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The effect of different ubiquinones on lifespan in *Caenorhabditis elegans*

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Abstract

Ubiquinone (UQ, Coenzyme Q, CoQ) transfers electrons from complexes I and II to complex III in the mitochondrial electron transport chain. Depending on the degree of reduction, UQ can act as either a pro- or an antioxidant. Mutations disrupting ubiquinone synthesis increase lifespan in both the nematode (*clk-1*) and the mouse (*mclk-1*). The mutated nematodes survive using exogenous ubiquinone from bacteria, which has a shorter isoprenyl tail length (UQ₈) than the endogenous nematode ubiquinone (UQ₉).

The mechanism underlying *clk-1*'s increased longevity is not clear. Here we directly measure the effect of different exogenous ubiquinones on *clk-1* lifespan and mitochondrial function. We fed *clk-1* engineered bacteria that produced UQ₆, UQ₇, UQ₈, UQ₉ or UQ₁₀, and measured *clk-1*'s lifespan, mitochondrial respiration, ROS production, and accumulated ROS damage to mitochondrial protein. Regardless of dietary UQ, *clk-1* animals have increased lifespan, decreased mitochondrial respiration, and decreased ROS damage to mitochondrial protein than N2. However *clk-1* mitochondria did not produce less ROS than N2. The simplest explanation of our results is that *clk-1* mitochondria scavenge ROS more effectively than wildtype due to the presence of DMQ₉. Moreover, when compared to other dietary quinones, UQ₁₀ further decreased mitochondrial oxidative damage and extended adult lifespan in *clk-1*.

Keywords

ubiquinone; mitochondria; respiration; reactive oxygen species; *C. elegans*; life span; genetics

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Author Contributions YY performed or helped with all experiments, analyzed the data and contributed to writing the manuscript. JAG contributed and performed a unique protocol and assisted in writing the manuscript. PGM and MMS designed the studies, oversaw all parts of the project, analyzed the data and contributed to writing the manuscripts.

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1. Introduction

Ubiquinone (UQ, CoenzymeQ, CoQ) is a redox-active lipid primarily found in the mitochondrial inner membrane (Battino *et al.*, 1990; Tran and Clarke, 2007). UQ functions in multiple ways within the cell. It is an essential link in mitochondrial electron transport from complex I and II to complex III in the mitochondrial electron transport chain. The reduced form of UQ can serve as a free radical scavenger to protect cells from oxidative damage (Kagan *et al.*, 1990; Lenaz *et al.*, 2002; Genova *et al.*, 2003), while the partially oxidized semiquinone generates the superoxide free radical (Lass *et al.*, 1997).

UQ consists of a modified benzoquinone ring which can be reversibly reduced and oxidized, and a hydrophobic isoprenyl tail which contains 6 to 10 isoprenyl units. The isoprenyl tail length of UQ is species-specific, being ten (UQ₁₀) in humans, nine (UQ₉) in rodents and *Caenorhabditis elegans* (*C. elegans*), and eight (UQ₈) in *Escherichia coli* (*E. coli*). It has been suggested that different isoprenyl tail lengths of UQs may correlate with the lifespan of different species (Olgun *et al.*, 2003). UQ analogues with shorter isoprenyl tails enhance superoxide formation more than UQs with longer tails (Lenaz *et al.*, 2002; Genova *et al.*, 2003). UQ can promote superoxide formation by mediating electron transfer from the iron-sulfur cluster N2 of complex I to oxygen, instead of transferring electrons directly to complex III.

In *C. elegans*, *clk-1* mutants lack the evolutionarily conserved enzyme necessary for UQ biosynthesis (Miyadera *et al.*, 2001; Jiang *et al.*, 2001). *C. elegans clk-1* mutants accumulate demethoxyquinone-9 (DMQ₉) instead of UQ₉ (Miyadera *et al.*, 2001). For survival the animals rely on both endogenous DMQ₉ and exogenous UQ₈ from the bacterial food source (Jonassen *et al.* 2001). With dietary UQ₈ from standard nematode bacteria OP50 or K12, *clk-1* mutants display the abnormal “clock” (Clk) phenotype. This includes slow and desynchronized embryonic and postembryonic development, decreased brood size, slower egg production rate, and most interestingly, a prolonged lifespan as well as less oxidative damage to their mitochondrial proteins (Wong *et al.*, 1995) (Kayser *et al.*, 2004a). However, the underlying cause for the increased lifespan of *clk-1* remains a point of debate.

