Metabolism and bioenergetics in long-lived mutants of *Caenorhabditis elegans*

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Summary
Samenvatting

Summary

With advancing age, detrimental changes accumulate in cells and tissues; these changes enhance the risk of disease and death. A widely accepted theory explaining the cause of aging is Harman's Free Radical/Oxidative Damage theory of aging (1956; 1972). This theory postulates that the major determinants of lifespan are intracellular reactive oxygen species (ROS). These ROS are assumed to cause damage to cell components and consequently, to advance the aging process.

Most aspects of the aging process are expected to be conserved throughout evolution. Interference with mitochondrial function, alterations in the insulin/IGF-1 pathway, and treatments such as dietary restriction positively influence lifespan in many model organisms. These manipulations have often been associated with reductions in metabolic rate and in oxidative stress. Low metabolic rates and reduced oxidative stress are seen as candidate mechanisms linking the Free Radical/Oxidative Damage theory with prolonged lifespan.

The free-living nematode *Caenorhabditis elegans* is an excellent tool to study aging. Among the advantages of working with *C. elegans*, its short lifespan and generation time, and the availability of genetic and molecular techniques to modulate its lifespan are particularly important for aging research. Remarkably, many of the mutations and manipulations known to extend its lifespan are associated with enhanced stress resistance and/or reduced rates of metabolism. This association seems to support the Free Radical/Oxidative Damage theory of aging. To test this theory, we assessed metabolic rates and antioxidant defenses in the dauer diapause stage, Clock mutants, dietary restricted worms and nematodes carrying the *daf-2(e1370)* mutation, which are all long-lived nematode strains.

The dauer is an alternative, non-feeding stage in the development of *C. elegans* which occurs in conditions of overcrowding or nutrient shortage and which shows a remarkable longevity. Clock mutations cause a slowing-down of physiological rates; some of these mutations are linked to mitochondrial functioning. Lifespan can also be extended by dietary restriction, the reduction of food intake without malnutrition. Mutation in *daf-2(e1370)* disrupts Insulin/IGF signaling, a pathway that affects dauer diapause, stress resistance and longevity through regulation of the forkhead transcription factor DAF-16. These longevity strains did not consistently show reductions in metabolic rate or enhanced antioxidant defenses, and therefore do not support the Free Radical/Oxidative Damage theory of aging.

To assess the role of energy metabolism in aging, we chose to study the reduction-of-function mutation *daf-2(e1370)*. Though *daf-2(e1370)* nematodes were not hypometabolic, we confirmed that they do exhibit a distinct energy metabolism: while their respiration decreased with age at a similar rate to the reference strain, their heat dissipation was considerably lower than in wild-type. This observed shift in metabolism may indicate a higher metabolic efficiency, as standing ATP levels were significantly elevated in this strain.

Expression or proteomic studies failed so far to provide an explanation for this alteration in aerobic energy production. To explore this further, we used different approaches to investigate the effect of aging and Ins/IGF-1 pathway disruption on mitochondrial function. Firstly, we developed a suitable protocol to isolate functional mitochondria from aging *C. elegans* cohorts; we were able to

show that damage to the mitochondria inflicted by the isolation process was limited and proportionate at all ages. We used this protocol to study mitochondrial characteristics at the proteome level. In wild-type worms, the abundance of key mitochondrial proteins decreased with age while the mitochondrial mass, inferred from the mitochondrial DNA copy number, remained unaltered. In contrast, the age-dependent decline of key mitochondrial proteins and electron transport chain complexes was considerably attenuated in *daf-2(e1370)* adult animals.

Next we showed that the *daf-2(e1370)* mutation alters mitochondrial aerobic energy production. Whereas in mitochondria isolated from the wild-type, a dramatic decrease in energy production occurred with age, in *daf-2* mutants the decrease in mitochondrial bioenergetic competence was considerably attenuated, suggesting a higher energetic efficiency. Concomitantly, mitochondria isolated from *daf-2(e1370)* animals had higher membrane potentials.

Though several aspects of *daf-2(e1370)* mitochondrial function were higher or better preserved with age, some of our findings pointed out that maintained bioenergetic efficiency is not sufficient to explain *daf-2*'s distinct energy metabolism. In contrast to whole-worm respiration rates, oxygen consumption in activated mitochondria showed no age-specific fall, and heat dissipation from isolated mitochondria did not reflect the low heat production of *daf-2* nematodes. Instead, these findings suggested that extra-mitochondrial regulatory mechanisms are important in control of whole-worm metabolism, and that mitochondrial malfunction is unlikely a primary cause of ageing.

As high mitochondrial membrane potentials have been linked to elevated ROS levels, we measured ROS production capacity by isolated mitochondria and determined concurrent *in vivo* damage to mitochondrial protein and DNA. We were able to show that, in line with their higher membrane potentials, mitochondria isolated from the *daf-2(e1370)* mutant produce more ROS than those of wild-type. In contrast, little damage to mitochondrial protein or DNA was observed in these mutants. Damage to mitochondrial proteins was elevated in wild-type worms only in the oldest age classes. Combined with the knowledge that reducing levels of antioxidant defenses does not negatively influence lifespan, a logical conclusion drawn from these findings is that in standard circumstances, ROS levels do not limit lifespan.

Some important conclusions can be drawn from our studies. Data on metabolic rate and antioxidant defense from a variety of long-lived *C. elegans* strains, together with assessments of ROS production and oxidative damage in the *daf-2(e1370)* mutant strain, contribute to disproving the validity of the Free Radical/Oxidative damage theory of aging, at least in this species. We studied the Ins/IGF-1 pathway mutation *daf-2(e1370)* in order to assess the putative role of energy metabolism in longevity. Though we confirmed that disruption of the Ins/IGF-1 pathway alters aerobic energy production, and we were able to show that the *daf-2(e1370)* allele results in high bioenergetic competence throughout the adult life of the animals, we could not assert that these characteristics are essential to the longevity of the mutant. The main argument against this assertion is the fact that certain aspects of *daf-2'*s energy metabolism were also observed in the *daf-16(mgDf50)* mutant. It is the forkhead transcription factor DAF-16 that transduces longevity signals when the Ins/IGF-1 pathway is disrupted; similarities in metabolic phenotype between the two mutants imply that the metabolic changes imparted by *daf-2(e1370)* are auxiliary, rather than essential, mechanisms of lifespan extension. Moreover, whole-worm metabolism is most likely influenced considerably by extra-mitochondrial regulatory mechanisms. Therefore we propose that the *daf-2(e1370)*

mitochondrial phenotypes are not likely primary mechanisms of *daf-2(e1370)* longevity, and that low *daf-2* function alters the overall rate of aging by a yet unidentified mechanism, with an indirect protective effect on mitochondrial function. A candidate mechanism for maintained bioenergetic competence is enhanced autophagy of mitochondria and subsequent mitogenesis, which together could ensure sustained presence of competent mitochondria.

Samenvatting

Met het toenemen van de leeftijd accumuleren schadelijke veranderingen in cellen en weefsels. Deze veranderingen verhogen het risico op ziekte en dood. Een algemeen aanvaarde verouderingstheorie is de Vrije Radikaal/Oxidatieve Schade Theorie van Harman (1956; 1972). Deze theorie stelt dat intracellulaire reactieve zuurstof species ('Reactive Oxygen Species' of ROS) een belangrijk aandeel hebben in levensduurbepaling. Van deze ROS wordt aangenomen dat ze schade aan celcomponenten veroorzaken en bijgevolg het verouderingsproces sturen.

Van de meeste aspecten van het verouderingsproces wordt verwacht dat ze evolutionair geconserveerd zijn. Wijzigingen in mitochondriale functie en in de Insuline/IGF-1 signaaltransductie, en ingrepen zoals diëtaire restrictie hebben een positieve invloed op de levensduur in vele modelorganismen. Deze manipulaties worden vaak geassocieerd met een afname in metabole snelheid en in oxidatieve stress. Lage metabole snelheden en verminderde oxidatieve stress worden aanzien als kandidaat-mechanismen voor de link tussen de Vrije Radikaal/Oxidatieve Schade Theorie en verlenging van de levensduur.

De vrijlevende nematode *Caenorhabditis elegans* is een bijzonder geschikt model voor verouderingsstudies. Vooral zijn korte levensduur en generatietijd, en de beschikbaarheid van genetische en moleculaire technieken die zijn levensduur kunnen wijzigen, zijn belangrijk voor verouderingsonderzoek. Opvallend is dat vele van de levensduurverlengende mutaties en manipulaties geassocieerd zijn met verhoogde stressresistentie en/of verlaagde metabole snelheid. Deze associatie lijkt de Vrije Radikaal/Oxidatieve Schade verouderingstheorie te bevestigen. Om deze theorie te testen, heben we de metabole snelheid en antioxidant-verdedigingsmechanismen onderzocht in het dauer diapause stadium, Clock-mutanten, diëtair gerestricteerde wormen en *daf-2(e1370)* mutanten. Al deze nematodestammen zijn langlevend.

Het dauerstadium is een alternatief stadium in de ontwikkeling van *C. elegans*, dat zich niet voedt en dat voorkomt bij 'overcrowding' (het aanwezig zijn van grote aantallen wormen in een beperkte ruimte) of bij tekorten aan nutriënten. De dauer heeft een opvallend lange levensduur. Clock-mutaties vertonen trage fysiologische ritmes; sommige van deze mutaties zijn geassocieerd met het functioneren van de mitochondriën. Levensduur kan ook verlengd worden door diëtaire restrictie, met andere woorden een reductie van de voedselopname zonder tekorten aan essentiële voedingsstoffen. Mutatie in *daf-2(e1370)* verstoort de Ins/IGF- signaaltransductie. Dit signaaltransductiepad beïnvloedt dauer diapause, stressresistentie en levensduur door het reguleren van de forkhead transcriptiefactor DAF-16. Deze langlevende wormen vertoonden geen algemene reducties in metabole snelheid, noch een algemene verhoging van antioxidant-defensiemechanismen. Bijgevolg kunnen zij de Vrije Radikaal/Oxidatieve Schade verouderingstheorie niet bevestigen.

Om de rol van het energiemetabolisme in veroudering te bepalen, bestudeerden we de reductie-in-functie mutatie *daf-2(e1370)*. Hoewel nematoden met deze mutatie niet hypometabool zijn,

konden we bevestigen dat zij een bijzonder energiemetabolisme vertonen: de leeftijdsgerelateerde afname van de respiratie was gelijkaardig aan die van de referentiestam, maar de warmteproductie was aanzienlijk lager dan in het wild-type. Deze verschuiving in hun metabolisme kan wijzen op een hogere metabole efficiëntie, daar de ATP-inhoud van deze stam significant hoger was dan in het wild-type.

