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Selection on the timing of adult emergence results in altered circadian clocks in fruit flies *Drosophila melanogaster*

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Summary

To investigate whether circadian clocks in fruit flies *Drosophila melanogaster* evolve as a consequence of selection on the timing of adult emergence, we raised four replicate populations each of early (*early*$_{1..4}$) and late (*late*$_{1..4}$) emerging flies by selecting for adults that emerged during the morning and the evening hours. We estimated the percentage of flies that emerged during the two selection windows to evaluate the direct response to selection, and the circadian phenotypes of adult emergence and locomotor activity rhythms under light/dark (LD) and constant darkness (DD) to assess the correlated response to selection. After 55 generations, the percentage of flies emerging during the morning window increased in the *early* populations, but decreased in the *late* populations. The percentage of flies emerging during the evening window increased in the *late* populations, but decreased in the *early* populations. The time course and waveform of emergence and locomotor activity rhythms of the selected populations diverged from each other as well as from the controls. Further, the circadian periodicity of the *early* populations was significantly shorter than the controls, while that of the *late* populations was significantly longer than the controls. The light-induced phase response curve of the selected populations differed significantly within groups as well as from the controls. Such modifications in the circadian phenotypes of the selected populations due to heritable changes in genetic architecture, in response to imposed selection pressure, suggest that the circadian clocks underlying emergence and locomotor activity rhythms in *D. melanogaster* evolve as a correlated response to selection on the timing of adult emergence.

Key words: *Drosophila*, selection, circadian, emergence, locomotor activity, circadian period, PRC.

Introduction

Adult emergence in a variety of insect species is bimodal; i.e. most adult individuals emerge close to 'lights-on' (resulting in a primary peak), while a small number of them emerge throughout the day, displaying a small peak close to 'lights-off' (forming a secondary peak) (Pittendrigh, 1954; Jackson, 1983; Saunders, 1992; Sheeba et al., 2001). Such emergence patterns have facilitated efforts to derive the 'early' and 'late' strains of *Drosophila pseudoobscura* (Pittendrigh, 1966), *D. melanogaster* (Clayton and Paietta, 1972) and *Pectinophora gossypiella* (Pittendrigh and Minis, 1971). The early and late strains of these species were derived by selecting for individuals that emerged during the morning (lights-on) and the evening (lights-off) hours under 12 h:12 h light:dark (LD) cycles. As a result, the primary emergence peak of the early and late strains diverged by about 4–5 h, and their circadian periodicities (τ) differed by about 2.5 h, after 50 generations of selection in *D. pseudoobscura*, 16 generations in *D. melanogaster*, and after 9 generations in *P. gossypiella*. Furthermore, in *D. pseudoobscura* and *P. gossypiella*, the early–late differences were maintained under a wide range of photoperiods (Pittendrigh, 1981). Both species showed similar changes in circadian period; i.e. the early strains had longer τ than the parental strains, whereas the late strains had shorter τ than the parental strains. Although the phase and τ of emergence rhythm differed among the selected strains, their light-induced phase response curve (PRC, a plot of phase shift in the rhythm as a function of phase of light pulse exposure) were strikingly similar, suggesting that the circadian pacemakers of the selected and control strains did not diverge. Pittendrigh interpreted these results in the light of his 'master–slave oscillator model' (Pittendrigh, 1981) and argued that the phase and period differences between the selected strains were not because of the differences in their circadian pacemakers, but due to altered coupling between the circadian pacemaker (master) and the driven (slave) oscillators that govern adult emergence rhythm. Although it is possible to obtain phase separation due to altered coupling between two oscillators, it is hard to imagine how similar circadian pacemakers can generate oscillations with widely different τ.

In a separate study, bimodality in locomotor activity rhythm was used to derive early and late strains of an Indian population of *Drosophila* (*D. rajashekari*) (Joshi, 1999). In this study, the selected strains were initiated from a single isofemale line. Such selection schemes involve the highest degree of inbreeding and linkage disequilibrium, which can lead to inbreeding depression and elimination of variation from the population (reviewed in Sharma and Joshi, 2002). Surprisingly, despite a high degree of homozygosity (due to inbreeding), the early and late strains not only survived for over 59 generations, but also continued to respond to selection.

Of the few empirical studies on the selection for early and late emergence, many suffer from numerous shortcomings that we can now identify and appreciate with the benefit of hindsight gained through decades of empirical studies in evolutionary genetics. For example, previous selection studies used individuals as replicates within the selection regime. Individuals live, reproduce and die, and as a consequence of heritable differences in reproductive output among individuals, populations evolve. Hence, the unit of replication in any study addressing evolutionary questions should be population, not individuals. Therefore, it is not possible to rule out that the changes in circadian phenotypes reported in early selection studies may be a consequence of genetic drift or inbreeding that the populations may have undergone (reviewed by Prasad and Joshi, 2003; David et al., 2005; Miller and Hedrick, 2001).

It is believed that circadian clocks have evolved as a consequence of natural selection under the influence of periodic selection pressures present in our geophysical environment (reviewed in Aschöff, 1964; Hastings et al., 1991; Saunders, 1992; Pittendrigh, 1993; Sharma, 2003a; Dunlap et al., 2004). This suggests that temporal scheduling of behaviour and physiology is central to understanding the evolution of circadian clocks. Therefore, the most appropriate way of empirically addressing this issue would be to carry out rigorous and systematic long-term laboratory selection studies on the timing of rhythmic behaviours, and then to supplement it with a critical evaluation of its consequence on circadian clocks. In our opinion, this is the approach that will provide us with meaningful insights into the possible evolutionary processes that may have been instrumental in the fine-tuning of circadian clocks.

In this paper, we report the results from the first 55 generations of our ongoing laboratory selection experiment aimed at studying whether circadian clocks in fruit flies *D. melanogaster* evolve as a consequence of selection on the timing of adult emergence. For this purpose, four replicates each of *early*, *control* and *late* populations were derived from four baseline populations of *D. melanogaster*. To assess the direct as well as correlated responses of selection on the timing of adult emergence, adult emergence rhythm of the selected and control populations was assayed every 15 generations under LD cycles, and emergence and locomotor activity rhythms were assayed under LD and constant darkness (DD) conditions at the 55th generation.