Several studies have indirectly investigated whether the large amount of DMQ in *clk-1* mutants supports mitochondrial function and is the cause of their extended lifespan. In yeast, *clk-1* mutants have been constructed which contain only DMQ₆ (*Coq7/Cat5*). Without added exogenous UQ₆, these cells grew slowly, failed to maintain respiratory activity, and lost antioxidant activity (Padilla *et al.*, 2004). *C. elegans clk-1* mutants also require UQ₈ from their diet to become fertile adults; *clk-1* fed UQ-less bacteria arrested as L2 larvae (Jonassen *et al.*, 2001; Jonassen *et al.*, 2002). Mouse *clk-1* null mutants (*mclk1*^{-/-}) arrest development around embryonic day 10, but partial loss of activity extends lifespan in mice (Jiang *et al.*, 2001; Liu *et al.*, 2005). Interestingly, in embryonic stem cells from *mclk1*^{-/-}, both NADH- and succinate-cytochrome c reductase activity were significantly decreased (Levavasseur *et al.*, 2001; Liu *et al.*, 2005; Stepanyan *et al.*, 2006), suggesting that DMQ is a relatively poor electron carrier compared to UQ. Using direct measurements of mitochondrial function, we found that *clk-1* mitochondria display a specific defect in complex I-dependent respiration, whereas complex II-dependent respiration remains normal (Kayser *et al.*, 2004b). More recently, it was shown that only UQ₉, and not DMQ₉, can be reduced by NADH in the plasma membrane of *C. elegans* (Arroyo *et al.*, 2006). These studies suggest that DMQ is not able to substitute for UQ function completely, and the UQ profile is an important factor in establishing the *clk-1* mutant phenotype.

Two groups have shown that brood size, gonad morphology and rate of development of *clk-1* animals are sensitive to the length of the side chain of exogenous UQ (Hihi *et al.* 2003, Jonassen *et al.*, 2003). However, neither group studied the effect of the ubiquinone species on

lifespan or mitochondrial function. We hypothesized that differences in UQ isoprenyl tail length are functionally important *in vivo*, and that variation in UQ tail length would affect lifespan in the nematode. Changes in lifespan may directly result from changes in generation or scavenging of reactive oxygen species (ROS), as well as from altered rates of mitochondrial respiration. The role of scavengers in determining the lifespan of *clk-1* has been questioned (Yang *et al.*, 2007, Rea *et al.*, 2007). However, in neither of these studies were mitochondrial respiration, ROS production, or oxidative damage to proteins directly studied.

We directly studied mitochondrial function in *clk-1(qm30)* fed different UQ species to understand the mechanism underlying its increased lifespan, and to test the effects of different exogenous UQs on lifespan. The results suggest that the lifespan extension caused by different tail-lengths of UQ is not caused by differences in ROS generation, but by variations in ROS scavenging.

2. Materials and Methods

2.1 Nematode strains

N2 (Bristol) and *clk-1 (qm30)* were obtained from the *Caenorhabditis* Genetics Center (Minneapolis, MN, USA). *clk-1 (qm30)* is the canonical null allele. Worms were grown on nematode growth media (NGM) agar with a lawn of the desired *E. coli* as food source and maintained at 20°C. The *E. coli* strains (Okada *et al.*, 1997) used in this study were the kind gift of M. Kawamukai, Shimane University, Japan. Normally worms are fed either *E. coli* K12 (growth in liquid culture) or *E. coli* OP50 (growth on agar plates), each of which produce UQ₈.

2.2 Lifespan study

Surface-sterilized eggs were obtained by dissolving gravid *qm30* or N2 adults in hypochlorite/NaOH solution (Wood, 1988). Eggs were washed three times with S-basal and transferred to plates containing a lawn of bacteria producing either UQ₆, UQ₇, UQ₈, UQ₉ or UQ₁₀, or the standard bacteria strain OP50 (producing UQ₈) and kept at 20°C. Worm lifespans were determined by recording the number of alive or dead worms over time; day 0 was defined as the day the worms started to lay eggs. Worms were scored as dead if they did not move after touch with a platinum wire. Worms that crawled off the plate, died because of bagging or protrusion of the gonad through the vulva were discarded from the initial data set. Each lifespan study was performed with over 100 worms. Animals were raised at 20°C. No FUDR was used for lifespan study. Lifespans were reported as the day that 50% of the animals had died.