Genexpressiestudies of studies van het proteoom hebben totnogtoe geen verklaring geboden voor deze wijziging in aerobe energieproductie. We gebruikten verschillende invalshoeken om het effect van veroudering en verstoring van het Ins/IGF-1- signaaltransductiepad op mitochondriale functie verder te onderzoeken. Ten eerste ontwikkelden we een geschikt protocol om functionerende mitochondriën te isoleren uit leeftijdssynchrone *C. elegans*-populaties. We konden aantonen dat schade aan mitochondriën ten gevolge van het isolatieproces beperkt bleef, en niet proportioneel verergerde met het toenemen van de leeftijd. We gebruikten dit protocol om mitochondriale karakteristieken op het proteoom-niveau te bestuderen. In wild-type wormen daalde de abundantie van belangrijke mitochondriale proteïnen met toenemende leeftijd, terwijl de mitochondriale massa, afgeleid van het aantal mitochondriaal-DNA-kopijen, onveranderd bleef. Daarentegen bleef de leeftijdsgerelateerde afname in mitochondriale proteïnen en complexen van de elektronentransportketen beperkt in *daf-2(e1370)* adulten.

Vervolgens toonden we aan dat de *daf-2(e1370)* mutatie de aerobe energieproductie door de mitochondriën wijzigt. Terwijl mitochondriën geïsoleerd uit het wild-type een sterke daling in energieproductie vertoonden met toenemende leeftijd, bleef deze daling in mitochondriale bioenergetische competentie beperkt in *daf-2* mutanten. Dit suggereert een hogere energetische efficiëntie. Hiermee gepaard vonden we een hogere membraanpotentiaal in mitochondriën geïsoleerd uit *daf-2(e1370)* nematoden.

Hoewel bleek dat, met toenemende leeftijd, verscheidene aspecten van mitochondriale functie in *daf-2(e1370)* hoger waren of beter behouden bleven dan in het wild-type, wezen sommige van onze bevindingen erop dat behoud van bioenergetische efficiëntie niet volstaat om het bijzondere energiemetabolisme van *daf-2* te verklaren. In tegenstelling tot de respiratie van wormen vertoonde het zuurstofverbruik door mitochondriën geen leeftijdsgerelateerde daling. Ook werd de lage warmteproductie van *daf-2* wormen niet weerspiegeld in de warmteproductie door geïsoleerde mitochondriën. Wat deze bevindingen wel suggereren is dat extramitochondriale regulerende mechanismen belangrijk zijn voor het sturen van het metabolisme in de worm, en dat mitochondriale malfunctie waarschijnlijk geen primaire oorzaak van veroudering is.

Daar aangetoond is dat een hoge mitochondriale membraanpotentiaal verhoogde ROS-niveaus kan veroorzaken, bepaalden we de ROS-productiecapaciteit van geïsoleerde mitochondriën en de schade aan mitochondriale proteïnen en DNA tengevolge ervan. We konden aantonen dat mitochondriën geïsoleerd uit de *daf-2(e1370)* mutant, gelijklopend met hun hogere membraanpotentiaal, meer ROS produceren dan die van het wild-type. Daarentegen vonden we slechts een beperkte schade aan mitochondriale proteïnen en DNA in deze mutanten. In het wild-type was schade aan mitochondriale proteïnen enkel verhoogd in de oudste leeftijdsstadia. Sinds geweten is dat

het verlagen van antioxidant-defensiemechanismen geen negatieve invloed heeft op de levensduur, kunnen we stellen dat ROS niet beperkend zijn voor de levensduur in normale omstandigheden.

Enkele belangrijke conclusies volgen uit onze studies. Gegevens over metabole snelheid en antioxidant-defensiemechanismen van verscheidene langlevende *C. elegans* stammen en bepalingen van ROS-productie en oxidatieve schade in de *daf-2(e1370)* mutant dragen bij tot het ontkrachten van de Vrije Radikaal/Oxidatieve Schade Theorie, tenminste in deze soort. We bestudeerden de Ins/IGF-1 signaaltransductie mutatie *daf-2(e1370)* om de mogelijke rol van het energiemetabolisme in verlenging van de levensduur te bepalen. Hoewel we bevestigden dat verstoring van het Ins/IGF-1 signaaltransductiepad de aerobe energieproductie wijzigt, en hoewel we konden aantonen dat het *daf-2(e1370)*-allel resulteert in hoge bioenergetische competentie gedurende de hele adulte levensduur, konden we uit de resultaten niet afleiden dat deze karakteristieken essentieel zijn voor de lange levensduur van de mutant. Het belangrijkste argument hiertegen is dat bepaalde aspecten van het energiemetabolisme van *daf-2* ook teruggevonden werden in de *daf-16(mgDf50)* mutant. Het is deze forkhead transcriptiefactor DAF-16 die levensduurverlengende signalen doorgeeft wanneer het Ins/IGF-1 signaaltransductiepad verstoord is. Gelijkenissen in het metabole fenotype tussen deze twee mutanten impliceren dat de metabole veranderingen veroorzaakt door *daf-2(e1370)* eerder bijkomstige dan essentiële levensduurverlengende mechanismen zijn. Daarenboven wordt het metabolisme in de worm hoogstwaarschijnlijk aanzienlijk beïnvloed door extramitochondriale regulerende mechanismen. Bijgevolg stellen we dat de mitochondriale fenotypes van *daf-2(e1370)* hoogstwaarschijnlijk geen primaire levensduurverlengende mechanismen zijn, en dat verlaagde *daf-2* functie de snelheid van veroudering wijzigt door een nog ongekend mechanisme dat een indirect beschermend effect heeft op de mitochondriale functie. Een kandidaat-mechanisme voor het behoud van bioenergetische competentie is verhoogde autofagie van mitochondriën, gepaard gaande met mitogenese; deze twee factoren zouden samen kunnen zorgen voor het behoud van competente mitochondriën.

PART I: INTRODUCTION

Chapter 1 General introduction and outline of the thesis

1.1. Definition and theories of aging

Understanding the processes that determine aging and lifespan is one of the most difficult issues in biology. It is also a highly fascinating topic, of interest not only to the scientists who dedicate their life trying to unravel its mysteries, but to everyone, as we all will eventually experience its effects. In order to understand aging, it must first be defined. Numerous definitions have been formulated; Arking (1998) has summarized them in his comprehensive book 'Biology of Aging' and has distilled from them the following definition:

'Aging is the time-dependent series of *cumulative, progressive, intrinsic, and deleterious functional and structural changes* that usually begin to manifest themselves at reproductive maturity and eventually culminate in death.'

Scientists have attempted to formulate their views on aging from the end of the 19th century onwards, first in a purely theoretical, non-experimental way, while at the present time, they rely on the most advanced molecular tools to add to their understanding of aging. During the past century, two major approaches to studying aging emerged: both proximate (mechanistic) and ultimate (evolutionary) causes of aging have been debated. Mechanistic theories of aging will be discussed later; first we summarize the most important evolutionary theories of aging.

1.1.1. Evolutionary theories of aging

In 1859, Charles Darwin published his theory of biological evolution (Darwin, 1859), based on the idea that biological evolution acts to increase the fitness and performance of species evolving in successive generations. Initially, this idea of increased fitness seemed to complicate the understanding of aging in view of evolution, as aging entails late-life degeneration, not immortality. Also, many manifestations of aging happen at ages beyond the reach of natural selection, after reproduction is halted. However, it was this problem with timing that ultimately shaped evolutionary theories of aging as they are known at present. One of the first to formulate his views was August Weismann who, at the end of the 19th century, spoke of a specific programmed-death-mechanism designed by natural selection to eliminate the old members of a population, thereby freeing up resources for younger generations (reviewed in Ljubuncic and Reznick, 2009). Later, Weismann renounced the idea of old organisms being detrimental to the population; instead, he considered them as neutral for the biological species. Nevertheless, with his Theory of Programmed Death, Weismann was the first to use evolutionary arguments to explain aging. His theory, while controversial, was revived a few years ago in the form of the Programmed and Altruistic Aging Theory (Longo et al., 2005) to explain a phenomenon seen in *S. cerevisiae* where populations seem to die to the benefit of a few mutants.

In 1941, Haldane (Haldane, 1941) observed that in Huntington patients, the effects of this dominant lethal mutation, inducing a deadly genetic disease, only became apparent after reproduction had ended. This led to the suggestion that aging is due to mutations that affect the organism at later ages, thereby escaping natural selection. The idea that, as a

result of extrinsic mortality, there is a progressive weakening in the force of selection with increasing age is generally accepted (Charlesworth, 2000). The following theories are based on this idea.

1.1.1.1. Mutation Accumulation Theory

In his Mutation Accumulation Theory, Medawar (1952) considered aging as a byproduct of natural selection, or an inevitable result of the declining force of natural selection with age. Deleterious mutations expressed at a young age are severely selected against due to their negative impact on fitness, while those expressed only later in life are relatively neutral to selection as their bearers have already transmitted their genes to the next generation. This theory predicts that the latter mutations can passively accumulate in successive generations, and that the frequency of genetic diseases should increase at older ages.

1.1.1.2. Antagonistic Pleiotropy Theory

Williams' Antagonistic Pleiotropy Theory of Aging (Williams, 1957) assumes not only that genes may affect several traits of an organism (pleiotropy) but also that these pleiotropic effects may influence individual fitness in antagonistic ways. In this way, harmful late-acting genes can remain in a population, or be actively accumulated, if they have a beneficial effect early in life, such as increasing fitness at early ages or increasing reproductive success, and are favored by selection. It is the *active* accumulation of pleiotropic genes that sets the Antagonistic Pleitropy Theory apart from the Mutation Accumulation Theory (Le Bourg, 2001).

1.1.1.3. Disposable Soma Theory

Weismann, the author of the first evolutionary theory of aging, is also credited with formulating the Germ Plasm Theory, stating that the body is strictly divided into two types of cells: germ cells and somatic cells. This is reiterated in the Disposable Soma Theory by Kirkwood (Kirkwood, 1977). In essence, this theory is not new when compared to the Antagonistic Pleiotropy Theory; they both focus on the idea of a life-history trade-off (Promislow et al., 2006). However, it attempts to specify in more detail how one and the same gene could have both deleterious and beneficial effects. Essential to this theory is the emphasis on the optimal balance between somatic maintenance and repair versus reproduction. Investment in the germ line is vital for viability across the generations, whereas the soma needs only to support the survival of a single generation. Mutations that fit in both the Antagonistic Pleiotropy Theory and the Disposable Soma Theory have effects that save energy for reproduction by partially disabling molecular proofreading and other accuracy promoting devices in somatic cells.

