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ORIGINAL PAPER

Circadian stress tolerance in adult *Caenorhabditis elegans*

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Abstract Circadian rhythms control several behaviors through neural networks, hormones and gene expression. One of these outputs in invertebrates, vertebrates and plants is the stress resistance behavior. In this work, we studied the circadian variation in abiotic stress resistance of adult *C. elegans* as well as the genetic mechanisms that underlie such behavior. Measuring the stress resistance by tap response behavior we found a rhythm in response to osmotic (NaCl $LC_{50} = 340$ mM) and oxidative (H_2O_2 $LC_{50} = 50$ mM) shocks, with a minimum at ZT0 (i.e., lights off) and ZT12 (lights on), respectively. In addition, the expression of *glutathione peroxidase* (*C11E4.1*) and *glycerol-3-phosphate dehydrogenase* (*gpdh-1*) (genes related to the control of stress responses) also showed a circadian fluctuation in basal levels with a peak at night. Moreover, in the mutant *osr-1* (AM1 strain), a negative regulator of the *gpdh-1* pathway, the osmotic resistance rhythms were masked at 350 mM but reappeared when the strain was treated with a higher NaCl concentration. This work demonstrates for the first time that in the adult nematode, *C. elegans* stress responses vary daily, and provides evidence of an underlying rhythmic gene expression that governs these behaviors.

Keywords *Caenorhabditis elegans* · Circadian rhythm · Stress · Osmotic · Oxidative

Abbreviations

CGC	Caenorhabditis Genetics Center
CT	Circadian time
DAF	Abnormal Dauer formation
DD	Dark–dark (constant darkness)
FuDR	Fluorodeoxyuridine
GPDH	Glycerol-3-phosphate dehydrogenase
GPX	Glutathione peroxidase
HSP	Heat shock protein
LC_{50}	Lethal concentration 50
LD	Light–dark
MSRA	Methionine sulfoxide-*S*-reductase
NGM	Nematode growth medium
OSR	Osmotic stress-resistant
ZT	Zeitgeber time

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Introduction

Daily rhythms in physiological variables are ubiquitous in nature in most organisms from cyanobacteria to humans. These rhythms are entrained to environmental cues, and sustained in constant conditions by an endogenous circadian clock, which is based on a central pacemaker, conformed of cellular (neural) and molecular networks. Circadian time is transmitted to the whole organism through neural/neuroendocrine signals. Rhythmic output is manifested as locomotor activity, sleep/wake patterns and a

variety of physiological and metabolic functions (Dunlap et al. 2004; Kohsaka and Bass 2007). Indeed, one of the adaptive functions of circadian rhythmicity is the prediction of periodic environmental signals that are of significant value for life on Earth.

Environmental stress plays a key role in the evolution and ecology of most organisms; since stress-inducing agents vary along the day, it is logical that organisms must be able to predict these periodic changes in order to survive.

Several environmental cues, including daily variations in temperature, UV light intensity or humidity, might guide or entrain rhythmic cellular responses to environmental stress. Since the stress machinery is conserved among many organisms, it is possible to find similar patterns of circadian regulation among different species. For example, heat shock proteins (mainly HSP70 and HSP16) are regulated by a circadian clock in *Synechocystis*, *Neurospora*, *Gonyaulax* and *Drosophila* (Rensing and Monnerjahn 1996; Ceriani et al. 2002), at transcriptional or induction levels. Rhythms in antioxidant enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, haloperoxidase and glutathione peroxidase are regulated at transcriptional or activity levels in many different organisms spanning various taxa, such as plants and humans (Hardeland et al. 2003).

Circadian rhythms in abiotic stress resistance were previously reported in *Caenorhabditis elegans* (*C. elegans*) larvae (Kippert et al. 2002). In addition, locomotor activity has also been shown to be rhythmic in adult worms (Saigusa et al. 2002; Simonetta and Golombek 2007). This animal provides an excellent model for genetics and neurobehavioral studies (Brenner 1974), and due to mutant availability, ease of culture and several neurobiological approaches, it could be used as a novel and fruitful model for circadian studies.

