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Circadian rhythms in metabolic variables in *Caenorhabditis elegans*

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ABSTRACT

Circadian rhythms govern a wide variety of physiological and metabolic functions in most organisms through neural networks, hormones and gene expression.
In this work, we studied the circadian variation in metabolic variables of adult *C. elegans* such as food consumption, pharyngeal contractions, defecation and oxygen consumption. Feeding behavior was clearly rhythmic under LD conditions, with a non-significant trend under DD conditions. In addition, a daily and circadian variation in muscle contraction of the pharynx was observed. Oxygen consumption also showed a circadian fluctuation with a maximum in the middle of the night (a peak was found around ZT18/CT18). Furthermore, defecation behavior also showed a daily variation in the N2 strain (wild type). This work demonstrates that in the adult nematode *C. elegans* metabolic variables vary daily. In summary, our results will allow us to take full advantage of this widely used animal model (including research in genetics, ageing and developmental biology) for studies in Chronobiology.

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1. Introduction

Circadian clocks are autonomous internal timekeeping mechanisms that allow organisms to adapt to external daily rhythms of light, temperature and other environmental factors. The circadian clock is based on a central pacemaker, composed of cellular and molecular networks, which transmit circadian time through neural/neuroendocrine signals to the whole organism. Rhythmic output is manifested as locomotor activity, sleep/wake patterns and a variety of physiological and metabolic functions [1,2].

Metabolism is the set of chemical reactions that allow organisms to grow and reproduce, maintain their structures, and respond to their environments. It has been known for many years that numerous aspects of physiology exhibit daily rhythmicity, including many types of circulating and intracellular metabolites, feeding-related hormones, and ingestive behaviors. Many of these rhythms are driven, at least in part, by circadian clocks [3]. For example, gluconeogenic genes such as phosphoenolpyruvate carboxykinase and glucose 6-phosphatase are typical clock-controlled metabolic-related genes [4]. In *Drosophila melanogaster*, feeding behavior is under circadian control and clocks in metabolic tissues regulate the phase of the feeding rhythm [5]. Several other invertebrate species exhibit diurnal or circadian changes in metabolism; for example, *Nephrops norvegicus* kept under constant conditions in darkness shows an endogenous rhythm in its respiratory activity with a nocturnal peak activity phase [6].

C. elegans is a free-living nematode that lives in soil and feeds on bacteria. This animal provides an excellent model for genetics and neuro-behavioral studies [7] and, due to mutant availability and ease of culture, it could be used as a novel and fruitful model for circadian studies.

Biological rhythms have been characterized in *C. elegans* based mainly on the study of the ultradian defecation clock (period less than 24 hours) [8,9]. However, in recent years rhythms in swimming behavior [10] and response to osmotic stress in starved larvae (L1) and adults have been reported[11,12]. Furthermore, locomotor activity has been shown to be rhythmic in adult worms by 2 different approaches [10,13,14], one consisted in assessing swimming speed throughout the day using an automated bug tracker system and the other one measures gross overall locomotor activity in liquid medium.

In this work, we studied the circadian variation in metabolic variables of adult *C. elegans* such as food consumption, pharyngeal contractions, defecation and oxygen consumption and show that in the adult nematode metabolic exhibits significant daily variations. These data provide additional support in the sense that the chronobiological study of *C. elegans* might provide fundamental information about the basis of circadian rhythmicity in eukaryotes.

Abbreviations: CGC, Caenorhabditis Genetics Center; CT, Circadian Time; DD, Dark-Dark (Constant Darkness); FUDR, Fluorodeoxyuridine; LD, Light-Dark; NGM, Nematode Growth Medium; ZT, Zeitgeber Time.