Materials and methods

Fly population maintenance and selection protocol

The *early* and *late* populations were initiated from four ancestral baseline populations of *D. melanogaster* that have been maintained in the laboratory for several generations as separate entities without any gene flow between them. The maintenance protocol and ancestry of these baseline populations are described in detail elsewhere (Sheeba et al., 1998). Briefly, they were maintained as large outbred populations under alternating 12:12 h LD cycles (light intensity 15±5 μW cm^{-2} s^{-1}) with banana-jaggery food and water available *ad libitum*. Temperature (25±1°C) and humidity (75±5%) were maintained constant throughout the study. A total of 1200 breeding adults per population, with roughly equal number of males and females, were maintained in Plexiglas^{TM} cages (25 cm×20 cm×15 cm) on a 21-day discrete generation cycle. Eggs were collected by placing Petri dishes with food into these cages during the light phase of the LD cycle, dispensed at a density of about 300 eggs into vials (18 cm height×2.4 cm diameter) containing 10 ml of food. Such a high density of eggs resulted in staggered adult emergence for several cycles. Flies emerging between the 9th and 13th days after egg collection were collected into Plexiglas^{TM} cages containing a Petri dish of food. On the 18th day, a generous smear of yeast-acetic acid paste was applied on the food plates and kept in the cages. Three days later, eggs were collected to initiate the next generation. These four populations (referred as the baseline populations) served as the founder populations for the initiation of the selection lines. From these four baseline populations, four early ($early_{1..4}$) and four late ($late_{1..4}$) populations were initiated by imposing selection for adult emergence during the morning (05:00–09:00 h) and the evening (17:00–21:00 h) hour (henceforth referred to as the morning and evening selection windows) under 12:12 h LD cycles, where lights came on at 08:00 h and went off at 20:00 h (Fig. 1). Four control populations ($control_{1..4}$) were also initiated along with the selected populations, where no conscious selection pressure was applied on the timing of adult emergence. Each *early*, *control* and *late* population was derived from one baseline population, thus forming matched selected and control pair ($early_i$, $control_i$ and $late_i$ are more closely related than $early_j$, $control_j$ and $late_j$, i,j=1–4). For example, the $early_1$, $control_1$ and $late_1$ populations were initiated from baseline population 1. The four replicate populations with identical subscripts were treated as random factor in the analysis of variance (ANOVA). The selected and control populations were maintained under similar conditions, except that in each generation the adult flies for the *early* populations were collected between 05:00–09:00 h (M, morning window), for the *late* populations between 17:00–21:00 h (E, evening window), and the *controls* were collected through out the day, for 4–5 successive days. Care was taken to maintain a large out-bred structure (N~1200 with roughly equal numbers of males and females) of the populations.

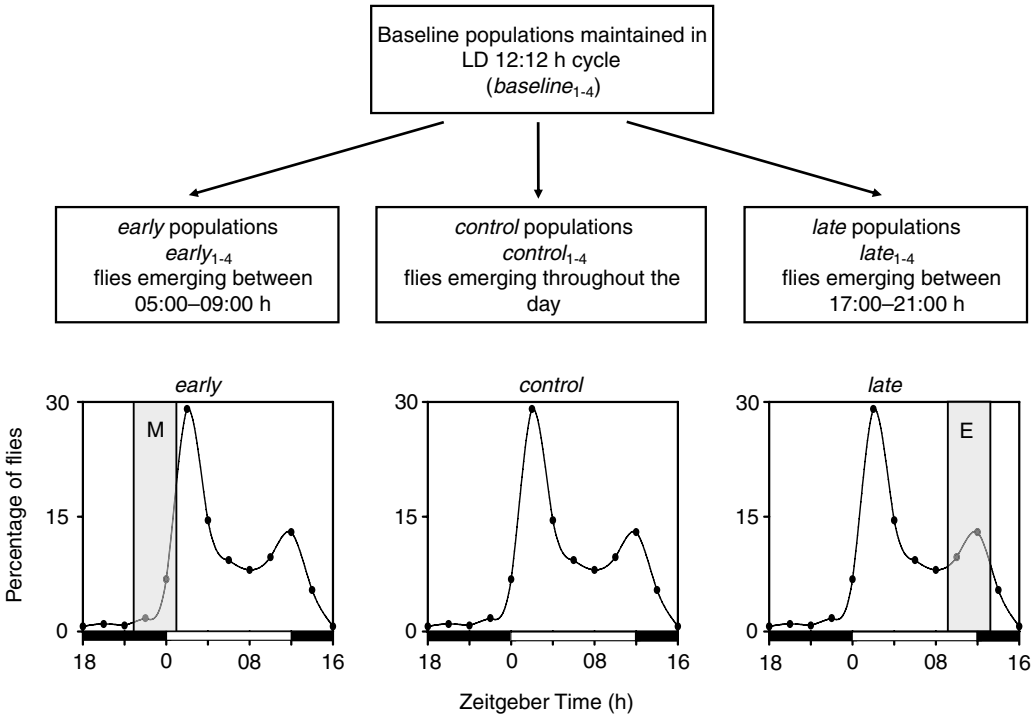

Fig. 1. Schematic representation of the selection protocol. The selection experiments were carried out under 12:12 h light:dark (LD) cycles ['lights-on' at 08:00 h and 'lights-off' at 20:00 h, where Zeitgeber Time 0 (ZT0) denotes lights-on]. Four baseline populations ($baseline_{1..4}$) maintained for over 75 generations under 12:12 h LD cycles were used to derive four early ($early_{1..4}$) and four late ($late_{1..4}$) populations of flies by imposing selection for early and late adult emergence. Four control populations ($control_{1..4}$) were also raised in a similar manner, except that they did not experience any conscious selection pressure. Flies emerging between 05:00–09:00 h formed the *early* populations, while those emerging between 17:00–21:00 h made it to the *late* populations. Flies emerging through out the day were used to raise the *control* populations. The morning (M) and evening (E) selection windows are shown in the grey boxes in the *early* and *late* panels.

Imposition of different maintenance regimes may induce nongenetic parental effects. Therefore, all selected and control populations were subjected to one generation of common rearing conditions prior to the assays, during which no conscious selection pressure was imposed. Such treatment for one generation has been shown to eliminate nongenetic parental effects (Prasad et al., 2001). Eggs were collected from the running cultures and dispensed into vials with about 10 ml of food at a density of about 300 eggs per vial. After the 12th day of egg collection, adult flies were collected into Plexiglas^{TM} cages with abundant food. For the assays, flies were supplied with yeast-acetic acid paste for 2 days prior to the egg collection. The progeny of these flies hereafter will be referred as standardized flies.

Eclosion assay

The percentage of flies emerging during the M and E windows of selection, phase-relationship between the eclosion peak and LD cycle, waveform of emergence rhythm under LD cycles were estimated at 5th, 10th, 25th, 40th and 55th generations, and the waveform and τ of the emergence rhythm under DD were assessed at the 55th generation. For these assays, eggs of approximately same age were collected from the standardized flies and dispensed at approximately 300 eggs per vial into vials with 10 ml of food. These vials were kept under LD and DD conditions. Ten such vials were set up per population for assays under each light condition. These vials were monitored for the first emergence and thereafter checked regularly at 2 h intervals for 10 consecutive days, and the number of flies was recorded. The percentage of flies emerging during the M and E windows was estimated by normalizing the total number of flies emerging during these windows by the total number of flies that emerged in one complete cycle. The phase-relationship of the emergence rhythm was estimated as the average time interval between the peak of eclosion and lights-on in the LD cycle. The phase-relationship values were considered to be negative if the peak followed lights-on and were taken to be positive when the peak preceded lights-on. Under DD conditions, adult emergence was monitored under dim red light (λ>640 nm) at 2 h intervals for 10 consecutive days.