2.3 Mitochondrial studies

Worms were grown for mitochondrial isolation as previously published (Kayser *et al.*, 2001), with the exception that the different bacterial strains in Table 1 were used as food. *clk-1* animals lay very few eggs when they have been grown on UQ₆ or UQ₇ from hatching. To obtain large quantities of UQ₆ or UQ₇ fed *clk-1* adults, *clk-1* were first grown in S-basal with K12 until dauer larvae were obtained. Dauers were isolated with 2% SDS treatment and washed several times with S-basal. *clk-1* dauers were collected and allowed them to exit dauer stage in S-basal with UQ₆ or UQ₇. Mitochondria preparation and assays of oxidative phosphorylation (OXPHOS) were performed as previously described. (Kayser *et al.*, 2004b)

2.4 Western blots

Mitochondrial protein concentration was determined by the Lowry assay (Lowry *et al.*, 1951). Western blots of 30ug of mitochondrial protein from *clk-1* fed with wildtype bacteria K12 (producing UQ₈), or bacteria producing UQ₆, UQ₇, UQ₈, UQ₉ or UQ₁₀ were performed

by electrophoresis on 15% SDS polyacrylamide tris-glycine gels. Proteins were then transferred electrophoretically to 0.45- μ m polyvinylidene difluoride membranes (Francis and Waterston, 1985). Membranes were blocked overnight in 5% milk-PBST and incubated for 2 h with primary antibodies. Anti-HNE (Calbiochem, San Diego, CA) was used at a 1:5000 dilution. Anti-complex I subunit NDUFS3 (Mitosciences, Eugene, Oregon) was used to ensure equal loading. After three washes with PBS-0.05% Tween 20, the membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotech, Santa Cruz, CA) diluted in 5% milk-PBST. This secondary antibody was detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

2.5 Quantification of reactive oxygen species

ROS were measured as H_2O_2 by the Amplex Red assay (Invitrogen, Carlsbad, CA, USA) as described in Chen *et al.* (Chen *et al.*, 2003). The concentration of fluorogenic indicator amplex red and horseradish peroxidase used were 50 μ M and 0.5 unit/ μ l, respectively.

2.6 Quantification and identification UQ species

Samples were prepared by adding a known fixed amount of the internal standard di-propoxy-UQ₁₀ (diPrQ₁₀, a generous gift from Iain P. Hargreaves, Institute of Child Health, London, United Kingdom) and 1-propanol (0.5mL for 400 μ g mitochondrial protein). 20 μ L of 2mg/mL *p*-benzoquinone were added to ensure that all the quinones were in the oxidized form. All the quinones; ubiquinones (UQ), demethoxyquinones (DMQ) and rhodoquinones (RQ), were ionized by Atmospheric Pressure Chemical Ionization (APCI) and analyzed using a triple quadrupole mass spectrometer. Specific multiple reaction monitoring mode (MRM) transitions were performed, corresponding to m/z 864 $\rightarrow$ 197 (UQ₁₀), 920 $\rightarrow$ 253 (diPrQ₁₀), 795.5 $\rightarrow$ 197 (UQ₉), 765.5 $\rightarrow$ 166.9 (DMQ₉), 780.9 $\rightarrow$ 181.9 (RQ₉), 727.5 $\rightarrow$ 197 (UQ₈), 659.5 $\rightarrow$ 197 (UQ₇) and 591.5 $\rightarrow$ 197 (UQ₆), respectively. The samples were delivered to the API 4000 tandem mass spec (Applied Biosystems, Foster City, California, USA) using an Agilent 1200 series HPLC binary pump (Agilent technologies, Waldbronn, Germany) delivering 50% methanol:isopropanol at 0.5mL/min in flow injection analysis (FIA) mode. Samples were quantified against a 6 concentration levels calibration curve made with known amounts of UQ₆, UQ₉ and UQ₁₀ and the internal standard (diPrQ₁₀) spanning the concentration range found in the samples. UQ₇ was quantified assuming the same response in the detector as UQ₆. UQ₈, DMQ₉ and RQ₉ were quantified assuming the same response in the detector as UQ₉.

2.7 Statistical analysis

All data are presented as the mean of n experiments $\pm$ SD, with $n \geq 3$. Comparison of lifespan data were performed with logrank. All other comparisons were first analyzed by ANOVA, using Prism software. If ANOVA indicated a statistical difference, data sets were individually compared using a students t-test. Two sets of data were considered as statistically different when $p < 0.05$.

