Book: Aging and Oxidants in Animals and Plants

Aging and oxidants in the nematode *Caenorhabditis elegans*

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1.1 Testing the oxidative damage theory of aging in *C. elegans*

The biology of aging remains poorly understood. For example, it remains unclear what sort of biological processes are the primary determinants of aging, or of the differences in aging rate between different animal species. The extent to which aging involves the same or different processes in different animal taxa also remains unclear. Many forms of pathology are associated with increased levels of molecular damage (involving, for example, oxidation), and aging is no exception (Halliwell and Gutteridge, 2007).

Among contemporary mechanistic theories of aging, many view accumulation of molecular damage, particularly oxidative damage, as the primary cause. It has been postulated that a major cause of such damage is reactive oxygen species (ROS), particularly the free radical O_2^- (superoxide) and its derivatives (reviewed by (Balaban et al., 2005, Raha and Robinson, 2000). O_2^- is generated in the cell by diverse processes, for example, as a by-product of the activity of the mitochondrial electron transport chain. Such mitochondrial ROS has been viewed as a possible mechanistic basis of another hypothetical aging mechanism. The rate of living theory postulates that the rate of energy metabolism determines the rate of aging. It has been suggested that this could be because the rate of production of ROS by mitochondria, and therefore the rate of accrual of molecular damage, is higher when metabolic rate is higher (Sohal and Weindruch, 1996). In the following discussion, I will critically assess in turn whether metabolic rate, mitochondria, and reactive oxygen species are determinants of aging in the nematode *Caenorhabditis elegans.* To this end, I will survey the numerous investigations of these issues that have been carried out using this model organism.

C. elegans is a free-living nematode that can be found in soil rich in organic matter, particularly compost. Experimentally, it has the advantage of being a complex animal, with a nervous system, reproductive system, and alimentary canal, yet one that is so small (adults reach only ~1.2 mm in length) that it may be handled like a micro-organism, with the convenience and low cost that this implies. For studying aging it has two particular advantages: its has a very short lifespan (usually 2-3 weeks), and its lifespan are unaffected by inbreeding effects which have complicated studies of the genetics of aging in *Drosophila* and the mouse (Johnson and Hutchinson, 1993). There

are also the many advantages associated with an established genetic model system, including well-characterised mutations in large numbers of genes, availability of a well-annotated genome sequence, and powerful molecular genetic methodologies. The latter include construction of transgenic animals, use of fluorescent proteins to visualise gene expression within the transparent body of the nematode, and RNA-mediated interference to knock down gene expression.

Several types of approach have been taken to investigate the role of oxidative stress in aging in *C. elegans*. These include testing for correlations between aging and various aspects of oxidative metabolism. Studies of this sort typically either examine age changes in wild-type nematodes, or differences between wild-type nematodes and mutants with altered aging rates. Some attempts have also been made to test theories of aging more directly by manipulating selected aspects of the relevant biology (e.g. antioxidant defence) and examining the resulting effects on aging. Most studies have been of the first type which is, arguably, the least informative.

One of the strengths of *C. elegans* as a model for studying aging is the ease with which classical genetic approaches may be applied. Many genes have been identified where loss of function due to mutation or RNAi leads to altered lifespan. A problem with studies of short-lived strains is that a reduction in lifespan can result either from accelerated aging (progeria) or from pathologies unrelated to normal aging, and it can be difficult to distinguish the two. However, methods have been developed to identify likely instances of progeria (Garigan et al., 2002, Gerstbrein et al., 2005), and some short-lived mutant studies have been informative. For example, the gene *mev-1* encodes a subunit of Complex II in the electron transport chain (Ishii et al., 1998). Mutation of *mev-1* results in hypersensitivity to oxidative stress, elevated production of mitochondrial ROS and shortened lifespan (Ishii et al., 1990, Senoo-Matsuda et al., 2001), reviewed by (Ishii et al., 2006).

Many more studies have focused on genes where mutation increases lifespan, e.g. those encoding elements of the insulin/IGF-1 signaling (IIS) pathway, which include the *daf-2* insulin/IGF-1 receptor and the *age-1* phosphatidylinositol 3-kinase (Kimura et al., 1997, Morris et al., 1996). Mutation of *age-1,* for example, can increase the mean and maximum lifespan of *C. elegans* adults by up to 10-fold (Ayyadevara et al., 2007). Long-

lived IIS mutants also show resistance to oxidative stress and increased levels of the antioxidant enzymes superoxide dismutase (SOD) and catalase (Larsen, 1993, Vanfleteren, 1993, Vanfleteren and De Vreese, 1995). Numerous studies, in *C. elegans* and other species, report correlations between oxidative damage and aging. While this might seem to suggest that the oxidative damage theory must be true, it is not safe to conclude this. To date, there is little direct evidence demonstrating, for example, control of normal aging in *C. elegans* by superoxide or SOD, or hydrogen peroxide (H_2O_2) or catalase. In fact, relatively few studies have been conducted that directly test oxidative damage theories of aging in *C. elegans*. Many more studies of this sort have been conducted in other models. For example, numerous studies of the effects on aging of over-expression of SOD and catalase have been conducted in *Drosophila* (Sun and Tower, 1999, Parkes et al., 1998, Orr and Sohal, 2003). Ultimately, theories of aging in *C. elegans* may only be verified or falsified reliably by means of such direct testing.

1.2 Metabolic rate and aging in *C. elegans*

Central to many discussions of the rate of living theory is the idea that increased metabolic rate will lead to increased production of O_2^- and, consequently, an increased rate of accumulation of molecular damage and of aging (Beckman and Ames, 1998, Finkel and Holbrook, 2000). This view has informed many studies of metabolic rate and aging in *C. elegans*, as elsewhere. However, this view is not necessarily correct. At lower rates of metabolism, the inner mitochondrial membrane potential increases, which can increase O_2^- production. As metabolic rate increases, membrane potential and O_2^- production drop (Brand, 2000). Thus, all else being equal, one might expect lifespan to increase with increasing metabolic rate.

Effects of temperature on lifespan

C. elegans do show striking rate of living effects insofar as lifespan is shorter at higher temperatures. For example, the median lifespan of wild-type hermaphrodites is 24 days at 15˚C compared to 16 days at 22.5˚C (Gems et al., 1998). This implies that processes whose rate determines the rate of aging occur faster at higher temperatures; but the

identity of these critical processes remain undetermined. To date, studies of rate of living effects have focussed on energy metabolism and production of O_2^- by mitochondria. In *C. elegans*, metabolic rate does increase with increasing temperature (Van Voorhies and Ward, 1999), but a causal role of metabolic rate in determining aging rate has not been demonstrated.

Metabolic rate in long-lived nematodes

In order to test the rate of living theory, metabolic rate has been measured in long-lived nematodes, including *age-1* and *daf-2* mutants, *clk-1* mutants (with defects in ubiquinone biosynthesis, see below), and nematodes subjected to various forms of dietary restriction. The instructive power of such tests is rather limited, however, for the following reasons. If metabolic rate were the sole determinant of longevity in *C. elegans*, then one should see a reduction in metabolic rate in long-lived nematodes, though such an observation would give no indication of causality. If long-lived nematodes show no change in metabolic rate, or even a small increase, this does not demonstrate that metabolic rate is not a determinant of aging, since more than one mechanism may contribute to mutant longevity.

