

Clock mutations alter developmental timing in *Drosophila*

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The developmental time of *period* mutants in *Drosophila melanogaster* was monitored under different environmental conditions. We observed that the *per^s* mutants, which have short 19 h circadian cycles, develop faster from eggs to adult than the wild-type: *per^L* mutants, which have long 28 h circadian rhythms, complete development more slowly than the wild-type. These results suggest that endogenous timers may be involved in regulating the development time of *D. melanogaster*.

INTRODUCTION

The *period* (*per*) gene plays an important role in biological timekeeping within *Drosophila* (Hall and Rosbash, 1988). Mutations at this locus in *D. melanogaster* can either speed up, slow down, or obliterate various types of biological rhythms. The *per^s* mutation for example shortens the normal free-running circadian period in both pupal/adult eclosion and individual adult locomotor activity patterns from 24 to 19 h (Konopka and Benzer, 1971). Also, *per^s* shortens the male fly's lovesong cycle, from 55 to 40 s (Kyriacou and Hall, 1980). The *per^{L1}* mutation lengthens both of these cycles, to 28 h and 80 s respectively, and the *per⁰¹* mutants appear arrhythmic in both characters (Konopka and Benzer, 1971; Kyriacou and Hall, 1980). Furthermore, *per⁰¹* mutants are reported to have arrhythmic pupal heartbeats (Livingstone, 1981; Dowse *et al.*, 1988). As the *per* mutations appear to affect a spectrum of temporally programmed phenotypes, we wondered whether these clock mutants would exhibit differences in development time, which at 25°C is approximately 10 days.

METHODS

Strains

The *per^s*, *per^{L1}* and *per⁰¹* and *per⁺* alleles were initially on genetic backgrounds in which the *per*

alleles were coisogenic with respect to their autosomes, but not with respect to their X chromosome. We therefore repeatedly backcrossed the three mutants and the wild-type to a chromosome carrying a deletion of the *period* gene, DF(1)64j4 (Hamblen *et al.*, 1986). After 12 backcross generations we re-extracted the *per* alleles and made homozygous stocks of *per^{L1}*, *per^s*, *per⁰¹* and *per⁺*. These stocks are therefore coisogenic except at the *per* locus.

Environmental conditions

The development time of period mutants in *D. melanogaster* was monitored under five different environmental conditions, four of which were under a constant temperature of 25±1°C. These were constant darkness (DD), 12 h light, 12 h dark (LD12:12), constant bright light (LL) and constant very bright light (VLL). A fifth condition used was alternating 12 h light, dark cycles with a 12 h 25±1°C/18±1°C temperature cycle superimposed (LD12:12T). The 25°C was synchronised to the 12 h light phase. The light/dark and dark/light transitions were at 9 p.m. and 9 a.m. respectively.

Monitoring development

Eggs were collected from fertilised females under LD12:12 conditions. Virgin females had been

collected simultaneously from the four *per* genotypes and aged for 3 days before being paired with males. They were allowed to mate for two days and then 50 fertilised females were placed in a bottle and allowed to lay eggs for 48 h in order to acclimatize them to their egg-laying media. During this period fresh egg-laying plates were provided every 3 h during the light phase. After this "pre-laying" period it was found that enough eggs could be obtained simultaneously from each genotype in one 2 h egg laying session to set up each experiment. Five experimental conditions were used, LD12:12, LD12:12T, DD, LL and VLL (see above). To examine the duration of the embryonic stage, rows of 20 freshly laid eggs were placed on glass plates containing agar. The eggs were monitored every 2 h until all larvae had hatched. Eggs were monitored under LL and approximately 200 eggs were observed for each genotype.

To examine the duration of the larval period, first instar larvae were obtained simultaneously from the four *per* genotypes by taking only those individuals which had hatched within 2 h of each other. Consequently our larval duration measure is largely uncontaminated by any effects of the *per* alleles on egg hatching rate. Using a plastic drinking straw, 10 larvae were scooped up from an egg laying plate, and placed in a food vial containing a standard amount of *Drosophila* medium according to Kyriacou (1985). As many vials as possible were set up depending on the number of larvae available, but a minimum of 100 larvae from each genotype were plated out in this way for the LD12:12, DD and LL experiments, and 250 for the LD12:12T and VLL experiments. The vials were then placed in one of the five environmental conditions, and monitored until puparium formation, and adult eclosion. When observations were made in darkness as in the LD12:12, LD12:12T and DD conditions a lamp using a filter (Wratten No. 87C) which transmitted wavelengths greater than 600 nm was used. *Drosophila* are insensitive to red light of this wavelength (Frank and Zimmerman, 1969; Kyriacou and Burnet, 1979).