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2. Materials and methods

2.1. General methods and strains

The animals used in these experiments were of the species *Caenorhabditis elegans*, TJ1060 [spe9(hc488); fer15(b26)] strain, previously described [15]. This strain carries temperature-sensitive alleles at two loci that result in sterility at 25 °C, but allow reproduction at 20 °C. For the defecation rate experiment, wild-type *C. elegans* (N2) and JT73 (itr-1(sa73)) strain were used (all strains were provided by the Caenorhabditis Genetics Center, University of Minesotta, MN, USA). Nematode stocks were maintained in NGM medium (0.3% NaCl, 0.25% Peptone, 5 μg/ml cholesterol, 1 mmol/l $CaCl_2$, mmol/l $MgSO_4$, 1.7% Agar in 25 mmol/l of potassium phosphate buffer pH 6.0) with thick bacterial lawns of *E. coli* OP50 strain [7] under LD 12:12 h (light intensity 400 lux) photoperiodic cycling conditions at 16 °C. For experimental testing, a population of worms was synchronized to the same developmental stage by the chloride method [16] and cultured in liquid medium composed of 3.5 ml of M9 (42 mM Na_2HPO_4, 22 mM KH_2PO_4, 85,5 mM NaCl, 1 mM $MgSO_4$) + Antibiotic-antimycotic (AB) 1x (Gibco, USA) in 50 ml baffled erlenmeyer flasks, at 110 rpm and 18.5 °C. Eggs were hatched overnight and L1 larvae were maintained without food for 2 days and entrained to LD 12:12 h conditions. At the third day (at the time of lights on, defined as zeitgeber time 0 or ZT0) worms were transferred to NGM plates seeded with *E. coli* OP50 or *E. coli* NA22 depending on the experiment and the temperature was raised to 25.3 °C to avoid self reproduction. For non-TJ1060 strains, 20 μM of Fluorodeoxyuridine (FuDR) was added at L4 stage to avoid self-reproduction [17]. Sampling and recordings under constant conditions were performed under dim (<1 lux) red light, which we have found does not affect circadian behavior in *C. elegans* [14].

Unless otherwise specified, all chemicals were purchased from Sigma Chemical Co. (St Louis, MO).

2.2. Feeding Assay

The food consumption of nematodes was determined in an indirect way by measuring the decrease in optical density at 600 nm (OD_{600}) of an *E. coli* OP50-supplemented medium. Growth-synchronized worm populations of TJ1060 late L1 larvae were transferred to plates seeded with *E. coli* OP50. Nematodes were grown to L4 stage under 12:12 h light-dark cycles. Once nematodes reached the L4 stage, they were washed off the plates with 5 ml of complete S medium and then were fed with medium (*E. coli* 10^{10}/ml with complete S medium and AB; adapted from [15]) and kept at a final concentration of 6 worms/20 μl. 24 hours later (one-day old adults) nematodes where either kept under the same photoperiod LD or placed in constant darkness (DD) for sampling according to each assay. Samples were taken for 1.5 days every 6 hours under LD and DD conditions and the optical density of the sample was determined by means of a spectrophotometer (UV-160A, UV-Visible Recording Spectrophotometer, Shimadzu). Experiments were performed at two independent times with three biological replicates at each time.

In addition, for the feeding assays we controlled for OD600 decay without the addition of nematodes and found that this value remains constant throughout the experimental assay time course, indicating no bacterial contribution to this variable.

2.3. Pharyngeal pump recording

Growth-synchronized worm populations of TJ1060 late L1 larvae were transferred to plates seeded with *E. coli* OP50. Once the nematodes reached to L4 stage a single animal was transferred with a pick to each well of a 24-well plate filled with 1 ml of NGM medium and seeded with *E. coli* OP50. 24 hours later nematodes where either kept under the same photoperiod (LD 12:12 h) or placed in constant darkness (DD) for sampling according to each assay. Pumping rate of nematodes were quantified by counting the number of pharyngeal muscles contractions for a specific time period. Recordings were made for 1.5 days every 6 hours by observing the same animals in their wells using a dissection microscope (Olympus SZ61). Experiments were performed at two independent times with n = 20 worms.

2.4. Defecation Assay

Growth-synchronized worm populations of N2 and JT73 late L1 larvae were independently transferred to plates seeded with *E. coli* OP50. Once the nematodes reached to L4 stage a single animal was transferred with a pick to each well of a 24-well plate filled with 1 ml of NGM medium supplement with 20 μM 5-fluorodeoxyuridine (FuDR) and seeded with *E. coli* OP50. 24 hours later nematodes where either kept under the same photoperiod (LD 12:12 h) or placed in constant darkness (DD) for sampling according to each assay. Defecation frequencies were recorded for two days every 6 hours by observing the animals in their wells using a dissection microscope (Nikon SMZ645). The defecation cycle length was defined as the duration between the pBoc steps (posterior body muscle contraction) of two consecutive defecations. Each animal was scored for five consecutive cycles (six successive pBoc steps). Experiments were performed at two independent times with n = 20 worms.

