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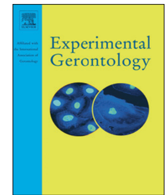

Short report

High transcript levels of heat-shock genes are associated with shorter lifespan of *Caenorhabditis elegans*

X. Manière [a], A. Krisko [a,b], F.X. Pellay [a,c], J.-M. Di Meglio [d], P. Hersen [d,e], I. Matic [a,*]

[a] *Inserm Unit 1001, Université Paris-Descartes, Sorbonne Paris Cité, Faculté de Médecine Paris Descartes, 75014 Paris, France*
[b] *Mediterranean Institute for Life Sciences (MedILS), 21000 Split, Croatia*
[c] *NAOS group/Jean-Noël Thorel, 13855 Aix-en-Provence, France*
[d] *Laboratoire Matière et Systèmes Complexes, UMR7057, CNRS & Université Paris Diderot, 75013 Paris, France*
[e] *MechanoBiology Institute, National University of Singapore, Singapore*

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ABSTRACT

Individual lifespans of isogenic organisms, such as *Caenorhabditis elegans* nematodes, fruit flies, and mice, vary greatly even under identical environmental conditions. To study the molecular mechanisms responsible for such variability, we used an assay based on the measurement of post-reproductive nematode movements stimulated by a moderate electric field. This assay allows for the separation of individual nematodes based on their speed. We show that this phenotype could be used as a biomarker for aging because it is a better predictor of lifespan than chronological age. Fast nematodes have longer lifespans, fewer protein carbonyls, higher heat-shock resistance, and higher transcript levels of the *daf-16* and *hsf-1* genes, which code for the stress response transcription factors, than slow nematodes. High transcript levels of the genes coding for heat-shock proteins observed in slow nematodes correlate with lower heat-shock resistance, more protein carbonyls, and shorter lifespan. Taken together, our data suggests that shorter lifespan results from early-life damage accumulation that causes subsequent faster age-related deterioration.

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1. Introduction

One of the central tenets of biology is that genotype interacts with the environment to produce a phenotype. The same is expected for aging. However, the lifespans of genetically identical inbred laboratory organisms, e.g., *Caenorhabditis elegans* nematodes, fruit flies, and mice, vary greatly between individuals even under identical environmental conditions (Finch and Tanzi, 1997). While the role of genetic and environmental factors in aging has long been under intense scrutiny, the molecular mechanisms responsible for lifespan variation, independent of these two factors, are largely unknown. To study these molecular mechanisms, individual animals of different biological ages must first be identified. Biological age can be determined using biomarkers of aging, which are physiological and physical properties that indicate that the body is aging. However, biomarkers are not simply things that change with age, such as gray hair in humans. A useful biomarker must be a better predictor of lifespan than chronological age. Different biomarkers have been used to determine biological age of *C. elegans* nematodes: accumulation of lipofuscin in the gut, rate of pharyngeal pumping, pharyngeal morphology, defecation rates, internal growth of food bacteria, ability to induce heat-shock response, induction of certain genes (e.g., *sod-3*), and muscle-function decline that results in reduction of motility with age (Eckley et al., 2013; Pincus and Slack, 2010).

Separation of individual nematodes of the same chronological age based on their spontaneous motility demonstrated that the lifespan could vary greatly between different motility categories (Herndon et al., 2002; Hosono et al., 1980). However, the study of freely crawling nematodes is complicated by their erratic behavior, e.g., alternation between active foraging and resting periods, and their irregular trajectories. We have developed an assay based on the stimulation of nematode movement by a moderate electric field, which can evaluate an individual nematode crawling speed without the aforementioned confounding effects. Using this assay, we have previously observed that the capacity of *C. elegans* to respond to an electric field by moving declines with age (Maniere et al., 2011). In this study, we decided to evaluate if this method could be used to sort nematodes based on their biological age. To this end, we sought to trigger animal locomotion by electricity and sort animals as a function of their speed. This approach allowed us to separate and study groups of isogenic animals of the same chronological age. Biological age of different groups of animals was evaluated by measuring survival, protein carbonyls, which are irreversible oxidative damage to proteins, and heat-shock resistance, because it was previously shown that protein oxidation increases (Adachi et al.,

* Corresponding author.
E-mail address: ivan.matic@inserm.fr (I. Matic).

