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Tyrosol, a main phenol present in extra virgin olive oil, increases lifespan and stress resistance in *Caenorhabditis elegans*

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ABSTRACT

Extra virgin olive oil (EVOO) consumption has been traditionally related to a higher longevity in the human population. EVOO effects on health are often attributed to its unique mixture of phenolic compounds with tyrosol and hydroxityrosol being the most biologically active. Although these compounds have been extensively studied in terms of their antioxidant potential and its role in different pathologies, their actual connection with longevity remains unexplored. This study utilized the nematode *Caenorhabditis elegans* to investigate the possible effects of tyrosol in metazoan longevity. Significant lifespan extension was observed at one specific tyrosol concentration, which also induced a higher resistance to thermal and oxidative stress and delayed the appearance of a biomarker of ageing. We also report that, although tyrosol was efficiently taken up by these nematodes, it did not induce changes in development, body length or reproduction. In addition, lifespan experiments with several mutant strains revealed that components of the heat shock response (HSF-1) and the insulin pathway (DAF-2 and DAF-16) might be implicated in mediating tyrosol effects in lifespan, while caloric restriction and sirtuins do not seem to mediate its effects. Together, our results point to hormesis as a possible mechanism to explain the effects of tyrosol on longevity in *C. elegans*.

1. Introduction

Extra virgin olive oil (EVOO), a central ingredient of the Mediterranean diet, is unique among other vegetable oils due to its particular extraction process by mechanical procedures that preserve minor compounds originally present in the olive fruit (*Olea Europea* L.) (Visioli and Galli, 1998). In the last decades, increasing evidence has suggested that regular EVOO intake might have multiple beneficial effects on health, as it has been associated with a lower incidence of atherosclerosis, cardiovascular disease and certain types of cancer (Gerber, 2003; Stoclet et al., 2004). Likewise, in numerous epidemiologic studies, Mediterranean diet and, more specifically, olive oil consumption have also been associated to a higher longevity in the human population (Buckland et al., 2011; Lagiou et al., 2006; Trichopoulou et al., 2005). Although health-protective effects of olive oil have been traditionally attributed to its high content of monounsaturated fatty acids, mainly oleic acid, it is now widely accepted that the particular abundance of different antioxidants, especially phenolic compounds, is also highly implicated in these effects. The main families of phenols present in olive oil are: simple phenols, lignans, flavonoids and sercoiridoids (Carrasco-Pancorbo et al., 2005; García-Villalba et al., 2009). Among the first group, tyrosol and hydroxytyrosol, in simple forms or as conjugates, are considered the most important for their contribution to flavor, stability and nutritional value of oil, with also demonstrated bioavailability in humans (Bendini et al., 2007). In addition, these compounds have shown a strong antioxidant activity as well as the ability to inhibit pro-oxidation processes on human LDL particles in numerous *in vitro* experiments (Caruso et al., 1999; Correa et al., 2009; Visioli and Galli, 1998). Even though the protective role of EVOO phenols in different pathologies is currently the object of extensive research, to our knowledge, there have been very few studies that explore in depth the molecular mechanisms by which olive oil may influence longevity (Jacomelli et al., 2010), being often

Abbreviations: EGCG, epigallocatechin gallate; EIC, extracted ion chromatogram; EVOO, extra virgin olive oil; HSF-1, heat shock transcription factor-1; LC-TOFMS, liquid chromatography time-of-flight mass spectrometry; NGM, nematode growth medium; ROS, reactive oxygen species; *sHsp*, small heat shock proteins.

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assumed that these effects are the result of the antioxidant potential of its phenolic compounds and other free-radical scavengers such as vitamin E (Frankel, 2011).

Recent studies suggest that the combination of antioxidant/anti-inflammatory polyphenols found in many fruits and vegetables may show efficacy in slowing aging (Joseph et al., 2005), although many of them seem to act through molecular mechanisms in part independent to their antioxidant properties. In this sense, resveratrol present in red wine has emerged as an important mediator of longevity, as it has proved capable of acting through the homeostasis regulator sirtuin in different animal models (Baur et al., 2006; Howitz et al., 2003; Valenzano et al., 2006; Viswanathan et al., 2005; Wood et al., 2004). Other dietary flavonoids, such as quercetin and blueberry polyphenols among others, have been shown to modulate the lifespan in simple model organisms also by activating molecular mechanisms separable from their direct antioxidant capacity (Saul et al., 2008; Wilson et al., 2006). Surprisingly, despite their high structural resemblance with some of these polyphenols cited above, none of the phenolic components present in EVOO have been studied previously in terms of their potential to extend lifespan.

In order to investigate the effects of olive oil phenols on parameters related to aging, we designed a study to determine whether tyrosol, one of the most abundant phenols in EVOO, was able to affect longevity in the context of a complete organism. For this purpose, we chose the nematode *Caenorhabditis elegans*, a multicellular, well characterized model organism with a relatively short lifespan that can be reproducibly assayed (Guarente and Kenyon, 2000). The fact that several important aspects influencing aging, such as genetic and environmental factors, are conserved between nematodes and mammals, including humans, makes this organism the ideal choice for our study (Garigan et al., 2002; Gems and Riddle, 2000; Herndon et al., 2002).

The main objective of this work was to determine if tyrosol is able to affect the lifespan of *C. elegans*. We also aimed to examine the effects of tyrosol on different parameters related to longevity in this organism, in particular, resistance to thermal and oxidative stress, body length, reproduction and pharynx pumping rate. Finally, we have attempted to identify the molecular pathways or genes which could be involved in the physiological effects of tyrosol by analyzing some of the mechanisms that have been shown to mediate longevity in response to other dietary phenolic compounds. Our findings show, for the first time, that one simple phenol present in EVOO can provide anti-aging benefits *in vivo* in a whole organism and, although these effects might be in part related to tyrosol antioxidant properties, other additional molecular mechanisms seem to be implicated in this protective response.

2. Materials and methods

2.1. Strains and growth conditions

C. elegans strains were routinely propagated at 20 °C on Nematode Growth Medium (NGM) plates containing a lawn of *Escherichia coli* strain OP50 as a food source, unless stated otherwise (Brenner, 1974). Strains used in this study were: N2 (var. Bristol); DH26, *fer-15(b26)II*; TJ356, *zls356[(Pdaf-16::daf-16::GFP)*; *rol-6(su1006)]*; TK22, *mev-1(kn1)III*; VC199, *sir-2.1(ok434)IV*; PS3551, *hsf-1(sy441)I*; GM11, *daf-16(m26)I*; *fer-15(b26)II and* GM6, *fer-15(b26)II*; *daf-2(e1370)III*. The strains GM11 and GM6 were a kind gift from M. Muñoz (Universidad Pablo de Olavide, Spain). The rest of *C. elegans* strains used as well as the OP50 bacteria were obtained from the *Caenorhabditis* Genetics Center (http://www.cbs.umn.edu/CGC/).

2.2. Treatment with tyrosol

For all the analysis, tyrosol (2-(4-hydroxyphenyl) ethylalcohol; Extrasynthese, France) was dissolved in ethanol and added in its final concentrations to the liquid NGM medium previously autoclaved and cooled to 50 °C. The media was immediately dispensed into Petri dishes that were kept protected from light and stored at 4 °C until use. A final ethanol concentration of 0.1% (v/v) was maintained in control and tyrosol containing plates. All the experiments involving tyrosol were always carried out in parallel with a control group that was not exposed to this compound. In all the experiments, nematodes were exposed to the treatment starting from the egg stage unless stated otherwise.

2.3. Lifespan assays

We used the strain *fer-15(b26)*, which is sterile at 25 °C, as wild type control in our lifespan experiments since the sterility phenotype facilitated the analysis by avoiding progeny interference and minimizing internal hatching during lifespan determinations. The lack of significant lifespan differences between this strain and the N2 *wild type* strain at 25 °C has been well documented (Klass, 1977). For longevity assays, synchronous populations were obtained by egg hypochlorite preparation and incubation on fresh NGM plates unless stated otherwise. Animals were raised at 25 °C until the L4 molt (0-day adult) and lifespan scoring was initiated on the first day of adulthood at the same temperature. When using fertile strains, adult nematodes were transferred daily to new treatment plates during the first 4 days of adulthood to prevent progeny overgrowth and then transferred to new treatment plates every 3–4 days. For all the assays the number of surviving animals was monitored daily until death. Nematodes were considered to be dead when they did not respond to a mechanical stimulus with a platinum wire or no pharyngeal pumping was observed. Animals that became desiccated on the side of the plate, died by internal hatching or exploded were censored and incorporated as such in the analysis. Statistical analyses and survival plots of lifespan data were performed with GraphPad Prism 5 software (GraphPad Software Inc.).

2.4. Thermotolerance assays

Synchronized *fer-15(b26)* hermaphrodite nematodes, incubated in the presence or absence of tyrosol, were grown as described for the lifespan assays. At the fifth day of adulthood, about 100 nematodes per treatment and assay were moved to 35 °C for 8 h. For thermotolerance assays with other strains the period of time at 35 °C was adapted accordingly, depending on the strain resistance. After the temperature shift, nematode survival was assessed by responsiveness to a gentle touch with a platinum wire and pharyngeal pumping. Survival was scored as the fraction of animals alive from the original number of animals on the plate. Animals lost from desiccation on the sides of the plate were censored from the analysis. Each assay was performed three times.

2.5. Paraquat assays

For paraquat-induced oxidative stress assays, hypochlorite-isolated eggs were seeded onto control or treatment 60 mm NGM agar plates and then allowed to hatch at 20 °C. After two days, L4 larvae were selected and transferred to 35 mm NGM plates containing paraquat (Paraquat dichloride; Sigma–Aldrich, Germany) to a final concentration of 4 mM, with or without tyrosol, and subsequently incubated at 20 °C. Survival scoring was initiated on the first day of adulthood. For each experimental condition 5 plates were used with at least 100 nematodes in total. Alive and dead animals were scored daily until all had died. Animals were considered dead when no response was observed following a gentle touch by a platinum wire and no pharyngeal pumping was observed. Nematodes which had escaped the plates, died after internal hatching or exploded were excluded from the assay. Each assay was performed at least three times. We used the strain *mev-1(kn-1)* as paraquat-sensitive control (Ishii et al., 1998).

