



## Circadian rhythms of locomotor activity in *Drosophila*

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### Abstract

*Drosophila* is by far the most advanced model to understand the complex biochemical interactions upon which circadian clocks rely. Most of the genes that have been characterized so far were isolated through genetic screens using the locomotor activity rhythms of the adults as a circadian output. In addition, new techniques are available to deregulate gene expression in specific cells, allowing to analyze the growing number of developmental genes that also play a role as clock genes. However, one of the major challenges in circadian biology remains to properly interpret complex behavioral data and use them to fuel molecular models. This review tries to describe the problems that clockwatchers have to face when using *Drosophila* activity rhythms to understand the multiple facets of circadian function.

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### 1. Introduction

*Drosophila* has been used as a model organism for the study of circadian rhythms since the 1950s with the pioneer work of C. Pittendrigh. Pittendrigh's studies on fruit flies' circadian rhythms started by the observation that in the wild, *Drosophila persimilis* was mostly active in the morning whereas *Drosophila pseudoobscura* preferred the drier evening hours (Pittendrigh, 1993). However, most of Pittendrigh experiments were done by using another output of the *D. pseudoobscura* circadian clock, namely the emergence of young adults from their pupal case at the end of metamorphosis. Indeed, in the absence of an automatic setup it is certainly much easier to count how many adults eclose during a defined time window in the day, than to quantify locomotor activity

of adult flies in the same time window. Pupal emergence experiments allowed Pittendrigh to confirm the endogenous nature of the circadian clock and to define some of the main properties of these clocks such as temperature compensation and light-induced resetting. The paucity of *D. pseudoobscura* genetics (although selection for early and late eclosion in this species showed that such rhythm parameters were indeed genetically determined; Pittendrigh, 1981) induced clock researchers to turn to *D. melanogaster* to look for clock mutants. This led to the discovery of the first *Drosophila* clock mutants, obtained out of a chemical mutagenesis by Konopka and Benzer in the early 1970s (Konopka and Benzer, 1971). This landmark study opened a new era in circadian research, demonstrating that even complex behaviors could be tackled by classical genetic analysis, and making it possible to subsequently identify the molecular components that constitute a circadian clock (Allada, 2003; Allada et al., 2001; Blau, 2003; Stanewsky, 2003; Young and Kay, 2001).

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## 2. Locomotor activity as a circadian output

### 2.1. A simple and efficient way to analyze clock function

Among the dozen of genes that, so far, have been shown to play a role in the *Drosophila* circadian clock, seven were isolated by genetic screens based on pupal emergence (*period*, *timeless*) or locomotor activity (*clock*, *cycle*, *double time*, *shaggy*, *casein kinases 2alpha* and *beta*). The analysis of clock function through pupal emergence requires a large number of emerging pupae whose imagos will be manually collected from the culture at defined time points during a few days. Automated devices have been recently developed but they require the manual handling of the pupae (Konopka et al., 1994). In such eclosion monitors, pupae are glued on a plastic disk placed on top of a silicon-coated funnel. The disk will be tapped at defined time intervals by a computer-controlled tapping system, which precipitates the newly eclosed flies through the infrared emitter-detector located at the bottom of the funnel. Only one genotype is counted in each eclosion monitor, making these devices useful for analyzing a handful of mutants but not for testing hundreds of mutant lines out of a genetic screen.

In contrast, large numbers of genotypes can be easily tested in activity recording monitors. Different types of devices recording *Drosophila* activity were developed in the past 20 years (Hamblen et al., 1986; Helfrich, 1986) but the commercial TriKinetics ([www.trikinetics.com](http://www.trikinetics.com)) *Drosophila* activity monitors are the most widely used. In practice, individual flies are introduced into small glass tubes half-filled with sucrose-containing gelose. Each tube is monitored by an infrared emitter-detector that will record how many times the walking fly has interrupted the light beam during a defined period of time (bin). Each bin of the experiment (usually 1 or 2 weeks long) will be associated with an activity value and the resulting file will be analyzed to detect rhythmic patterns. Current *Drosophila* setups allow the testing of several thousands of individual flies in less than 2 weeks in the laboratory and have been largely used to screen for clock mutants as well as for the detailed analysis of their locomotor activity rhythms in various light and temperature conditions.

Activity recording of individual flies has been extremely powerful for the isolation of clock mutations. F1 genetic screens can be conducted, in which the progeny of mutagenized flies is directly monitored without establishing homozygous lines. This is particularly useful for X-linked mutations for which recessive hemizygous alleles can be detected in the F1 male progeny. In addition, many autosomal clock mutations are semi-dominant and can therefore be detected in heterozygous individuals. In particular, alleles that modify the period length have always been found to be semi-dominant, generating intermediate periods in heterozygous animals, in *Drosophila* as well as in other species (Allada et al., 2001; Stanewsky, 2003; Young and Kay, 2001). Importantly, looking at individual flies clearly identifies rhythmic and arrhythmic animals. This is not the case for eclosion experiments where the population will be seen as arrhythmic (no preferential emergence time during the day) if constituted of unsynchronized rhythmic individuals. Of course, looking at different clock outputs is required to understand the cellular organization of the circadian clock and recent results indicate that the circadian control of eclosion, activity and response to olfactory cues relies on both common and distinct pathways (Blanchardon et al., 2001; Emery et al., 1997; Krishnan et al., 2001).

Whereas new alleles of already characterized genes are regularly isolated in genetic screens, new genes appear to resist the classical screens based on adult locomotor activity. This is likely to be a consequence of such genes also having an essential role in *Drosophila* development. Indeed, the most recent genes that have been implicated in the *Drosophila* clock were already known for their developmental role. Although their complete loss-of-function is lethal, semi-dominant hypomorphic alleles of *dbt* or *ck2alpha* have been isolated in locomotor activity screens made in adult flies (Lin et al., 2002; Price et al., 1998; Suri et al., 2000).