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1998), while heat-shock resistance decreases with age (Ben-Zvi et al., 2009). Finally, to gain insight into molecular mechanisms responsible for different phenotypes characterizing groups of nematodes having different biological ages, we analyzed the transcriptional profiles of genes coding for chaperones and co-chaperones, antioxidant enzymes, stress response transcription factors, and proteasome subunits.

2. Materials and methods

2.1. Nematode and bacterial strain

C. elegans N2 strain, kindly provided by J. J. Ewbank (Centre d'Immunologie de Marseille-Luminy (CIML), UM2 Aix-Marseille Université, Marseille, France), was used for all experiments, unless otherwise indicated. The *Escherichia coli* strain used in this study was the uracil-deficient strain OP50.

2.2. Nematode maintenance and synchronization

Nematodes were maintained at 25 °C on nematode growth medium (NGM) agar plates, which were previously seeded with the stationary-phase bacterial culture and incubated at 37 °C. Age-synchronized populations of nematodes were initiated from eggs recovered following sodium hydroxide (0.5 M final) and sodium hypochlorite (0.96% final) treatment of gravid adults maintained at 25 °C and fed by OP50 bacteria. All assays were carried out with nematodes synchronized twice: once before development by sodium hydroxide/hypochlorite treatment as described above and a second time at the end of development by selecting exclusively nematodes at the end of the 4th larval (L4) stage based on vulva morphology.

2.3. Survival assays

At L4 stage, 60 nematodes were transferred to Petri dishes of 9 cm in diameter where almost all the surfaces were covered by a lawn of OP50 bacteria. Petri dishes necessary for the six first days of experiments were prepared one day before day 0. Nematodes were transferred to new plates every day during the first 5 days to avoid progeny contamination, and every 2–3 days thereafter. Dead nematodes were scored every 24 h. A nematode was considered dead when it failed to respond to plate tapping or a gentle touch with a platinum wire. Only nematodes that died as a result of getting stuck to the wall of the plate were censored. Lifespan was measured as the time from the end of L4 larval stage (beginning of adulthood) until death.

2.4. Electrotaxis assays

For each experiment, approximately 60 nematodes 5 days after L4 stage were transferred from a cultivation plate and rinsed with electrotaxis buffer. They were then transferred on an agar gel in a drop of electrotaxis buffer. After the electrotaxis run, the agar gel was cut into three in order to isolate the 25% slowest nematodes, from the intermediate group and the 25% fastest nematodes. Each piece of agar was then rinsed and nematodes were transferred onto a fresh lawn of OP50 bacteria.

The agar gel was composed of: deionized water, 2% of Bacto-Agar, glycerol (6 mL of glycerol 60% for 1 L), and NaCl (0.250 mmol/L) as previously described in Gabel et al. (2007). The gel was cast by pouring a first layer of agar and adding a PDMS (polydimethylsiloxane) block onto it, so that it will shape the future cavity where nematodes will crawl. A second layer of gel was then poured around the PDMS block. Once solidified, the PDMS block was removed. The resulting agar pad was then placed in an electrophoresis box filled with buffer composed of de-ionized water, glycerol (6 mL of glycerol 60% for 1 L) and NaCl (0.250 mmol/L) as previously described (Gabel et al., 2007). We used a PS305 electrophoresis power supply (APELEX, France) and the Wide Mini-Subtm Cell electrophoresis box (Biorad, USA). Electric field strength in the gel was 4 V/cm. See Supplementary movie for a step-by-step description of the electrotaxis assay protocol.

2.5. Measurements of nematode heat-shock resistance

After the electrotaxis assay, different categories of nematodes were transferred onto a bacterial lawn of OP50 bacteria ($\approx$25 nematodes per bacterial lawn). After allowing nematodes to recover for 12 h at 25 °C, the Petri dishes were transferred to 35 °C. In the survival kinetic, dead nematodes were scored every hour for 10 h. In the 2-hour heat-shock, dead nematodes were censored at the end of the heat-shock.