2.6. Pharynx pumping assays

The pharyngeal-pumping rate of *fer-15(b26)* nematodes was quantified at room temperature by video recording individual animals using a Nikon Coolpix 5000 camera under 80× magnification on a Leica MZ10F dissection microscope. A total of 15 nematodes from each day of adulthood analyzed were randomly selected and the pumping frequency was determined at least three times per nematode over a 15 s interval. Videos were scored by individuals blinded to the experimental group.

2.7. Length measurements and reproduction assay

Synchronized hermaphrodites were cultured as in lifespan assays at 25 °C. At adult days 0, 1 and 5 respectively, 20 nematodes per experimental condition were killed by heat exposure and the length of each individual nematode was measured using a microscope with a graduated eyepiece. For reproduction assays, 8 L4 larvae were transferred to individual plates and moved to a fresh plate every 2 days until reproduction ceased. The offspring of each animal was counted at the L4 stage. The experiment was performed twice.

2.8. Nuclear DAF-16::GFP localization

Synchronized cultures of the transgenic strain TJ356 stably expressing a DAF-16::GFP fusion protein (Henderson and Johnson, 2001) were raised from L1 on 35 mm NGM plates and incubated at 20 °C either in the presence or absence of tyrosol until they reached the L4 stage. For fluorescence identification purposes, L4 larvae were directly observed under a fluorescence stereomicroscope (Leica MZ10F). Nematodes were classified according to the subcellular DAF-16 distribution into "cytosolic", "intermediate" and "nuclear". We performed 3 independent trials with an average of 150 nematodes in total for each trial. For micrographs, animals were mounted on agarose pads, anaesthetized with 10 mM levamisole (Levamisole hydrochloride, Sigma–Aldrich, Germany) and photographed in an Axiolab fluorescence microscope (Zeiss, Germany) at a 20× magnification. Animals previously incubated at 37 °C for 2 h were used as positive controls.

2.9. Analysis of gene expression by quantitative RT-PCR

Synchronized *fer-15(b26)* nematodes were grown at an approximate density of 100 animals per plate in the presence or absence of tyrosol. Animals were maintained at 25 °C until they reached the L4 stage or adult days 1 or 4. At this time, nematodes were washed from plates, rinsed 3 times through a 45 μm nylon strainer (BD Falcon, USA), collected into Eppendorf tubes and allowed to settle on ice. The worm pellet was resuspended in 350 μl of lysis buffer and sonicated on ice for total disruption. RNA was isolated using the RNeasyPlus Mini kit (Qiagen, USA) and cDNA was prepared with Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Fermentas Intl.). Real-time PCR was performed in a MxPro thermal cycler (Stratagene, USA) using SYBR Fast Master Mix (2x) Universal (KAPABiosystems, USA) with 1 μM primers and 1 μl cDNA in a 10 μl reaction volume using the following gene-specific primers: *act-1* (NM_073418), GTGTGACGACGAGGTTGCCGCTCTTGTTGTAGAC (F) and GGTAAGGATCTTCATGAGGTAATCAGTAAGATCAC (R); *hsp-1* (NM_070667); CCATTGAGGACGAGAAGCTC (F) and CTTGGAAATGATTGGGTTGG(R); *hsp-12.6* (NM_069267); ATGATGAGCGTTCCAGTGATGGCTGACG(F) and TTAATGCATTTTTCTTGCTTCAATGTGAAGAATTCC(R); *hsp-70* (NM_060084); CGTTTCGAAGAGCTCTGTGCTGATCTTTTCCGC (F) and TTAATCAACTTCCTCTACAGTAGGTCCTTGTGG (R); *hsp-16.1* (NM_072953); GTCACTTTACCACTATTTCCGTCCAGCTCAACGTTC (F) and CAACGGGCGCTTGCTGAATTGGAATAGATCTTCC (R); *mtl-1* (NM_072295); ATGGCTTGCAAGTGTGACTG (F) and TTTCTCACTGGCCTCCTCAC (R); *sod-3* (NM_078363); CCAACCAGCGCTGAAATTCAATGG (F) and GGAACCGAAGTCGCGCTTAATAG (R); *gst-4* (NM_069447); TTTTCTATGGAAGTGACGCTGA (F) and TCACAATATCAGCCCAAGTCA (R); *aip-1* (NM_074078); TTCCCGAATGAATCCATGTT (F) and TGGAATTGGAATTTCTTGTTTG (R); *aqp-1* (NM_063109); ATGGTCCAGGTGTTTTCCAC (F) and GAACAAAAATATCGTCGGCTTC (R). Experiments were performed in triplicates, and the relative quantities of target genes, corrected with the normalizing gene *act-1*, were calculated using the Stratagene MxProTM QPCR Software.

2.10. Analysis of tyrosol uptake by Liquid chromatography time-of-flight mass spectrometry (LC-TOFMS)

Synchronized *fer-15(b26)* nematodes were incubated on 100 mm solid NGM agar plates containing 250 μM tyrosol as in lifespan assays. After 5 days, adult nematodes were collected from the plates and rinsed 3 times through a 45 μm nylon mesh (BD Falcon, USA) with deionized water in order to eliminate bacterial contamination. After centrifugation, the worm pellet was washed 2 more times and finally resuspended in 100 μl of deionized water. Worms were disrupted by 3 cycles of thaw-freezing in liquid nitrogen and then sonicated on ice for complete cuticle disruption. After sonication, the sample was centrifuged and the supernatant was frozen at −20 °C until analysis.

Chromatographic analysis was carried out using an HPLC system (Agilent Series 1290 Infinity, Agilent Technologies, Santa Clara, CA, USA) equipped with a reversed phase C18 analytical column of 100 mm × 4.6 mm and 1.8 μm particle size (Agilent Zorbax XDB-C18). 20 μl of extract was injected in each study. Mobile phases A and B were water with 0.1% formic acid and acetonitrile respectively. The chromatographic method held the initial mobile phase composition (10% B) constant for 5 min, followed by a linear gradient to 100% B at 30 min. The flow-rate used was 0.5 mL min^{-1}.

The HPLC system was connected to a time-of-flight mass spectrometer Agilent TOF 6220 (Agilent Technologies, USA) equipped with an electrospray interface operating in negative ionization mode, using the following operation parameters: capillary voltage: 2500 V; drying gas: 9 L min^{-1}; gas temperature: 325 °C; nebulizer pressure: 40 psig; fragmentor voltage: 140 V. LC-TOFMS accurate mass spectra were recorded across the range 50–1000*m/z*. Agilent MassHunter software was used for full-scan data acquisition and data processing.

2.11. Statistical analysis

Data analysis by Log-Rank (Mantel-Cox) tests and unpaired *t*-tests were performed using GraphPad Prism version 5 for Windows (GraphPad Software, USA). For all the experiments, the effects of tyrosol treatment were compared to untreated controls assayed in parallel.

3. Results

3.1. Tyrosol extends lifespan in C. elegans

In order to characterize the effects of tyrosol on longevity, we first performed a dose–response analysis where we tested several concentrations of this compound added in the nematode medium. Adult *fer-15(b26)* nematodes grown at 25 °C, have a mean lifespan of 12.16 days and an average maximum lifespan of 18.67 days. Among the four different concentrations initially tested, tyrosol at 250 μM was the one that displayed the most significant effect on both, median and mean lifespan (Fig. 1A). Lower and higher concentrations resulted in more subtle increases in median lifespan, although mean lifespan values were not statistically different from those of the control group (Fig. 1B). To corroborate the effects of 250 μM tyrosol, we analyzed a total of 976 animals in 5 independent trials and we found that, indeed, 250 μM tyrosol significantly extended both mean and median lifespan in all of the 5 trials (Table 1). In addition, this tyrosol concentration induced a significant increase in maximum lifespan from 18.67 to 20.68 days (10.76% increase; $p \leq 0.0051$). When applied only from adult day 1, tyrosol treatment (250 μM) also induced a significant lifespan extension in *fer15(b26)* nematodes, suggesting that the observed effect was not the result of a delay in the nematode development in response to tyrosol (Fig. 1C).

3.2. Tyrosol does not inhibit growth of E. coli OP50

C. elegans lifespan is significantly increased when fed with UV-killed bacteria or live bacteria unable to proliferate upon antibiotic treatment (Garigan et al., 2002). Tyrosol and other phenolic compounds in olive oil are known to present antimicrobial effects against several types of bacteria (Tuck and Hayball, 2002). In order to rule out that the concentration of tyrosol used in our experiments had noxious effects on the *E. coli* strain OP50, we monitored the growth of this strain in liquid culture at 37 °C in the presence or absence of tyrosol at the different concentrations previously tested in the lifespan experiments (from 100 μM to 2.5 mM). We also examined the growth of *E. coli* lawns on NGM agar plates by reproducing identical conditions to those used in *C. elegans* lifespan assays. Since we did not observe differences in the bacterial population growth due to the presence of this phenol, either in liquid culture or on solid NGM plates (Fig. 2A and B), we conclude that the effect of tyrosol increasing worm lifespan is not due to a decrease in food availability, also excluding the possibility that this lifespan extension might be indirectly caused by a reduced infection rate in the nematodes by living bacteria.

3.3. Tyrosol postpones the decline in pharyngeal pumping frequency, but does not affect body length, developmental rate or reproduction

One biomarker of aging in *C. elegans* is the speed of the pharynx contraction. Thus, young adult animals pump at an average of 250–300 times per minute and this frequency decreases gradually with age (Huang et al., 2004). We chose to evaluate this marker in order to assess if tyrosol-induced lifespan extension was accompanied of a lower rate of aging in these nematodes. Tyrosol treatment was efficient at slowing aging in *C. elegans*, as it postponed the age-related decline in the pharynx pumping rate compared to non treated controls. This difference was evident at all the time points analyzed from adult day 2 to adult day 9 (Fig. 3A).

