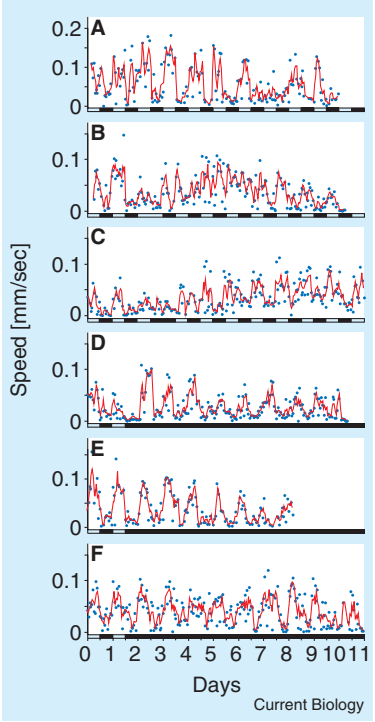

Figure 2. Fluctuation of moving speeds in *C. elegans*.
Circles show moving speeds calculated from the length of footprints. Each consecutive data triplet was smoothed with a three point sliding mean to obtain the mean velocity (mm sec^{-1}) at the central time of each data triplet. Solid lines depict the fluctuations of mean velocity thus obtained: in (A) LD, and reversed LD, beginning with (B) L and (C) D, at 20°C; (D) in DD at 15°C; (E) 20°C; and (F) 25°C. Black bars on abscissa mark dark time.

Acknowledgements

Supplementary material
Supplementary material for this article is available at http://current-biology.com/supmat/supmatin.htm.

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Caenorhabditis elegans has a circadian clock

Fred Kippert, David S. Saunders, Mark L. Blaxter

Circadian clocks, biological timing mechanisms that serve to synchronize organismal behavior and physiology with the day/night cycle, have been found in most eukaryotic organisms where they have been sought. Loci which when mutated disrupt clock function have been identified and the molecular dissection of circadian clocks has provided valuable insights into the underlying mechanisms of this universal biological phenomenon [1]. Much of this work has been carried out on a small number of model organisms such as *Neurospora* [2], *Arabidopsis* [3], *Drosophila* [4,5] and mouse [5]. However, as we recognize that clock mechanisms are more complex than previously thought, many new questions have arisen [6]. For example, the same molecules are used in very different ways in the fly and mammalian circadian clocks [4,5]. Here a second invertebrate model would be very valuable, i n particular one with a capacity for genome-wide functional analysis. The ‘other’ invertebrate model is *Caenorhabditis elegans* [7,8], in which no circadian behavior has yet been described.

Why has no circadian clock been described in *C. elegans*? In standard laboratory, mixed stage cultures *C. elegans* has a short generation time, less than

3 days at 20°C. Thus the non-circadian rhythms of the lifecycle — embryogenesis, molting and gonadal development — may mask circadian periodicity. While adults can be prevented from reproducing, by blocking fertilization or embryonic develop-ment, these treatments are insufficiently tight or may interfere with a circadian clock (our unpublished observations). In the wild, *C. elegans* will rarely meet conditions as found in the laboratory — 20–25°C and excess food — and mean lifecycle time is likely to be significantly extended. *C. elegans* has a facultative diapausal stage, the third stage larval dauer, which is formed under conditions of starvation or overcrowding [9]. Entering dauer allows the nematodes to survive over extended time spans. From an experimental perspective, however, dauer larvae are a potentially difficult tool as they have resistant cuticles and do not feed and thus may be refractory to external application of pharmacological agents or reporter gene substrates.

We therefore investigated whether other stages could be maintained as uniform populations for extended time spans. When larvae hatch from eggs in the absence of food they cannot develop further. We examined these starved first stage (L1) larvae and found that on standard plates they maintain a high level of viability for several weeks at 25°C, and several months at 15°C or 5°C. L1 could therefore be an alternative to the dauer for surviving prolonged periods of conditions not favorable for growth and development, in particular through winter.

In many organisms resistance to various stresses fluctuates through the daily cycle [10,11]. We found that the resistance of L1 to heat and hyperosmotic stress showed a daily rhythm. Hyperosmotic stress proved to be a more tractable experimental system and was investigated in more detail. Figure 1 shows the rhythm in hyperosmotic stress resistance during the last light phase of a light/dark cycle and subsequent constant darkness at 20°C. Larvae from the same culture were exposed to (Figure 1A) 1.5 M NaCl or (Figure 1B) 1.3 M NaCl for 3 hours and percentage survival was assayed when they had developed into late larvae or young adults. A sine wave was fitted by eye to the data from exposure to 1.3 M NaCl, and the period determined to be 21.9 hours. Periodogram analysis revealed a period of 22.1 hours ($p<0.0005$) which we consider to be a slight overestimation (see below). Exposure to higher salt concentrations consistently resulted in rhythms with higher amplitudes, but this was always accompanied by much more noisy data (as seen in Figure 1A), not very suitable for further analysis.