2.6. Image analysis

Experiments were imaged with a 6.6 Mpixels CMOS monochrome camera (Pixelink) with a close focus zoom lens 10× (136130 mm FL, Edmund Optics Ltd, UK). A white, bright field/dark field ring light (Edmund Optics Ltd.) was used to enhance the contrast. Since the nematode trajectories are nearly straight, image analysis was straightforward. Trajectories of nematodes were computed from images using FIJI including the plugin M2Track and analyzed with a macro in Microsoft excel.

2.7. Preparation of protein extracts and protein carbonylation measurement

Nematodes were collected just after the electrotaxis assay or the heat-shock assay and they were rinsed in M9 before proceeding with protein extraction. Samples of nematodes were pelleted by a 5 minute centrifugation at 4000 ×*g* and resuspended in the lysis buffer of the OxyElisa protein carbonylation detection kit (Millipore) supplemented with a mixture of protease inhibitors (Roche). Approximately 60 animals were resuspended in 100 μL of the lysis buffer. Resuspended nematodes were broken using a mechanical homogenizer with glass beads of 150–202 μm (Sigma) and then centrifuged for 20 min at 12,000 ×*g*. Samples were supplemented with 10 mg/100 μL lipid removal agent (Sigma 13360-U), kept 1 h at room temperature (RT) with shaking and centrifuged for 15 min at 10,000 ×*g*. Genomic DNA fragments were removed by using carboxylated nanobeads (Shanghai Ehoo Biotechnology Co., Ltd., average particle size: 80 nm, surface carboxyl volume ≥ 0.25 mmol/g) at the final concentration of 2 mg/mL, kept at RT for 15 min followed by centrifugation of 15 min at 10,000 ×*g*. The amount of protein in the supernatant was measured by the BCA kit (Pierce) and total protein carbonylation using the OxyElisa kit according to the producer's manual. Briefly, protein extracts diluted to 10 μg/mL were loaded into ELISA wells (Nunc, provided in the kit) and incubated overnight at 4 °C to allow proteins to adsorb to the surface. This step was followed by DNPH (component of the kit) derivatization of adsorbed proteins and detection of derivatized dinitrophenol (DNP)-carbonyl by a rabbit anti-DNP primary antibody and goat anti-rabbit secondary antibody conjugated to HRP (included in the kit). Subsequent incubation with enzyme substrate 3,3′,5,5′-tetramethylbenzidine resulted in a colored product that was quantified using a microplate reader with maximum absorbance at 450 nm.

2.8. RNA isolation and quantitative RT-PCR

After electrotaxis, assay nematodes from each category were divided into aliquots of ~80 individuals. First, nematodes were rinsed in M9 to minimize contamination and then suspended in TRIzol (Invitrogen), 90% of final volume. DNA was removed with a Turbo DNase (Ambion) treatment and the Superscript III (Invitrogen) was used to synthesize cDNA. Quantitative PCR was performed using the SYBRgreen method. Relative levels of mRNA were normalized to the level of act-1. Experiments were repeated at least three times. Primers used in this study are published elsewhere (Supplementary material Table 2).

2.9. Statistical analyses

Statistical analyses and graphic displays were made using Prism 5.0c from GraphPad Software, Inc.

3. Results and discussion

3.1. Electrotaxis allows sorting of nematodes and prediction of lifespan

Nematodes were exposed to a mild electric field on day 5 of adulthood due to the following rationale (Fig. 1A): (i) Previous work suggested that spontaneous motility of nematodes during reproductive period may not be a relevant criterion to predict life expectancy (Herndon et al., 2002). Noteworthy, gravidity could also have confounding effects on our assays. (ii) Over 99% of nematodes are alive at this time point. (iii) The amount of protein carbonyls in a cohort of isogenic nematodes rapidly increased after the end of the reproductive period (Fig. 1A). Innocuousness of electric field on nematode longevity was first verified. The survival curves for nematodes with and without exposure to an electric field exhibited no difference (Supplementary material Fig. 1). This indicated that mild electric field had neither deleterious nor hormetic effect on nematodes, unlike what was previously observed with mild heat-shock or oxidative stress (Cypser and Johnson, 2002; Link et al., 1999).

