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The Nematode *Caenorhabditis elegans*: Oxidative Stress and Aging in the Nematode *Caenorhabditis elegans*

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Summary The senescent decline that leads inevitably to death in most animal species is accompanied by a massive increase in molecular damage. Yet, the chain of events that initially causes this process, and the determinants of the rate at which it happens, remain poorly understood. For many years, much research on this topic has been guided by an interrelated set of theories that view oxidative damage as a potential primary cause of aging. These theories have framed the construction and interpretation of many studies in the nematode *Caenorhabditis elegans*. In this chapter, we critically survey these studies. Overall, these investigations have either disproved or, at least, failed to find clear evidence for many of the oxidative damage theories. In particular, they have failed to demonstrate any role of metabolic rate or mitochondrial superoxide (O_2^-) in aging. However, they have revealed a powerful influence of mitochondria on the rate of aging in *C. elegans*. This may or may not have something to do with mitochondrial O_2^- production.

Keywords *Caenorhabditis elegans*, aging, oxidative stress, molecular damage, metabolism, mitochondria, antioxidant.

1 Introduction

Theories relating oxidative damage to aging, which have been reviewed previously [1, 2] and in Chapter 1 of this book, have motivated a large number of studies using *C. elegans*. These theories have been linked conceptually to form a theoretical framework (Fig. 6.1A). Briefly, aging is the result of molecular damage. This results in particular from reactions with reactive oxygen species (ROS), such as O_2^- (superoxide) and its derivatives, which is produced mainly as a by-product of the activity of the mitochondrial electron transport chain (ETC). The rate of oxidative metabolism is a determinant of aging, because it affects the rate of production of ROS.

Although this is sometimes represented as a unified theory, it contains a number of distinct and testable propositions that, individually, may or may not be

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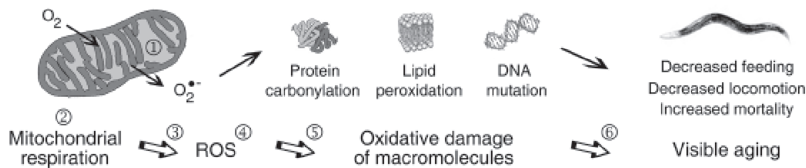
A. Oxidative Damage Theory of Aging



B. Support For Theory in *C. elegans*

Hypothesis	Likelihood	Evidence
① Mitochondrial function influences aging	Definitely	Many mitochondrial defects increase lifespan
② Metabolic rate determines aging rate	Unclear	Conflicting; support is correlative only
③ Mitochondrial superoxide causes aging	Unlikely	SOD mimetics do not increase lifespan
④ ROS-mediated damage is a cause of aging	Unclear	Weak; best evidence: *gst-10* studies
⑤ ROS are a cause of molecular damage	Likely	None
⑥ Molecular damage causes aging	Likely	Consistent, but correlative

Fig. 6.1 Oxidative stress and aging in the nematode *C. elegans*. (A) Oxidative damage theory of aging, as it pertains to *C. elegans*. This theory proposes that mitochondrial ROS (specifically O_2^-) are directly responsible for oxidative damage to cellular macromolecules. This damage ultimately manifests itself as deterioration of tissues, resulting in observable changes in behavior, morphology, and mortality rate associated with aging. Protein carbonylation, lipid peroxidation, and DNA mutation can all be assayed biochemically in *C. elegans*. In contrast, direct measurements of ROS are difficult, especially in vivo. Numbers pertain to hypotheses given in B. (B) Support for the oxidative damage theory of aging based on studies using *C. elegans* as a model system. Likelihood: overall assessment based on information given in this chapter. Evidence: brief comment on the nature of the evidence (i.e., not a full justification of likelihood). Note that much evidence is merely correlative, limiting the strength of proof or disproof for most hypotheses

true (Fig. 6.1B). For example, aging may or may not be caused by molecular damage, and this damage may or may not be caused by ROS. The main source of ROS may or may not be mitochondrial oxidative phosphorylation, and, more broadly, mitochondria may or may not be critical determinants of aging rate. Metabolic rate may or may not affect aging, and any links between metabolic rate and aging may or may not reflect effects on O_2^- production. The use of *C. elegans* as an experimental model introduces another dimension to each of these questions, namely, Does the role of any of these factors show evolutionary conservation? As a hypothetical example, O_2^- might be a major cause of the damage that underlies aging in mammals, but not in *C. elegans*.

In the following discussion, we critically assess each of these questions in turn by examining relevant experimental studies using *C. elegans*. There is a rich and complex scientific literature in this field, particularly due to the work of Siegfried Hekimi (McGill University, Canada), Naoaki Ishii (Tokai University, Japan), and Jacques Vanfleteren (Ghent University, Belgium), and their collaborators. Overall, these studies imply that some of the propositions above are true, some are half-truths, and some are false, at least as far as *C. elegans* is concerned.

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2 Why Test Theories of Aging in *C. elegans*?

2.1 *C. elegans* as a Model for Studies of Aging

Caenorhabditis elegans is a free-living nematode of little economic importance, found in soil rich in organic matter. 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 are ~1.2 mm in length) that it may be handled like a microorganism, with the convenience and low cost that this implies. For studying aging, it has two particular advantages: its life span is very short (usually 2–3 weeks; Fig. 6.2), and there are no inbreeding effects on life span, which have complicated studies of the genetics of aging in *Drosophila* and the mouse. There are also the obvious advantages of an established genetic model system: availability of a well-annotated genome sequence, well-characterised mutations in large numbers of genes, and powerful molecular genetic methodologies. The latter include RNA-mediated interference to knock down gene expression, construction of transgenic animals, and use of fluorescent proteins to visualize gene expression within the transparent body of the nematode. The existence of a well-coordinated community of *C. elegans* researchers has led to the creation of central research resources. For example, information on *C. elegans* is collated into a central Web facility, WormBase (http://www.wormbase.org), and cooperatively written books on paper [3, 4], and, more recently, freely available online (http://www.wormbook.org). Experimental resources include strain distribution via the Caenorhabditis Genetics Center (http://biosci.umn.edu/CGC/CGChomepage.htm), the Fire Lab plasmid vector kit for preparation of transgenic lines (currently distributed commercially by Addgene), and a library of RNA interference (RNAi) feeding clones that includes most of the genes in the *C. elegans* genome [5].

