Genes, Brain and Behavior
Official publication of the International Behavioural and Neural Genetics Society

Genes, Brain and Behavior (2015) ***14:*** *493–501* doi: 10.1111/gbb.12231

Pigment-dispersing factor signaling in the circadian system of *Caenorhabditis elegans*

A. Herrero[†,1], A. Romanowski[†,1], E. Meelkop[‡,1], C. S. Caldart[†], L. Schoofs[‡] and D. A. Golombek[†,*]

[†] Laboratorio de Cronobiología, Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Buenos Aires, Argentina, and [‡] Animal Physiology and Neurobiology Section, Department of Biology, KU Leuven, Leuven, Belgium
[1] These authors contributed equally to this work.

**Corresponding author: Dr D. A. Golombek, Laboratorio de Cronobiologia, Departamrnto de Ciencia y Tecnologia, Universidad Nacional de Quilmes, Roque Saenz Peña 352, Bernal B1876BXD, Buenos Aires, Argentina. E-mail: dgolombek@unq.edu.ar*

The neuropeptide pigment-dispersing factor (PDF) is important for the generation and entrainment of circadian rhythms in the fruitfly *Drosophila melanogaster*. Recently two *pdf* homologs, *pdf-1* and *pdf-2*, and a PDF receptor, *pdfr-1*, have been found in *Caenorhabditis elegans* and have been implicated in locomotor activity. In this work, we have studied the role of the PDF neuropeptide in the circadian system of *C. elegans* and found that both *pdf-1* and *pdf-2* mutants affect the normal locomotor activity outputs. In particular, loss of *pdf-1* induced circadian arrhythmicity under both light–dark (LD) and constant dark (DD) conditions. These defects can be rescued by a genomic copy of the *pdf-1 locus*. Our results indicate that PDF-1 is involved in rhythm generation and in the synchronization to LD cycles, as rhythmic patterns of activity rapidly disappear when *pdf-1* mutants are recorded under both entrained and free-running conditions. The role of PDF-2 and the PDF receptors is probably more complex and involves the interaction between the two *pdf* paralogues found in the nematode.

Keywords: *C. elegans*, circadian, clock genes, locomotor activity, nematode, neuropeptide, pdf, *pdf-1*, *pdf-2*, pigment-dispersing factor

Received 28 January 2015, revised 18 June 2015, accepted for publication 24 June 2015

Circadian rhythms are ubiquitous in nature and their molecular background appears to be relatively conserved throughout the Animal Kingdom (Rosbash 2009; Yu & Hardin 2006). Central and peripheral circadian clocks depend on the activity of 'clock genes' underlying a transcription–translation molecular feedback loop that generates endogenous rhythmicity with a period close to 24 h, which, in turn, is entrained to the solar day by environmental signals (Golombek & Rosenstein 2010).

The nematode *Caenorhabditis elegans* exhibits circadian rhythms in locomotor activity, abiotic and biotic stress tolerance, olfaction and metabolism, among other physiological processes (Hasegawa *et al.* 2005; Kippert *et al.* 2002; Migliori *et al.* 2011; Olmedo *et al.* 2012; Romanowski *et al.* 2011; Saigusa *et al.* 2002; Simonetta & Golombek 2007; Simonetta *et al.* 2008, 2009). In addition, the *C. elegans* genome contains homologs to clock genes present in well-known circadian models such as *Drosophila melanogaster*, including *lin-42* (*period*), *tim-1* (*timeless*), *kin-20* (*doubletime*), *sma-9* (*clock*), *aha-1* (*cycle*) and *atf-2* (*vrille*) (Banerjee *et al.* 2005; Chan *et al.* 2003; Hasegawa *et al.* 2005; Jeon *et al.* 1999; Tennessen *et al.* 2006, 2010). While these genes have shown to be involved in developmental processes in the worm, the molecular circadian clock of *C. elegans* still remains elusive. A recent genome-wide analysis, although identifying a large subset of light- and temperature-entrained oscillating transcripts from the *C. elegans* genome (with just two genes in common between the two sets), failed to find significant rhythms in the expression of clock gene homologs (Van Der Linden *et al.* 2010).

A promising candidate for the *C. elegans* circadian clockwork is the pigment-dispersing factor (PDF) and its receptor. In *D. melanogaster*, *pdf* is expressed in the relatively small subset of lateral neurons that comprise the central circadian clock (Chang 2006; Nitabach & Taghert 2008; Taghert & Shafer 2006) and its expression is required in order to maintain adequate cycling of locomotor activity (Renn *et al.* 1999) and oscillator cell coupling (Lin *et al.* 2004; Peng *et al.* 2003). Two PDF-encoding genes have been identified in *C. elegans* (*pdf-1* and *pdf-2*) (Janssen *et al.* 2009), which resemble the crustacean PDH system (Meelkop *et al.* 2012a) and whose products interact with different splice forms of the specific PDF receptor (Janssen *et al.* 2008). Besides playing a role in male reproduction (Barrios *et al.* 2012), the nematode PDF system is involved in the regulation of locomotion (Meelkop *et al.* 2012b) and behavioral states of arousal (Flavell *et al.* 2013) and, therefore, we hypothesized that this peptide family plays an important role in the genesis and entrainment of locomotor activity circadian rhythms in *C. elegans*.

Materials and methods

General methods and strains

The following *C. elegans* strains were used: N2 (Bristol strain, *wild type*), LSC27 *pdf-1(tm1996)*, bearing a 588-bp deletion that affects the first exon of both products PDF-1a and PDF-1b; LSC40 *pdf-2(tm4393)*, bearing a 401-bp deletion that affects the first exon of the gene; LSC39 *pdfr-1(lst34)*, bearing an 855-bp deletion that affects the three

splicing variants of the only PDF receptor encoding gene known to date (*pdfr-1*); LSC10 *pdf-1(tm1996);lstIs1[pdf-1(+);Pelt-2::GFP];* the double mutant LSC41 *pdf-1(tm1996); pdf-2(tm4393); LSC84 = N2; lstEx2[pdf-1 +] and LSC54 = N2; lstEx3 [pdf-2 +]*. The N2 strain was provided by the Caenorhabditis Genetics Center, University of Minnesota, MN, USA. The mutant strains, rescue strains, overexpressing strains and double mutants of PDF-related genes were generated in Dr Liliane Schoofs' laboratory (Janssen *et al.* 2008, 2009; Meelkop *et al.* 2012b).