Light pulse phase-response curve (PRC) for emergence rhythm

Light pulse-induced phase-response curves (PRC) were constructed for the selected as well as the control populations to estimate the extent of clock sensitivity to light. To estimate the emergence rhythm PRC, flies from selected and control populations were subjected to brief light stimuli at circadian time 2 (CT2), CT8, CT14 and CT20. For this assay, eggs of

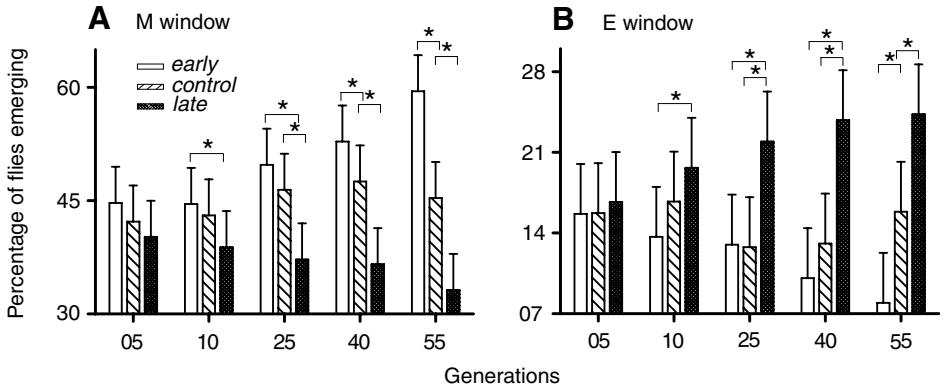

Fig. 2. Percentage of flies emerging during (A) the morning (M) window (05:00–09:00 h) and (B) the evening (E) window (17:00–21:00 h) in selected and control populations during the 5th, 10th, 25th, 40th and 55th generations. Error bars represent 95% confidence intervals (95%CI) around the mean across four replicate populations (10 vials per populations) for visual hypothesis testing.

approximately same age were collected from the standardized populations and dispensed into vials containing 10 ml of food at an egg density of ~300 per vial and maintained under 12 h:12 h LD cycles. After 5 days, flies were transferred to DD and exposed to light stimuli of 1000 lux intensity and 15 min duration at CT2, CT8, CT14 and CT20, in the first circadian cycle. Ten such vials were used for light exposure from each replicate population at each phase and ten more vials served as the experimental controls. The control vials at each tested CT were transported in light-tight containers (wrapped additionally with black cloth) along with the experimental vials to ensure that light pulse *per se* and not the disturbances associated with handling, transfer and human interference, cause phase shift. From the primary data, we estimated the mean phase of primary eclosion peak under LD as well as DD conditions for the experimental as well as control vials. Phase shifts were estimated using the method suggested previously (Sharma and Daan, 2002). Briefly, two regression lines were drawn through the peaks of emergence, one immediately following the light pulse and the other preceding it. The phase shift values were obtained by subtracting control phase shift values (obtained for the control vials, which were not subjected to the light pulse) from the experimental phase shift values.

Locomotor activity assay

The phase of the morning and evening activity peaks, activity levels during the M and E selection windows, waveform of locomotor activity rhythm under LD, τ and waveform of locomotor activity rhythm under DD were estimated at the 55th generation. For the assays, eggs were collected from the standardized populations and dispensed into vials containing 10 ml of food at a density of about 300 eggs per vial. Freshly emerged adult flies were transferred individually into activity monitors within 24 h of their emergence (Sharma, 2003b). The locomotor activity behaviour of the flies was monitored for the first 10 days under 12:12 h LD cycles and for about 15 days in DD. The percentage of activity during the M and E windows of selection was estimated

Table 1. *Results of ANOVA on the percentage of flies emerging during the morning and evening selection windows at different generations*

Effect	d.f. effect	MS effect	d.f. error	MS error	*F*	*P*
Generation (G)	4	0.001	12	0.009	0.166	0.951
Population (P)	2	0.004	6	0.0001	27.764	**0.001**
Window (W)	1	2.363	3	0.001	3423.085	**0.001**
Replicate (R)	3	0.002	0	0	–	–
G×P	8	0.001	24	0.0003	2.043	0.084
G×W	4	0.003	12	0.006	0.456	0.766
P×W	2	0.127	6	0.001	147.531	**0.001**
G×R	12	0.009	0	0	–	–
P× R	6	0.0001	0	0	–	–
W×R	3	0.001	0	0	–	–
G×P×W	8	0.012	2	0.001	11.833	**0.001**
G×P×R	24	0.0004	0	0	–	–
G×W×R	12	0.006	0	0	–	–
P×W×R	6	0.001	0	0	–	–
G×P×W×R	24	0.0004	0	0	–	–

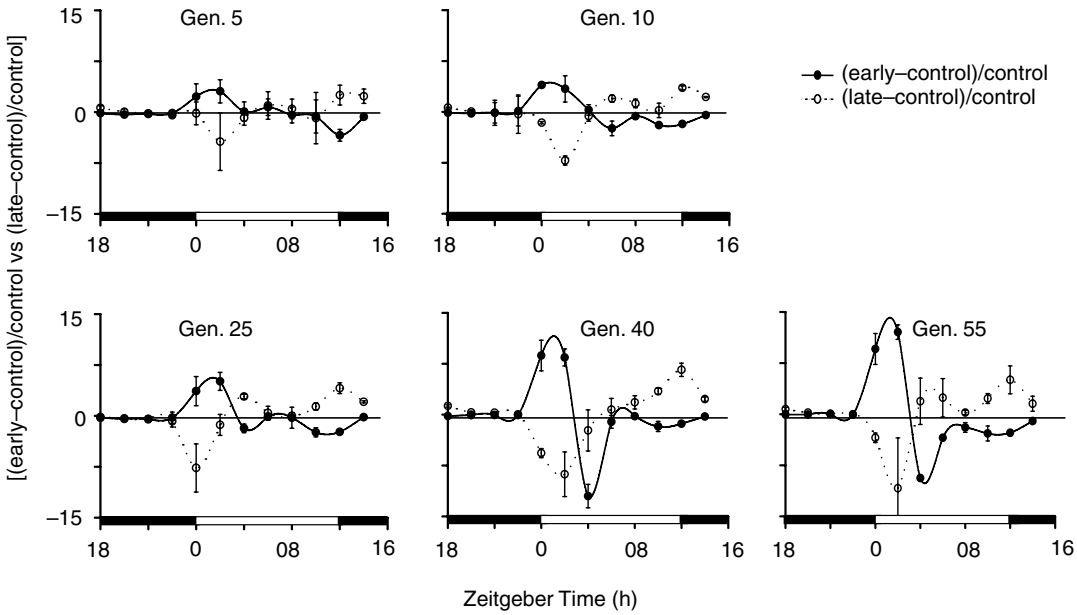

Fig. 3. The average 'difference waveform' of emergence rhythm of the *early* and *late* populations [(*early–control*)/*control*] and [(*late–control*)/*control*] were assayed under a 12:12 h LD cycle at the 5th, 10th, 25th, 40th and 55th generations (Gen.). The 'difference waveforms' were estimated by first subtracting the average emergence waveforms of the *early* and *late* populations from the *controls* and then scaling it by the average waveform of the *controls*. The filled and empty bars denote the dark (20:00–08:00 h) and the light (08:00–20:00 h) phases of the LD cycle and Zeitgeber Time 0 (ZT0) denotes the time at which lights came on.

by normalizing the amount of activity exhibited during these windows by the total amount of activity in one complete cycle. The phase of the morning and evening activity peaks under LD cycles were estimated using CLOCKLAB (Actimetrics, Evanston, IL, USA) taking activity data collected for 10 successive days.