1.1.2. Mechanistic theories of aging

While evolutionary theories have tried to clarify *why* aging has evolved, mechanistic theories explore *how* genetic and physiological processes affect lifespan. By predicting that aging is caused by a progressive accumulation of molecular and cellular damage (Kirkwood and Austad, 2000), the Disposable Soma Theory blurs the distinction between evolutionary and mechanistic theories. Like Kirkwood, the majority of mechanistic theories of aging focus on the accumulation of various forms of damage as causal to aging; among these, the Free Radical Theory of Aging has received the most attention.

1.1.2.1 Free Radical/Oxidative damage theory of aging

1.1.2.1.1 History

It has long been known that small mammals generally have higher specific metabolic rates and shorter lifespans than large mammals. At the beginning of the 20th century, Rubner concretized this observation, calculating the life-time energy potential (the energy intake per gram per life span) of several homeotherms (Rubner, 1908). According to Rubner, the animals tested expended similar amounts of metabolic energy per gram body weight per lifetime, despite a large variation in body mass. This led to the conclusion that among homeothermic animals, there is an inverse correlation between lifespan and specific metabolic rate. A decade later, Loeb and Northrop (1917) found that in *Drosophila*, a poikilotherm, an inverse correlation was seen between adult lifespan and ambient temperature; from this temperature-dependence in lifespan they deduced that 'the duration of life is determined by the production of a substance leading to old age and natural death or by the destruction of a substance or substances which normally prevent old age and natural death'. These studies in homeo- and poikilotherms were incorporated into the Rate-of-Living Theory by Pearl (1928), assuming that there is an inverse relationship between life span and metabolic rate. This theory predicts that a fixed amount of energy is available to organisms. The organism perishes when this 'metabolic potential' is consumed; therefore, lifespan is determined by the rate at which available energy is expended. Though the Rate-of-Living Theory has since been discredited (Arking, 1998; Austad and Fischer, 1991; Finch, 1990; Speakman et al., 2004), some investigators struggle to abandon this appealing idea (Lapointe et al., 2009).

Based on the work of Gerschman and co-workers (1954), who discovered that oxygen free radicals are formed in situ in response to radiation and oxygen poisoning, Denham Harman published his Free Radical Theory of Aging in 1956 (Harman, 1956). In his publication 'Aging: a theory based on free radical and radiation chemistry', he drew on previous observations to state that aging is potentially linked to metabolic rate, and suggested the existence of a universal phenomenon causing aging. As free radicals are present in living cells, and as their concentration is seemingly increased with increasing metabolic activity, he pointed to free radicals as potential candidates causing aging in all living things, and designated respiratory enzymes as likely sources of these free radicals. Produced as a side-effect of cellular metabolism, free radicals may cause aging by attacking cell constituents, eventually impairing the functional efficiency and reproductive ability of the cell. Moreover, he suggested that extension of lifespan is possible by chemical

intervention. The Free Radical Theory of Aging was strengthened further by the discovery of superoxide dismutase (SOD) (McCord and Fridovich, 1969), which provided evidence of *in vivo* generation of superoxide, and the existence of defense mechanisms against free radicals. In 1972, Harman proposed an important modification of the Free Radical Theory, pointing out mitochondria as the main source of free radicals (Harman, 1972). Stating that over 90% of the oxygen taken up is reduced in the process of mitochondrial electron transport, he concluded that the rate of oxygen utilization would determine the rate of accumulation of damage by free radicals; this damage would be located mainly in the mitochondria, potentially increasing their fragility. A relatively recent adaptation of Harman's theory incorporates other forms of activated oxygen besides free radicals as causal agents of oxidative damage; the theory is now often referred to as the Oxidative Stress (or Oxidative Damage) Theory of Aging (Sohal and Allen, 1990; Sohal and Weindruch, 1996). Most importantly, the inclusion of mitochondria and metabolic rate linked the Free Radical/Oxidative Damage Theory to the aforementioned Rate-of-Living Theory, which found a molecular basis for its 'live fast, die young' principle in the accumulation of damage caused as a side-effect of mitochondrial metabolic rate (Beckman and Ames, 1998; Sohal and Allen, 1990). It is no surprise then that the 'live fast, die young' concept, directly linking free radical production to metabolic rate, still is adhered to by gerontologists (Branicky et al., 2000; Feng et al., 2001; Finkel and Holbrook, 2000; Foksinski et al., 2004; Giorgio et al., 2007; Philipp et al., 2005; Sohal et al., 2002), despite numerous studies opposing it (see 1.1.2.1.5.).

1.1.2.1.2 Free radicals and reactive oxygen species

Free radicals are defined as any species capable of independent existence that contains one or more unpaired electrons (Halliwell and Gutteridge, 2007). Due to the presence of unpaired electrons, free radicals can be highly reactive, resulting in potentially harmful modifications. The collective term reactive oxygen species includes oxygen radicals and non-radical derivatives of oxygen which can also take part in radical-type reactions (Table 1). Likewise, the term reactive nitrogen species unites radical and non-radical N_2 derivatives.

Table 1. Oxygen radicals and non-radical derivatives of oxygen

Radicals	**Non-radicals**
Superoxide $O_2^{\cdot -}$	Hydrogen peroxide H_2O_2
Hydroxyl $OH^{\cdot}$	Hypochlorite HOCl
Peroxyl $RO_2^{\cdot}$	Ozone O_3
Alkoxyl $RO^{\cdot}$	Singlet oxygen
Hydroperoxyl $HO_2^{\cdot}$	Peroxynitrite $ONOO^-$

Oxygen is a relatively weak univalent electron acceptor and most organic molecules are poor univalent electron donors, ensuring that O_2 cannot efficiently oxidize amino acids and nucleic acids. Yet it interacts with the unpaired electrons of transition metals and organic radicals. Most transition metals contain unpaired electrons and can accept or donate single electrons. They can function as catalysts for oxidation/reduction reactions.

However, if their availability is uncontrolled, they can catalyze unwanted free radical reactions.
The reduction of O_2 to water requires four single electron transfers; O_2 can become partially reduced, with one-electron reduction leading to the superoxide radical, two-electron transfer to hydrogen peroxide and three-electron reduction yielding the hydroxyl radical.

1.1.2.1.2.1 Biologically important ROS

-hydroxyl radical

The most potent oxygen species in biological systems is probably the hydroxyl radical. Instantly after it is formed, it reacts with surrounding molecules (Czapski, 1984; Sies, 1993), damaging sugars, amino acids, phospholipids, DNA bases and organic acids (Halliwell and Gutteridge, 1984). Its mechanism of action can be hydrogen atom abstraction, addition and electron transfer, leading to formation of other, less reactive, radicals (Halliwell and Gutteridge, 1984; 2007) which contribute to the cytotoxicity of the hydroxyl radical. The hydroxyl radical can be generated from hydrogen peroxide through Fenton chemistry (Fenton, 1894), as such:

$$Fe^{2+} + H_2O_2 \text{->intermediate oxidizing species ->} Fe^{3+} + OH^{\cdot} + OH^{-}$$

This reaction of transition metal ions with hydrogen peroxide is probably the most biologically relevant mechanism of hydroxyl radical generation; other sources of hydroxyl radical production have also been described (Halliwell and Gutteridge, 2007).

-superoxide radical

The superoxide radical contains one unpaired electron and is moderately reactive (Halliwell and Gutteridge, 2007). It can be submitted to three types of reactions: dismutation, oxidation and reduction. In aqueous solutions, it does not react at all with most biological molecules, though it can be protonated, forming the more reactive hydroperoxyl radical which is uncharged, allowing it to cross membranes. Superoxide, which does not readily cross membranes, can react with other radicals like $NO^{\cdot}$ (nitric oxide, see later in this section) or iron ions in iron-sulphur proteins, resulting in selective biological damage. Also, it contributes to the formation of the hydroxyl radical via the Fenton reaction (Gutteridge, 1990). Its involvement in the mechanism of this reaction is uncertain; possibly, $O_2^{\cdot-}$ facilitates release of iron needed for Fenton chemistry (Halliwell and Gutteridge, 2007; Imlay, 2003).

Superoxide is generated through one-electron reduction of O_2. Several enzymes contribute to $O_2^{\cdot-}$ production. One of them is xanthine oxidase, which normally acts as a dehydrogenase, transferring electrons to NAD^+ rather than O_2, but in certain pathological conditions, the active site of the enzyme is oxidized and the enzyme acts as an oxidase, producing $O_2^{\cdot-}$ (Magder, 2006). Phagocytic cells contain NAD(P)H oxidase, which can produce a burst of $O_2^{\cdot-}$ as a defense against invading microorganisms (Babior, 1999). Cytochrome P450 enzymes are also capable of producing $O_2^{\cdot-}$ as a side reaction of the

breakdown of target molecules (Halliwell and Gutteridge, 2007). Non-enzymatic production of $O_2^{\cdot-}$ can occur through single electron transfer to oxygen by reduced coenzymes or prosthetic groups like flavins or Fe-S clusters. The most important source of $O_2^{\cdot-}$ is believed to be the mitochondrial electron transport chain; the mechanisms responsible for generation of $O_2^{\cdot-}$ will be discussed in section 1.2.5..

-<u>hydrogen peroxide</u>

Hydrogen peroxide contains no unpaired electrons and is not a radical. It is constitutively produced in many, if not all, tissues *in vivo* (Halliwell and Gutteridge, 2007). On itself, it is a moderately reactive ROS. However, it is more stable than $O_2^{\cdot-}$ and it readily crosses membranes through diffusion and via aquaporins (Halliwell and Gutteridge, 2007); as a consequence, its impact reaches further than its immediate surroundings. Only a limited number of enzymes can be inactivated directly by H_2O_2. Nevertheless, it can – indirectly- cause considerable oxidative damage: in the presence of iron, it can be reduced to the highly reactive hydroxyl radical. Contributors to cellular H_2O_2 generation include dismutation of $O_2^{\cdot-}$ and peroxisomal ß-oxidation of fatty acids; due to the presence of the enzyme catalase (which dismutates H_2O_2 to water and oxygen; see section 1.1.2.1.3.) in peroxisomes, the net contribution of peroxisomes to H_2O_2 production is uncertain (Halliwell and Gutteridge, 2007).