The observed tyrosol-mediated lifespan extension did not affect other fitness parameters in the nematodes such as growth and reproduction. Thus, although treated nematodes showed a slight decrease in body length at the L4 larval stage, the body size at adult days 1 and 6 was not affected by tyrosol, suggesting that this

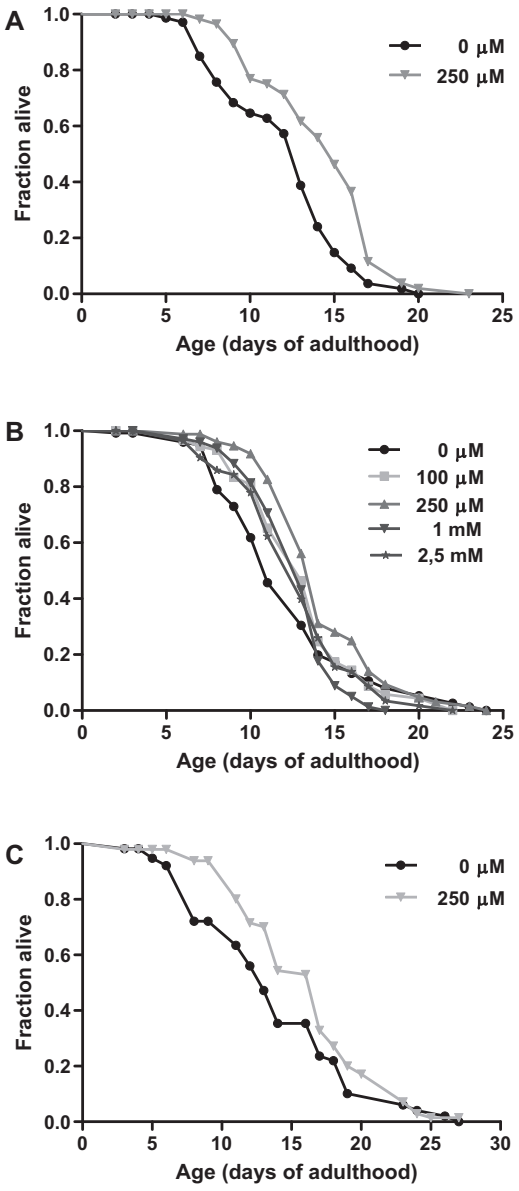

Fig. 1. Analysis of tyrosol on *C. elegans* lifespan at 25 °C. (A) Representative graph of a single trial with 250 µM tyrosol. Combined data from five independent trials are presented in Table 1. (B) Dose–response analysis on *fer-15(b26)* hermaphrodites. Nematodes were cultured with different tyrosol concentrations from conception until death and monitored for survival. The mean lifespan for 0 µM, 100 µM, 250 µM, 1 mM and 2.5 mM tyrosol were [days (worms assayed)]: 11.95 (80), 12.77 (71), 14.23 (65), 12.60 (83) and 12.53 (60), respectively. (C) Tyrosol exposure from adult day 1 also induced a significant lifespan extension in *fer-15(b26)* nematodes grown at 25 °C. The mean lifespan values for 0 µM and 250 µM tyrosol experimental groups in two independent trials were [days (worms assayed)]: 12.81 (204) and 14.84 (195), respectively. Statistical significance of the difference between the curves in each independent trial was demonstrated by the Log-Rank test ($p < 0.05$).

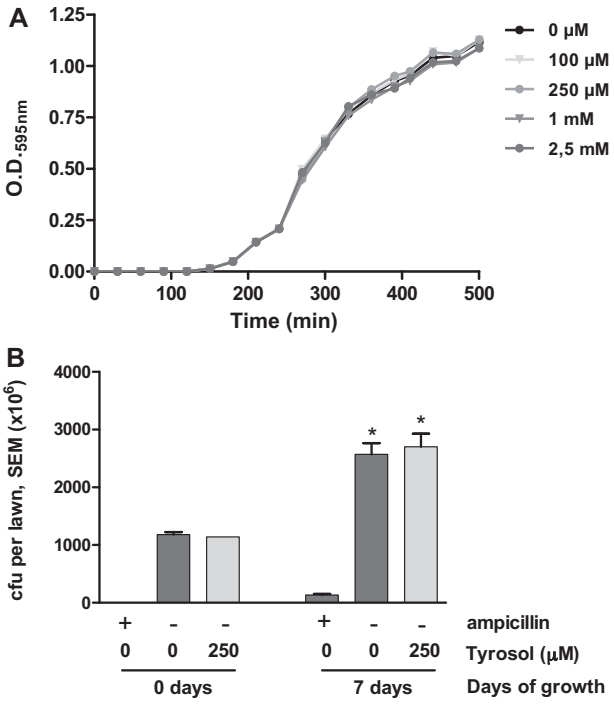

Fig. 2. Tyrosol treatment does not inhibit bacterial growth at the concentration used in the lifespan assays. (A) *E. coli* OP50 growth in liquid culture was monitored for up to 8 h at 37 °C after inoculation of a preculture in LB containing 4 different tyrosol concentrations (100 µM to 2.5 mM). (B) Bacterial lawn growth on NGM agar was also examined for a 7-day period under the same conditions of those used for *C. elegans* survival assays. Average number of colony forming units (cfu) per lawn is shown after the indicated treatments in three independent trials. Error bars indicate SEM among individual animals scored. The differences compared to 0 days were considered significant at $^{*}p < 0.05$, unpaired *t*-test with two tail distribution.

compound does not impair the overall growth in these nematodes (Fig. 3B). Likewise, neither daily reproductive output nor total brood size, were significantly altered by tyrosol treatment (Fig. 3C). When we examined the time for synchronized L1 larvae to develop into adulthood, we did not find significant development differences that could account for the observed increase in lifespan in response to tyrosol (Supplemental Fig. 1A). In line with this result, tyrosol did not postpone the egg-laying starting time in these nematodes, confirming the absence of a negative effect of this treatment on the developmental rate of *C. elegans* (Supplemental Fig. 1B).

3.4. Tyrosol treated nematodes exhibit enhanced thermotolerance and resistance to oxidative stress

The high correlation between stress resistance and longevity in *C. elegans* is well documented (Johnson et al., 2002; Muñoz, 2003). To analyze if tyrosol was able to increase stress resistance in *C. elegans*, we first evaluated how this treatment affects the response to thermal stress by monitoring nematode survival after an 8 h

Table 1
Tyrosol mediated longevity in *C. elegans*.

Treatment	Mean lifespan[a]		Maximum lifespan[a]		Median lifespan[a]		n[b]	Trials
	Days ± SEM	Change in %	Days ± SEM	Change in %	Days ± SEM	Change in %		
Control	12.16 ± 0.29	21.13***	18.67 ± 0.64	10.76*	12.60 ± 0.4	20.63**	493	5
Tyrosol (250 µM)	14.73 ± 0.34		20.68 ± 0.15		15.20 ± 0.58		480	

[a] Mean values of the median, maximum and mean lifespans of 5 independent trials; SEM, standard error of the mean. Changes compared to control (0 µM) were considered significant at $p < 0.01$(*), $p < 0.005$(**) and $p < 0.0005$(***) by unpaired *t*-test with two tail distribution. Statistical significance of the difference between the curves in each independent trial was demonstrated by Log-Rank test (p values: 0.0006; 0.0001; 0.0037; 0.0004 and 0.01, respectively).
[b] Number of hermaphrodites analyzed.

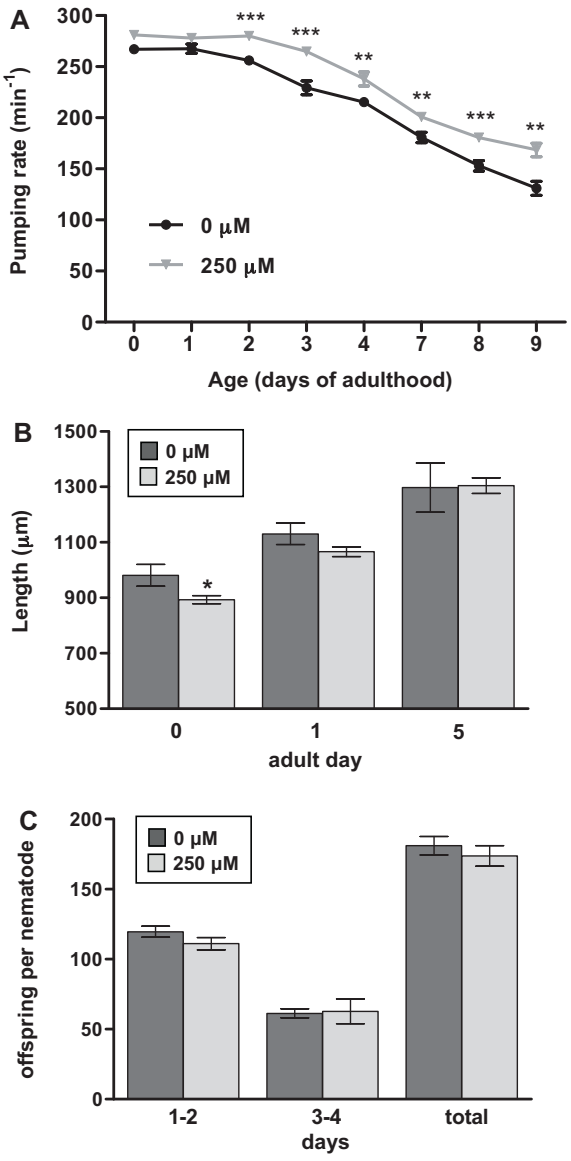

Fig. 3. Effect of tyrosol on pharynx pumping rate, body length and reproduction. (A) Tyrosol treatment slowed the decline in pharynx pumping during aging compared to non-treated controls. Results are expressed as average pumping rate (pumps per minute) in 15 animals scored in 3 trials; error bars indicate SEM among individual animals scored. Differences compared to control were considered significant at $^{**}p < 0.05$; $^{***}p < 0.005$, unpaired *t*-test with two tail distribution. (B) The effect of 250 μM tyrosol on the nematodes length at adult days 0, 1 and 5. Shown are mean ± SEM values of 20 nematodes per experimental condition. Differences compared to control (0 μM) were considered significant at $^{*}p < 0.05$, unpaired *t*-test with two tail distribution. (C) Daily and total reproduction output was determined from 8 animals per concentration. The errors represent the SEM. No value reached the significance limit of $p < 0.05$ when compared to its untreated control, unpaired *t*-test with two tail distribution.

exposure to 35 °C. As shown in Fig. 4A, thermotolerance was increased by two-fold in adult *fer-15(b26)* animals treated with 250 μM tyrosol. In detail, three independent assays yielded survival rates increased by 61.5%, 55.8% and 49%, respectively (total of 182 untreated and 214 treated nematodes).