The age-synchronized isogenic cohorts of *C. elegans* nematodes were placed on the agar gel. They were subjected to an electric field for 8 min, and their displacements were monitored. Some nematodes crawled more than 6 cm, while others hardly moved from the starting point (Fig. 1B). The distance that nematodes crawled from the starting line correlated well ($R^2 = 0.912$) with their speed in spite of the fact that they sometimes changed direction or made pauses (Supplementary material Table 1). Separation of different categories was performed by cutting electrophoresis gel in the three sections, which allowed us to divide cohorts of approximately 60 individuals per experiment in 3 categories: 25% fast, 50% intermediate and 25% slow. We then studied the lifespan of nematodes from the different categories. Nematodes from the fast category lived 15% longer ($T_{50} = 15$ days) than nematodes from the intermediate category ($T_{50} = 13$ days). Nematodes from the slow category exhibited a 31% shorter lifespan ($T_{50} = 9$ days) than nematodes from the intermediate category (Fig. 1C).

3.2. Heat-shock resistance is an earlier predictor of lifespan than protein carbonyl content in the absence of stress

The mortality curves for the three categories of nematodes show that the initial mortality (intercept with the Y axis) of the fast, intermediate, and slow categories ranged from low to intermediate to high, respectively (Supplementary material Fig. 2). We hypothesized that the high initial mortality could result from the accumulation of damage and/or reduced stress resistance already early in life (Gavrilov and Gavrilova, 2001). For this reason, the protein carbonyl level and heat-shock resistance for different nematode categories were measured. The level of protein carbonyls was significantly higher in the slow category than in the fast and intermediate categories, 43% vs. 33%, respectively (Fig. 2A). There was no difference in the level of protein carbonyls between the fast and the intermediate categories. On the contrary, the heat-shock resistance of the nematodes from the 3 categories was significantly different. After 10 h of heat-shock at 35 °C, 80% of the nematodes from the fast category, 54% from the intermediate category and 27% from the slow category were still alive (Fig. 2B). As stress can reveal hidden heterogeneity within population (Rea et al., 2005), nematodes from the 3 categories were exposed to 35 °C for 2 h, and protein carbonyl content was measured. We observed a significant increase in the levels of protein carbonyls in the 3 categories. Nematodes from the slow group had significantly more protein carbonyls (26%) than the fast group (Fig. 2C). In summary, while physiological level of protein