2.5. Measurement of Oxygen Consumption

Growth-synchronized worm populations of 3,000 aprox. TJ1060 late L1 larvae were transferred to plates seeded with *E. coli* NA22 (2 plates per timepoint per experiment). Once the nematodes reached the adult day-1 stage they were either maintained under the same photoperiod LD 12:12 h or changed to a constant darkness (DD) regime for sampling according to each assay. For oxygen consumption recordings, approximately 3,000 nematodes were transferred to 15 ml tubes and washed three times with 14 ml of M9 buffer. The supernatant of the last wash was assessed for oxygen consumption; under these conditions we found no detectable O_2 consumption that could be linked to any remaining *E. coli* in the M9. After washing, nematodes were transferred to the oxygen measuring chamber and the readings of oxygen consumption were done at 25.3 °C usually for 10 – 30 min, depending on the oxygen consumption rates. The zero calibration of oxygen concentration was adjusted with sodium sulfite (Biopack®, Argentina), which completely deoxidized the solution and the maximal concentration was calibrated with water containing dissolved air. Oxygen consumption rates are reported as pl O2 per min and per worm and were measured for 2 days every 6 hours. Oxygen concentration was measured using a polarographic O_2 electrode (Qubit systems: OX1LP Dissolved Oxygen Package).

2.6. Data analysis

One-way ANOVA was applied in physiological variables rhythms followed by a Tukey test for group comparison. Each pattern of daily variation was characterized by performing a waveform regression with Sigma Plot (Jandel Scientific, Erkrath, Germany). Profiles were fitted with the following equation: $y = y0 + a * \sin(2 * pi * x / b + c)$, where y is *n*th data point, x is the time of the *n*th data point, y0 is the baseline value, a is the amplitude, b is the period of the wave and c is the phase. Acrophases were determined by Cosinor analysis of the data sets, by fitting the data to 24-h cosine waveforms in order to obtain the best-fit amplitude and phase values. In order to control for age or developmental-related changes, for circadian analysis data series were detrended. All data were expressed as the mean ± *SEM* of n values.

3. Results

3.1. Daily variation of the food consumption and pharyngeal pumping in adult nematodes

To determine if the feeding behavior of adult worms varies throughout the day, food consumption was recorded indirectly by measuring the optical density of *E. coli* OP50. When a population of adult worms was fed *E. coli* OP50 and the density of bacteria was checked daily for turbidity measurement (550 nm), a rhythmic feeding pattern was observed under LD conditions (ANOVA test $p=0.013$) (Fig. 1A). A peak was found at late day-early night and a minimum value at ZT21 ($p<0.05$, Tukey Comparison Test; acrophase 6.76 h, Cosinor analysis). When adult worms were grown under constant dark conditions there was no significant temporal change in feeding behavior, probably due to the increased variability in the data (Fig. 1B). However, there is a trend towards a slightly delayed phase in DD conditions (compared to LD), which could suggest that a putative endogenous rhythm of feeding is not necessarily of exactly 24 hours or that it can be masked by the environmental zeitgeber.

On an agar surface in the presence of abundant bacteria, normal worms exhibit a continuous pharyngeal pump activity at an average rate of about 260 pumps / min [18]. In order to determine whether contraction of the pharynx is under circadian control, we recorded the pharyngeal pumping rate throughout the day in both LD and DD conditions. When adult worms were transferred to plates of 24 wells with bacteria *E. coli* OP50 as a food source, a daily variation in muscle contraction of the pharynx was observed under LD conditions ($n=20$, ANOVA test $p<0.0001$). As observed in Fig. 2A, a peak was found at ZT0, and a minimum value around ZT12-18 ($p<0.001$, Tukey's Test; acrophase 1.81 h, Cosinor analysis). When adult worms were grown under constant dark conditions, the circadian rhythm in muscle contraction of the pharynx was maintained ($n=20$, ANOVA test $p<0.0001$). A peak was found at CT0, and a minimum value around CT12-18 ($p<0.001$, Tukey's Test) (Fig. 2B).