In the main, reductions in metabolic rate in long-lived *C. elegans* have not been detected in insulin/IGF-1 signaling mutants or animals subjected to dietary restriction, but they have been in some strains with mitochondrial defects. In *age-1* and *daf-2* mutants, oxygen consumption rate shows no reduction and even slight increases (Vanfleteren and De Vreese, 1996, Braeckman et al., 2002a, Braeckman et al., 2002b, Houthoofd et al., 2005a, Houthoofd et al., 2005b). Lower levels of heat production and higher levels of ATP were also seen in *daf-2* mutants, which might imply a higher level of mitochondrial coupling in this mutant. Consistent with this, levels of O_2^- production in isolated mitochondria are higher in *daf-2(e1370)* mutants than in wild type (Brys et al., 2007). One study also reported a decline in metabolic rate in *age-1* and *daf-2* mutants, and concluded that the rate of living theory is supported (Van Voorhies and Ward, 1999). This study measured CO_2 production, which could explain the discrepancy with other studies (for further discussion of methodological issues in metabolic studies of *C. elegans*, see (Braeckman et al., 2002a, Braeckman et al., 2002b, Van Voorhies, 2002).

How dietary restriction (DR) affects metabolic rate in *C. elegans* depends on how DR is exerted. For example, DR by bacterial dilution has no effect on oxygen consumption rate, while DR by means of axenic culture (i.e. no microbial food source) or an *eat-2* mutation (which reduces feeding rate) increases oxygen consumption rate and heat production (Houthoofd et al., 2002b, Houthoofd et al., 2002c).

Strains with alterations in mitochondrial function vary in terms of metabolic rate, and *clk-1* has little effect on metabolic rate (Braeckman et al., 2002c, Braeckman et al., 1999, Felkai et al., 1999). However, metabolic rate was reduced in *isp-1* mutants, which affect Complex III of the electron transport chain (ETC) (Feng et al., 2001) and animals subjected to RNAi knockdown of several ETC genes (Dillin et al., 2002). Thus, it is possible that reduced metabolic rate somehow contributes to the increased longevity of long-lived forms of *C. elegans* in some cases. However, the effect of metabolic rate on aging in *C. elegans* remains unknown.

Differences in energy metabolism between* C. elegans *and vertebrates

As always with studies of aging in *C. elegans* a major concern is whether any given findings are relevant to mammalian aging. In terms of energy metabolism, *C. elegans* is clearly different from higher animals in several respects. For example, *C. elegans* possess the glyoxylate pathway, absent in higher animals, which makes possible the conversion of acetyl CoA to glucose. Expression of the main glyoxylate enzyme, which has both malate synthase and isocitrate lyase activity, is up-regulated in *daf-2* and *age-1* mutant adults, and in the very long-lived, diapausal dauer larva stage (McElwee et al., 2006a, McElwee et al., 2006b, Vanfleteren and De Vreese, 1995). *C. elegans* also synthesize the disaccharide trehalose, also lacking in higher animals.

Nematodes are also capable of anaerobic respiration using an alternative electron acceptor, rhodoquinone (Takamiya et al., 1999), and the malate dismutation pathway (Tielens et al., 2002). When cultured under anoxic conditions, *C. elegans* excrete lactate, acetate, succinate and propionate (Foll et al., 1999). It has been suggested that such anaerobic respiration might reduce O_2^- production levels, thereby increasing lifespan (Rea and Johnson, 2003). Transcript profile studies suggest that this pathway might be upregulated in dauer larvae and *daf-2* mutants (Holt and Riddle, 2003, McElwee et al.,

2006a, McElwee et al., 2006b). However, increased anaerobic respiration in *daf-2* mutants would be expected to generate heat, which would increase their calorimetric/respirometric (C/R) ratio. In fact, the C/R ratio is reduced in mutants with reduced insulin/IGF-1 signaling (Houthoofd et al., 2005b).

In conclusion: While rate of living effects are seen in *C. elegans*, the biochemical processes whose rate is so strongly determinative of aging remain unclear. The evidence for the importance of O_2 consumption is weak, to say the least. One alternative aging rate-determining process that is affected by temperature is protein synthesis. Several studies have recently shown that reduction of function of various genes linked to protein biosynthesis increases lifespan in *C. elegans* (Hansen et al., 2007, Henderson et al., 2006, Pan et al., 2007, Syntichaki et al., 2007).

1.3 Mitochondria and aging in *C. elegans*

Mitochondria, superoxide and aging

Investigations of the cause of cellular oxidative damage often focus on O_2^- produced as a by-product of the reduction of O_2 by the electron transport chain (ETC) of mitochondria. Isolated mitochondria or sub-mitochondrial particles can generate substantial amounts of O_2^-. For example, O_2^- production by isolated rat liver mitochondria respiring in State 4 accounts for around 1-2% of oxygen consumed (Boveris, 1977). However, levels of mitochondrial O_2^- production *in vivo* are much lower, in the 0.1-0.3% range (St-Pierre et al., 2002, Staniek and Nohl, 2000), and the relevance of mitochondrial O_2^- to aging remains unclear (Imlay and Fridovich, 1991, Nohl and Hegner, 1978). In addition, the relative importance of other sources of ROS as contributors to molecular damage and aging is unknown; ROS, including O_2^- and H_2O_2, are also produced in other ways, such as O_2^- by cytochrome P450 oxidases, xanthine oxidase and membrane-associated NADPH oxidase. The hypothesis that mitochondrial O_2^- causes aging is far from proven.

The mitochondria of *C. elegans* are largely similar to those of higher animals. For example, their mitochondrial DNA is similar in terms of gene content and overall size (Murfitt et al., 1976, Okimoto et al., 1992). However, there are some significant differences (see below) so, as always with *C. elegans*, once should generalize cautiously.

Little is known about levels of mitochondrial O_2^- production *in vivo* in *C. elegans* and whether it contributes to aging though it is at least clear that isolated *C. elegans* mitochondria do produce O_2^- (Senoo-Matsuda et al., 2001).

In mammalian cells, levels of mitochondrial O_2^- production increase with age, e.g. a 25% increase with age in isolated rat heart mitochondria (Nohl and Hegner, 1978). One study has reported that there is no age increase in mitochondrial O_2^- in *C. elegans* (Yasuda et al., 2006). A more recent study has even reported a decline with age in mitochondrial ROS production (measured as H_2O_2) (Brys et al., 2007). Consistent with this, complex I activity drops by 60% between day 4 and day 12 (Yasuda et al., 2006).