### 2.2. Linking behavioral phenotypes with molecular functions

Mutations inducing changes in the circadian period have been isolated for several components of the core clock, namely *per*, *tim*, *dbt*, *ck2alpha*, *ck2beta* (Akten et al., 2003; Konopka and Benzer, 1971; Lin et al.,

2002; Rothenfluh et al., 2000; Suri et al., 2000). However, linking period changes with the molecular function of the gene product faces serious limitations, as illustrated for the long and short period alleles of *per* (Curtin et al., 1995). This is even worse when the complete loss-of-function does not allow survival of the adults, as is the case for several of the clock genes (*ck2alpha*, *ck2beta*, *dbt*, *sgg*, *smb*, *pdp1*, *vri*). This difficulty is likely to be a consequence of the biochemical functioning of the circadian oscillator, based on the controlled increase (cytoplasmic compartment) and subsequent decrease (nuclear compartment) of the levels of the clock proteins PER and TIM. For example, the loss-of-function of a gene encoding a PER kinase that promotes PER degradation would be expected to speed up the oscillator by preventing PER to be degraded during its cytoplasmic accumulation or to slow down the oscillator by delaying PER nuclear degradation at the end of the cycle. Because regulatory proteins affecting these molecular oscillations may act in both the accumulation and degradation phases of the cycle, as recently shown for DBT (Kloss et al., 2001), it is nearly impossible to identify mutations as losses or gains of function from the circadian period changes that they induce. In addition, the semi-dominant effect of period-altering mutations prevents their use for epistasis studies, with most combinations giving intermediate phenotypes.

Other limitations arise from the possible differences between the molecular mechanisms driving the brain clock that controls activity rhythms, and the peripheral clocks such as the one running in the eyes. Unfortunately, important features such as phosphorylation of the proteins and fine quantization of their levels require the biochemical analysis of head extracts. This has to be taken into account when trying to connect molecular data, based on head extracts that mostly reflect the eye oscillator, with behavioral data that depend upon the brain. A well known example is the function of the *cry* gene product, which appears to be both a photoreceptor and a core clock protein in the antennae and probably the eyes, but only a photoreceptor in the brain (Emery et al., 2000; Krishnan et al., 2001; Levine et al., 2002a; Stanewsky et al., 1998). Thus, in the *cry<sup>b</sup>* mutant, levels of the PER and TIM proteins do not oscillate in the eyes, but their oscillations appear normal in at least a subset of the brain clock neurons (Stanewsky et al., 1998).

As frequently observed in behavioral studies (e.g. learning and memory in *Drosophila*), behavioral defects can also be the consequence of developmental defects that may be rather subtle. The CLOCK and CYCLE proteins act in the core oscillator by activating the transcription of the *per* and *tim* genes. In addition, *Clk<sup>irk</sup>* and *cyc<sup>0</sup>* mutations have been shown to be associated with abnormal axonal projections from the pacemaker neurons in the adult brain. This phenotype is independent of the *per* gene, since it is not observed in *per<sup>0</sup>* null mutants, and could well be responsible for some aspects of the *Clk<sup>irk</sup>* and *cyc<sup>0</sup>* circadian phenotypes.

A final difficulty is common to all genetics studies, namely that any phenotype results from the interaction between a given genotype and a given environment. Such interactions are obviously even more complex, and less predictable, for behavioral phenotypes. Indeed, it was recently found that genotypes considered arrhythmic may prove behaviorally rhythmic under certain conditions (see Section 4.2(c)).

### 2.3. Understanding the neuronal bases of rhythmic behavior

Analysis of PER expression in the brain has defined several groups of neurons that cyclically express the PER protein (Blanchardon et al., 2001; Frisch et al., 1994; Helfrich-Forster, 1996; Kaneko and Hall, 2000). The understanding of the contribution of these different neurons is beyond the analysis of loss-of-function mutations affecting the core clock genes. However, the use of locomotor activity rhythms has allowed to reveal more subtle phenotypes that arise from mutations affecting the function of some of these neurons. The *disco* mutants, which have strongly disorganized optic lobes and do not display PER-expressing lateral neurons, are mostly arrhythmic in constant darkness (DD), but show a strong and advanced light-OFF anticipation in light–dark cycles (LD) (Blanchardon et al., 2001; Dushay et al., 1989; Helfrich-Forster, 1998). The recently isolated *pdf<sup>0</sup>* mutant (PDF stands for pigment-dispersing factor) has a very similar circadian phenotype, indicating that this neuropeptide is the major circadian neurotransmitter and that a PDF-independent oscillator is still running in these flies at least in LD conditions (Renn et al., 1999). The DD behavior is rather

interesting, showing a progressive loss of rhythmicity for the majority of the flies during the course of the experiment whereas a variable fraction of them stay rhythmic with a short period (Renn et al., 1999).

The development of the dual Gal4-UAS system to modify gene expression in specific cells (Brand and Dormand, 1995) makes possible the study of specific groups when appropriate drivers are available. This has been used to induce apoptosis of the PDF-expressing lateral neurons and to overexpress the *per* gene in the same cells (Blanchardon et al., 2001; Renn et al., 1999). The same expression system was also used to show that the electrical activity of these cells is an essential element of their free-running molecular clock (Nitabach et al., 2002), but that they do not use tetanus toxin-sensitive transmitter release to drive rhythmic behavior (Blanchardon et al., 2001; Kaneko et al., 2000). Unfortunately, specific Gal4 drivers are not currently available to target the other PER-expressing neuronal groups and this remains the limiting factor for such analyses. The possibility of manipulating gene expression has been used to analyze the phenotype of genes whose loss-of-function is lethal. Overexpression of *vri* and *sgg* restricted to the lateral neurons modifies the behavioral circadian period, whereas their overexpression in all *tim*-expressing cells has allowed the analysis of their effects on the molecular clock (Blau and Young, 1999; Martinek et al., 2001). Finally, transgenes expressing the *sgg* and *smb* genes under the control of heat-shock promoters have been used to rescue the corresponding lethal mutants up to adulthood, allowing their behavioral analysis in activity monitors (Grima et al., 2002; Martinek et al., 2001). The use of RNAi-mediated targeted gene extinction will certainly strongly enhance our ability to decipher the circadian function of vital genes in clock cells.