Nematode stocks were maintained in Nematode Growth Medium (NGM) plates (0.3% NaCl, 0.25% peptone, 5 μg/ml cholesterol, 1 mmol/l $CaCl_2$, 1 mmol/l $MgSO_4$, 1.7% agar in pH 6.0 25 mmol/l potassium phosphate buffer) covered with thick bacterial lawns of *Escherichia coli* HB101, in light:dark (LD) cycles 12:12 h, with lights on at 0900 h (defined as *zeitgeber time 0* or ZT0). Temperature conditions were kept constant at 16°C.

All chemical compounds were bought from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise specified.

Locomotor activity assay and experimental conditions

The locomotor activity of nematode populations was recorded as previously described (Simonetta *et al.* 2009) with some minor modifications. In order to perform the locomotor activity assays, nematode populations were synchronized to the same developmental stage by the chlorine method (Lewis & Fleming 1995). The harvested eggs were cultured overnight in a 50-ml Erlenmeyer flask with 3.5 ml of M9 buffer (42 mM Na_2HPO4, 22 mM KH_2PO4, 85,5 mM NaCl, 1 mM MgSO4) + antibiotic–antimycotic 1× (Gibco, Carlsbad, CA), at 110 rpm, 18.5°C and under LD 12:12 h conditions. The next day, L1 larvae were transferred to NGM plates with *E. coli* OP50. When nematodes reached the L4 stage, they were transferred to a 96-well plate (approximately five to seven nematodes per well) in 40 μl of L15 medium (Gibco) supplemented with 1.5× antibiotic–antimycotic (Gibco) + 40 μM 5-fluoro-2′-deoxyuridine (FUdR) + cholesterol 5 mg/ml + 2% v/v skim milk. Plates were covered with an optic film to avoid evaporation and in each well the film was perforated twice to avoid condensation. Nematode population recordings are preferred to single worm recordings because of the higher amplitude of activity due to a higher number of infrared beam crosses. Individual recordings, although rhythmic, exhibit a low signal-to-noise ratio and are therefore not suitable for accurate circadian analysis.

When nematodes had to be handled in dark conditions, a red light with intensity lower than 1 lux was used. This type of light has no effects over *C. elegans* synchronization (Simonetta *et al.* 2009).

For light synchronization experiments, nematodes were subjected to LD cycles (400:0 lux 12:12 h) under constant temperature (17.5°C). Stability of the incubator temperature was checked with an IButton sensor model DS1921H-F5 (Maxim Integrated Products Inc., Sunnyvale, CA, USA), programmed to take one sample every 5 min. This sensor provides a resolution of 0.125°C. There was no temperature variation in the recording chamber throughout the experiments (within the resolution of the temperature sensor). The light source employed throughout the experiments was a Compact Fluorescent Lamp Philips Essential PLE15W230, and it is interesting to note that the spectrum peaks in the green and blue zones, coincident with what has been reported for *C. elegans*' photic sensitivity (Burr 1985; Edwards *et al.* 2008).

For experiments in constant conditions, nematodes were kept at a constant temperature (17.5°C) under constant darkness (DD).

Locomotor activity data acquisition and analysis

Locomotor activity (swimming behavior) data were sampled at 1-min intervals and binned in 30-min blocks. The raw data were detrended and normalized by dividing each data point by the corresponding point of a trend curve (fitted by a 72-h low-pass Butterworth filter), as described (Levine *et al.* 2002b). The Butterworth filter, similar to a low- and high-pass filtering with moving average, is widely applied in noisy data (such as the analysis of fly courtship songs) or for the analysis of locomotor activity of flies in order to remove the high-frequency components and avoid possible artifacts in peak detection (Levine *et al.* 2002b; Wright 1997). High frequencies were removed by a 4 h moving average window. Animals with very low activity levels (<20% of average population activity) were discarded.

For synchronization experiments, the filtered data were analyzed by Cosinor analysis (best fit to a 24-h cosine waveform). Nematodes presenting a statistically significant rhythm were used for this analysis; actograms were constructed (with the scale adjusted according to the lower threshold) for better visualization of rhythms. The acrophases (time at peak) for each group of five to seven worms were scored and grouped for histogram plotting and then further analyzed by circular statistics (Rayleigh test) (Levine *et al.* 2002a).

In order to determine the percentage of truly entrained nematodes, the acrophase of the population in the last 3 days of entrained conditions was compared to that of the first day of free-running conditions and phase transition (PTC) plots were constructed (Pittendrigh 1981).

The circadian period in each phase of the assay (entrained and free-running conditions) was determined by arithmetic-averaging of the period of each population of five to seven nematodes, obtained by Lomb–Scargle periodogram analysis.

Nematode populations are visually inspected after each locomotor activity assay. Wells that suffered contamination or that did not have live populations were discarded from further analysis. In addition, we performed lifespan assays for the different wild-type and mutant populations under study, by using the same equipment. All of the strains exhibited a similar, and relatively low, decrease in activity as nematodes age (data not shown).

All values are expressed as the mean value ± SE, unless otherwise noted.

Results

Circadian rhythms of locomotor activity require functional pdf-1 expression

To understand the role of the PDF neuropeptide in the generation and light synchronization of locomotor activity rhythms of *C. elegans*, the entraining capability of several mutant strains in genes related to PDF was studied. Because of the great variability previously observed in the activity pattern of individual nematodes, population studies were performed (Simonetta & Golombek 2007; Simonetta *et al.* 2009). The locomotor activity of adult nematodes was assayed for 7 days under light:dark (LD) 12:12 h conditions and then for a further 7 days under constant darkness (DD).

As previously reported, more than one third of the N2 strain nematode populations recorded exhibited robust circadian rhythms under an LD cycle, which were maintained under constant conditions for at least 1 week, with an average circadian period of 23.9 ± 0.18 h (Fig. 1a). However, the *pdf-1* mutant did not show diurnal or circadian rhythms under LD or DD conditions, respectively (Fig. 1b). As shown in Fig. 1c, the *pdf-1* rescue strain recovered normal circadian rhythms with parameters that were similar to the N2 strain (with a circadian period of 24.08 ± 0.15 h). Although with a higher level of variability, *pdf-2* mutants tended to entrain to the LD cycle but exhibited weaker rhythms under DD (with a 23.9 ± 0.29 h period, Fig. 1d). Even though the LD rhythmicity pattern in this strain is not self-evident in the actograms depicted in Fig. 1d, the raw data analysis shows a clear rhythmic pattern close to 24 h under entraining conditions that is lost upon transfer to constant conditions, as determined by Lomb-Scargle periodogram analysis. Interestingly, the double *pdf-1;pdf-2* mutant showed normal rhythms, as shown in the corresponding actograms in Fig. 1e (with a circadian

period of 24.1 ± 0.34 h). Mutants defective in *pdfr-1* tended to entrain to LD cycles (see Fig. 3) but also presented very weak, although significant, rhythms under constant conditions (circadian period of 23.1 ± 0.44 h, Fig. 1f). In the case of this strain, even though the general activity remains high, there is a subtle amplitude modulation of the activity with a period close to 24 h, as determined by Lomb-Scargle periodogram analysis.