In the LL and VLL conditions, flies were monitored under constant bright light. In the LL condition, the light was generated from an incubator and the photon fluence rate (pfr) measured as $3 \mu\text{mol m}^{-2} \text{s}^{-1}$. In the VLL condition the pfr was $100 \mu\text{mol m}^{-2} \text{s}^{-1}$. Normal laboratory lighting has a pfr of $1.5 \mu\text{mol m}^{-2} \text{s}^{-1}$. From the experimental design described above we were able to obtain measurements for the duration of the embryonic, the larval and a combined measure for the duration of the larval and pupal stages.

Statistical analysis

Statistical analyses of the development time of *per* mutants compared to the wild-type for different developmental stages, were performed using the Kolmogorov Smirnov two sample test (Siegel, 1956) to compare the developmental speed of each mutant vs. the wild-type. As an additional test, a two-way ANOVA was performed with genotypes as one factor and time as the other. The cumulative number of individuals per egg laying tray, or per vial, which completed each developmental stage was used as the dependent variable. Although the cumulative data are not independent, the ANOVA can pick out genotype and interaction effects and, when used in addition to the non-parametric test above, is useful in interpreting the results. Analysis of variance was also performed on the final numbers of larvae pupating in order to examine larval viability.

RESULTS

Egg-hatching rate

The results of the Kolmogorov-Smirnov tests, where each *per* mutant was compared to the wild-type are shown in table 1. The *per^s* eggs hatched significantly faster than the wild-type eggs ($P < 0.01$). In the ANOVA the genotype ($F = 4.13$ $P < 0.01$) and interaction effects ($F = 2.2$ $P < 0.05$) were significant. The Newman-Keuls *a posteriori* procedure confirms that the *per^s* genotype hatches significantly faster than *per⁺* ($P < 0.01$) with no significant differences between the other genotypes. Table 2 gives the mean (\pm SEM) number of

Table 1 Statistical analysis of the development time of each *per* mutant compared to the wild-type

Developmental stage	Conditions	<i>per^s</i>	<i>per^L</i>	<i>per⁰¹</i>
Embryonic	LL	fast*	ns	ns
Larval	DD	ns	slow**	ns
	LD12:12	ns	slow**	ns
	LD12:12T	fast*	slow**	slow*
	LL	ns	slow**	slow**
	VLL	fast**	slow**	fast*
Combined larval + pupal	DD	fast*	slow**	fast**
	LD12:12	fast*	slow**	ns
	LD12:12T	fast**	slow**	slow**
	LL	fast*	slow**	fast**
	VLL	fast**	slow**	fast**

* $P < 0.05$.

** $P < 0.01$.

Table 2 The mean number (\pm SEM) of individuals out of 10 completing embryonic, larval, and pupal development at specific time points. The results for the three time points given in the LD12:12T for the combined larval and pupal condition illustrate a significant interaction of genotypes \times time (see text)