As worms grow old, their oxygen consumption rate drops dramatically (De Cuyper and Vanfleteren, 1982, Vanfleteren and De Vreese, 1996, Suda et al., 2005, Yasuda et al., 2006). For example, a recent study measured a drop in oxygen consumption from ~200 pl/min/worm in early adulthood to ~25 pl/min/worm by 9 days of age (Suda et al., 2005). Taken together, these results suggest that *in vivo* levels of mitochondrial O_2^- production decrease substantially with age in *C. elegans*. It therefore seems unlikely that the age increase in oxidative damage in *C. elegans* is due to increased O_2^- production later in life.

Effects of mitochondrial electron transport chain (ETC) defects on aging

The oxidative damage theory could imply that mutations affecting components of the ETC would either decrease or increase lifespan. Defects in electron transport might increase production of O_2^- and reduce lifespan; alternatively, overall reduction in electron flow might lower O_2^- and increase lifespan. In *C. elegans*, both effects of disruption of ETC genes on lifespan have been seen. However it remains unclear whether this has anything to do with O_2^- generation; reviewed by (Anson and Hansford, 2004).

Genes encoding mitochondrial proteins predominated in several large scale RNAi screens for genes with effects on lifespan (Dillin et al., 2002, Lee et al., 2003a, Hansen et al., 2005, Hamilton et al., 2005). In particular, RNAi of many mitochondrial and nuclear genes encoding proteins of ETC Complexes I - V caused substantial increases in lifespan. RNAi affecting other mitochondrial proteins, such as mitochondrial carriers also increased lifespan. The combination of mitochondrial defects and increased lifespan is

sometimes referred to as the Mit phenotype (Rea, 2005). In most cases Mit animals also show delayed development, reductions in body size, fertility, activity level and feeding rate (Dillin et al., 2002), as well as abnormalities in mitochondrial morphology (Lee et al., 2003a). For some genes, Mit animals have normal body size and feeding rates, but increased lifespan (Hansen et al., 2005), implying that life extension is not causally connected to reduced body size or feeding rate. The loss of a single protein component of the large ETC protein complexes may cause accumulation of unfolded proteins in the mitochondria, and in many cases, Mit animals accumulate the mitochondrial chaperone HSP6 (Yoneda et al., 2004, Hamilton et al., 2005). Mit mutants have also been identified with mutations that either affect ETC genes directly (Feng et al., 2001, Tsang et al., 2001) or in the case of *lrs-2*, indirectly. *lrs-2* encodes a unique mitochondrial leucyl-tRNA synthetase which is required for the expression of the twelve mitochondrially-encoded polypeptides. The *lrs-2* mutation is predicted to block expression of all twelve of these polypeptides; maternally rescued mutants form small, sterile, long-lived adults (Lee et al., 2003a). Severe loss of ETC function often causes larval arrest and lethality (Lee et al., 2003a, Tsang et al., 2001).

What mechanisms might underlie the extension of lifespan in Mit animals? One interpretation is that it is due to reduced metabolic rate and perhaps also lowered production of O_2^-. Several observations are consistent with the first view: in Mit animals there is usually a reduction in O_2 consumption rate (Lee et al., 2003a) and ATP levels can be reduced to as little as 20% of wild-type (Dillin et al., 2002). This might suggest that ATP levels limit the rate of processes that promote aging. However, an interesting study by Dillin et al. (2002) suggests that something more complex is going on. To test the timing of effects of Mit defects on aging, expression of ETC genes was selectively knocked-down during larval development or in adulthood. Knockdown in larvae alone increased adult lifespan (Dillin et al., 2002). This is perhaps not surprising since mitochondrial number may be programmed during development: in the transition from L4 to adulthood alone there is a 6-fold increase in the number of mitochondria (Tsang and Lemire, 2002).

More surprisingly, adult-specific knockdown of ETC gene expression reduced ATP levels but did not increase lifespan. The authors postulated that there exists in *C.*

elegans a system that registers the rate of respiration during development, and adjusts the rate subsequent of aging accordingly (Dillin et al., 2002). The reason this is quite surprising is that the timing of action of insulin/IGF-1 signaling and dietary restriction are exactly the opposite: during adulthood and not development. This finding warrants further investigation: for example, how does life-long, larva-specific and adult-specific RNAi of ETC genes compare in terms of effects on mitochondrial O_2^- production, O_2 consumption, mitochondrial number and morphology, and HSP-6 expression?

It is not likely that life extension in Mit animals involves the insulin/IGF-1 pathway, since knockdown of ETC genes increases lifespan both in *daf-16* and *daf-2* mutant animals (Dillin et al., 2002, Lee et al., 2003a, Hansen et al., 2005, Hamilton et al., 2005). Lifespan is also increased by RNAi of several genes encoding glycolytic enzymes such as phosphoglycerate mutase (F57B10.3) (Lee et al., 2003a) and glucose-6-phosphate isomerase (Y87G2A.8) (Hansen et al., 2005), suggesting that glycolysis somehow reduces lifespan. This appears to involve different mechanisms relative to Mit animals, since animals develop normally, body size is not reduced, mitochondria show normal morphology, and the extension in lifespan requires DAF-16 (Lee et al., 2003a, Hansen et al., 2005).

Ubiquinone (coenzyme, or CoQ) plays a major role in the ETC. Production of O_2^- by the mitochondrial ETC appears to be largely the result of transfer of electrons from ubisemiquinone to oxygen (Boveris, 1977, Raha and Robinson, 2000). Deficiency in CoQ can also increase lifespan. For example, *clk-1* encodes a mitochondrial protein necessary for the final step in CoQ biosynthesis (Ewbank et al., 1997, Stenmark et al., 2001, Felkai et al., 1999); reviewed by (Stepanyan et al., 2006). Mutation of *clk-1* causes accumulation of the precursor of nematode CoQ, demethoxy-ubiquinone-9 (DMQ9) (Miyadera et al., 2001), and increased lifespan (Wong et al., 1995). CoQ varies between species in the number of isoprene units in its side chain. *E. coli* have an eight unit side chain (CoQ_8), *C. elegans* have CoQ_9, and mammals CoQ_{10}. Likewise, if *C. elegans* are fed on *E. coli* lacking CoQ_8, this increases their lifespan, too (Larsen, 2002). The above findings might suggest that lowering CoQ levels reduces flux through the ETC, thereby lowering ROS production and increasing lifespan (but see below).

Mutations affecting ETC proteins lead to a shortening of lifespan in a minority of

cases. *mev-1(kn1)* is a point mutation in the gene for succinate dehydrogenase cytochrome *b* in Complex II, and causes hypersensitivity to oxidative stress and shortened lifespan (Ishii et al., 1990). The mutation compromises electron transfer from succinate to ubiquinone and results in increased electron leakage to oxygen. In wild-type mitochondria, O_2^- production results from electron leak at complex I and particularly III (Raha and Robinson, 2000). *mev-1(kn1)* disrupts complex II and results in O_2^- production from complex II (Senoo-Matsuda et al., 2001). *gas-1* encodes a subunit of Complex I and, like *mev-1*, mutation of *gas-1* results in hypersensitive to oxidative stress and reduced lifespan under normoxia (Hartman et al., 2001). Unlike *mev-1, gas-1* does not increase nuclear mutation rate.