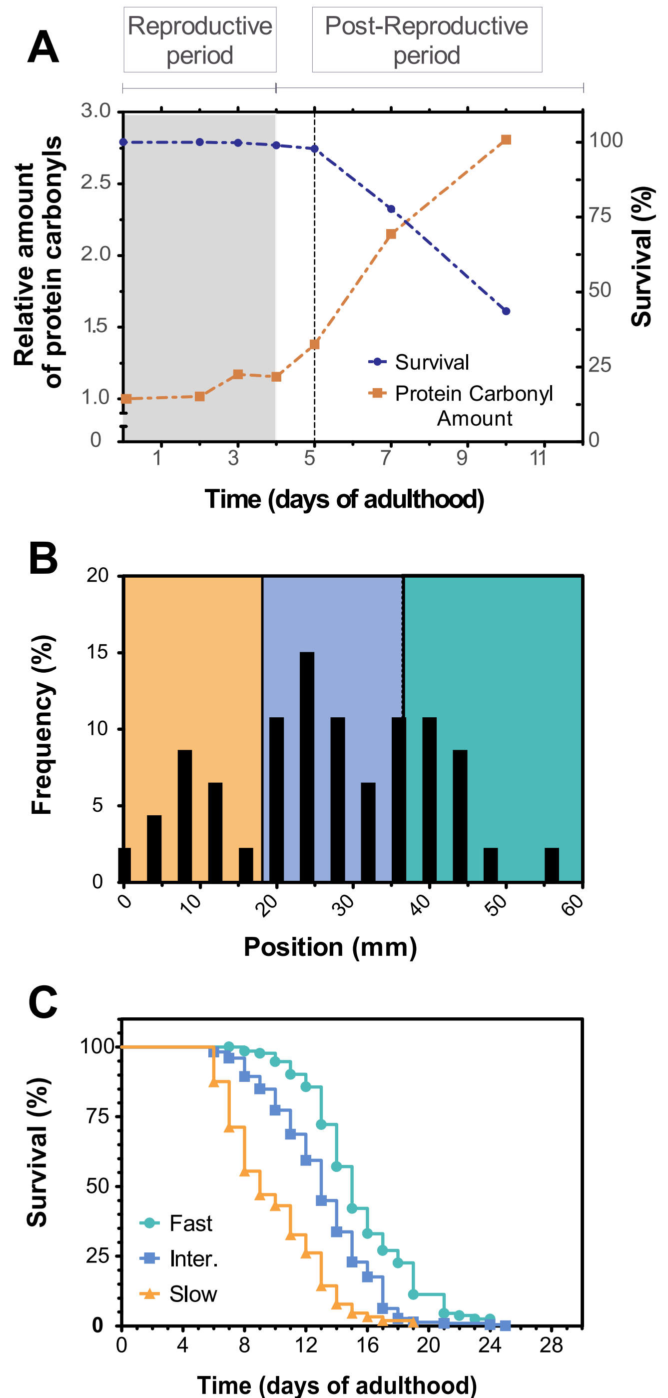

Fig. 1. Electrotaxis at day 5 of adulthood allows sorting of nematodes and prediction of lifespan. (A) The orange curve represents the amount of protein carbonyls in nematodes at different time points relative to the amount of protein carbonyls in nematodes at day 0. Day 0 represents the end of developmental stage. Reproductive period is indicated in gray. The blue curve represents survival (%) of adult nematodes over time. (B) Positions of individual day 5 adult nematodes (N = 47) on the gel after 8 min of the electrotaxis run. The ~25% slowest nematodes belong to the slow category, the ~25% fastest nematodes to the fast category, and the rest of the nematodes to the intermediate category. (C) Survival of different categories of nematodes: Fast group (green), intermediate group (blue) and slow group (orange). The difference between the life expectancies of nematodes from the three groups was significant (Log-rank (Mantel–Cox) Test, P value < 0.0001). Graph represents pooled results of 3 different experiments with similar results (N = 503).

carbonylation did not distinguish between the 3 categories of nematodes with different life expectancies, the ability to withstand heat-shock significantly varied between the 3 nematode groups. Resistance