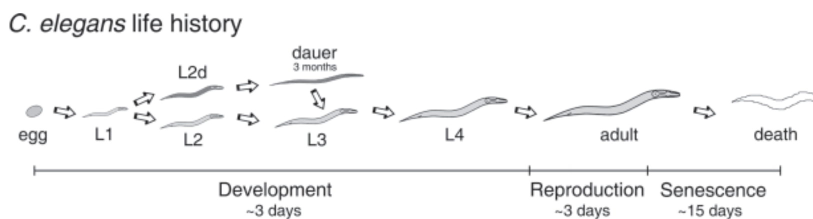

Fig. 6.2 *C. elegans* life history. This is broadly divisible into embryonic and larval development, reproduction, and senescence. Larval development has four stages of growth (e.g., L1, larval stage 1). Total life span is a mere 3 weeks at 20 °C (*C. elegans* life span is dependent on ambient temperature). Typically, life span measurements represent adult life span only, the mean being approximately 18 days. In contrast, at the L2 stage, larvae can enter an alternative, dormant state known as dauer. Dauer larvae can survive for at least 3 months without food, essentially a fourfold increase in longevity. After exposure to a food source, dauer larvae resume development and subsequently reproduce and senesce as normal

2.2 Approaches to Testing Oxidation-Related Theories of Aging

The role of oxidative stress in aging in *C. elegans* has been investigated in several different ways. First, correlations between aging and various aspects of oxidative metabolism have been examined. Such studies typically either examine age changes in wild-type nematodes, or differences between wild-type nematodes and mutants with altered rates of aging. Attempts also have been made to test theories of aging more directly by manipulating individual aspects of the relevant biology (e.g., antioxidant defense) and looking at effects on aging. The majority of studies have been of the less informative first type.

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 life span. A problem with studies of short-lived strains is that a reduction in life span 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 [6, 7], and some studies of short-lived mutants have been informative. For example, the gene *mev-1* encodes a subunit of complex II in the electron transport chain (ETC) [8]. Mutation of *mev-1* results in hypersensitivity to oxidative stress, elevated production of mitochondrial ROS, and shortened life-span [9, 10]; for a recent review on *mev-1*, see Ishii et al. [11]. Most studies have focused on mutations that increase life span, such as those affecting the insulin/insulin-like growth factor (IGF)-1 signaling (IIS) pathway, which can more than double the adult life span [12]. For example, long-lived IIS mutants show resistance to oxidative stress and increased levels of the antioxidant enzymes superoxide dismutase (SOD) and catalase [13–15].

Many correlative studies of this type suggest a link between oxidative damage and aging, from which it is sometimes tempting to conclude: there is no smoke without fire, i.e., surely the oxidative damage theory must be true? However, it is not safe to conclude this. As yet, there is no 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 overexpression of SOD and catalase have been conducted in *Drosophila* [16–18]. Ultimately, it is likely that only by means of such direct testing will theories of aging be verified or falsified in *C. elegans*. In the overview that follows, the evidence for and against each individual oxidation-related theory (Fig. 6.1B) is examined.

3 Is Aging in *C. elegans* Caused by Molecular Damage?

3.1 Age Increases in Damage to Protein, DNA, and Lipid

As in other organisms, levels of molecular damage increase with age in *C. elegans* (Fig. 6.1A). Age increases in levels of oxidized (carbonylated) proteins are seen in whole *C. elegans* extracts [19, 20]. One protein showing large age increases in carbonylation is the yolk protein vitellogenin 6 [21]. Vitellogenins accumulate to high levels during aging in *C. elegans* [6, 22]. Properties of some *C. elegans* vitellogenins suggest that they may form part of lipoprotein particles akin to mammalian apoB-dependent low-density lipoprotein (LDL) particles [23]. Oxidation of LDLs by ROS contributes to atherosclerosis in mammals. Together, this suggests distant molecular parallels between protein aging in *C. elegans* and mammalian atherosclerosis.

Recently, it was discovered that levels of carbonylated proteins increase with age in mitochondria but not the cytoplasm [20] (F. Matthijssens and J. R. Vanfleteren, personal communication). This intriguing result suggests various possibilities: that damage to mitochondria is critical in *C. elegans* aging, that molecular damage occurs more readily within mitochondria (perhaps due to O_2^-), and that damaged proteins are repaired or replaced more efficiently in the cytosol than in mitochondria.

Levels of DNA damage also increase with age in *C. elegans*. There are age increases in numbers of single-strand DNA breaks [24] and of deletions in mitochondrial DNA [25]. Age changes in other forms of molecular damage such as lipid peroxidation and glycation remain largely unexplored in *C. elegans*.

3.2 Age Increases in Blue Fluorescence

Accumulation of fluorescent material occurs during aging in a wide range of animal species, including humans. Such age pigment, or lipofuscin, is a complex agglomeration of damaged lipids, proteins, and carbohydrates [26]. Lipofuscin is thought to represent the residuum of damaged molecular matter that the cell is unable to dispose of, which typically accumulates in lysosomes and may contribute to aging [27, 28].

In *C. elegans*, age increases in fluorescent material also have been observed, either by spectrophotometric assays of nematode extracts [29–32] or whole animals [7] (Fig. 6.3A), or visual examination of animals by using epifluorescence microscopy [6, 33] (Fig. 6.3B–E). The latter approach reveals punctate blue fluorescence, particularly in the intestine (gut granules) (Fig. 6.3C). These puncta are probably secondary lysosomes [34].

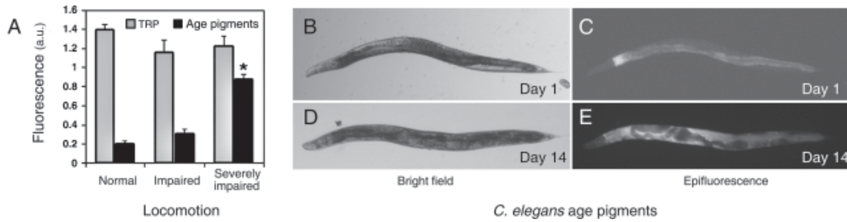

Fig. 6.3 Blue fluorescence as a biomarker of aging in *C. elegans*. (**A**) Age pigment fluorescence reflects physiological, rather than chronological age. As worms age, normal spontaneous locomotion progressively deteriorates to movement only when touched. Before death, only the head moves feebly when touched. Senescent adults of identical age were sorted as class A (normal), class B (impaired), and class C (severely impaired) based on locomotion, and blue fluorescence level was measured for each class. Note that all animals are of the same chronological age, suggesting that age pigment levels correlate with impending death rather than age. TRP, tryptophan fluorescence. Adapted from Gerstbrein et al. [7], with permission of Blackwell Publishing. (**B–E**) Before death, blue fluorescent material seems to be redistributed from the intestine to the pseudocelom. (**C**) Fluorescent gut granules in the intestine of a young (day 1) adult. (**E**) Prior to death (day 14), fluorescence increases dramatically with a rapid, redistribution of fluorescent material into the pseudocelomic space, apparently accompanying an organism-wide breakdown in tissue and organ integrity. Overall, these results suggest that in *C. elegans*, the age increase in blue fluorescence does not reflect the slow age increase in molecular damage, but rather is an indicator of impending death in individual nematodes

