RESEARCH ARTICLE

Clock accuracy and precision evolve as a consequence of selection for adult emergence in a narrow window of time in fruit flies *Drosophila melanogaster*

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SUMMARY

Although circadian clocks are believed to have evolved under the action of periodic selection pressures (selection on phasing) present in the geophysical environment, there is very little rigorous and systematic empirical evidence to support this. In the present study, we examined the effect of selection for adult emergence in a narrow window of time on the circadian rhythms of fruit flies *Drosophila melanogaster*. Selection was imposed in every generation by choosing flies that emerged during a 1 h window of time close to the emergence peak of baseline/control flies under 12 h:12 h light:dark cycles. To study the effect of selection on circadian clocks we estimated several quantifiable features that reflect inter- and intra-individual variance in adult emergence and locomotor activity rhythms. The results showed that with increasing generations, incidence of adult emergence and activity of adult flies during the 1 h selection window increased gradually in the selected populations. Flies from the selected populations were more homogenous in their clock period, were more coherent in their phase of entrainment, and displayed enhanced accuracy and precision in their emergence and activity rhythms compared with controls. These results thus suggest that circadian clocks in *D. melanogaster* evolve enhanced accuracy and precision when subjected to selection for emergence in a narrow window of time.

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INTRODUCTION

In fruit flies, *Drosophila melanogaster*, the act of emergence of adults from the pupal case is 'gated'. Every day, adults emerge in large numbers within an 8–10 h interval, starting 1–2 h prior to 'dawn', when humidity in the environment is high and temperature is low (Pittendrigh, 1954; Saunders, 2002). This duration of emergence is referred to as the 'gate of emergence' and it is believed that flies that are developmentally mature enough to emerge during or before the gate is open do emerge, while others who mature after the gate closes need to wait until the next day's gate opens. The intervening hours of the day when little or no emergence occurs constitute the 'forbidden zone' of emergence. In a recent study, Mukherjee et al. (Mukherjee et al., 2012) successfully modelled such gating in emergence, taking into account accumulation of a developmental substance and its rhythmic threshold. It is believed that such gating of emergence during high humidity morning hours increases the chances of survival of flies in the wild by preventing desiccation under harsh conditions in the afternoon (Saunders, 2002). The adaptive benefit of circadian clocks in *Drosophila* is not restricted to adult emergence alone as these clocks are involved in the regulation of a wide variety of physiological processes that need to be co-ordinated with cycling environmental factors (Pittendrigh, 1993; Sharma and Joshi, 2002; Sharma, 2003). It is commonly believed that circadian clocks may have evolved under the influence of such cyclic environmental factors to enable organisms to maintain temporal order in their metabolism and behaviour.

It seems intuitive that precisely timed rhythmic activities with less variability would confer greater adaptive advantage to organisms than randomly occurring ones (Roenneberg et al., 2003; Sharma, 2003). Moreover, previous studies have reported significant variation in the precision of circadian rhythms between individuals and suggested that precision in rhythmic processes is a function of clock period, which implies that precision can be taken as a marker of adaptive evolution of circadian clocks (Aschoff et al., 1971; Pittendrigh and Daan, 1976; Sharma and Chandrashekaran, 1999). Notwithstanding the importance of stability of daily rhythms for a wide range of organisms living under periodic environmental conditions, our understanding of evolutionary aspects of accuracy and precision in circadian time-keeping systems is almost negligible.

Several studies in the past have examined the effect of artificial selection for timing of adult emergence on circadian clocks in insects (Pittendrigh and Minis, 1971; Clayton and Paietta, 1972; Kumar et al., 2007a). Although these studies suggest that circadian clocks respond to selection on timing of emergence, it is difficult to conclude that changes in clocks were due to adaptive evolution, because of certain critical limitations that some of these studies suffered from. For example, in two of the previous studies (Pittendrigh and Minis, 1971; Clayton and Paietta, 1972) there is no mention of whether replicates at the level of population were used, and what the population size of each breeding population was. Therefore, the fact that outcome of selection in these studies suffered from inbreeding or random genetic drift cannot be ruled out (Sharma and Joshi, 2002). In a relatively recent study by Kumar et

al. (Kumar et al., 2007a), four large random mating populations of *D. melanogaster* were subjected to selection for early and late emergence, and it was shown that the selected populations evolved circadian clocks that differed from controls in their circadian period and photic sensitivity (Kumar et al., 2007a; Kumar et al., 2007b; Vaze et al., 2012). The early and late populations comprised flies that emerge at two extremities of the day, and therefore do not represent the 'normal' flies, which begin emerging 1–2h after lights-on and continue to emerge until lights-off. The rationale of the current study was that artificial selection for emergence during the peak emergence time of control/baseline populations would provide insights into the genetic variation underlying the timing of emergence in these populations and its relationship with the circadian phenotype. Such variation in circadian phenotypes may also have evolutionary advantages under varying environmental conditions. Hence we undertook this separate artificial laboratory selection experiment where only flies within the mode of the distribution of emergence were allowed to contribute to the next generation.

Here we report the results of our long-term (80 generations) study aimed at elucidating the effect of selection for adult emergence during a narrow window of 1h coinciding with the emergence peak of the baseline fruit fly *D. melanogaster* populations under 12h:12h light:dark (LD) cycles. To assess the response of selection on circadian clocks, two well-studied circadian rhythms – adult emergence and locomotor activity – were used as read-outs of circadian clocks. We found that with increasing generations, flies selected for emergence in a narrow window of time evolved circadian rhythms with enhanced accuracy and precision.

MATERIALS AND METHODS
Selection protocol
Four replicates of precision populations (PP$_{1-4}$) were derived from four ancestral control populations (CP$_{1-4}$) of *D. melanogaster* Meigen 1830 which had been maintained under 12h:12h LD cycles at constant temperature (~25°C) and relative humidity (~75%) on banana-jaggery (BJ) food medium for more than 250 generations. The PP populations were initiated by selecting for flies that emerged during zeitgeber time 01–02 (ZT01–02; henceforth will be referred to as selection window) under 12h:12h LD cycles, where time of lights-on is considered as ZT00 and of lights-off as ZT12 (supplementary material Fig. S1). Flies that emerged during this selection window, on four successive days (9th–12th day after egg collection) comprised the breeding adults for initiating the next generation. The purpose behind choosing the ZT01–02 window for our selection was twofold: (1) to avoid flies that emerge in response to lights-on (startle), and (2) to choose flies that comprise the emergence peak of baseline/control CP populations. Four CP$_{1-4}$ were maintained along with PP$_{1-4}$, which otherwise experienced all conditions similar to the selected populations but were not under any conscious selection pressure for timing of emergence. Flies emerging throughout the day were used as breeding adults for the next generation for CP populations. For both PP and CP, 1200 breeding adults per replicate population, with approximately equal numbers of males and females, were maintained in Plexiglas cages with BJ medium in Petri dishes. Five days post-emergence (i.e. on the 18th day after eggs were collected), yeast–acetic acid paste was provided on a Petri dish containing BJ medium to induce greater mating and enhanced egg production. Three days later, eggs laid over a period of 3h (ZT01–04) were collected on BJ medium and approximately 300 eggs were transferred into glass vials (18cm height × 2.4cm diameter) containing 10ml of BJ medium. Forty-eight such vials per replicate were used for PP and sixteen for CP, and both populations were maintained on a 21-day non-overlapping generation cycle. This selection experiment has now been continuing for over 80 generations.

Standardization of selected stocks
Altered physiology of the parents due to environmental conditions that they experience is known to influence the phenotypes of their progeny (Prasad et al., 2001). Similarly, selection regimes experienced by the populations can potentially influence the phenotype of their progeny through non-genetic effects of the imposed selection. In our selection regimes, the timing of emergence of parents differed between populations and hence could influence the phenotype of their progeny. Therefore, in order to eliminate non-genetic parental effects and to unambiguously test the genetic differences between selected and control populations, we used one generation of common rearing conditions for selected populations. Such protocol eliminates non-genetic parental effects and is not known to affect circadian rhythms in *Drosophila*.