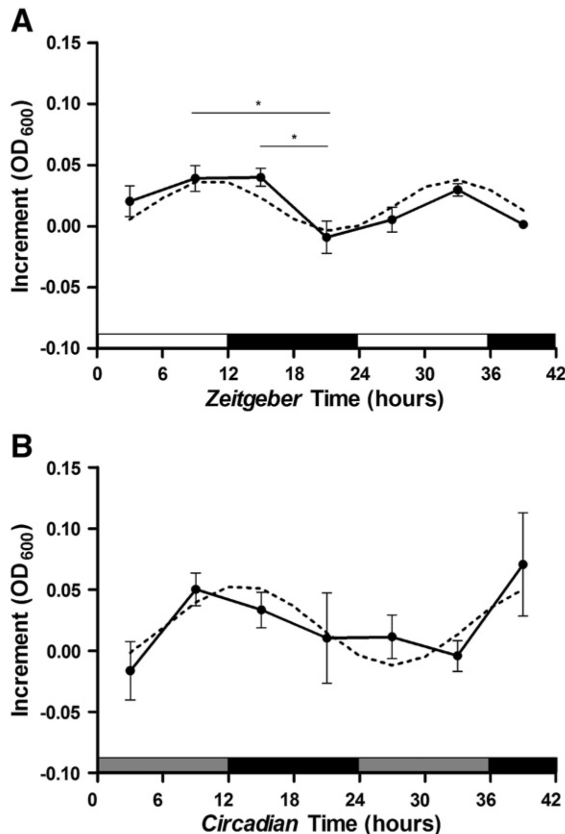

Fig. 1. Daily variation of the food consumption of *C. elegans* in A) the presence of LD cycles (ANOVA $p=0.013$) and B) DD conditions. The increment (OD_{600}) was calculated as the derivative function between two successive time points of raw data, and corresponds to the speed of OD change between those two points. Experiments were performed at two independent times with three biological replicates at each time. Dotted lines correspond to a sinusoidal function fit to the data. * $p<0.05$.

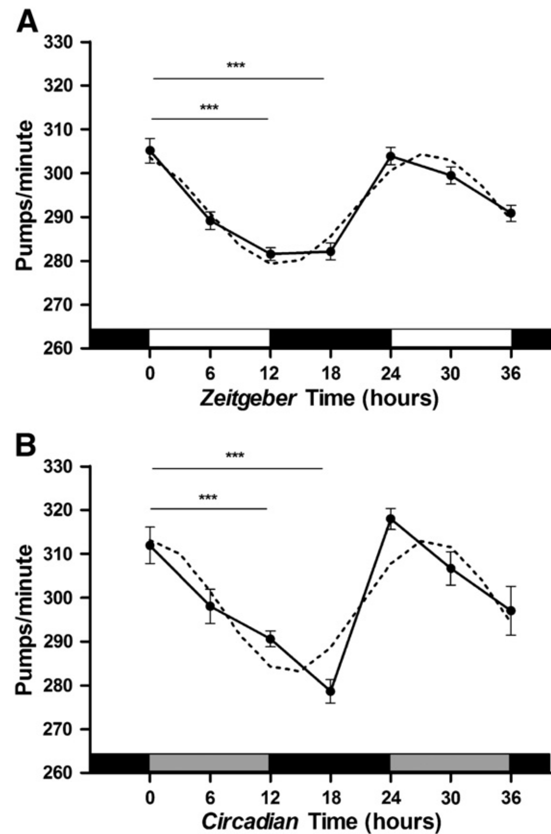

Fig. 2. Daily variation in muscle contraction of the pharynx of *C. elegans* in A) the presence of LD cycles (ANOVA $p<0.0001$) and B) DD conditions (ANOVA $p<0.0001$). Experiments were performed at two independent times with $n=20$ worms. Dotted lines correspond to a sinusoidal function fit to the data. *** $p<0.001$.

3.2. Defecation behavior

Defecation in *C. elegans* is achieved by a cyclical stereotyped motor program. At 20-25º, in the presence of abundant food, the defecation cycle period is 40 to 45 seconds, with very little variation [19]. To determine whether the ultradian period of defecation is influenced by the circadian system of the nematode, we measured the rate of defecation in adult animals through day in both LD and DD conditions. We found a daily variation in defecation behavior with an increase in the defecation cycle length during the night ($n=20$, ANOVA $p=0.0002$). A peak was found around ZT18 ($p<0.05$, Tukey´s Test; acrophase 22.04 h, Cosinor analysis) (Fig. 3). Autocorrelation analysis (not shown) also indicated a 24 h periodicity for these data. Detrended data for the DD experiment indicated no clear variation throughout the circadian cycle.