The age increase in blue fluorescence could reflect the broader age accumulation of molecular damage and might, in principle, contribute to aging. However, neither of these possibilities stands up well to scrutiny. Although fluorescent gut granules are highly visible even in late larvae and young adults, during early and mid-adulthood the population mean increases in blue fluorescence are modest [7] (A. Taylor and D. Gems, unpublished). More significantly, if aging nematodes are graded on the basis of impaired locomotion (which reflects declining life expectancy), only the most impaired show increased blue fluorescence (Fig. 6.3A; [7]) (D. Gems, unpublished). Moreover, this increase is not due to increased gut granule fluorescence, but rather to a sudden, large increase in fluorescent material in the pseudocelom of the worm in the days preceding its death (compare Fig. 6.3C and E) (A. Taylor and D. Gems, unpublished). In addition, *Escherichia coli*-fed *C. elegans* maintained in liquid culture, conditions that result in a normal life span, showed only marginal increases in blue fluorescence with age, yet they showed a normal life span [7]. That animals age normally in the absence of substantial increases in blue fluorescence suggests that it contributes little to aging. Another study in liquid culture saw a substantial age increase in blue fluorescence [32], perhaps due to higher food levels used (J. R. Vanfleteren, personal communication).

Together, these findings cast some doubt on the view that age increases in blue fluorescence reflect overall age increases in molecular damage that cause aging. The age increase in blue fluorescence in *C. elegans* may instead be a culture condition-dependent effect reflecting terminal pathology in nematodes as they approach death.

3.3 Molecular Damage in Mutants with Altered Life Span

Studies of whole *C. elegans* homogenates show that *daf-2* and *age-1* mutants (long-lived) accumulate protein carbonyls at a lower rate than wild type, whereas *mev-1* and *daf-16* mutants (short-lived) accumulate them more quickly [19, 35, 36]. Accumulation of protein carbonyls was also slower than wild type in isolated mitochondria from *daf-2(e1370)* animals [37]. *mev-1(kn1)* mutants also show increased levels of DNA damage (8-oxo-7,8-dihydro-2′-deoxyguanosine) and elevated nuclear mutation rate [38]. Thus, there is a clear general correlation between rate of damage accumulation and aging. Mutations which affect life span also affect age increases in blue fluorescence. In long-lived *daf-2* mutants, the age increase is slowed down, whereas in short-lived *daf-16* mutants it is accelerated [6, 7].

3.4 Conclusions

Overall, these findings are consistent with the view that accumulation of molecular damage causes aging. Yet, it remains unclear whether the accumulation of damage is really the cause of aging (i.e., of increased morbidity and mortality rate) or merely a noncausal correlate either of a different sort of damage that is causal, or some other age-associated change. If these or other sorts of damage *are* causal, it is not clear whether they are the primary cause of aging, or downstream, knock-on effects of some unknown primary cause.

4 Do Reactive Oxygen Species Cause Aging in *C. elegans*?

4.1 Alterations of Prooxidant Levels

If aging is caused by ROS, then manipulating ROS levels should affect aging rate. The effects of ambient oxygen concentration on life span and mortality rate have been tested in wild-type and *mev-1* mutant populations (Fig. 6.4A) [39]. In wild-type, these parameters were unaltered in 2, 8, and 40% oxygen relative to 21%. This is a striking result, because it implies either that levels of O_2^- production are unaltered over this range, or that ROS are not a determinant of aging. However, very large changes in O_2 concentration can affect aging in wild type. In 1% O_2, mean life span was increased by 15% (Fig. 6.4A), and the Gompertz component of mortality was decreased [39]. 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 life span was slightly reduced (by 14%) (Fig. 6.4A), perhaps due to increased oxidative damage. In contrast to wild type, in *mev-1* populations there is a direct relationship between oxygen concentration and life span (Fig. 6.4A); the mutation rate in *mev-1* mutants is also hypersensitive to effects of elevated oxygen [38].

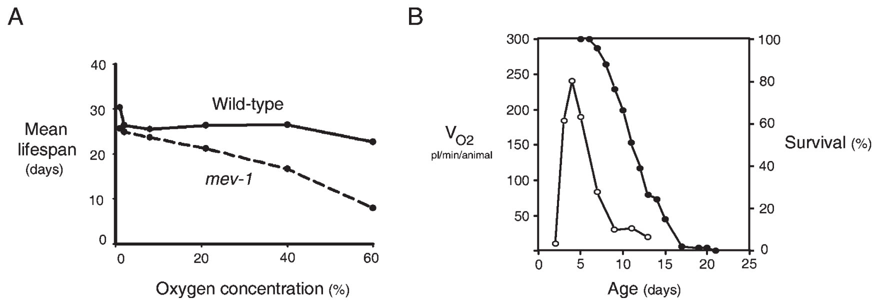

Fig. 6.4 Oxygen and aging in *C. elegans*. (**A**) Effect of ambient oxygen concentration on mean life span. Wild-type and *mev-1* mutants were cultured under various levels of ambient oxygen relative to atmospheric oxygen concentration (21%). Although increasing ambient oxygen presumably leads to increased oxidative stress, wild-type life span is relatively insensitive to ambient oxygen levels. This suggests that ROS are not a determinant of the rate of normal aging. In contrast, *mev-1* mutants are acutely sensitive to ambient oxygen concentration, consistent with the finding that *mev-1* mutants have defective electron transport and elevated ROS production. Adapted from Honda et al. [39], with permission of the Gerontological Society of America. (**B**) Rate of oxygen consumption (V_{O2}) (open circles) and survival (closed circles) of aging wild-type animals at 25 °C. Note that oxygen consumption drops dramatically after day 5 of adulthood. This suggests that age increases in oxidative damage are unlikely to be the result of age increases in mitochondrial superoxide production. Adapted from Suda et al. [101], with permission of Elsevier Publishing

Overall, these results suggest that, under normoxic conditions, O_2^- levels determine aging in *mev-1* but not wild type. The possibilities that O_2^- does not cause normal aging, whereas elevated O_2^- levels can accelerate aging, are not necessarily contradictory. Aging in both cases may involve molecular damage, but with damage resulting from different causes. Indeed, other observations suggest mechanistic differences between aging in *mev-1* and wild type. In otherwise wild-type *C. elegans*, prevention of apoptosis (programmed cell death) by mutation of *ced-3* does not extend life span [6]. Thus, apoptosis does not contribute to normal aging. By contrast, mutation of *ced-3* increases life span of *mev-1* populations, apparently by preventing O_2^--induced apoptosis [40]. 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 [10].