3. Results

3.1 Quinone profile in dietary bacteria and mitochondria of *clk-1* fed with UQ₆₋₁₀

First we showed that all bacteria primarily contained their designated quinone (Figure S1), consistent with the results previously reported (Jonassen *et al.*, 2001). After feeding *clk-1* (*qm30*) with UQ₈, UQ₉, or UQ₁₀ for 3 or more generations, we isolated mitochondria protein from them to verify whether dietary UQ accumulated in the mitochondria. *clk-1* fed with UQ₆ or UQ₇ is nearly sterile and will not grow in sufficient quantities to isolate mitochondria. Therefore, for studies of mitochondria containing UQ₆ or UQ₇, liquid cultures of *clk-1* were

harvested as dauers and fed with UQ₆ or UQ₇ to let them exit dauer stage. Synchronous cultures of young adults which had been fed UQ₆ or UQ₇ for approximately 7 days were harvested for mitochondrial isolation. The endogenous UQ which did accumulate in *clk-1* mitochondria was enriched by the primary species produced in the dietary bacteria (Figure 1). The amount of different UQs from dietary bacteria delivered to *clk-1* mitochondria was similar for all UQs, ranging from 40 pmol/mg to 65 pmol/mg mitochondrial protein. The amount of DMQ₉ species was similar in all *clk-1* cultures and significantly increased from that of N2 ($p < 0.01$ for each *clk-1* culture compared to N2, Figure 1) though our measurements were lower than those reported previously (Jonassen *et al.*, 2001).

3.2 Lifespan is affected by different ubiquinones

clk-1 mutants reach maturity later and lay fewer viable offspring than N2, regardless of which ubiquinone is provided (Figure S2). In order to interpret *clk-1* lifespan in the face of developmental delay, we examined the lifespan data by defining day 0 as the day the worms reached adulthood (began to lay eggs). In addition, we wanted to correlate these results to measurements of mitochondrial function (see below). *clk-1* animals grown on UQ₆ and UQ₇ did not have enough offspring to allow isolation of mitochondria. For these UQ₆ and UQ₇ lifespans (Figure 2), to correlate the data to the mitochondrial assays, we transferred *clk-1* animals grown on UQ₈ as dauers to UQ₆ and UQ₇ bacteria.

clk-1 mutants lived longer than wild-type (N2) animals, regardless of which ubiquinone they were fed. As shown in Figure 2, the overall and adult lifespan of *clk-1* fed with OP50 and UQ₈ were similar, indicating the animal's lifespan is not influenced by the different parental strain of bacteria. The adult lifespans of *clk-1* fed with OP50, UQ₆, UQ₇, UQ₈ and UQ₉ are similar (16 ± 0.7 , 15 ± 0.5 , 16 ± 0.7 , 17 ± 0.3 days and 17 ± 0.4 days, respectively). UQ₁₀ fed worms show a longer adult lifespan of 19 ± 0.4 days ($p < 0.05$).

3.3 Mitochondria respiration rates are affected by different ubiquinones

Different UQs may affect *C. elegans* lifespan by changing mitochondrial respiration. We examined mitochondrial respiratory activity, since UQ is an essential link of mitochondrial electron transport. Mitochondria were isolated from *clk-1* fed with UQ₆, UQ₇, UQ₈, UQ₉, UQ₁₀ or K12 (producing UQ₈), and N2 fed with K12. Oxidative phosphorylation studies were performed to evaluate the integrated mitochondrial function of intact mitochondria. The state 3 respiration rates shown reflect the near-maximal rate of oxygen consumption and the capacity for electron flux through the respiratory chain. The complex I dependent respiration rate was significantly decreased in *clk-1* regardless of the species of dietary quinone (Figure 3A) when compared with N2 ($p < 0.05$). *clk-1* fed with UQ₆, UQ₇, or UQ₁₀ each displayed a more decreased complex I-dependent respiration rate when compared to *clk-1* fed with UQ₈ or UQ₉ ($p < 0.05$). When using the complex II specific electron donor succinate, the respiration rates of *clk-1* fed with engineered bacteria expressing UQ₆₋₁₀ also decreased when compared to the wildtype worm, N2 ($p < 0.05$) (Figure 3B), but not as severely as complex I dependent respiration rates. *clk-1* worms fed with UQ₇ or UQ₁₀ showed lower complex II-dependent respiration rates than those fed K12, UQ₆ or UQ₈ ($p < 0.05$). *clk-1* worms fed with UQ₇ also showed lower complex II-dependent respiration rates than those fed with UQ₉ ($p < 0.05$), in agreement with our earlier report (21).

