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RESEARCH ARTICLE

A model based on oscillatory threshold and build-up of a developmental substance explains gating of adult emergence in *Drosophila melanogaster*

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SUMMARY

Adult emergence (eclosion) of fruit flies *Drosophila melanogaster* under constant laboratory conditions follows a circadian pattern with bouts of eclosion recurring at approximately 24 h intervals. Under periodic light:dark (LD) cycles, adults emerge only during a specific time of the day followed by little or no emergence for the rest of the day. This phenomenon is therefore equated to a gate of emergence that, when open, allows adults to emerge and when closed, no emergence takes place. In this study, we attempt to understand the mechanism underlying adult emergence rhythm in *D. melanogaster* using a model based on interplay between developmental and circadian clock systems. The model is composed of an oscillatory threshold of a substance that builds up during pre-adult development. Computer simulations based on this model enabled us to make specific predictions about the 'gate width' of the adult emergence rhythm under conditions of fast/slow pre-adult development and short/long circadian periods, which we subsequently tested empirically. The main predictions from the simulations are: (1) flies with faster development have greater gate width and *vice versa*, and (2) flies with faster circadian clocks have shorter gate width and *vice versa*. To empirically validate these predictions, we carried out experiments on *D. melanogaster* populations known to have fast/slow pre-adult development, short/long circadian periods and narrow/wide gate width. Additionally, we manipulated the rate of pre-adult development of the above flies by increasing/decreasing ambient temperature to further examine the influence of developmental rates on gate width of adult emergence rhythm by a complementary approach. The results show that gate width is greatly influenced by the duration of pre-adult development and the length of circadian cycles. This suggests that the adult emergence rhythm of *D. melanogaster* may be based on mechanisms involving oscillatory threshold and build-up of a developmental substance.

Key words: *Drosophila*, adult emergence, development, circadian, gating, simulation.

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INTRODUCTION

The *Drosophila* adult emergence (eclosion) rhythm is amongst the best studied population-level rhythms, and several early experiments have shown that although emergence is an event that occurs only once in the lifetime of an organism, its timing is controlled by an ongoing oscillation in the developmental process (Pittendrigh and Skopik, 1970). Adult emergence in a population of flies raised under light:dark (LD) cycles is found to be rhythmic, with well-defined peaks occurring close to lights on. This rhythm free-runs in constant darkness (DD), displaying a period close to 24 h, provided the population of flies is exposed to a stimulus during any stage of their development, in the form of transfers from constant light (LL) to DD, from LD to DD, or even in the form of a brief non-recurrent light pulse (Saunders, 2002). The timing of the adult emergence rhythm depends upon the developmental state of the flies, the phase, the period of their developmental and circadian clocks, and the ambient environmental condition. A certain interval of the day acts as the 'forbidden zone' for emergence, and a brief interval during early morning as the 'allowed zone' or the 'gate' of adult emergence. It is believed that a continuously consulted circadian clock 'reads' the developmental states of the flies and only those flies that are mature enough to emerge during the gate are allowed to come out of the pupae, whereas others who mature after the gate has closed are forced to wait until the next gate opens (Pittendrigh and Skopik, 1970; Qiu and Hardin, 1996).

In the present study, we propose and empirically test a model of the *Drosophila* emergence rhythm in order to understand the nature of its gating. Our model is adopted from an earlier model proposed by Winfree (Winfree, 1980), who used it to explain cyclic sleep–wake behaviour in humans and subsequently implemented it in the study of the *Drosophila* emergence rhythm. Winfree's model is based on linear accumulation of a sleep substance, which promotes wakefulness. The model assumes that an individual wakes up from sleep when this substance has accumulated beyond a certain threshold. The rhythmicity in sleep and wakefulness is simulated by proposing that the threshold necessary for waking up from sleep is oscillatory in nature, or that this required threshold varies periodically with the time of the day. We considered the process of a human waking up from sleep and a fruit fly emerging from its pupal case to be functionally equivalent. This enabled us to adopt Winfree's (Winfree, 1980) model, wherein we incorporated a developmental substance in the fly instead of a sleep substance to elucidate emergence rhythm. We built our model with two key components, one that involves pre-adult development and the other,

circadian clocks. We followed this up using the model to simulate the emergence rhythm of *Drosophila melanogaster* Meigen 1830. Our model takes note of the fact that circadian clocks control the emergence rhythm by assuming that the threshold level of a developmental substance oscillates, thus adding a mechanistic touch to the idea of the opening and closing of the gate. In essence, we chose an oscillatory threshold model because it may reveal the manner in which circadian clocks gate adult emergence rhythm by linking it with pre-adult development of the fly seamlessly to produce the rhythm. The simulations of the model show that gate width of adult emergence is greater in populations that develop faster than in those which develop slower. It further predicts that gate width is greater in flies with a longer circadian period than in those with a shorter period.

MATERIALS AND METHODS

The model

Our model is composed of a two-step control of the timing of adult emergence. One is exerted by development, as emergence can occur only if the pre-adult development of the fly is complete. The other is exerted by circadian clocks, as it is known that emergence is a clock-controlled process. We incorporated the developmental control into the model by considering a substance '*X*' that builds up in the fly as development proceeds. When the level (concentration) of *X* crosses a particular threshold, a series of steps are initiated that result in the emergence of the adult fly from its pupal case. To integrate circadian clock control into the model, we propose that threshold of *X* that is necessary for the fly to emerge as an adult is a periodic function of the time of day. In other words, at different times of the day, flies would require different levels of *X* to be able to eclose. To facilitate simulation of the model, we assumed that the accumulation of *X* begins as soon as a fly pupates. Because the *D. melanogaster* emergence rhythm is normally observed only in a mixed-age population, our model assumes that different individuals in the population under consideration pupate at different times. Consequently, as all the individuals in a population do not pupate at the same time, accumulation of *X* in different individuals in the population would start at different times. Further, we assume that inter-individual variance in the rate of accumulation of *X* is negligibly small, or that *X* accumulates at the same rate in every individual.