### 3. Method of analysis and measure of arrhythmicity: from raw activity data to quantitative rhythmicity parameters

#### 3.1. Periodogram analysis

Monitoring the locomotor activity of an individual fly results in a raw data file with a number of bins—a

bin is a 30-min recording interval—that are associated with an activity value (the number of times the fly crosses the infrared beam). To extract the circadian period from this file, several methods of analysis can be performed such as autocorrelation (correlogram) analysis (Dowse et al., 1989; Levine et al., 2002b), periodogram analysis (Hamblen et al., 1986; Levine et al., 2002b), and maximum entropy spectral analysis (MESA; Dowse et al., 1989; Levine et al., 2002b). Autocorrelation is based on the relationship between the values of a signal and the same signal some time later, and gives information about the periodicity of the signal. Periodogram analysis uses traditional Fourier techniques such as fast Fourier transform (FFT) to decompose the signal into sinusoidal waves and to determine the period of the rhythm as well as its robustness. MESA gives the same information but is based on another mathematical method to filter any cyclical components from data sets. Periodogram analysis works best for identifying cycles whose periods are a small fraction of the length of the data samples. Moreover, it needs to have data that are stationary (non-shifting) over the observation period. By contrast, MESA can find a cycle in a sample only as long as the cycle itself (Levine et al., 2002b), as is indeed the case for DNA microarray hybridization data (Langmead et al., 2002) or market oscillations (Ehlers, 2002). All three methods were used to determine the periodicity of biological rhythms such as circadian locomotor activity rhythmicity and give similar results (Dowse et al., 1989; Levine et al., 2002a,b).

In our lab, we use the chi-squared periodogram analysis (Sokolove and Bushell, 1978). Data analysis is done on a Macintosh computer running the Faas (Fly activity analysis suite) software developed by M. Boudinot (available upon request) and which is derived from the Brandeis Rhythm Package (<http://hawk.bcm.tmc.edu>) written by D. Wheeler. For various aspects of the use of BRP, see Ederly et al. (1994), Hamblen et al. (1986), Hamblen-Coyle et al. (1992), and Konopka et al. (1994).

The periodogram (Fig. 1A) is a plot of the estimation of the rhythm power as a function of the test period (we use period values ranging from 10 to 45 h). The main parameters of the periodogram analysis obtained after treatment of the raw data are the period of the rhythmic flies (the period that corresponds to the highest power of the periodogram) and their associated

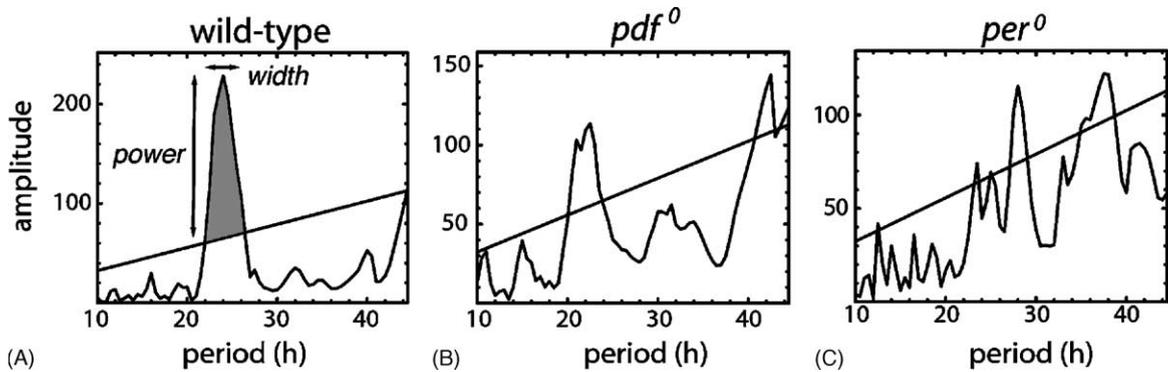


Fig. 1. Representative periodograms derived from activity records of individual flies in constant darkness. Flies were first entrained to LD cycles for 3–5 days, then kept in constant darkness for at least 6 additional days. The periodograms correspond to at least 5 days of activity, beginning 24 h after the last light-OFF transition. Power of the rhythm is defined as the height of the main peak above the line corresponding to the confidence limit (here a 95% confidence limit was used). The width of a peak is also defined relative to that line. Notice that the vertical scale differs between the three graphs. The *per*<sup>0</sup> periodogram is much more ragged than the other two. Its main peak, although in the circadian range (28 h), is very much narrower than for wild-type or *pdf*<sup>0</sup>. The *per*<sup>0</sup> periodogram also displays several additional peaks above the 95% confidence limit.

power (which reflects the robustness of the rhythm). The power value is given as the height of the periodogram peak above the 1 or 5% significance threshold which is arbitrarily chosen for the chi-squared analysis, and strongly depends upon the number of bins that are used for the analysis. Other parameters of the periodogram are the width and the complexity of the peak, as well as the total number of significant peaks that are detected. Multiple peaks indicate that several rhythmic components are detected giving rise to more complex rhythmicity. It is of course important to consider mean values of sets of tested flies, rather than individual flies. However, although activity levels are highly variable, individual period values are remarkably similar within a given genotype (at least for the genuinely rhythmic ones, see Section 3.2).

Different parameter effects can be observed and influence the result of the periodogram analysis. ‘Internal’ effects, that are linked to the experimental procedure or to the method of analysis, should be considered. The number of bins that are used, as well as the position of the starting bin as regard to the phase of the activity, influence the results of the experiment. Typically, 5 or 6 days are taken into account to test the period of the flies and longer experiments will be better for long period mutants. For fewer days, either the program will not detect any rhythm because of in-

sufficient data, or it will find a period but with a higher degree of variability. It may however be difficult to lengthen the experiment because of the increasing mortality of flies in the monitors. In addition, because the LD bimodal activity profile (see Fig. 4) does not disappear immediately after lights-OFF, the time during which the flies are left in constant darkness before the beginning of the analysis often influences the DD data.