-<u>other reactive species</u>

Besides oxygen-based radicals, other derivatives are known such as reactive nitrogen species, sulphur-based molecules and carbon-centered molecules. Nitric oxide ($NO^{\cdot}$) is a relatively stable free radical that can diffuse to targets distant from its production site. It is synthesized by nitric oxide synthase enzymes; as $NO^{\cdot}$ plays important neurological and vascular roles, the activity of these enzymes is carefully regulated. $NO^{\cdot}$ can function as a free radical scavenger; when it reacts with $O_2^{\cdot-}$ it can outcompete SOD (Beckman and Koppenol, 1996). The product of this essentially irreversible reaction however is the highly reactive nitrogen species peroxynitrite ($ONOO^-$). Peroxynitrite, when protonated, can cause cytotoxic processes like lipid peroxidation, inactivation of enzymes by the formation of nitrotyrosine residues, depletion of glutathione and DNA damage (Magder, 2006).

1.1.2.1.2.2 Sources of ROS in vivo

Several systems produce reactive oxygen species. Some systems are beneficial and use ROS as a defense mechanism, such as in macrophages and neutrophils (Halliwell and Gutteridge, 2007), as well as the ROS produced by xanthine oxidase (Kayyali et al., 2001), or as signaling molecules (e.g. blood pressure regulation by nitric oxide synthases in endothelial cells of the vascular system (Remacle et al., 1995)). Sometimes ROS are generated under pathological conditions or as side-effects of other reactions (e.g. by the enzymes involved in detoxification of xenobiotics through the Cytochrome P450 system (Caro and Cederbaum, 2004), and by peroxisomal oxidases needed for ß-oxidation of fatty acids (Jezek and Hlavata, 2005)). Some biologically important molecules that are prone to auto-oxidization, are

known to release ROS in this process (such as glyceraldehyde, $FMNH_2$, $FADH_2$, adrenalin and thiol compounds such as cysteine; Halliwell and Gutteridge, 2007). Finally, the major source of ROS production in the cell is the mitochondrial electron transport chain; it is believed to generate ROS continuously.

As the main ROS generator, the mitochondrial ETC has been studied extensively. Reportedly, it is not the only mitochondrial source of ROS; the Krebs cycle enzyme alpha-ketoglutarate dehydrogenase is said to be able to generate $O_2^{\cdot-}$ from its flavoprotein constituents under certain conditions (Starkov et al., 2004). In contrast, ROS production by the ETC is said to be a constant process. Energy carriers originating from the TCA cycle are oxidized by enzyme complexes of the ETC; abstracted electrons are passed on through a series of redox centers in the ETC, ultimately reducing O_2. Electron transfer through the ETC is accompanied by translocation of protons out of the mitochondrial matrix and into the intermembrane space. The electrochemical gradient formed is used to drive ATP synthesis. While the cytochrome *c* oxidase complex (complex IV) uses the majority of the electrons delivered at the ETC to reduce O_2 to water, electrons may escape from the ETC at earlier stages, generating $O_2^{\cdot-}$. Mitochondria convert approximately 0.1%-0.3% of the consumed oxygen to superoxide which can further react to generate other ROS (Fridovich, 2004; St-Pierre et al., 2002). Estimated amounts of mitochondrial ROS production vary widely, depending on the species or tissues examined (Halliwell and Gutteridge, 2007). ROS generated by the ETC are mainly released towards the mitochondrial matrix, though the intermembrane space may also contain ROS originating from the ETC. The mechanisms involved in ROS generation by the ETC will be discussed in section 1.2.5..

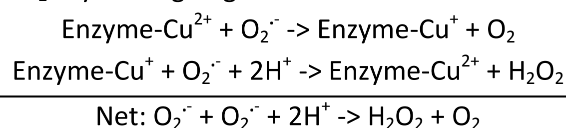
1.1.2.1.3 Antioxidant defenses

Organisms have adapted to life in the current level of O_2 in the atmosphere by developing antioxidant defense mechanisms. Halliwell and Gutteridge (2007) define antioxidants as any substance that delays, prevents or removes oxidative damage to a target molecule. This definition includes, in broad terms, actions that inhibit the production of ROS, prevent generated ROS from damaging macromolecules, adapt to generated levels of ROS by upregulating defense mechanisms, and repair damage inflicted by ROS. The main focus of this section is on antioxidant systems that counteract or neutralize generated ROS, preventing them from exerting pro-oxidant effects on proteins, lipids and DNA.

Among the antioxidant defense mechanisms developed by organisms, four important enzymes were found that are present in all eukaryotic cells. One of them is superoxide dismutase (SOD), which is capable of catalytically removing $O_2^{\cdot-}$. In fact, multiple structurally different metallo-enzymes constitute the SODs, each class containing (a) different metal ion(s) in their active center. Here we discuss SODs containing copper and zinc, or manganese.

Figure 1a: Sources of ROS and antioxidant defenses (adapted from Kamata and Hirata, 1999); figure 1b: schematic model of the electron transport system including sites of ROS production (Balaban et al., 2005)

SODs containing iron can be found in some bacteria, algae, trypanosomes, yeasts and higher plants. The first SOD of which the function was discovered (McCord and Fridovich, 1969), is CuZnSOD. CuZnSOD, present in almost all eukaryotic cells, is found predominantly in the cytosol, but also in lysosomes, the nucleus, the mitochondrial intermembrane space and in peroxisomes. A variant of CuZnSOD is also found extracellularly. The zinc contained in the active center stabilizes the enzyme but takes no part in its antioxidant capacity; the Cu ions catalyze dismutation of $O_2^{\cdot-}$ by undergoing alternate oxidation & reduction:



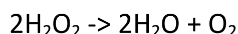
$$\text{Enzyme-}Cu^{2+} + O_2^{\cdot-} \rightarrow \text{Enzyme-}Cu^{+} + O_2$$

$$\text{Enzyme-}Cu^{+} + O_2^{\cdot-} + 2H^{+} \rightarrow \text{Enzyme-}Cu^{2+} + H_2O_2$$

$$\text{Net: } O_2^{\cdot-} + O_2^{\cdot-} + 2H^{+} \rightarrow H_2O_2 + O_2$$

Manganese SOD (MnSOD), first isolated from *E. coli* , contains Mn(III) at its active site. While MnSOD is structurally different from CuZnSOD, it catalyzes essentially the same reaction. It is found in bacteria, plants and animals; in animal tissues it is located in mitochondria. Though there are structural dissimilarities between MnSODs originating from bacteria and higher organisms, the amino acid sequences in animals, plants and bacteria are similar and unrelated to CuZnSOD (see also section 1.2.2.1.).

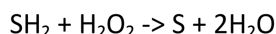
Another major antioxidant enzyme is catalase. This haem-containing enzyme catalyzes direct decomposition of H_2O_2 to O_2 by dismutation, where one H_2O_2 molecule is reduced to H_2O, while the other H_2O_2 is oxidized to O_2 :



$$2H_2O_2 \rightarrow 2H_2O + O_2$$

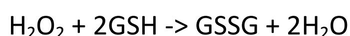
The peroxisomes (Baudhuin et al., 1965; De Duve and Baudhuin, 1966) are the main (often the only) site where catalases are found. Together with the mitochondria, these organelles host the process of ß-oxidation of fatty acids. Both organelles contain flavoprotein dehydrogenases; in mitochondria, these enzymes donate electrons to the electron transport chain while in peroxisomes, electrons react with O_2 and ultimately yield H_2O_2. In this way, peroxisomes include both H_2O_2-generating and H_2O_2-consuming enzymes. The yeast *S. cerevisiae* (Seah and Kaplan, 1973) and the nematode *C. elegans* (Togo et al., 2000) contain both peroxisomal & cytosolic catalase; in *S. cerevisiae*, the peroxisomal form is also found in mitochondria under conditions of respiratory growth (Petrova et al., 2004). So far, catalase has been detected in mitochondria of rat heart (Radi et al., 1991) and liver (Salvi et al., 2007) as well but this could not be confirmed in mice (Zhou and Kang, 2000).

A group of enzymes, known as peroxidases, are capable of removing H_2O_2 by using peroxide to oxidize another substrate (written as SH_2 in following reaction):



$$SH_2 + H_2O_2 \rightarrow S + 2H_2O$$

Many peroxidases have a broad specificity in the substrates they can oxidize, while others are specific for a single substrate. The glutathione peroxidases (GPx) all contain selenium at their active site; they remove H_2O_2 by coupling its reduction to H_2O with oxidation of reduced glutathione (GSH) (Cohen and Hochstein, 1963), a thiol-containing tripeptide.



$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

While less common in plants and bacteria, these enzymes are widely distributed in animal tissues. Most GPxs are specific for GSH as the reductant; some can reduce peroxides other than H_2O_2 like fatty acid hydroperoxides and various synthetic hydroperoxides (Thomas et al., 1990).

GSH, the most abundant low-molecular-weight thiol compound synthesized in cells, is not only a cofactor for GPx but also a redox agent in itself, capable of reducing various RS (Halliwell and Gutteridge, 2007). In doing so, it becomes oxidized to form GSSG, which can be re-reduced by glutathione reductase. GSH also chelates copper ions, diminishing their ability to generate ROS (Hanna and Mason, 1992); conjugation of xenobiotics with GSH, catalyzed by glutathione S-transferase enzymes is a crucial step in the metabolism of xenobiotics (Booth et al., 1961). Synthesized by the cytoplasm, GSH reaches mitochondria via transporters in the inner mitochondrial membrane (Fernandez-Checa and Kaplowitz, 2005).

A fourth, widely distributed, enzymatic system with antioxidant capacities is composed of peroxiredoxin enzymes and thioredoxins. Peroxiredoxins are a family of peroxidases that are capable of reducing H_2O_2 and organic peroxides; their active sites contain thiols in the form of cysteine (Wood et al., 2003). In mammalian cells, they constitute 0.1 to 0.8% of the total soluble protein. Different types of peroxiredoxins are found in different cellular locations: in mitochondria, the cytosol, the ER, in peroxisomes and extracellularly. Some peroxiredoxins rely on thioredoxin to return to the reduced state (Miranda-Vizuete et al., 2000). These small ubiquitous proteins can also undergo redox reactions with other proteins: they possess oxidoreductase activity through their cysteine-rich active site, which enables them to reduce protein disulphide bridges (Holmgren, 1989). They reduce methionine sulphoxide reductase, a key enzyme in removal of oxidative damage to proteins (Lowther et al., 2000). Re-reduction of oxidized thioredoxins is performed by thioredoxin reductases.