An early study proposed that the short lifespan and sensitivity to pro-oxidants of *mev-1* animals was due to the fact that SOD levels are half that of wild-type (Ishii et al., 1990). Consistent with this, deletion of *sod-1*, the major Cu/Zn SOD in *C. elegans*, shortens lifespan (J.J. McElwee and D. Gems, unpublished). However, although it was reported that *mev-1* lifespan can be extended by administration of chemical mimetics of SOD (Melov et al., 2000), a further study was unable to replicate this finding (F. Matthijssens and J.R. Vanfleteren, personal communication).

In-so-far as they shorten rather than increase lifespan, *mev-1* and *gas-1* are atypical among genetic interventions affecting mitochondria and lifespan. Here it is worth bearing in mind that *mev-1(kn1)* is a reduction-of-function allele, and not a null; RNAi of *mev-1* results in a high level of embryonic lethality (Ichimiya et al., 2002). *mev-1(kn1)* reduces activity of the ETC by 80%, but does not affect succinate dehydrogenase (SDH) activity (Ishii et al., 1998). The MEV-1 subunit of complex II contains a binding site for CoQ. Potentially, reduced affinity of CoQ to MEV-1 protein leads to increased mobility of CoQ and electron leak to oxygen. By contrast, in most cases knockdown of expression of genes encoding ETC proteins may simply reduce electron flow and O_2^- formation.

Superoxide production in mitochondrial effects on aging

As mutational studies have demonstrated, mitochondria can influence aging in *C. elegans*. One interpretation is that this reflects altered electron flux through the ETC and altered O_2^- levels. If this were true, one would expect an accompanying alteration in

metabolic rate. Conversely, one would not expect an increase in somatic maintenance mechanisms (e.g. antioxidant defence).

Is retarded aging in Mit animals attributable to reduced electron flux and reduced O_2^- production? This possibility has been extensively investigated in studies of *clk-1* and CoQ. Several findings suggest that the above view is an oversimplification. Firstly, if the longevity of *clk-1* mutants were due to an effect of lowered CoQ levels on electron flux, then this strain should have a reduced metabolic rate. In fact, neither metabolic rate nor ATP levels are lower in *clk-1* animals (Braeckman et al., 1999, Felkai et al., 1999, Braeckman et al., 2002c), though RNAi of other mitochondrial genes does lower ATP levels (Dillin et al., 2002). Moreover, succinate-cytochrome *c* reductase activity is almost normal in *clk-1* mutants, implying that $DMCoQ_9$ (perhaps supplemented with bacterially-derived CoQ_8) functions as well as CoQ_9 (Felkai et al., 1999, Miyadera et al., 2001). It has been suggested that $DMCoQ_9$ produces less O_2^- than CoQ_9 (Miyadera et al., 2001), but this has not been tested directly.

Interpretation of the role of *clk-1* and CoQ in aging is complicated by the fact that the reduced (quinol) form of CoQ can act as a lipid-soluble antioxidant which protects against lipid peroxidation (Lass and Sohal, 2000, Kwong et al., 2002, Miyadera et al., 2002), which explains why it is marketed as a human dietary supplement. Consistent with this, CoQ_{10} supplementation increases lifespan in *C. elegans* in both wild-type and *mev-1* animals (Ishii et al., 2004), and also reduced O_2^- production in isolated mitochondria.

These results appear to conflict with the finding that feeding *C. elegans* with *E. coli* lacking CoQ_8 increases their lifespan (Larsen, 2002). How may these findings be reconciled? One possibility is that different forms of CoQ have different effects on lifespan. The increases in lifespan seen by Ishii *et al.* resulted from supplementation with CoQ_{10}, which may somehow promote longevity more than CoQ_9 (Ishii et al., 2004); possibly CoQ_8 increases superoxide production more than CoQ_9. To explore this, *E. coli* strains were engineered which produce CoQ_7, CoQ_8, CoQ_9, or CoQ_{10}. *E. coli* producing CoQ_9, or CoQ_{10} partially suppressed the reduced fertility of a weak *clk-1* mutant, but effects of these *E. coli* strains on lifespan were not reported (Jonassen et al., 2003).

An alternative scenario is that $DMCoQ_9$ generates less O_2^- than CoQ_9 (Miyadera et al., 2002). RNAi of *sod-1* (cytosolic Cu/Zn SOD) and mutation of *sod-4* (putative

extracellular Cu/Zn SOD) can partially suppress some *clk-1* mutant phenotypes. This, it has been suggested, may reflect reduced O_2^- production by $DMCoQ_9$ which interferes with signaling pathways in which O_2^- acts as a secondary messenger (Shibata et al., 2003, Stepanyan et al., 2006). However, it seems unlikely that the presence of $DMCoQ_9$ causes increased lifespan, since mutation of *rte-2* suppresses *clk-1* longevity without reducing levels of $DMCoQ_9$ (Branicky et al., 2006).

If the increase in lifespan associated with the Mit phenotype reflects a reduction in metabolism and ROS production, one would not expect any associated increase in stress resistance. However, this prediction is not well supported. For many genes encoding mitochondrial genes, long-lived animals subjected to RNAi proved to be resistant to H_2O_2 and heat stress, although not paraquat (Lee et al., 2003b). Moreover, mutation of *isp-1* in Complex III elevates *sod-3* expression and increases paraquat resistance (Feng et al., 2001), and *clk-1* mutant animals show resistance to ultraviolet light (Murakami and Johnson, 1996) and increased catalase levels (but reduced SOD activity levels) (Braeckman et al., 2002c). This could imply that disruption of mitochondria stimulates stress resistance pathways, perhaps due to increased O_2^- production, a mechanism dubbed "mitohormesis" (Rea, 2005). The terms hormesis usually refers to situations where brief or low level exposure to stressors induces a response that results in stress resistance.

Other findings are consistent with the occurrence of mitohormesis. For example, treatment with the drug antimycin A, which blocks Complex III, increases O_2^- production from isolated *C. elegans* mitochondria (Senoo-Matsuda et al., 2001) and, interestingly, appears to increase lifespan (Dillin et al., 2002), although the effect is not large. Mitochondrial ROS production (measured as H_2O_2) is also elevated by mutation of *daf-2* (Brys et al., 2007). There is also evidence that the SKN-1-dependent antioxidant system is activated in *clk-1* mutants (Rea, 2005). The most direct evidence that increased mitochondrial O_2^- production can contribute to longevity comes from a recent study by Schulz *et al*. (2007) who induced a state resembling dietary glucose restriction in *C. elegans* by means of a chemical inhibitor of glycolysis, 2-deoxy-D-glucose (DOG). Treatment with DOG was found to increase both ROS production, resistance to oxidative stress (paraquat and sodium azide) and lifespan. Additional treatment with the antioxidant

N-acetyl-cysteine (NAC) blocked the DOG-induced increases in ROS levels and stress resistance and lifespan (Schulz et al., 2007).