Direct effects of ROS on *C. elegans* are most often examined by administration of redox cycling compounds such as juglone, or, more commonly, paraquat (methyl viologen) [14, 41–44], which generate O_2^- in vivo. O_2^- production by redox cyclers can be measured as an increase in cyanide-independent O_2 consumption. Although 1 mM paraquat does not detectably increase cyanide-independent O_2 consumption by *C. elegans* [45], 2 mM paraquat does increase it, and this concentration is just sufficient to decrease adult life span [42]. 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* [45].

In conclusion, although several treatments predicted to increase intracellular ROS have been shown to reduce life span, it remains unclear whether such effects reflect accelerated aging, or whether effects of ROS limit normal aging.

4.2 Does Elevated ROS Accelerate Age Changes in Molecular Damage?

If ROS causes normal aging, one would expect that experimental elevation of ROS would accelerate age changes in molecular damage seen in normal aging (Fig. 6.1). This prediction has been little explored, although one report described increased blue fluorescence under hyperoxia [33].

Isolated mitochondria from *mev-1* animals show elevated levels of O_2^- production [10]. Thus, O_2^- production might be elevated in vivo and it might account for the shortened life span of *mev-1* under normoxia (Fig. 6.4A). The increased levels of protein oxidation in *mev-1* animals supports this hypothesis [19, 36]. *mev-1* also has been reported to elevate levels of blue fluorescence [33]. However, a recent study saw no such effect either in *mev-1* or *gas-1* animals [7].

4.3 Antioxidant Defense and Aging

Organismal defenses against oxidative damage include chemical and enzymatic antioxidants. If oxidative damage causes aging, then one might expect a correlation between antioxidant defense and longevity. Moreover, experimental enhancement of antioxidant defense 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 life span, the possibility remains that this occurs by some other mechanism than protection against molecular damage. Moreover, if increases in life span are not seen, it remains possible that multiple antioxidant defense mechanisms act in concert to protect against aging or that antioxidant mechanisms act in concert with other prolongevity mechanisms.

A range of genes and processes contribute to protection against oxidative damage [46, 47], 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 defense are enzymes that detoxify primary prooxidant molecules. For example, SODs convert O_2^- into H_2O_2 [48], 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 counteract this production. 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, oxidized 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.

4.4 SOD and Catalase

The biology of SOD and catalase in *C. elegans* is unusual in several respects. For example, *C. elegans* has 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* [13, 49, 50], and instead of one mitochondrial Mn SOD there are also two, encoded by *sod-2* and *sod-3* [51–53]. 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, whereas *sod-3* and *sod-5* are dauer up-regulated isoforms [50, 54, 55] (J. J. McElwee, R. Doonan, and D. Gems, unpublished). Why there should be dauer-specific isoforms is unclear. Because SOD-2 and SOD-3 Mn SODs have similar specific activities [52], and either SOD-1 or SOD-5 Cu/Zn SOD can rescue the paraquat sensitivity of SOD-deficient yeast [50], which suggests that reproductive and dauer isoforms are not functionally different.

The SOD-1 and SOD-5 Cu/Zn SODs are unusual in other respects. To mature, Cu/Zn SODs must incorporate copper, and in all other eukaryotes, whether animals, fungi, or plants, this requires the copper chaperone of SOD (CCS) protein. Uniquely, *C. elegans* does not possess a CCS, and Cu/Zn SOD maturation does not require it, but instead depends on an unidentified glutathione-dependent pathway [50]. Studies of SOD-1 and SOD-5 expressed in yeast also hint that, in contrast to other eukaryotes, *C. elegans* might not have Cu/Zn SOD in the mitochondrial intermembrane space, although the evidence here is not conclusive [50].

The Cu/Zn SOD encoded by *sod-4* is similar to mammalian extracellular Cu/Zn SODs [56]. However, it is also different 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 from the cell, but then it remains tethered at the cell surface [56].

The *C. elegans* genome contains a tandem array of three genes encoding catalases (*ctl-1, ctl-2*, and *ctl-3*; [57]). By contrast, other metazoans have only a single catalase, whereas *Saccharomyces cerevisiae* have a peroxisomal and a cytosolic catalase. CTL-2 is a peroxisomal catalase, and it is responsible for ~80% of total

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catalase activity. It also has a lower pH optimum for activity and higher peroxidase activity than mammalian peroxisomal catalases [57–59]. Much of the *ctl-1* and *ctl-3* gene sequences are 100% identical. Studies of a CTL-1::green fluorescent protein (GFP) fusion protein imply that CTL-1 is a cytosolic catalase [58]. Although this paper was retracted (see below), it was for reasons unrelated to the CTL-1::GFP finding. One possibility is that CTL-1 acts as a cytosolic H_2O_2 scavenger because *C. elegans* lacks an H_2O_2-scavenging glutathione peroxidase [14] (J. R. Vanfleteren, personal communication). A promoter fusion test implies that *ctl-3* is expressed in pharyngeal muscle and neurons [57]. More work is needed to confirm and define the cellular localization of CTL-1 and CTL-3. In summary, compared, for example, with humans, *C. elegans* has a more elaborate armoury of SODs (six) and catalases (three) to detoxify ROS; yet, its life span is a mere few weeks.

Long-lived *daf-2* and *age-1* mutants show age increases in SOD and catalase activity levels, and in resistance to oxidative stress (e.g., paraquat and H_2O_2), increases that are not seen in the wild type [13–15, 54]. Northern blot analysis reveals a large increase in *sod-3* mRNA in *daf-2* mutants [54, 60], and microarray studies reveal additional, smaller increases in *sod-1* and *sod-5* mRNA [61–63]. *sod-3* levels are elevated throughout the life course in *daf-2* mutants, even in the developing embryo [54]. Microarray studies also show increases in expression of at least one catalase gene in *daf-2* mutants, but because of the high degree of similarity between *ctl* gene sequences, one cannot say which gene(s). This also complicates interpretation of RNAi studies [63]. Levels of SOD and catalase also are 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* [64]. In dauer larvae (Fig. 6.2), levels of SOD activity are four- to fivefold higher than in young adults, and levels of *sod-3* mRNA are elevated [13, 54, 65]. Catalase levels also seem to be elevated in dauer larvae [66], although here there is conflicting evidence [13].