### 3.2. How can you tell a rhythmic fly?

Determining when a fly can be considered as arrhythmic is a non-trivial problem when analyzing data from activity monitors. A good example to study such a question is the *per*<sup>0</sup> mutant flies, which carry a mutation leading to a non-functional PER protein and giving rise to constitutive levels of the different mRNAs and proteins involved in the circadian clock. These flies should not be able to sustain any circadian rhythmicity, although weak circadian components were detected in their activity profiles, suggesting the existence of other oscillators (Helfrich and Engelmann, 1987; Helfrich-Forster, 2001). In our hands, the periodogram analysis detects more than 30% of *per*<sup>0</sup> flies as rhythmic. When we look at the different parameters obtained by the analysis of each individual ‘rhythmic’ *per*<sup>0</sup> fly, some main differences

can be detected in comparison to normal rhythmic flies such as the wild-type (compare Fig. 1A and C). The first parameter we will consider is the power of these flies. Flies with negative power values have already been classified as arrhythmic by the periodogram analysis. Rhythmic *per*<sup>0</sup> flies show a much wider distribution of the power values than the wild-type flies. We arbitrarily chose to define a new power threshold of 20, a value that best discriminates between wild-type and *per*<sup>0</sup> rhythmic flies, according to the visual inspection of the individual periodograms. However, not only do a lot of *per*<sup>0</sup> flies have a power less than 20 but the remaining ones have a mean value of the power very low compared to wild-type. The width of the peak of the periodogram is another very useful tool to determine arrhythmic flies. When the flies are robustly rhythmic, the width of the peak is large (5–6 h), but for “rhythmic” *per*<sup>0</sup> flies it decreases to lower values (2 h or less). A third way to distinguish truly rhythmic flies is the number of peaks obtained in the periodogram. Whereas a single peak corresponds clearly to a unique circadian period, the presence of several peaks very often represents arrhythmicity. However, because a 12-h rhythmicity is displayed by the flies in LD conditions, peaks corresponding to 12-h multiples are sometimes detected in addition to the circadian period. A last way to detect if a genotype is arrhythmic is correlated to the dispersion of the periods of all the nominally rhythmic flies around the mean value of the period for all the tested flies of that same genotype. A typical result for *per*<sup>0</sup> shows periods ranging from 12 to 30 h, whereas dispersion for the wild-type is less than 1 h around the mean. We thus consider a fly as circadianly arrhythmic if its period differs from the genotype mean by more than 4 h. Finally, it is noteworthy to point out that direct eye inspection of the data can very often easily dissociate rhythmic from non-rhythmic patterns when the computer cannot.

Another interesting arrhythmic mutant to examine is the *pdf*<sup>0</sup> mutant. Although it is considered to lose completely its rhythmicity after a few days in DD (see Section 2.3 above), a closer look at the periodogram shows that it is in fact a lot more rhythmic than *per*<sup>0</sup> flies as the periodogram of *pdf*<sup>0</sup> is more related to wild-type than *per*<sup>0</sup> (Fig. 1A–C). Indeed, rather variable circadian phenotypes have been described for this mutant (Renn et al., 1999).

### 3.3. Phase determination

The phase of the oscillations is also an important parameter of circadian rhythmicity. This phase corresponds to an identifiable recurring reference point in the cycle (peak, trough, offset, etc.). It is important not only to correlate the phase of locomotor activity with peak levels of the clock proteins, but also to study the influence of perturbations to the system (i.e. light or temperature pulses, mutations affecting clock or photoreception components). The phase of the rhythm can be depicted in a circle (360°) representing the duration of the period (24 h in the wild-type or 20 h for *per*<sup>S</sup> flies) where the phase is the angle of a vector and the coherence of the phase is depicted by its length, i.e. the longer, the more coherent (Fig. 2). To examine the circular distributions of all the vectors (one fly, one phase vector), we use circular statistics that give us the circular mean (mean phase value), the length of the mean vector (distribution of the sample), the circular standard deviation and the standard error of the Rayleigh’s test of uniformity, which is a criterion for rejecting a uniform distribution (random phase) (see also Levine et al., 2002b for a similar approach). This test is however limited to a large number of individual vectors. Moreover, it only works for unimodal distributions because, otherwise, opposites modes balance each other, so that there is no significant mean phase even if the distribution is non-random.

With the Faas software, the accuracy of phase determination may vary depending on whether the phase of reference that is used is the trough or the peak of the activity rhythms. In constant darkness conditions, the peak region (centered in the second half of the subjective day) is rather broad, and sometimes contains two or more sub-peaks, whereas troughs are less ambiguous. Another possibility is to use the middle of the offset, i.e. the region located between the last maxima of the subjective day and the next trough. Comparing the different results obtained with these three methods show crucial differences in phase measurement with large variability for the peak, medium variability for the trough, and the highest precision for the middle of the offset.

The influence of light on the clock is often measured by the amplitude of phase shifts induced by short (usually a few minutes) light pulses. As the phase shift (absolute value and sign) depends on the time

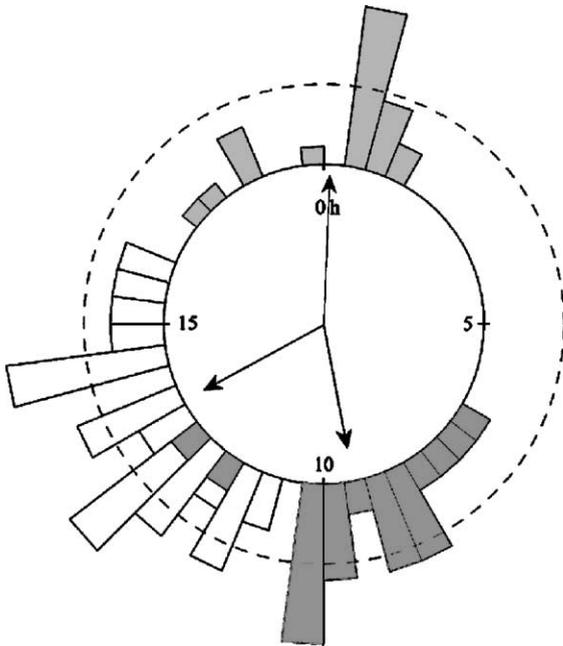


Fig. 2. Phase determination of locomotor activity of *per<sup>S</sup>* flies after a pulse of light. The inside circle is a clock dial where one full turn corresponds to the period of the *per<sup>S</sup>* flies, i.e. approximately 20 h. The circular histograms represent the phase distribution of three groups of flies. For easier comparison, the histogram for each group of flies is normalized to the corresponding peak value,  $n_{\max}$  (maximum number of flies at a given phase).