We also tested strains overexpressing each of the two *C. elegans pdf* paralogues. The *pdf-1* overexpressing strain exhibited normal rhythms, as shown in Fig. 1g (circadian period of 23.83 ± 0.15). A similar result was obtained for the *pdf-2* overexpressing strain (circadian period of 23.5 ± 0.27 h, Fig. 1h).

In all cases where significant rhythms were found under an LD cycle, onset phase tends to be maintained upon transfer to constant conditions, suggesting that true entrainment, rather than masking, was achieved.

The right panel in Fig. 1 shows the distribution of circadian periods along the strains (only for rhythmic populations), which was adjusted to Gaussian curves in the cases where significant circadian rhythms were found. Again, there is a clear dispersion of activity for the *pdf-1* mutants as compared with the rest of the strains.

Figure 2 shows the percentage of samples that exhibited significant diurnal (i.e. under LD conditions) or circadian (DD) rhythms in locomotor activity. Again, the most striking result is the lack of rhythmicity in the *pdf-1* strain, completely rescued by the microinjection of a genomic fragment containing the regulatory and coding regions of the *pdf-1* locus, and the failure of *pdf-2* mutants to sustain circadian cycles in constant conditions. It is interesting to note that both of the *pdf-1* or *pdf-2* overexpression strains are very similar to either N2 or the *pdf-1* rescue strain. The percentages of rhythmic and synchronized animals are statistically similar to those of the N2 strain.

The phase of the circadian rhythm was calculated as the onset for locomotor activity. As seen in Fig. 3, in the case of the N2 (Fig. 3a), *pdf-1* rescue (Fig. 3c), the double mutant (Fig. 3e), *pdfr-1* (Fig. 3f), *pdf-1*+ (Fig. 3g) and *pdf-2*+ (Fig 3h), a clear nocturnal phase can be found. The phase clustering is not clear in the *pdf-2* mutant strain (Fig. 3d), while the relatively low proportion of animals from the *pdf-1* strain that did exhibit diurnal rhythms did not show a tendency toward a coherent locomotor activity onset (Fig. 3b). It should be noted that the *pdf-1* rescue strain did recover circadian rhythmicity (as shown before) but the nematodes now exhibit a ∼4 h phase delay with respect to the N2 controls and the phase appears to be more homogeneous among the population. It is interesting to note that even though the *pdfr-1* strain exhibits a shorter period, the preservation of rhythms in the receptor mutant is consistent with the preservation of rhythms in the *pdf-1;pdf-2* ligands double mutant.

Discussion

PDF is involved in conveying rhythmic information in *D. melanogaster* (Hardin 2005; Peschel & Helfrich-Forster 2011; Renn *et al.* 1999), and the presence of two homologues in *C. elegans* led us to question whether this peptide is also involved in rhythm generation in this organism. In order to do so, we chose to study locomotor activity rhythms, which allow a continuous readout of activity (Simonetta & Golombek 2007). As the locomotor activity rhythms of *C. elegans* show a high interindividual variability, probably because this parameter does not constitute a very robust output of the biological clock of this organism, nematode populations conformed of five to seven individual nematodes were used.

The results obtained with the N2 strain are in accordance with what was previously published (Simonetta *et al.* 2009), with clear circadian rhythms both under entrained (LD) and free-running (DD) conditions. The acrophase histogram analysis indicated nocturnal activity and, moreover, as the onset phase was maintained in the LD–DD transition, masking from the *zeitgeber* could be ruled out.

When we characterized the locomotor activity of the *pdf-1* mutant strain, we found that the loss of this peptide induced circadian arrhythmicity under both LD and DD conditions. These defects can be rescued by a genomic copy of the *pdf-1 locus* (Figs. 1c and 3c). The rescue strain even outperforms the N2 strain in terms of the percentage of rhythmic worms, which could be an effect of the strain bearing multiple copies of the transgene that are usually associated with the microinjection technique, therefore causing a transgene expression different from that of the wild-type strain (Evans 2006). In other words, this 'better' performance of the *pdf-1* rescue strain might be due to a slightly different expression of the transgene when compared with that from the wild-type strain, which normally occurs with genomic rescues of mutant *C. elegans* due to the multiple copies that constitute the extrachromosomal arrays that are formed in microinjection-based transgenic lines. Several regulatory processes govern the expression of these extrachromosomal arrays, resulting in a slightly different expression and regulation than that of wild-type nematodes. However, although the pdf-1 rescue populations exhibit a slightly higher percentage of rhythmic animals in both LD and DD, the differences are not statistically significant from the controls, as determined by Fisher's *G*-test ($P > 0.05$). Therefore, the effect that can be seen in the actograms appears to be related to an increase in amplitude of the locomotor activity rhythm and not on the overall percentage of rhythmic populations.

The *pdf-2* mutant population, although entrained by the LD cycle, exhibited a decrease in the percentage of animals with significant free-running rhythms. The *pdf-1*+ and *pdf-2*+ overexpressing strains were shown to be remarkably similar to the wild-type nematodes and showed no statistical differences in terms of the percentage of rhythmic and synchronized populations. It is interesting to note that the *pdf-1; pdf-2* double mutant strain showed activity patterns strikingly similar to the N2 strain. Indeed, this has been previously reported in two other behaviors, *swimming activity* and *number of reversals* (Meelkop *et al.* 2012b). In this work, it was shown that *pdf-1* and *pdfr-1* mutants had lower *swimming speed* than the N2 strain, but both *pdf-2* and *pdf-1;pdf-2* mutants showed a wild-type-like speed. Although this might be considered a limitation, as swimming speed and reversal number could affect beam crossing and, therefore, rhythmicity