It seems likely that the elevated levels of antioxidant enzymes contributes to oxidative stress resistance, at least to some extent, but what about longevity? The effects on aging of manipulations of SOD and catalase levels have been investigated in *C. elegans*, although not as systematically as in *Drosophila*. RNAi knockdown of expression of *sod-3* has been reported to very weakly suppress *daf-2* longevity [63], but, surprisingly, RNAi of *sod-5* had the opposite effect [61]. More surprisingly, deletion of *sod-2* and *sod-3*, alone or in combination, has no effect on adult life span (J. J. McElwee and D. Gems, unpublished).

Whereas deletion of *ctl-1* (cytosolic catalase) has no effect on life span, deletion of *ctl-2* (peroxisomal catalase) shortens life span [57]. The authors interpreted this as progeria, although 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 [57]. Mutation of *ctl-1* was also at one time thought to suppress the longevity of *daf-2* mutants [58], but the study concerned was subsequently retracted [67].

The effects of overexpression of *sod* genes has not been studied in any detail. Overexpression of a *sod-3::gfp* fusion protein did not affect life span, but, as the

authors stressed, SOD activity level was not examined in this strain [68]. 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 [69]. This suggests, at least, that elevated *sod-3* expression does not increase life span in animals deficient in HSF-1.

Administration of the SOD mimetic salen manganese compounds EUK-8 and EUK-134 to *C. elegans* results in significant increases in SOD activity levels (e.g., a fivefold increase in mitochondrial SOD activity) and resistance to paraquat [42, 70]. Although one study reported that these compounds also increased life span in *C. elegans* [71], other workers were unable to reproduce this effect, either in *C. elegans* [42, 72] or in *Drosophila* [73] or house flies [74]. Levels of EUK-8 that were optimal for protection against paraquat had no effect on life span. Higher levels of EUK-8 actually shortened life span [42, 72]. These results imply that O_2^- does not contribute to normal aging in *C. elegans* (Fig. 6.1B).

4.5 Other Antioxidant Defenses

If studies of SOD and catalase in *C. elegans* aging are somewhat fragmented, the role of other antioxidant defense processes is a surface that has barely been scratched. In one study glutathione peroxidase (GPX) activity was not detected in *C. elegans* by using either *tert*-butyl hydroperoxide [14] or H_2O_2 (J. R. Vanfleteren, personal communication). However, the *C. elegans* genome contains a number of GPX-like proteins (e.g., C11E4.2, F26E4.12, F55A3.5, R03G5.5, R05H10.5, T09A12.2, and Y94H6A.4); possibly some or all of these are lipid hydroperoxidases.

There are hints of a possible role of metal trafficking proteins in longevity assurance. Exogenous iron shortens life span in *C. elegans* [75], and *daf-2* and *age-1* mutants are resistant to heavy metals (e.g., cadmium and copper), and they show elevated expression of *mtl-1*, which encodes a metallothionein [76]. RNAi of *mtl-1* slightly reduces *daf-2* mutant longevity [63]. *ftn-1*, which encodes a ferritin heavy chain, is also strongly up-regulated in *daf-2* mutants (Table 6.1) [62].

Global changes in gene expression in *daf-2* and *age-1* mutants have been studied using whole genome DNA microarrays [61–63, 77]. By one estimate, 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 [62]. This finding weakens the conclusions of studies showing correlations of expression of individual genes (e.g., *sod-3*) with IIS mutant longevity. In fact, the number of genes that are regulated by IIS is so high that it is possible to find evidence supporting most theories of aging [78].

One way to lessen the problem of bias in data interpretation is to screen for gene classes overrepresented among differentially expressed genes. One study combined this approach with a comparison of array data from *daf-2* mutants (compared with *daf-16; daf-2*) and dauer larvae (compared with recovered dauer larvae) [55, 62, 78]. The rationale here was that it is likely that *daf-2* mutants are long-lived by dint of

Table 6.1 Changes in mRNA levels in *daf-2* versus *daf-16; daf-2* of selected genes linked to antioxidant defense

Gene	Protein	Log-2 FC	p
sod-1 (C15F1.7)	Cu/Zn superoxide dismutase	**0.80**	**0**
sod-2 (F10D11.1)	Mn superoxide dismutase	0.37	0.096
sod-3 (C08A9.1)	Mn superoxide dismutase	**4.15**	**0**
sod-4 (F55H2.1)	Cu/Zn superoxide dismutase	0.44	0.16
sod-5 (ZK430.3)	Cu/Zn superoxide dismutase	**1.66**	**0.0015**
gst-4 (K08F4.7)	Glutathione *S*-transferase	**1.78**	**0.00013**
gst-10 (Y45G12C.2)	Glutathione *S*-transferase	*0.76*	*0.032*[a]
C46F11.2	Glutathione reductase	*−0.51*	*0.024*
mtl-1 (K11G9.6)	Metallothionein	**3.45**	**0**
mtl-2 (T08G5.10)	Metallothionein	**−0.80**	**0.00097**
cuc-1 (ZK652.11)	Copper chaperone	*−0.75*	*0.044*
F40G9.2	Copper chaperone	−0.29	0.46
ftn-1 (C54F6.14)	Ferritin heavy chain	**5.19**	**0**
ftn-2 (D1037.3)	Ferritin heavy chain	*−0.45*	*0.030*
F20D6.11	Ferredoxin reductase	**0.87**	**0.0098**
aco-1 (ZK455.1)	Aconitase/iron-regulatory protein	−0.40	0.24
pcs-1 (F54D5.1)	Phytochelatin synthase	−0.103	0.704
trxr-1 (C06G3.7)	Thioredoxin reductase	0.097	0.63
trxr-2 (ZK637.10)	Thioredoxin reductase	0.055	0.82
F43E2.5	Methionine sulfoxide reductase (MsrA)	−0.49	0.091

Data derived from mRNA profile analysis by using whole genome microarrays to compare *daf-2* (long-lived, DAF-16 on) with *daf-16; daf-2* (not long lived, DAF-16 off) strains [62]. Values where $p < 0.01$ are shown in bold; values where $0.001 < p < 0.05$ are in italics. Note that of the 8/20 genes where $p < 0.01$, all but one are up-regulated in *daf-2. ctl* (catalase) genes are not shown because similarity between the genes makes microarray data uninterpretable. However, one or more *ctl* gene shows up-regulation in *daf-2* animals.