3.4 The level of oxidative damage to mitochondrial protein is strikingly decreased in mitochondria from *clk-1* fed with UQ₁₀

ROS are byproducts of mitochondrial electron transport and are proposed to be a major cause of aging (Balaban *et al.*, 2005). Since there were differences in lifespan between N2 and *clk-1* fed with different UQs, we examined whether there were differences in accumulated ROS damage between these animals. The toxic aldehyde, 4-hydroxynonenal (HNE), is produced by

lipid peroxidation triggered by ROS (Esterbauer *et al.*, 1991). HNE is capable of damaging multiple cell components. We examined mitochondrial proteins for the presence of the HNE-adduct (Tsai *et al.*, 1998; Kayser *et al.*, 2004a) as a proxy for overall cumulative ROS-induced damage to mitochondria. *clk-1* exhibits much less damage to mitochondrial protein than controls, regardless of its UQ species (Figure 4). However, *clk-1* fed with UQ₁₀ showed a further reduction in accumulated damage to mitochondrial protein. The differences seen in specific bands in the *clk-1* cultures (*e.g.* compare 55 kDa and 32 kDa bands in UQ₇ and UQ₈ cultures), were not uniformly observed in every experiment, though the overall decrease in HNE staining was always observed.

3.5 H₂O₂ production is decreased in mitochondria from *clk-1* fed with UQ₁₀

ROS damage is likely dependent on the rate of ROS production from the respiratory chain (Adam-Vizi and Chinopoulos, 2006). We therefore quantified the amount of H₂O₂ released from intact mitochondria from *clk-1* fed with bacteria producing UQ₆₋₁₀, or wildtype bacteria K12. We compared those amounts to each other and to that released from mitochondria from N2 fed with K12. In the absence of inhibitors, *clk-1* and N2 mitochondria generate similar amounts of H₂O₂ with either malate (Figure 5A) or succinate as a substrate (Figure 5B). Since ROS released into the mitochondrial matrix could be scavenged and not measured externally from intact mitochondria, we also measured ROS production from submitochondrial particles from N2 and *clk-1* fed K12 or UQ₁₀. We limited our studies to three cultures due to the difficulty in isolating enough mitochondria to study as SMPs. The three cultures studied represent the three groups with differing amounts of ROS damage seen on Western blot and thus give information about the underlying cause for these differences. The amounts of ROS production by *clk-1* SMPs stimulated with NADH was increased compared to N2 (Figure 5C). With succinate, SMPs from *clk-1* generated the same or more H₂O₂ than N2 (Figure 5D). In no case was the ROS production lower in *clk-1* mitochondria than in N2 mitochondria. The sites of potential ROS release from the electron transport chain were similar to those previously reported in mammalian mitochondria (Chen *et al.*, 2003) (Figures S3, S4).

4. DISCUSSION

To understand how UQ tail lengths affect lifespan, we directly tested the effects of different UQ tail lengths on: 1) *C. elegans* lifespan, 2) respiration and electron transport from complex I and II to complex III in the mitochondrial respiratory chain, 3) free radical damage to mitochondrial proteins, 4) generation of reactive oxygen species (ROS).

4.1 Lifespan

Regardless of which UQ species *clk-1* animals ingest, they live longer than do N2 animals. However, animals fed UQ₁₀ live the longest. Saiki *et al.* recently reported that the lifespan extension in wildtype *C. elegans* fed on Q-less bacteria was not influenced by the addition of a water-soluble UQ₁₀ (Saiki *et al.*, 2008). They concluded that the respiratory deficiency of the Q-less *E. coli* extended the lifespan of the nematode, rather than the precise nature of the UQ ingested. It is likely that a Q-less diet will alter metabolism in a way not seen in the bacteria used in the present study, and may independently affect lifespan. In addition, they did not study the effects of the water soluble UQ₁₀ on *clk-1* lifespan. Clearly our studies show that different exogenous UQs from otherwise genetically identical bacteria can have an effect on lifespan in *clk-1*.