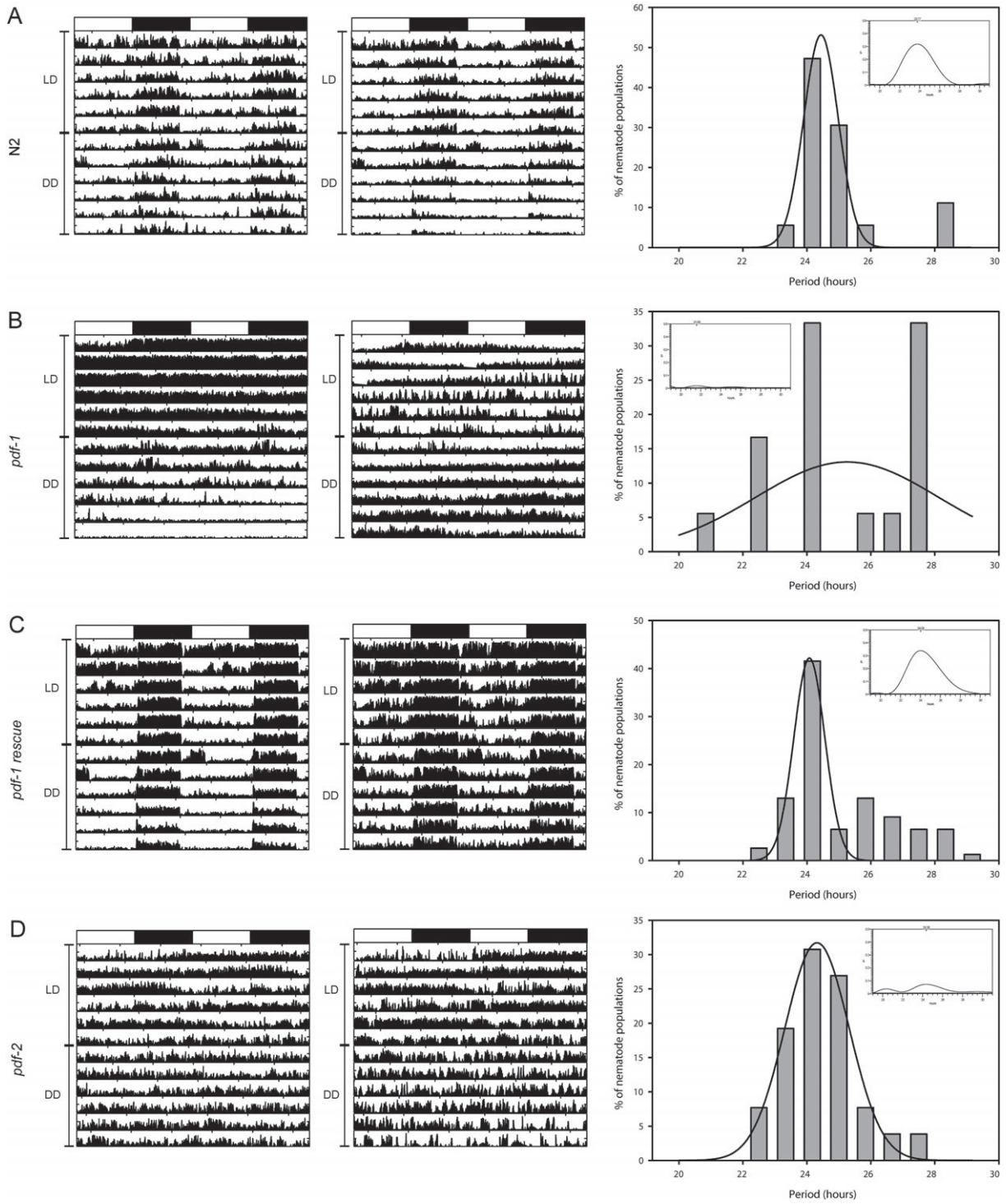

Figure 1: Representative actograms of the different *C. elegans* strains assayed in this study. The representative actograms of two populations of (a) N2, (b) *pdf-1*, (c) *pdf-1 rescue*, (d) *pdf-2*, (e) *pdf-1;pdf-2*, (f) *pdfr-1*, (g) *pdf-1*+ and (h) *pdf-2*+ strains are shown. *pdf-1* mutants lost synchronization and appeared arrhythmic under constant conditions; this phenotype was recovered in a *pdf-1* rescue strain. *pdf-2* mutants tended to lose rhythmicity under constant conditions, while *pdf-1;pdf-2*, *pdfr-1* and overexpressing mutants did not show significant changes in their circadian parameters. The right panels show the frequency distribution of circadian periods for all strains and a representative periodogram of a single population under DD conditions (inset).