Adult emergence assay
Beginning at the fifth generation of selection, percentage of flies emerging during the selection window (ZT01–02), emergence waveform, gate-width of emergence, 'synchrony' and 'accuracy' of adult emergence rhythm were estimated every five to 10 generations. Percentage emergence during the selection window was expressed in terms of PP/CP emergence ratio, estimated by dividing the percentage of flies that emerged during ZT01–02 in PP by that in CP. Circadian period, 'homogeneity' and 'precision' of emergence rhythm was assayed only at the 80th generation. For adult emergence rhythm assay, eggs were collected from standardized populations and transferred at a density of approximately 300 eggs per vial into vials containing 10ml of BJ medium. These vials were kept under LD cycles and 10 such vials per population were set up for the assay. The vials were monitored regularly for emergence of the first fly and thereafter at 2h intervals for five successive days, and the number of adults that emerged in the preceding 2h duration was recorded. To have a better resolution of emergence during morning hours we monitored emergence between ZT23–04 every 1h.

Locomotor activity assay
The circadian period (τ) of locomotor activity rhythm under constant darkness (DD) and its homogeneity were estimated every 10 generations starting at the 30th generation. Daily waveform, percentage activity, synchrony in phase of entrainment and accuracy of locomotor activity rhythm under 12h:12h LD was assayed in the 70th generation. For this assay, 2-day-old virgin males were introduced individually into activity tubes and their locomotor activity behaviour was monitored for the first 10days under LD cycles and then for ~10days in DD using Drosophila Activity Monitors (TriKinetics, Waltham, MA, USA).

Measures of variance in emergence and activity rhythms
How robust a rhythm is can be assessed by the extent of its inter- and intra-individual variations. We used four separate measures of variance to characterize emergence and activity rhythms under LD (synchrony and accuracy) and DD (homogeneity and precision) (Daan and Beersma, 2002). Synchrony was estimated as the inverse of standard deviation (s.d.) of the phase relationship (ψ) between emergence (or activity rhythm) and lights-on under LD. To estimate synchrony in emergence rhythm of each replicate population, we first obtained mean ψ of emergence peak for each of the 10 vials

by averaging it across five cycles, and then estimated the reciprocal of s.d. taking 10 such average ψ values. Similarly, to estimate synchrony of activity rhythm of each replicate population, we first obtained mean ψ of morning activity peak for each of the 32 individuals by averaging it across ten cycles, and then estimated the reciprocal of s.d. taking 32 such average ψ values. Accuracy of emergence rhythm in each replicate population was estimated as the inverse of s.d. of daily ψ of emergence peak across 5 days. Similarly, accuracy of activity rhythm was estimated as the reciprocal of s.d. of daily ψ of morning activity peak across ten days. Homogeneity in τ of each replicate population was estimated as the inverse of s.d. of τ (10 vials for emergence, and 32 flies for activity). Precision of emergence or activity rhythm of each replicate population was estimated as the reciprocal of s.d. of cycle-to-cycle τ. Because all the four measures of variance are estimated as 1/(s.d. of phase or period expressed in hours), they all have a unit of h^{-1}.

Statistical analyses

The waveform of emergence and activity rhythms under LD cycles was obtained by dividing emergence or activity data collected in every reading interval (1 or 2 h, respectively) by the total number of flies or total amount of activity during one complete cycle. The τ of adult emergence and activity rhythms under DD was estimated by subjecting time series data collected for 5 days for emergence and 10 days for activity to Lomb Scargle Periodogram in ClockLab (Actimetrics, Wilmette, IL, USA). The gate-width of emergence rhythm under LD was estimated as the time interval between the start and end of emergence on each day, with start and end defined as time when emergence becomes greater than or less than an arbitrary cut-off of 5% of the total daily emergence. The emergence and activity data collected under LD and DD were analysed separately using a mixed-model ANOVA treating replicate population as random factor, and generation (G), time point (T) and population (P) as fixed factors crossed with replicates. ANOVA was followed by *post hoc* multiple comparisons using a Tukey's test. Ninety-five per cent comparison intervals [calculated using minimum significant difference in Tukey's test (Sokal and Rohlf, 1995)] were used in the figures as error bars to facilitate visual hypothesis testing (Gabriel, 1978). Thus overlapping error bars would imply that the means are not different. The generation-wise waveforms of emergence rhythm and frequency distribution of τ of activity rhythm of PP and CP populations were additionally analysed using a Kolmogorov–Smirnov two-sample test to assess whether the two waveforms were different. All statistical analyses were implemented on STATISTICA for Windows Release 5.0B (StatSoft, 1995).

RESULTS
Adult emergence rhythm
Selected populations show increased emergence during selection window

To assess the direct response to selection, we estimated PP/CP emergence ratio during the selection window (ZT01–02). PP/CP emergence ratio equal to 1 implies that emergence during the selection window from PP and CP populations are equal. With increasing generations, a greater percentage of flies began to emerge during this time in PP compared with CP, which was observed as an increase in PP/CP emergence ratio (Fig. 1). ANOVA on the PP/CP emergence ratios showed a statistically significant effect of generation (G) ($F_{8,24}$=6.11, $P<0.0002$; Fig. 1). *Post hoc* multiple comparisons using Tukey's test showed that PP/CP emergence ratio was significantly higher compared with 1 in all except the 15th,
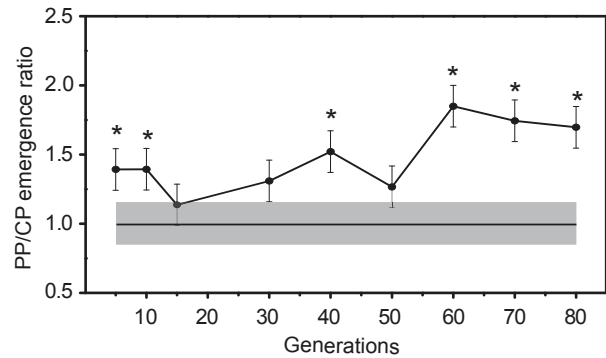

Fig. 1. Generation-wise PP/CP emergence ratio in *Drosophila melanogaster*. Generations are plotted along the *x*-axis and ratio of percentage emergence during the selection window (ZT01–02) in selected (PP) over control (CP) flies along the *y*-axis. Error bars indicate 95% confidence intervals (95% CI) around the mean for visual hypothesis testing. PP/CP emergence ratio equal to 1 implies that emergence during selection window from PP and CP populations are equal. The width of the grey box parallel to the *x*-axis represents an error bar of 95% CI around the PP/CP emergence ratio of 1. Response to selection can be seen in PP populations as an increase in the PP/CP emergence ratio. Asterisks denote statistically significant deviation of PP/CP emergence ratio from 1.

30th and 50th generations, which indicates that a significantly greater percentage of PP flies emerge during the selection window compared with CP. Percentage of CP flies emerging within the selection window did not vary significantly across generations, whereas PP flies started showing significantly higher percentage emergence during the selection window by the fifth generation (Fig. 1, Table 1, supplementary material Fig. S2a). Thus, as a signature of direct response to selection, flies emerging during the selection window increased in the selected populations.