Resistance to oxidative stress was examined by exposing nematodes to paraquat, an intracellular free-radical generating pesticide. Tyrosol treatment induced a significantly increased survival in the presence of paraquat 4 mM in three independent trials ($p < 0.0001$ Log-Rank (Mantel-Cox) test) (Fig. 4B).

To further examine if tyrosol conferred protection against acute oxidative stress, we conducted the same experiments with *mev-1(kn1)* mutants, which have a defect in the succinate dehydrogenase subunit C that results in overproduction of superoxide, leading to an excessive oxidative stress and reduced lifespan (Ishii et al., 1998; Senoo-Matsuda et al., 2001). Although the effect of tyrosol was not as prominent as the one observed in *fer-15(b26)* nematodes, it was sufficient to induce a slight increase in both, median and mean survival of *mev-1(kn1)* animals exposed to paraquat (Fig. 4C). In the absence of paraquat, *mev-1(kn1)* mutants showed a reduced lifespan compared to that of *fer-15(b26)* nematodes; however, this shorter life span was not significantly extended by tyrosol treatment (Table 2).

3.5. Effect of tyrosol on the mRNA expression of heat shock proteins

Considering the high impact exerted by tyrosol on thermal stress resistance, we next examined the effect of the same tyrosol treatment on the expression level of several well known stress-inducible genes in *C. elegans*. Thus, we decided to analyze transcripts of four heat shock proteins which levels have been previously shown to increase during the course of nematode aging (Golden and Melov, 2004; Lund et al., 2002). By using quantitative RT-PCR, we confirmed that the expression of *hsp-16.1*, *hsp-12.6*, *hsp-70* and *hsp-1* increased significantly from day 0 to day 4 of adulthood (Fig. 5) and, as expected, it was also induced at both ages after a 2-h incubation at 37 °C (data not shown). Interestingly, tyrosol treatment induced a significant 3.5-fold increase in *hsp-12.6* mRNA expression at adult day 4 in wild type nematodes cultured at 25 °C (Fig. 5A). By contrast, we did not observe significant changes in the levels of *hsp-16.1*, *hsp-70* or *hsp-1* mRNA in response to tyrosol at any of the two stages analyzed (Fig. 5B–D).

3.6. Genetic requirements for increased lifespan and stress resistance mediated by tyrosol

In order to gain insight into the mechanisms underlying the observed effects of tyrosol, we conducted lifespan experiments with mutants of various genes that act as key regulators in several stress response pathways known to affect *C. elegans* lifespan (Hsu et al., 2003; Lee et al., 2003; Murphy et al., 2003; Trapp and Jung, 2006). Table 2 shows the changes in mean and median lifespan in *C. elegans* mutant strains for the sirtuin gene *sir-2.1*, for the transcription factors *daf-16* and *hsf-1* and for the insulin receptor *daf-2*. Tyrosol treatment induced a significant increase in median and mean lifespan compared to untreated controls only in *sir-2.1* mutants. By contrast, the lifespan of *daf-2(e1370)*, *daf-16(m26)* and *hsf-1(sy441)* mutants was unaffected by tyrosol exposure, suggesting that the molecular pathways where these genes participate could mediate the tyrosol effects on longevity. We next examined if these genes were also required for the increased oxidative and thermal stress resistance observed in response to tyrosol. Our results in this sense show that, although *daf-2*, *daf-16* and *hsf-1* are required for the increased resistance to paraquat upon tyrosol treatment (Fig. 6A and B), only *daf-16* and *hsf-1* seem to be also required for the enhanced thermotolerance (Fig. 7C and D), whereas *daf-2(e1370)* mutants displayed an increased resistance to thermal stress in response to tyrosol very similar to the one observed for *wild type* nematodes (Fig. 7A and B).

3.7. Effect of tyrosol on subcellular DAF-16 localization

DAF-16 is a FoxO transcription factor which plays a central role in the control of the stress response and longevity both, in *C. elegans* and in mammals (Schaffitzel and Hertweck, 2006). Hence, downregulation of the insulin signaling pathway is known to increase *C. elegans* lifespan in a DAF-16-dependent manner (Kenyon et al., 1993; Kimura et al., 1997). To get insights into the

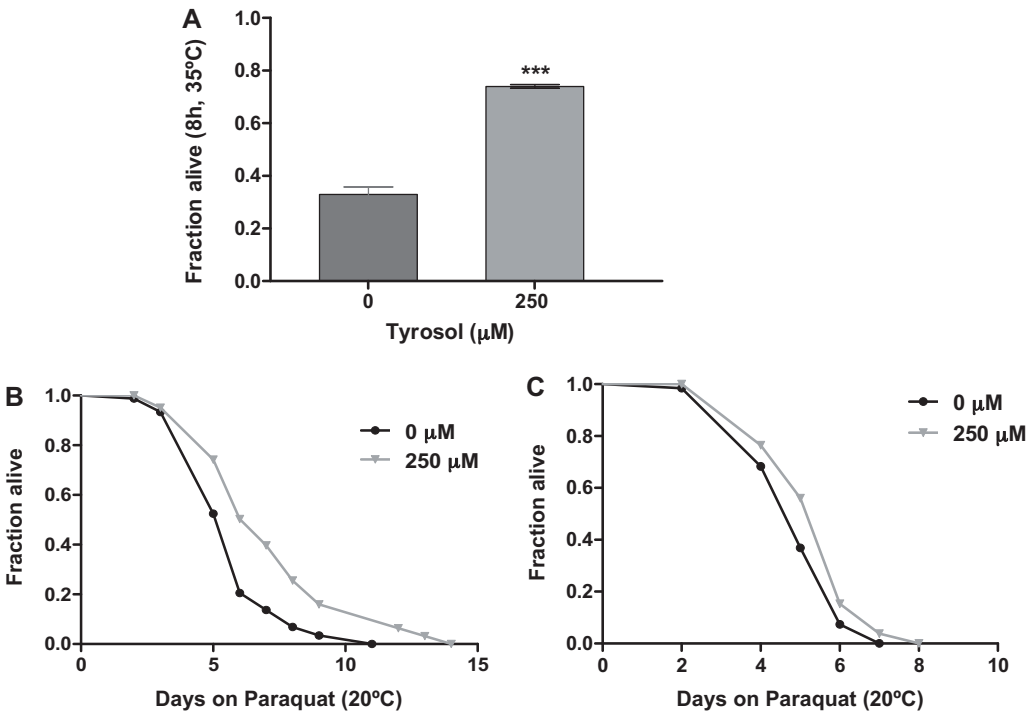

Fig. 4. Treatment with tyrosol increases thermotolerance and oxidative stress resistance. (A) Survival at 35 °C of *fer-15(b26)* nematodes with the indicated treatment (tyrosol 0 μM or 250 μM). Shown is the average survival of 3 experiments with 130 animals/experiment. Error bars, SEM; $^{***}p < 0.0001$, unpaired *t*-test with two tail distribution. (B) Survival on 4 mM paraquat was increased significantly in 250 μM tyrosol treated *fer-15(b26)* nematodes compared to untreated controls. A representative experiment out of 4 independent trials with similar results using an average of 90–100 nematodes/trial is shown ($p < 0.0005$, Log-Rank (Mantel-Cox) test). (C) Tyrosol also induced an increased survival to 4 mM paraquat in *mev-1(kn)* mutants. Representative experiment out of 2 independent trials with similar results using an average of 90-100 nematodes/trial is shown (($p < 0.05$, Log-Rank (Mantel-Cox) test).

molecular mode of action of tyrosol, we analyzed its influence on the subcellular distribution of DAF-16 by using the DAF-16::GFP reporter strain TJ356 (Henderson and Johnson, 2001).

Translocation of DAF-16::GFP from the cytosol into the nucleus in tyrosol treated animals was only apparent in a fraction of the nematodes and it was not completely nuclear in any of them under the conditions used in our lifespan experiments (Fig. 8A). However, tyrosol resulted in an increase of the fraction of worms showing intermediate DAF-16 nuclear translocation by nearly 2-fold compared with non-treated controls ($^{*}p < 0.005$, Student's *t*-test) (Fig. 8B). Thermal stress, used as a positive control, induced full-nuclear DAF-16 localization in 100% of the nematodes.

3.8. Identification of tyrosol in worm samples by LC-TOFMS

Incubation of nematodes in liquid medium is often used to increase the availability of small compounds, whereas incubation using solid medium might decrease the chance that the same compounds are taken up in the intestine. In order to confirm that tyrosol was efficiently incorporated by the nematodes, we performed HPLC analysis of extracts from treated nematodes after incubation for several days in solid medium containing tyrosol at the effective concentration used in our study (250 μM). The identification and confirmation of tyrosol was performed by comparing both retention time and accurate mass spectra obtained from worm samples with those corresponding to the analytical standard of tyrosol. For identification purposes we used the extracted ion chromatogram (EIC) of the deprotonated molecule ($[M-H]^-$) using a mass-window width of 10 ppm. Worm samples showed a chromatographic peak at 8.7 min (Fig. 9B), matching with retention time of analytical standard peak of tyrosol (Fig. 9A). Furthermore, accurate mass spectra of the obtained chromatographic peak from worm sample matched up with the accurate mass spectra of the analytical standard (insets in Fig. 9A and B). Therefore, the presence of tyrosol in worm samples is confirmed.