In this work, we studied the rhythmic resistance to osmotic, oxidative and temperature stress in adult *C. elegans* measuring the tap behavior after a stressing condition. The tap behavior consists of the induction of swimming escape movement when the worm culture is subjected to mechanical shock and it easily allows for the scoring of responsiveness of the nematode. It is widely used in memory, locomotor mutant screening and stress tolerance assays (Chalfie and Sulston 1981; Larsen 1993; Hardaker et al. 2001; Hart 2006).

In addition, we studied the circadian expression of heat shock proteins, antioxidative enzymes and osmotic shock-induced proteins at the transcriptional level. Taken together, this work demonstrates for the first time that in the adult nematode *C. elegans* stress responses vary daily, and provides evidence of an underlying rhythmic gene expression that governs these behaviors.

Materials and methods

C. elegans culture

The animals used in these experiments were of the species *C. elegans*, TJ1060 [*spe9*(*hc488*); *fer15*(*b26*)] strain, previously described (Fabian and Johnson 1994). This strain carries temperature-sensitive alleles at two loci that result in sterility at 25°C, but allows reproduction at 20°C. For some experiments, AM1 [*osr-1*(rm1)] or RB1373 [*gpdh-1*(ok1558)] strains 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 $CaCl_2$, 1 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 (Brenner 1974) under 12:12 h light:dark (LD, light intensity 400 lux) conditions at 16°C. For experimental testing, a population of worms was synchronized to the same developmental stage by the chloride method (Lewis and Fleming 1995) and cultured in liquid medium composed of M9 (42 mM Na_2HPO_4, 22 mM KH_2PO_4, 85,5 mM NaCl, 1 mM $MgSO_4$) + antibiotic–antimycotic (AB) 1× (Gibco, USA) in 25 ml baffled erlenmeyer flasks, at 110 rpm and 18.5°C. Eggs were hatched overnight and L1 larvae were maintained without food for 3 days and entrained to LD conditions. On the fourth day (at the time of lights on, defined as zeitgeber time 0 or ZT0), worms were fed with medium (*E. coli* 10^{10}/ml with complete S medium and AB; adapted from Fabian and Johnson 1994) and kept at a final concentration of 15 worms/10 µl. Temperature was raised to 25°C to avoid self reproduction. On the first day of adult stage, the medium was renewed at ZT0. Unless otherwise specified, all chemicals were purchased from Sigma Chemical Co. (St Louis, MO).

Stress resistance behavioral assay

To determine the response to different types of stress, 20 µl of worm liquid culture was mixed with 30 µl of the solution containing the stimuli, or M9 as control, for 2 h. After treatment, tap responsive worms were assayed (as described by Larsen 1993) per triplicate. Total and tap responsive animals were counted visually. The LC_{50} (concentration to get 50% of tap responsive worms) for NaCl solution (200, 250, 300, 350, 400, 450 and 500 mM), H_2O_2 (0, 25, 50, 70, 90 mM) and temperature (29, 30, 31, 32, 33, 34, 35 °C) was calculated.

For circadian experiments, 20 µl of worm liquid culture (20–35 individuals) was treated with the stressors for 2 h at LC_{50} concentration (or M9 as a control), from ZT18 of the first day of adult, every 6 h for 1.5 days. Samples were

taken by triplicate, counted twice and all the experiments were conducted from three paired independent cultures. When nematodes were cultured in dark–dark (DD) conditions, the samples were handled under dim red light. All samples were scored live after the treatment at each time point. For non-TJ1060 strains, 50 µM of fluorodeoxyuridine (FuDR) was added at L4 stage to avoid self-reproduction (Mitchell et al. 1979).