Selected populations display altered daily emergence waveform

To study the effect of selection on the daily waveform of emergence rhythm under LD we estimated percentage of flies that emerge in 1 h bins prior to, during and after the selection window (supplementary material Fig. S3a). We observed an increase in emergence during the selection window in PP compared with CP, which reached consistent statistical levels of significance by the 40th generation. This increase was associated with a concurrent reduction in emergence prior to lights-on (ZT23–00, 40th generation onwards), before the selection window (ZT00–01, 70th generation onwards) and an increase after the selection window (ZT02–03, 40th generation onwards), which eventually resulted in the divergence of emergence waveform in PP from CP (Fig. 2, supplementary material Fig. S2b, Fig. S3a). ANOVA showed a statistically significant effect of G ($F_{8,24}$=3.01, $P<0.01$), time of the day (T) ($F_{14,42}$=132, $P<0.0001$) and population (P)×G ($F_{8,24}$=2.9, $P<0.01$), P×T ($F_{14,42}$=56.3, $P<0.0001$), G×T ($F_{112,336}$=20.4, $P<0.0001$) and P×G×T interactions ($F_{112,336}$=7.4, $P<0.0001$), while the effect of P was statistically not significant ($F_{1,3}$=2.9, $P<0.1$). *Post hoc* multiple comparisons using Tukey's test revealed that emergence in PP was significantly higher than in CP during ZT02–03 (40th generation onwards) and lower than CP during ZT23–00 (40th generation onwards) and ZT00–01 (70th generation onwards; supplementary material Fig. S2b). Kolmogorov–Smirnov's test for two sample comparison revealed that emergence waveform of PP and CP were significantly different at the 60th ($P<0.01$), 70th ($P<0.05$) and 80th ($P<0.01$) generations (Fig. 2). Consistent changes in circadian waveform of PP compared with CP, observed in

Table 1. Percentage of *Drosophila melanogaster* flies emerging during the selection window, gate-width, synchrony and accuracy of adult emergence under light:dark cycle at several assay generations

Generation	Flies emerging in selection window (%)	Gate-width (h)	Synchrony (h^{-1})	Accuracy (h^{-1})
Control populations				
05	10.1±1.3	11.8±0.7	1.6±0.5	5.2±0.7
10	11.4±1.3	11.8±0.7	2.0±0.5	4.8±0.7
15	11.9±1.3	11.9±0.7	1.6±0.5	4.3±0.7
30	8.8±1.3	12.6±0.7	2.5±0.5	6.0±0.7
40	11.5±1.3	12.6±0.7	2.4±0.5	3.8±0.7
50	11.5±1.3	12.3±0.7	2.7±0.5	5.3±0.7
60	9.5±1.3	12.3±0.7	2.1±0.5	6.0±0.7
70	12.5±1.3	11.9±0.7	1.8±0.5	5.4±0.7
80	12.7±1.3	11.6±0.7	2.7±0.5	4.8±0.7
Precision populations				
05	13.9±1.3	11.8±0.7	2.1±0.5	6.5±0.7
10	15.8±1.3	10.8±0.7	2.0±0.5	6.1±0.7
15	13.4±1.3	10.6±0.7	1.9±0.5	5.6±0.7
30	11.4±1.3	10.5±0.7	5.0±0.5	9.0±0.7
40	17.4±1.3	10.5±0.7	3.6±0.5	8.2±0.7
50	14.5±1.3	9.8±0.7	4.2±0.5	7.2±0.7
60	17.5±1.3	8.5±0.7	4.7±0.5	8.7±0.7
70	21.8±1.3	9.2±0.7	3.8±0.5	7.7±0.7
80	21.7±1.3	9.5±0.7	4.3±0.5	7.6±0.7

All values are means ± 95% confidence intervals.

successive assay generations, suggest that selection for emergence in a narrow window of time results in the evolution of daily emergence waveform.

Selected populations have reduced emergence gate-width

With increasing generations, gate-width of emergence in PP became gradually narrower and by the 80th generation it was reduced to 9.5±0.7h (mean ± 95% CI), while it remained more or less unchanged at 11.6±0.7h in CP (Fig. 3A, Table 1). ANOVA showed a statistically significant effect of P ($F_{1,3}$=492.5, $P<0.0002$), G ($F_{8,24}$=5.09, $P<0.0008$) and P×G interaction ($F_{8,24}$=3.77, $P<0.005$). *Post hoc* multiple comparisons using Tukey's test revealed that in the 30th generation onwards, gate-width of PP became significantly narrower than CP, which suggests that selection for emergence in a narrow window of time results in a significant reduction in gate-width of emergence rhythm.

Selected populations display enhanced synchrony in emergence peak

Synchrony of emergence rhythm in each replicate population was estimated as the reciprocal of s.d. of ψ across 10 vials. By the 30th generation PP flies started exhibiting enhanced synchrony in emergence rhythm compared with CP flies, which was consistently seen in all the assay generations (Fig. 3B, Table 1). ANOVA showed a statistically significant effect of P ($F_{1,3}$=25.33, $P<0.01$), G ($F_{8,24}$=2.04, $P<0.02$) and P×G interaction ($F_{8,24}$=2.23, $P<0.03$). *Post hoc* multiple comparisons using Tukey's test revealed that PP flies display enhanced synchrony in emergence rhythm from the 30th generation onwards, which suggests that selection for emergence in a narrow window of time yields populations with coherent emergence rhythm.

Selected populations display enhanced accuracy in emergence rhythm

Accuracy of emergence rhythm was estimated as the inverse of s.d. of daily ψ. By the 30th generation of selection, accuracy of emergence rhythm of PP increased compared with CP (Fig. 3C, Table 1). ANOVA showed a statistically significant effect of P

($F_{1,3}$=38.5, $P<0.008$), G ($F_{8,24}$=2.65, $P<0.03$) and P×G interaction ($F_{8,24}$=7.5, $P<0.03$). *Post hoc* multiple comparisons using Tukey's test revealed that in the 30th generation onwards, accuracy of emergence rhythm in PP was significantly greater than CP, which suggests that selection for emergence in a narrow window of time makes emergence rhythm more stable under LD.

Selected populations have faster running clocks

By the 80th generation, τ of emergence rhythm in PP became significantly shorter than CP (Fig. 3D). ANOVA followed by *post hoc* multiple comparisons using Tukey's test revealed that τ of PP was significantly shorter than CP ($F_{1,3}$=19.3, $P<0.02$; Fig. 3D). PP flies also exhibited enhanced amplitude of emergence rhythm compared with controls under DD (supplementary material Fig. S3b). This suggests that selection for emergence in a narrow window of time results in populations with a faster clock period.

Selected populations display increased homogeneity in clock period

To study the effect of selection on inter-vial variance we examined homogeneity in τ of emergence rhythm at the 80th generation. Homogeneity in τ in each replicate population was calculated as the inverse of s.d. of τ across all 10 vials. PP exhibited increased homogeneity in τ compared with CP. ANOVA followed by *post hoc* multiple comparisons using Tukey's test showed that homogeneity in τ of PP was significantly greater compared with CP ($F_{1,3}$=43.12, $P<0.007$; Fig. 3E), suggesting that selection for emergence in a narrow window of time makes circadian clocks of selected populations more homogenous.