Developmental stage	Conditions	Time point	<i>per</i> ⁺	<i>per</i> ^s	<i>per</i> ⁰¹	<i>per</i> ^{L1}
Embryonic		2	2.95 \pm 0.41	4.90 \pm 0.66	3.40 \pm 0.50	2.70 \pm 0.24
Larval	DD	3	8.20 \pm 0.29	9.00 \pm 0.36	8.60 \pm 0.30	5.00 \pm 0.79
	LD12:12	3	4.10 \pm 0.73	3.10 \pm 0.48	4.20 \pm 0.44	2.20 \pm 0.82
	LD12:12T	2	5.28 \pm 0.43	6.44 \pm 0.64	4.00 \pm 0.47	1.84 \pm 0.33
	LL	3	4.90 \pm 0.64	4.10 \pm 0.67	2.30 \pm 0.30	2.30 \pm 0.80
	VLL	2	1.60 \pm 0.37	3.96 \pm 0.31	2.20 \pm 0.42	0.08 \pm 0.06
Combined larval + pupal	DD	3	4.50 \pm 0.50	5.20 \pm 0.44	6.30 \pm 0.52	0.50 \pm 0.27
	LD12:12	3	6.22 \pm 0.72	8.30 \pm 0.50	8.29 \pm 0.68	5.80 \pm 0.61
	LD12:12T	1	0.44 \pm 0.14	2.68 \pm 0.50	1.84 \pm 0.39	0
	LD12:12T	2	3.40 \pm 0.39	4.68 \pm 0.64	3.88 \pm 0.46	0.20 \pm 0.10
	LD12:12T	3	7.12 \pm 0.36	5.56 \pm 0.69	5.32 \pm 0.51	1.36 \pm 0.13
	LL	3	5.40 \pm 0.54	6.10 \pm 0.66	7.00 \pm 0.47	4.20 \pm 0.27
	VLL	3	4.20 \pm 0.55	5.40 \pm 0.44	6.28 \pm 0.56	1.56 \pm 0.66

eggs which had hatched by the second observation period (20.5 h after egg laying). At this point one can see clearly that more *per*^s eggs have hatched compared to the other genotypes, this difference being significant when comparing *per*^s to *per*⁺ ($P < 0.01$). The significant interaction is due to all the genotypes having the same scores at the last observation period, *i.e.*, all eggs had hatched. Therefore there is some evidence that even in the first 20–24 h of development, the *per* mutants may be having some effect on the duration of the embryonic stage. The direction of the difference produced in *per*^s eggs parallels the effects of this mutation on circadian and ultradian cycles.

Larval duration

The Kolmogorov–Smirnov test reveals that *per*^{L1} flies remained significantly longer in the larval stage than the wild-type under all environmental conditions (see table 1 and fig. 1 for examples). The *per*^s mutants appear to develop slightly faster than the wild-type during the larval stage under some conditions (see table 1 and fig. 1) whereas the *per*⁰¹ larval development time, appears quite variable when compared to *per*⁺. The ANOVAS for these data all give significant genotype and interactions effects (except for the interaction term in the LD12:12 condition). Inspecting the means for some of the salient time points in table 2, it is clear that *per*^{L1} flies lag far behind the wild-type during the larval period under all conditions. The *per*^s flies appear to develop faster than the wild-type in the LD12:12T ($P < 0.01$), VLL, ($P < 0.01$) and also in DD, but this latter effect narrowly misses significance using the conservative New-

man–Keuls test. This pattern of results confirms that of the Kolmogorov–Smirnov test. In addition, in the *per*⁰¹ vs. *per*⁺ comparison, the results of the Newman–Keuls test supports those of the non-parametric test with *per*⁰¹ developing significantly more slowly than the wild-type in LD12:12T and LL ($P < 0.01$) and significantly more quickly in VLL ($P < 0.05$). The significant interaction terms in the ANOVA are due to the *per*^{L1} genotype “catching up” with the others at the later time points. No significant interactions are observed in the early time points between the four genotypes.

Combined larval and pupal duration

When the combined measures of larval and pupal duration are examined we see that *per*^{L1} flies take significantly longer than the wild-type to complete development, and *per*^s flies develop significantly faster than wild-type using the Kolmogorov–Smirnov procedure (table 1 and fig. 1). The development of *per*⁰¹ flies again appears variable, being significantly slower under LD12:12T, and faster under VLL, LL and DD. The ANOVA results again largely confirm the non-parametric test. Significant genotype effects ($P < 0.01$) are observed in all conditions, and interaction effects in the DD, LD12:12T ($P < 0.01$) and VLL ($P < 0.05$) conditions. The LD12:12T interaction is due to the *per*⁺ means criss-crossing with the *per*⁰¹ and *per*^s means in the first three time points: *per*^s and *per*⁰¹ eclose significantly faster than *per*⁺ in the first two observations periods, but *per*⁺ “catches up” and overtakes them at the third observation (see table 2 and fig. 1). All the other interactions are caused by the slower *per*^{L1} genotypes “catching