4.2 Respiration and electron transport

Complex I displayed more marked UQ-specific differences in respiratory capacity when compared with complex II, with the effect of UQ₁₀ the most notable. This agrees with our previous study of *clk-1* fed wildtype *E. coli* (Kayser *et al.*, 2004a). Lenaz and colleagues

proposed that the length of isoprenyl tails of UQs relates to their location and diffusion in the membrane (Lenaz *et al.*, 2002; Genova *et al.*, 2003). UQs with shorter isoprenyl tails are hypothesized to be relatively more exposed to the surface of the inner mitochondrial membrane and the intermembrane space. Consequently, they may be less favorably positioned to receive electrons from complex I. In addition, since virtually all complex I appears to exist in supercomplexes in the nematode (Suthammarak *et al.*, *J Biol Chem*, In Press), it may be that less supplemental UQ is accessible to complex I, even with multiple generations of feeding. We saw no correlation between the length of isoprenyl tails and the rate of mitochondrial respiration. Decreased (Lee *et al.*, 2003) or increased (Bonawitz *et al.*, 2007) mitochondrial activity have both been shown to extend lifespan. We found no simple correlation between mitochondrial respiratory capacities and lifespan; *e.g.* the effects of UQ species on ROS damage seem to outweigh the effects on respiration *per se* (see below).

4.3 ROS damage to mitochondrial proteins

Strikingly, *clk-1* mitochondrial protein had much lower levels of oxidative damage than N2, regardless of which UQ had been fed to the animal. The decreases in ROS damage in the *clk-1* animals as a group correlated with their longer lifespans, as compared to N2. Protein from *clk-1* mitochondria with UQ₁₀ was consistently the least labeled by HNE. The remarkable lack of ROS damage to *clk-1* animals fed UQ₁₀ is accompanied by a further, significant lengthening of *clk-1*'s already prolonged lifespan. We allowed the animals to all reach young adulthood before isolating the mitochondria. The similar developmental stage should indicate similar total amounts of metabolic flux during development.

4.4 Generation of ROS

Since animals with varied UQs differ in their accumulation of ROS damage, we hypothesized that different UQ species cause a decrease in either ROS production or scavenging. No significant differences in ROS released from intact mitochondria were seen when either malate or succinate were used as substrates. Interestingly, the amounts of H₂O₂ produced from *clk-1* SMPs with K12 (producing UQ₈) or UQ₁₀ were both higher than that in N2 using NADH as a substrate. Thus, the decrease in ROS damage in the *clk-1* mitochondrial proteins is seen despite equal to higher rates of ROS production. The rates of ROS production did not correlate either with lifespan or with ROS damage, as measured by HNE staining. The simplest interpretation of these data is that ROS are more effectively scavenged in *clk-1* animals than in N2. This results in less accumulated oxidative damage to mitochondrial proteins, and prolongs lifespan.

Recently, Yang *et al.* reported that knock down of the cytoplasmic scavenger *sod-1*, or the two mitochondrial scavengers, *sod-2* and *sod-3*, by RNAi does not shorten the lifespan of *clk-1* (Yang *et al.*, 2007). In addition, Van Raamsdonk and Heikimi show that deletion of *sod-2* actually extends lifespan of *clk-1* animals (Van Raamsdonk and Heikimi, 2009). Thus, these ROS scavengers are unlikely to be important in scavenging ROS in *clk-1* animals or ROS are not important in determining lifespan. The roles of other potential ROS scavengers such as glutathione S-transferase or catalase have not been studied. It would be of interest to see which, if any, of the ROS scavengers are upregulated in *clk-1*.

How do large amounts of DMQ₉ in *clk-1* mitochondria affect our results? When the electrochemical properties of UQ₂ and DMQ₂ were evaluated by Miyadera and colleagues, they concluded that UQ₂ is a stronger catalyst than DMQ₂ in terms of the O₂ reduction into O₂^{•-} (Miyadera *et al.*, 2002). Given that the total amount of ubiquinone species (DMQ plus UQ) between *clk-1* and N2, one candidate for the increased scavenging across all the *clk-1* groups is DMQ₉. The large amount of DMQ₉ as a potential ROS scavenger may explain why all *clk-1* groups had lower oxidative damage and longer lifespans than N2. Heikimi and

colleagues have isolated tRNA suppressor mutations which reverse many of the phenotypes of *clk-1* (including longevity) but which do not change DMQ levels (Branicky et al., 2006). This would argue that DMQ₉ is not an important scavenger in *clk-1*. However, no report of the lifespans of the suppressors alone was given nor was of ROS production measured. Clearly further studies are necessary to resolve this issue. As noted above, other scavengers may be of primary importance in *clk-1* as well.