Table 2
Effect of 250 μM tyrosol treatment on different *C. elegans* strains lifespan compared to untreated controls.

Strain	Median survival		Mean lifespan		N[a]
	Days		Days ± SEM		
	Control	Treated	Control	Treated	
N2(wild type)	13	15*	13.17 ± 0.62	15.35 ± 0.72*	125
fer-15(b26)	12.6	15.2**	12.16 ± 0.29	14.73 ± 0.34***	973
mev-1(kn-1)	9	10	9.59 ± 0.62	10.04 ± 0.72	94
sir-2.1(ok434)	11	12*	11.33 ± 0.36	12.39 ± 0.30*	270
daf-16(m26); fer-15(b26)	13	14	12.99 ± 0.35	13.18 ± 0.38	355
hsf-1(sy441)	12	12	12.92 ± 0.33	13.88 ± 0.40	245
daf-2(e1370); fer-15(b26)	41	42	38.17 ± 2.46	40.51 ± 3.21	189

Statistical significance of the difference between the curves in each independent trial was demonstrated by Log-Rank test; $p < 0.05$(*), $p < 0.005$(**) and $p < 0.0005$(***). Each trial was conducted at least twice.
[a] Number of hermaphrodites analyzed.

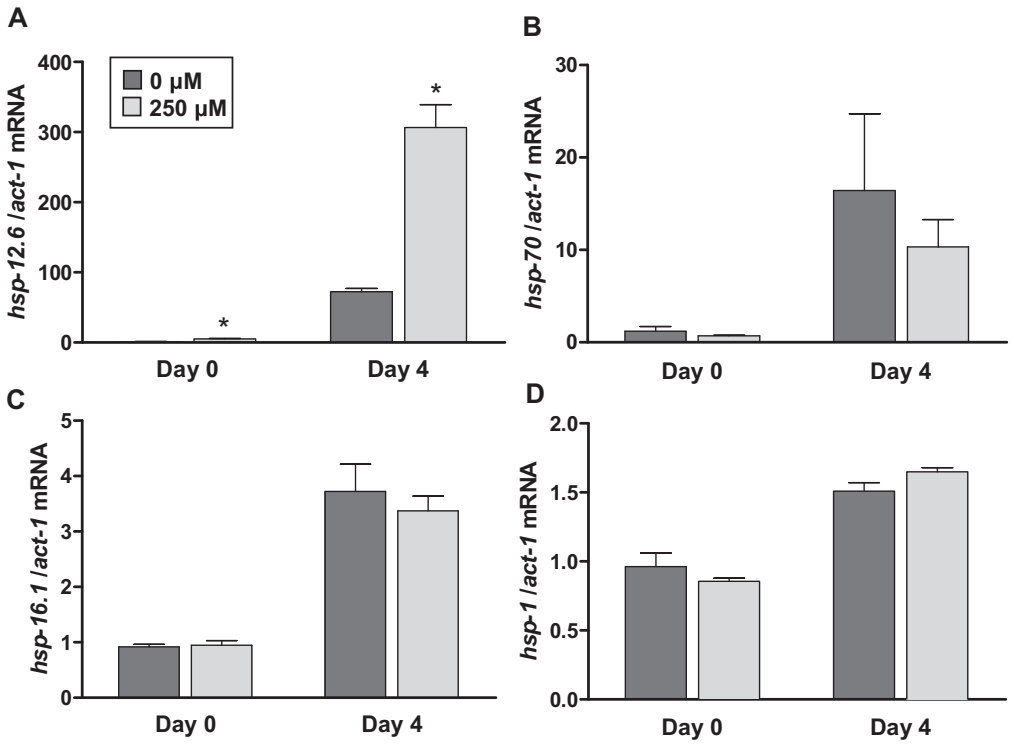

Fig. 5. Effects of tyrosol on inducible *sHsps* transcription. (A–D) Expression levels of small heat-shock proteins related to *act-1* in *fer-15(b26)* populations in response to 250 µM tyrosol. Total RNA samples were obtained at adult days 0 and 4. Graphs show the mean of 3 biological replicates with SEM (*$p < 0.01$, unpaired *t*-test with two tail distribution).

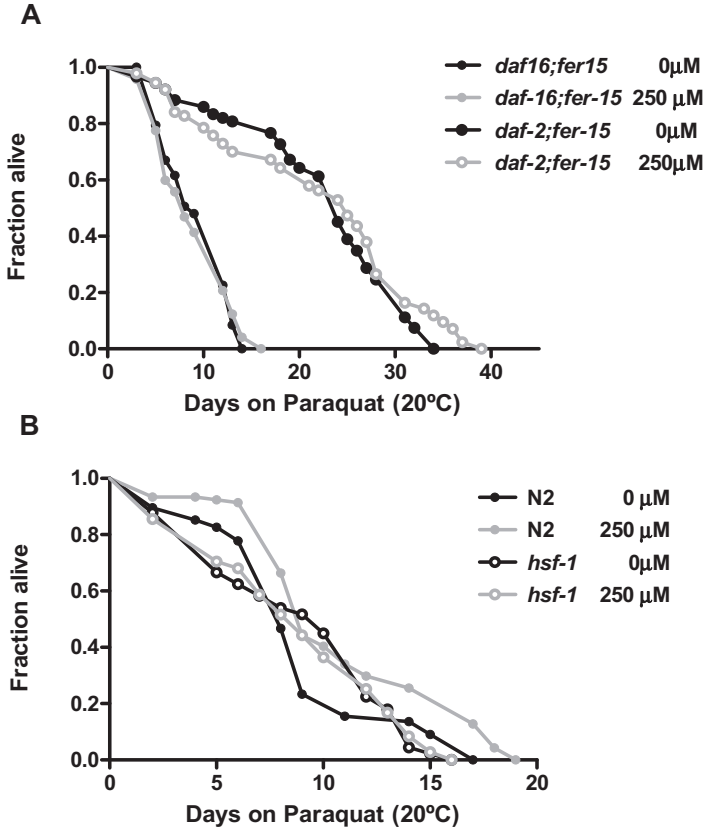

Fig. 6. HSF-1, DAF-2 and DAF-16 are required for enhanced resistance to paraquat in response to tyrosol. (A) *fer-15(b26)*; *daf-16(m26)* and *fer-15(b26); daf-2(e1370)* double mutants fail to show increased survival to paraquat when treated with tyrosol. (B) Tyrosol treatment also fails to increase survival to paraquat of *hsf-1(sy441)* mutants, but significantly increases survival of *wild type* (N2) nematodes ($p < 0.0005$, Log-Rank (Mantel-Cox) test). Representative graphs of 2 independent trials with similar results using an average of 100 nematodes per trial.

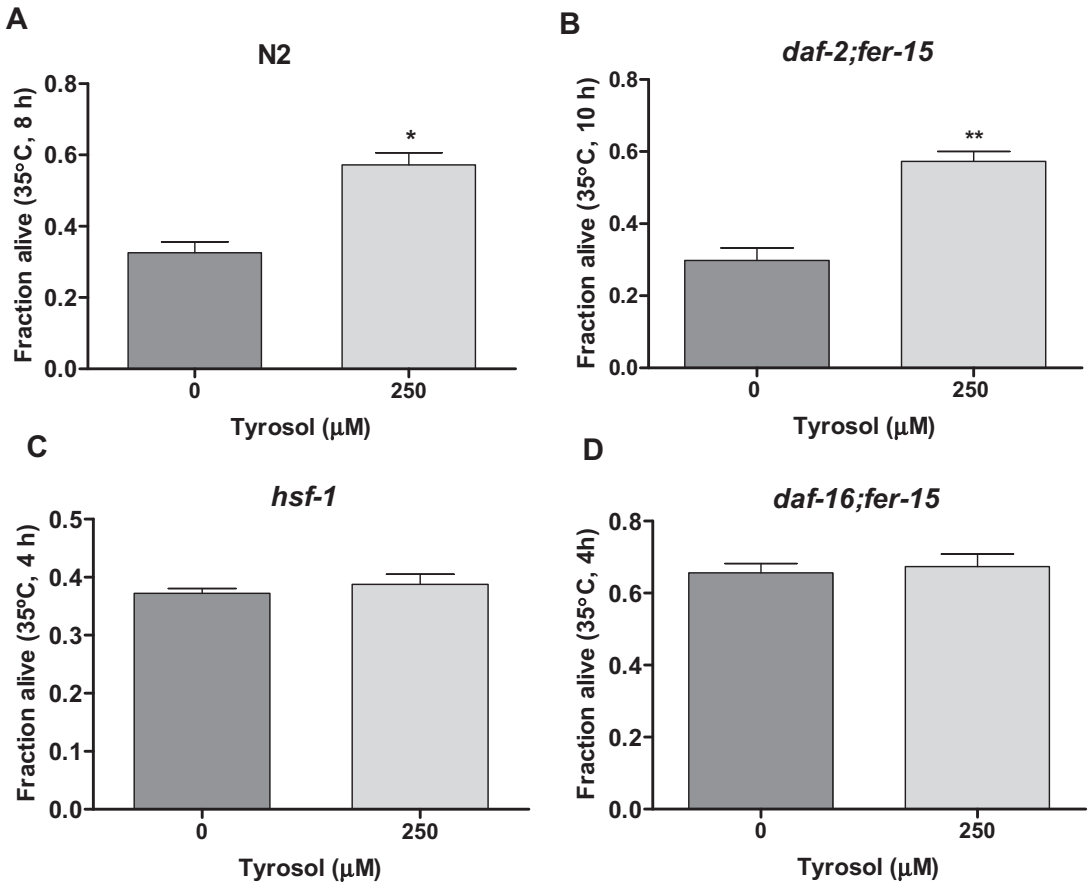

Fig. 7. HSF-1 and DAF-16 are required for enhanced thermotolerance in response to tyrosol at 35 °C. (A) Tyrosol significantly increases *wild-type* (N2) thermotolerance. (B) *daf-2(e1370); fer-15(b26)* mutants showed a significant increase in thermotolerance in response to tyrosol treatment. (C, D) *hsf-1(sy441)* and *daf-16(m26); fer-15(b26)* mutants failed to show increased survival when subjected to tyrosol treatment. The graphs represent the average survival in 3 trials with 60 animals per trial. Error bars, SEM; $^{*}p < 0.01$; $^{**}p < 0.05$, unpaired *t*-test with two tail distribution.