In order to determine whether mutations affecting the ultradian clock period have any effect on the circadian clock, we studied the mutant strain JT73 whose period of defecation is about 95 seconds [20]. The behavior of defecation in the mutant strain under LD conditions presented a daily pattern, although the phase appeared to be shifted toward the photophase of the daily photoperiod ($n=10$, ANOVA $p=0.012$) (Supplementary Fig. 1).

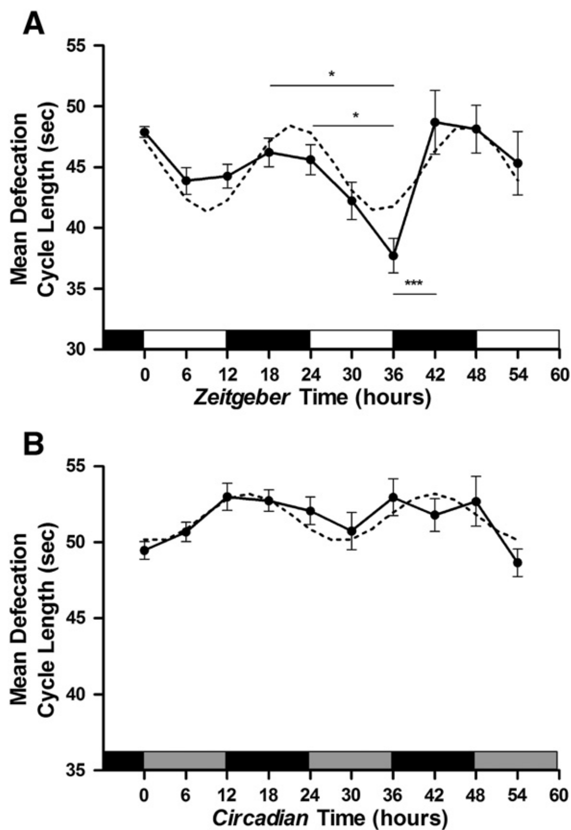

Fig. 3. Defecation behavior of *C. elegans* in A) the presence of LD cycles (ANOVA $p=0.0002$) and B) DD conditions. Experiments were performed at two independent times with $n=20$ worms. Dotted lines correspond to a sinusoidal function fit to the data. * $p<0.05$, *** $p<0.001$.

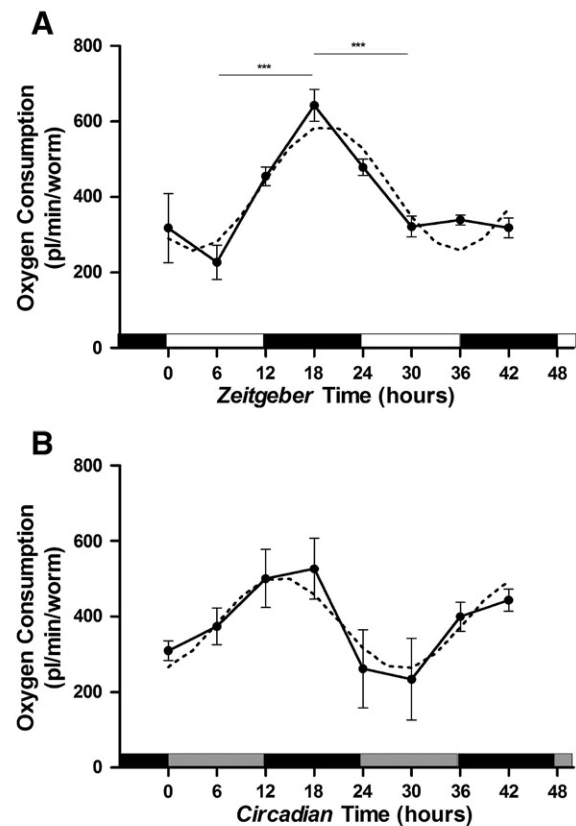

Fig. 4. Daily variation in the oxygen consumption of *C. elegans* in A) the presence of LD cycles (ANOVA $p<0.0001$) and B) DD conditions (ANOVA $p=0.0434$). Dotted lines correspond to a sinusoidal function fit to the data. *** $p<0.001$.