Semiquantitative RT-PCR

Large-scale liquid cultures of worms were grown as described before, and a sample of 2,000 worms was taken every 6 h from ZT18 of the first day of adulthood for 2 days. RNA was isolated by Trizol method, checked by spectrophotometry and an agarose denaturing gel. Five micrograms of RNA was treated with DNAse I MAXI-script™ (AMBION, Foster City, CA), mixed with oligo dT and reversely transcripted (M-MLV RT, Superscript™ RT, Invitrogen, Carlsbad, CA). Conditions for linearity of the semiquantitative PCR were adjusted for annealing temperature, Mg^{2+} and cycling conditions for each set of primers. The primers used to amplify were (5′–3′): *hsp-6* (heat shock protein 70 kDa) (Fw: GGACAAACCAAAGGGAC ATG, Rev: AACGAATGCTCCAACCTGAG), *hsp-16.1* (heat shock protein 16 kDa) (Fw: TATTTCCGTCCAGCT CAACG, Rev: ATCGCTTCCTTCTTTGGTGC), *gpdh-1* (glycerol-3-phosphate dehydrogenase) (Fw: CAAGCTAC AAAAGGGAGCCC, Rev: GGATTTCACGTTGTAGG CCC), *c11e4.1* (*gpx*) (gluthathione peroxidase) (Fw: ATG GCACTTTGGCAGCTCA, Rev: ACGCGCAAAAAGTA GCAACG) and *msra* (methionine-S-sulfoxide reductase) (Fw: TGAGCCATTGGACAAGTTCTA, Rev: TTAAGC ATGACAGTTTCTTGGA). We used the gene *act-4* (actin) for housekeeping purposes (Fw: TGAAGATCCTCACTG AGCGC, Rev: AGCACTTGCGGTGGACAATC). The PCR program was 2′ 94°C, 30 cycles of 30″ 92°C, 30″ 60°C, 1′ 72°C, and final extension 5′ 72°C, except for gpdh-1, which was run for 31 PCR cycles. All primers were purchased from Invitrogen.

Quantitative real-time PCR

The ICycler IQ system (Bio-Rad) was used to perform the real-time PCR reactions. For each ICycler reaction, a mastermix of the following reaction components was prepared to the indicated end-concentration: 12.4 µl water, 1.2 µl $MgCl_2$ (3 mM), 0.4 µl forward primer (0.4 µM), 0.4 µl reverse primer (0.4 µM), 2.0 µl Buffer (1×) (Invitrogen, Carlsbad, CA), 0.4 µl dNTPs (0.2 mM), 0.2 µl Platinum TAQ DNA polymerase (1 unit) (Invitrogen, Carlsbad, CA) and 2 µl SybrGreen Type I (1×) (Invitrogen, Carlsbad, CA). ICycler mastermix (19 µl) was filled in each of the 96 wells of a PCR plate and 1 µl cDNA was added as PCR template. The PCR program was 2′ at 94°C, 45 cycles of 30″ at 92°C, 30″ at 60°C (single fluorescense measurement), 1′ at 72°C, final extension of 5′ at 72°C, melting curve program (60–95°C with a heating rate of 0.5°C per second and a continuous fluorescence measurement) and finally a cooling step to 15°C. The results were quantified using the standard curve method. To do so, a six-point standard curve of a series of two-fold dilutions was constructed for each gene assayed (Karsai et al. 2002). Gene levels were normalized to the housekeeping gene actin (*act-4*) levels.

Data analysis

The LC_{50} was calculated by the Spearman–Karber method (LC50 software V1.0, U.S. EPA Release). One-way ANOVA was applied on stress resistance rhythms, followed by a Dunnet's test for group comparison (control group: ZT12 day 2 and CT12 day 2, for LD and DD, respectively). 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 (dampened sine): $y = y_0 + a \exp(-x/d) \sin(2\pi x/b + c)$, where y is nth data point, x is the time of the nth data point, y_0 is the baseline value, a is the amplitude, b is the period of the wave, c is the phase and d is the dampening parameter of the waveform. Acrophases were determined by Cosinor analysis (i.e., best fit to cosine waveforms) of the data sets; RT-PCR results were grouped by day (ZT0.5 and ZT6)–night (ZT12.5 and ZT18) and analyzed by means of two-tail Student's t test. Osmotic stress resistance assays of mutant strains were analyzed by one-way ANOVA followed by a Dunnet's test for group comparison (control group: ZT0). All data were expressed as the mean ± *SEM* of n values.