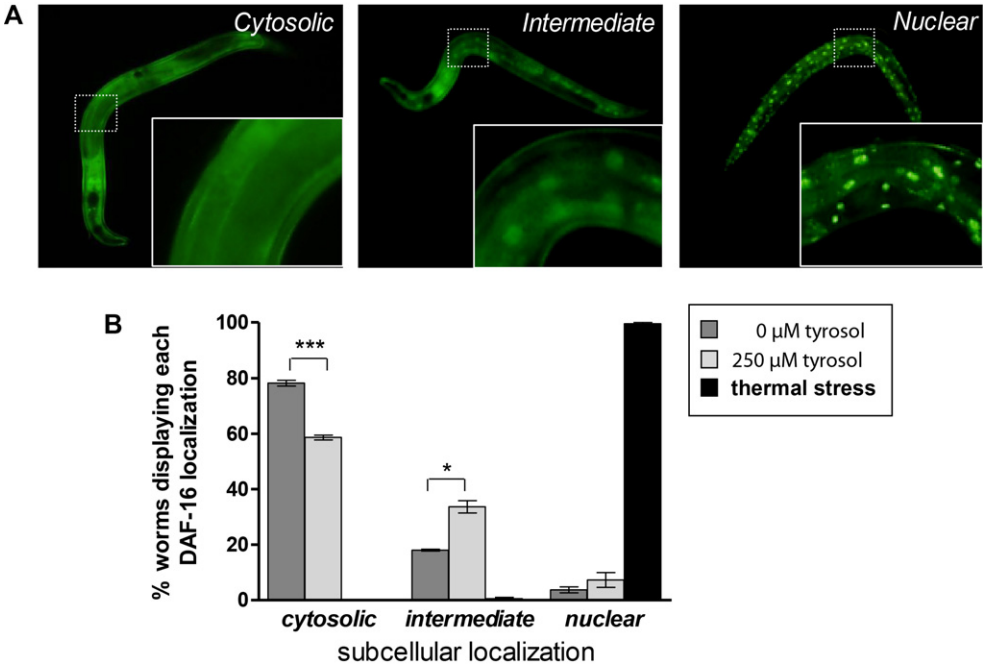

Fig. 8. Effect of tyrosol on the subcellular DAF-16 localization. Subcellular distribution of DAF-16 in three independent trials with an average of 150 nematodes per experiment was analyzed under a fluorescence stereomicroscope. (A) Each individual worm was classified according to its localization phenotype as "cytosolic", "intermediate" or "nuclear". (B) The results are presented as mean ± SEM of the percentages of nematodes with respective DAF-16 localization in three trials. Under thermal stress, almost 100% of the nematodes displayed complete nuclear localization. Differences compared to untreated controls were considered significant at $^{***}p < 0.005$; $^{*}p < 0.05$, unpaired *t*-test with two tail distribution.

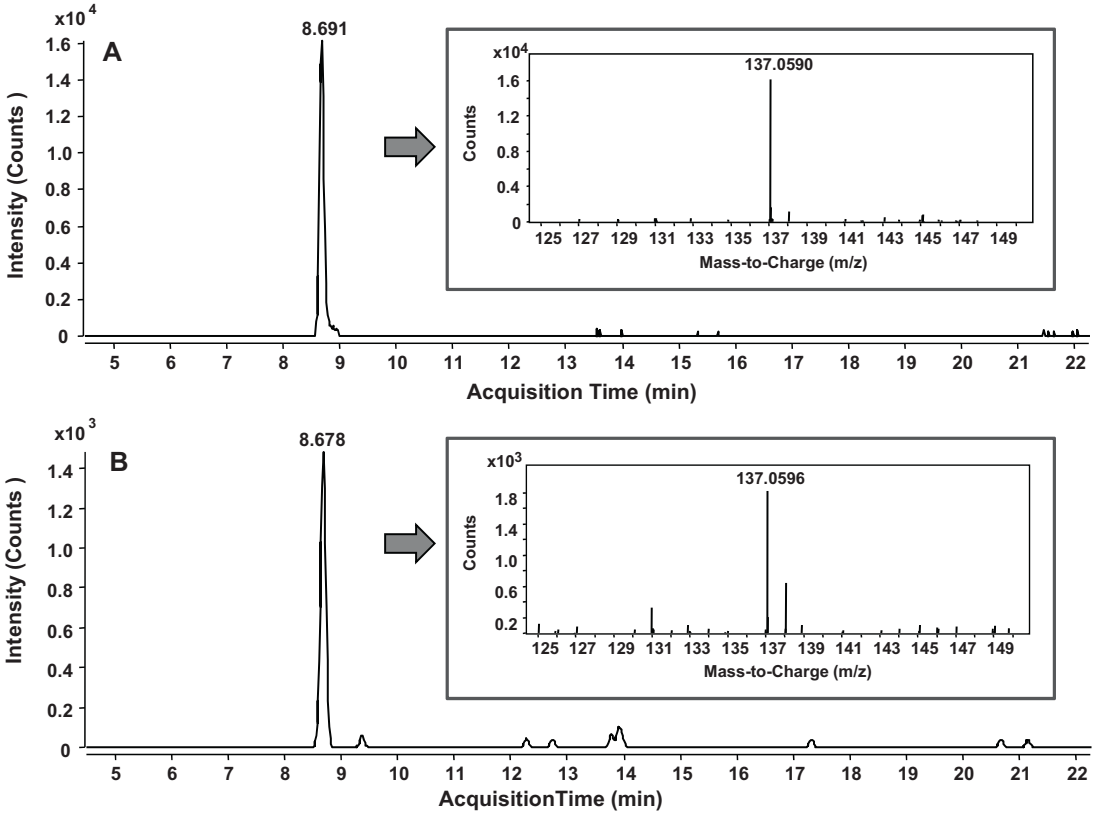

Fig. 9. Uptake of tyrosol by *C. elegans*. (A) Extracted ion chromatogram (EIC) and accurate mass spectrum (inset) corresponding to a tyrosol standard. (B) Extracted ion chromatogram (EIC) and accurate mass spectrum (inset) corresponding to the identified tyrosol peak of a worm sample.

4. Discussion

In recent years, there has been a growing interest in the identification of molecules capable of slowing the deleterious effects of aging *in vivo*. Dietary phenolic compounds have emerged as promising candidates mainly due to their antioxidant and anti-inflammatory properties, but also because a variety of them have demonstrated an effective bioavailability in humans (Manach et al., 2005).

This study shows that treatment with a moderate concentration of tyrosol is able to increase the longevity of *C. elegans* based on the observation that the treatment with this phenol induced significant extensions of median, mean and maximum life span in these nematodes. Interestingly, of the 4 different concentrations tested, an intermediate one (250 μM) resulted the most effective in terms of lifespan extension. Although higher and lower concentrations also induced lifespan extension, this effect was not as strong or significant as the observed at 250 μM, suggesting a dose-dependent effect of tyrosol in these animals. The observed life span extension was accompanied by a postponed age-associated decline in pharyngeal pumping rate, indicating that tyrosol might prolong youthfulness of the nematodes and did not act by simply improving survival of senescent individuals. These effects are significant and robust, considering that they were produced by the administration of a single chemical substance. Nonetheless, they are not as large as the differences achieved by mutations in longevity genes (Kenyon et al., 1993). Although other phenolic compounds have shown efficacy in life span modulation in *C. elegans*, especially quercetin, resveratrol, blueberry polyphenols and catechin (Kampkötter et al., 2008; Saul et al., 2009; Wilson et al., 2006; Wood et al., 2004), this is the first time that one of the main phenols present in EVOO is reported to have these effects in an animal model.

As a part of this study, we also investigated the possible mechanisms that might be implicated in the effects of tyrosol on longevity. Tyrosol could influence *C. elegans* lifespan by several indirect mechanisms. On the one hand, olive oil phenols have been reported to possess antimicrobial effects under certain conditions (Bisignano et al., 1999; Tuck and Hayball, 2002). In this scenario, lifespan extension in treated nematodes could be achieved by a decreased bacterial viability caused by a tyrosol-induced arrest of bacterial growth, mimicking conditions of caloric restriction which have been also reported to increase lifespan (Walker et al., 2005). To test this possibility, we monitored growth of the OP50 *E. coli* strain in the presence or absence of different tyrosol concentrations, both on liquid culture and on solid NGM agar plates. Our results demonstrate that tyrosol had no effect on the bacterial population growth at the doses used in our lifespan assays. On the other hand, tyrosol might confer bad tasting to the bacterial lawn and media, reducing food intake and, consequently, inducing calorie restriction in the worms which, in turn, would be responsible for the observed lifespan extension. This option can be also ruled out based on the fact that the pharyngeal pumping rate in tyrosol treated nematodes was not reduced compared to control ones. Taken together, it appears that caloric restriction dependent mechanisms do not mediate tyrosol lifespan extension. Moreover, the fact that we were able to detect tyrosol in nematode extracts after incubation in treated NGM plates confirms that this compound is efficiently taken up or ingested by the nematodes, indicating that it does not undergo significant metabolic transformations by living bacteria. Finally, we also discarded a possible "disposable soma effect" of tyrosol because we did not observe any impairment in development, growth or reproduction that could account for a redistribution of energy from growth to maintenance in the nematodes.