To study the dynamics of the model, we looked at the periodic function representing oscillation of the threshold of *X* (Fig. 1). It is evident that because the threshold function is periodic, it necessarily increases for a part of the day and then decreases during the rest of the day. We examined this in conjunction with accumulation of *X*. If the increasing portion of the threshold function rises faster than the rate of accumulation of *X*, then the level of *X* would cross its threshold only when the threshold function starts decreasing. The level of *X*, therefore, would not be able to cross the threshold during the increasing portion as the higher rate of increase of the threshold function would ensure that the accumulation function does not intersect it. As specified previously, emergence of an adult can occur only when the level of *X* crosses its threshold value. Consequently, this ensures that adults are able to emerge out of pupae only during that part of the day when the threshold of *X* is falling. Adult emergence thus becomes restricted to only a certain part of the day. The model is illustrated in an illustrative example in Fig. 1. In this example, to keep the model simple and easy to program on a computer, we assumed that *X* accumulates linearly as development proceeds and its threshold oscillates sinusoidally with a mean of 5.0 and amplitude of 1.0. When the level of *X* in a fly reaches the threshold, it emerges out of its pupal case and *X* is reset to –1, to indicate an emergence event in the program. Bouts of emergence are separated by intervals of time when no emergence occurs. In this case, bouts of adult emergence are found to occur at every 24 h interval, because the threshold itself oscillates with a period of 24 h. In the simulations of the model, we have fixed the phase and shape of oscillations (Fig. 1). This was done to keep the simulations simple, but this is not a natural situation because it is well known that individuals (in the present study, populations) do have a range of phase and speed of circadian clocks even under LD conditions. So, even though flies may complete development at the same time, the timing of their emergence may not be same. However, if our specific simulation of the oscillatory threshold model, with its simplistic assumptions, is able to capture features of real fly populations and thereby garner evidence for the model, a more realistic simulation will be able to do the same.

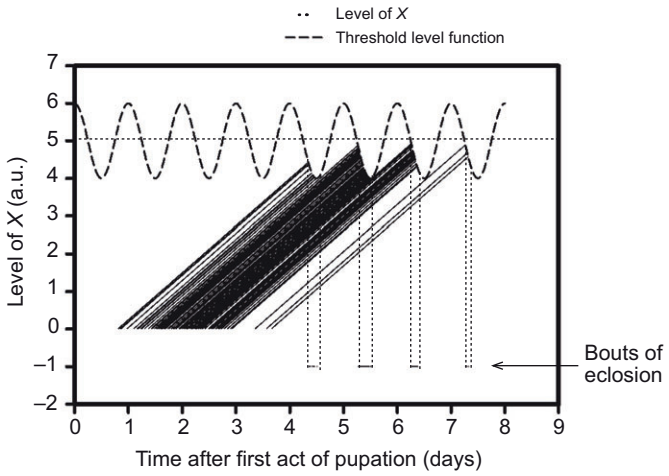

Fig. 1. Implementation of developmental and circadian clock controls of adult emergence in a computer simulation resulting in gating of adult emergence in *Drosophila melanogaster*. An adult fly emerges out of the pupa when the level of substance *X* exceeds the oscillating threshold. Once an adult fly emerges, its level of *X* is reset to –1. Bouts of emergence (which, as shown, happen when the level of *X* in an individual reaches the threshold) can be seen separated by time intervals during which no emergence occurs; thus, the model is able to capture the phenomenon of gating of adult emergence of flies. This specific simulation assumes that *X* accumulates linearly in the fly and its threshold oscillates sinusoidally with a mean of 5.0 and amplitude of 1.0.

Simulations of the model

As specified previously, we assume that the fly population under consideration has a mixed-age distribution with a constant rate of accumulation of the developmental substance *X*. To begin simulation of the model, we incorporated these two aspects into the program. Some experimental assays of adult emergence rhythms use larval crowding to desynchronize development, and thus create a mixed-age distribution (Sheeba et al., 2001). In such a crowded population, the time distribution of pupation can be assumed to be approximately bell-shaped (normal) (Bonnier, 1926), with variance as a function of larval crowding. Additionally, the initial number of eggs in a population determines the number of individuals in the population and, thereby, decides the extent of larval crowding in the population. A such, we built the property of mixed-age distribution into the model by deriving the time of pupation of each individual from a normal distribution with parameters dependent on the initial number

of eggs from which the population is built up. We incorporated a constant rate of accumulation of X in the model by assuming that this process of accumulation begins as soon as a fly pupates. It is obvious that the accumulation of X would continue until it reaches its threshold and the adult fly emerges. The time interval for the accumulation of X thus stretches from pupation to adult emergence. During this time, X builds up linearly from 0 to its threshold value. With these conditions in mind, we built in the following expression for the linear rate of accumulation of X:

$$\text{Accumulation rate} = \frac{\text{Mean threshold of } X}{\text{Duration of pupal development}} . \quad (1)$$

Having incorporated this property (linear accumulation of the development substance) into the model populations in which adult emergence rhythm is being simulated, we went on to add the effect of oscillation in the threshold of X. To achieve this, we assumed that all individuals in the model population have the same clock period and that the circadian clock of each individual readily entrains to externally imposed LD cycles, adopting the same phase. This allowed us to use the same time counter to simulate oscillation of the threshold in all flies of the population. We used a time counter that counted time after the first pupation takes place in the population. To keep the model simple, we assumed that oscillation of the threshold of X is sinusoidal. Taking all this into account, we modelled oscillatory threshold by the threshold level function (TLF), which we define as:

$$\text{TLF} = \text{Mean threshold of } X + \cos\frac{2\pi t}{\tau} , \quad (2)$$

where t refers to the time counter used, i.e. time after the first fly pupated, and τ refers to the period of oscillations of the threshold of X (equivalently, the circadian period of flies). As a result, the term t/τ can be taken as a measure of the number of oscillations of the threshold completed in time t.

While running the simulation, as X accumulated in the fly, its level was compared with the corresponding value of TLF. The fly was allowed to emerge as adult only if the level of X exceeded the value of TLF. Concomitantly, the program kept track of each act of emergence by adding to an hourly counter of the frequency of emergence. We reset the level of X in an individual fly to –1 as soon as it emerged to make sure that the program did not access flies that had already emerged so that each fly could emerge only once during the entire simulation.