Results

Daily variation of resistance to osmotic and oxidative stress in adult nematodes

To assay the existence of a rhythmic pattern of stress resistance in adult nematodes, we quantified the tap response behavior throughout the day or circadian cycle. When a population of adult nematodes is treated with increasing concentrations of NaCl, a correlation in tap response is found (Fig. 1a). We chose the LC_{50} (340 mM) for this test. When different samples of a population of adult nematodes were treated with this NaCl concentration, a rhythmic pattern was observed under both light–dark and constant dark conditions ($n = 27$ for LD and

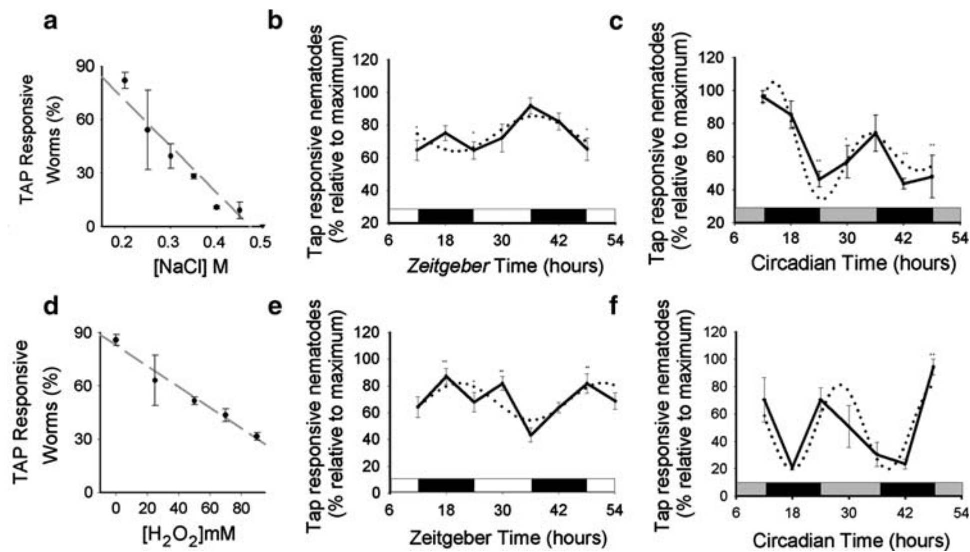

Fig. 1 Rhythmic stress tolerance in adult *C. elegans*. Osmotic stress tolerance: **a** LC50 assay for NaCl; **b** the percentage of tap responsive nematodes after NaCl treatment varies rhythmically along the day in LD 12:12 conditions ($n = 27$ for each time point); **c** circadian rhythm in osmotic stress tolerance under DD conditions ($n = 9$ for each time point). Oxidative stress tolerance: **d** LC50 assay for H_2O_2; **e** nematodes kept in LD 12:12 conditions show a daily rhythm in oxidative stress tolerance ($n = 27$ for each time point); **f** circadian rhythm of oxidative stress tolerance under DD conditions ($n = 9$ for each time point). Data was analyzed by one-way ANOVA followed by Dunnet post hoc tests (*$P < 0.05$ vs. ZT/CT 12). In all cases, means ± SEM are shown. *Dotted lines* correspond to a dampened sine fit to the data

$n = 9$ for DD, $P < 0.05$, ANOVA, in both cases) with a minimum tolerance at ZT0 or CT0 in LD cycles or continuous darkness, respectively ($P < 0.05$) (Fig. 1b, c). A peak was found around ZT12/CT12 ($P < 0.05$, Dunnet multiple comparisons test). LD and DD data sets were then fitted to a dampened sine equation (best fit for circadian conditions corresponded to a 20.6 h period) (Fig. 1b, c—dotted line). Acrophases were determined to be ZT13.74 (LD) and CT13.19 (DD), as shown by Cosinor analysis ($P < 0.05$).

When the same procedure was applied for a H_2O_2 oxidative shock (Fig. 1d, $LC_{50} = 50$ mM), adult nematodes also showed a daily pattern in LD and DD ($n = 27$ for LD and $n = 9$ for DD, ANOVA test $P < 0.05$ in both cases) but with an inverted phase in comparison to the osmotic shock (maximum = ZT0 or CT0 in DD, $P < 0.05$, Dunnet multiple comparisons test) (Fig. 1e, f). LD and DD data sets were fitted to a dampened sine equation; the circadian period under DD was found to be of 20.3 h. Acrophases were determined to be ZT23.42 (LD) and CT2.49 (DD), as shown by Cosinor analysis ($P < 0.05$).