Selected populations display marginally enhanced precision in emergence rhythm

To study the effect of selection on clock precision, we assayed precision of emergence rhythm under DD at the 80th generation. Precision of emergence rhythm was estimated as the inverse of s.d. of daily period. Although PP exhibited enhanced precision in emergence rhythm, this difference was statistically not significant ($F_{1,3}$=2.37, $P=0.09$; Fig. 3F), which suggests that selection for
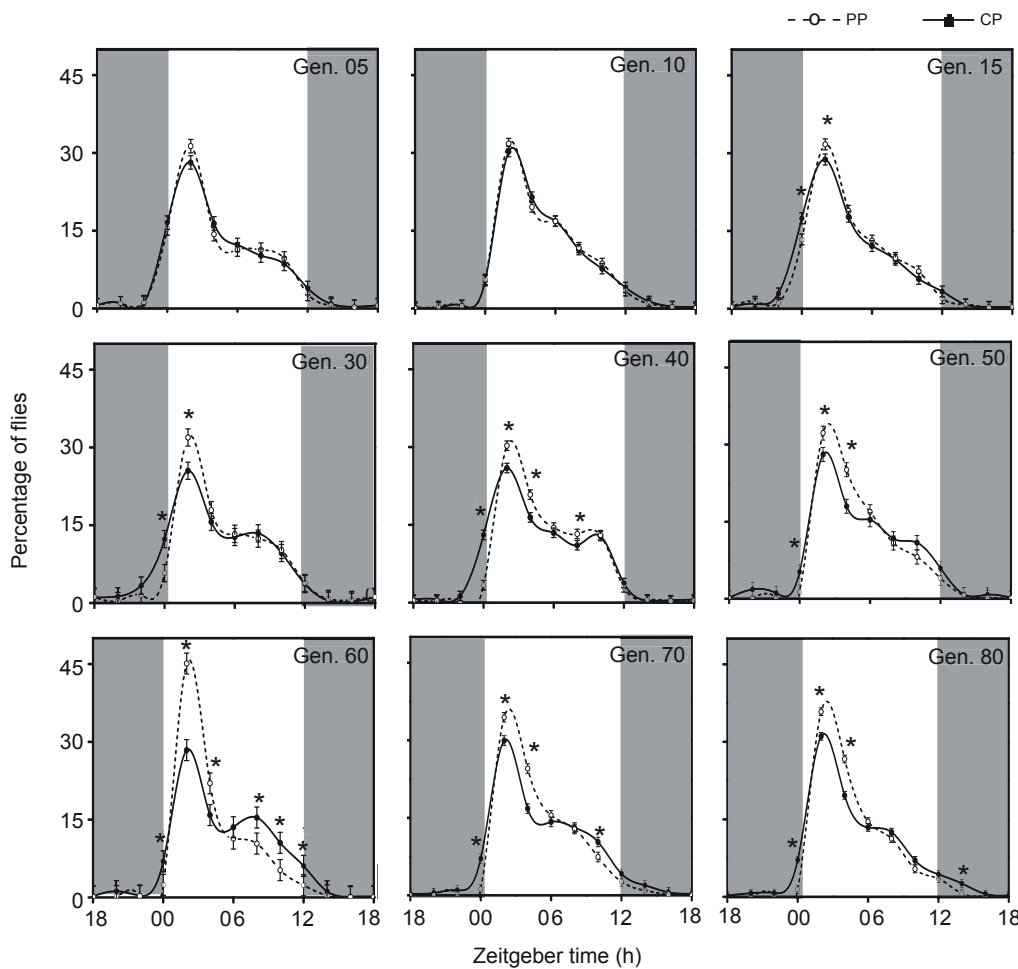
Fig. 2. Waveform of adult emergence rhythm in selected and control populations. Percentage of flies that emerged in 2 h bins are plotted along the *y*-axis and zeitgeber time in hours along the *x*-axis. The shaded areas represent duration of darkness in the light/dark (LD) cycles and empty areas the duration of light. Adult emergence rhythm assays were performed under LD cycles every five to 10 generations starting at the fifth generation. With increasing generations the overall waveform of emergence is gradually altered with the emergence of a prominent morning peak and reduction of evening peak. Error bars indicate 95% CI around the mean for visual hypothesis testing. Asterisks denote statistically significant differences between PP and CP.

emergence in a narrow window of time improves the precision of clocks underlying emergence rhythm.

Activity rhythm

To study if effects of selection for emergence in a narrow window of time observed at the populational level rhythm percolate down to individual level circadian rhythms, we assessed locomotor activity rhythm of selected and control flies under LD and DD.

Selected populations display altered daily activity waveform

By the 70th generation of selection, total within selection window and daytime activity of PP were enhanced and night-time activity reduced compared with CP (Fig. 4A,B). Furthermore, morning activity peak of PP was significantly greater, and these flies were more active during the morning compared with CP flies (Fig. 4A,B). ANOVA on hourly collected activity data showed a statistically significant effect of P ($F_{1,3}$=36.9, P<0.009), T ($F_{23,69}$=210, P<0.0001) and P×T interaction ($F_{23,69}$=9.3, P<0.0001). *Post hoc* multiple comparisons using Tukey's test revealed that activity of PP was significantly higher than CP during early morning (ZT01–02) and lower during late night (ZT19–21; Fig. 4A,B). This suggests that selection for emergence in a narrow window of time results in altered circadian waveform of activity rhythm.

Selected populations show increased synchrony in activity rhythm

Synchrony of activity rhythm in each replicate population was estimated as the reciprocal of s.d. of ψ across 32 individual flies. By the 70th generation, flies from PP exhibited significantly

enhanced synchrony in activity rhythm compared with those from CP (Fig. 4C). ANOVA followed by *post hoc* comparisons using Tukey's test revealed that synchrony in activity rhythm of PP was significantly higher than CP ($F_{1,3}$=21.2, P<0.01; Fig. 4C), which suggests that selection for emergence in a narrow window of time makes flies more coherent.

Selected populations display enhanced accuracy in activity rhythm

Accuracy was estimated as the reciprocal of s.d. of daily ψ of morning activity peak across 10 days. By the 70th generation of selection, PP flies started exhibiting significantly higher accuracy in activity rhythm compared with CP flies (Fig. 4D). ANOVA followed by *post hoc* comparisons using Tukey's test revealed that accuracy of activity rhythm in PP was significantly greater than that in CP ($F_{1,3}$=36.3, P<0.009; Fig. 4D), which suggests that selection for emergence in a narrow window of time results in increased day-to-day stability in activity rhythm.

Selected populations have faster running clocks

To study if selection for emergence in a narrow window of time has any impact on the clock period, we assayed activity rhythm of PP and CP flies under DD. ANOVA showed a statistically significant effect of P ($F_{1,3}$=33.27, P<0.01), G ($F_{4,12}$=9.92, P<0.003) and P×G interaction ($F_{4,12}$=38.18; P<0.0001). *Post hoc* multiple comparisons using Tukey's test revealed that in the 50th generation onwards, τ of PP was significantly shorter than CP (Fig. 5A). A significantly greater proportion of PP flies began displaying rhythmicity with τ significantly shorter than CP (Fig. 5B). The Kolmogorov–Smirnov
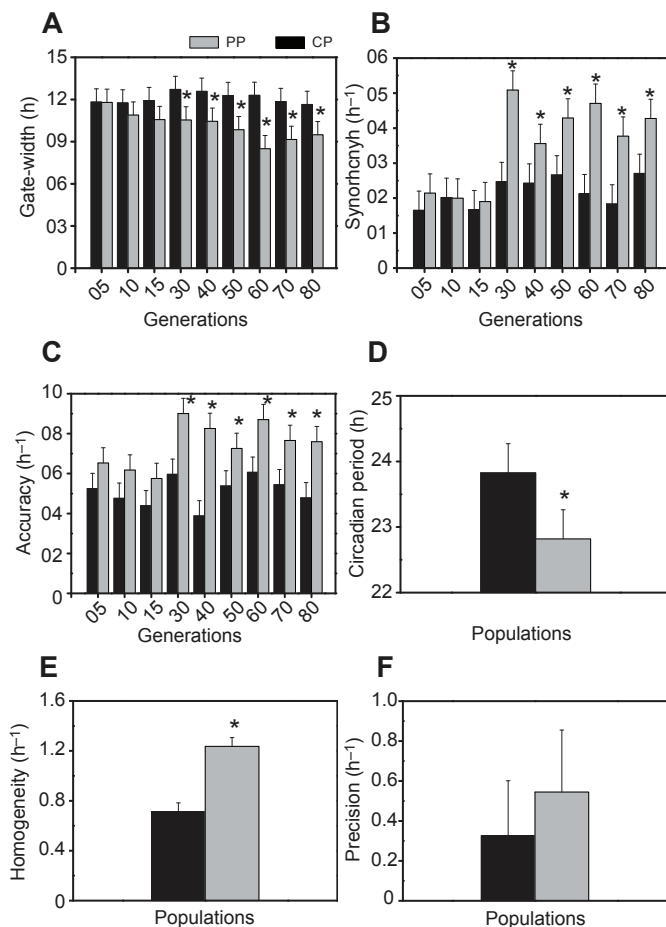
Fig. 3. (A) Gate-width of adult emergence rhythm in generation-wise assays. Generation is plotted along the x-axis and gate-width along the y-axis. With increasing generations gate-width became narrower in selected (PP) populations compared with controls (CP). (B) Synchrony of adult emergence rhythm under LD cycles in generation-wise assays. Generation is plotted along the x-axis and synchrony along the y-axis. From the 30th generation onwards, synchrony in emergence rhythm enhanced in PP compared with CP. (C) Accuracy of adult emergence rhythm in generation-wise assays. By the 30th generation PP evolved greater accuracy in their adult emergence rhythm compared with CP. (D) Circadian period (τ) of adult emergence rhythm in PP and CP. The τ of adult emergence rhythm of PP was significantly shorter than CP. (E) Homogeneity in τ of adult emergence rhythm. PP exhibit enhanced homogeneity in τ compared with CP. (F) Precision in τ of adult emergence rhythm in PP and CP populations. Error bars indicate 95% CI around the mean for visual hypothesis testing. Asterisks denote statistically significant differences between PP and CP.

two-sample test also revealed that the distribution of τ in the 50th, 60th and 70th generation assays was significantly advanced in PP relative to CP ($P<0.001$; Fig. 5B). These results suggest that selection for emergence in a narrow window of time yields flies with faster running clocks.