[a]Up-regulation of *gst-10* in *daf-2* mutants has also been demonstrated in an independent study [84].

misexpressing dauer longevity assurance processes. The few gene classes identified as associated with longevity included three involved in the phase 1, phase 2 biotransformation system (i.e., the xenobiotic metabolism or drug detoxification system). In addition, GSTs were strongly overrepresented among genes up-regulated in *daf-2* mutants, but not dauers. The biotransformation is a complex system of enzymes involved in multiple processes, particularly detoxification and clearance of a wide spectrum of endobiotic and xenobiotic toxins [79].

The comparison of transcript profiles from *daf-2* mutants and dauers implies that the biotransformation system is activated in these long-lived milieus and suggests the possibility that these detoxification processes contribute to longevity [78]. Recently, a comparison of transcript profiles from long-lived IIS mutant *C. elegans, Drosophila*, and mouse discovered evolutionary conservation in the up-regulation of three classes of biotransformation enzymes (particularly GSTs) and longevity [80].

GSTs are a highly diverse, rapidly evolving enzyme class (there are 51 putative GST-encoding genes in *C. elegans*). Among other things, GSTs use glutathione conjugation to detoxify endobiotic and xenobiotic toxins, including the products of

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oxidative damage [81]. A screen for genes up-regulated upon exposure to the O_2^- generator paraquat identified *gst-4* [82]. Overexpression of *gst-4* resulted in increased resistant to paraquat, but not increased life span [83]. However, RNAi of *gst-4* slightly reduces *daf-2* mutant longevity [63], and microarray data implies that *gst-4* expression is increased in *daf-2* mutants (Table 6.1).

The *gst-10* gene is also up-regulated in *daf-2* mutants [84] (Table 6.1). GST-10 protein detoxifies 4-hydroxynon-2-enal (HNE), an abundant lipid peroxidation product resulting from oxidative stress [85]. RNAi of *gst-10* increased sensitivity to HNE toxicity, and it reduced life span in both wild-type and *daf-2* mutant populations. The effect of *gst-10* RNAi on *daf-2* mutant life span has been confirmed by us (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 life span [86]. Overexpression 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 life span, by 22 and 13%, respectively [43]. Arguably, this is the most robust proof to date of a role in *C. elegans* longevity assurance of an enzyme involved in protection against oxidative damage.

Another enzyme contributing to oxidative stress resistance is mitochondrial nicotinamide nucleotide transhydrogenase (NNT). This catalyses 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 GPX. Reduced glutathione 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 greatly lowered glutathione (GSH)/glutathione disulfide ratio (58 *vs* 12 in wild type *vs* mutant) [44]. The large magnitude of this effect implies that cytosolic as well as mitochondrial GSH pools are affected. This results in increased sensitivity to paraquat but, oddly, not H_2O_2, and there is no effect on life span [44].

4.6 Noncatalytic Antioxidants

There is a long history of studies of the effects on aging of noncatalytic antioxidants (principally vitamin E), often generating inconclusive findings. Vitamin E studies have used 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 life span of *C. briggsae* (a sister species to *C. elegans*) by 31% [87]. Similarly, vitamin E increased life span in *C. elegans* [88]. However, in both studies nematodes were cultured in an axenic medium (i.e., without *E. coli*), which is nutritionally suboptimal; moreover, the effects of vitamin E on life span 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* life span by around 20%, but it also reduced fecundity and delayed the timing of reproduction [89]. 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. Although α-tocopherol had no effect, tocotrienols had a protective effect against damage and stress, and they caused a slight increase in mean but not maximum life span [90]. A more recent report described a single trial where vitamin E increased life span in wild-type (+11%) but not *mev-1* animals [91]. 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.

4.7 Conclusions

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. If molecular damage causes aging, it is unclear how important damage caused by O_2^- is. Arguably, the strongest evidence that O_2^- does contribute to *C. elegans* aging is that overexpression of HNE-conjugating GSTs can increase longevity, because O_2^- contributes to HNE formation. The fact that SOD mimetics do not increase life span seems to contradict this; an alternative interpretation is that there exists a proportion of O_2^- in cells whose level, by some unknown mechanism, is unaffected by increases in levels of SOD activity.

5 Do Mitochondria Play a Role in *C. elegans* Aging?

5.1 Does Superoxide Production by Mitochondria Contribute to Aging?

Studies of the source of oxidative damage in the cell have often focused on O_2^- produced as a by-product of the reduction of O_2 by the mitochondrial ETC. Isolated mitochondria or submitochondrial 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 [92]. However, levels of mitochondrial O_2^- production in vivo are much lower, in the 0.1–0.3% range [93, 94], and the relevance of mitochondrial O_2^- to aging remains unclear [95, 96]. 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, also are produced in other ways,

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for example O_2^- by membrane-associated NADPH oxidase, cytochrome P450 oxidases, and xanthine oxidase. The notion that mitochondrial O_2^- causes aging remains very much a hypothesis.

5.2 Mitochondria, Superoxide, and Aging in *C. elegans*

C. elegans mitochondria are similar in many respects to those of higher animals. For example, their mitochondrial DNA is similar in terms of gene content and overall size [97, 98]. 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. However, isolated *C. elegans* mitochondria do produce O_2^- [10].

In mammalian cells, levels of mitochondrial O_2^- production increase with age, e.g., a 25% increase with age in isolated rat heart mitochondria [96]. One study has reported that there is no age increase in mitochondrial O_2^- in *C. elegans* [20]. A second recent study has even reported a decline with age in mitochondrial ROS production (measured as H_2O_2) [37]. Consistent with this, complex I activity drops by 60% between day 4 and day 12 [20].

As worms age, their rate of oxygen consumption drops dramatically (Fig. 6.4B) [20, 99–101]. 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 [101]. Taken together, these results suggest that in vivo levels of mitochondrial O_2^- production decrease substantially with age in *C. elegans*. Thus, the age increase in oxidative damage in *C. elegans* seems unlikely to be due to increased O_2^- production later in life.

5.3 Mitochondrial ETC Defects Can Increase or Reduce Life Span

Based on the oxidative damage theory, one might predict that mutations affecting components of the ETC could, in principle, either decrease or increase life span. Abnormalities in electron flow might increase production of O_2^- and reduce life span; alternatively, overall reduction in electron flow might lower O_2^- and increase life span. In *C. elegans*, both effects of disruption of ETC genes on life span have been seen, but any role of O_2^- in this remains undemonstrated (for review, see [102]).