The adult emergence rhythm of *D. melanogaster* is often studied in populations of approximately 300 individuals (Sheeba et al., 2001). It has been observed that in such assays, individuals in the population normally pupate over a period of approximately 6 days. Keeping these details in mind, we ran the simulation of the model assuming a population of 300 eggs. Coupled with this, as specified earlier, the program assumed that there is a normal distribution of pupation time in this population. We took the mean of this distribution as 3 days and the standard deviation as 1 day. This choice of parameters ensured that 99% of flies in the population pupate within a period of 6 days (because of the 3σ limit of the normal distribution).

As the idea of an oscillatory threshold controlling adult emergence in fruit flies has not been previously examined, we are not aware of studies aimed at measuring the parameters of such a periodic function. Consequently, we made some arbitrary assumptions about the TLF while performing the illustrative simulations in this study. Firstly, we assumed that the TLF is sinusoidal in nature – this assumption, however, does not preclude the possibility of the TLF being some other periodic function of time. Secondly, we assume the mean threshold of X to be 5.0 and the amplitude of oscillation of the TLF to be 1.0, 1.5 or 2.0. Additionally, the assumption of a linear accumulation of X is also illustrative in nature – as specified previously, it only aids in writing a computer program to simulate a specific instance of the model. Very similar results would emerge if X was assumed to accumulate as a specific non-linear function of time. The quantitative results obtained from the simulations, therefore, should not be seen as actual attempts to predict the quantitative results of the experiments. Instead, in this study, the qualitative trends observed from simulations carried out using a certain set of parameters turn out to be of actual consequence in the experimental studies. The quantitative results yielded by the simulations only serve to highlight the trends to be looked at in the experimental results.

Predictions from the simulations

Gate width of flies with faster/slower development

While running the simulations, we assumed that accumulation of X in an individual fly initiates as soon as the fly pupates. We simulated adult emergence rhythm in populations having different pupal development times. In a population of 300 eggs kept at 25°C, pupal development of an individual fly normally takes approximately 4 days. Fig. 2A shows the simulated emergence profiles of flies with two different pupal development times, 3 days (faster than normal) and 5 days (slower than normal), with the TLF assumed to be a sinusoid with a mean of 5.0 and amplitude of 1.0. From the simulated emergence profile itself, it is clear that the peaks of adult emergence are broader in the population that has a shorter duration of pupal development. In addition, the gate width of adult emergence from the simulations is quantified as a function of pupal development time (varying from 3 to 5 days) and amplitude (1.0, 1.5 and 2.0) of the TLF (Fig. 3A). As specified previously, the exact values of the gate width obtained from these simulations depend crucially on the arbitrarily assumed parameters used to construct the TLF. However, using a certain parameter set, the simulations yield the important experimentally testable prediction that gate width of adult emergence decreases with an increase in the pupal development time at specific amplitudes of the threshold oscillations of X. The simulations concomitantly reveal that the gate width of adult emergence decreases with an increase in the amplitude of the threshold oscillations of X at a specific duration of pupal development.

Gate width of flies with faster/slower clocks

Fig. 2B shows the adult emergence profiles of flies with a τ of 19.0 or 28.0 h (with 4 days as the pupal development time; seen in usual assays of adult emergence performed at 25°C) with the TLF assumed to be a sinusoid with a mean of 5.0 and amplitude of 1.0. The simulated gate width of adult emergence is quantified as a function of τ (varying from 18 to 30 h) and amplitude (1.0, 1.5 and 2.0) of the TLF shown in Fig. 3B. The quantitative values obtained for gate width of adult emergence serve to indicate the trend of greater gate width in the population with a longer τ at a specific amplitude of threshold oscillations of X. As noted previously, we observe that the gate width of adult emergence decreases with an increase in the amplitude of the threshold oscillations of X at a specific τ. Additionally, these simulated values also reveal that the difference in gate widths of adult emergence, using the same parameter set for the TLF, is larger between flies with a τ of 28 or 18 h than between flies that develop faster/slower by 2 days as pupae. Once again, although the quantitative values obtained from the simulations depend on the exact parameter set used, we obtain

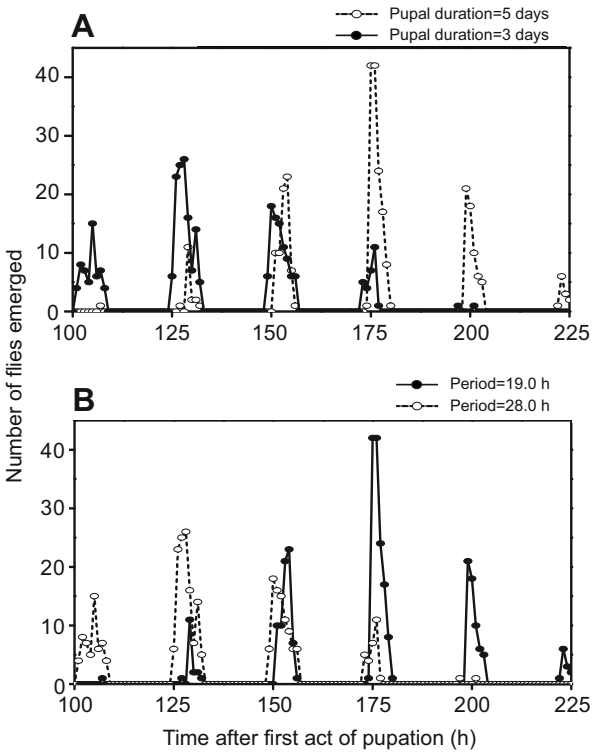

Fig. 2. (A) Simulated adult emergence profiles of populations of flies developing faster (pupal duration=3 days) or slower (pupal duration=5 days). Faster and slower development are modelled using a higher and lower constant rate of accumulation of the developmental substance *X*, respectively. The model predicts a wider gate of emergence in the faster developing population compared with the slower developing population. (B) Simulated emergence profiles of populations of flies with short (19 h) and long (28 h) circadian clock periods. The difference in the clock periods is reflected in the period of oscillations of the threshold of *X*. The model predicts a wider gate of adult emergence for the long period population compared with the short period population. Both simulations assume that the threshold level function (TLF) is sinusoidal with a mean of 5.0 and amplitude of 1.0.

qualitative trends in the results that are subjected to experimental scrutiny.