The dashed circle indicates height at half maximum. The arrows correspond to the mean vectors (see text for definition). The white histogram corresponds to unperturbed flies, i.e. that did not receive light pulses. The total number of flies tested,  $n$ , is 20 and  $n_{\max}$  is 3. Their mean period is  $20.4 \pm 0.1$  h and their mean phase (activity peak) is  $13.7 \pm 0.8$  h (95% confidence limit). The size of the mean vector (black arrow) is 0.90. The light gray histogram corresponds to flies having a 1-h light pulse at maximum intensity (around 400 lx) 4 h after lights-OFF ( $n = 21$ ,  $n_{\max} = 9$ ). Mean period is  $20.4 \pm 0.1$  h and mean phase is  $0.1 \pm 0.6$  h, corresponding to a phase delay of approximately 6.8 h, as observed in the phase–response curve shown in part B of Fig. 3 (bottom arrow). The dark gray histogram corresponds to flies receiving a 1-h light pulse at maximum intensity 8 h after lights-OFF ( $n = 21$ ,  $n_{\max} = 5$ ). Mean period is  $20.3 \pm 0.1$  h and mean phase is  $9.5 \pm 0.7$  h, corresponding to a phase advance of approximately 4.2 h, as observed in the phase–response curve shown in part B of Fig. 3 (top arrow).

The size of the mean vectors are equal to 0.90, 0.94 and 0.91 for the three cases described above, respectively. All phasings are significant with  $P < 0.001$  (from Rayleigh's circular statistics), and so are the differences between them (from Watson's non-parametric test).

the pulse is given, phase–response curves or PRCs are commonly used to describe how the clock responds to light (Fig. 2). Typical PRCs for most organisms show phase delays at the beginning of the subjective night, followed by phase advances in the second half of the night, whereas the subjective day contains a region with no phase shifts, also called the 'dead zone' (Saunders et al., 1994). Two different types of PRC can be obtained (Fig. 3): Type 1 showing small phase shifts, and Type 0 for stronger ones (amplitude is greater than half of the period). The question remains, however, to determine whether shifts are delays or advances when the amplitude is important: what differentiates a 6-h advance (orthodromic phase shifting) from a 18-h delay (antidromic phase shifting)? Although many flies prefer advancing their clocks, mutants such as *so* (*sine oculis*) delay it when using a particular wavelength, possibly because of the lack of a particular photoreceptor structure in this genotype (Helfrich-Forster et al., 2002).

A small region of the PRCs is particularly interesting to note: the region of transition between delays and advances for Type 0 PRCs where all the phases of the cycle can be reached, or in other words, where no phase can be determined. This region was often examined to study the possibility of permanently suppressing circadian rhythmicity (Winfree, 1970, 1980). However, because these experiments involved eclosion rhythms in a population and not individual fly rhythmicity (see Section 2.1 above), the question remains whether the molecular clock stops in each individual, thus abolishing any notion of phase, or whether it goes on, but with a different phase in each individual, thus making the population as a whole appear arrhythmic.

When the flies are entrained by LD cycles, the activity pattern is quite different from flies that are kept in constant darkness (DD). Whereas a broad unimodal peak is observed in DD, a bimodal rhythm is often observed in LD with a morning peak around lights-ON, an evening peak at approximately lights-OFF and a trough between them (Fig. 4). The circadian nature of the morning peak is still debated (Helfrich-Forster, 2000). Characterizing the phase in LD conditions anyway requires the proper identification of the morning and evening peaks. The restriction of activity to the early morning and late evening is well-documented for various *Drosophila* species in the wild (Pittendrigh,

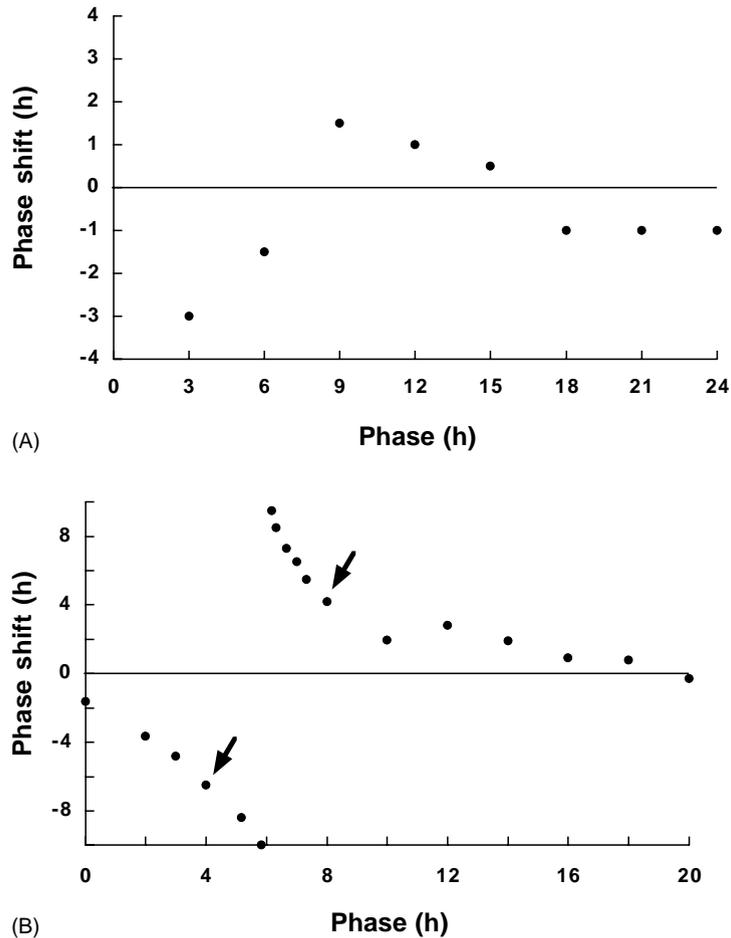


Fig. 3. Phase-response curves (PRC) for wild-type (Type 1) and *per<sup>S</sup>* (Type 0) mutant flies. These curves were obtained for a pulse of 15 min at maximum light intensity (around 400 lx) for wild-type flies (A) and for a 1-h pulse of the same intensity for *per<sup>S</sup>* flies (B). Flies were entrained for 3 days in 12:12 h light–dark conditions then put in constant darkness (DD). Pulses are always given after the last lights-OFF transition. The arrows in part B correspond to the phase delays (light gray histogram) and advances (dark gray histogram) shown in Fig. 2.

1993), and probably contributes to limit desiccation during the drier midday hours.