However, the increased DMQ₉ levels do not explain the differences between *clk-1* fed UQ₁₀ and *clk-1* fed UQ₆, UQ₇, UQ₈ or UQ₉. Since UQ₁₀ fed animals should have similar DMQ₉ levels as the *clk-1* animals fed other UQ species, our results may indicate that UQ₁₀ provides stronger protection against oxidative damage than the other UQs used in this study.

Though mitochondria have been implicated as the major ROS production site, studies have questioned whether lifespan, oxidative damage and mitochondrial activity are independently regulated (Rea et al., 2007; Yang et al., 2007). Recently, Rea et al. (Rea et al., 2007) blocked mitochondrial function by knocking down expression of subunit of F₀F₁ ATPase *atp-3* to different levels. There was no correlation between degree of lifespan extension and level of oxidative damage to total worm protein, which appeared to differ by less than 50% from controls for any level of RNAi treatment. However, the results of Rea et al. blocked metabolism in a way that does not necessarily alter ROS production or scavenging. In their study, lifespan may have been extended by specifically affecting development. In addition, our studies measure oxidative damage to mitochondrial proteins, not total worm protein, which may be a better predictor of lifespan.

Based on our data, UQ₆, UQ₇, and UQ₁₀ fed *clk-1* animals shared low respiration rates but were diverse in lifespan. *clk-1* animals fed with UQ₁₀ have longer adult lifespans than those fed with UQ_{6,9}. By comparing adult lifespan, we intended to eliminate effects of UQs on developmental delay. The decreased oxidative damage in mitochondria with UQ₁₀ makes us speculate that UQ₁₀ is a better ROS scavenger than other UQ homologues used in this study. These results demonstrated that, in these animals, oxidative damage is an important factor in lifespan determination. In support of this conclusion, the *C. elegans gas-1* mutant, which has a defective mitochondrial complex I subunit, also displays a low complex-I specific mitochondrial respiratory capacity, similar to *clk-1*. However, it accumulates intense oxidative damage in mitochondrial protein and has a very short lifespan (Kayser et al., 2001; Kayser et al., 2004a). By virtue of its role as a key subunit of complex I itself, it may have effects on ROS production that are not seen in *clk-1* mutants. Studies to understand the differences between *clk-1* and *gas-1* are in progress.

In all cases, the distributions of UQ species were broader in *clk-1* mitochondria than were the distributions in the bacteria on which they were raised. In the cases of *clk-1* raised on UQ₆ and UQ₇, this was primarily the result of methodology. As stated in the Results, *clk-1* animals raised on UQ₆ or UQ₇ alone have very few offspring which precludes isolation of their mitochondria. To obtain enough nematodes for those studies, we allowed animals raised on K12 (which produce UQ₈) to reach dauer stage and then fed them bacteria producing UQ₆ or UQ₇. As a result, the animals were exposed to UQ₆ or UQ₇ for only one period of mitochondrial proliferation (dauer to young adult). However, even *clk-1* animals grown several generations on bacteria producing almost entirely UQ₉ or UQ₁₀ had distributions of quinones more broad than those of the bacteria. Earlier studies found that null allele strains of *clk-1* accumulated UQ₉ from OP50 bacteria, despite the finding that such bacteria made no measureable UQ₉ (Arroyo et al., 2006). Thus, the distribution of quinones in *clk-1* mitochondria may result from a “leakiness” in the production of UQ₉ or UQ₁₀ species in the animal and from unequal uptake of the different quinones in their food.

Exogenous UQ₁₀ and related quinone supplementation has been shown to support and improve mitochondrial oxidative phosphorylation (Marriage *et al.*, 2004; Yen *et al.*, 2005), supply protective benefits in oxidative stress (Matthews *et al.*, 1998) and apoptosis (Moon *et al.*, 2005). Our findings, which show that supplemental UQ₁₀ increases lifespan and minimizes oxidative damage to mitochondrial proteins, may substantiate therapies that use it to treat mitochondrial and aging related diseases.