3.3. Oxygen consumption

C. elegans requires oxygen to move, develop and survive [21]. To study the existence of a rhythmic pattern in the respiratory activity of adult worms, oxygen consumption was measured throughout the day. Our results show that under conditions of LD oxygen consumption shows a daily variation (ANOVA test $p<0.0001$) with a maximum in the middle of the night (Fig. 4A). A peak was found at ZT18 and a minimum value at ZT6 ($p<0.001$, Tukey's Test; acrophase 18.02 h, Cosinor analysis). Also, to determine whether this rhythm is endogenous, oxygen consumption recordings were repeated under conditions of constant darkness. As shown in Fig. 4B, our results indicate that the circadian rhythm in oxygen consumption is maintained in DD DD (ANOVA test $p=0.0434$), peaking around the late subjective day (acrophase 15.12 h, Cosinor analysis).

4. Discussion

We previously reported the presence of circadian behaviours in *C. elegans* such as resistance to osmotic stress [12] and locomotor activity [13,14]. In this work, we focused on the study of the circadian oscillations in metabolic activity in this nematode. Here we report significant daily variations in several metabolic variables of *C. elegans*. The fact that physiology exhibits a tight chronobiological control gives the species an adaptive advantage in terms of optimizing its energy use.

4.1. Feeding behavior

Because feeding is an activity closely related to energy metabolism, we first studied potential circadian changes in feeding behavior *in C. elegans*. We found that *C. elegans* feeding behavior varies throughout the day. When a population of adult worms was fed with *E. coli* OP50 and the density of bacteria was checked daily for turbidity measurement, we found that feeding rate of *C. elegans* tends to decreases during the middle of the night in the presence of LD cycles. This feeding rate is not clearly driven by circadian clock, since in constant darkness no significant changes in feeding were recorded. However, there is a trend towards a circadian variation, with a phase that is slightly delayed in DD (compared to LD), which could relate to a low-amplitude DD rhythms not necessarily of exactly 24 hours.

Pharyngeal behavior in *C. elegans* is composed of two motions: pumps and isthmus peristalses [18,22]. Both types of movements allow the food to pass through the lumen of the pharynx, grinding nutrients up along the path into the intestine [22]. On an agar surface in the presence of abundant bacteria, normal worms pump nearly continuously at an average rate of about 260 pumps / min [18]. To determine whether contraction of the pharynx correlates with the feeding rate, we measured the pharyngeal pumping rate throughout the day. Our results show a daily variation in the contraction of the pharynx that is maintained in constant darkness. As shown in Fig. 2, the contraction of the pharynx of *C. elegans* tends to decrease towards the late day/early night in both LD and DD cycles. In the wild, animals tend to eat at specific times of day that may vary from one species to another. *C. elegans* largely depends on the chemosensation to move in its natural habitat and to detect and feed on bacteria. This soil nematode is capable of sensing a large repertoire of olfactory cues and a variety of attractant chemicals, many of which are byproducts of bacterial metabolism, and therefore might represent chemical signals in the animal's natural environment to find food [23,24]. Also, sensing of metabolic products of bacteria may be regulated by the bacterial cell division cycle. A microenvironment in which the density of bacteria is high could be an enabling environment for *C. elegans* feeding. Despite

not having many studies demonstrating the existence of circadian rhythms in bacteria, it is known that many microorganisms have a internal biological clock for measuring daily time. In cyanobacteria daily oscillations in gene expression and cell divisions is controlled by circadian clock [25].

4.2. Defecation behavior

We also studied the rate of defecation in adult animals throughout the day. We found a daily variation of defecation behavior with an increase in the defecation cycle length during the night under LD cycles. These results indicate that the worms defecate less frequently in the night. In *C. elegans*, the flow of bacteria inside the digestive tract is controlled by two muscle complexes, one in the pharynx and another one in a small group of muscles located in the posterior end of the intestine which control the opening of the anus; this in turn facilitates the removal of intestinal contents by high internal pressure [24]. Indeed, these findings correlate with the results for feeding and contraction of the pharynx.