Uncoupling proteins and aging

Mitochondrial O_2^- production is predicted to be highest when the ETC is fully reduced, in State 4. Uncoupling proteins (UCPs) or chemical protonophores such as dinitrophenol can uncouple electron transport from ATP synthesis, which increases heat production and lowers O_2^- production (Brand, 2000). A prediction of the oxidative damage theory is that increased uncoupling should reduce ROS production, thereby increasing lifespan. This has been investigated a little in *C. elegans*. The worm genome contains a single gene encoding a protein with sequence homology to mammalian UCPs, *ucp-4,* which is strongly expressed in muscle (Iser et al., 2005). Absence of *ucp-4* function resulted in increased levels of ATP and cold sensitivity, consistent with function as an uncoupling protein. However, only a very slight increase in mitochondrial membrane potential was seen, and lifespan was not affected.

It has also been suggested that mitochondria from *daf-2* mutants have a higher level of coupling, given the lower calorimetric/respirometric ratio and the higher levels of ATP and ROS production (Houthoofd et al., 2005a, Houthoofd et al., 2005b, Brys et al., 2007). However, it is worth noting that this lowering of the calorimetric/respirometric ratio is not suppressed by mutation of *daf-16*, which does suppress *daf-2* longevity (Houthoofd et al., 2005a, Kenyon et al., 1993). Thus, one can at least say that this metabolic shift is not enough in itself to increase lifespan. Further studies seem warranted to establish the effects of mitochondrial uncoupling on aging in *C. elegans*.

In conclusion: A number of independent screens for genes with effects on aging have all pointed to the importance of mitochondria in aging. Disruption of mitochondrial function usually increases lifespan in *C. elegans*, but the mechanisms involved are unknown. One possibility is that O_2^- production in Mit animals is reduced, but this remains largely unexplored. In principle, reduced ATP production might seem a strong candidate mechanism, potentially linking the Mit phenotype, dietary restriction and rate of living effects; for example, ATP feeds growth, including protein synthesis, which promotes aging (Pan et al., 2007, Hansen et al., 2007, Syntichaki et al., 2007). However,

the importance of ATP levels in aging is not experimentally supported; see e.g. (Dillin et al., 2002). An alternative possibility that is finding increasing empirical support is that disruption of mitochondrial function activates somatic maintenance processes via the hormetic effects of increased ROS production (Rea, 2005). The support for the mitohormesis theory provided by Schulz *et al.* (2007), in particular, suggests that the standard oxidative damage theory of aging may be very wrong, at least as far as *C. elegans* is concerned.

1.4 Reactive oxygen species and aging in *C. elegans*

Alterations of pro-oxidant levels

According to the standard oxidative damage theory, aging is caused by ROS. If this is correct, then manipulating ROS levels should affect aging rate; i.e. lowering ROS should increase lifespan and *vice versa*. The effects of ambient oxygen concentration on lifespan and mortality rate have been tested in wild-type and *mev-1* mutant populations (Honda et al., 1993). In wild-type, these parameters were unaltered in 2%, 8% and 40% oxygen relative to 21%. This is a striking result, as it implies either that levels of O_2^- production are unaltered over this range, or that ROS are not a determinant of aging. Nevertheless, if large enough, changes in O_2 concentration can affect aging in wild-type. In 1% O_2, mean lifespan was increased by 15% and the Gompertz component of mortality was decreased (Honda et al., 1993). Whether this effect is mediated by changes in O_2^- production, metabolic rate, or some other factor is unclear. In 60% O_2, wild-type mean lifespan was slightly reduced (by 14%), probably due to increased oxidative damage. In contrast to wild-type, in *mev-1* populations there is a direct relationship between oxygen concentration and lifespan; the mutation rate in *mev-1* mutants is also hypersensitive to effects of elevated oxygen (Hartman et al., 2004).

Taken together, these findings imply that, under conditions of normoxia, O_2^- levels determine aging in *mev-1* but not wild-type. The possibilities that O_2^- does not cause normal aging, while elevated O_2^- levels can accelerate aging, are by no means contradictory. Aging in both cases may involve molecular damage, but resulting from different causes. Indeed, other observations suggest mechanistic differences between

aging in *mev-1* and wild-type, as follows. In otherwise wild-type *C. elegans*, prevention of apoptosis (programmed cell death) by mutation of the gene *ced-3* does not extend lifespan (Garigan et al., 2002). Thus, apoptosis does not contribute to normal aging. By contrast, mutation of *ced-3* increases lifespan of *mev-1* populations, apparently by preventing O_2^--induced apoptosis (Senoo-Matsuda et al., 2003). However, this extension is the result of suppression of early mortality, and late-life survival was unchanged. *mev-1* mutants also have elevated lactic acid levels, suggesting that lactic acidosis might contribute to their mortality (Senoo-Matsuda et al., 2001).

A common means to test the effects of ROS on *C. elegans* is administration of redox cycling compounds such as juglone or, more commonly, paraquat (methyl viologen) (Henderson and Johnson, 2001, Vanfleteren, 1993, Keaney et al., 2004, Ayyadevara et al., 2005b, Arkblad et al., 2005), which generate O_2^- *in vivo*. *In vivo*, redox cyclers receive electrons from NADH or NADPH via the action of diaphorase enzymes, and this activity has been detected in *C. elegans* (Blum and Fridovich, 1983). O_2^- production by redox cyclers can be measured as an increase in cyanide-independent O_2 consumption. While 1 mM paraquat does not detectably increase cyanide-independent O_2 consumption by *C. elegans* (Blum and Fridovich, 1983), 2 mM paraquat does increase it, and this concentration is just sufficient to decrease adult lifespan (Keaney et al., 2004). These results strongly imply that *C. elegans* lifespan can be shortened by elevated levels of O_2^-, but the extent to which this effect involves an acceleration of processes occurring during normal aging is unknown.

Effects of ROS on age changes in molecular damage

If ROS causes normal aging, experimental elevation of ROS should accelerate age changes in molecular damage seen in normal aging. This prediction has been little explored, though one report described increased blue fluorescence under hyperoxia (Hosokawa et al., 1994).

Isolated mitochondria from *mev-1* animals show elevated levels of O_2^- production (Senoo-Matsuda et al., 2001). Thus, O_2^- production might be elevated *in vivo* and might account for the shortened lifespan of *mev-1* under normoxia. The increased levels of protein oxidation in *mev-1* animals supports this (Adachi et al., 1998, Yasuda et al.,

1999). *mev-1* has also been reported to elevate levels of blue fluorescence (Hosokawa et al., 1994). However, a recent study saw no such effect either in *mev-1* or *gas-1* animals (Gerstbrein et al., 2005).

Effects of antioxidant defence on aging

Cellular defences against oxidative damage include both chemical and enzymatic antioxidants. If oxidative damage causes aging, then one might expect a correlation between antioxidant defence and longevity. Moreover, experimental enhancement of antioxidant defence should retard aging. Many studies have tested both of these expectations; yet in each case, establishing a causal role of oxidative damage in aging is difficult. For example, a correlation between level of an antioxidant agent and longevity could be coincidental. If experimentally induced elevation in levels of an antioxidant agent increases lifespan, the possibility remains that this occurs by some other mechanism than protection against molecular damage. Moreover, if increases in lifespan are not seen, it remains possible that multiple antioxidant defence mechanisms act in concert to protect against aging, or that antioxidant mechanisms act in concert with other prolongevity mechanisms.