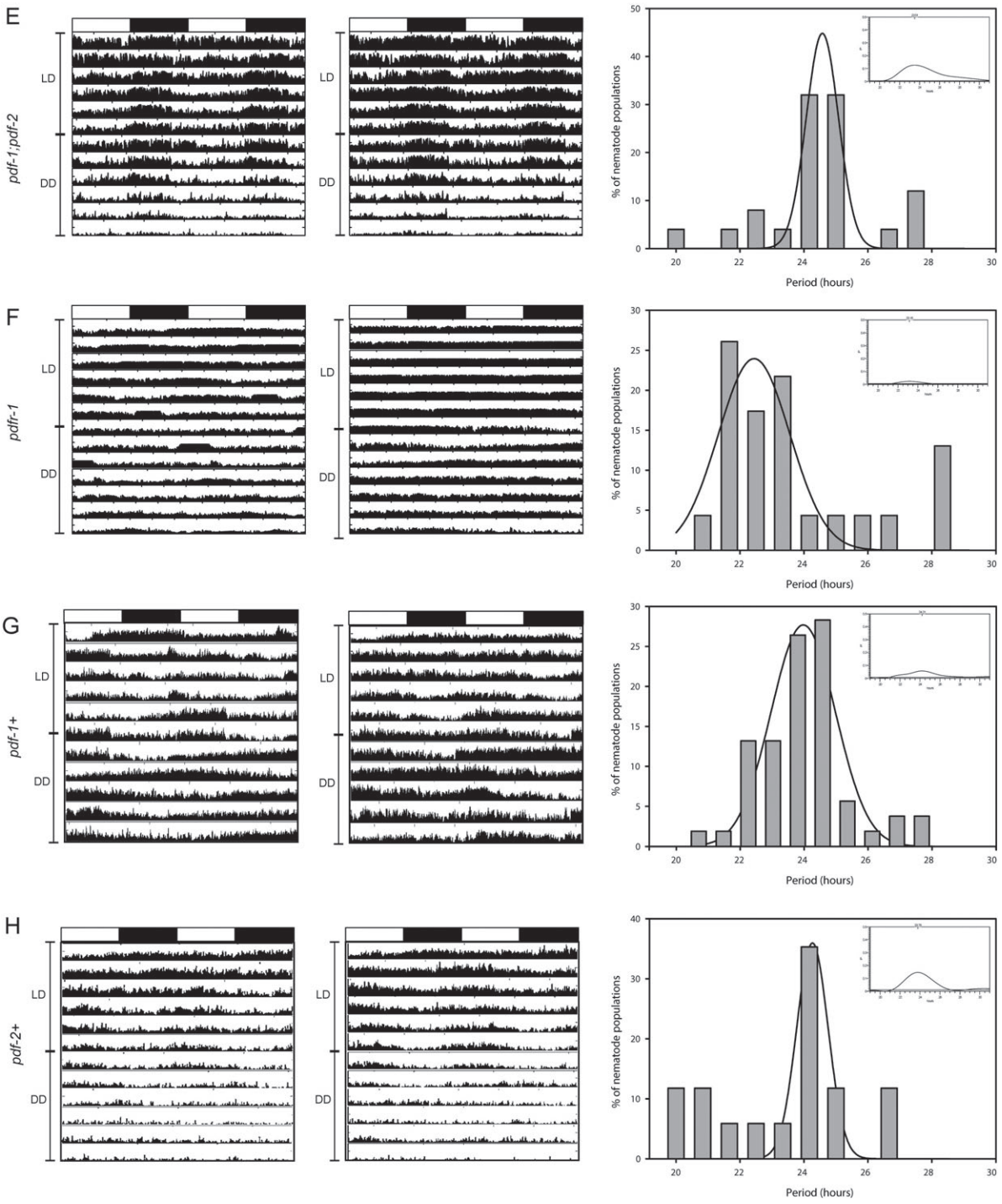

Figure 1: Continued.

recordings, we found no significant changes in overall locomotor activity assay recordings. Also, the *pdf-2* strain exhibited fewer reversals than the N2, whereas *pdf-1* and *pdfr-1* showed more reversals; the double mutant was similar to the wild-type controls. This fact, as well as our results suggest an antagonistic role for the PDF-1 and PDF-2 peptides, as was previously proposed (Janssen *et al.* 2008; Meelkop *et al.* 2012b).

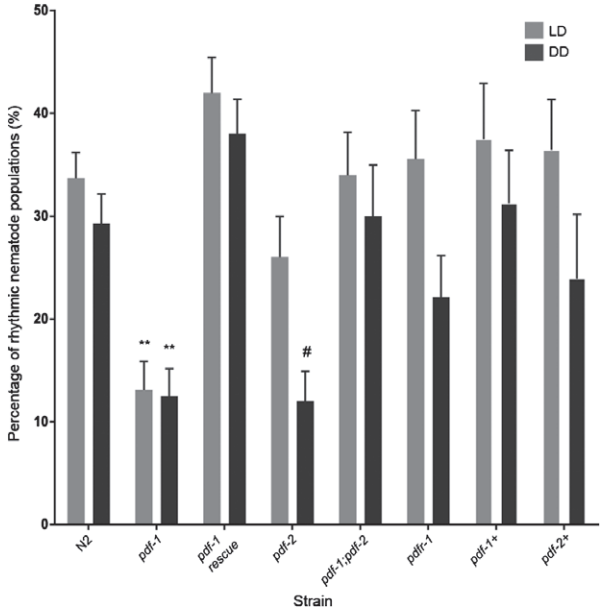

Figure 2: Percentage of rhythmic animals under entrained (LD) and free-running (DD) conditions. The bars indicate the percentage of rhythmic populations under LD 12:12 h conditions and after transfer to constant conditions (DD) [$^{*}P < 0.01$; $^{\#}P < 0.08$ vs. respective (LD or DD) controls from the N2 strain, two-tailed Fisher's exact test]. The error bars indicate the standard error of percentage (SEP).

Animals lacking a functional *pdfr-1* did not show significant differences with the N2 controls in terms of statistical analysis, although the locomotor pattern appears much noisier than in the controls, as suggested by visual inspection of the actograms. These results suggest that there might be alternate routes for PDF signaling other than the known *C. elegans* PDF receptors, a possibility that remains to be further investigated.

In the case of the *pdf-1* and the *pdfr-1* mutants, as well as with the other strains, the results could be indicative of aging-related differences. However, when we performed lifespan assays we found no differences among the strains under study and, moreover, no overall changes in general activity (data not shown).

In *D. melanogaster*, PDF is required to maintain activity rhythms under constant conditions (Renn *et al.* 1999) and to synchronize the amplitude and phase of individual oscillator cells (Lin *et al.* 2004; Peng *et al.* 2003; Peschel & Helfrich-Forster 2011). *Drosophila melanogaster* has a bimodal activity rhythm with morning and afternoon–night transition peaks. *Pdf* mutant flies maintain rhythmicity under LD cycles, although they lose their morning anticipatory behavior and show a 2-h phase advance in their maximum activity in the afternoon to night transition. After 3 days in DD conditions, *pdf* mutants completely lose their rhythmicity, which reflects the gradual loss of synchronization by PDF (Peschel & Helfrich-Forster 2011; Renn *et al.* 1999). In fruit flies, the loss of the PDFR affects rhythms at the individual and not at the population level. Under laboratory conditions, *D. melanogaster* activity patterns exhibit two main components, a morning component (M) and an evening component (E). PDFR mutants completely lack the M component, showing a unimodal pattern of activity instead of the wild-type bimodal pattern (Hyun *et al.* 2005; Lear *et al.* 2005; Renn *et al.* 1999).

The effect of *pdf* mutants on circadian rhythms could be due to an underlying cycle in the expression of the genes. However, we determined by qRT-PCR that the mRNA levels of *pdf-1*, *pdf-2* and the PDF receptor *pdfr-1* do not cycle in *C. elegans* subjected to 12:12 h LD cycles and then released into free-running conditions (data not shown). This reveals another similarity with *D. melanogaster* (Park & Hall 1998). Different reports have shown the existence of a daily rhythm in the immunoreactivity of PDF, limited only to the axon terminals projected from the small ventrolateral neurons (s-LNvs) (Park *et al.* 2000; Wu *et al.* 2008). *Gryllus bimaculatus* and *D. melanogaster* show a daily rhythm in PDF release (Abdelsalam *et al.* 2008; Park *et al.* 2000; Wu *et al.* 2008), meanwhile other insects such as *Apis mellifera* do not (Bloch *et al.* 2003). It still has to be determined which is the case for *C. elegans*.

Overall, this study allows us to conclude that the PDF role in the generation of circadian rhythms is evolutionary conserved, from crustaceans to arthropods and also in *C. elegans*. In this nematode, PDF-1 peptides are involved in rhythm generation and in the synchronization to LD cycles. Rhythmic patterns of activity rapidly disappear when *pdf-1* mutants are recorded under both entrained and free-running conditions. The role of PDF-2 and the PDF receptors is probably more complex and involves the interaction between the two *pdf* paralogues found in the nematode.

A recent study has reported large-amplitude oscillations of gene expression in *C. elegans* larvae, although only short periods (i.e. 8 h) were considered. Moreover, these oscillations were phase-locked, suggesting robust synchronization of gene expression along development (Hendriks *et al.* 2014). However, the influence of such oscillations on overt behavior, including locomotion, remains to be determined.