4.3. Endogenous control of oxygen consumption

We also studied oxygen consumption of adult worms under both LD and DD conditions. Our results show that *C. elegans* oxygen consumption exhibits a daily and circadian rhythm, with maximal values during the night (ZT18/CT18). We have previously reported that these nematodes are more active during at night [13,14]. This pattern of activity correlates with our current data of oxygen consumption, an indirect calorimetric measurement of metabolic rate.

5. Conclusions

In this work we have shown that several physiological variables in *C. elegans* such as food consumption, pharyngeal contractions, defecation and oxygen consumption exhibit daily rhythmicity and are sustained under constant environmental conditions. We and others [10,13,14] have found that these nematodes are more active during at night. This pattern of activity correlates with the rhythm we found in the oxygen consumption, as well as a higher resistance to stressful stimuli during the night [12]. However, food consumption, pharyngeal contractions and defecation to show lower activity values during the night. Because oxygen diffuses rapidly through *C. elegans*, it does not become a limiting factor for respiration until it reaches external concentrations below 4% [21,26]. *C. elegans* exhibits a strong behavioural preference for 5–12% oxygen, avoiding higher and lower oxygen levels [27]. These behavioural preference for lower oxygen concentrations is not only a behavioral mechanism of protection against oxidative stress but also signal the presence of food in the form of actively growing bacteria that consume oxygen more quickly than the oxygen can diffuse through soil. Therefore, in a microenvironment rich in nutrients (bacteria), worms tend to move less since there is food in abundance; in addition, oxygen consumption might decrease since there is less oxygen available. Finally, feeding and defecation rates increase because of the relatively high-resource environment.

However, the molecular mechanisms involved in the generation of metabolic rhythms is not well understood. It is known for other species that the circadian clock is linked to many aspects of metabolism, including energy, carbohydrate, amino acid, lipid or protein metabolism and heme biosynthesis [25,28]. For example, gluconeogenic genes such as phosphoenol pyruvate carboxykinase and glucose 6-phosphatase are typical clock-controlled metabolic-related genes [4]. *C. elegans* expresses ortholog genes for most of the key enzymes involved in eukaryotic intermediary metabolism, suggesting that the major metabolic pathways are probably present in this species [29]. To identify possible genes that are under circadian control, we used microarrays containing whole genome tiling array *C. elegans* (Affymetrix). Our preliminary data indicate that out of the top 75 genes that show a circadian transcription profile, about 8% correspond to genes related to metabolic pathways (data not shown).

Another question that remains to be answered is how these rhythms are entrained by light. It is known that *C. elegans* is photoresponsive to visible light (540 nm) to intensities as low as 40 lux [30]. Also, recent reports have shown that *C. elegans* may sense ultraviolet light, and a photic stimulus (ultraviolet, blue or light) induces a negative phototaxic response in the nematode [31,32]. Moreover, these worms can sense light through a group of photoreceptor cells, some of which respond to light by opening cGMP-sensitive CNG channels [32]. Particularly, a phototransduction cascade was identified involving the participation of a "taste receptor" [33]. The presence of pigment dispersing factor (*pdf*) and its receptors has been recently described in *C. elegans*, with a significant degree of homology with the *Drosophila pdf* (one of the genes responsible for the origin of circadian rhythms in the fly). Furthermore, overexpression of this factor in the nematode significantly affected circadian rhythms in *C. elegans*, suggesting that it is also essential for the circadian clock in this species [34,35]. Indeed, this output pathway is relevant for *C. elegans*' circadian rhythms, which appears to be synchronized by light [10,11,13,14] and temperature [14] through unknown input mechanisms.

In addition, several genes with diverse degrees of homology to clock genes have been described in this species [36]. However, several of these genes have been proposed to be involved in other regulatory mechanisms, such as developmental processes, including *lin-42* (a period homolog) that is expressed during molting in several cells [37], or a *timeless* homolog which appears to be involved in chromosomal cohesion [38,39]. The relationship of these genes to circadian rhythmicity is currently unknown, although we have previously reported changes in circadian period in *lin-42(mg152)* or *lin-42 (n1089)* mutants and found a significant increase in circadian period as compared to wild type controls [14].

In summary, this study takes additional advantage of an extremely useful model system which has only recently been exploited for circadian studies and will certainly be subject to more research in order to understand the elusive basis of the molecular clock machinery.

Supplementary materials related to this article can be found online at doi:10.1016/j.physbeh.2011.01.026.

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