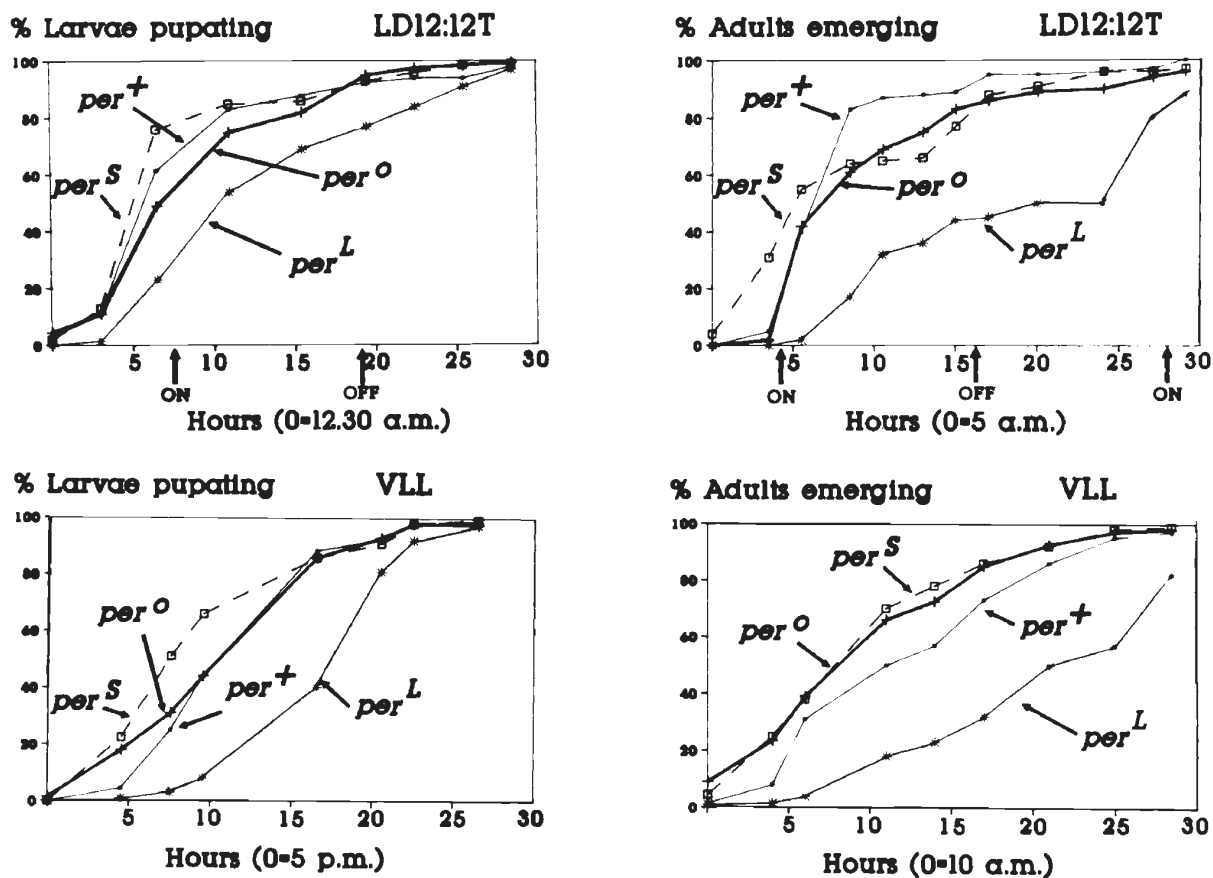


Figure 1 Developmental time in *per* variants. The cumulative percentage of larvae pupating and adults emerging are plotted over time. The data for the LD12:12T and VLL conditions are shown. The arrows at the bottom of the graph in the LD12:12T condition denote the times of lights "on" and lights "off" (on at 9 a.m. off at 9 p.m.). The real time corresponding to ON is also given. Note the sudden emergence of adult flies through "gates" between 7-10 a.m. and their sudden pupation between 3-7 a.m., in LD12:12T. This can be compared with the VLL profiles, where flies pupate and emerge more gradually throughout the day, because eclosion and pupation rhythms have been damped out by the bright light.

up" with the others in the later time points. Inspecting table 2, it is clear that *per^S* flies eclose significantly faster than *per⁺*, and *per^{L1}* eclose significantly more slowly than *per⁺* ($P < 0.01$ all *a posteriori* comparisons). *per^{O1}* flies eclose significantly faster than *per⁺* in DD, LL, and VLL, ($P < 0.01$), but they are slower in LD12:12T ($P < 0.01$ at one third observation). These results also confirm the Kolmogorov-Smirnov pattern.