Besides locomotor activity patterns, there are other oscillating behavioral changes in *C. elegans* that appear to be regulated by neuropeptides. These nematodes exhibit a periodic alternation between quiescent and active states (Fujiwara *et al.* 2002; Raizen *et al.* 2008); in addition, there is a clear alternation between dwelling (i.e. local movements) and roaming (i.e. active migration) (Ben Arous *et al.* 2009). Recent reports have emphasized the role of neuromodulators in the regulation of these behavioral states. For example, the neuropeptide NLP-22 is expressed rhythmically in consonance with lethargic state and, more interestingly, it is regulated by the clock gene period orthologue *lin-42*. Moreover, forced expression of *nlp-22* causes quiescence in freely moving and feeding nematodes (Nelson *et al.* 2013). In addition, the neuropeptide receptor NPR-1 is also related to the regulation of lethargus; NPR-1 knockout mutants did not exhibit quiescent behavior (Choi *et al.* 2013).

This changes in quiescence and locomotion across larval stages has also been reported to depend on PDF-1. Indeed, an increase in its secretion induced lethargic-like

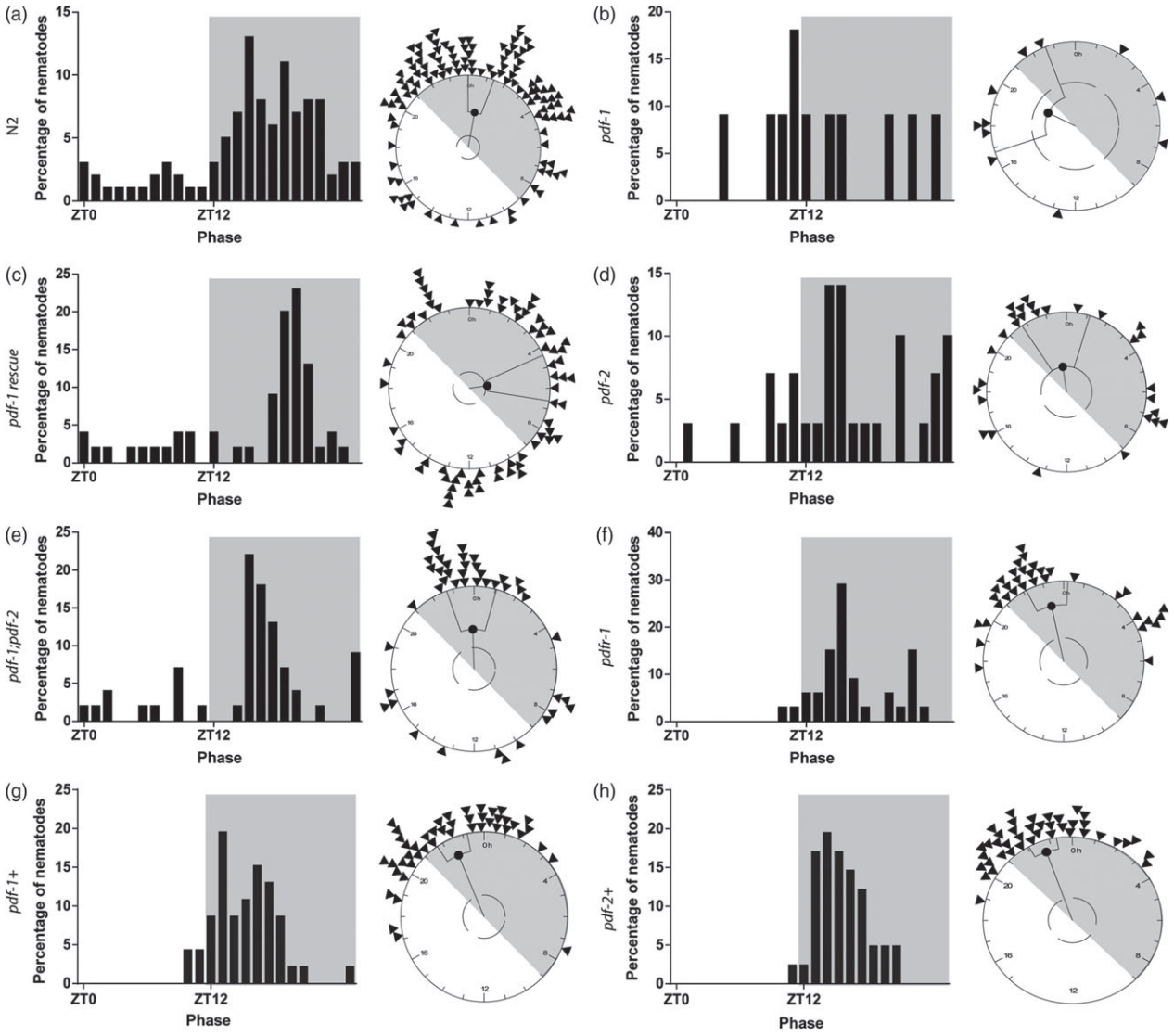

Figure 3: Phase distribution of rhythmic nematodes under LD conditions. The histograms represent the percentage of rhythmic populations with their respective phases (measured as locomotor activity onset) throughout the day [with zeitgeber time (ZT) – 12, defined as the time of lights off). (a) N2, (b) *pdf-1*, (c) *pdf-1 rescue*, (d) *pdf-2*, (e) *pdf-1;pdf-2*, (f) *pdfr-1*, (g) *pdf-1*+ and (h) *pdf-2*+. All nematode strains with the exception of the pdf-1 mutant exhibited nocturnal activity (i.e. locomotor activity onset). The relatively few pdf-1 mutants that did exhibit significant rhythmicity appear to have their onset phase distributed randomly throughout the day. The right panels show the Rayleigh analysis (phase plots) for all strains.

behavior, probably mediated by the PDF receptors (Choi *et al.* 2013). However, PDF also promotes locomotion in an exploration-like behavioral paradigm (Barrios *et al.* 2012; Janssen *et al.* 2008). Recently, this PDF role was incorporated into a neural modulatory circuit that also includes serotonergic neurons: while PDF promoted roaming, serotonin release induced dwelling-type locomotion (Flavell *et al.* 2013). Of course, there is a lot of variation in these behaviors, which depend on feeding and substrate conditions and age, among other variables, but the important message here is that locomotor activity patterns throughout development are regulated by specific neuromodulatory circuits. While several homologues or orthologues of circadian clock genes play a developmental role in nematodes (e.g. Jeon *et al.* 1999; Monsalve *et al.* 2011), the PDF appears to have retained its circadian functionality, in terms of the *Drosophila* circadian system and, as described in this study, in *C. elegans*.