Selected populations display increased homogeneity in clock period

Homogeneity of period in each replicate population was calculated as the inverse of s.d. of τ across all 32 individuals. The homogeneity in τ was significantly increased in PP compared with CP (Fig. 5C). ANOVA showed a statistically significant effect of P ($F_{1,3}=28.4$, $P<0.01$), G ($F_{4,12}=5.4$, $P<0.01$) and P×G interaction ($F_{4,12}=4.38$, $P<0.01$). *Post hoc* multiple comparisons using Tukey's test revealed
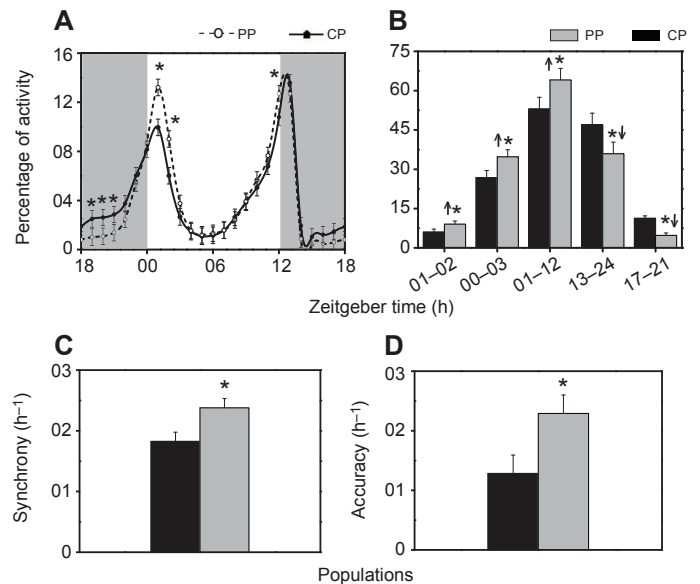

Fig. 4. Flies from selected populations exhibit enhanced activity during selection window. Activity profiles of selected (PP) and control (CP) populations under light/dark (LD) cycles. (A) The percentage of activity averaged over 10 successive cycles is plotted along the y-axis and zeitgeber time in hours along the x-axis. An overall increase in daytime activity and reduction in night-time activity can also be seen. (B) Percentage activity during selection window (ZT01–02), morning peak (ZT00–03), daytime (ZT01–12) and night-time (ZT13–24 and ZT17–21) in flies from selected and control populations. Flies from PP populations were more active than those from CP populations. (C) Synchrony in the phase of morning activity peak under LD cycles. PP flies exhibited enhanced synchrony in the phase of morning activity peak. (D) Accuracy in the morning activity peak of activity under LD cycles. PP flies had enhanced accuracy in activity rhythm compared with CP flies. Error bars indicate 95% CI around the mean for visual hypothesis testing. Asterisks denote statistically significant differences between PP and CP.

that with increasing generations, homogeneity in τ began to increase in PP compared with CP, and it eventually became significantly higher by the 50th generation (Fig. 5C), which suggests that selection for emergence in a narrow window of time makes flies homogenous in terms of their clock period.

Selected populations evolve precise circadian clocks

Precision was estimated as the inverse of s.d. of daily τ of activity rhythm across 10 cycles. PP flies exhibited enhanced precision in activity rhythm compared with CP (Fig. 5D). ANOVA revealed a statistically significant effect of P ($F_{1,3}=13.03$, $P<0.03$); however, the effect of G ($F_{4,12}=1.38$, $P=0.20$) and P×G interaction was statistically not significant ($F_{4,12}=1.52$, $P=0.20$). This suggests that selection for emergence in a narrow window of time yields populations with precise circadian clocks.

DISCUSSION

We examined the effect of selection for adult emergence in a narrow window of time on circadian rhythms in four large outbred populations of *D. melanogaster*. With increasing generations, more and more flies started emerging during the selection window; as a result, the emergence waveform of selected populations diverged from controls. From the generation-wise emergence profiles it appears that increment in the number of flies emerging during the selection window, particularly during early generations of selection,
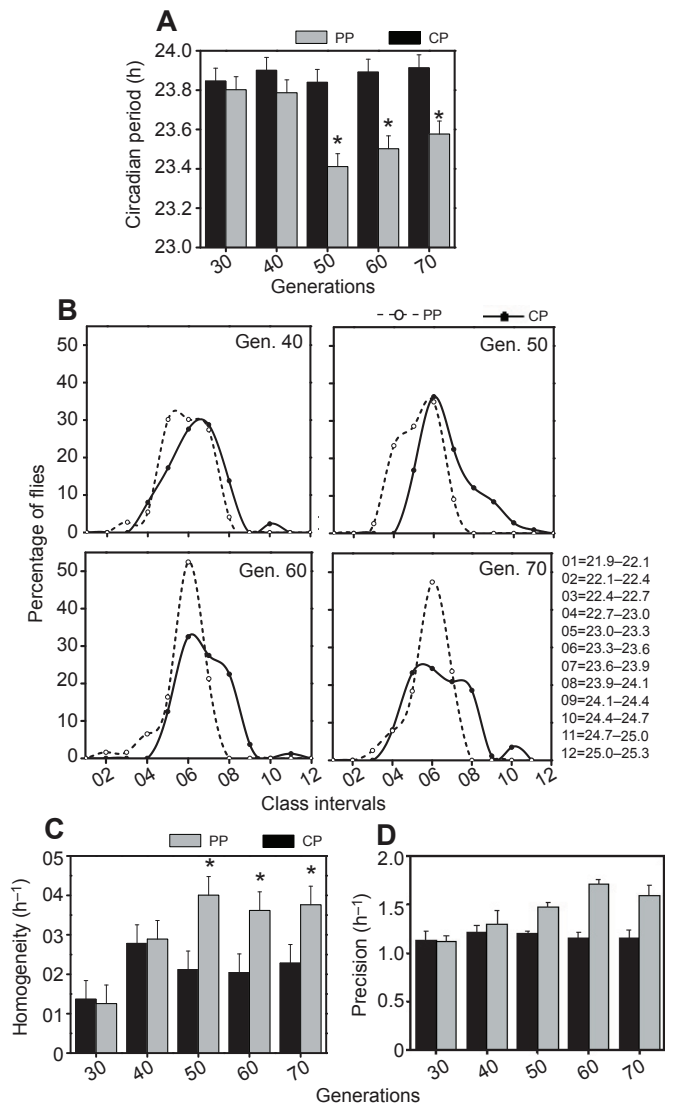
Fig. 5. (A) Circadian period (τ) of activity rhythm of selected (PP) and control (CP) flies. τ of activity rhythm in PP and CP flies is plotted along the *y*-axis and generation along the *x*-axis. Flies from PP populations had significantly shorter τ compared with those from CP populations. (B) Frequency distribution of τ of PP and CP populations. Class interval of τ (h) is plotted along the *x*-axis and percentage of flies along the *y*-axis. The class intervals are defined in the grid to the right of the figure. By the 60th generation, ~50% flies from PP had shorter τ with a narrower distribution between 23.3 and 23.6 h, while in CP populations the distribution was much broader and included longer τ. (C) Homogeneity in τ of PP and CP populations. With increasing generation, homogeneity in τ enhanced in PP populations. (D) Precision in τ of PP and CP populations. PP flies displayed enhanced clock precision compared with CP flies. *Post hoc* multiple comparisons could not be carried out because the P×G interaction was statistically not significant. Therefore, error bars used in this panel are s.e.m. Asterisks denote statistically significant differences between PP and CP.

was at the cost of flies emerging in anticipation to lights-on and/or selection window, and in subsequent generations those with propensity to emerge later in the day began to contribute (supplementary material Fig. S2b). As shown by a significant reduction in gate-width, increase in emergence peak and PP/CP emergence ratio in the selected populations, selection seems to have acted against the two extremities of emergence distribution,

favouring flies that emerge during the peak emergence time (Fig. 2). This suggests that populations subjected to selection for emergence in a narrow window of time experience stabilizing selection, where selection seems to act against the two extremities of the adult emergence waveform. These findings are consistent with those of previous studies, which showed that quantitative traits experiencing stabilizing selection do not show any change in the mean value, but undergo depletion in genetic variation (Barton and Keightley, 2002; Hurst, 2009).