Larval viability

Table 3 gives the mean numbers of larvae per vial which pupated. Two-way ANOVA gave significant effects of environmental conditions ($F = 16.13$, $df = 4$, $418 P < 0.01$) and genotypes ($F = 3.33$, $df = 3$, $418 P < 0.05$). From inspection of the means in table 2 it appears that the VLL conditions are acting

to reduce larval viability, and that the *per^S* larvae are generally the most viable genotype, although this advantage is reduced under the two "entrainment" conditions, LD12:12 and LD12:12T. Interestingly, *per^{O1}* larvae are no less viable than *per⁺*.

Table 3 Viability of larvae under different environmental conditions. The numbers represent the mean number of larvae out of 10 which reached the pupal stage

Conditions	<i>per⁺</i>	<i>per^{L1}</i>	<i>per^S</i>	<i>per^{O1}</i>	Grand means
LD12:12T	8.76	8.72	8.72	9.0	8.80
LL	8.36	8.54	9.96	9.13	9.00
VLL	6.32	6.84	7.80	7.28	7.06
LD12:12	8.56	8.36	8.44	8.2	8.39
DD	9.00	8.40	9.70	9.10	9.05
Grand means	8.20	8.17	8.92	8.54	8.46

DISCUSSION

The different environmental conditions we used were designed to bypass interference from the circadian clock on our measures of development times. The circadian clock "gates" adult emergence from the pupa in that the majority of adults which eclose in a given 24 h period emerge in a narrow time band, or "gate", around dawn (Pittendrigh, 1974). When flies are exposed to light and dark then placed under DD, the period of the circadian clock free-runs with the genotype specific period (Konopka and Benzer, 1971). Thus although the first instar larvae were all gathered within two hours of hatching, by the time they eclose as adults, (i.e., 10 days later at 25°C) their emergence may be spread around two eclosion "gates". These will occur 19 h apart in *per^s* flies, 24 h apart in *per⁺* flies and 28 h apart in *per^{L1}* flies. Consequently we would predict that compared to the wild-type, *per^{L1}* flies would on average develop more slowly, and *per^s* flies would develop more quickly. In LD12:12, the three genotypes, *per^s*, *per^{L1}* and *per⁺* are all entrained to the 24 h cycle (Konopka, 1972), so, any differences between the mutants development time and the wild-type will not be due to the different periodicities of the mutants, although the rhythm of each mutant may lock on to a slightly different phase (Konopka, 1972). The eclosion of *per⁰¹* mutants does not entrain under LD12:12 but it will entrain if a temperature cycle is also imposed (Konopka, 1972). Therefore the LD12:12T condition is designed to entrain *per⁰¹* flies, and in this environment all four *per* genotypes have prominent eclosion gates which occur every 24 h.

Under LL and VLL conditions *per⁺*, *per^{L1}* and *per^s* become arrhythmic (Konopka, 1972; Kyriacou and Hall, 1980), and the absence of eclosion "gates" allows the development time of these three variants to be directly compared with *per⁰¹*. Thus under these conditions, differences in the period and the phase of cycles of the four *per* variants are eliminated. We tested all four strains in the LL and VLL conditions in preliminary experiments to see whether they produced a "damping" out of the eclosion rhythm. Both lighting conditions succeeded, giving eclosion profiles of *per⁺*, *per^s* and *per^{L1}* flies which were very similar to *per⁰¹* (data not shown).