The simulated emergence profiles shown in Fig. 2 indicate that gate width of adult emergence may vary from cycle to cycle, indicating that our model based on linear build-up and oscillatory threshold is a good representation of reality as it captures features that would be seen in any real fruit fly population. The small gate width in the last cycle of emergence is an artifact of very few flies remaining to emerge by that time. Consequently, although the gate was open for the usual period, the supply of pupae was soon exhausted and no more flies emerged. As a result, a very narrow peak of emergence can be seen in the last cycle.

Empirical validation of the model

As the results of our simulations predict larger gate width in flies that develop faster, we used laboratory populations of *D. melanogaster* that were selected to develop faster as pre-adults (P.Y. and V.K.S., unpublished) to elucidate the effect of faster or slower development on the gating of adult emergence. In addition, we predicted a narrow gate of adult emergence in a population that develops slowly based on the hypothesis that a population that has a small gate width will develop slowly during the pre-adult stage. We attempted to corroborate this prediction by additionally measuring the pre-adult development time in *D. melanogaster* populations selected for narrow gate width (Kannan et al., 2012). All adult emergence rhythm assays were performed under 12 h:12 h LD cycles (where lights came on at 10:00 h and went off at 22:00 h). This ensured that flies adopted the same period by entraining to 24 h LD cycles, and hence their clock periods are similar.

Apart from this attempt to test the model by varying the rate of development but keeping the circadian oscillations unaffected, we examined the predictions of our model in fly strains with widely different circadian periods but by-and-large similar rates of development. This was achieved by carrying out adult emergence rhythm assays in *per* mutant flies (per^S and per^L) with short (~19 h) and long (~28 h) periods. Although these flies were initially reared under 12 h:12 h LD cycles (until pupation), the assays were performed under constant darkness (DD) at 25°C so that their circadian clocks would free-run with different circadian periods.

To further test the predictions of our model, we assayed adult emergence rhythm at high (29°C) and low temperatures (18°C). Fruit flies are known to develop faster at high temperatures (29°C) and slower at low temperatures (18°C) (Powsner, 1935). Consequently, based on the predictions from the model, it is expected that in any given population, gate width would be larger at higher temperatures and smaller at lower temperatures. As circadian clocks compensate for changes in temperature, such manipulations in temperature will not bring about any appreciable change in clock period (Pittendrigh, 1954; Bruce, 1960). Temperature thus provides a protocol for manipulating 'developmental' control of emergence without disturbing the period of the 'circadian clock' control.

Populations with fast/slow development

Selected populations were initiated from baseline developing (BD; control) populations of *D. melanogaster* that have been maintained for hundreds of generations under DD at 25°C on banana–jaggery food medium. From these populations, faster developing (FD) populations were initiated by selecting for the fastest 20–25% flies emerging in each generation. BD populations were also maintained along with the selected populations, in which no conscious selection pressure was applied. BD populations were derived from four parental outbred populations maintained under LL as genetically independent populations for 600 generations. Maintenance and history of these parental populations are described in Sheeba et al. (Sheeba et al., 1998). Selected and control populations were derived from one BD population, thus forming a matched selected–control pair. A total of 1200 breeding adults per population, with a roughly equal number of males and females, were maintained as large outbred populations in Plexiglas cages with banana–jaggery medium as the food source. After 7 days, yeast acetic acid paste was applied on a food plate and provided as the food source. Three days later (on the eleventh day), approximately 300 eggs were collected and transferred into vials (18×2.4 cm, height × diameter) containing 10 ml of food. In the BD populations, flies emerging between 9 and 12 days after egg collection were collected into Plexiglas cages containing a Petri dish of banana–jaggery medium. In the case of the FD population, however, only the first 20–25% of emerging adults was collected. Both selected as well as control populations were maintained on a 21 day discrete generation cycle. The FD and BD populations were always maintained under DD. After 30 generations of selection, under their usual maintenance conditions, circadian periods of FD and BD flies were 23.77±0.03 and 24.10±0.03 h, respectively (P.Y. and V.K.S., unpublished). We found that the pre-adult development time of FD

under these conditions was 209.13±1.3 h, whereas that of BD was 227.70±2.2 h (all values are means ± s.e.m.).

Populations with fast/slow circadian clocks

Stock maintenance and the adult emergence rhythm assay on the per^S and per^L (Konopka and Benzer, 1971) populations was performed in a manner similar to that of FD populations. The per^S and per^L populations were, however, maintained on corn meal food instead of banana–jaggery food and were reared in 12 h:12 h LD cycles. The circadian period of per^S and per^L flies under DD is ~19 and ~28 h, respectively.

Populations selected for narrow gate of adult emergence

From the BD populations (Kumar et al., 2006), precision populations (PPs) were initiated using flies that emerge between 11:00 and 12:00 h under 12 h:12 h LD cycles, where lights came on at 10:00 h and went off at 22:00 h. Control populations (CPs), without any conscious selection pressure, were also initiated from the BD populations and maintained along with the selected (PP) populations. The remaining aspects of their maintenance are similar to that of FD and BD populations, except that in each generation for four to five successive days, adult flies for the PPs were collected between 11:00 and 12:00 h and for CPs throughout the day. After 80 generations of selection, the gate width in PP and CP flies was 9.24±0.3 and 11.89±0.15 h, respectively (Kannan et al., 2012). The circadian period of the two populations was 23.50±0.03 and 23.90±0.05 h, respectively (all values are means ± s.e.m.).

Adult emergence rhythm assay

The gate width under 12 h:12 h LD cycles and DD (only for per^S and per^L flies) was estimated by performing adult emergence assays. Eggs were collected from various laboratory populations of *D. melanogaster* and transferred at a density of approximately 300 eggs per vial with 10 ml of food. Several such vials per population were kept under LD cycles, which were monitored for first emergence and thereafter checked regularly at 2 h intervals for 4–5 days, during which the number of flies was recorded. Gate width was estimated as the time interval between the start and end of emergence in a cycle with a cut-off at 5% of overall emergence during the cycle. For the estimation of gate width, start of a new day/cycle was assumed to occur at 04:00 h (midway through the dark phase of the LD cycle) because adult emergence is at its minimum around this time. In each of the replicate vials used in the adult emergence rhythm assays, average gate width was calculated as an average across cycles in which more than 25 flies emerged. In this protocol, we excluded cycles of adult emergence in which a very small number of flies eclosed. Finally, the gate width for a population was obtained by taking an average across its representative vials.