Adult emergence rhythm is masked by light, as seen in terms of sudden increase in emergence immediately (within 10 min) after lights-on (McNabb and Truman, 2008). To avoid selecting flies that emerge in startle response to lights-on, we positioned our selection window during ZT01–02. The fact that emergence profile of selected populations under DD displayed higher amplitude compared with controls, confirmed that the response to selection for emergence in a narrow window of time is primarily mediated through changes in the endogenous circadian clocks and not due to startle effect of emergence to lights-on. However, the fact that amplitude of emergence in the first cycle of DD is enhanced does not necessarily imply that the period is shortened. Although changes in emergence and activity waveforms of selected populations were primarily due to altered circadian pacemakers, it seems quite plausible that several neural and endocrinal events, participating in timing the response of the clock's output signal, may also be involved. Such mechanisms might include circadian-regulated release of neuropeptide eclosion hormone, which is known to play a significant role in the regulation of gating in adult emergence rhythm (Truman, 2005).

One of the striking features of the impact of selection for emergence in a narrow window of time is that until the 40th generation, it primarily alters the emergence waveform without affecting the clock period, and then suddenly the average period shortens. This is probably due to the fact that direct response to selection is visible within a few generations of selection when processes downstream to the circadian pacemaker start responding to selection. In contrast, it took over 40 generations of selection to produce a statistically significant effect in the circadian pacemaker, which can be seen as a slow response to selection. However, the most interesting result of this selection is that it also resulted in an overall change in emergence waveform, shortening of clock period and in a consistent increase in accuracy, synchrony, homogeneity and precision of emergence and activity rhythms.

We observed that flies selected for emergence in a narrow window of time evolve shorter period of emergence and activity rhythms. This is consistent with the notion that phase of the entrained rhythm relative to a reference phase in the environmental cycle is a function of two basic properties of the circadian oscillator–circadian period and phase response curve (Pittendrigh and Daan, 1976; Roenneberg et al., 2003). A shorter circadian period is associated with an earlier phase of entrainment, and longer period with later circadian phase. Thus for a given phase response curve, phase of entrainment of rhythms with shorter period would occur earlier than those with longer period. Since the phase of selection window in our study was earlier than the mean emergence time of flies, it is expected that such selection would favour individuals with shorter circadian period based on the relationship between phase of entrainment and clock period (Pittendrigh and Daan, 1976; Hamblen-Coyle et al., 1992). Our finding is consistent with those of Kumar et al. (Kumar et al., 2007a), who showed that circadian period of emergence rhythm became shorter in populations that were selected to emerge in the morning, and longer in populations selected to emerge in the evening. In the study of Kumar et al. (Kumar et al., 2007a) the

morning selection window spanned ZT21–01, whereas in our present study the phase of selection window was later (ZT01–02). Considering the emergence waveform of CP, this selection window coincides with the morning hours, and hence it is expected that such selection would favour individuals with shorter circadian period.

The synchrony in phase of entrainment and homogeneity of clock period in the selected populations was significantly enhanced, which suggests that selection for emergence in a narrow window of time makes flies more similar to each other in terms of their circadian clocks. In addition, accuracy of emergence and activity rhythms under LD, and precision in DD was also increased, which suggests that selected populations evolve accurate and precise circadian clocks. Furthermore, given that the amplitude of emergence and activity rhythms is also increased significantly in the selected populations, our results suggest that selection for emergence in a narrow window of time results in the evolution of robust and precise circadian clocks. Previous studies in *D. melanogaster* have shown that proteins of some core clock genes go through a series of phosphorylation steps, which are critical for the maintenance of precision of circadian clocks (Bae and Edery, 2006). The period of circadian cycle is also dependent upon the efficiency of a cocktail of kinases, which determine its day-to-day precision (Kloss et al., 1998; Price et al., 1998). In the light of the role of kinases involved in the molecular clockwork, it is likely that post-translational mechanisms involved in the circadian regulation may have diverged in the selected flies.

All our assays were performed under identical environmental conditions, which ensured that direct response to selection in the form of consistent changes in circadian rhythms in four replicate large outbred selected populations relative to their ancestral controls, are the result of adaptive evolution and not due to inbreeding or random genetic drift. Intense response to selection with rapid decline in phenotypic variance is likely to be due to strong stabilizing selection pressure that populations experience over generations (Hunt et al., 2007). In our study also, enhanced synchrony and homogeneity in circadian rhythms is likely to be due to decline in underlying genetic variation. Further genetic analyses of these populations are likely to help us unravel the possible role of additive, dominance and maternal effects, which may contribute to their circadian architecture.

In summary, our study showed that selection for emergence in a narrow window of time resulted in reduced inter- and intra-individual variance in circadian clocks underlying emergence and activity rhythms in the fruit fly *D. melanogaster*. Selection pressure, which was applied on the populational rhythm, not only makes the rhythm more coherent and precise, it also trickled down to rhythms at the individual level. This is shown by enhanced synchrony, accuracy, homogeneity and precision in locomotor activity rhythm of the selected flies. This suggests that stringent selection for emergence to occur in a narrow window of 1h close to emergence peak results in enhanced accuracy and precision of circadian clocks. This, to the best of our knowledge, is the first study of its kind demonstrating that stabilizing selection for timing of adult emergence results in reduced inter- and intra-individual variance in circadian rhythms.

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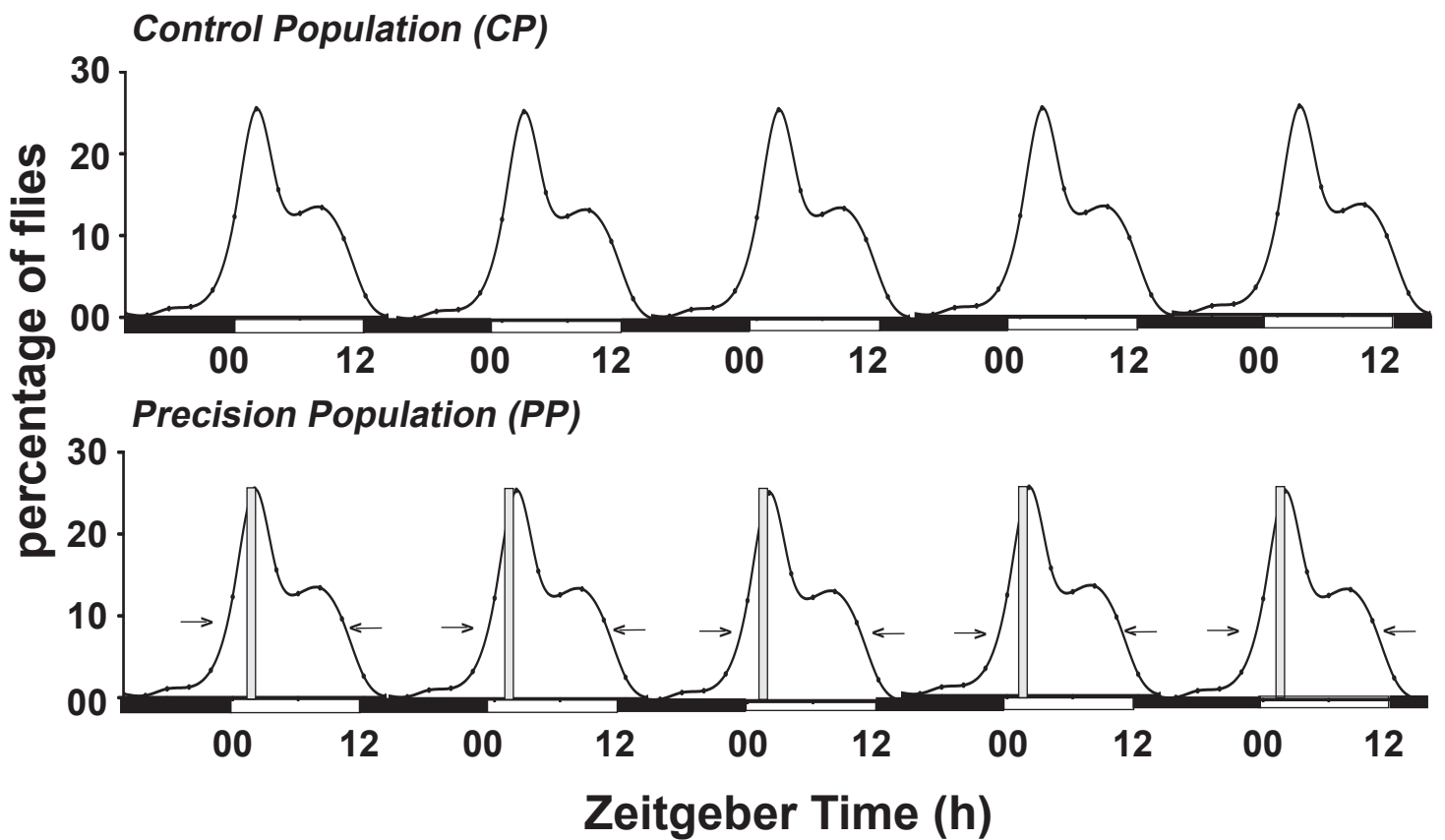
Figure S1. Schematics of the laboratory selection protocol.