Non-catalytic antioxidants

Over the years many studies have been conducted examining the effects on aging of non-catalytic antioxidants (particularly vitamin E), often generating inconclusive findings. Vitamin E studies have employed its constituents α-tocopherol and tocotrienols, and the α-tocopherol derivative α-tocopherolquinone (α-TQ). An early study found that α-tocopherol and α-TQ both increase lifespan of *C. briggsae* (a sister species of *C. elegans*) by 31% (Epstein and Gershon, 1972). Similarly, vitamin E increased lifespan in *C. elegans* (Zuckerman and Geist, 1983). However, in both studies nematodes were cultured in an axenic medium (i.e. without *E. coli*), which is nutritionally sub-optimal; moreover, the effects of vitamin E on lifespan were exerted during development, not adulthood. Thus, these findings may reflect a nutritional effect on growth in axenic medium. In another study, vitamin E increased *C. elegans* lifespan by around 20%, but also reduced fecundity and delayed the timing of reproduction (Harrington and Harley, 1988). Here,

the authors concluded that effects on aging could reflect slight toxicity, which slowed development, growth and aging. Yet another study compared the effects of α-tocopherol and tocotrienols on levels of protein oxidation, resistance to oxidative damage (exerted by ultraviolet B irradiation) and longevity. While α-tocopherol had no effect, tocotrienols had a protective effect against damage and stress, and caused a slight increase in mean but not maximum lifespan (Adachi and Ishii, 2000). A more recent report described a single trial where vitamin E increased lifespan in wild-type (+11%) but not *mev-1* animals (Ishii et al., 2004). Overall, and taking into account the tendency to publish only results showing positive effects, these studies provide little persuasive evidence that vitamin E supplementation protects against aging.

A large number of genes and processes contribute to protection against oxidative damage (Halliwell and Gutteridge, 2007, Mathers et al., 2004), any one of which may limit the rate of age accumulation of molecular damage, and its impact on homeostasis and survival. In the first line of defence are enzymes which detoxify primary pro-oxidant molecules. For example, superoxide dismutases (SOD) convert O_2^- into H_2O_2 (Fridovich, 1995), and this is converted into water and O_2 by catalases and glutathione peroxidases (GPX). Numerous proteins affect ROS production levels, such as metal trafficking proteins. Free metal ions such as Fe^{3+} stimulate production of very damaging forms of ROS such as OH^-, and metallothioneins and ferritins will counteract this. The forms of molecular damage that can occur are extremely diverse, as are the enzymes that detoxify, repair or remove damaged moieties. For example, peroxidised lipids are targets for numerous glutathione lipid hydroperoxidases and glutathione S-transferases (GSTs). In proteins, oxidation of just the amino acid methionine can be repaired by methionine sulfoxide reductase. Effects of oxidative damage to protein on protein function can, to some extent, be restored by the action of molecular chaperones. Finally, oxidised proteins can be removed by cellular turnover processes such as proteasome-dependent protein degradation and autophagy. Any of these enzymes and processes could, in principle, contribute to longevity assurance by protecting against oxidative damage.

Antioxidant enzymes: Superoxide dismutase and catalase

The superoxide dismutases and catalases of *C. elegans* are unusual in a number of ways.

For one, *C. elegans* possesses more isoforms of these enzymes than higher animals. Instead of one cytosolic Cu/Zn SOD there are two, encoded by *sod-1* and *sod-5* (Giglio et al., 1994, Larsen, 1993, Jensen and Culotta, 2005), and instead of one mitochondrial Mn SOD there are also two, encoded by *sod-2* and *sod-3* (Giglio et al., 1994, Hunter et al., 1997, Suzuki et al., 1996). A combination of SOD activity assays in *sod* mutants, and studies of levels of mRNA and reporter expression imply that *sod-1* and *sod-2* are the major isoforms expressed during reproductive development, while *sod-3* and *sod-5* are dauer up-regulated isoforms (Honda and Honda, 1999, Jensen and Culotta, 2005, Wang and Kim, 2003) (R. Doonan, J.J. McElwee and D. Gems unpublished). Why there should be dauer-specific isoforms is unclear. SOD-2 and SOD-3 Mn SODs have similar specific activities (Hunter et al., 1997), and either SOD-1 or SOD-5 Cu/Zn SOD can rescue the paraquat sensitivity of SOD-deficient yeast (Jensen and Culotta, 2005), suggesting that reproductive and dauer isoforms are not functionally different.

There are other ways in which the SOD-1 and SOD-5 Cu/Zn SODs are different. To mature, Cu/Zn SODs need to incorporate copper, and in all other eukaryotes, whether animals, fungi or plants, this requires the copper chaperone of SOD protein (CCS). Unusually, *C. elegans* does not possess a CCS, and Cu/Zn SOD maturation does not require it, but instead depends on an unknown glutathione-dependent pathway (Jensen and Culotta, 2005). Studies of SOD-1 and SOD-5 expressed in yeast also hint that, unlike other eukaryotes that have been looked at, *C. elegans* may not have Cu/Zn SOD in the mitochondrial inter-membrane space, though the evidence here is not conclusive (Jensen and Culotta, 2005).

sod-4 encodes a Cu/Zn SOD that resembles mammalian extracellular Cu/Zn SODs (Fujii et al., 1998). However, SOD-4 is also distinctive in that there are two predicted isoforms, products of alternative splicing of mRNA. SOD4-1 resembles a typical secreted Cu/Zn SOD, but SOD4-2 has an additional C-terminal sequence resembling a transmembrane domain. This suggests that this unique SOD is secreted through the plasma membrane, but then remains tethered at the cell surface (Fujii et al., 1998).

The catalases of *C. elegans* are also unusual. The *C. elegans* genome contains a tandem array of three genes encoding catalases, *ctl-1, ctl-2* and *ctl-3* (Petriv and

Rachubinski, 2004). By contrast, other metazoans have only a single catalase, while *S. cerevisiae* has a peroxisomal and a cytosolic catalase. CTL-2 is the *C. elegans* peroxisomal catalase, and is responsible for ~80% of total catalase activity; it also has a lower pH optimum for activity and higher peroxidase activity than mammalian peroxisomal catalases (Taub et al., 1999, Togo et al., 2000, Petriv and Rachubinski, 2004). Much of the *ctl-1* and *ctl-3* gene sequences are 100% identical. Studies of a CTL-1::GFP fusion protein imply that CTL-1 is a cytosolic catalase (Taub et al., 1999). One possibility is that CTL-1 evolved as a cytosolic H_2O_2 scavenger because *C. elegans* lacks an H_2O_2-scavenging glutathione peroxidase (Vanfleteren, 1993) (J.R. Vanfleteren, personal communication) (see below). A promoter fusion test implies that *ctl-3* is expressed in pharyngeal muscle and neurons (Petriv and Rachubinski, 2004). More work is needed to confirm and define the cellular localization of CTL-1 and CTL-3. In summary, given its very short lifespan *C. elegans* has an surprisingly elaborate arsenal of SODs (six) and catalases (three) to protect itself against ROS.