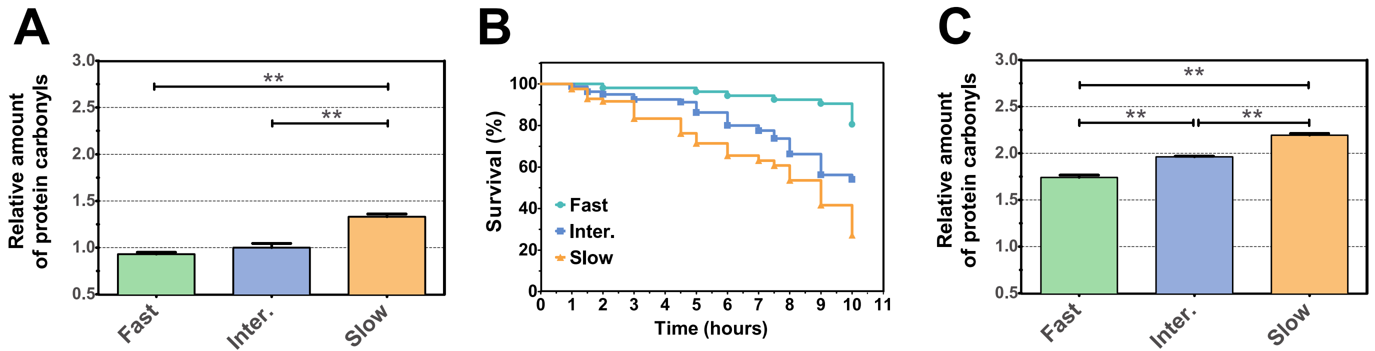

Fig. 2. Heat-shock resistance is an earlier predictor of lifespan than protein carbonyl content. (A) Relative amount of the protein carbonyls in groups of nematodes sorted by their speed. Data are normalized according to the values of the intermediate category. Presented data are mean values (±SEM) from 4 independent experiments. (B) Survival of the different groups of nematodes over time at 35 °C. Fast group (green), intermediate group (blue) and slow group (orange). Difference between the survivals of nematodes from different groups was significant (Log-rank (Mantel–Cox) Test, P value < 0.0001). Graph represents pooled data from 3 different experiments with similar results (N = 217). (C) Relative amount of protein carbonyls after a 2 hour heat-shock at 35 °C in different nematode categories. Data are normalized relative to the intermediate category of the unstressed nematodes as in (A). Presented data are the mean values (±SEM) from 4 independent experiments. (P-values were calculated using Student's t-test: ** represents a P-value < 0.001).

to heat-shock and the ability to limit protein carbonyl accumulation during heat-shock seem to be early biomarkers of long lifespan.

3.3. High transcript levels of genes coding for small heat-shock proteins in the absence of stress are associated with short lifespan of post-reproductive adults

The transcriptional profiles of genes coding for chaperones and co-chaperones, antioxidant enzymes, stress response transcription factors, and proteasome subunits in the 3 nematode categories were analyzed. This analysis was performed in order to gain further insight into molecular mechanisms responsible for variation in biological age within a cohort of isogenic age-synchronized nematodes. Nematodes from the fast category, which had longer lifespan, fewer protein carbonyls and higher heat-shock resistance, had significantly higher transcript levels of the *daf-16*, *hsf-1,* and *gcs-1* genes than nematodes from the slow category (Fig. 3B, C). Conversely, nematodes from the slow category had significantly higher transcript levels of the genes coding for the two small