Estimation of the difference waveforms of emergence and locomotor activity rhythms

The average waveform of emergence rhythm under LD and DD conditions was estimated by dividing the total number of flies emerging every 2 h by the total number of flies emerging in one cycle. Similarly, the waveform of locomotor activity rhythm was obtained by dividing hourly collected activity data by the total amount of activity during one complete cycle. The mean waveform of emergence and locomotor activity rhythms were estimated from time series data obtained for a minimum of 10 days. In order to compare the emergence and activity waveforms of the selected populations we estimated 'difference waveform' of each population by calculating the difference

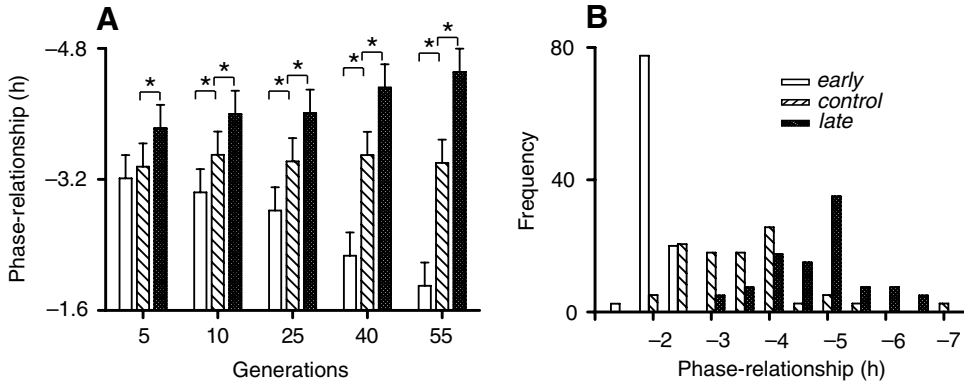

Fig. 4. (A) The phase-relationship between the emergence rhythm and the LD cycle of the selection and control populations at the 5th, 10th, 25th, 40th and 55th generations. The phase-relationship was estimated as the time interval between the primary peak of the emergence and 'lights-on' in the LD cycle, averaged over 10 consecutive cycles. Error bars represent 95% confidence intervals (95%CI) around the mean across four replicate populations (10 vials per populations) for visual hypothesis testing. (B) The frequency distribution of phase-relationship of the *early*, *control* and *late* populations under 12:12 h LD cycles. Time (h) is plotted along the *x*-axis and percentage frequency along the *y*-axis.

between the emergence or activity waveform of a selected population and its respective control. For example, the difference waveform of the replicate 1 of the selected populations would be [($early_1$–$control_1$)/$control_1$] and [($late_1$–$control_1$)/$control_1$].

Statistical analyses

The τ of emergence rhythm under DD was estimated by subjecting time series data collected over 10 consecutive cycles to Fourier spectral analysis using Statistica™ (rel.5.0B) (Statistica, 1995). Statistical significance of rhythmic contributions from different frequencies in the periodogram was tested using Siegel's modification of the Fischer test (Siegel, 1980). This method delineated the frequency components present in the time series by defining a threshold value (Rao and Sharma, 2002). The τ of locomotor activity rhythm under DD was estimated by subjecting time series data collected over ten consecutive cycles to Lomb-Scargle Periodogram analysis using CLOCKLAB (Actimetrics, Evanson, IL, USA).

The circadian parameters of emergence and locomotor activity rhythms were subjected to separate mixed model analysis of variance (ANOVA) treating replicate as random factor, whereas generation and population as fixed factors crossed with replicates. The percentage of flies emerging during the M and E windows was used as fixed factor crossed with generation, population and replicate. In all statistical analyses, population means were used as the unit of analysis. Multiple comparisons were done using 95% confidence intervals (95%CI) around the mean. The error bars used throughout the text as well as figures, unless otherwise specified are 95%CI to facilitate visual hypothesis testing. Therefore, overlapping error bars would imply that the values do not differ significantly.

The phase-relationship, timing of morning and evening activity peak and τ of emergence and locomotor activity rhythms were used as data in a mixed model ANOVA crossed with replicate and population. The 'difference waveforms' of emergence and locomotor activity rhythms of the selected and control populations were analyzed using Kolmogorov–Smirnov two-sample test. All analyses were implemented using Statistica for Windows (rel.5.0B) (Statistica, 1995).

Results

Percentage of flies emerging during the morning and evening windows of selection

In order to assess the direct response to selection on the timing of adult emergence, we estimated the percentage of flies emerging during the M and E selection windows. With increasing generations, the percentage of flies that emerged during the M window increased gradually in the *early* populations, and steadily decreased in the *late* populations (Fig. 2A; Table 1). On the other hand, the percentage of flies emerging during the E window increased steadily in the *late* populations, while it decreased gradually in the *early* populations (Fig. 2B; Table 1). The percentage of flies emerging during the M and E windows remained unchanged in the *controls*. Though the differences between the *early* and *late* populations reached statistically significant levels as early as the 10th generation, those between the *early* and *controls* took 40 generations to become statistically significant. After 55 generations, the percentage of flies emerging during the M window was about 60%, 45% and 33% in the *early*, *control* and *late* populations, while that emerging during the E window was about 8%, 16% and 24% (Fig. 2A,B).

Emergence rhythm under LD

The 'difference waveforms' of the *early* and *late* populations are shown generation-wise in the Fig. 3. With increasing generations, a peak began to emerge during the morning in the 'difference waveform' of the *early* populations, while in the *late* populations a similar peak emerged in the evening, indicating that the waveforms of the selected populations are gradually diverging from the controls. In addition, a prominent trough appeared (especially in the 40th and the 55th generation assays) in the *early* populations, immediately after the morning peak, suggesting that the percentage of flies emerging at this phase decreases significantly in the *early* populations compared to the *controls*. Interestingly, this also happens to be the phase of maximum emergence in the *controls* (Fig. 3). The Kolmogorov–Smirnov two-samples revealed that the 'difference waveforms' of the *early* and *late* populations were significantly different in the 40th ($P<0.05$) and the 55th ($P<0.01$) generation assays.

The primary peak of emergence occurred early in the morning in the *early* populations, followed by the *controls*, and then the *late* populations, and the separation between the peaks of the three populations gradually increased with generation (Fig. 4A; Table 2). Though the emergence peak of the *late* populations diverged significantly from the *early* and *controls* by the 10th generation, those of the *early* and *controls* took 25 generations to move away from each other (Fig. 4A). The divergence in the emergence peaks of the selected and control populations is clearly evident in the frequency distribution plot of the phase-relationship of their adult emergence rhythm obtained at the 55th generation (Fig. 4B).

Emergence rhythm under DD

At the 55th generation, we assayed the adult emergence rhythm of the selected and control populations under DD. The Kolmogorov–Smirnov test for two samples revealed that the average 'difference waveforms' of the *early* and *late* populations were significantly different ($P<0.01$ for each replicate pair).