The selection experiments for narrow gate of adult emergence were carried out under 12:12 h light/dark (LD) cycles where lights came on at 10:00 h and went-off at 22:00 h. Zeitgeber Time 00 (ZT00) denotes lights-on and ZT12 lights-off under LD cycles. Four baseline populations maintained under LD cycles were used to derive four Precision Populations by selecting for flies emerging between ZT01 and ZT02. Therefore, for four selected Precision populations (PP_{1-4}) only flies that emerged within this narrow window of time were allowed to be the part of the breeding pool for the next generation. Four Control Populations (CP_{1-4}) were also maintained in a similar manner, except that they were not subjected to any conscious selection pressure and flies emerging throughout the day were made to be the part of the breeding population for the next generation. Selection windows (ZT01-02) are shown in the lower panel as grey boxes. The horizontal filled and empty bars represent the duration of dark and light phases of the LD cycles. Zeitgeber Time (hours) is plotted along the *x*-axis and percentage of flies emerging in an interval of 2 h along the *y*-axis.
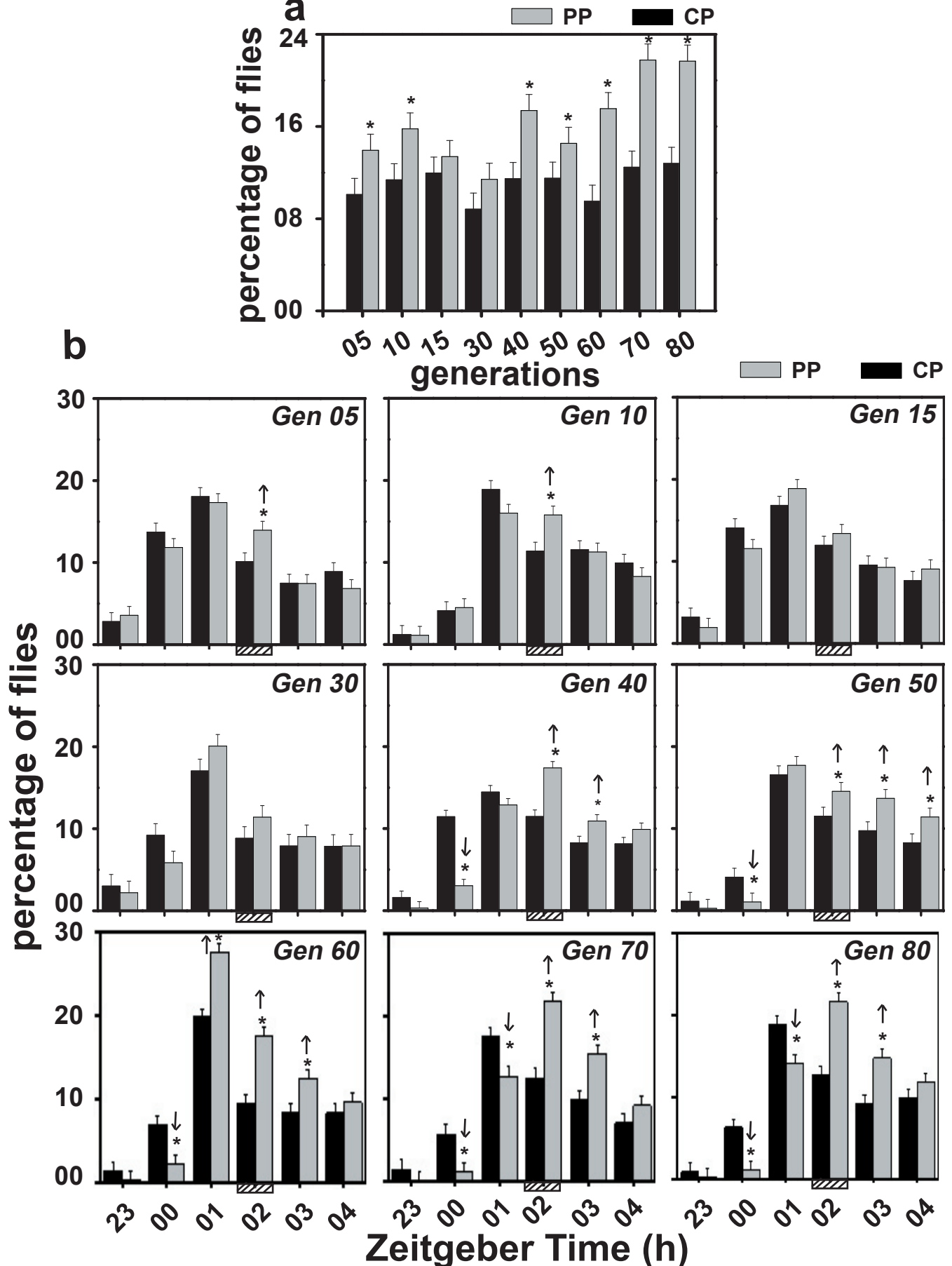
Figure S2. (a) Percentage of flies emerging during the selection window (ZT01-02). Generations are plotted along the *x*-axis and percentage of flies emerging during the selection window (ZT01-02) along the *y*-axis. With increasing generations, increased percentage of flies emerged during the selection window in PP compared CP. (b) Primary peak of emergence of PP and CP populations is shown in 1 h resolution. Concurrent to increase in the percentage of flies emerging during selection window there is a decrease in the percentage emergence prior to the selection window, with reduced anticipation to lights-on. Upward arrows denote increase and downward arrows decrease in the percentage emergence in PP populations in 1 h interval. Shaded box on the *x*-axis denotes the selection window. Asterisks denote statistically significant difference between PP and CP. All other deatils are same as in figure 1.
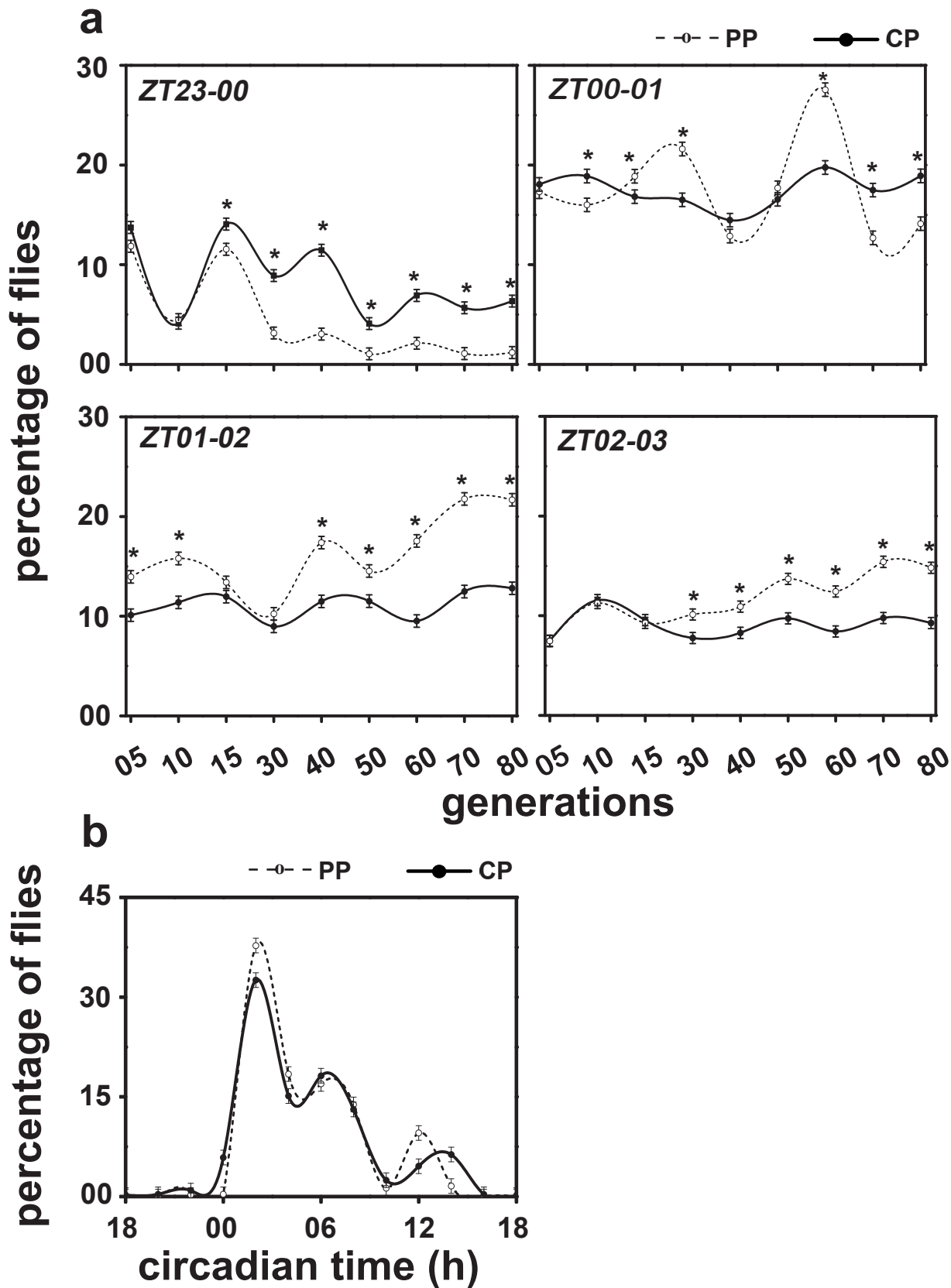
Kannan et al. Figure S2