#### 4. Experimental conditions: the importance of being constant

The relevance of laboratory conditions to those in the wild is a very general issue in biology. Obviously, neither LD cycles with sudden lights-ON and lights-OFF transitions, nor complete darkness in constant temperature, are common features of natural environments. Whatever the choice of conditions made

by the experimenter, however, controlling them tightly will be critical to achieve meaningful results in the circadian field. Moreover, the results may depend, sometimes in unexpected ways, on the precise combination of conditions. For instance, changes in one parameter may completely reverse the response to changes in another.

##### 4.1. Ensuring stable and uniform experimental conditions

Temperature changes and light variations are the two major environmental inputs for the circadian

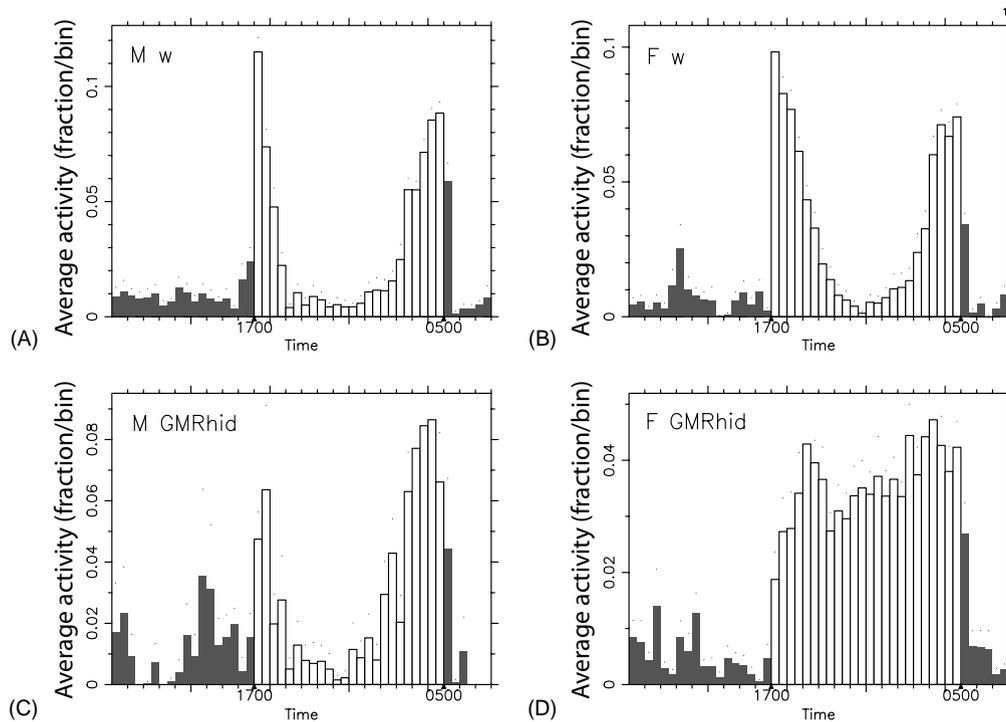


Fig. 4. Average distribution of activity for groups of flies in an LD cycle. Histograms represent the distribution of the relative activity of flies (fraction of daily activity per 0.5-h bin) during their 4th full day in a 12:12h light–dark cycle. The relative activities per bin were first computed for all flies, and the values for each bin were then averaged for all flies of a given genotype ( $n = 16$  except for C,  $n = 6$ ). The shaded bars indicate the dark part (night) of the LD cycle. All genotypes are more active during the day. Lights-ON is followed by a rapid surge in activity, which can be described as a “morning peak”, and corresponds mainly to an acute response to light. Activity then usually decreases to low levels, before rising again several hours before lights-OFF, reaching an “evening peak” and then dropping abruptly. The evening peak is clearly clock-controlled, since it is advanced in short-period flies, and delayed in long-period ones. Note that females tend to remain more active than males during the day. This well-known sexual dimorphism (Helfrich-Forster, 2000) is particularly striking for the GMR-*hid* flies (compare C and D). It makes it often difficult to distinguish between the morning and evening peaks of the females.

clock. Whereas circadian rhythms are temperature compensated (Pittendrigh, 1993), ensuring that the clock keeps running at the same period within a physiological range of temperatures, some clock mutants lose this property, and will show different free-running periods in constant darkness depending on temperature (activity has been measured in temperature conditions ranging from 15 to 30 °C). In addition, temperature appears to affect the phase of the activity even in wild-type flies (Majercak et al., 1999). Finally, the wide use of the temperature-sensitive Gal4-UAS system for modifying gene expression in transgenic flies requires a strict control of temperature in behavioral experiments. Various systems are in use for temperature control, which usually limit fluctuations

to below  $\pm 0.3$  °C. Ventilators can also be used to homogenize temperature within large incubators.

Light is more difficult to homogenize, since its intensity varies as the square of the distance to the light source. It is thus particularly important to check for uniformity of light intensity at the level of the flies whose rhythms are being analyzed. Even with one light source for each platform within an incubator, and with careful positioning of the activity monitors, the intensity seen by individual flies may vary by more than 30%. When switching from one model of incubator to another, it is difficult to avoid variations of close to a factor of 2. This is of course no problem in experiments performed at saturating light intensity (e.g. usual entrainment in LD, where the intensity is

several orders of magnitude above the threshold). It may also be acceptable when measuring action spectra on log scales, but it might hamper finer comparisons between mutants. When using monochromatic light, ensuring uniform illumination becomes even more difficult (see below).

Several biological parameters are known to strongly influence circadian behavior. As an example, females clearly show more robust rhythms than males, and their mating status (virgins or mated) may affect their rhythmic pattern (Helfrich-Forster, 2000). Another main difference between males and females resides in the locomotor activity pattern of flies kept in LD cycles (Helfrich-Forster, 2000), with the activity of females being more evenly distributed throughout the day. The extent of this dimorphism varies between strains (see Fig. 4 for an extreme example). The smaller size of males presumably makes them more sensitive to desiccation, so that decreasing their activity in the middle of the day should be even more adaptive than for females. Sexual dimorphism has also been reported in finer scale analyses of *Drosophila* locomotor activity (see J.-R. Martin, this volume). Age is another biological parameter that affects analyses, because old flies can be either more or less rhythmic at the end of their lives compared to younger ones (because females live longer than males, sex will thus also influence circadian behavior indirectly).