In several large scale RNAi screens for genes with effects on life span, genes encoding mitochondrial proteins predominated [103–106]. In particular, RNAi of many mitochondrial and nuclear genes encoding proteins of ETC complexes I–V caused substantial increases in life span. RNAi affecting other mitochondrial proteins, such as mitochondrial carriers, also increased life span. The combination

of mitochondrial defects and increased life span is sometimes referred to as the Mit phenotype [107]. In most cases, Mit animals also show delayed development, reductions in body size, fertility, activity level, and feeding rate [103], and abnormalities in mitochondrial morphology [104]. For some genes, Mit animals have normal body size and feeding rates, but increased life span [105], 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 [106, 108]. Mit mutants also have been identified with mutations that either affect ETC genes directly [109, 110] 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 12 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 [104]. Severe loss of ETC function often causes larval arrest and lethality [104, 110].

By what mechanisms might life span be extended in Mit animals? One interpretation is that it is due to reduced metabolic rate and perhaps also to 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 [104], and ATP levels can be reduced to as little as 20% of wild type [103]. This might suggest that ATP levels limit the rate of processes that promote aging. However, a challenging study by Dillin et al. [103] suggests that something more complex is occurring. 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 life span [103]. This is perhaps not surprising because mitochondrial number may be programmed during development: in the transition from L4 to adulthood alone there is a sixfold increase in the number of mitochondria [111].

More surprisingly, adult-specific knockdown of ETC gene expression reduced ATP levels but did not increase life span. 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 [103]. 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?

Life extension in Mit animals is unlikely to involve the insulin/IGF-1 pathway, because knockdown of ETC genes increases life span both in *daf-16* and *daf-2* mutant animals [103–106]. Life span is also increased by RNAi of several genes encoding glycolytic enzymes such as phosphoglycerate mutase (F57B10.3) [104] and glucose-6-phosphate isomerase (Y87G2A.8) [105], suggesting that glycolysis somehow reduces life span. This seems to involve different mechanisms relative to

Mit animals, because animals develop normally, body size is not reduced, mitochondria show normal morphology, and the extension in life span requires *daf-16* [104, 105].

Coenzyme Q (CoQ, or ubiquinone) plays a major role in the ETC. Production of O_2^- by the mitochondrial ETC seems to be largely the result of transfer of electrons from ubisemiquinone to oxygen [2, 92]. Deficiency in CoQ can apparently also increase life span. For example, *clk-1* encodes a mitochondrial protein necessary for the final step in CoQ biosynthesis [112–114, for review, see 115]. Mutation of *clk-1* causes accumulation of the precursor of nematode CoQ, demethoxy-ubiquinone-9 [116], and increased life span [117]. 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 life span, too [118]. The above-mentioned findings might suggest that lowering CoQ levels reduces flux through the ETC, thereby lowering ROS production and increasing life span (but see below).

In a few cases, mutations affecting ETC proteins lead to a shortening of life span. *mev-1(kn1)* is a point mutation in the gene for succinate dehydrogenase cytochrome *b* in complex II, and it causes hypersensitivity to oxidative stress and shortened life span [9]. 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 [2]. *mev-1(kn1)* disrupts complex II and results in O_2^- production from complex II [10]. *gas-1* encodes a subunit of complex I and, like *mev-1*, mutation of *gas-1* results in hypersensitivity to oxidative stress and reduced life span under normoxia [119]. Unlike *mev-1, gas-1* does not increase nuclear mutation rate.

The short life span and sensitivity to prooxidants of *mev-1* animals was first attributed to the fact that SOD levels are half that of wild type [9]. Consistent with this, deletion of *sod-1*, the major Cu/Zn SOD in *C. elegans*, shortens life span (J. J. McElwee and D. Gems, unpublished). However, although it was reported that *mev-1* life span can be extended by administration of chemical mimetics of SOD [71], a further study was unable to replicate this finding (F. Matthijssens and J. R. Vanfleteren, personal communication).

mev-1 and *gas-1* are atypical among genetic interventions affecting mitochondria and life span in that they shorten rather than increase life span. 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 [120]. *mev-1(kn1)* reduces activity of the ETC by 80%, but it does not affect succinate dehydrogenase activity [8]. 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.

5.4 Is Superoxide Production Important for Mitochondrial Effects on Aging?

Mutational studies have demonstrated that 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. Moreover, one would not expect an increase in somatic maintenance mechanisms (e.g., antioxidant defense).

Is it the case that in Mit animals there is reduced electron flux and reduced O_2^- production leading to retarded aging? This question has been extensively investigated in studies of *clk-1* and CoQ. Several findings suggest that the above-mentioned view is an oversimplification. First, 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 [31, 114, 121], although RNAi of other mitochondrial genes does lower ATP levels [103]. 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 [114, 116]. It has been suggested that $DMCoQ_9$ produces less O_2^- than CoQ_9 [116], but this has not been tested directly.

What really complicates interpretation of the role of *clk-1* and CoQ in aging is that the reduced (quinol) form of CoQ can act as a lipid-soluble antioxidant that protects against lipid peroxidation [122–124], which is why it is marketed as a human dietary supplement. Consistent with this, CoQ_{10} supplementation increases life span in *C. elegans* in both wild-type and *mev-1* animals [91] and also reduces O_2^- production in isolated mitochondria.

Thus, these results seem to conflict with the observation that feeding *C. elegans* with *E. coli* lacking CoQ_8 increases their life span [118]. How may these findings be reconciled? One possibility is that different forms of CoQ have different effects on life span. The increases in life span seen by Ishii et al. [91] resulted from supplementation with CoQ_{10}, which may somehow promote longevity more than CoQ_9; possibly, CoQ_8 increases superoxide production more than CoQ_9. To explore this, *E. coli* strains were engineered that 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, surprisingly, effects of these *E. coli* strains on life span were not reported [125].

Another possibility is that $DMCoQ_9$ generates less O_2^- than CoQ_9 [124]. 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 [23, 115]. However, it seems unlikely that the presence of $DMCoQ_9$ causes increased life span, because mutation of *rte-2* suppresses *clk-1* longevity without reducing levels of $DMCoQ_9$ [126].