Some endogenously produced molecules with (non-enzymatic) antioxidant potential are known, including coenzyme Q. *In vitro*, coenzyme Q, an essential part of the electron transport chain (see section 1.2.4.1.1.), can act as an antioxidant in its reduced form (ubiquinol) (Ernster and Dallner, 1995). In contrast, coenzyme Q is often described as a source of $O_2^{\cdot -}$, therefore its role as an *in vivo* antioxidant remains uncertain (Halliwell and Gutteridge, 2007).

Antioxidant capacities have been ascribed to several low-molecular-mass agents taken up from the diet. This applies to ascorbic acid (vitamin C), tocopherols and tocotrienols (combined under the nutritional term vitamin E), carotenoids and certain plant phenols. Though there is little doubt about their reactive species (RS) scavenging capacity, the benefits of administration of these compounds are not always clear as there are few studies supporting their role as antioxidants *in vivo* (Halliwell and Gutteridge, 2007). An antioxidant role has been proposed for resveratrol, a polyphenol found in a variety of plant species (Belguendouz et al., 1997). However, it has many other physiological effects (Pirola and Frojdo, 2008); as a consequence, any beneficial effect by dietary uptake could not be ascribed to its antioxidant capacity alone.

1.1.2.1.4 Forms of oxidative damage

Even with this extensive array of defense mechanisms against attack by RS, oxidative damage still occurs *in vivo* and affects DNA, lipids and proteins (Sies, 1993). It is possible that the capacity of antioxidants to scavenge RS is not absolute or demands excessive energy; alternatively, removal of all RS could be detrimental to the organism in view of the role of RS in redox regulation and signaling. When antioxidant defense functions inadequately, damage to macromolecules can occur.

DNA damage

While physiologically relevant levels of $O_2^{\cdot -}$, H_2O_2, $NO^{\cdot}$ and organic peroxides are not reactive enough to cause oxidative damage to DNA, the highly reactive $OH^{\cdot}$ radical can affect

nuclear bases and sugars. $OH^{\cdot}$-dependent damage to DNA bases results in formation of 8-hydroxyguanine and thymine and cytosine glycols (Bergamini et al., 2004). Deoxyribose is susceptible to fragmentation after hydrogen abstraction by $OH^{\cdot}$; this process leads to formation of sugar peroxyl radicals. DNA-base-derived radicals can crosslink with protein radicals or with amino acid residues (Evans et al., 2004). Oxidative damage to bases and sugars can be aggravated by the presence of transition metals; through Fenton chemistry, H_2O_2 can increase DNA strand breakage and base modification products (Imlay and Linn, 1988; Mello-Filho and Meneghini, 1991). Ultimately, damaged DNA results in mutation and changes in gene expression (Halliwell and Gutteridge, 2007).

Due to its proximity to the ETC, the lack of histones and introns, and lower rates of DNA repair (Croteau et al., 1999; Lim et al., 2005; Richter et al., 1988; Yakes and Van Houten, 1997), mtDNA is expected to suffer greater oxidation than nuclear DNA, yet so far, results are contradictory (Anson and Bohr, 2000; Anson et al., 2000; Beckman and Ames, 1999; Lim et al., 2005; Mandavilli et al., 2002; Richter et al., 1988).

Errors in DNA can be repaired before and after replication. Recognition, removal and replacement can correct oxidative damage to DNA nucleosides in two ways. Nucleotide excision repair (NER) acts by excision of oligonucleotides containing bulky oxidative lesions, typically caused by UV light, after which the resulting gap is filled in by a polymerase and closed by ligase (de Laat et al., 1999). In base excision repair (BER), a glycosylase removes an altered DNA base by hydrolyzing the bond between base and sugar-phosphate backbone; the DNA is resealed after insertion of the correct base (Lindahl et al., 1997). Wrongly-paired bases or DNA base modifications that escaped proofreading of DNA polymerases can be corrected after replication by mismatch repair; after recognition of mismatches, the DNA strand containing incorrect information is selectively removed and re-synthesized (Harfe and Jinks-Robertson, 2000). When double-strand breaks occur, they can be repaired by homologous recombination (West, 2003) or non-homologous DNA end-joining (Lieber et al., 2003). In mammals, it was found that mitochondria are capable of repairing oxidative damage to mtDNA, mostly by BER but potentially also by mismatch repair (Hashiguchi et al., 2004; Larsen et al., 2005).

Lipid damage

Double bonds in polyunsaturated fatty acids (PUFAs) are prone to be attacked by RS. Again, $OH^{\cdot}$ is presumably the main source of oxidative damage to lipids, as $NO^{\cdot}$ and $O_2^{\cdot-}$ are not sufficiently reactive; moreover, the charge carried by $O_2^{\cdot-}$ prevents it from crossing membranes (Halliwell and Gutteridge, 2007). Initiation of lipid peroxidation is the formation of carbon radicals, most often by hydrogen atom abstraction from the methylene moiety close to a double bond. In the presence of O_2, carbon radicals can lead to peroxyl radicals which, by abstraction of $H^{\cdot}$ from an adjacent fatty acid side chain, result in a new carbon radical together with a lipid hydroperoxide. Through this chain reaction (fig 2), multiple peroxide molecules originate from a single initiation event (Halliwell and Gutteridge, 2007).

In addition, lipid hydroperoxides can break down to reactive aldehyde products, including 4-hydroxy-2-nonenal (HNE) (Uchida, 2003). As lipid molecules (in the form of phospholipids) make up between 30 and 80% of biological membranes by mass, lipid

peroxidation influences membrane fluidity and integrity; consequently, membrane proteins like enzymes and ion channels can also be affected (Catala, 2009). The phospholipid cardiolipin, an essential component of the inner mitochondrial membrane and rich in unsaturated fatty acids, is a possible target for lipid peroxidation (Chicco and Sparagna, 2007).

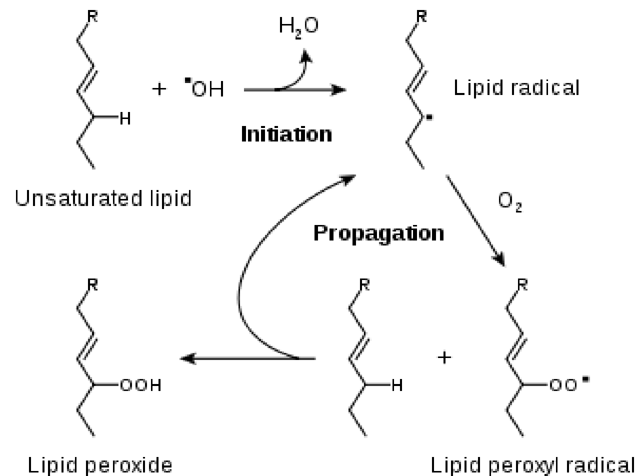


Figure 2: Representation of the initiation and propagation reactions of lipid peroxidation (Wikimedia commons).

A form of repair for oxidative damage to lipids is the reduction of peroxides within membranes to alcohols by phospholipid hydroperoxide glutathione peroxidases; alternatively, glutathione peroxidases remove fatty acid peroxides released when peroxides are cleaved from membranes by phospholipases (Halliwell and Gutteridge, 2007).

Protein damage

Proteins can not only be directly attacked by RS, they are also prone to secondary damage by end-products of lipid peroxidation (Catala, 2009) and by glycation (Wautier and Schmidt, 2004). At physiological levels, $NO^{\cdot}$ or $O_2^{\cdot -}$ cannot directly damage proteins; H_2O_2 selectively damages accessible –SH groups. Amino acid side-chain oxidation leads to production of carbonyl moieties by introduction of aldehyde or ketone functions into these side-chains (fig 3); particularly proline, arginine and lysine side chains are susceptible (Bergamini et al., 2004). Amino acid peroxides can decompose to $RO^{\cdot}$ and $RO_2^{\cdot}$ radicals in the presence of transition metals. Transition metals are also capable of directly binding thiol groups in cysteine and methionine (Brot and Weissbach, 1983); oxidation of methionine –S causes formation of methionine sulphoxide (Schoneich et al., 1993). While oxidative damage to proteins is generally irreversible, methionine sulphoxide can be re-reduced; likewise, peroxiredoxin inactivation and glutathionylation can be reversed (Halliwell and Gutteridge, 2007). Consequences of oxidative protein damage are not limited to proteins like receptors, antibodies, signaling and transport proteins; interference with enzyme activity may also affect DNA polymerases and repair enzymes (Halliwell and Gutteridge, 2007).

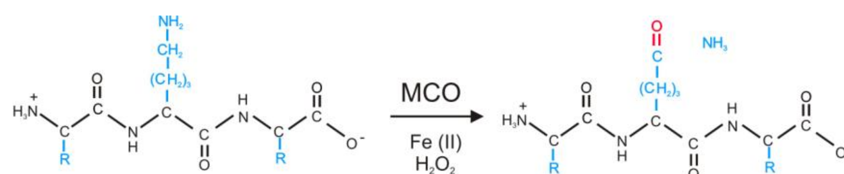


Figure 3: carbonylation of an amino acid side chain by metal catalyzed oxidative (MCO) attack.

As mentioned, oxidation of methionine can be reversed; methionine sulphoxide is reduced by methionine sulphoxide reductases who gain their reducing power from thioredoxin (Stadtman, 2004). Because this formation of methionine sulphoxide is reversible, it is seen as a means to protect proteins from oxidative damage (Levine et al., 1996). Removal of irreversible protein damage is regulated at different cellular locations. Lysosomes hydrolyze cytoplasmic proteins and organelles; this process is called autophagy (Kiffin et al., 2006). They can also degrade proteins taken into the cells by endocytosis. Lon protease, an ATP-dependent proteinase, is located in peroxisomes and mitochondria (Lee and Suzuki, 2008); it recognizes and degrades oxidized mitochondrial proteins. Aconitase, an enzyme particularly sensitive to inactivation by superoxide (Gardner and Fridovich, 1991), is a candidate for degradation by Lon protease (Bota and Davies, 2002). Eukaryotic cells contain the proteasome, a cytoplasmic and nuclear system capable of removing unwanted proteins, including oxidatively damaged proteins (Poppek and Grune, 2006). Targets for removal can be recognized by the presence of hydrophobic patches (Grune et al., 2003) or tagged by attachment of ubiquitin (Hershko and Ciechanover, 1998).