Is the increased longevity of *daf-2* and *age-1* attributable to any degree to increased levels of SOD and catalase? Long lived IIS mutants do show age increases in SOD and catalase activity levels, and in resistance to oxidative stress (e.g. paraquat and H_2O_2), increases which are not seen in the wild type (Vanfleteren, 1993, Larsen, 1993, Vanfleteren and De Vreese, 1995, Honda and Honda, 1999). Northern blot studies have shown a large increase in *sod-3* mRNA levels in *daf-2* mutants (Honda and Honda, 1999, Yanase et al., 2002), and microarray studies reveal additional, smaller increases in *sod-1* and *sod-5* mRNA (McElwee et al., 2003, McElwee et al., 2004, Murphy et al., 2003). *sod-3* levels are elevated throughout the life course in *daf-2* mutants, even in the developing embryo (Honda and Honda, 1999). Microarray studies also show increases in expression of at least one catalase gene in *daf-2* mutants, but because of the similarity between *ctl* gene sequences, one cannot say which. This also complicates interpretation of RNAi studies (Murphy et al., 2003). Levels of SOD and catalase are also elevated in *C. elegans* subjected to dietary restriction and, in contrast to insulin/IGF-1 signaling mutants, this increase does not depend on *daf-16* (Houthoofd et al., 2003). In dauer larvae, levels of SOD activity are 4-5-fold higher than in young adults, and levels of *sod-3* mRNA are elevated (Honda and Honda, 1999, Anderson, 1982, Larsen, 1993). Catalase

levels also seem to be elevated in dauer larvae (Houthoofd et al., 2002a), though here there is conflicting evidence (Larsen, 1993).

It seems likely that the elevated levels of antioxidant enzymes contribute to oxidative stress resistance, at least to some degree, but whether they contribute to longevity remains unclear. The effects on aging of manipulations of SOD and catalase levels has been investigated in *C. elegans*, though not as systematically as in Drosophila. RNAi knockdown of expression of *sod-3* has been reported to very weakly suppress *daf-2* longevity (Murphy et al., 2003) but, surprisingly, RNAi of *sod-5* had the opposite effect (McElwee et al., 2003). More surprisingly, deletion of *sod-2* and *sod-3*, alone or in combination, has no effect on adult lifespan (J.J. McElwee and D. Gems, unpublished).

Deletion of *ctl-1* (the cytosolic catalase) has no effect on lifespan, while deletion of *ctl-2* (the peroxisomal catalase) shortens lifespan (Petriv and Rachubinski, 2004). The authors interpreted this life shortening as progeria, though more evidence would be required to establish this with certainty. *ctl-2* mutants show abnormalities in peroxisomal morphology. Surprisingly, protein oxidation (protein carbonyl levels) increases more rapidly with age in wild-type than in *ctl-1* or *ctl-2* animals (Petriv and Rachubinski, 2004). It was at one point claimed that *ctl-1* is required for the longevity of *daf-2* mutants (Taub et al., 1999), but the study concerned was subsequently retracted (Taub et al., 2003).

There have been few reports of the effects of over-expression of *sod* genes. Over-expression of a *sod-3::gfp* fusion protein did not affect lifespan but, as the authors made clear, SOD activity level was not examined in this strain (Henderson et al., 2006). In one study, it was observed that loss of heat shock factor 1 (HSF-1) suppressed *daf-2* mutant longevity without suppressing the elevation in *sod-3* expression (Hsu et al., 2003). This suggests, at least, that elevated *sod-3* expression does not increase lifespan in HSF-1 deficient animals.

Several studies have examined the effects on *C. elegans* of administering the SOD mimetic salen manganese compounds EUK-8 and EUK-134. This results a significant increases in SOD activity levels (e.g. a 5-fold increase in mitochondrial SOD activity) and resistance to paraquat (Keaney et al., 2004, Sampayo et al., 2003). Although one study reported that these compounds also increased lifespan in *C. elegans* (Melov et al.,

2000), other workers did not see this effect, either in *C. elegans* (Keaney and Gems, 2003, Keaney et al., 2004), or in *Drosophila* (Magwere et al., 2006) or houseflies (Bayne and Sohal, 2002). Levels of EUK-8 that were optimal for protection against paraquat had no effect on lifespan, suggesting again (c.f. ambient oxygen studies) that O_2^- does not contribute to normal aging in *C. elegans*.

Other antioxidant defences

While the role of SOD and catalase in *C. elegans* aging remains poorly understood, even less is known about the role in aging of other antioxidant defence mechanisms. One possibility is that metal trafficking proteins play a role in longevity assurance. Exogenous iron shortens lifespan in *C. elegans* (Gourley et al., 2003), and *daf-2* and *age-1* mutants are resistant to heavy metals (e.g. cadmium and copper) and show elevated expression of the metallothionein *mtl-1* (Barsyte et al., 2001). RNAi of *mtl-1* slightly reduces *daf-2* mutant longevity (Murphy et al., 2003). The *ftn-1* ferritin heavy chain is also strongly up-regulated in *daf-2* mutants (McElwee et al., 2004).

In many species, glutathione peroxidase (GPX) is a major hydrogen peroxide scavenger. In one study GPX activity was not detected in *C. elegans* using BuOOH (Vanfleteren, 1993) and H_2O_2 (J.R. Vanfleteren, personal communication). However, the *C. elegans* genome contains a number of GPX-like proteins, some or all of which could, in principle, be lipid hydroperoxidases. The possible function of these proteins, e.g. in stress resistance and aging, remains unexplored.

In recent years, global changes in gene expression in *daf-2* and *age-1* mutants have been studied using DNA microarrays (McElwee et al., 2003, McElwee et al., 2004, Murphy et al., 2003, Golden and Melov, 2004). One study showed that at least 2,348 genes are up- or down-regulated in *daf-2* animals relative to normal-lived *daf-16; daf-2* controls: in other words, some 12% of genes in the *C. elegans* genome (McElwee et al., 2004). That so many genes are regulated by IIS constitutes a difficult obstacle to understanding how IIS controls aging. For one, it renders relatively uninformative studies that show correlation of expression of individual genes (e.g. *sod-3*) with IIS mutant longevity. In fact, among such large lists of IIS regulated genes, it is possible to find evidence supporting most theories of aging (Gems and McElwee, 2005).