For the calculation of gate width in adult emergence assays of per^S and per^L flies performed under DD, we chose to consider circadian period as the duration of each cycle. Consequently, the length of each cycle of adult emergence was taken to be 19 h for per^S and 28 h for per^L.

Statistical analyses and programming language

The gate width of adult emergence was statistically analyzed using two-factor ANOVAs where population and temperature were treated as fixed factors. *Post hoc* multiple comparisons were performed using Tukey's test, and $P<0.05$ was considered as the level of statistical significance. All statistical analyses were implemented using the statistical module of SigmaPlot for Windows (version 11.0, Systat Software, San Jose, CA, USA) and STATISTICA for Windows (version 5.0, StatSoft, Tulsa, OK, USA). The program used to simulate the model of emergence was written using the freely available programming language Python for Windows (version 2.5.1) (van Rossum, 2003). All values are presented as means ± s.e.m.

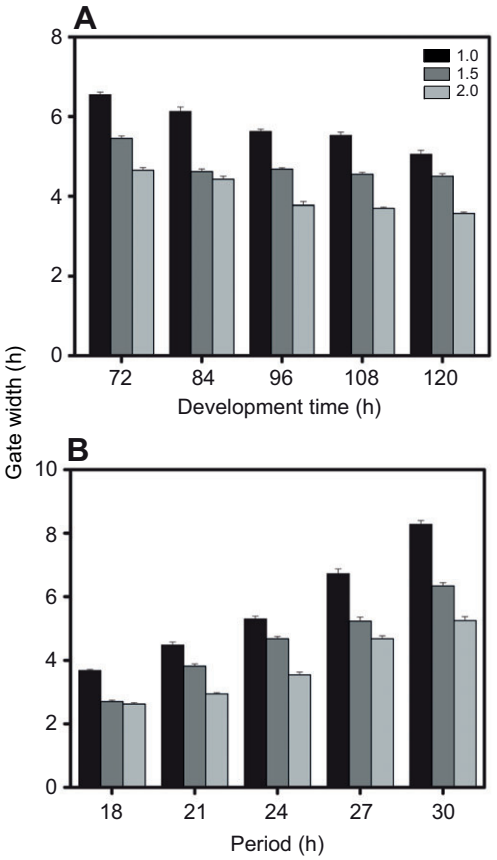

Fig. 3. (A) Simulated gate width of adult emergence as a function of pupal development time and amplitude of oscillations (1.0, 1.5 and 2.0) of the threshold of *X*. The simulations indicate that gate width decreases with an increase in development time at a specific amplitude and with an increase in amplitude for a specific duration of pupal development. In the simulation, it is assumed that *X* accumulates linearly and its threshold oscillates sinusoidally with a mean of 5.0. (B) Simulated gate width of adult emergence as a function of circadian period and amplitude of oscillations (1.0, 1.5 and 2.0) of the threshold of *X*. The simulations indicate that gate width increases with an increase in circadian period at a specific amplitude and with a decrease in amplitude at a specific circadian period. In the simulation, it is assumed that *X* accumulates linearly and its threshold oscillates sinusoidally with mean of 5.0. All values are reported as means ± s.e.m.

RESULTS

Gate width of fast/slow developing flies

The gate width of adult emergence of FD and BD flies under LD cycles is shown in Fig. 4A. As shown by our simulations, which predicted a greater gate width for populations that develop faster, we found that at 25°C (their usual maintenance temperature) under LD cycles, gate width was larger in FD flies than in BD flies ($P<0.0002$). At 25°C, the gate width of adult emergence of FD and BD populations was 18.62±0.65 and 14.61±0.73 h, respectively. To further test the predictions of our simulations, we assayed adult emergence rhythm in FD and BD populations at 18 and 29°C. In concurrence with the selection for faster development, gate width of adult emergence in FD flies was found to be significantly greater