ANOVA on τ showed a significant main effect of population ($F_{2,6}=9.51$; $P<0.01$). Multiple comparisons using 95%CI revealed that the mean τ of the *early* populations was significantly shorter than the *controls*, while that of the *late* populations was significantly longer than the *controls* (Fig. 5A). The frequency distribution of the τ of emergence

Table 2. *Results of ANOVA on the phase-relationship between the primary eclosion peak and light/dark cycle at different generations*

Effect	d.f. effect	MS effect	d.f. error	MS error	*F*	*P*
Generation (G)	4	0.106	12	0.502	0.210	0.928
Population (P)	2	11.059	6	0.377	29.310	**0.001**
Replicate (R)	3	0.763	0	0	–	–
G×P	8	0.713	24	0.162	4.401	**0.002**
G×R	12	0.009	0	0	–	–
P×R	6	0.0001	0	0	–	–
G ×P×R	24	0.0004	0	0	–	–

rhythm differed among the selected and control populations (Fig. 5B).

Light pulse phase response curve (PRC)

Light exposure at CT14 caused a significantly smaller phase delay in the *early* populations compared to the *controls*, while it evoked a significantly greater phase delay in the *late* populations compared to the *controls* (Fig. 6; Table 3). On the other hand, light exposure at CT20 evoked a significantly greater phase advance in the *early* populations compared to the *controls*, while it caused a significantly smaller phase advance in the *late* populations compared to the *controls* (Fig. 6; Table 3). The phase shift in the emergence rhythm of the selected and control populations did not differ at CT2 and CT8.

Locomotor activity rhythm under LD

To estimate the correlated response to selection on the timing of adult emergence we assayed the locomotor activity rhythm of individual flies from the selected and control populations. Activity levels during the M and E selection windows were compared among the *early*, *control* and *late* populations. The *early* flies were more active in the morning, the *late* flies were more active in the evening, while the *controls* were as active in the morning as in the evening (Fig. 7A–H; Table 4). The average activity level during the M window was about 26.2%, 21.0% and 20.1% in the *early*, *control* and *late* populations (Fig. 7A), while that during the E window was about 21.0%, 25.7% and 30.0% (Fig. 7B). The differences in the locomotor activity patterns of the selected and control flies persisted even in the first cycle of DD, following a LD to DD transfer. In DD, the activity peak of the *early* flies was restricted to the mid subjective day, while that of the *control* and *late* flies was shifted towards the late subjective day (Fig. 7I–K). The morning activity peak occurred significantly earlier in the *early* flies compared to the *late* and *controls*, while the evening activity peak occurred significantly later in the *late* flies compared to the *early* and *controls* (Fig. 8A,B; Table 5). The frequency distribution of phase of the morning and evening activity peaks differed among the selected and control populations (Fig. 8C,D).

Locomotor activity rhythm under DD

The circadian period (τ) of locomotor activity rhythm of the selected populations was altered in response to selection (Fig. 9A–D). ANOVA showed a significant main effect of population on τ ($F_{2,6}$=0.005; P<0.001). Multiple comparisons revealed that the mean τ of the *early* populations was significantly shorter than the *controls*, while that of the *late* flies was significantly longer than the *controls* (Fig. 9D). As illustrated in the frequency distribution plot, a greater

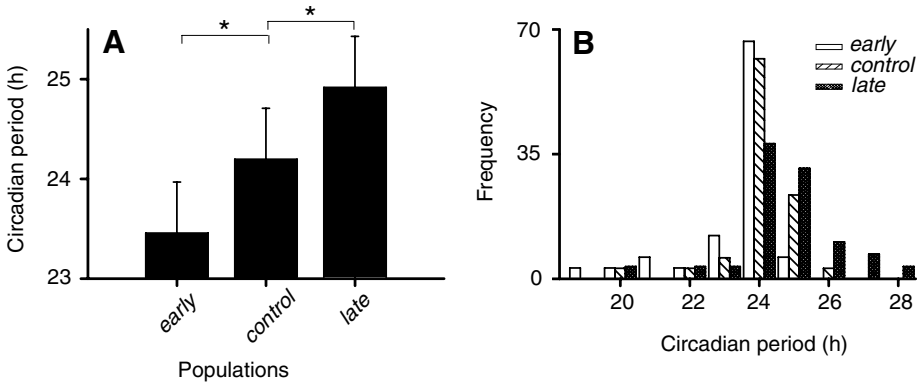

Fig. 5. (A) The mean circadian periodicities (τ) of emergence rhythm of the selected and control populations at the 55th generation. Error bars represent 95% confidence intervals (95%CI) around the mean across four replicate populations (10 vials per populations) for visual hypothesis testing. (B) The frequency distribution of the τ of emergence rhythm of the *early, control* and *late* populations. Time (h) is plotted along the *x*-axis and percentage frequency along the *y*-axis.

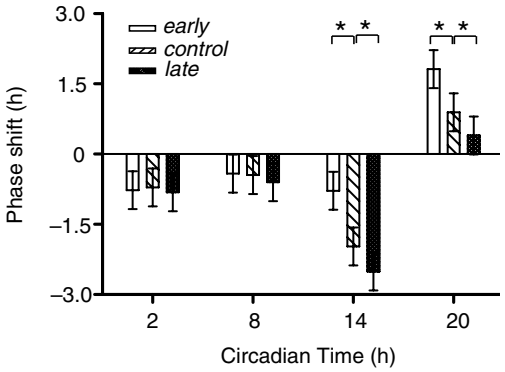

Fig. 6. Light pulse-induced phase shift (h) of the emergence rhythm at four phases (CT2, 8, 14 and 20) of the selected and control populations. Circadian Time 0 (CT0) indicates the onset of locomotor activity. Error bars indicate 95% confidence intervals (95%CI) around the mean for visual hypothesis testing. A total of 40 vials were used, of which 10 were used for each replicate population at each phase.

percentage of *early* flies had shorter τ compared to the *controls*, while a greater percentage of *late* flies had longer τ compared to the *controls* (Fig. 9E). Further, the τ of the locomotor activity and adult emergence rhythms showed a significant positive correlation (r=+0.76; P<0.003) (Fig. 10).

Discussion

We imposed artificial selection pressure on the timing of adult emergence on four large (N>1200), genetically independent, random mating populations of *D. melanogaster* and derived four populations each of early ($early_{1..4}$), control ($control_{1..4}$) and late ($late_{1..4}$) flies. After 55 generations of selection, the percentage of flies emerging during the M window increased in the *early* populations, while it decreased in the *late* populations. The percentage of flies emerging during the E window increased in the *late* populations, while it decreased in the *early* populations. The waveform of emergence rhythm in individual flies from the *early*, *control* and *late* populations differed significantly, particularly in the 40th and 55th generations. The emergence pattern of the *early* populations was left-skewed compared to the *controls*, while that of the *late* populations was marginally right-skewed (Fig. 3), indicating that the time course and waveform of emergence rhythm is altered due to selection. This suggests that *D. melanogaster* populations respond to selection on the timing of adult emergence by gradually enhancing the percentage of flies emerging during the selection windows and by modifying the overall emergence waveform.