Although the temperature shock assay showed a linear correlation between the number of locomotive animals and temperature values (data not shown), we could not apply the tap assay approach because when the nematodes are transferred from the shock temperature ($LC_{50} = 32.5$°C) to the recording temperature (25°C), locomotion is significantly affected and the result is not reproducible over time. No statistical significant rhythms were found in tap responsiveness when worms were treated with M9 buffer (data not shown).

Expression of stress-related genes

To relate stress resistance to gene responses, we measured the expression level of stress-related genes in *C. elegans* when given a shock of heat, oxidative or osmotic stimuli. As shown in Fig. 2 and Table 1, osmotic, oxidative and temperature shocks were sufficient to induce *hsp6* (heat shock protein 70 kDa), *hsp16* (heat shock protein 16 kDa), *gpx* (gluthathione peroxidase) and *gpdh-1* (glycerol-3-phosphate dehydrogenase) transcription. The observed inductions correlate well with previous publications for hsp6 (Heschl and Baillie 1990), hsp16 (Link et al. 1999; Hong et al. 2004), gpdh-1 (Lamitina et al. 2006; Choe and Strange 2007).

Because rhythmic tolerance to a stressful environment could be genetically controlled, we studied the basal levels of stress-related characterized genes. Although not all of them showed a clear day versus night variation, we found that *gpx* (oxidative stress-related) and gpdh-1 (osmotic stress-related) showed a significantly higher expression level at night time (Fig. 3, Table 2, Student's *t* test, $P < 0.05$). These results were confirmed by real-time PCR using the standard curve method for *gpx* and *gpdh-1* (ZT18/ZT6: *gpx* = 1.7×; *gpdh-1* = 5.2×; $n = 3$ with technical duplicates).

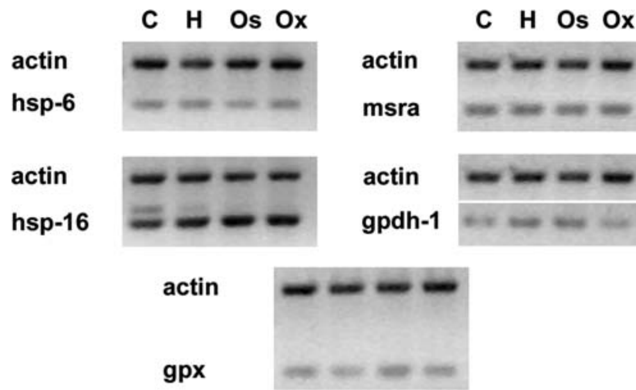

Fig. 2 Expression of stress-related genes. Representative gels of RT-PCRs of stress-related genes. Reactions were performed as described after nematodes were subjected to shocks of 350 mM NaCl (osmotic), 50 mM H_2O_2 (oxidative) or 32°C (temperature) and compared to basal levels. *hsp-6* expression was induced by heat and oxidative shocks; *hsp-16* and *gpx* were induced by any of the three shocks; *gpdh-1* was induced by either heat and osmotic shocks; *msra* levels remained without change after the shocks, as can be seen in the table

Table 1 Quantification of the RT-PCR reactions for stress-related genes

Gene	Type of shock		
	Heat	Osmotic	Oxidative
hsp-6	++	–	–
hsp-16	++	++	++++
gpx	++	+++	++
msra	–	–	–
gpdh-1	+	+	–

+: ≥30% increase in mRNA levels; ++: ≥60% increase in mRNA levels; +++: ≥90% increase in mRNA levels; ++++: ≥120% increase in mRNA levels. In all cases, the increase is relative to controls (i.e., buffer or no temperature change)

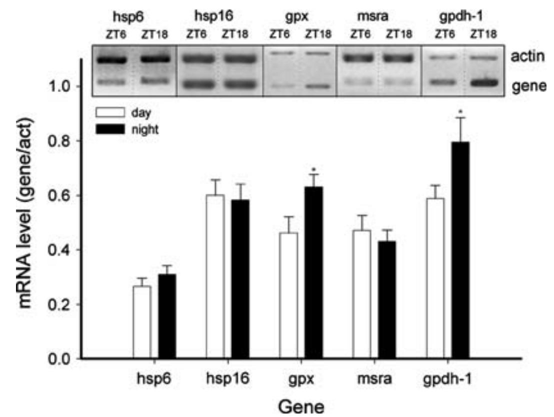

Fig. 3 Diurnal variation in stress-related gene expression. RT-PCRs of stress-related genes were performed as described and day–night expression was quantified. RT-PCR products show significant variation in day versus night expression of glutathione peroxidase (*gpx*) and glycerol-3-phosphate dehydrogenase (*gpdh-1*), their levels being higher during the night. Difference in band intensities between the assayed time points is shown in a representative gel above each *column*. **P* < 0.05, Student's *t*-test