1.1.2.1.5. Evaluation of the Free Radical/Oxidative Damage Theory of Aging

First formulated in the 1950's, the Free Radical/ Oxidative Damage Theory of Aging is seen by many as the most plausible aging theory proposed to date. However, in past decades, a myriad of studies have tried to verify it while even now, over 50 years after it was first conceived, scientists have not succeeded in obtaining a consensus about the accuracy of this theory. What's more, they disagree on the strictness with which the theory should be interpreted. Some investigators claim that, for the Free Radical/ Oxidative Damage Theory to be of value, it must imply that oxidative stress is the cause of aging and consequently, the determinant of lifespan; if this criterion is not fulfilled, then oxidative stress may be merely a consequence of aging. Others view the theory as valuable if oxidative damage is proven to be a major contributor to aging (Beckman and Ames, 1998; Gems and Doonan, 2009; Muller et al., 2007). Though very few studies unambiguously support the more stringent view of the theory, there are plenty of indications that oxidative damage is associated with aging. Support for the Free Radical/ Oxidative Damage Theory of Aging was readily found in correlations between lifespan, levels of ROS, oxidative damage and/or antioxidant defense. Enhanced ROS production with increasing age was observed in mitochondria or submitochondrial particles of the housefly (Farmer and Sohal, 1989), mice (Sohal et al., 1994) and rats (Muscari et al., 1990), and in intact cells from rat liver (Hagen et al., 1997; Sastre et al., 1996). Two long-lived mouse species belonging to the genus *Peromyscus* produce less ROS and have higher stress resistance than the house mouse *Mus musculus* (Ungvari et al., 2008). Also, mitochondria from birds seemingly produce lower levels of ROS

than those of rodents, consistent with their difference in life expectancy (Barja and Herrero, 1998; Herrero and Barja, 1998). Sohal and co-workers (1990b) and Ku and co-workers (1993) found an inverse relationship between rates of mitochondrial ROS generation and maximum lifespan potential in a range of mammals. Age-related increases in oxidative damage were observed for proteins (Sohal et al., 1993; Stadtman, 1992; Toroser et al., 2007), and mitochondrial DNA (Ames et al., 1993; Barja and Herrero, 2000) of various animal model systems. Likewise, oxidative damage to lipids was observed to increase with age (Barata et al., 2005; Sawada and Carlson, 1987); gradual accumulation of lipofuscins, consisting of cross-links of lipid and protein residues, was reported in a range of species (Beckman and Ames, 1998) and is seen by some as a useful biomarker for aging (Yin, 1996). Often, a positive relationship between antioxidant levels and lifespan is regarded as supportive of the Free Radical/oxidative damage Theory of Aging (Arking, 1998). However, these observations fail to show the causal role of oxidative stress in aging. A more convincing link between aging and oxidative stress is supplied by intervention studies. These studies aim to manipulate ROS levels in order to observe their effect on lifespan. This can be approached by increasing or decreasing ROS levels, expecting lifespan shortening or extension, respectively. Both approaches often entail manipulation of antioxidant defenses. Lifespan was shortened by loss of CuZnSOD and MnSOD in *Drosophila* (Duttaroy et al., 2003; Kirby et al., 2002; Paul et al., 2007; Reveillaud et al., 1994). Mice lacking cytoplasmic CuZnSOD had a 30% reduced lifespan accompanied by high levels of oxidative damage (Elchuri et al., 2005); absence of MnSOD in mice led to severe oxidative damage to mitochondria and early postnatal death (Lebovitz et al., 1996; Li et al., 1995). Apparently, a different way of manipulating ROS levels is by changing partial oxygen pressure; rat liver subcellular fractions produced more ROS under hyperoxia than under normoxia (Chance et al., 1979). Deleting CuZnSOD and/or MnSOD accelerated chronological aging in yeast, an effect that could be partially reversed by decreasing oxygen tension (Longo et al., 1996; Longo et al., 1999). Efforts to decrease ROS production by manipulating antioxidant levels have led to lifespan increase in several model organisms. Overexpression of MnSOD extended yeast chronological lifespan (Harris et al., 2003). *Drosophila* lifespan was extended by overexpression of peroxiredoxin 5, methionine sulfoxide reductase, SOD1 or glutamate-cysteine ligase (Martin et al., 2009; Orr et al., 2005; Radyuk et al., 2009; Ruan et al., 2002) and by overexpression of glucose-6-phosphate dehydrogenase which enhances reductive capacity (Legan et al., 2008). According to Schriner and co-workers (2005), overexpression of catalase in mitochondria extended lifespan in mice, accompanied by reduced oxidative damage; likewise, human cytosolic thioredoxin overexpression in transgenic mice had a beneficial effect on lifespan (Mitsui et al., 2002). In some studies, administering antioxidant mimetics was sufficient to prolong lifespan: according to Anisimov and co-workers (2008), the mitochondria-targeted antioxidant mimetic skQ1 can prolong lifespan in the fungus *Podospora anserina*, in *Drosophila*, in mice and in the crustacean *Ceriodaphnia affinis*; Ali and co-workers (2004) and Quick and colleagues (2008) described a synthetic SOD mimetic that could extend lifespan in mice, not only in *SOD2* knockouts but also in mice wild-type for SOD (strain C57BL6, 'normal' mice).

As discussed, arguments in favor of the Free Radical/ Oxidative Damage Theory of Aging are abundant; however, the number of studies opposing this theory is increasing. The

link between lifespan, ROS production, oxidative damage and/or antioxidant defense is far from straightforward in and between certain species. In the fruitfly, extension of lifespan by caloric restriction was not associated with lower ROS production, and lowering ROS production by overexpressing adenine nucleotide translocase could not prolong life (Miwa et al., 2004). In a wild population of sheep, it was shown that oxidative damage did not increase with age (Nussey et al., 2009). The naked mole rat has higher levels of lipid peroxidation, protein carbonylation and DNA oxidative damage than laboratory mice, even at a young age, yet lives 8 times longer (Andziak et al., 2006); on the other hand, age-related oxidative damage to thiol groups is attenuated in this rodent, together with levels of ubiquitinated proteins (Perez et al., 2009). In mice, a mutation in mtDNA polymerase γ led to accumulation of mtDNA mutations and faster aging, without affecting ROS production or oxidative damage (Kujoth et al., 2005). Ant queens, who are considerably long-lived, expressed cytosolic SOD (SOD1) at lower levels and contained lower SOD1 activity than the short-lived worker ants (Parker et al., 2004). (Sohal et al., 1990a) looked for correlations between levels of different antioxidants and maximum lifespan potential in various organs of six mammalian species; some positive and negative correlations were found, but no clear relationship was detected between overall antioxidant defense and lifespan. Increase or decrease of antioxidant levels does not necessarily lead to a positive or negative effect on lifespan, respectively. According to Mockett and co-workers (1999, 2003), Orr and colleagues (2003), and Shchedrina and co-workers (2009), overexpression of MnSOD, CuZnSOD, catalase, methionine-R-sulfoxide reductase B[1] or thioredoxin reductase could not increase lifespan in *Drosophila*. Others claim that overexpression of MnSOD can extend *Drosophila* lifespan (Sun et al., 2002), however, this seems to occur without affecting oxidative stress resistance or oxidative damage (Curtis et al., 2007). According to Orr and Sohal (2003), lifespan extension by overexpression of CuZnSOD in the fruitfly is only effective in compromised genetic backgrounds. Overexpression of several genes coding for antioxidant enzymes, specifically CuZnSOD, MnSOD, and/or catalase, were found to have no lifespan-prolonging effect in mice (Huang et al., 2000; Jang et al., 2009; Perez et al., 2008b), even when, in the case of MnSOD (SOD2) overexpression, less oxidative damage was produced (Jang et al., 2009). Very high overexpression of glutathione peroxidase, CuZnSOD or MnSOD can be deleterious (Jaarsma et al., 2000; McClung et al., 2004; Raineri et al., 2001; Rando et al., 1998). Treatment with antioxidant mimetics failed to extend lifespan in *Drosophila* and the housefly (Bayne and Sohal, 2002; Magwere et al., 2006). Clinical trials testing antioxidants on humans have not been able to consistently show their benefits for decreasing mortality; some trials were stopped early due to increased incidence of disease and mortality (Howes, 2006). Attempts to increase ROS production by knocking out genes coding for antioxidant enzymes did not always result in the expected shortening of lifespan. In mice, the heterozygous knockout of *Sod2* (*Sod*$^{+/-}$) had approximately 50% reduction in MnSOD activity, some elevated oxidative stress markers and increased cancer incidence, yet lifespan was unaltered (Mansouri et al., 2006; Van Remmen et al., 2003; Van Remmen et al., 1999). Heterozygous knockouts of mitochondrial thioredoxin (*Trx2*$^{+/-}$) showed higher oxidative damage (Perez et al., 2008a) but no significant decrease in lifespan (Pérez et al., 2009). Likewise, lifespan was not different from wild-type in glutathione peroxidase

[1] catalyzes the reduction of the R epimer of methionine sulfoxide (Moskovitz et al., 2002).

knockouts *Gpx1*$^{-/-}$ and *Gpx4*$^{+/-}$, and, contrary to previous reports (Moskovitz et al., 2001), in methionine sulfoxide reductase A (*MsrA*$^{-/-}$) knockouts (Pérez et al., 2009).

The majority of the studies claiming to support the Free Radical/oxidative damage Theory of Aging delivered only correlative evidence; they demonstrated the toxicity of superoxide but did not provide a direct link to aging. Through progress in genetic technology came the possibility to alter expression of antioxidant enzymes, thereby manipulating levels of ROS and oxidative damage. However, alteration of antioxidant enzymes is not always a straightforward intervention. First of all, the consequences of knockout or overexpression of genes coding for antioxidant enzymes on ROS production or oxidative damage aren't always assessed. Also, antioxidant enzyme knockdown – due to the inherent toxicity of ROS - can result in early death instead of shortening of lifespan through faster aging. If lowering antioxidant levels does not shorten lifespan, this may be through compensation for the lack of one antioxidant by enhancement of another. Also, lowering antioxidant levels most likely has an impact on signaling via ROS (see section 1.2.4.2.3.), the consequences of which are hard to predict. The same is true for overexpression of antioxidant genes leading to lifespan extension, where other effects of the genetic intervention besides increasing antioxidant levels may be beneficial for lifespan. Failure to extend lifespan through increase of a specific antioxidant may illustrate the importance of balancing the total range of antioxidant defenses. In all, we can state that many studies that comply with or refute the Free Radical/oxidative damage Theory of Aging could be interpreted wrongly. Even now, more than fifty years after it was first formulated, Harman's theory cannot be proven or disproven. However, as the counter-evidence – derived from many model organisms, including *C. elegans* (which will be discussed at length in section 7.1.) - keeps growing, it may be appropriate for future research to 'seek alternatives to the oxidative damage theory' as proposed by Gems and Doonan (2009).