Under LD cycles, the morning peak of activity in the *early* populations occurred earlier than the *controls*, while that of the *late* populations occurred later than the *controls*, thus unerringly mimicking the adult emergence patterns (Fig. 7C–H). Although the total amount of daily activity did not differ among the *early*, *control* and *late* populations, the waveforms of their locomotor activity rhythm were significantly different (Fig. 7C–H; Fig. 8A,B). The *early* flies started activity earlier than the *controls* and were generally more active in the morning than evening, while the *late* flies started activity later than the *controls* and were more active in the evening than morning. The *control* flies showed bimodal activity pattern and were as active in the morning as in the evening. Further, the *early* flies showed greater anticipation to lights-on, while the *late* flies showed greater anticipation to lights-off (Fig. 7C–E), which is consistent with their faster and slower circadian periods. Interestingly, the differences between the activity patterns of the *early* and *late* flies were retained in the first cycle of DD, following an LD to DD transfer, indicating that the changes in the locomotor activity patterns are inherent (Fig. 7I–K). Further, the τ of emergence and locomotor activity rhythms showed a significant positive correlation (Fig. 10), suggesting that these two rhythms are genetically correlated. Such correlations between adult emergence and locomotor activity rhythms have been previously reported in an early study on the *period* mutants of *D. melanogaster* (Konopka and Benzer, 1971).

Given that the phase-relationship between a circadian rhythm and LD cycle depends upon the τ, and the light pulse PRC of the underlying circadian clocks (Pittendrigh and Daan, 1976; Sharma and Chidambaram, 2002), the gradual divergence in the phase-relationship of emergence peaks of the *early* and *late* populations (Fig. 3, Fig. 4A, Fig. 8A,B) can be ascribed to gradual changes in (i) τ, or (ii) PRC, or (iii) both τ and PRC. We observed that both τ and the PRC of the selected populations have diverged from each other as well as from the *controls*. Compared to the *controls*, the *early* populations had shorter τ, smaller phase delay at CT14, and larger phase advance at CT20, while the *late* populations had longer τ, greater phase delay at CT14, and smaller phase advance at CT20 (Fig. 6). While interpreting the differences in the PRC

Table 3. *Results of ANOVA for phase shift of eclosion rhythm*

Effect	d.f. effect	MS effect	d.f. error	MS error	F	P
Population (P)	2	2.89	6	0.39	7.27	**0.024**
Phase (Ph)	3	16.01	9	0.72	22.29	**0.001**
Replicate (R)	3	0.17	0	0	–	–
P×Ph	6	0.78	18	0.25	3.16	**0.027**
P×R	6	0.39	0	0	–	–
Ph×R	9	0.72	0	0	–	–
P×Ph×R	18	0.25	0	0	–	–

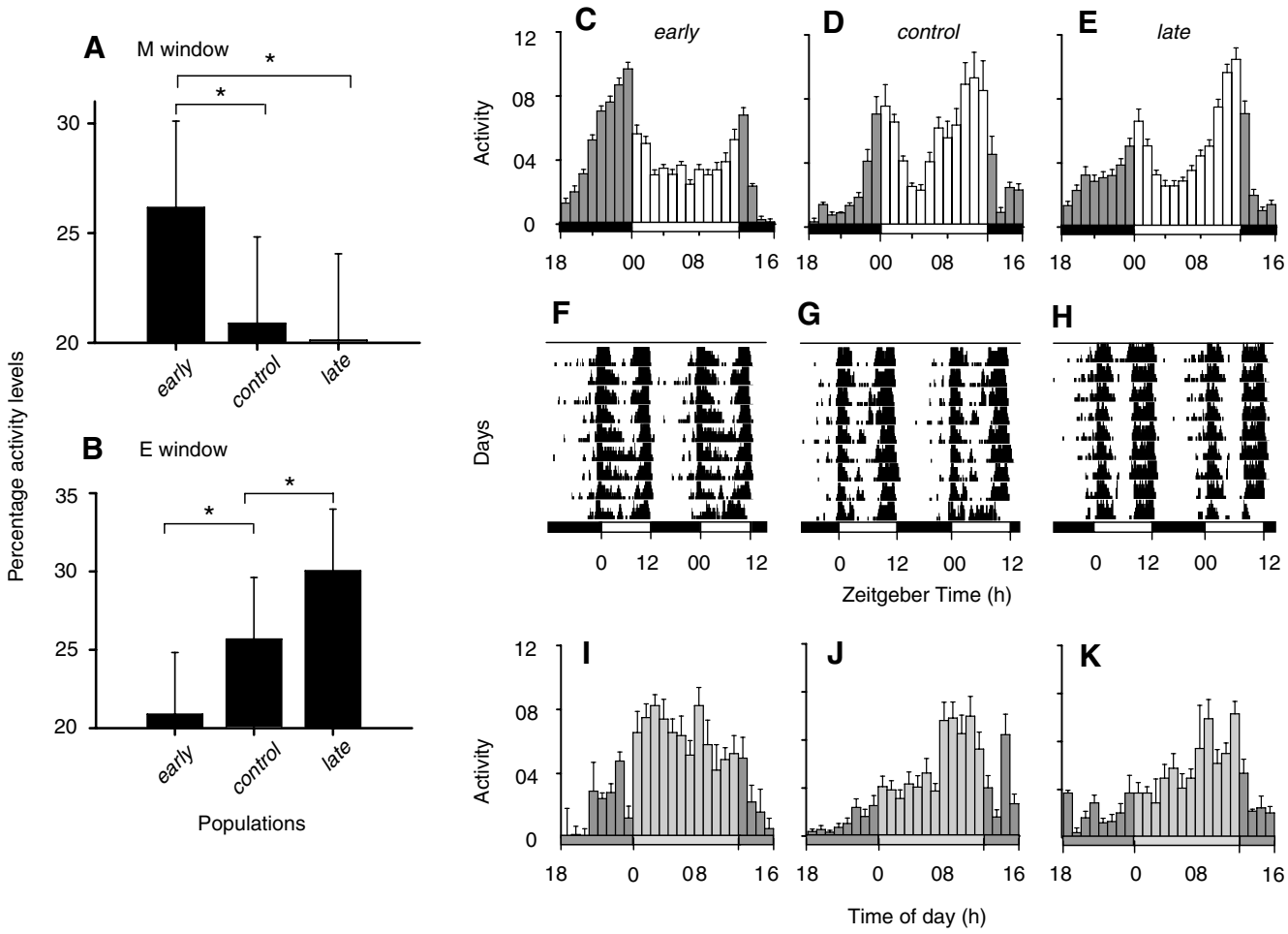

Fig. 7. Activity levels of the selected and control flies during the morning (05:00–09:00 h) and evening (17:00–21:00 h) windows of selection. (A) Percentage activity during the morning (M) window in the selected and control populations. (B) Percentage activity during the evening (E) window in the selected and control populations. Error bars represent 95% confidence intervals (95%CI) around the mean for visual hypothesis testing. The average locomotor activity plots of the (C) *early* (N=161), (D) *control* (N=171) and (E) *late* (N=156) flies, monitored under a 12:12 h LD cycle after 55 generations of selection. Locomotor activity profiles are plotted as the mean activity during 1 h bins, averaged over 10 consecutive cycles. The percentage of activity, averaged over 10 successive cycles, is plotted along the ordinate and time of the day (h) along the abscissa. Values are means ± s.e.m., constructed using the variations among the replicate populations within each selection regime. The white and grey vertical bars denote activity levels during the day and night, respectively. Additionally, one representative locomotor activity pattern each of flies from the (F) *early*, (G) *control* and (H) *late* populations are shown. The horizontal black bars denote the dark phase (20:00 h–08:00 h) and white bars represent the light phase of the LD cycle. Zeitgeber time 0 (ZT0) denotes the time at which lights come on under the LD cycle. The average locomotor activity plots of the (I) *early* (N=32), (J) *control* (N=27) and (K) *late* (N=37) flies, during the first cycle of DD following a LD/DD transfer. Horizontal dark grey bars denote the subjective night and light grey bars the subjective day under DD conditions. Vertical dark and light grey bars denote activity during the subjective night and subjective day, respectively, under DD.