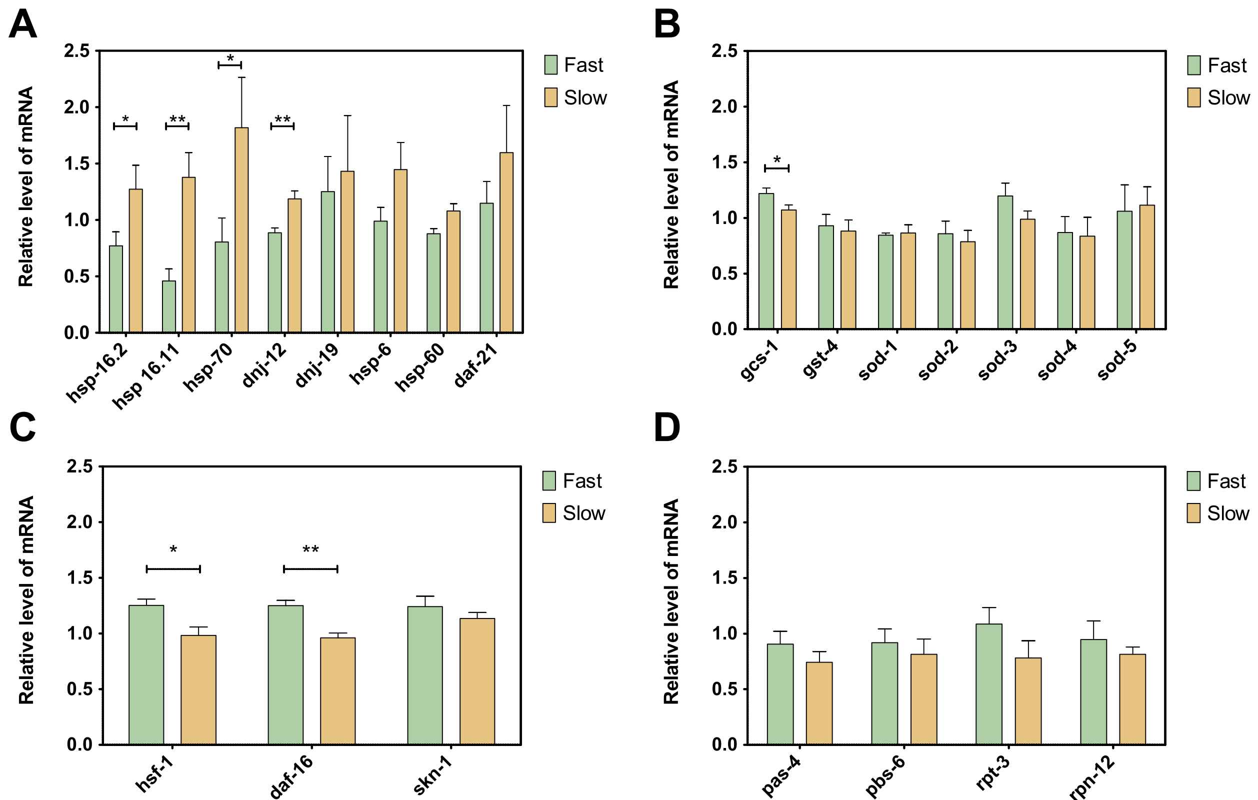

Fig. 3. Transcript levels in the different categories of nematodes. Transcript levels were measured by qRT-PCR. Transcript levels were first normalized to *act-1* as an internal control and then normalized to the values of the intermediate category for each experiment. Genes are grouped according to the Gene Ontology. (A) Chaperones and co-chaperones: *hsp-16.2* and *hsp-16.11* are small heat-shock proteins; *hsp-70*, *hsp-60* and *hsp-6* are heat-shock proteins; *daf-21* is an HSP-90; and *dnj-12* and *dnj-19* are co-chaperones. (B) Antioxidant defense enzymes: *gcs-1* and *gst-4* are related to glutathione metabolism and *sod-1*, *sod-2*, *sod-3*, *sod-4* and *sod-5* are the superoxide dismutases. (C) Stress response transcription factors: *hsf-1* regulates heat-shock and proteotoxicity stress response, *daf-16* regulates general stress response, and *skn-1* regulates oxidative stress response. (D) Proteasome subunits: *pas-4* and *pbs-6* are core proteasome subunits and *rpt-3* and *rpn-12* are proteasome regulatory subunit. Presented data are the mean values (±SEM) from at least 3 independent experiments. (P-values were calculated using one tailed Student's t-test: * represents a P-value < 0.05, ** represents a P-value < 0.001).

heat-shock proteins, *hsp-16.2* and *hsp-16.11*, the heat-shock protein *hsp-70* and the co-chaperone *dnj-12*, than nematodes from the fast category (Fig. 3A). Other tested genes related to antioxidant defense and protein degradation showed no statistical difference of transcript levels between the different nematode categories (Fig. 3B, D).

In our study high transcript levels of small heat-shock proteins genes are associated with short lifespan. The same negative correlation between high heat-shock reporter gene expression early in life and lifespan was also observed in *Drosophila* (Yang and Tower, 2009). The results presented in this study contrast with previous reports that associated to high transcript levels of small heat-shock proteins genes with long lifespan and/or increased tolerance to proteotoxic stress in nematodes (Fonte et al., 2008; Rea et al., 2005; Walker and Lithgow, 2003). These seemingly conflicting results can be explained by differences in the experimental procedures, which preclude direct comparisons: different temperatures (20 °C vs 25 °C), *hsp-16.2* transcriptional control (spontaneous, stress-induced or artificial overexpression), and nematode age (young vs post-reproductive adults).