This type of problem of bias in data interpretation can, to a degree, be avoided by identifying gene classes showing a significant level of over-represented among differentially expressed genes. One study combined this approach with a comparison of array data from *daf-2* mutants (compared to *daf-16; daf-2*) and dauer larvae (compared to recovered dauer larvae) (McElwee et al., 2004, Gems and McElwee, 2005, Wang and Kim, 2003). This was based on the plausible supposition that *daf-2* mutants are long-lived because they heterochronically express dauer longevity assurance mechanisms. Among the small number of longevity-associated gene classes were several specifying the phase 1, phase 2 biotransformation system (i.e. xenobiotic metabolism or drug detoxification). Glutathione S-transferases (GSTs) were also strongly over-represented among genes up-regulated in *daf-2* mutants, but not dauers. Biotransformation involves a complex system, of enzymes and pumps involved in detoxification and clearance of a wide spectrum of endobiotic and xenobiotic toxins, and some biosynthetic processes (Gibson and Skett, 2001). Thus, comparisons of transcript profiles from *daf-2* mutants and dauers implies that the biotransformation system is activated in these long-lived milieus. This suggests that these detoxification processes might contribute to longevity, a possibility that is easy to rationalize, given that biotransformation provides defence against molecular damage (Gems and McElwee, 2005). A recent comparison of transcript profiles from long-lived IIS mutant *C. elegans*, Drosophila and mice showed up-regulation of three classes of biotransformation enzymes (particularly GSTs) in all three species (McElwee et al., 2007). Such evolutionary conservation in the correlation of increased biotransformation and increased longevity could reflect a role for this system in longevity assurance.

The glutathione S-transferases are a highly diverse, rapidly evolving enzyme class; the *C. elegans* genome includes 51 putative GST-encoding genes. Among other things, GSTs use glutathione conjugation to detoxify endobiotic and xenobiotic toxins, including the products of oxidative damage (Hayes and McLellan, 1999). A screen for genes up-regulated upon exposure to the O_2^- generator paraquat identified *gst-4* (Tawe et al., 1998). Overexpression of *gst-4* resulted in increased resistant to paraquat, but not increased lifespan (Leiers et al., 2003). However, RNAi of *gst-4* slightly reduces *daf-2* mutant longevity (Murphy et al., 2003), and microarray data implies that *gst-4* expression

is increased in *daf-2* mutants.

One of the few *C. elegans* GSTs to have been well characterized is GST-10 which detoxifies 4-hydroxynon-2-enal (HNE), an abundant lipid peroxidation product resulting from oxidative stress (Engle et al., 2001). *daf-2* mutants show up-regulation of *gst-10*, and RNAi of *gst-10* increased sensitivity to HNE toxicity, and reduced lifespan in both wild-type and *daf-2* mutant populations (Ayyadevara et al., 2005a). The effect of *gst-10* RNAi on *daf-2* mutant lifespan has been independently verified (D. Weinkove and D. Gems, unpublished). RNAi of *gst-5, gst-6, gst-8* or *gst-24* also increased sensitivity to HNE toxicity, but of these genes only RNAi of *gst-5* reduced lifespan (Ayyadevara et al., 2007). Over-expression in *C. elegans* of either *gst-10* or murine *mGsta4* (which also detoxifies HNE) lead to increased levels of HNE-conjugating activity, increased resistance to oxidative stress (e.g. paraquat and H_2O_2) and lowered levels of HNE-protein adducts. Interestingly, overexpression of *gst-10* or *mGsta4* increased median lifespan by 22% and 13%, respectively (Ayyadevara et al., 2005b). This is a rare example of evidence that robustly supports a role for oxidative damage in *C. elegans* aging, though effects on aging of HNE in its capacity as a secondary messenger have not been excluded.

Mitochondrial nicotinamide nucleotide transhydrogenase (NNT) is another enzyme that contributes to oxidative stress resistance. It does so by catalysing the reduction of $NADP^+$ by NADH, providing NADPH for reduction of glutathione within mitochondria. This is important in animal mitochondria, which lack catalase. H_2O_2 generated by SOD is usually detoxified instead by mitochondrial glutathione peroxidase (GPX). Reduced glutathione (GSH) in mitochondria is also a substrate for phospholipid hydroperoxidases. In *C. elegans*, *nnt-1* is widely expressed (e.g. in intestinal, hypodermal and neuronal cells). Deletion of *nnt-1* leads to a lowered ratio of reduced to oxidized GSH (58 *vs* 12 in wild type *vs* mutant) (Arkblad et al., 2005). The large magnitude of this effect implies that cytosolic as well as mitochondrial GSH pools are affected. This results in increased sensitivity to paraquat though not H_2O_2. Interestingly, there is no effect on lifespan, which implies that a shift in cellular redox state towards a more oxidative conditions does accelerate aging(Arkblad et al., 2005).

In conclusion: Aging in *C. elegans* is accompanied by an accumulation of

molecular damage, but why this accumulation occurs is unclear. It is also unclear to what extent this damage is caused by ROS, or O_2^- in particular, or how important is damage caused by O_2^- (as opposed to other agents of molecular damage). Perhaps the strongest evidence that O_2^- does contribute to *C. elegans* aging is that over-expression of HNE-conjugating GSTs can increase longevity, since O_2^- contributes to HNE formation. If O_2^- can contribute to aging, then why do SOD mimetics not increase lifespan? One possible explanation is that in *C. elegans* intramitochondrial O_2^- does not contribute to aging: mutants lacking Mn SOD have a normal lifespan (J.J. McElwee and D. Gems, unpublished), and SOD mimetics are concentrated within mitochondria (Keaney et al., 2004).

1.5 Conclusions

The possibilities that damage from mitochondrial O_2^- is a determinant of aging, and that metabolic rate affects the rate of aging, have been tested extensively in *C. elegans*. The results of these tests have, in the main, been either negative, inconclusive or mixed. In particular, there is little clear evidence that metabolic rate is a determinant of aging, though the possibility that metabolic rate can affect aging has not been excluded. Molecular damage clearly accumulates with age, but it remains uncertain whether this is a primary cause of aging, and the mechanisms that determine the rate of damage accumulation remain unclear.

Mitochondria can clearly exert powerful effects on aging in *C. elegans*, yet it is far from clear that O_2^- production plays any role in this. Besides oxidative phosphorylation and ATP production, mitochondria play many roles in the cell, including calcium homeostasis, steroid biogenesis, pyrimidine biosynthesis and fatty acid metabolism. The effects of CoQ on aging are especially difficult to interpret, since it also affects many processes both in mitochondria and elsewhere, and can act either as a pro-oxidant or antioxidant (Miyadera et al., 2002). The idea that damage from O_2^- causes of aging remains unclear, and a recent study even implies that increased mitochondrial can retard aging by inducing stress defense mechanisms (so called mitohormesis) (Schulz et al., 2007).

One possibility is that oxidative damage plays a major role in some organisms but not others. There are reasons for being suspicious that aging in *C. elegans* may involve at least some mechanisms that are not evolutionarily conserved, i.e. are private rather than public (Martin et al., 1996). For example, instead of causing rapid death, as one might expect, disruption of the electron transport chain usually extends lifespan in *C. elegans*, raising a worry that this is a nematode peculiarity; in *C. elegans* O_2 consumption and O_2^- production decreases rather than increases with age; and age accumulation of protein oxidation is largely restricted to the mitochondria. Arguably, though, these concerns should not be taken as an argument against using *C. elegans* as a model for studies of aging. Rather, they underscore the importance of distinguishing public and private mechanisms of aging to maximize the utility of model organisms.

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