The sensitivity in rhythmic osmotic stress resistance is affected by the gpdh-1 pathway

To characterize the role of *gpdh-1* in the circadian variation of stress tolerance, we studied the behavior of mutants of the osmotic stress response pathway. As shown in Fig. 4a, mutants for *osr-1* (a negative regulator of the osmotic pathway) were in general less sensitive to stress (the percentage of tap responsive nematodes percentage at ZT0 and ZT24 was significantly higher for *osr-1* mutant (AM1 strain) when compared to the control strain (TJ1060), ($P < 0.05$, Dunnet's comparisons test) and lacked a circadian variation in response to 340 mM NaCl ($P > 0.05$, Dunnet's comparisons test. When this experiment was performed with a higher concentration of NaCl (corresponding to the LC_{50} = 450 mM obtained for the *osr-1* mutant), a daily variation appeared in the *osr-1* mutant and still persisted in the control strain as shown in Fig. 4b ($P < 0.05$, Dunnet's comparisons test).

Discussion

The nematode *C. elegans* was originally isolated from compost, mushroom beds, garden soil and water, and is also associated with millipedes, isopods, insects, snails and slugs (Kiontke and Sudhaus 2006). As in other organisms,

Table 2 Quantification of the diurnal variation in the expression of stress-related genes (RT-PCR reactions) at ZT0.5, ZT6, ZT12.5 and ZT18

Gene	Zeitgeber time				*P* (ANOVA)
	ZT0.5	ZT6	ZT12.5	ZT18	
hsp-6	0.24 ± 0.04 (5)	0.28 ± 0.04 (9)	0.33 ± 0.04 (5)	0.30 ± 0.05 (7)	>0.05
hsp-16	0.58 ± 0.13 (5)	0.61 ± 0.06 (9)	0.61 ± 0.14 (4)	0.56 ± 0.05 (6)	>0.05
gpx	0.42 ± 0.08 (5)	0.43 ± 0.08 (7)	0.57 ± 0.07 (5)	0.69 ± 0.06 (7)	<0.05
msra	0.55 ± 0.11 (5)	0.42 ± 0.05 (8)	0.35 ± 0.07 (5)	0.48 ± 0.05 (7)	>0.05
gpdh-1	0.52 ± 0.06 (5)	0.63 ± 0.07 (8)	0.57 ± 0.04 (4)	0.81 ± 0.06 (7)	<0.05

The number of samples assayed in each time point is enclosed within parentheses. All values shown are normalized to *act-4*

