.

Activity-rest rhythm under semi-natural conditions (SN) and constant darkness in semi-natural conditions (SN-DD)

Raw activity counts in 1-h bins under SN for all the three stocks are shown in Fig. 4a. ANOVA on activity-rest profile did not reveal a statistically significant effect of stock ($F_{2,6} = 3.43$, $p > 0.05$), while the effect of time-point ($F_{23,69} = 226.06$, $p < 0.05$) and stock × time-point interaction was statistically significant ($F_{46,138} = 2.04$, $p < 0.05$). Further, a trend of higher activity at several time-points was seen in DD stocks as compared to LL stocks and LD stocks (local time 0 to 10 h; Fig. 4a), and post hoc comparisons revealed that DD stocks show a significantly higher evening peak as compared to the LL stocks (local time 19 h, $p < 0.05$). However, ANOVA on activity counts in 6-h intervals revealed no statistically significant effect of stock ($F_{2,6} = 3.43$, $p > 0.05$) or stock × time interval interaction ($F_{6,18} = 1.54$, $p > 0.05$), whereas the effect of time interval was statistically significant ($F_{3,9} = 80$, $p < 0.05$). ANOVA on the change in the peak activity level between DD and SN for the three stocks revealed that the effect of stock was not statistically significant ($F_{2,6} = 0.55$, $p > 0.05$; Fig. 4c, Online resource 2).

We also examined the profiles of the three stocks under DD of otherwise SN-like conditions with temperature and humidity cycling since the light intensity in SN conditions was much higher than the other conditions under which we recorded activity of these flies. Raw activity profiles for all the three stocks under constant darkness in otherwise SN-like conditions (SN-DD) of temperature and humidity are shown in Fig. 4a. ANOVA revealed a statistically significant effect of stock ($F_{2,6} = 6.81$, $p < 0.05$), time-point ($F_{23,69} = 128.69$, $p < 0.05$), and stock × time-point interaction ($F_{46,138} = 4.90$, $p < 0.05$). Post hoc comparisons revealed significantly higher activity in DD stocks as compared to LD stocks and LL stocks at several time-points (local time 0800 to 1500 h, $p < 0.05$; Fig. 4a). Likewise, ANOVA on activity counts in 6-h intervals also demonstrated statistically significant effect of stock ($F_{2,6} = 6.81$, $p < 0.05$), time interval ($F_{3,9} = 124.29$, $p < 0.05$), and stock × time interval interaction ($F_{6,18} = 7.37$, $p < 0.05$; Fig. 4b). Post hoc comparisons revealed significantly higher activity of DD stocks as compared to LL and LD stocks in ZT06-12 and ZT12-18 time intervals ($p < 0.05$; Fig. 4b). ANOVA on the change in peak activity level between DD and SN-DD for the three stocks revealed that the effect of stock was not statistically significant ($F_{2,6} = 0.03$, $p > 0.05$; Fig. 2c, Online resource 2).

These results suggest that DD stocks retain higher evening activity peak and a trend of higher activity levels under SN at some of the time-points. Thus, DD stocks might still show robust enhancement of activity levels under SN conditions relative to other stocks though these differences appear to be somewhat mitigated by high light intensity experienced during the day.

Discussion

Robustness is considered to be a fundamental feature of biological systems that can facilitate evolvability (Kitano 2004). However, robustness of a system is also associated with trade-offs such as fragility or sensitivity to unexpected perturbations (Kitano 2004). Thus, a feature more pronounced under certain environments may not necessarily be robust across other environments. Therefore, it is essential that the phenotype is assessed under multiple environmental conditions to unequivocally establish that it is robust. Results from our previous study (Shindey et al. 2016) demonstrated that DD stocks have evolved higher activity levels of free-running activity-rest rhythm in DD, and higher power of free-running rhythms as compared to LD and LL stocks. Higher activity levels and power of free-running rhythm are suggestive of DD stocks having evolved robust circadian rhythms. In this study, we wished to test if the robust free-running activity-rest rhythm in DD stocks stem from robust circadian clocks that underlie these rhythms, and therefore, we studied the activity-rest rhythm under multiple environmental conditions.