We measured spontaneous transcription level of the *hsp-16.2* gene in post-reproductive adults at 25 °C. Johnson's group showed that the level of *hsp-16.2* induction with the heat-shock (2 h at 35 °C) in young nematodes was correlated with long lifespan at 20 °C. However, they did not measure the transcript levels of *hsp-16.2* in post-reproductive adults (Rea et al., 2005). Link's group observed that constitutive overexpression of *hsp-16.2* could delay toxic effect of β-amyloid peptide synthesis in young nematodes at 25 °C. Therefore, the expression of *hsp-16.2* well above the physiological level attenuates proteotoxic stress, but does not provide full resistance as these nematodes die rapidly after the induction of the β-amyloid peptide synthesis (Fonte et al., 2008). Walker and Lithgow showed that multiple copies of *hsp-16A* extend nematode lifespan maintained lifelong at 20 °C. However, they did not measure *hsp-16.A* transcript levels in post-reproductive adults (Walker and Lithgow, 2003). Therefore, it cannot be excluded that transcript levels of *hsp-16.A* in post-reproductive adults could have been negatively correlated with subsequent lifespan within the population of those nematodes, even though they lived longer than nematodes with less *hsp-16.A* copies. The same could be true for the nematodes from Johnson's study.

Two nonexclusive possibilities could explain why high transcript levels of small heat-shock proteins are not beneficial in our case. (i) High transcript levels of small heat-shock proteins genes can be induced in slow nematodes as a consequence of the irreversible protein damage accumulation. In such cases, induction of these genes can be considered as a proxy of advanced senescence. Our results indeed showed that slow nematodes have more protein carbonyls and lower heat-shock resistance than the nematodes from the fast category. Notwithstanding the fact that proteins are more carbonylated in the slow group, genes coding for the antioxidant response are not induced. This may be because there are more misfolded proteins that are prone to oxidation rather than higher level of reactive oxygen species in these nematodes. Misfolded proteins could accumulate because of high translation error rates (Dukan et al., 2000), folding issues, and/or because of inefficient protein degradation. (ii) High transcript levels of the small heat-shock protein genes could be induced in order to compensate for the reduction of the amount of functional small heat-shock proteins. This induction may be due to the aggregation of insoluble small heat-shock proteins, which increases with the nematode age (David et al., 2010).

In agreement with our hypothesis that the induction of these genes can be considered as an indication of advanced senescence, published transcriptome analysis of the aging *C. elegans* showed that the expression of heat-shock protein genes increased after the reproductive period, even at 20 °C (Eckley et al., 2013; Lund et al., 2002). On the other hand, genes coding for the transcription factors DAF-16 and HSF-1 were more expressed in the fast than in the slow category. Overexpression of *hsf-1* in transgenic nematodes increases their lifespan (Morley and Morimoto, 2004); moreover, high expression of these genes could result in an enhanced response to stress. For example, it was shown that the ability to strongly induce *hsp-16.2*, which is regulated by DAF-16 and HSF-1, after a heat-shock predicts longer lifespan in nematodes (Cypser et al., 2013; Rea et al., 2005). Importantly, these studies also showed that the transcription level of the *hsp-16.2* gene correlated with the *hsp-16.2* promoter driven GFP protein levels, as well as the HSP-16.2 protein levels (Link et al., 1999). Such correlation remains to be demonstrated under our experimental conditions. Similarly, it remains to be determined which molecular mechanisms are responsible for the lower levels of small heat-shock protein gene transcripts in fast nematodes despite the fact that they have increased levels of *hsf-1* and *daf-16* transcripts. Finally, the higher transcript level of the *gcs-1* gene coding for the limiting step of the synthesis of glutathione in the fast category of nematodes could indicate increased protection against reactive oxidative species.

4. Conclusions

The data presented in this study demonstrates that electrotaxis can be used as a biomarker of aging, because nematode speed in an electric field is a better predictor of lifespan than chronological age. Fast nematodes display longer lifespan, fewer protein carbonyls, and higher heat-shock resistance, as well as significantly higher transcript level of the *daf-16*, *hsf-1*, and *gcs-1* genes than nematodes from the slow category. The high transcript level of the genes coding for the small heat-shock proteins observed in the slow nematodes correlates with short-lifespan. This correlation indicates that these genes may be induced as a consequence of advanced senescence. Therefore, long lifespan could be associated with a higher reactivity to stress, rather than with overprotection, which is speculated to be physiologically costly.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.exger.2014.09.005.

Conflict of interest

The authors have no conflict of interest.

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