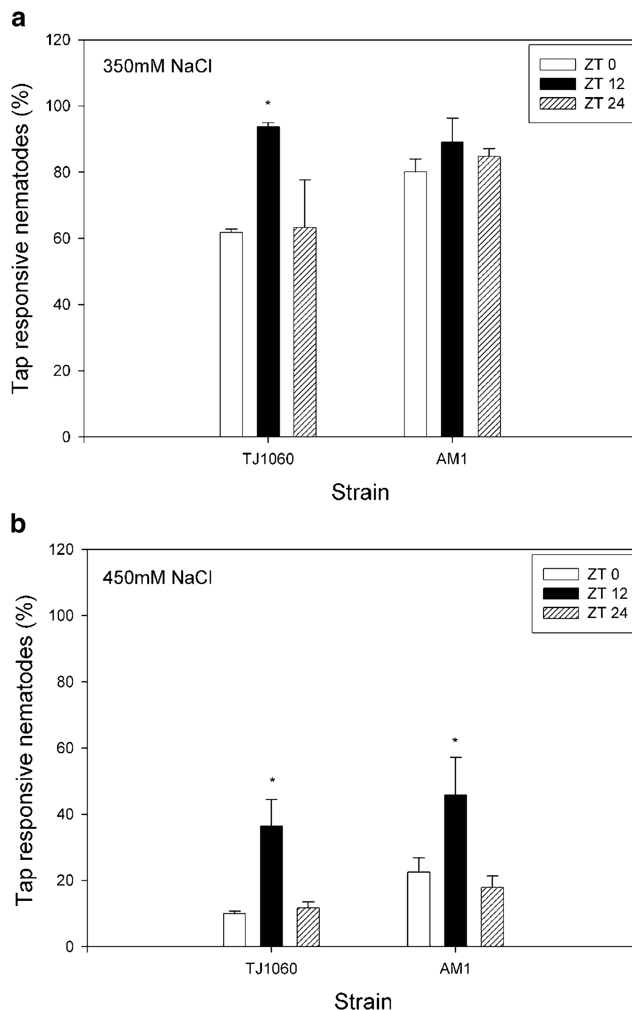

Fig. 4 Diurnal variation in stress tolerance in mutant strains. Different strains were assayed at the maximum and minimum tolerance time points according to the osmotic resistance rhythm and then compared to the control strain. **a** AM1, which is a mutant for the negative regulator in the osmotic stress response (*osr-1*), shows a high tolerance to a 350 mM NaCl shock at both time points. **b** When a higher NaCl concentration (450 mM) is used, AM1 shows a diurnal variation of osmotic stress tolerance. $*P < 0.05$ from ZT0, one-way ANOVA followed by Dunnet post hoc tests

rhythmic temperature, humidity, salinity and UV light environmental conditions could have influenced the appearance and selection of biological circadian controls to not only respond to these changes but also to be able to foresee such conditions in advance (Anokhin 1974; Marques et al. 1997; Engelmann 1988; Pittendrigh 1993).

Daily variations in osmotic stress tolerance

Kippert et al. (2002) provided the first evidence of a circadian clock in *C. elegans* larvae, by studying the response to 1.3 M NaCl throughout the day, which was rhythmic under LD and DD conditions and could be entrained to an experimental photoperiod. We have successfully replicated these results in larvae (data not shown) and considered it worthwhile to study this behavior in adult nematodes. However, since the presence of hermaphrodites is usually considered a problem for long-term studies because of the continuous laying and hatching of eggs, we used two different strategies to prevent reproduction. First, we used the TJ1060 strain, which is infertile at temperatures above 25°C, and in addition, in other experiments, we added FuDR to the culture, which prevents egg viability (Mitchell et al. 1979). Therefore, we were able to work with pure adult cultures of the different strains under study.

The finding of a minimum in the tolerance to osmotic stress at the beginning of the day is in accordance with the reported circadian variation in larvae (Kippert et al. 2002 and our own unpublished results). However, a major difference is that the tolerance range (in terms of NaCl concentration) varies significantly among developmental stages: larvae are able to resist 1.3 M NaCl shocks, while adults do not survive when exposed to concentrations above 0.5 M (in nematodes grown on enriched peptone growth medium with 21 mM NaCl; Solomon et al. 2004). In the field, as temperature rises, desiccation increases, and in accordance with our results, a higher osmotic tolerance would be expected during the day.

We aimed to further describe this response by analyzing the behavior of the AM1 mutant strain, which corresponds to *osr-1* gene and is characterized as high osmotic stress-resistant (Solomon et al. 2004). This phenotype correlates with our results, and indeed, the daily rhythm in osmotic stress (in response to 350 mM) is abolished in *osr-1* mutants. To study whether rhythmicity was truly abolished or if the concentration was insufficient to allow for daily variations, an LD_{50} curve was constructed for this strain (data not shown) and the assay was repeated with 450 mM NaCl. At this concentration, daily variations still persisted in the control strain (with a lower percentage of tap responsive nematodes in each time point when compared to the 350 mM assay) but also appeared in the *osr-1* mutant, suggesting that circadian rhythmicity was not abolished but rather masked, because 350 mM was below the threshold needed for daily variations to appear. This change in sensitivity in response to 350 mM NaCl, with the concomitant masking of the rhythmic behavior, indicates a role of the corresponding gene in the input pathway of the response, which, although not causal to the generation of such response, might be considered important in terms of the signalling machinery of stress responses in *C. elegans*. It is tempting to speculate that circadian changes in the expression of *osr-1* might participate in the circadian gating of stress-induced behavior.