In this sense, the PDF system of *C. elegans* has many similarities with the system described in *D. melanogaster* and fulfils an important role in *C. elegans* adaptation to its surrounding environment, allowing this organism to adequately predict daily changes that occur in nature and, in addition, is

part of the molecular oscillator that originates endogenous circadian rhythms.

References

Abdelsalam, S., Uemura, H., Umezaki, Y., Saifullah, A.S., Shimohigashi, M. & Tomioka, K. (2008) Characterization of PDF-immunoreactive neurons in the optic lobe and cerebral lobe of the cricket, *Gryllus bimaculatus*. *J Insect Physiol* **54**, 1205–1212.

Banerjee, D., Kwok, A., Lin, S.Y. & Slack, F.J. (2005) Developmental timing in *C. elegans* is regulated by kin-20 and tim-1, homologs of core circadian clock genes. *Dev Cell* **8**, 287–295.

Barrios, A., Ghosh, R., Fang, C., Emmons, S.W. & Barr, M.M. (2012) PDF-1 neuropeptide signaling modulates a neural circuit for mate-searching behavior in *C. elegans*. *Nat Neurosci* **15**, 1675–1682.

Ben Arous, J., Laffont, S. & Chatenay, D. (2009) Molecular and sensory basis of a food related two-state behavior in *C. elegans*. *PLoS One* **4**, e7584.

Bloch, G., Solomon, S.M., Robinson, G.E. & Fahrbach, S.E. (2003) Patterns of PERIOD and pigment-dispersing hormone immunoreactivity in the brain of the European honeybee (Apis mellifera): age- and time-related plasticity. *J Comp Neurol* **464**, 269–284.

Burr, A.H. (1985) The photomovement of *Caenorhabditis elegans*, a nematode which lacks ocelli. Proof that the response is to light not radiant heating. *Photochem Photobiol* **41**, 577–582.

Chan, R.C., Chan, A., Jeon, M., Wu, T.F., Pasqualone, D., Rougvie, A.E. & Meyer, B.J. (2003) Chromosome cohesion is regulated by a clock gene paralogue TIM-1. *Nature* **423**, 1002–1009.

Chang, D.C. (2006) Neural circuits underlying circadian behavior in *Drosophila melanogaster*. *Behav Processes* **71**, 211–225.

Choi, S., Chatzigeorgiou, M., Taylor, K.P., Schafer, W.R. & Kaplan, J.M. (2013) Analysis of NPR-1 reveals a circuit mechanism for behavioral quiescence in *C. elegans*. *Neuron* **78**, 869–880.

Edwards, S.L., Charlie, N.K., Milfort, M.C., Brown, B.S., Gravlin, C.N., Knecht, J.E. & Miller, K.G. (2008) A novel molecular solution for ultraviolet light detection in *Caenorhabditis elegans*. *PLoS Biol* **6**, e198.

Evans, C.T. (2006) Transformation and microinjection. In WormBook (ed), *WormBook*. The *C. elegans* Research Community, doi/10.1895/wormbook.1.108.1, http://www.wormbook.org.

Flavell, S.W., Pokala, N., Macosko, E.Z., Albrecht, D.R., Larsch, J. & Bargmann, C.I. (2013) Serotonin and the neuropeptide PDF initiate and extend opposing behavioral states in *C. elegans*. *Cell* **154**, 1023–1035.

Fujiwara, M., Sengupta, P. & McIntire, S.L. (2002) Regulation of body size and behavioral state of *C. elegans* by sensory perception and the EGL-4 cGMP-dependent protein kinase. *Neuron* **36**, 1091–1102.

Golombek, D.A. & Rosenstein, R.E. (2010) Physiology of circadian entrainment. *Physiol Rev* **90**, 1063–1102.

Hardin, P.E. (2005) The circadian timekeeping system of *Drosophila*. *Curr Biol* **15**, R714–R722.

Hasegawa, K., Saigusa, T. & Tamai, Y. (2005) *Caenorhabditis elegans* opens up new insights into circadian clock mechanisms. *Chronobiol Int* **22**, 1–19.

Hendriks, G.J., Gaidatzis, D., Aeschimann, F. & Grosshans, H. (2014) Extensive oscillatory gene expression during *C. elegans* larval development. *Mol Cell* **53**, 380–392.

Hyun, S., Lee, Y., Hong, S.T., Bang, S., Paik, D., Kang, J., Shin, J., Lee, J., Jeon, K., Hwang, S., Bae, E. & Kim, J. (2005) *Drosophila* GPCR Han is a receptor for the circadian clock neuropeptide PDF. *Neuron* **48**, 267–278.

Janssen, T., Husson, S.J., Lindemans, M., Mertens, I., Rademakers, S., Ver Donck, K., Geysen, J., Jansen, G. & Schoofs, L. (2008) Functional characterization of three G protein-coupled receptors for pigment dispersing factors in *Caenorhabditis elegans*. *J Biol Chem* **283**, 15241–15249.

Janssen, T., Husson, S.J., Meelkop, E., Temmerman, L., Lindemans, M., Verstraelen, K., Rademakers, S., Mertens, I., Nitabach, M., Jansen, G. & Schoofs, L. (2009) Discovery and characterization of a conserved pigment dispersing factor-like neuropeptide pathway in *Caenorhabditis elegans*. *J Neurochem* **111**, 228–241.

Jeon, M., Gardner, H.F., Miller, E.A., Deshler, J. & Rougvie, A.E. (1999) Similarity of the *C. elegans* developmental timing protein LIN-42 to circadian rhythm proteins. *Science* **286**, 1141–1146.

Kippert, F., Saunders, D.S. & Blaxter, M.L. (2002) *Caenorhabditis elegans* has a circadian clock. *Curr Biol* **12**, R47–R49.

Lear, B.C., Merrill, C.E., Lin, J.M., Schroeder, A., Zhang, L. & Allada, R. (2005) A G protein-coupled receptor, groom-of-PDF, is required for PDF neuron action in circadian behavior. *Neuron* **48**, 221–227.

Levine, J.D., Funes, P., Dowse, H.B. & Hall, J.C. (2002a) Advanced analysis of a cryptochrome mutation's effects on the robustness and phase of molecular cycles in isolated peripheral tissues of *Drosophila*. *BMC Neurosci* **3**, 5.

Levine, J.D., Funes, P., Dowse, H.B. & Hall, J.C. (2002b) Signal analysis of behavioral and molecular cycles. *BMC Neurosci* **3**, 1.

Lewis, J.A. & Fleming, J.T. (1995) Basic culture methods. *Methods Cell Biol* **48**, 3–29.