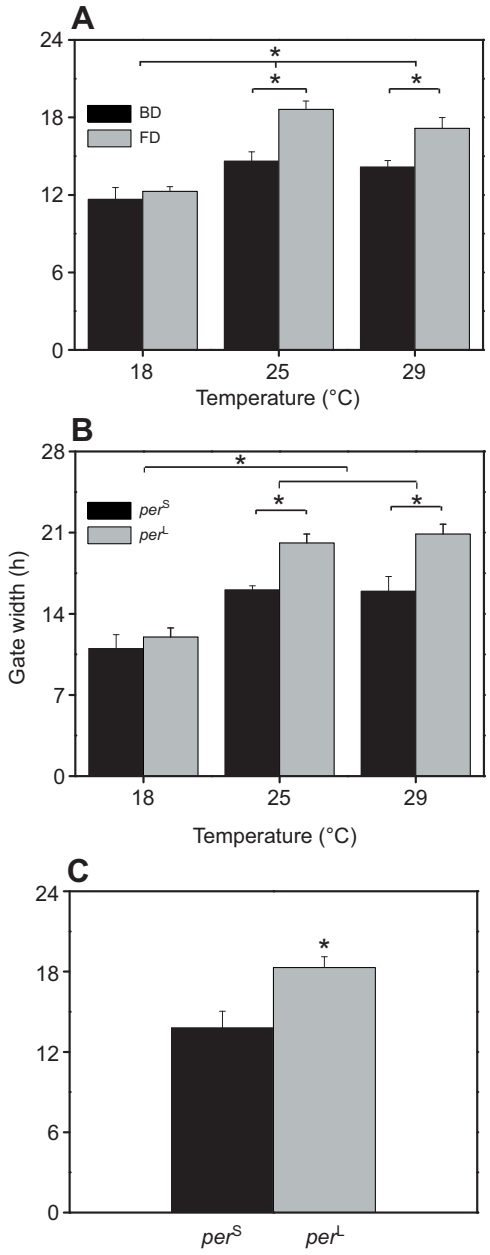

Fig. 4. (A) Empirically obtained width of emergence gate under 12 h:12 h light:dark (LD) cycles in populations selected for faster development (FD) and their baseline control populations (BD) at 18, 25 and 29°C. The wider gate of adult emergence of FD compared with that of BD at 25°C corroborates the basic prediction of the model of larger gate width in populations that develop faster. A total of 17, 20 and 13 vials of FD flies were used at 18, 25 and 29°C, respectively. A total of 4, 20 and 15 vials of BD flies were used at 18, 25 and 29°C, respectively. (B) Width of emergence gate in per^S and per^L flies at 18, 25 and 29°C under 12 h:12 h LD cycles. The wider gate of adult emergence of both per^S and per^L flies at 29°C supports the model's predictions. A total of 10, 10 and 8 vials of per^S flies were used at 18, 25 and 29°C, respectively, whereas a total of 10, 9 and 8 vials of per^L flies were used at 18, 25 and 29°C, respectively. (C) Width of the gate of adult emergence under constant darkness (DD) of per^S and per^L flies at 25°C. The wider gate width of adult emergence in per^L flies is in accordance with the model's prediction of a larger gate width in the population with a longer circadian period. A total of 19 vials of per^S flies and 20 vials of per^L flies were used for this assay. For A–C, each vial was started off with approximately 300 eggs, and adult emergence from the resultant set of pupae was observed. All values are reported as means ± s.e.m.

than in BD flies at 29°C (P<0.04). On a similar note, comparing across temperatures, a statistically significant increase in gate width of FD flies was seen at 29°C compared with 18°C (P<0.001). We also observed an analogous increase in the gate width of FD populations at 25°C compared with at 18°C (P<0.001). These results additionally lend support to the model's prediction of a wider gate width of adult emergence at higher temperatures (where pre-adult development is faster) and *vice versa* (Fig. 3A).

Gate width in *per* mutants

The gate width of adult emergence of per^S and per^L flies under 12 h:12 h LD cycles is shown in Fig. 4B. At 25°C, gate width of adult emergence is found to be greater in per^L as compared with per^S flies (P<0.03). In agreement with the model's prediction of a wider gate of adult emergence at higher temperatures, we saw significant increase in gate width of both per^S and per^L flies at 25°C (P<0.01) and 29°C (P<0.01) when compared with their respective gate widths at 18°C. The gate width of per^L was, once again, found to be greater than that of per^S at 29°C (P<0.009). We did not, however, find any statistically significant difference in the gate widths of per^S and per^L at 18°C.

The simulations of the model had predicted a greater gate width of emergence for flies with longer circadian period than in flies with shorter periods. We tested this by performing adult emergence rhythm assays of per^S and per^L flies under DD at 25°C. Consistent with the predictions of the simulations of the model, we observed that gate width of adult emergence was significantly larger in per^L flies than in per^S flies (P<0.001; Fig. 4C).

Gate width and development time in PP flies

We studied the effect of fast and slow development on the gate width of emergence in *D. melanogaster* populations selected for a narrow gate of adult emergence by performing adult emergence rhythm assays under LD cycles at 18, 25 and 29°C. The gate width of adult emergence of PP and CP flies is shown in Fig. 5B and the emergence profiles of the two populations at different temperatures are shown in Fig. 5A. In concurrence with the selection for narrow gate of adult emergence imposed on the PP flies at 25°C, the gate width of the PP flies was significantly smaller than that of the CP flies at 25°C (P<0.001). Additionally, the PP flies had a narrower gate of adult emergence than the CP flies at 29°C (P<0.02). However, we did not see a similar difference between PP and CP flies at 18°C. Comparing gate widths across temperatures, in agreement with the simulations, which predicted wider gate of adult emergence at higher temperatures, gate width of the CP flies was significantly greater at both 25°C (P<0.01) and 29°C (P<0.003) than at 18°C. However, there was no difference in the gate widths of PP flies at 18, 25 and 29°C.

As stated previously, simulation of the model of emergence predicted that flies that develop faster will have a wider gate of adult emergence and *vice versa*. This prediction implies that flies with a narrow gate of adult emergence would develop slower than controls which have a wider gate width. We measured the pre-adult development time of the PP and CP flies in an attempt to test this hypothesis. The pre-adult development time of PP and CP flies under their usual maintenance conditions at 25°C was 198.4±0.76 and 195.5±0.82 h, respectively (Fig. 5C). In concurrence with the predictions of the model, PP flies, which have smaller gate width of adult emergence than CP flies (Fig. 5A,B), developed significantly more slowly as pre-adults compared with CP flies (P<0.004). The difference in the pre-adult development time between the two populations, though statistically significant, seems somewhat small given the difference between the widths of their emergence gates at 25°C.