We also studied the *gpdh-1* mutant, which bears a 1,227 bp deletion. Recent studies (Lamitina et al. 2006)

demonstrate that although the rate of hypertonicity-induced glycerol accumulation was slowed in the gpdh-1-deletion mutant, steady-state glycerol levels under control and hypertonic conditions were similar to those in wild-type animals. Accordingly, the mutants exhibited a similar diurnal rhythm as the control strain (data not shown).

Daily variations in oxidative stress tolerance

In addition, we studied the daily variation in response to oxidative stress (hydrogen peroxide). Oxidative reactive species (ROS) could be generated by exposition to UV light and/or by the mitochondria. In this sense, ROS generation could be increased when the animals are highly active, since underground animals are usually not exposed to sunlight. We and others (Simonetta and Golombek 2007; Saigusa et al. 2002) have found that these nematodes are more active during the late night/dawn hours, while the minimum of locomotor activity occurs at the end of the day/early night. This pattern of activity correlates with the rhythm we found in oxidative stress resistance, which might be related to ROS production in response to metabolic activity.

Endogenous control of stress tolerance rhythms

Both rhythmic patterns appear to have an endogenous circadian period of less than 24 h (20.6 and 20.3 h, osmotic and oxidative patterns, respectively) as determined by waveform fitting. However, increasing the sample rate used in the assays could reveal a more precise estimation of circadian period.

It is interesting that these two rhythmic behaviors in response to different stressful stimuli are in antiphase. Cosinor analysis indicated an acrophase of the osmotic stress resistance rhythm at around ZT13 (LD) and CT13 (DD), while peak times for oxidative stress responses were at ZT24 (LD) and CT2 (DD), suggesting a differential mechanism of control for both variables, which might be located at the level of an unknown circadian clock or its output pathway. This also suggests that the rhythms are not affected by an underlying feeding rhythm, which would place both responses under the same phase.

Indeed, the stress response machinery involves many changes at transcriptional, translational and enzymatic levels. In this sense, we aimed to study the transcription of genes related to stress in these species and found that mRNA levels of *gpdh-1* and *gpx* are higher at night. When compared to the observed behaviors, the variation in *gpdh-1* mRNA levels is in accordance with osmotic tolerance rhythms, while *gpx* levels are inversely related to what is seen in terms of oxidative stress tolerance. Indeed, more studies are needed to better understand the link between mRNA rhythms and behavioral rhythms. If rhythms do exist at the protein and/or activity level, mRNA levels need not be in the same phase as their products levels or activity rhythms, which would directly affect the behavior.

Future perspectives

In this work, we have shown the existence of daily rhythms of stress tolerance in the adult nematode *C. elegans*. However, the mechanism responsible for how circadian timing is transduced into stress responses is not well understood. It is known that the cellular stress response machinery is regulated at the neuronal level, through neuroendocrine pathways (Guarente and Kenyon 2000; Kops et al. 2002; Lee et al. 2003; Hwangbo et al. 2004; Liang et al. 2006). In *C. elegans*, several genes, including DAF-16 and TPH-1 (implicated in the production of serotonin), are involved in the neuroendocrine regulation of stress (Liang et al. 2006; Baumeister et al. 2006). Whether these or other genes are related in the circadian control of stress-related behavior remains to be established.

Another question that remains to be answered is how the 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 (Burr 1985). Since no ocelli nor cryptochromes nor opsins are known, the mechanism responsible for light perception is still a mystery to be solved. Indeed, this entrainment pathway is relevant for *C. elegans*' circadian rhythms, which appear to be synchronized by light (Kippert et al. 2002; Saigusa et al. 2002; Simonetta and Golombek 2007) and temperature (Simonetta, unpublished) through unknown input mechanisms. Circadian rhythms are found in organisms living under extreme conditions, including underground and underwater; so the problem of sensing an environment, which gives weak cyclic cues, is definitely relevant for survival.

In summary, we have demonstrated a clear diurnal and circadian rhythm of stress tolerance to two different stimuli in adult *C. elegans*, which appear to be related to specific genes in the metabolic stress pathway. 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.

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