Table 4. *Results of ANOVA on the morning and evening activity levels*

Effect	d.f. effect	MS effect	d.f. error	MS error	F	P
Population (P)	2	0.001	6	0.001	3.924	0.081
Window (W)	1	0.006	3	0.002	3.958	0.141
Replicate (R)	3	0.001	0	0	–	–
P×W	2	0.012	6	0.001	23.102	**0.002**
P×R	6	0.0001	0	0	–	–
W×R	3	0.002	0	0	–	–
P×W×R	6	0.001	0	0	–	–

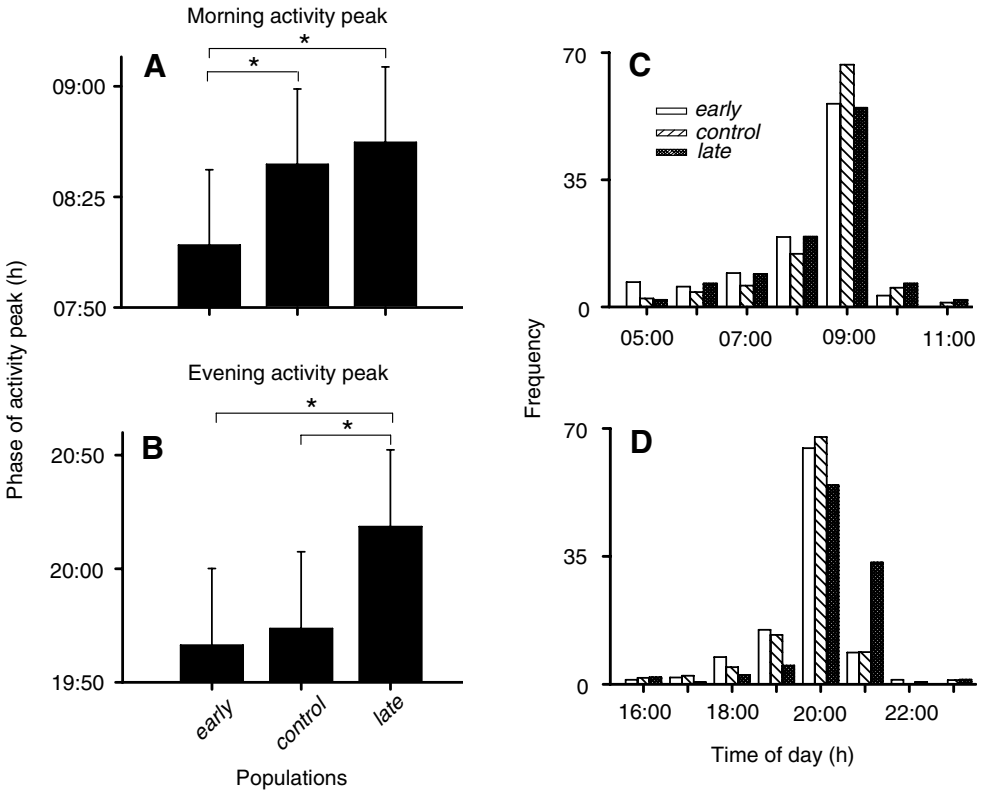

Fig. 8. (A) Mean phase of the morning activity peak of the selected and control flies under 12:12 h LD cycle. The mean phase of the morning activity peak was estimated as the time interval between the morning peaks and lights-on, averaged over 10 consecutive cycles. (B) Mean phase of the evening activity peak was estimated as the average time interval between the evening peaks and light-off, averaged over 10 consecutive cycles. Error bars represent 95% confidence intervals (95%CI) around the mean for visual hypothesis testing. A total of *early* (N=161), *control* (N=171), and *late* (N=156) flies were used to estimate the mean phase of the morning and evening activity peaks. (C) The frequency distribution of mean phase of morning, and (d) evening activity peak of the *early*, *control* and *late* populations under 12:12 h LD cycles. Time (h) is plotted along the x-axis and percentage frequency along the y-axis.

among the selected and control populations, one should consider the differences in their τ values. The mean τ of the emergence rhythm of the *early* and the *late* populations differs by about 1.5 h, which could lead to a difference of about an hour or so in the phase of light exposure in their clocks. While having little effect during the subjective day, this could have a major impact during the subjective night when the PRC slopes are steeper. For example, faster clocks in the *early* populations would allow the light pulse to fall at a later phase than the *controls*, and as a result during the early subjective night phase delays would be larger, and phase advances during the late subjective night would be smaller, than the *controls*. The slower clocks in the *late* flies would allow the light pulse to fall at an earlier phase than the *controls*, and therefore during the early subjective night the phase delays would be smaller and phase advances during the late subjective night would be larger than the *controls*. However, such limitations do not weaken the strength of our conclusions on the PRC, since compared to the *controls* the *early* populations undergo smaller phase delay at CT14 and larger phase advance at CT20, while the *late* populations undergo larger delay at CT14 and smaller phase advance at CT20. This suggests that the actual PRC

Table 5. *Results of ANOVA on the phase of morning and evening activity peaks*

Effect	d.f. effect	MS effect	d.f. error	MS error	F	P
Population (P)	2	0.743	6	0.079	9.378	**0.014**
Phase (Ph)	1	933.889	3	0.024	38292.109	**0.001**
Replicate (R)	3	0.068	0	0	–	–
P×Ph	2	0.115	6	0.046	2.487	0.163
P×R	6	0.079	0	0	–	–
Ph×R	3	0.024	0	0	–	–
P×Ph×R	6	0.046	0	0	–	–