#### 4.2. Interactions between parameters

For a given type of analysis, each lab usually uses a specific combination of conditions (e.g. LD12:12 at 20°C rather than LD 10:14 at 25°C). This is of course required for reproducibility. It would also be impractical to test all possible combinations. However, one should be aware that several aspects of behavior, or even of rhythmicity proper, may be quite different between seemingly similar conditions. Here are a few examples:

(a) Flies may display different patterns of activity in LD cycles at different temperatures (Majercak et al., 1999). Tomioka et al. (1998) observed higher daytime activity at 25°C than at 30°C, for wild-type and three *per* mutant strains. In fact, *per<sup>0</sup>* flies displayed a complete reversal of activity, being mostly day-active at 25°C but strikingly

night-active at 30°C. A similar reversal, although less marked, was also found for *per<sup>S</sup>* flies. One may speculate that reducing daytime activity may be more important during hot spells, in order to minimize desiccation further.

(b) Temperature cycles in different lighting conditions may also yield different activity patterns. For instance, in DD, wild-type flies entrain to 24-h-long T-cycles (alternations of 25 and 30°C), but do not entrain to 20 or 28 h cycles (Yoshii et al., 2002). This result probably parallels that obtained by Tomioka et al. (1998) for *per<sup>S</sup>* and *per<sup>L</sup>* flies, which do not entrain to 24 h T-cycles in DD. However, entrainment occurs in all such cases when the flies are kept in LL rather than DD (Tomioka et al., 1998; Yoshii et al., 2002). This may seem surprising given the well-known behavioral (Konopka et al., 1989) and molecular (Zerr et al., 1990) arrhythmicity elicited by constant light in constant temperature. It does however represent true entrainment of a rhythm, and not a masking (non-circadian) effect of temperature cycles, because of both the dependency of phase on cycle length (at least for wild-type flies), and the advance and delay of the activity peak for the *per<sup>S</sup>* and *per<sup>L</sup>* mutants, respectively, in 24-h T-cycles.

Note that these last examples should not be taken as artifactual, as they may reveal some important underlying aspects of circadian clocks. Indeed, such experiments tend to support the multi-oscillatory view of the clock initially proposed by C. Pittendrigh (Dowse and Ringo, 1987; Helfrich-Forster, 2001; Pittendrigh et al., 1958). As the latter remarked, when one deal with complex adaptations, one should also bear in mind that focusing on an isolated environmental variable, and an isolated aspect of the organism's response, can be misleading.

(c) Even more striking results were reported recently on mammalian clocks. Mutant mice which lack either one of several clock proteins (PER homolog mPER2, or CLK) are either arrhythmic or lose rhythmicity in a few days, when the animals are transferred from LD to DD (Vitataerna et al., 1994; Zheng et al., 1999). However, their behavior was rhythmic when they were transferred to LL, contrary to wild-type animals which tended to become arrhythmic for high enough light intensities

(Spoelstra et al., 2002; Steinlechner et al., 2002). It was suggested that such genotype–environment interactions are consistent with the organization of the circadian system as a network of regulatory loops (Roenneberg and Merrow, 2003). Although these effects might not be directly relevant to clock function in the wild, they would still be useful to dissect the network. Similarly, the persistent behavioral rhythmicity of adult *cry<sup>b</sup>* flies in LL, and their intensity-dependent increase in period length, may lead to unexpected insights into clock function (e.g. what makes lateral neurons different from other clock gene-expressing cells in the fly).

#### 4.3. Lighting the clock: how much light? of what kind?

Light intensity is obviously a crucial parameter when testing the light sensitivity of fly strains which are defective in visual system anatomy or function, or in other light-sensitive pathways. In addition, to obtain action spectra (which show the sensitivity of a response as a function of wavelength), one requires as monochromatic a light as possible, with precisely measured intensities. When searching the circadian literature, one encounters several different units for light intensity (see Section 5.1), which sometimes makes comparisons difficult.

In practice, to test for wavelength dependency, one may simply cover the fly bottles (or the activity monitors) with a broad-band Plexiglas filter, which will not affect the uniformity of illumination. The problem becomes more complex for experiments with monochromatic light. Narrow-band interference filters are small and expensive. For that reason, the filtered light initially comes out as a narrow beam, requiring a relatively sophisticated optical arrangement to illuminate uniformly the larger surface occupied by the flies (Helfrich-Forster et al., 2002). Powerful halogen lamps are used to generate enough light within each narrow bandwidth to be tested, so that care must be taken to prevent the lamp from overheating the incubators.

Such experiments showed very early on that the clock had its own specific photosensitivity: a pulse of red light (above 600 nm) did not phase shift the *Drosophila* circadian clock at all, although the flies visually perceive such light (Frank and Zimmerman,

1969). Indeed, wild-type *Drosophila* are entrained by red LD cycles but visual-system depleted flies are not (AK and FR, unpublished), showing that the visual system can mediate entrainment, which involves prolonged (usually 12-h long) and recurrent exposures to light. In contrast, phase shifts effected by short light pulses were shown to depend primarily if not exclusively on cryptochrome, a member of a blue-light sensitive family of proteins (Stanewsky et al., 1998), consistent with the early action spectra. Similarity between the action spectra for a molecular and a behavioral response to light pulses suggested that the former may play a part in the latter (Suri et al., 1998). More recently, detailed action spectra were obtained for entrainment of various photoreceptive single and double mutants, to provide further evidence for the role of the several candidate light input pathways into the clock (Helfrich-Forster et al., 2002).

## 5. Perspectives

The fruit fly obviously offers an extremely fruitful system to study circadian clock function at both the molecular, cellular and organismal levels. Together with the unicellular mold *Neurospora* (Feldman and Hoyle, 1973), it showed against the then-prevailing opinion that clocks were amenable to genetic dissection. A further quarter-of-a-century was necessary to obtain evidence supporting a remarkable conservation of the clock molecules from flies to humans.