Similar to that under LD12:12, we observed that DD stocks exhibited higher activity levels at majority of the time-points

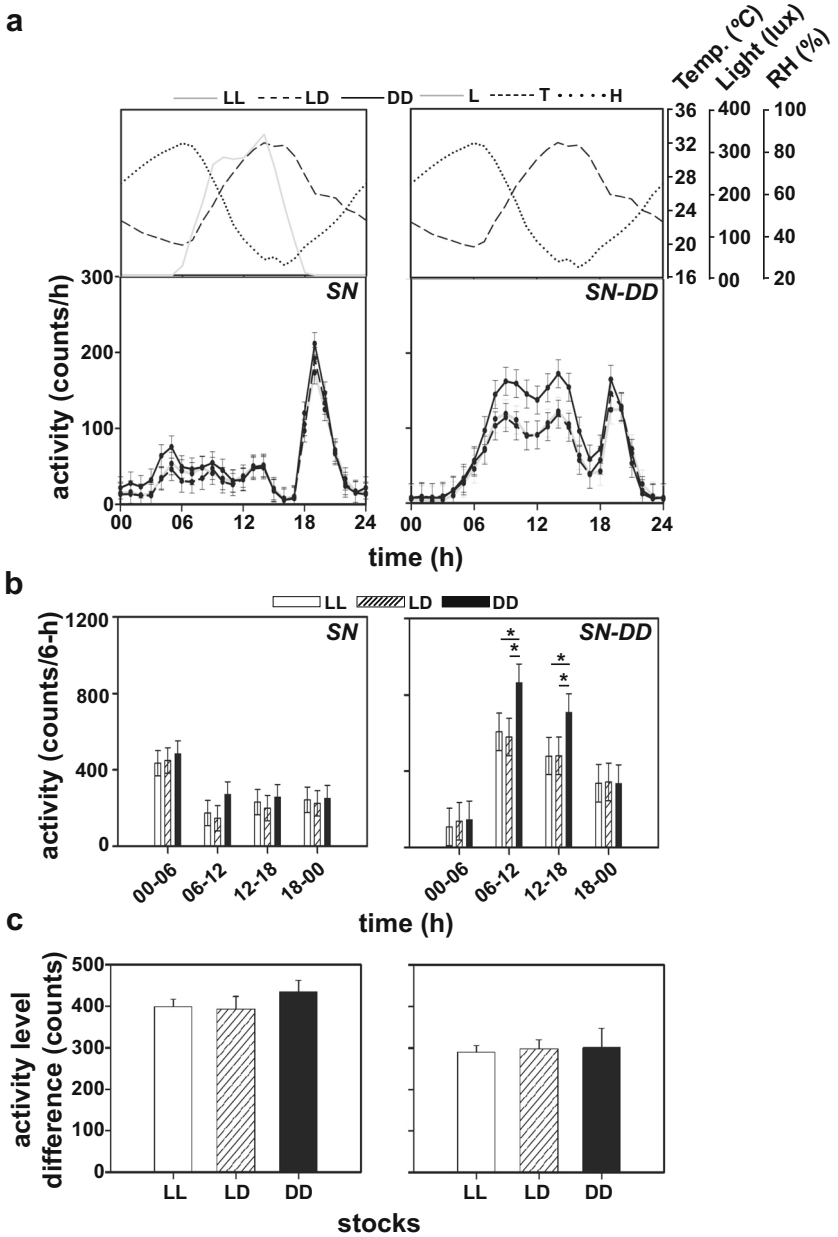

Fig. 4 **a** The activity-rest profile of LL, LD, and DD stocks under semi-natural (SN—*left*) and constant darkness in SN condition (SN-DD—*right*). The raw activity counts averaged over seven cycles are plotted in 1-h bins along the *y*-axis and local time in h along the *x*-axis. Environmental factors such as light, temperature, and humidity were also simultaneously monitored for both the conditions. **b** Comparison of 6-h activity counts for the three stocks in SN (*left*) and SN-DD (*right*) conditions. **c** Peak activity level differences between DD and SN (*left*) and between DD and SN-DD (*right*) for all three stocks. All other details are the same as in Fig. 1