Aside for these mechanisms, another plausible explanation for the effects of tyrosol on longevity is that it may increase intrinsic resistance to stress in the nematodes. Indeed, we showed that tyrosol not only did prolong lifespan but also increased the thermotolerance and resistance to oxidative stress induced by the pesticide paraquat, demonstrating an improved survival of these nematodes to adverse conditions. These results are in line with those previously obtained with other compounds in this animal model such as quercetin (Kampkötter et al., 2007; Saul et al., 2008), Gingko biloba extract (Wu et al., 2002), catechin (Saul et al., 2009) and epigallocatechin gallate (EGCG) (Brown et al., 2006). In contrast, blueberry polyphenols, failed to protect nematodes from oxidative stress induced by paraquat, although they conferred protection to thermal stress (Wilson et al., 2006). Exposure to tyrosol, not only promoted a significant increase in oxidative stress resistance in *wild type* nematodes. Furthermore, when *mev-1(kn1)* mutants were exposed to paraquat, a slight but significant increase in survival compared to non-treated *mev-1* mutants was detected, suggesting an amelioration of the oxidative damage caused by a ROS overproduction.

In order to investigate if known longevity pathways were involved in the tyrosol effects, we performed lifespan assays with the mutants strains *daf-16(m26)*, *daf-2(e1370)*, *sir-2.1(ok434)* and *hsf-1(sy441)* (Kenyon et al., 1993; Hsu et al., 2003; Murphy et al., 2003; Viswanathan et al., 2005). Interestingly, out of these four mutants only *sir-2.1(ok434)* did not suppress the extended longevity of tyrosol treatment. The sirtuin gene *sir-2.1* encodes a histone deacetylase-like protein known to integrate the metabolic status with lifespan in different animal models and its role in longevity has been associated to caloric restriction, although this fact is currently a matter of intense debate (Greer and Brunet, 2009; Guarente, 2005). In this context, one of the mechanisms that have been shown to increase *C. elegans* lifespan in response to certain dietary polyphenols such as the trans-stilbene resveratrol, involves *sir-2.1* (Wood et al., 2004). We thus decided to investigate the possibility that tyrosol might have the ability to increase the activity of sirtuins in a similar fashion as it had been described with resveratrol. Our results, using a specific screening assay, showed that tyrosol does not seem to activate SIRT1 activity *in vitro* under none of the 4 concentrations tested (Supplemental Fig. 3). Together with our survival assays with *sir-2.1(ok434)* mutants indicating that this gene is not required for the effects of tyrosol, at least under our experimental conditions, our data seem to discard a direct implication of *SIR-2.1* as a mediator of the effects of tyrosol on longevity and further supports our results excluding caloric restriction-dependent mechanisms in tyrosol mediated lifespan extension.

Since first identified in *C. elegans*, the insulin/IGF-1 signaling pathway has emerged as the most well-characterized regulator of longevity across species (Barbieri et al., 2003; Antebi, 2007). This pathway is comprised of many genes including the insulin/IGF-1 receptor (DAF-2) that signals through a conserved PI3-kinase/AKT pathway and ultimately down-regulates DAF-16, a forkhead transcription factor (FOXO) (Hsin and Kenyon, 1999). Our lifespan experiments with the *daf-2(e1370)* mutant suggest that this receptor might be required for both, lifespan-extending and stress resistance effects of tyrosol, whereas it appears to be dispensable for the tyrosol-induced thermotolerance observed in wild type nematodes. Downstream DAF-2, the *C. elegans* transcription factor DAF-16 constitutes the end point of several signaling pathways integrating longevity, the stress response and other important biological functions in a similar way as FoxO transcription factors in mammals (Lee et al., 2003; Schaffitzel and Hertweck, 2006; Murphy et al., 2003). Our results in this sense show that tyrosol fails to extend the lifespan of nematodes lacking a functional *daf-16* gene and demonstrate that DAF-16 seems to be also required for the increased survival to oxidative and thermal stress in response to tyrosol, pointing to a possible implication of this transcription factor and the insulin pathway in the tyrosol-mediated lifespan extension. As for other transcription factors, the nuclear localization of DAF-16 is a prerequisite for its gene transactivation function that is normally induced in response to thermal and oxidative stress. Consistent with this, tyrosol was able to induce a moderate level of DAF-16 nuclear localization in the nematodes suggesting that, although this transcription factor might be involved in the observed effects, it may not be the only mechanism responsible for them. Other natural substances such as quercetin and fisetin have been reported to induce strong nuclear translocation of DAF-16, although in these experiments, nematodes were cultured in liquid medium (Kampkötter et al., 2007, 2008) and not on solid NGM. It is worth noting that our analysis of the expression of several DAF-16 target genes in young and adult nematodes revealed a significant upregulation of only a fraction but not all of these genes (Supplemental Fig. 2), suggesting a moderate but consistent impact of tyrosol on this pathway. Nonetheless, taking into account that DAF-16 also receives input from several other pathways that regulate life span such as the JNK pathway (Oh et al., 2006), the precise mechanisms by which tyrosol interacts with the insulin pathway to affect lifespan would require further analysis.

Survival and adaptation to severe environmental conditions requires the activity of the heat-shock transcription factor-1 (HSF-1), which induces activation of various heat-shock genes or chaperones involved in maintaining the conformational homeostasis of proteins, among other important functions. Indeed, HSF-1, has been shown to modulate longevity in *C. elegans* and other organisms (Putics et al., 2008; Tatar et al., 1997) and, recently, it has been suggested that it might function in cooperation with DAF-16 to activate the expression of common target genes, including the family of *sHsp* (small heat shock proteins) genes (Hsu et al., 2003). Our results show that HSF-1 is required to attain the beneficial effects of tyrosol and, interestingly, *hsp-12.6* (one out of the 4 shsp genes analyzed) was significantly up-regulated in adult nematodes in response to the tyrosol treatment. The higher level of *hsp-12.6* mRNA in treated adult nematodes might be related to the observed increases in thermotolerance and lifespan induced by tyrosol. In this sense, Hsu et al. (2003) have proposed that this gene, among other *shsp* genes, may possess specific regulatory sequences for DAF-16 and HSF-1 consensus binding sites so that its expression would be under the control of both transcription factors. The same authors have also reported that the activity of sHSPs, including that of *hsp-12.6*, was able to extend lifespan and delay polyglutamine protein aggregation in *C. elegans* (Hsu et al., 2003).

It has been proposed that low-level stresses early in life might induce a complex stress response that involves changes in gene expression and metabolism resulting in lifespan extension (Rattan et al., 2007). This response is mainly characterized by the induction of heat shock proteins that function as molecular chaperones, preventing damaged proteins from aggregating before they can be refolded or degraded (Giese and Vierling, 2002; Verbeke et al., 2001). This hypothesis, known as hormesis, has been postulated as a frequent model to explain the effects of many natural and pharmacological compounds on longevity. According to our results, tyrosol effect on *C. elegans* might respond, at least in part, to a hormetic mechanism. Thus, it is possible that tyrosol exerts a mild stress during the first stages of life sufficient to increase the activity of pathways that promote stress resistance, thereby causing a lifespan extension in these animals. Nevertheless, other factors may be involved and we cannot rule out the overlapping of other signaling pathways to explain the observed effects.

Regardless of the precise mechanisms involved, it is evident from our experiments that the tyrosol present in EVOO is able to extend lifespan and increase the stress resistance of a whole organism under laboratory conditions. Although we have started to pinpoint some of the genetic requirements for these effects, a more thorough analysis including a genomics/proteomics approach and the utilization of a larger set of mutants, would be mandatory in order to establish the precise molecular pathways connecting tyrosol and longevity.

5. Conclusions

Our results demonstrate, for the first time, that tyrosol has the ability to extend lifespan in the nematode *C. elegans* and that this effect may be mediated by an enhanced thermotolerance and resistance to oxidative stress. In addition, we show that two transcription factors converging in the regulation of the heat shock response, DAF-16 and HSF-1, might be involved in the systemic effects of tyrosol which, as we also confirmed, is efficiently taken up by the nematodes.

This work constitutes an advance in the study of the biological effects of natural compounds *in vivo* and suggests the convenience of testing other phenolic components in EVOO for their effects on longevity. For this purpose, we eagerly recommend the utilization of *C. elegans* as a model in further investigations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mad.2012.07.004.

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Supplemental material and methods