Since then, that conservation has been both strengthened and relativized. On the one hand, with the notable exception of *timeless*, the main components of the *Drosophila* clock have been found to participate in circadian pacemaking in all multicellular animals, with classical expansions into gene families in vertebrates (e.g. two *cry* and three *per* genes in mammals). When considering plants, *Neurospora* or cyanobacteria, the genes themselves are now different (Johnson, 2001; Loros and Dunlap, 2001; Yanovsky and Kay, 2001). However, common principles such as coupled positive and negative transcription–translation feedback loops, and/or progressive protein phosphorylation underlie the circadian clock of all organisms (Dunlap, 1999; Young and Kay, 2001). Interestingly, PAS protein–protein interactions domains are found in the transcriptional activators of *Neurospora*, flies

and mammals. The presence of PAS domains in many proteins involved in the perception of chemical signals, redox potential, or light has led to the hypothesis that clocks may have evolved from proteins whose function was to sense different aspects of the environment (Gu et al., 2000; Taylor and Zhulin, 1999). Another evolutionary link with environment sensing is cryptochrome, a blue light-sensitive FAD-containing photoreceptor. It is used by plants, along with the red-sensitive phytochromes, for light input into the clock and many other light-dependent processes, whereas it appears to be dedicated to circadian clocks in animals (Cashmore et al., 1999).

On the other hand, the same molecules have sometimes evolved to take on different roles. The absence of TIM in vertebrates seems compensated by cryptochromes, which have become an integral part of the core clock mechanism (van der Horst et al., 1999), whereas they function solely as photoreceptors in plants (Devlin and Kay, 2000), and probably in the *Drosophila* brain (Emery et al., 1998; Stanewsky et al., 1998). Indeed, in the mammalian central clock (the suprachiasmatic nuclei), CRYs are not even exposed to light. The fly may offer insights into this diversification of function, via the comparison of its central and peripheral clocks, where CRY may have different functions (Glossop and Hardin, 2002). Genetic approaches are generally much easier in *Drosophila* than in vertebrates. A further simplification is that all clock genes are present as single copies in the fly genome. However, the availability of cell lines displaying autonomous rhythms is a major advantage of mammalian systems for studies of clock biochemistry (Balsalobre et al., 1998). The *Drosophila* model is still bound to remain unsurpassed to link molecular defects to behavior, as predicted by the pioneering work of Benzer's lab in the 1970s. It may even offer unexpected perspectives on the supra-cellular organization of a central brain clock, once the specific roles of its various clock-gene expressing brain neurons will be understood. For obvious reasons, it has not been a great model for electrophysiological studies, but calcium imaging tools (Wang et al., 2003; Yu et al., 2003) may soon allow to study the activity of the clock neurons in different environmental conditions in the live animal. Finally, our understanding of the pathways processing the circadian information downstream of the clock is close to nothing. The

recent development of whole genome transcriptional analyses could be the starting point for such studies, in *Drosophila* as well as in mammals (Claridge-Chang et al., 2001; Delaunay and Laudet, 2002; McDonald and Rosbash, 2001).

### 5.1. Technical annex: measuring light

Light intensity is more precisely called irradiance, and defined as an amount of light received per unit of surface area (and, implicitly, per unit of time). Let us consider a source of light as a point which radiates electromagnetic energy equally in all directions (Fig. 5). Its power, i.e. the amount of energy radiated per unit of time, is expressed in the SI (Système International) unit of watts (W), the SI unit of time being the second. However, only part of that energy will be emitted as light visible to the human eye, i.e. as waves with wavelengths comprised roughly between 400 and 800 nm. Specific units were thus defined with respect to that very small and arbitrary, from the physicist's point of view, portion of the spectrum.

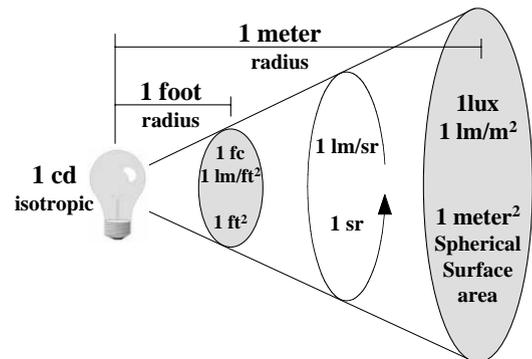


Fig. 5. Units of light. The candela (cd) is the SI unit for the total amount of light produced by the source (per unit of time). For obvious historical reasons, 1 cd roughly corresponds to the light emitted from a wax candle. One lux (1 lx) is the SI value of the amount of light received by a 1-m<sup>2</sup> area from a 1-cd source located 1 m away, whereas the lumen (lm) corresponds to the amount of light per unit of solid angle (called steradian, or sr) from a 1-cd source.

The foot-candle (fc), which is the light received by a 1 ft.<sup>2</sup> area from a 1-cd source located 1 ft. away, is the pre-metric homolog of the lux. The surface intersected by a given solid angle increases as the square of the distance to the origin. Thus 1 lm will correspond to 1 lx located 1 m away from the source, but to only 0.01 lx 10 m away, since the same amount of light is spread over a 100-fold larger surface.

The main photometric units are the candela (cd), the lumen (lm) and the lux (lx) (see the legend in Fig. 5).

The efficiency of the human eye's response to light varies greatly with wavelength, so that the relationship between photometric and energy units is quite complex. A monochromatic source emitting 1 W/sr is e.g. worth 25.9 or 73 lm if its wavelength is 450 nm (blue) or 650 nm (red), respectively, but a full 683 lm if it is 555 nm, corresponding to the sensitivity peak of human cones, the main players in daylight vision. A more objective alternative to the lux is thus an energy-based unit such as the W/m<sup>2</sup> or the mW/cm<sup>2</sup>. Sometimes both lux and mW/cm<sup>2</sup> are used in the same paper, the former for LD cycles, the latter for pulse experiments (Emery et al., 2000). When studying any biological impact of light as a function of wavelength, values given in W/m<sup>2</sup> allow to normalize the effects to the actual energy carried by the impacting light, irrespective of color. However, energy-based units do not take into account the quantal nature of light, which acts on biological molecules as a bombarding flux of photons. The number of incoming photons/cm<sup>2</sup>/s is thus the most rigorous unit when trying to produce an action spectrum, but it is relatively seldom used in circadian research (Frank and Zimmerman, 1969; Helfrich-Forster et al., 2002). One should also recognize that the corresponding correction factor is probably small relative to other uncertainties (see Section 4.1), since it is less than 2, i.e. the ratio of the relevant wavelength limits of 800 and 400 nm.

Here are a few useful conversion factors:

- 1 mW/cm<sup>2</sup> is worth 6830 lx (for 555 nm monochromatic light);
- 10<sup>14</sup> photons/cm<sup>2</sup>/s are worth 246 lx (for 555 nm monochromatic light);
- 1 fc (foot-candle) is worth 10.8 lx.

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