as compared to LL and LD stocks under winter-type/short photoperiod (LD04:20) and a similar trend for summer-type/long photoperiod (LD20:04) conditions. When assayed under LD12:12 at ambient temperatures of 18 and 28 °C which is also known to mimic winter and summer-type conditions, respectively, we observed that DD stocks exhibit higher activity across several time-points under both the temperatures (Fig. 2a). Interestingly, we observed that LL and DD stocks failed to suppress activity under long photoperiod as much as LD stocks, suggesting that evolution in constant conditions might have led to the reduced ability to adapt to summer-type photoperiods. However, we did not observe these differences in the midday activity under semi-natural conditions. This might probably be due to the very high light intensity in the semi-natural environment that might suppress activity. Previous studies have implicated two allelic forms of the core clock gene *timeless* (*tim*)—*ls-tim* and *s-tim* (Rosato et al. 1997) which are believed to play an important role in seasonal

adaptation (Tauber et al. 2007; Kyriacou et al. 2008). Furthermore, in a previous study, it was demonstrated that *early* and *late* emerging stocks of *D. melanogaster* differ in their circadian photosensitivity which the authors hypothesized to be associated with *cryptochrome (cry)* or *tim* expression (Nikhil et al. 2016a). Thus, examining *cry* expression and *tim* polymorphism is likely to provide some insight into the molecular basis of the differences between the three stocks.

We further examined the activity-rest behavior of all three stocks under LD cycles with periods of 18-h (LD09:09) and 30-h (LD15:15) and observed that under 18-h LD cycles, DD stocks exhibit significantly higher activity at several time-points as compared to LL stocks (Fig. 3a, b). Similarly, under 30-h LD cycles, DD stocks displayed significantly higher activity levels as compared to the other two stocks across various time-points, and a significantly higher evening activity peak as compared to LD stocks (Fig. 3a, b). Corroborating the results of our previous experiments performed under different photoperiods and ambient temperatures, activity-rest rhythm under non-24-h LD cycles also demonstrated the evolution of high activity level in DD stocks as compared to LL and LD stocks.

Since DD stocks exhibited higher levels of activity under multiple light and temperature regimes, we wished to assess if the phenotype persists under a harsher regime such as semi-natural conditions which is associated with simultaneous cycling of multiple zeitgebers. Consistent with our previous results, DD stocks displayed higher activity at certain time-points including the morning and evening activity peaks though statistically significant difference was observed only at the evening peak (Fig. 4a). Even though the DD stocks exhibited higher activity levels at these time-points in SN, the differences between populations appeared to be attenuated, and therefore, we examined the possible factors that might contribute to the reduction of these differences under SN by blocking light for one set of flies (SN-DD). Interestingly, under SN-DD, the differences in activity levels that were attenuated in SN conditions were restored, and DD stocks exhibited significantly higher activity during the day as compared to the other two stocks (Fig. 4a, b). This suggests that the reduction in differences between the stocks under SN might be due to the ability of high light intensity under SN to suppress activity in *D. melanogaster* (Rieger et al. 2007). Since the phenotype of enhanced activity was seen in most other experiments which were conducted with a light phase of 0.1 lx intensity, and even in temperature cycles (SN-DD), the suppression of activity by high light intensity is the most likely reason for mitigation of such differences in SN. Additionally, the persistence of higher activity of DD stocks in the evening when the light intensity is low under SN lends further support to this argument.

If this is true, then it can be hypothesized that DD stocks might have evolved higher light sensitivity as a consequence of adaptation to dark environments. At this stage, it is difficult to differentiate between whether such enhanced light sensitivity stems from the core clock itself or through differences in input/output pathways. However, comparisons of differences in peak activity levels between entrained conditions and constant conditions across the three stocks did not yield any statistically significant difference between the three stocks (Figs. 1c, 2c, 3c, 4c). This suggests that the higher activity levels in DD stocks seen across all these environmental regimes is probably not a consequence of differences in light sensitivity, but rather, is a consequence of enhanced intrinsic activity levels seen under constant conditions (Online resources 1 and 2).