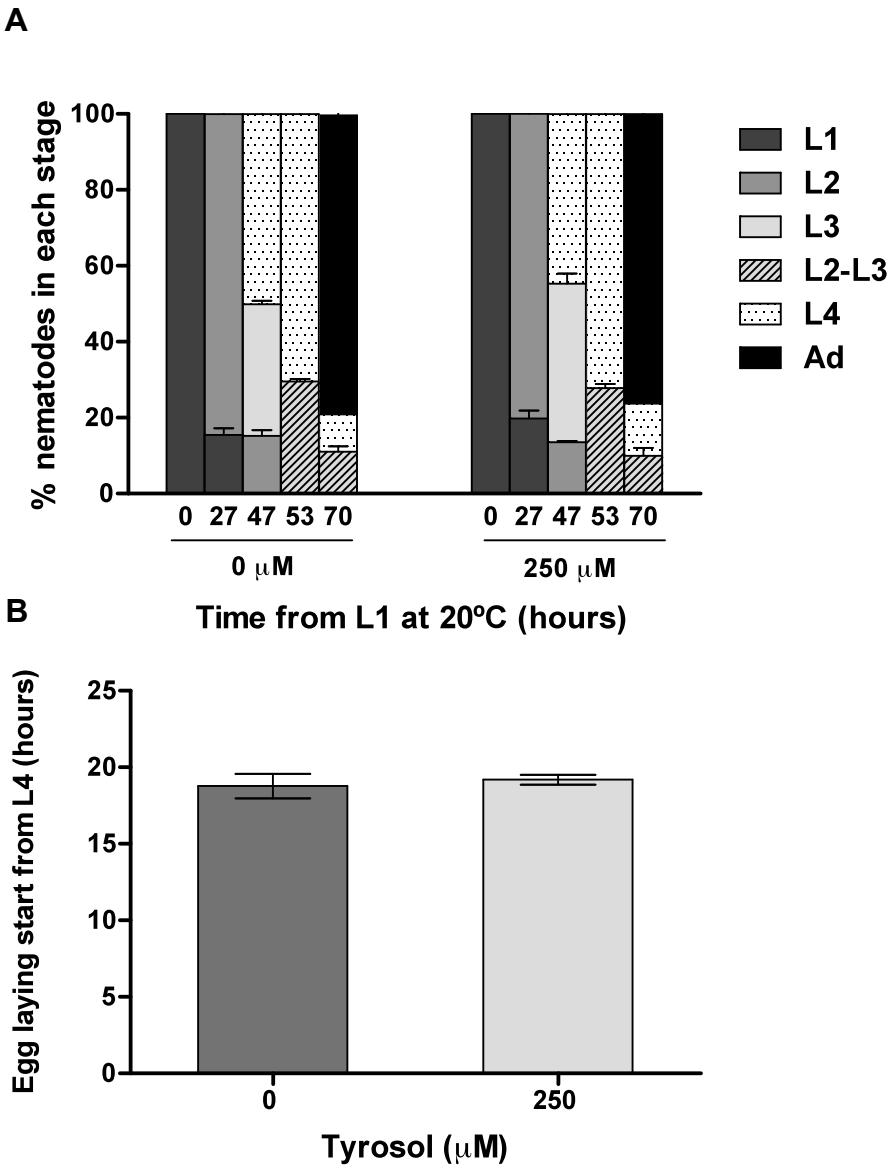
Development assays

Synchronized L1 larvae were obtained after hypochlorite treatment of *fer-15(b26)* gravid hermaphrodites followed by overnight incubation of embryos in M9 buffer. An average of 100 L1 larvae were seeded onto control and 250 µM tyrosol plates and the percentage of nematodes in each larval stage were determined after different incubation periods at 20°C until most individuals had reached the adult stage (70 hours). The experiment was performed in triplicate. For the egg-laying assay, we determined the average time for individual synchronized L4 larvae, incubated in control and 250 µM tyrosol from the L1 stage, to start laying eggs at 20°C.

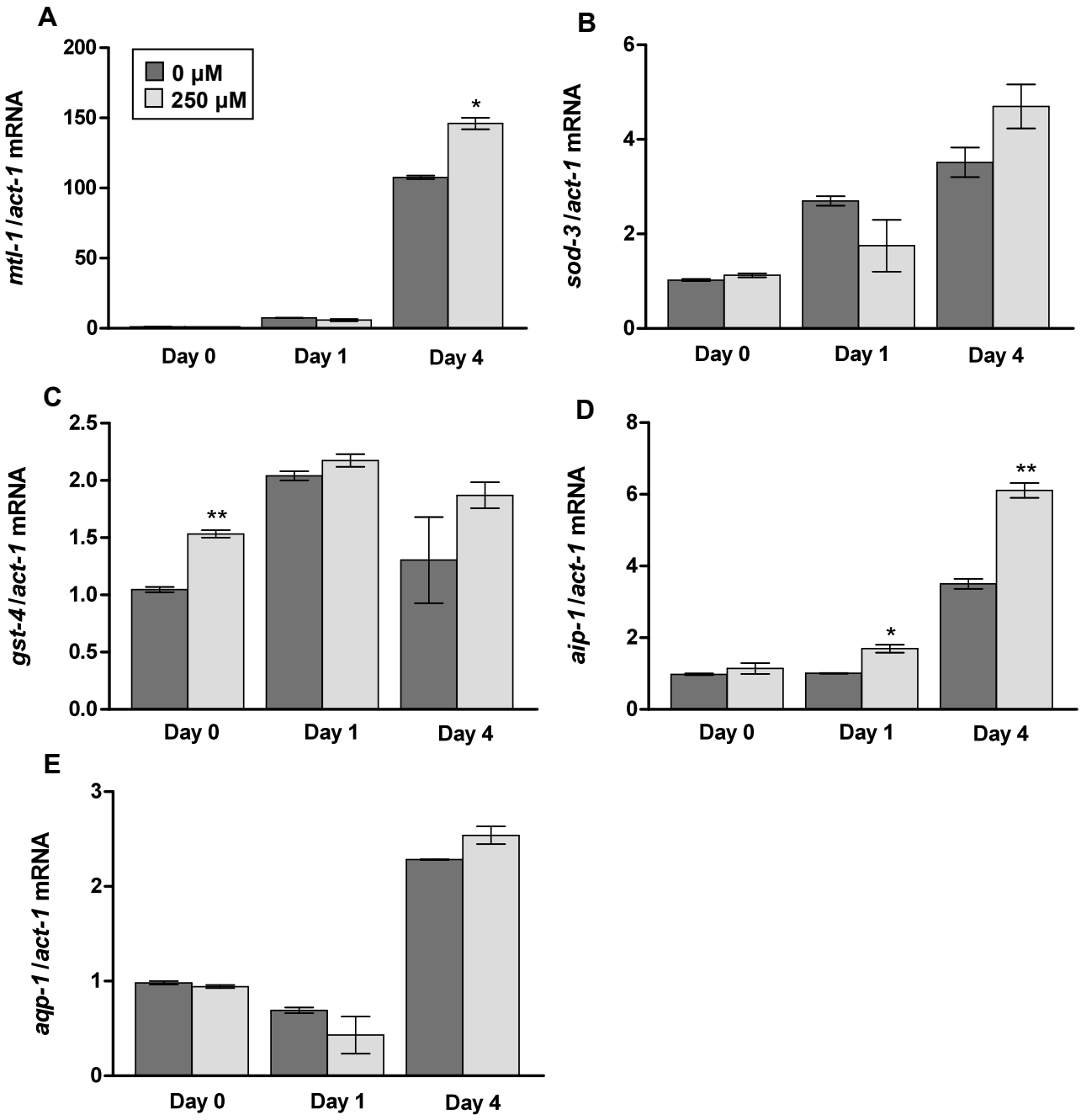
Recombinant SIRT1 activity assay

It has been shown that the polyphenol resveratrol is able to extend lifespan in *C. elegans* and other organisms by modulating sirtuins enzymatic activity (Viswanathan et al., 2005; Trapp and Jung, 2006; Zarse et al., 2010). In order to ascertain if tyrosol was able to induce sirtuin deacetylase activity *in vitro,* we used the SIRT1 Assay kit (Sigma-Aldrich, Germany) based on the deacetylation by SIRT1 of a synthetic substrate that contains an acetylated lysine side chain. The cleavage of the deacetylated substrate by a developer releases a fluorochrome, which is measured by a fluorometer. The assay was performed following manufacturer instructions. Final concentrations of tyrosol assayed were 100 µM, 250 µM, 1 mM and 10 mM. Control samples were incubated with solvent and without tyrosol. Resveratrol provided with the kit was used as SIRT1 activator control.

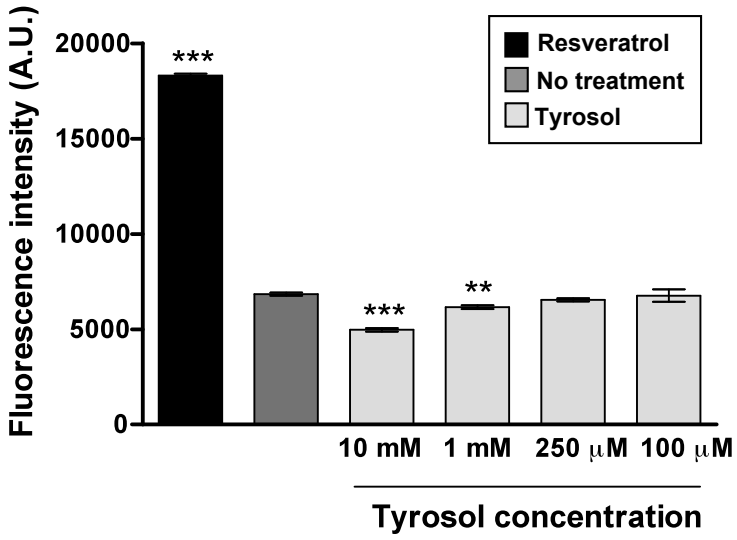
Supplemental Figure 1. Tyrosol does not impair the developmental rate of *fer-15(b26)* nematodes. A) Effect of tyrosol on the time for synchronized L1 larvae to develop into adulthood at 20°C. B) Tyrosol treatment does not delay the onset of egg-laying by synchronized adult hermaphrodites. The graph represents the average period of time for 10 individual animals from the L4 stage until they started laying eggs at 20°C. Error bars, SEM.



Supplemental Figure 2. Effect of tyrosol on the transcription of different DAF-16 target genes (Honda and Honda, 1999; Barsyte et al., 2001; Hsu et al., 2003; Tullet et al., 2008; Lee et al., 2009). (A-E) Individual mRNA expression levels of five DAF-16 activated genes in *fer-15(b26)* nematodes in response to tyrosol (250 μM). Total RNA samples were obtained from synchronized populations at the L4 larval stage and at adult days 1 and 4. Graphs show the mean values of 3 biological replicates. Error bars with SEM *(*p*<0.05*; **p*<0.005, unpaired *t*-test with two tail distribution). Differences compared to untreated controls (0 μM) were considered significant at *p*<0.05.



Supplemental Figure 3. Effect of tyrosol on SIRT1 deacetylase activity. All the reactions were performed simultaneously according to the kit indications using 5 µl of tyrosol at final concentrations of 10 mM, 1 mM, 250 µM and 100 µM. Equal volumes of either solvent or activator (resveratrol) were used for the control and resveratrol assays respectively. Each experimental condition was assayed in 4 replicates into the same plate and each assay was repeated two independent times. Differences compared to control were considered significant at ***p<0.005; ***p<0.0001*, unpaired *t*-test with two tail distribution.



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