Lin, Y., Stormo, G.D. & Taghert, P.H. (2004) The neuropeptide pigment-dispersing factor coordinates pacemaker interactions in the *Drosophila* circadian system. *J Neurosci* **24**, 7951–7957.

van der Linden, A.M., Beverly, M., Kadener, S., Rodriguez, J., Wasserman, S., Rosbash, M. & Sengupta, P. (2010) Genome-wide analysis of light- and temperature-entrained circadian transcripts in *Caenorhabditis elegans*. *PLoS Biol* **8**, e1000503.

Meelkop, E., Marco, H.G., Janssen, T., Temmerman, L., Vanhove, M.P. & Schoofs, L. (2012a) A structural and functional comparison of nematode and crustacean PDH-like sequences. *Peptides* **34**, 74–81.

Meelkop, E., Temmerman, L., Janssen, T., Suetens, N., Beets, I., Van Rompay, L., Shanmugam, N., Husson, S.J. & Schoofs, L. (2012b) PDF receptor signaling in *Caenorhabditis elegans* modulates locomotion and egg-laying. *Mol Cell Endocrinol* **361**, 232–240.

Migliori, M.L., Simonetta, S.H., Romanowski, A. & Golombek, D.A. (2011) Circadian rhythms in metabolic variables in *Caenorhabditis elegans*. *Physiol Behav* **103**, 315–320.

Monsalve, G.C., Van Buskirk, C. & Frand, A.R. (2011) LIN-42/PERIOD controls cyclical and developmental progression of *C. elegans* molts. *Curr Biol* **21**, 2033–2045.

Nelson, M.D., Trojanowski, N.F., George-Raizen, J.B., Smith, C.J., Yu, C.C., Fang-Yen, C. & Raizen, D.M. (2013) The neuropeptide NLP-22 regulates a sleep-like state in *Caenorhabditis elegans*. *Nat Commun* **4**, 2846.

Nitabach, M.N. & Taghert, P.H. (2008) Organization of the *Drosophila* circadian control circuit. *Curr Biol* **18**, R84–R93.

Olmedo, M., O'Neill, J.S., Edgar, R.S., Valekunja, U.K., Reddy, A.B. & Merrow, M. (2012) Circadian regulation of olfaction and an evolutionarily conserved, nontranscriptional marker in *Caenorhabditis elegans*. *Proc Natl Acad Sci USA* **109**, 20479–20484.

Park, J.H. & Hall, J.C. (1998) Isolation and chronobiological analysis of a neuropeptide pigment-dispersing factor gene in *Drosophila melanogaster*. *J Biol Rhythms* **13**, 219–228.

Park, J.H., Helfrich-Forster, C., Lee, G., Liu, L., Rosbash, M. & Hall, J.C. (2000) Differential regulation of circadian pacemaker output by separate clock genes in *Drosophila*. *Proc Natl Acad Sci USA* **97**, 3608–3613.

Peng, Y., Stoleru, D., Levine, J.D., Hall, J.C. & Rosbash, M. (2003) *Drosophila* free-running rhythms require intercellular communication. *PLoS Biol* **1**, E13.

Peschel, N. & Helfrich-Forster, C. (2011) Setting the clock – by nature: circadian rhythm in the fruitfly *Drosophila melanogaster*. *FEBS Lett* **585**, 1435–1442.

Pittendrigh, C.S. (1981) Circadian systems: entrainment. In Aschoff, J. (ed), *Handbook of Behavioral Neurobiology, Biological Rhythms*, New York, Plenum. pp. 95–124.

Raizen, D.M., Zimmerman, J.E., Maycock, M.H., Ta, U.D., You, Y.J., Sundaram, M.V. & Pack, A.I. (2008) Lethargus is a *Caenorhabditis elegans* sleep-like state. *Nature* **451**, 569–572.

Renn, S.C., Park, J.H., Rosbash, M., Hall, J.C. & Taghert, P.H. (1999) A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. *Cell* **99**, 791–802.

Romanowski, A., Migliori, M.L., Valverde, C. & Golombek, D.A. (2011) Circadian variation in Pseudomonas fluorescens (CHA0)-mediated paralysis of *Caenorhabditis elegans*. *Microb Pathog* **50**, 23–30.

Rosbash, M. (2009) The implications of multiple circadian clock origins. *PLoS Biol* **7**, e62.

Saigusa, T., Ishizaki, S., Watabiki, S., Ishii, N., Tanakadate, A., Tamai, Y. & Hasegawa, K. (2002) Circadian behavioural rhythm in *Caenorhabditis elegans*. *Curr Biol* **12**, R46–R47.

Simonetta, S.H. & Golombek, D.A. (2007) An automated tracking system for *Caenorhabditis elegans* locomotor behavior and circadian studies application. *J Neurosci Methods* **161**, 273–280.

Simonetta, S.H., Romanowski, A., Minniti, A.N., Inestrosa, N.C. & Golombek, D.A. (2008) Circadian stress tolerance in adult *Caenorhabditis elegans*. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* **194**, 821–828.

Simonetta, S.H., Migliori, M.L., Romanowski, A. & Golombek, D.A. (2009) Timing of locomotor activity circadian rhythms in *Caenorhabditis elegans*. *PLoS One* **4**, e7571.

Taghert, P.H. & Shafer, O.T. (2006) Mechanisms of clock output in the *Drosophila* circadian pacemaker system. *J Biol Rhythms* **21**, 445–457.

Tennessen, J.M., Gardner, H.F., Volk, M.L. & Rougvie, A.E. (2006) Novel heterochronic functions of the *Caenorhabditis elegans* period-related protein LIN-42. *Dev Biol* **289**, 30–43.

Tennessen, J.M., Opperman, K.J. & Rougvie, A.E. (2010) The *C. elegans* developmental timing protein LIN-42 regulates diapause in response to environmental cues. *Development* **137**, 3501–3511.

Wright, S. (1997) *The Scientist and Engineer's Guide to Digital Signal Processing*. California Technical Publishing, San Diego, CA, 626 p.

Wu, Y., Cao, G., Pavlicek, B., Luo, X. & Nitabach, M.N. (2008) Phase coupling of a circadian neuropeptide with rest/activity rhythms detected using a membrane-tethered spider toxin. *PLoS Biol* **6**, e273.

Yu, W. & Hardin, P.E. (2006) Circadian oscillators of *Drosophila* and mammals. *J Cell Sci* **119**, 4793–4795.

Acknowledgments

Supported by grants from ANPCyT, CONICET and the National University of Quilmes (Argentina), the Fund for Scientific Research-Flanders (FWO) and the KU Leuven Faculty Research Fund (Belgium). We would like to thank the Caenorhabditis Genetics Center, which is funded by the NIH National Centre for Research Resources (NCRR) for the wild-type strains, and Lic. Maria Eugenia Goya for her help with transgenic nematode lines maintenance and nematode picking. The authors declare no conflicts of interest.