From our results we can therefore conclude that in conditions where differences in the circadian periods of *per^s*, *per⁺*, *per^{L1}* and *per⁰¹* are experimentally bypassed (as in conditions LD12:12T, LL and VLL) the combined larval and pupal development times of the mutants appear

to have been altered. The most definitive results come from the arrhythmic LL and VLL conditions, because there are neither phase nor period differences between the *per* variants. The direction of differences in *per^{L1}* and *per^s* shows striking parallels with the effects of these mutations on circadian and ultradian cycles. The results with *per⁰¹* mutants suggest that under constant lighting conditions (either constant light or dark), they generally develop faster than the wild-type. However when entraining light and temperature cycles are imposed, they slow down compared to the wild-type.

An additional result which is worth noting is the larval viability measure. Clearly under VLL conditions larval viability is considerably reduced. Perhaps this is a byproduct of creating an "arrhythmic" phenotype in *per^s*, *per^{L1}* and *per⁺* flies, but this is unlikely given that the viability of the arrhythmic *per⁰¹* larvae is also reduced. This poor viability may simply be a consequence of a very bright light-induced physiological "stress". The generally superior *per^s* larval viability is not observed when *per^s* individuals are entrained to a LD12:12 cycle. When *per^s* flies are released from such environmental constraints as they are in DD, LL, and VLL, their viability increases, suggesting that the circadian clock may also play a role in this important fitness character.

Given that *per^s* flies appear to develop faster than the other genotypes, and also have superior larval viability, the question arises as to what would happen if the *per^s* allele was allowed to compete in a population cage with *per⁺*. Certainly one might expect *per^s* flies to compete quite successfully given this pre-imaginal fitness advantage. We have recently initiated a population study and will monitor changes in gene frequencies of *per^s*, *per^{L1}*, and *per⁰¹* in competition with *per⁺*.

The mutant circadian phenotypes observed in *per^s*, *per^{L1}* and *per⁰¹* flies are due to changes in the *per* gene's proteoglycan-like gene product (Reddy *et al.*, 1986; Jackson *et al.*, 1986; Yu *et al.*, 1987 and Baylies *et al.*, 1987). The mutant proteoglycans may alter the pattern of intercellular coupling between cells by affecting gap junctional communication (Spray *et al.*, 1987 and Bargiello *et al.*, 1987). Gap junctions appear to be important during development, because disturbing them can impair pattern formation (Warner, 1985). Furthermore restriction of the developmental fates of cells correlates with changes observed in their pattern of gap junctional communication (Serras and Van der Biggelaar, 1987; Lo and Gilula, 1979). Therefore it is conceivable that the rapid intercellular

communication demonstrated between *per*^s cells (Bargiello *et al.*, 1987) may also lead to the faster development we observe in our experiments, with obvious implications for *per*^{L1} development.

Alternatively, if the circadian clock of *Drosophila* is generated by a network of coupled oscillators, the *per* mutants may produce their circadian effects by altering the coupling between tonically active cells (Dowse, Ringo and Hall, 1987; Dowse and Ringo, 1987; Bargiello *et al.*, 1987). If this clock also regulates development time, then this may explain why *per*^s develop faster and *per*^{L1} develop slower than the wild-type. Perhaps the effects we see with *per* mutant development times might be due to the clock playing a role in the cell division mechanism. In lower eukaryotes, biochemical oscillations are important in cell cycle control and have been repeatedly suggested to represent the high frequency "ticks" of the circadian clock (Chance *et al.*, 1964; Borteaux *et al.*, 1975; Lloyd and Edwards, 1984). Thus there appears to be a relationship between biochemical oscillations, cell cycle and the circadian clock in these organisms. Furthermore, endogenous timers have also been invoked to explain the control of various developmental processes in higher organisms (Cooke and Zeeman, 1976; Snow and Tam, 1980).

The *per* transcript and gene product are observed at embryonic, pupal and adult stages (James *et al.*, 1986; Liu *et al.*, 1988; Siwicki *et al.*, 1988; Bargiello and Young, 1984). However, the *per* protein is required only at the adult stage in order to induce circadian rhythms (Ewer *et al.*, 1988). Thus the *per* gene may have other functions at the earlier developmental stages and our results suggest that one of these roles may be to regulate the timing of development. We detect effects of the *per* mutations at the embryonic, larval and also in the pupal stages, and further experiments are in progress to examine how the *per* mutants produce these changes in development time.

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