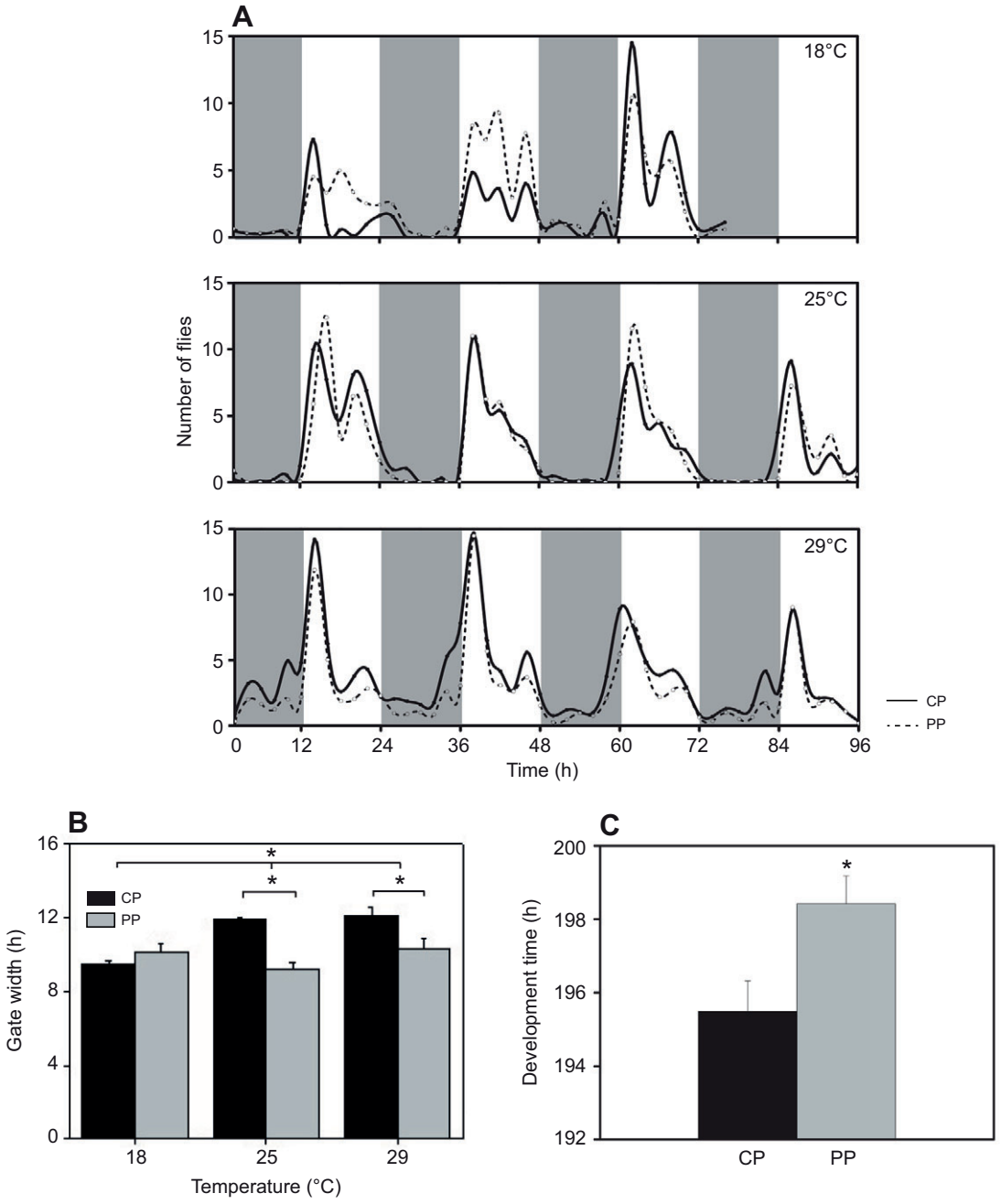

Fig. 5. (A) Empirically obtained adult emergence waveform of populations selected for narrow gate of adult emergence (PP) and their controls (CP) for three to four successive cycles. The number of flies emerged is plotted in 2 h bins over three to four successive cycles. Shaded areas represent the dark phase of LD cycles and white areas represent the light phase. PP flies emerged within a narrower gate compared with CP flies at 25 and 29°C. (B) Width of emergence gate under 12 h:12 h LD cycles in PP and CP flies at 18, 25 and 29°C. A total of 11, 20 and 16 vials of PP flies were used at 18, 25 and 29°C, respectively. A total of 7, 20 and 18 vials of CP flies were used at 18, 25 and 29°C, respectively. Each vial was started off with approximately 300 eggs, and adult emergence from the resultant set of pupae was observed. (C) Pre-adult development time of PP and CP flies at 25°C. The significantly longer pre-adult development time of PP flies, which shows a narrow gate of adult emergence, is in agreement with the model's prediction of slower development in populations that have a smaller gate width. A total of 20 vials each for both PP and CP flies were used in this assay. Each vial was started off with 30 eggs, and the pre-adult development time of each individual was noted. All values are reported as means ± s.e.m.

DISCUSSION

In this study, we analyzed and tested a model for adult emergence rhythm in *D. melanogaster* by simulating emergence rhythms using a computer program and performing adult emergence assays to validate the predictions of the model. The model was built on interplay between two controlling forces of emergence: pre-adult development and oscillations governed by circadian clocks. Such interplay of development and circadian oscillations has been previously used to explain temporal control of adult emergence in the moth *Manduca sexta* (Truman, 1984). In this moth, declining ecdysteroid titers (called the E system) provide excitatory inputs to the eclosion hormone releasing centres (called the G system).

Additionally, oscillations of the circadian clock maintain a gating effect in the release of eclosion hormone – it can only be released when this circadian 'gate' is open. The ecdysteroid titre is seen to provide excitatory inputs sufficiently high to cause the release of eclosion hormone and subsequent adult emergence only when it declines beyond a threshold. Once the ecdysteroid titer goes below its threshold, it interacts additively with the gating of eclosion hormone release, which eventually results in the gating of adult emergence. Our model, however, incorporates a major conceptual difference as compared with the *M. sexta* model through the idea of an oscillatory threshold of the developmental substance *X*. There is also no evidence that the threshold of the equivalent of *X* in the *M. sexta* model – ecdysteroid – oscillates in a similar fashion. Such a concept of an oscillatory threshold in addition to the build-up (or in a functionally equivalent sense, decline) of the same developmental substance is only seen previously in the ideas of Arthur Winfree with regards to the human sleep–wake cycle and insect emergence (Winfree, 1980).

The assumption of the build-up and not decline of a developmental substance during metamorphosis to simulate the developmental status of a fly is not a crucial one in our model. Our model can make the opposite assumption of decline of a developmental substance during metamorphosis to simulate the developmental status of a fly. The oscillatory threshold model can produce the very same results even if the substance *X* was declining instead of building up – the only difference in this case being that the decline of the substance *X* would then have to carry it below the threshold indicated by the oscillatory function. To simulate our model, we needed to fix a specific scheme of functional components; we decided to go with the idea of *X* building up, with a higher level of *X* indicating a more advanced stage of development in the pupal stage. In a corresponding manner, the assumption that *X* declines during metamorphosis is equally valid, and in this case a lower level of *X* indicates more advanced development during the pupal stage. Just as the function denoting the accumulation of *X* would be unable to cross the oscillatory threshold in its rising phase, a function showing the decline of *X* would also be unable to cross the oscillatory threshold when it is falling. Adult emergence in both cases would be restricted to a certain portion of the day. The questions that remain to be answered are: what is the physiological mechanism of determination of gate width, why is it important for insects, and what is the adaptive significance of the increasing gate width during faster development and at higher temperature?