Overall, the results of the present study are collectively suggestive of evolution of robust circadian clocks in DD stocks. This appears to be contrary to some of the previous studies demonstrating the regressive evolution of circadian clocks in organisms inhabiting constant dark habitats (Friedrich 2013). However, though the regressive evolution observed in such populations has been attributed to lack of light availability, the observed phenotypes might have also evolved due to other unidentified selective pressures in the habitat. Furthermore, lack of details pertaining to the populations' ancestry and the environmental history of the habitat makes it difficult to unequivocally conclude that absence of light is the primary reason for the regression of circadian systems in these populations. These issues are addressed in our study design as all populations were maintained under identical environments barring differences in their light regimes, and therefore the observed phenotypic divergence can directly be attributed to the presence or absence of photic cues in the environment of these populations. The results of our study, in addition to demonstrating the evolution of robust circadian rhythms in fruit fly *D. melanogaster* populations maintained under constant dark conditions over 330 generations, also highlight the advantage of using laboratory selection as a potential tool for the study of circadian clock evolution and associated properties. As discussed above, robustness is an important feature of biological systems as it prevents large phenotypic effects in response to environmental perturbations and facilitates evolvability of such systems. Therefore, the robust enhanced activity in DD stocks highlights the adaptive value of retaining such rhythms under constant dark environments which extends support to the adaptive advantage hypothesis discussed earlier in the context of these populations (Shindey et al. 2016). Future studies may shed more light on how higher levels of activity may be adaptive under constant conditions in contrast to cyclic

environments and whether these results lend credence to the adaptive advantage hypothesis of circadian rhythms.

Acknowledgments The authors thank Rutvij Kulkarni for his assistance in some of the experiments, and Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India for funding this project. We thank two anonymous reviewers for reading our manuscript and suggesting some very useful changes.

References

Akman OE, Ran DA, Brown PE, Millar AJ (2010) Robustness from flexibility in the fungal circadian clock. BMC Syst Biol 4:88

Cheng P, Yang Y, Liu Y (2001) Interlocked feedback loops contribute to the robustness of the Neurospora circadian clock. Proc Natl Acad Sci U S A 98:7408–7413

De J, Varma V, Saha S, Sheeba V, Sharma VK (2013) Significance of activity peaks in fruit flies, *Drosophila melanogaster*, under semi-natural conditions. Proc Natl Acad Sci U S A 110:8984–8989

Friedrich M (2013) Biological clocks and visual systems in cave-adapted animals at the dawn of speleogenomics. Integr Coml Biol 53:50–67

Gonze D, Halloy J, Goldbeter A (2002) Robustness of circadian rhythms with respect to molecular noise. Proc Natl Acad Sci U S A 99:673–678

Gurevich L, Cohen-Luria R, Wagner N, Ashkenasy G (2015) Robustness of synthetic circadian clocks to multiple environmental changes. Chem Commun 51:5672–5675

Hamblen-Coyle MJ, Wheeler DA, Rutila JE, Rosbash M, Hall JC (1992) Behavior of period-altered circadian rhythm mutants of *Drosophila* in light:dark cycles (Diptera: Drosophilidae. J Insect Behav 5:417–446

Helfrich-Förster C (2000) Differential control of morning and evening components in the activity rhythm of *Drosophila melanogaster*-Sex-specific differences suggest a different quality of activity. J Biol Rhythm 15:135–154

Ives PT (1970) Further genetic studies of the South Amherst population of *Drosophila melanogaster*. Evolution 24:507–518

Kannan NN, Mukherjee N, Sharma VK (2012) 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. Chronobiol Int 29:1312–1328

Kitano H (2004) Biological robustness. Nat Rev Genet 5:826–837

Konopka RJ, Benzer S (1971) Clock mutants of *Drosophila melanogaster*. Proc Natl Acad Sci U S A 68:2112–2116

Kyriacou CP, Peixoto AA, Sandrelli F, Costa R, Tauber E (2008) Clines in clock genes: fine-tuning circadian rhythms to the environment. Trends Genet 24:124–132

Low KH, Chen WF, Yildirim E, Edery I (2012) Natural variation in the *Drosophila melanogaster* clock gene *period* modulates splicing of its 3′-terminal intron and mid-day siesta. PLoS One 7:e49536

Majercak J, Sidote D, Hardin PE, Edery I (1999) How a circadian clock adapts to seasonal decreases in temperature and day length. Neuron 24:219–230