1.1.2.2 Other mechanistic theories of aging

Many other mechanistic theories of aging have been proposed in the past; some even date back to the end of the 19th century. At present, a lot of these theories have been countered but some may still have their value for future research directions. Five of them will be presented here; in the first four, the emphasis is on damage as a cause of aging but they differ in the type of damage that is put at the center of the theory. As most of them propose no alternative mechanism capable of inflicting molecular damage, the Free Radical/oxidative damage Theory of Aging is still implicated in the design of these theories. The fourth theory has a different view on aging: here, damage is not seen as a cause of aging; instead, according to the author, aging is a cause of damage.

1.1.2.2.1. Telomere shortening

In eukaryotes, the ends of linear chromosomes, the telomeres, consist of a repeated sequence of bases whose length decreases with each cell division. Primary cells isolated from mammals arrest cell division in culture after 50-90 divisions, when the chromosome reaches a critical length; this process is called *in vitro* replicative senescence and the limited

cellular capacity for replication was named the Hayflick limit (Hayflick, 1965). In immortal cell lines, the enzyme telomerase adds new sequences onto the ends of chromosomes at each DNA replication, maintaining the chromosome length and allowing cell division to continue. These observations led to the idea that organismal lifespan may be restricted by an *in vivo* limited capacity for cell division caused by telomere shortening (Campisi, 1997). In connection to the Free Radical/oxidative damage Theory of Aging, it was found that besides cell division, oxidative stress also has an impact on telomere length (von Zglinicki, 2002). However, the role of telomere shortening in aging can be refuted by a number of observations. There is no correlation between telomere length and potential lifespan. Telomeres in mice are considerably longer than in humans (de Lange et al., 1990; Kipling and Cooke, 1990); also, telomere length varies significantly in different species of mice with similar maximum lifespans (Greider, 1996). Most adult somatic cells are post-mitotic; no further telomere shortening occurs in these cells yet this does not prevent aging. In *C. elegans*, adult somatic cells are post-mitotic; Raices and co-workers (2005) found no correlation between telomere length and *C. elegans* lifespan. Knocking down telomerase activity in mice shortens telomeres but has no immediate impact on their lifespan; it does make them more cancer-prone (Goyns and Lavery, 2000). Crossing telomerase-overexpressor mice with transgenic mice that carry extra copies of tumor suppressor genes extended mean but not maximum lifespan, in other words: it improved health span (Tomas-Loba et al., 2008). Telomeres and their protection by telomerase definitely play a role in cancer development, but their role as a conserved mechanism of aging is doubtful, and it is far from certain that lifespan-extending effects similar to those of telomerase-overexpressor/tumor-suppressor mice will be observed in humans.

1.1.2.2.2. The mitochondrial theory of aging

Based on the Free Radical/Oxidative Damage Theory of Aging, (Miquel et al., 1980) proposed the mitochondrial theory of aging, suggesting that senescence is the result of damage caused by ROS to the mitochondrial genome in post-mitotic cells. This theory, while still regarding mitochondrial ROS as the primary cause of aging, narrows the focus of Harman's theory by stating that accumulation of somatic mutations in the mtDNA is the major contributor to aging. As discussed previously, proximity to the ETC, the lack of histones and introns, and lower rates of DNA repair would make mtDNA especially vulnerable to oxidant attack. Damage to mtDNA results in mutations, which lead to defective mitochondrial respiration, further increasing ROS generation, accumulation of new mutations and oxidative damage; this vicious cycle ultimately leads to cell death. Alternatively, existing mtDNA mutations caused by oxidative damage or errors of the mtDNA polymerase clonally expand to cause cellular dysfunction (Elson et al., 2001): because the mitochondrial genome has multiple copies in a single cell, the mutant molecule must be multiplied above a certain threshold level to result in biochemical deficiency. Suggested mechanisms for clonal expansion are replication advantage of mutated over wild-type molecules (Yoneda et al., 1992) or random genetic drift (Elson et al., 2001). Research in support of the theory reported age-related accumulation of mtDNA mutations (Wallace, 1999) and cytochrome *c* oxidase deficient cells (Krishnan et al., 2007). Arguments against this theory are in essence the same as those against the Free Radical/oxidative damage

Theory of Aging; particularly, it is put in perspective by two experimental approaches. Firstly, the phenotype of knock-in mice with elevated rates of mtDNA mutagenesis consisted of faster aging without enhanced ROS production (Kujoth et al., 2005; Trifunovic et al., 2004). Secondly, Vermulst and colleagues (2007) showed that heterozygous mutator mice were able to sustain a 500-fold increase in the frequency of point mutations compared to wild-type mice without showing an accelerated aging phenotype or a decreased lifespan.

1.1.2.2.3. The membrane pacemaker theory of aging

The membrane pacemaker theory of aging is an extension of the Free Radical/oxidative damage Theory of Aging. Three observations led to its conception. In the 1970's, it was discovered that membrane fatty acid composition in hearts of different mammal species was correlated with heart rate (Gudbjarnason et al., 1978). This systematic variation in membrane fatty acid composition was later found in other tissues of mammals as well (Couture and Hulbert, 1995). Shortly after, it was reported that in mammals, membrane composition was correlated with maximum lifespan (Pamplona et al., 1998). Hulbert united these findings in the membrane pacemaker theory of aging (Hulbert, 2005; Hulbert et al., 2007). This theory proposes that the acyl composition of membrane bilayers determines the rate of aging, based on the following findings. Firstly, fatty acids differ greatly in their susceptibility to oxidative damage. Saturated and mono-unsaturated fatty acids are resistant to peroxidation while polyunsaturated fatty acids are prone to oxidative attack (Halliwell and Gutteridge, 2007). Secondly, lipid peroxidation is a self-propagating process and many lipid peroxidation products are very reactive molecules themselves. Lastly, it was shown that membrane fatty acid composition differs in various situations (such as between species or diet-dependent). According to Hulbert (2005), this theory can explain the different lifespans of species (e.g. between birds and mammals), lifespan extension by caloric restriction and even differences in lifespan within species, for instance between honeybee workers and queens (Hulbert, 2008). Fatty acid susceptibility to peroxidation may even have predictive capacities when it comes to human lifespan expectancy (Puca et al., 2008).

1.1.2.2.4. Protein turnover hypothesis

The protein turnover hypothesis is based on the finding that aging is associated with the accumulation of post-translationally altered proteins (Stadtman, 1988). Proteins are constantly at risk of damage or alteration, either by genomic instability, by post-synthetic changes like oxidation and glycation, by denaturation or by conformational changes (Gafni, 1997; Hipkiss, 2006; Rothstein, 1979) . In order to maintain cellular homeostasis, proteins undergo repeated synthesis and degradation, or protein turnover. For this purpose, cells are equipped with proteolytic systems capable of protein degradation; the mechanisms of protein degradation, i.e. the lysosomal/autophagic system and the ubiquitin proteasome system are reviewed in (Martinez-Vicente et al., 2005). Age-related accumulation of aberrant proteins has been linked, in part, to a decrease in rates of protein degradation with increasing age (Carrard et al., 2002; Chondrogianni and Gonos, 2005; Cuervo and Dice, 2000;

Terman and Brunk, 2004); in this way, slowed protein turnover may contribute significantly to aging and age-related pathologies (Martinez-Vicente et al., 2005).

1.1.2.2.5. The TOR pathway as the lifespan determinant

Recently, a theory explaining aging was formulated centered around the Target-Of-Rapamycin (TOR) pathway by Blagosklonny (2006). The TOR pathway is a growth-promoting signaling pathway, activated by nutrients and growth factors, which drives synthesis of intracellular components. This pathway stimulates cell (mass) growth; in proliferating cells, this process is balanced by cell divisions, but when the cell cycle is blocked and TOR is still active, cells become hyperactive. In this theory, aging is seen as a consequence of TOR-driven cellular hyperfunction, which leads to cell and organ failure.

An important notion in this theory is the following: 'The aging process is not driven by damage. Aging causes damage, not damage causes aging'. Blagosklonny (2008) states that lifespan is not limited by accumulation of molecular damage. According to the author, oxidative damage does occur, but lifespan is restricted by TOR-driven aging before oxidative damage can cause the death of an organism. In addition, ROS-related findings can be fitted into the theory: though they are not required to explain the workings of the model, ROS may play signaling roles upstream and downstream of TOR.

Importantly, this theory fits into the evolutionary antagonistic pleiotropy theory of aging. The antagonistic pleiotropy theory postulates that there are genes which are beneficial early in life at the cost of aging. According to Blagosklonny (2008), genes implicated in the TOR pathway fit this description. As the TOR pathway constitutes a growth program in response to growth factors and nutrients, TOR is essential for development. However, the force of natural selection declines with age and TOR is not sufficiently switched off later in life; it is involved in aging and age-related diseases such as neurodegeneration and cancer. In other words, the developmental program driven by TOR becomes a quasi-program for aging. According to the author, a quasi-program is a purposeless continuation of a developmental program that was not switched off after completion (Blagosklonny, 2008). This 'quasi-program' has no purpose, but could be switched off genetically or pharmacologically, after completion of the developmental program.

Evidence supporting this theory is summed up in (Blagosklonny, 2006) and refers to lifespan extension by TOR pathway inhibition, either through mutations in the pathway, treatment with rapamycin (which is an inhibitor of the pathway), or reduction of calorie intake. Examples can be found in yeast, *Caenorhabditis elegans*, *Drosophila* and mammals. Also, age-related diseases in humans point to the 'quasi-program' of TOR as a cause of aging. As an example of an age-related disease, the author proposes osteoporosis and consequent bone fracture. At first sight, the cause of this disease could be classified as 'wear-and-tear'. However, osteoporosis results from hyper-function of cells that resorb bone (osteoclasts). Other diseases resulting from hyper-function are atherosclerosis, hypertension and cancer.