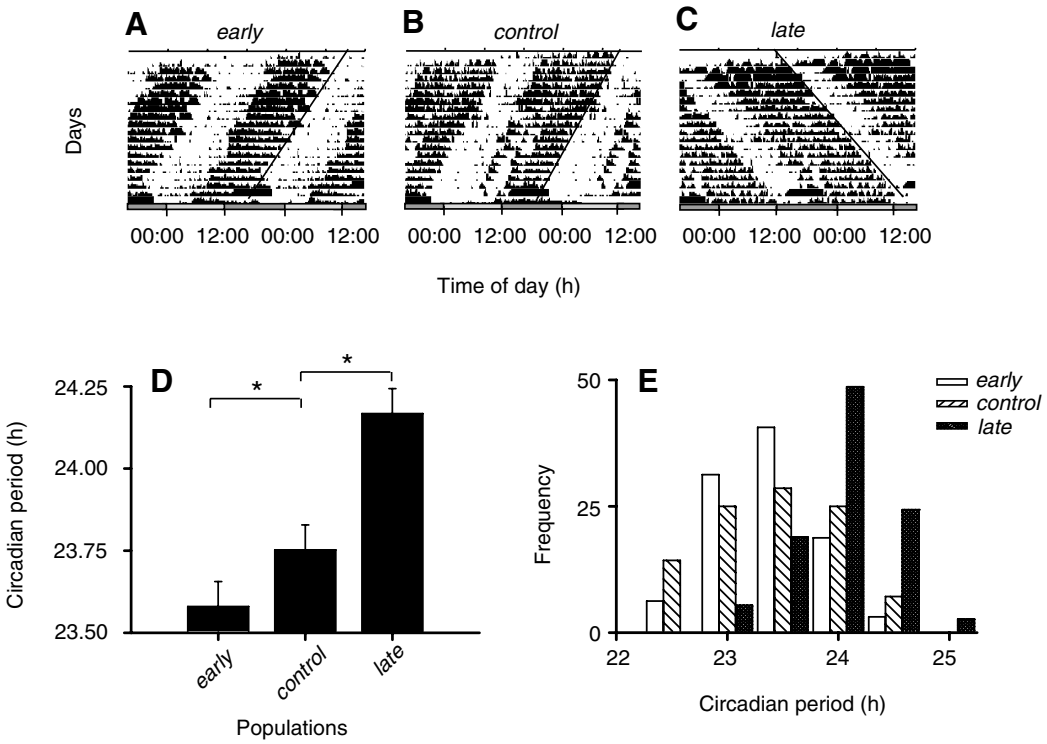

Fig. 9. Representative locomotor activity plots of individual (A) *early*, (B) *control*, and (C) *late* flies under constant darkness (DD). The vertical dark bars denote activity and their absence indicate rest. Activity for 10 consecutive cycles is plotted along the ordinate and time of the day in hours along the abscissa. (D) Mean circadian period (τ) (h) of the locomotor activity rhythm of the selected and control populations under constant darkness (DD). Error bars represent 95% confidence interval (95%CI) around the mean for visual hypothesis testing. A total of (N=32) *early*, (N=27) *control* and (N=37) *late* flies were used for the estimation of τ. (E) Frequency distribution of τ of the locomotor activity rhythm of the *early*, *control* and *late* populations. Time (h) is plotted along the *x*-axis and percentage frequency along the *y*-axis.

differences between the selected and control populations were even larger than those depicted in their PRCs (Fig. 6). Taken together, the results of our study indicate that circadian clocks of the *early* and *late* populations have diverged from the *controls* by altering their τ as well as PRC. These results are, however, in sharp contrast to a few early findings, where the early and late strains were reported to have a longer and a shorter τ compared to the parental controls (Pittendrigh, 1966; Pittendrigh and Minis, 1971). Further, in these studies the PRCs of the selected strains were also similar. On the other hand, in a separate study (Pittendrigh and Takamura, 1987) where a different species of *Drosophila* (*D. auraria*) was used to raise the early and late emerging strains, the results were just the opposite. In this case, the early strains had faster running clocks and the late strains had slower clocks, quite similar to the results of the present study. It is known that modes of evolutionary fine-tuning of a trait depend upon a number of factors such as the genetic architecture of the founder population, especially the available genetic variance for the trait in question, strength of selection, environmental conditions and population size. Therefore, it is possible that the differences in the outcome of studies on the early and late emerging strains were due to one or more such factors. Moreover, lack of replicates within selection lines, and

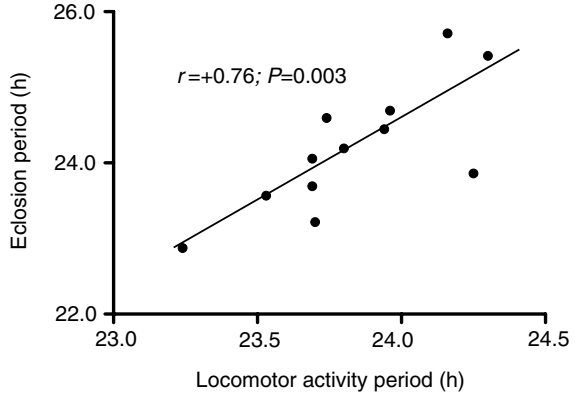

Fig. 10. Correlation between the circadian periods of the locomotor activity and emergence rhythms. The mean τ values of the locomotor activity and emergence rhythm are plotted along the *x* and *y*-axes, respectively.

inadequate information about the population size and rearing protocols, make it difficult to estimate the extent of genetic drift or inbreeding that the selected populations may have undergone in these studies. In addition, most previous studies were not continued for a long enough time to confirm whether the

selection responses had reached steady state. Some were terminated as early as the 9th generation and others lasted for not more than 15 generations.

The results of our study can also be taken as empirical evidence for the morning and evening (M–E) oscillator model proposed by Pittendrigh and Daan (Pittendrigh and Daan, 1976) and subsequently elaborated by Daan et al. (Daan et al., 2001). The model assumes that circadian clocks comprise two oscillators (the M and E oscillators), which track the 'dawn' and 'dusk' of the natural LD cycles by maintaining a precise and reproducible phase-relationship with them. The M oscillator was proposed to have shorter period and to rely more on phase advances than delays, whereas the E oscillator was considered to have longer period and to rely more on phase delays than advances. The *early* populations have evolved morning circadian phenotypes with faster running clocks and PRC with smaller phase delays and greater advances, while the *late* populations have evolved evening circadian phenotypes with slower running clocks and PRC with greater phase delays and smaller advances. The M–E oscillator model was also critically analyzed in a few recent studies in *Drosophila*, where flies with either morning or evening activity patterns were created by genetically manipulating a small group of clock neurons (Grima et al., 2004; Stoleru et al., 2004). These studies suggest that the morning and evening activity bouts in locomotor activity cycles are controlled by different sets of neurons. Given that the *early* and *late* flies have evolved morning and evening circadian phenotypes with almost all the features of the M and E oscillators proposed in the model, it would be interesting to investigate if the morning and evening activity patterns in these flies are regulated by different subgroups of clock neurons or by altered circadian waveforms of the core clock genes.

Our study is by far the most rigorous and unequivocal of all selection studies done so far on any rhythm or rhythm-related trait. The results are based on genetically independent, random mating, large populations of *Drosophila* derived from common ancestors, and clearly demonstrate that the time course and waveform of emergence and locomotor activity rhythms diverge from the controls in response to selection on the timing of adult emergence, and as a consequence circadian clocks of the selected populations evolve. The results are borne out of consistent heritable genetic changes in response to selection on the timing of adult emergence and not due to random genetic drift or due to some unknown environmental or non-genetic effect. The results further provide valuable functional insights into the genetic architecture of behavioral rhythms such as emergence and locomotor activity and the underlying genetic correlations between them. The results of our study can also be taken to suggest that one of the possible ways in which circadian clocks evolve, is through the process of adaptive evolution under the influence of periodic selection pressures present in the environment.

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