Majercak J, Chen WF, Edery I (2004) Splicing of the period gene 3′-terminal intron is regulated by light, circadian clock factors, and phospholipase C. Mol Cell Biol 24:3359–3372

Nikhil KL, Vaze KM, Sharma VK (2015) Late emergence chronotypes of fruit flies *Drosophila melanogaster* exhibit higher accuracy of entrainment. Chronobiol Int 32:1477–1485

Nikhil KL, Abhilash L, Sharma VK (2016a) Molecular correlates of circadian clocks in fruit fly *Drosophila melanogaster* populations exhibiting early and late emergence chronotypes. J Biol Rhythm 31:125–141

Nikhil KL, Vaze KM, Ratna K, Sharma VK (2016b) Circadian clock properties of fruit flies *Drosophila melanogaster* exhibiting early and late emergence chronotypes. Chronobiol Int 33:22–38

Rieger D, Stanewsky R, Helfrich-Förster C (2003) Cryptochrome, compound eyes, Hofbauer-Buchner eyelets, and ocelli play different roles in the entrainment and masking pathway of the locomotor activity rhythm in the fruit fly *Drosophila melanogaster*. J Biol Rhythm 18:377–391

Rieger D, Fraunholz C, Popp J, Bichler D, Dittmann R, Helfrich-Förster C (2007) The fruit fly *Drosophila melanogaster* favors dim light and times its activity peaks to early dawn and late dusk. J Biol Rhythm 22:387–399

Rosato E, Trevisan A, Sandrelli F, Zordan M, Kyriacou CP, Costa R (1997) Conceptual translation of *timeless* reveals alternative initiating methionines in *Drosophila*. Nucleic Acids Res 25:455–458

Sharma VK (2003) Adaptive significance of circadian clocks. Chronobiol Int 20:901–919

Sheeba V, Madhyastha NA, Joshi A (1998) Oviposition preference for novel versus normal food resources in laboratory populations of *Drosophila melanogaster*. J Biosci 23:93–100

Shindey R, Varma V, Nikhil KL, Sharma VK (2016) Evolution of circadian rhythms in *Drosophila melanogaster* populations reared in constant light and dark-regimes for over 330 generations. Chronobiol Int (*in press*).

Simunovic A, Jaenike J (2006) Adaptive variation among *Drosophila* species in their circadian rhythms. Evol Ecol Res 8:803–811

STATISTICA (1995) Statistica Vol. III, Statistics II. Statsoft Inc., TM Tulsa, OK, USA

Stelling J, Gilles ED, Doyle FJ (2004) Robustness properties of circadian clock architectures. Proc Natl Acad Sci U S A 101:13210–13215

Tauber E, Zordan M, Sandrelli F, Pegoraro M, Osterwalder N, Breda C, Daga A, Selmin A, Tauber E, Zordan M, Sandrelli F, Pegoraro M, Osterwalder N, Breda C, Daga A, Selmin A, Monger K, Benna C, Rosato E (2007) Natural selection favors a newly derived *timeless* allele in *Drosophila melanogaster*. Science 316:1895–1898

Thommen Q, Pfeuty B, Morant PE, Corellou F, Bouget FY, Lefranc M (2010) Robustness of circadian clocks to daylight fluctuations: hints from the picoeucaryote *Ostreococcus tauri*. PLoS Comput Biol 6:e1000990

Vaze KM, Sharma VK (2013) On the adaptive significance of circadian clocks for their owners. Chronobiol Int 30:413–433

Waddington CH (1957) The strategy of the genes. Allen, London

Wagner A (2005) Circuit topology and the evolution of robustness in two-gene circadian oscillators. Proc Natl Acad Sci U S A 102:11775–11780

Wheeler DA, Hamblen-Coyle MJ, Dushay MS, Hall JC (1993) Behavior in light-dark cycles of *Drosophila* mutants that are arrhythmic, blind, or both. J Biol Rhythm 8:67–94

Yoshii T, Vanin S, Costa R, Helfrich-Förster C (2009) Synergic entrainment of *Drosophila*'s circadian clock by light and temperature. J Biol Rhythm 24:452–464

Young MW (1998) The molecular control of circadian behavioral rhythms and their entrainment in *Drosophila*. Annu Rev Biochem 67:135–152