Figure S3. (a) Changes in the percentage of flies emerging prior to and after the selection window. Percentage of adult flies emerging between ZT23-03. Generations are plotted along the *x*-axis and percentage of flies emerging during 1 h interval along the *y*-axis. The percentage of flies emerging prior to lights-on (ZT23-00) is reduced in the selected (PP) population from 15 generation onwards. The percentage of flies emerging 1 h before the selection window (ZT00-01) is gradually increased by the 60th generation and decreased by the 70th generation. Percentage of flies emerging 1 h after the selection window (ZT02-03) is gradually increased from 30th generation onwards. Asterisks denote statistically significant difference between PP and CP. (b) First cycle of adult emergence rhythm waveform under DD. PP populations exhibit enhanced peak of emergence compared to Controls. All other details same as in Figure 1.
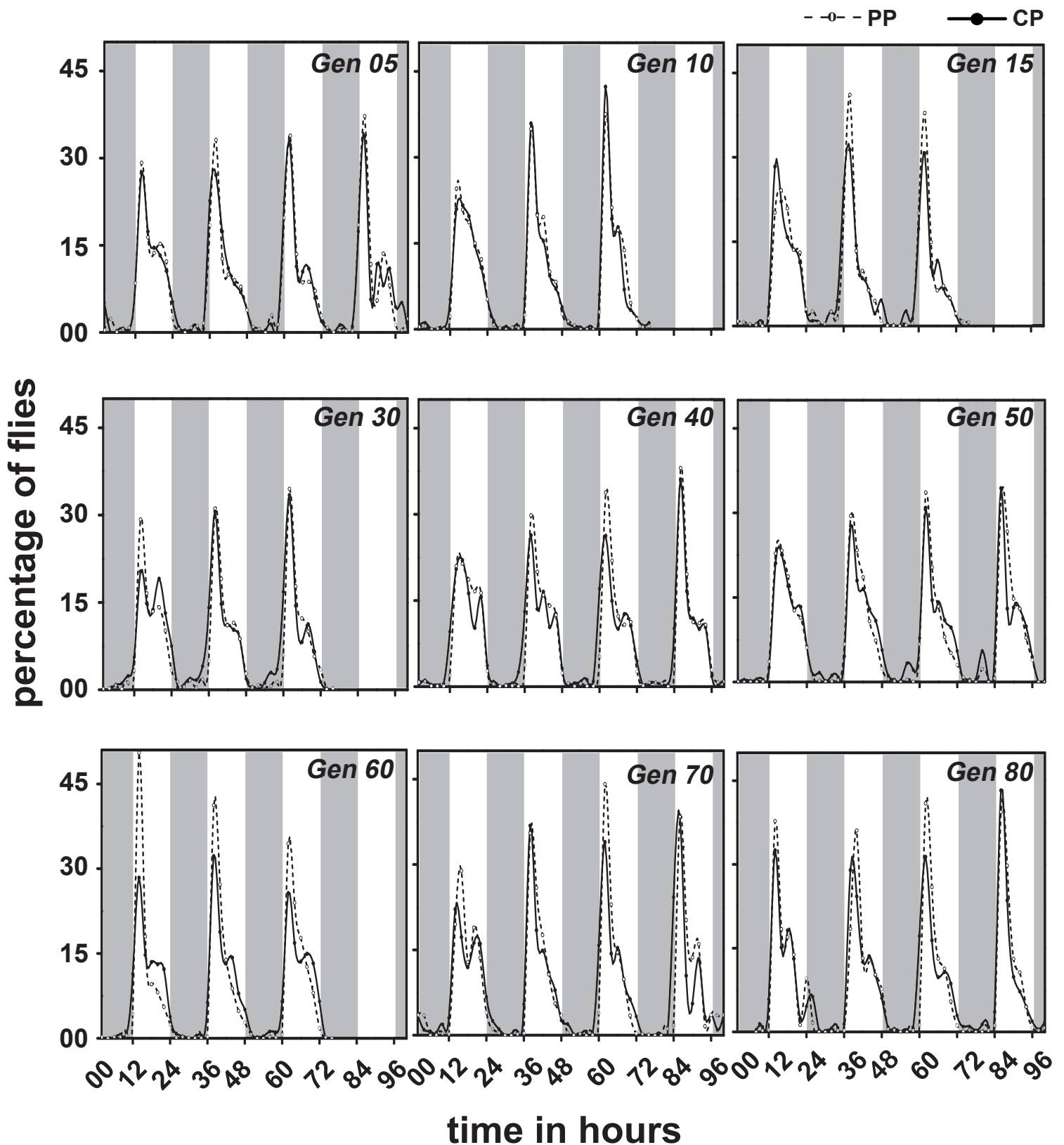
Figure S4. Waveform of adult emergence for four successive cycles.

Percentage of flies emerged 2 hourly bins over 3-4 successive cycles are plotted along the *y*-axis and time in hours along the *x*-axis. Shaded areas represent the dark phase of LD cycles and empty areas light phase. With increasing generations, waveform of adult emergence is altered in selected populations compared to controls.