If Mit longevity 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 [127]. Moreover, mutation of *isp-1* in complex III elevates *sod-3* expression and increases paraquat resistance [109], and *clk-1* mutant animals show resistance to ultraviolet light [128] and increased catalase levels (but reduced SOD activity levels) [31]. This could imply that disruption of mitochondria stimulates stress resistance pathways, perhaps due to increased O_2^- production [107]. Consistent with the latter idea, treatment with the drug antimycin A, which blocks complex III, increases O_2^- production from isolated *C. elegans* mitochondria [10], and, interestingly, it seems to increase life span [103], although the effect is not large. Mitochondrial ROS production (measured as H_2O_2) is also elevated by mutation of *daf-2* [37]. There is also evidence that the SKN-1-dependent antioxidant system is activated in *clk-1* mutants [107]. Thus, there is mounting evidence that increased mitochondrial O_2^- production can contribute to longevity.

5.5 Uncoupling Proteins and Aging in C. elegans

Production of O_2^- by mitochondria 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 [129]. A prediction of the oxidative damage theory is that increased uncoupling should reduce ROS production, thereby increasing life span. 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 [130]. 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 life span was not affected. It also has 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 [32, 37, 131]. 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 [32, 132]. Thus, one can at least say that this metabolic shift is not enough in itself to increase life span. Further studies seem warranted to establish the effects of mitochondrial uncoupling on aging in *C. elegans*.

5.6 Conclusions

Various genome-wide screens for genes with effects on aging have all pointed to the importance of mitochondria in aging. Disruption of mitochondrial function usually increases life span 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 [133–135]. However, the importance of ATP levels in aging is not experimentally supported (see [103]). One alternative is that disruption of mitochondrial function activates somatic maintenance processes [107].

6 Is Metabolic Rate a Determinant of Aging in *C. elegans*?

6.1 Metabolic Rate and Superoxide Production

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 [136, 137]. 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 [129]. Thus, all else being equal, one might expect life span to increase with increasing metabolic rate.

6.2 Effects of Temperature on Life Span

C. elegans do show striking rate-of-living effects insofar as life span is shorter at higher temperatures. For example, median life span of wild-type hermaphrodites is 24 days at 15 °C compared with 16 days at 22.5 °C [138]. This implies that processes whose rate determines the rate of aging occurs 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 [139], but a causal role of metabolic rate in determining aging rate has not been demonstrated.

6.3 Metabolic Rate in Long-Lived Nematodes

As a test of the rate-of-living theory, metabolic rate has been measured in long-lived nematodes, including *age-1* and *daf-2* mutants, *clk-1* mutants, and nematodes subjected to various forms of dietary restriction. The instructive power of such tests is

somewhat 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, although 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, because several mechanisms may contribute to mutant longevity.

In general, 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 [32, 100, 131, 140, 141]. Lower levels of heat production and higher levels of ATP also were 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 [37]. One study reported a decline in metabolic rate in *age-1* and *daf-2* mutants, and concluded that the rate of living theory is supported [139]. This study measured CO_2 production, which may explain the discrepancy with other studies (for further discussion of methodological issues in metabolic studies of *C. elegans*, see [140-142]).

The effects of dietary restriction (DR) on metabolic rate in *C. elegans* depends on how DR is exerted. DR by bacterial dilution has no effect on oxygen consumption rate, whereas DR by means of axenic culture or an *eat-2* mutation increases oxygen consumption rate and heat production [143, 144]. Strains with alterations in mitochondrial function vary in terms of metabolic rate. As mentioned, *clk-1* has little effect on metabolic rate [31, 114, 121]. However, metabolic rate was reduced in *isp-1* mutants [109] and animals subjected to RNAi knockdown of several ETC genes [103]. 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* really remains unknown.

6.4 Differences in Energy Metabolism between *C. elegans* and Vertebrates

One worry when using *C. elegans* as a model organism to investigate links between metabolism and aging is that its metabolism is different in several respects from that of higher animals. For example, *C. elegans* possess the glyoxylate pathway, absent in higher animals, that allows 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 dauer larvae [15, 145]. *Caenorhabditis elegans* also synthesize the disaccharide trehalose, also lacking in higher animals.

Nematodes are also capable of anaerobic respiration using an alternative electron acceptor, rhodoquinone [146], and the malate dismutation pathway [147]. When cultured under anoxic conditions, *C. elegans* excrete lactate, acetate, succinate, and propionate [148]. It has been suggested that such anaerobic respiration might reduce O_2^- production levels, thereby increasing life span [149]. Transcript profile studies suggest that this pathway might be up-regulated in dauer larvae and *daf-2* mutants [145, 150]. 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 [131].

6.5 Conclusions

Rate-of-living effects can occur in *C. elegans*, but the biochemical processes whose rates are 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 a number of genes linked to protein biosynthesis increases life span in *C. elegans* [68, 133–135].

7 Overall Conclusions

Tests of the various elements of the metabolic and oxidative damage theories of aging have in many cases failed to support these theories. In particular, there is little clear evidence that metabolic rate is a determinant of aging, although the possibility of effects of metabolic rate on aging have 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.

Trying to draw clear conclusions from work on these topics can sometimes be an exasperating occupation. Clearly, some aspects of the rate-of-living and oxidative damage theories of aging are wrong; yet, others seem to be supported—but usually weakly. It is as if these theories are somewhere near the truth, but not actually there: We are clearly missing something. For example, molecular damage seems to be important in aging; yet, the importance of atmospheric oxygen and O_2^- is highly uncertain. Likewise, mitochondria do seem to be important in aging, yet it is not clear that this has anything to do with O_2^-. Mitochondria play many roles in the cell beyond oxidative phosphorylation and ATP production, including calcium homeostasis, steroid biogenesis, pyrimidine biosynthesis, and fatty acid metabolism. The effects of CoQ on aging are particularly hard to interpret, because it also affects many processes both in mitochondria and elsewhere, and

is Janus faced in its effect on O_2^-, acting either as a prooxidant or antioxidant, depending on the cellular context [124].

A potential problem with the use of model organisms to study aging is the possibility that mechanisms of aging may differ across taxa, i.e., involve private rather than public mechanisms [151]. In the context of metabolic theories, there are a number of reasons for being suspicious that private mechanisms may be at play in *C. elegans*. For example, it is odd that disruption of the electron transport chain usually extends life span instead of causing death, raising a worry that this is a nematode peculiarity; in *C. elegans* O_2 consumption and O_2^- production declines with age; age accumulation of protein oxidation is largely restricted to the mitochondria; and the pattern of age increase of blue fluorescence does seem simply to reflect general age increases in molecular damage.

This survey clearly supports one particular conclusion: That more research in this field is needed. In particular, we need more direct testing of mechanisms by means of reverse genetic approaches, exemplified by the studies by Zimniak and co-workers on the glutathione *S*-transferase GST-10.

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