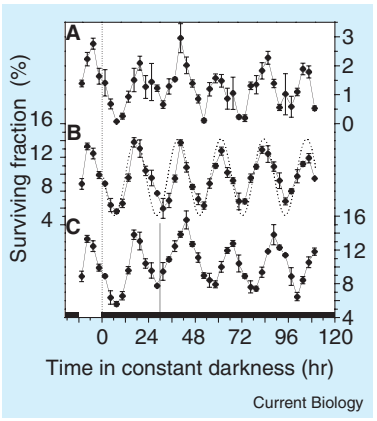

Figure 1. Circadian rhythm in resistance to hyperosmotic stress in starved larvae (L1) of *C. elegans*.
Eggs were prepared according to standard protocols and purified by Ficoll floatation. They were allowed to hatch overnight in the absence of food bacteria and then further purified by a second Ficoll flotation. The culture of starved larvae was maintained at a concentration of 10–50 larvae/µl in M9 buffer on a rotary shaker at 20°C (±0.2°C). A cocktail of antibiotics was added to prevent fungal and bacterial growth. The animals were subjected to 4–6 light/dark cycles of 12:12 hours. During the last light phase and in subsequent constant darkness 20 µl samples were taken in duplicate every 3 hours and added in a 96 well plate to 15 µl of M9 buffer containing NaCl to make up final concentrations of (A) 1.5 M or (B) 1.3 M. The larvae remained in the salt solution for 3 hr and 30 µl were then transferred into a 24 well plate with standard agar and *E. coli* as food source. The number of nematodes surviving the treatment was counted when they had developed into late larvae or young adults. The data shown are the mean of the duplicate samples with standard deviation. The period of the sine wave in (B) (fitted by eye) is 21.9 hr. (C) A part of the culture was removed and subjected to a 1 hr light pulse (shaded bar) at 500 lux.

C. elegans has been shown to be photoresponsive [12] and the action of light as a zeitgeber, or entraining stimulus, is evident from Figure 1A and B. The rhythm can be phase-shifted efficiently by moderate light pulses, such as 1 hour at 500 lux, which produced a delay of about 6 hours (Figure 1C). This change in phase following a light pulse and the free-running period, which is slightly but significantly shorter than the natural day, show the endogenous nature of the rhythm and exclude any uncontrolled exogenous effect.

A defining characteristic of circadian clocks is that the period is temperature compensated. We investigated the hyperosmotic stress resistance rhythm between 10 and 25°C and found little difference in period over this range. The periods derived from fitting sine waves by eye ranged from 21.1 hours at 25°C to 22.8 hours at 10°C (Figure 2). Using such a fit by 'expert eye' has been shown to be a useful method in biological rhythm research [13]. On the other hand, periodogram analysis, although important tool [14], is not accurate with shorter time series. It consistently overestimated the period by about 0.7 to 1 hours (Supplementary Figure S1A–E). Because the shape of the rhythm is quite regular, the error in periods derived from sine waves fitted by eye is unlikely to exceed ±0.5 hours (Supplementary Figure S1F–H). From the data shown in Figure 2, a Q_{10} of 1.06 can be calculated and this is

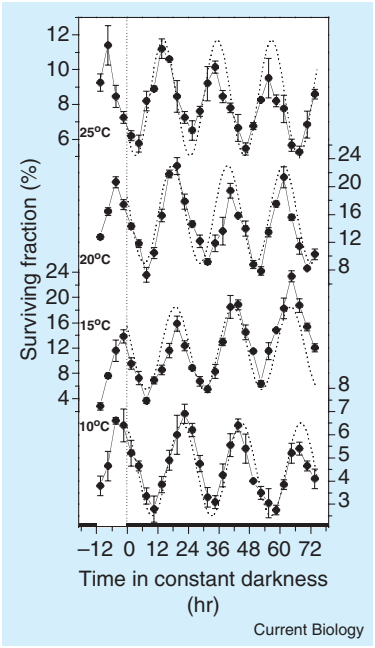

Figure 2. Temperature compensation of the circadian clock of *C. elegans* between 10 and 25°C.
Starved larvae were prepared, as for Figure 1, from cultures maintained at the indicated temperatures for at least 2 generations. Duplicate samples were withdrawn at the indicated time points and treated with 1.3 M NaCl for 3 hr. The data shown are the mean of the duplicate samples with standard deviation. The periods of the sine waves (fitted by eye) are 21.1 hr at 25°C, 21.5 hr at 20°C, 22.4 hr at 15°C and 22.8 hr at 10°C. Stress treatment and recovery after the hyperosmotic shock were at the experimental temperatures. The mean levels of stress tolerance observed reflect variability between individual cultures and not a systematic pattern with temperature. A dependence of phase on temperature was consistently observed.

consistent with observations from additional experiments (data not shown). However, the determination of an accurate Q_{10} will require much more data, in particular longer free-run experiments, and will therefore be addressed only once automated recording of *C. elegans* rhythms using appropriate reporter genes has been established. With a Q_{10} of about 1.06, the period is well compensated over the temperature range in which the organism grows and reproduces.

In contrast to the period, the phase relationship to the light/dark cycle showed a marked dependency on temperature. This may reflect the adaptive value of the rhythm as in the natural habitat of the worm, higher temperatures will cause hyper-osmotic stress earlier in the day.

The hyperosmotic stress resistance rhythm fulfils all the major criteria for a circadian clock. It has a free-running circadian period, is entrained by light and the period is temperature compensated. It is a robust and reproducible phenomenon which now allows the further exploration of the circadian system of *C. elegans*. Circadian rhythms in the expression of genes involved in conferring salt and heat resistance are being investigated and this should lead to the development of suitable reporter gene constructs. This will in turn enable the large-scale analysis of the *C. elegans* circadian clock, making use of the possibilities of functional genomics analysis such as the genome-wide study of gene function by RNAi [15].

Our results identify a second invertebrate system with the advantages of a completely sequenced genome and easy molecular genetic manipulation. Although it may be possible to extend the analysis to other stages, the L1 system offers substantial advantages: it is a minimal system with the animals having no activities other than waiting for better times to come; experiments can be performed over extended time spans and a wide temperature range; the completely transparent body of only 558 cells should ultimately enable us to follow cellular movements of clock molecules *in vivo*.

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Supplementary material
Supplementary material for this article is available at http://current-biology.com/supmat/supmatin.htm.

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