Many studies in worms make conclusions about lifespan as it relates to mitochondrial function and ROS production. These are seldom accompanied by measurements of either. We report the first *in vivo* characterization of the effects of different UQs on lifespan, and relate dietary supplementation of UQ₁₀ to mitochondrial function in an experimental model. Moreover, we provide evidence linking oxidative damage of mitochondrial proteins to lifespan, and support the roles of DMQ₉ and UQ₁₀ as antioxidants.

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Figure 1. Ubiquinone species from mitochondria of *clk-1* animals

Tandem mass spectrometry was used to identify the ubiquinone levels from mitochondria isolated from *N2* and *clk-1* adults fed the corresponding bacteria described in (A). The level of nmol quinone/g mitochondrial protein of different UQ analogues is displayed. Endogenous UQ₉ in *N2* approximated 1500 nmole/mg mitochondrial protein. Endogenous DMQ₉ in *clk-1* approximated 100-150 nmole/mg mitochondrial protein, regardless of which UQ diet was provided. The exogenous UQs accumulated in *clk-1* are much lower, ranging from 40~60 nmole/mg depend on the type of UQ from the bacterial diet. Note that *clk-1* animals grown on UQ₆ and UQ₇ bacteria were only fed the corresponding bacterial strain after being transferred from a dauer culture. The remaining cultures were grown several generations in the corresponding bacterial cultures.

Figure 2. Lifespan of N2 and *clk-1* fed with UQ₆ to UQ₁₀ were measured to verify the effects of different UQs analogues on aging

Survival curves defining day 0 as the day worms began to lay eggs are shown, to eliminate developmental effects on lifespan. The curves representing *clk-1* are all significantly different from N2 ($p < 0.05$). Only the curve representing *clk-1* fed with UQ₁₀ is different from the ones representing *clk-1* fed with UQ₆, UQ₇, UQ₈, UQ₉ and OP50. Animals were raised at 20°C. No FUDR was used for this lifespan study.

Figure 3. Oxidative Phosphorylation rates of mitochondria from N2 and *clk-1* fed with distinct UQ analogues

Malate (**A**) and succinate (**B**) were used as the electron donor for complex I and II, respectively. The respiration rates (state 3) were used to determine the integrated mitochondrial function. (**A**) Complex I dependent respiration rates significantly decreased in *clk-1* regardless of which UQ they were fed when compared to N2 ($p < 0.05$). *clk-1* fed with different UQs also showed different complex I dependent respiration rates. Each sample was measured in 3-5 independent experiments. Mean rates and standard deviations are shown. + indicates different compared to N2, $p < 0.05$. * indicates different compared to UQ₈, $p < 0.05$. # indicates different compared to UQ₉, $p < 0.05$. (**B**) Complex II dependent respiration in *clk-1* was not as severely affected as

complex I dependent respiration. *clk-1* fed engineered UQ₆ through UQ₁₀ showed a decreased complex II dependent respiration rate compared to N2 (p<0.05). Mean rates and standard deviations are shown. + indicates different compared to N2, p<0.05. * indicates different compared to K12, UQ₆, and UQ₈, p<0.05. # indicates different compared to UQ₉, p<0.05.

Figure 4. Oxidative damage is least in mitochondria of *clk-1* fed with UQ₁₀

The level of HNE modified mitochondrial protein was detected by antibody staining of Western blots and was used to indicate the level of accumulated oxidative damage. Each lane represents protein isolated from mitochondria of worms fed the corresponding UQ bacterial strains as labeled. N2 was grown on K12. Equal protein loading was confirmed by complex I subunit NDUF53 antibody staining. Similar mitochondrial proteins were modified by HNE in N2 and *clk-1*. However, the levels of oxidative damage were much lower in all *clk-1* samples; *clk-1* mitochondria containing UQ₁₀ were the least affected. This result was representative of 4 experiments.

Figure 5. H₂O₂ production from mitochondria from N2 fed with K12 and *clk-1* fed with K12 and engineered bacteria produce UQ₆, UQ₇, UQ₈, UQ₉ and UQ₁₀

H₂O₂ production was measured by the Amplex red assay (see Methods). H₂O₂ production was initiated from intact mitochondria with (A) malate (1.5 uM) or (B) succinate (1.5 uM). H₂O₂ production was also measured in submitochondrial particles (SMPs) induced by (C) NADH or (D) succinate. Each sample was measured in duplicate in 3-4 experiments. Mean rates and standard deviations are shown. * indicates different compared to N2 p<0.05. # indicates different compared to *clk-1* fed with K12 p<0.05.