Simulation of the model made two testable qualitative predictions about the gate width of adult emergence rhythm (a read-out of the model). Firstly, gate width of adult emergence would be greater in flies that develop faster and smaller in flies that develop slower (Figs 2, 3). Secondly, the model predicted that gate width of adult emergence would be greater in flies with a longer circadian period and smaller in flies with a shorter period (Figs 2, 3). We empirically tested these two predictions by performing adult emergence rhythm assays in fruit fly populations having (1) fast/slow pre-adult development and (2) fast/slow circadian clocks. In agreement with the model, we observed a wider gate of adult emergence at 25°C in populations of flies that have been exposed to selection for faster development than their controls at 25°C (Fig. 4A). Moving on to the model's prediction of wider gate of adult emergence in flies having longer circadian periods, we performed adult emergence rhythm assays for per^S and per^L flies under DD. In agreement with the predictions of the model, we observed a greater gate width of adult emergence in per^L flies compared with per^S flies (Fig. 4C). It has, however, been previously reported that per^S flies develop faster than per^L flies (Kyriacou et al., 1990). This would, according to the first prediction of the model, imply a larger gate width in per^S flies, in contrast to the observations, which instead are in agreement with the second prediction of the model. It seems, therefore, that the effect of shorter circadian period of per^S flies outweighs the influence of its faster development on the gate of adult emergence. This observation is in agreement with the results of the illustrative simulations of the model using a fixed set of parameters for the threshold function, which showed a much larger difference in the gate widths of fly populations with widely different circadian periods than in the gate widths of flies whose rates of pupal development differ widely (Fig. 3).

In the purview of our experiments, light is used to entrain the emergence rhythm of *per* mutants to 24 h LD cycles. This was done to examine the change in the gate width of adult emergence with temperature, as it keeps the period of the oscillatory threshold in our model fixed at 24 h. Upon comparison of LD gate-width data (at 25°C) with that of DD, we found that the mean values of the gate width of per^S and per^L are different under the two environmental regimes. Additionally, Fig. 3A shows that a particular combination of development time and amplitude (which in our experiments is modified by temperature) often produces the same gate width as that produced by a unique combination of circadian period and amplitude (shown in Fig. 3B). For instance, very similar gate-width values (around 5 h) are produced from a development time of 96 h and amplitude of 1.5 (Fig. 3A) as well as a circadian period of 24 h and amplitude of 1.5 (Fig. 3B). Therefore, although the results of our study suggest that light does have a role in the determination of gate width of emergence, individual experiments, depending upon the combination of development time, circadian period and amplitude, can display very similar gate widths in the presence or absence of light.

Because the simulations of the model show that flies that develop slowly have narrow gate widths, we surmised that flies that have narrow gate widths should develop slower than flies that have a wider gate. Indeed, at 25°C the pre-adult development time of populations selected for narrow gate of adult emergence was greater compared with controls (Fig. 5C). As development in *D. melanogaster* is known to become faster at higher temperature and slower at lower temperature (Powsner, 1935), and that circadian clocks are temperature compensated (Pittendrigh, 1954), using temperature as an agent to modulate developmental time allowed us to vary development rates of flies without a commensurate change in circadian period. However, circadian clocks are temperature compensated only relative to what is expected based on biochemical processes. In reality, the circadian period of *Drosophila* is not completely temperature compensated; rather it changes depending on the ambient temperature (Konopka et al., 2007). It is also noteworthy that *per* mutants had altered temperature compensation ability compared with wild-type flies. Non-compliance with the predictions of our model, if any, could be due to our assumption that amplitude of the oscillations of the threshold of *X* will not be affected by temperature to an extent that it will have an appreciable impact on the gate width of adult emergence. The results of our experiments at different temperatures, however, seem to indicate that this assumption is not entirely correct. They instead provide indirect support for another model that postulates that temperature-induced changes in the amplitude of the circadian oscillator are responsible for the phenomenon of temperature compensation (Lakin-Thomas et al., 1991; Majercak et al., 1999). Majercak et al. (Majercak et al., 1999) showed that the amplitude of oscillations of mRNA and proteins of two core clock genes *period* (*per*) and

timeless (*tim*) in *Drosophila* changed in a temperature-dependent manner: levels of *per* mRNA and TIM protein were higher at cooler temperature, whereas levels of PER protein and *tim* mRNA were higher at warmer temperature. In this scenario, as in the amplitude model of temperature compensation detailed in Lakin-Thomas et al. (Lakin-Thomas et al., 1991), the increase in the rate of development at higher temperature will tend to widen the gate of emergence – simultaneously, the rise in the amplitude of the threshold as temperature is raised will attempt to narrow the gate. These two effects will, thereby, cancel each other out to give an impression of stasis in the gate width with change in temperature.

Such changes in the amplitude of the threshold oscillations could be a vital difference between PP and CP flies as well; it could, for instance, account for the relatively small though statistically significant difference in the pre-adult development times of the two populations, given the large difference in their gate widths of adult emergence. In this case, it is pertinent to note that development time is a correlated response to the selection imposed on the PP flies. As in any artificial selection experiment, a correlated response to selection is never as strong as the response seen in the phenotype on which the selection is actually imposed (in this case, the gate width). Therefore, it is quite possible that the actual change in the gate width in PP flies, as compared with CP flies, is brought about by several factors acting in concert – for instance, it is possible that a difference in the amplitude of oscillations of the threshold of *X*, in addition to the difference in the pre-adult development times, plays a role in determining the gate width of adult emergence of the PP flies. Therefore, it is necessary to understand the nature of the substance *X* and its oscillatory threshold before any clear attempts to elucidate the role of its amplitude in adult emergence can be made. Consequently, though some of the fly populations do not meet the predictions of our model at all the temperatures tested, the fact that populations selected for faster pre-adult development show a wider gate of adult emergence and populations selected for narrow gate of adult emergence show a longer duration of pre-adult development despite the confounding effect of the amplitude of oscillations of the threshold of *X* lends extremely strong support to this model's basic prediction of a wider gate width with smaller pre-adult development time.

In essence, our attempt to explain the gating of adult emergence in fruit flies led us to postulate the existence of an oscillatory threshold of a marker of developmental status of the fly. The interaction between the build-up of this developmental substance and the periodic nature of its threshold was successful in capturing the phenomenon of gating of adult emergence in simulated runs on the computer and indicates that the gate width of adult emergence varies with pre-adult development time and the length of circadian cycles. Our experiments with fly populations with widely different development times and circadian periods corroborate these predictions and thus offer insights into a novel possible mechanism of control of adult emergence in fruit flies.

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