Since TOR is associated with many age-related diseases in humans, this underlines its potential importance for future research. According to Blagosklonny's theory (2006), it is a candidate target for pharmacological intervention against aging, for the following reasons: it is crucial for development but not essential after completion of development, and over-

activation accelerates aging while inhibition increases longevity. Blagosklonny suggests the TOR pathway inhibitor rapamycin, which has been used as an immunosuppressant in transplant patients and as an anti-cancer agent, as a potential anti-aging drug.

1.2. Oxidative metabolism, mitochondria and aging

Though the Free Radical Theory of Aging is as yet far from proven, the mitochondrion is seen by many as a major factor in the aging process. In this chapter, we will look into the potential link between aging and mitochondria. First, an overview is given of energy metabolism. The ultrastructure, organization and functions of mitochondria are discussed, together with the modes of mitochondrial ROS production. Finally, age-related changes in mitochondria and their importance for the aging process are reviewed.

1.2.1. Energy metabolism: overview (Voet et al., 2006)

Intermediary metabolism comprises all reactions concerned with storing and generating metabolic energy and with using that energy in biosynthesis of low-molecular-weight compounds and energy storage compounds. The part of intermediary metabolism consisting of pathways that store or generate metabolic energy is known as energy metabolism (Mathews and Van Holde, 1996). An important central pathway involved in energy metabolism is glycolysis; this catabolic pathway is situated in the cytosol and converts sugars, glucose in particular, to pyruvate through a series of enzymatic reactions. Free energy generated by the degradation of glucose to pyruvate is used for synthesis of ATP and reduction of nicotinamide adenine dinucleotide (NAD^+). In anaerobic organisms, pyruvate is subjected to fermentation; end products of fermentation include lactate (by homolactic fermentation) or ethanol and CO_2 (by alcohol fermentation). In oxidative metabolism, pyruvate is shuttled to the mitochondria and is decarboxylated and converted into acetyl–coenzyme A (acetyl-CoA), which in turn is oxidized in the tricarboxylic acid cycle (TCA cycle or Krebs cycle). Input of simple carbon compounds into the TCA cycle can also be derived from fatty acid or amino acid degradation; the TCA cycle is the point of convergence for all catabolic pathways of oxidative metabolism. Oxidation of these carbon compounds by the 8 enzymatically catalyzed reactions of the TCA cycle results in reduction of the nucleotide coenzymes NAD^+ to NADH and FAD (flavin adenine dinucleotide) to $FADH_2$, formation of (G/A)TP (guanosine/adenosine triphosphate) and release of CO_2. The reduced electron carriers NADH and $FADH_2$ donate their electrons to the mitochondrial electron transport system (ETS). Here, electrons are transferred through a series of redox proteins to O_2, the final electron acceptor. Electron transport along the ETS allows some of the redox proteins to shuttle protons out of the mitochondrial matrix, creating a transmembrane proton concentration gradient which is the driving force behind mitochondrial ATP synthesis through oxidative phosphorylation.

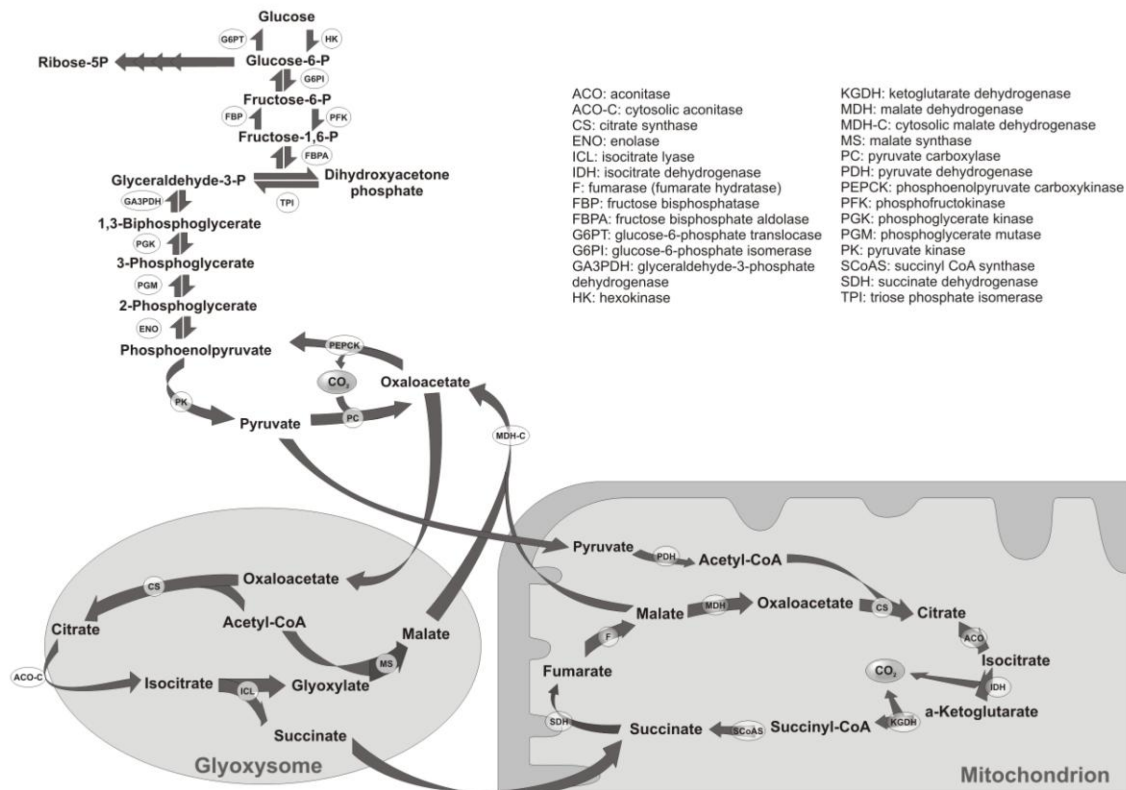


Figure 4: Schematic overview of some of the major pathways in energy metabolism. Shown are glycolysis, gluconeogenesis, the pentose phosphate pathway, the glyoxylate cycle and the TCA cycle. Adapted from (Castelein et al., 2008).

Control over glycolysis is exerted by regulation of the activity of key enzymes in the pathway. For instance, phosphofructokinase (PFK), which catalyzes the phosphorylation of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (FBP), is the most important point of control for glycolysis in muscle. ATP is both substrate and allosteric inhibitor of PFK. Other compounds like ADP, AMP and fructose-2,6-bisphosphate (F2,6P) can counter the inhibitory effect of ATP and are activators of PFK.

Another control mechanism for flux through the glycolysis pathway is substrate cycling. Control by substrate cycling is possible when two opposing reactions catalyzed by separate enzymes occur simultaneously; for instance, F6P is phosphorylated to FBP by PFK; the opposed reaction fructose-1,6-bisphosphatase (FBPase) catalyzes the hydrolysis of FBP to F6P. The combined reactions result in net ATP hydrolysis. Any intervention that lowers the activity of one enzyme and increases the activity of the other enzyme, influences flux through glycolysis. The rate of substrate cycling may be under neuronal or hormonal control; for example, levels of thyroid hormone can modify activities of PFK and FBPase (Shulman et al., 1985).

Regulation of the TCA cycle is exerted by supply of acetyl-CoA and demand for ATP. The decarboxylation of pyruvate to acetyl-CoA is strictly regulated as it is an irreversible reaction; this process can be inhibited by pyruvate dehydrogenase kinase. The activation state of the rate-determining enzymes of the TCA cycle (citrate synthase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase) sets the pace of this pathway. Also, intermediates of the TCA cycle are needed as biosynthetic precursors; for instance,

biosynthesis of certain amino acids requires α-ketoglutarate and oxaloacetate. Availability of certain intermediates exerts control over key enzymes of the TCA cycle, such as citrate, which competes with oxaloacetate and inhibits citrate synthase. Additional regulatory mechanisms include activation or inhibition of isocitrate dehydrogenase by ADP and ATP, respectively, and stimulation of the TCA cycle by Ca^{2+}.

An alternative to glycolysis is the pentose phosphate pathway; this pathway breaks down glucose derivatives for anabolic purposes. Generated products include NADPH, CO_2 and pentoses; pentoses are used in biosynthesis of nucleotides and nucleic acids, while NADPH is needed for maintenance of cellular redox balance (e.g. by re-reducing oxidized glutathione) and as reducing equivalents for biosynthetic reactions.

The glyoxylate pathway provides an alternative way to process acetyl-CoA, not oxidizing it completely to CO_2 but converting it to succinate and malate. This pathway is found in plants, bacteria, fungi and nematodes; it could also be operational in other animal species (like insects and mammals) under certain circumstances (Popov et al., 2005). Normally, its key reactions are performed by two separate enzymes but *C. elegans* has a single enzyme with a fused isocitrate lyase and malate synthase domain. In plants, and presumably also in some nematodes (Aueron and Rothstein, 1974; Patel and McFadden, 1977), key reactions of this pathway take place in glyoxysomes. Resulting succinate can be converted to malate in the mitochondria, thereby fueling the TCA cycle; malate can also be transported to the cytosol where it is oxidized to oxaloacetate for entry into gluconeogenesis, enabling conversion of fats to carbohydrates.

1.2.2. Origin and ultrastructure of mitochondria

1.2.2.1. Origin

As originally formulated in the generally accepted endosymbiotic theory, the presence of mitochondria in eukaryotic cells originates from when a primitive eukaryotic cell entered into a stable endosymbiotic relationship with a bacterium (Alberts et al., 2002). Certain features of mitochondria are regarded as evidence for this theory; for instance, mitochondria have their own DNA and protein synthetic machinery, and mitochondrial proteins and rRNA sequences show similarities with those of bacteria. Extensive sequence homology was found between mitochondrial and bacterial MnSODs (Fridovich and Poole, 2008). In recent years, this theory has met with opposition. According to Martin and Muller (1998), the mitochondrion arose by interaction between two prokaryotic cells. This view of evolution, tracing the origin of mitochondria to the same time frame as the origin of the nucleus, fits with recent findings that the oxygenation of oceans took place long after eukaryotes originated and diversified, and explains why many anaerobic eukaryotes contain mitochondrial proteins which are used for ATP generation in the absence of oxygen (Martin and Muller, 1998; Mentel and Martin, 2008).

1.2.2.2. Ultrastructure

High-resolution images of mitochondria date back to the 1950's (Palade, 1964); mitochondria were seen as vesicle-like structures consisting of an outer membrane, an inner membrane with invaginations, termed cristae, an intermembrane space and the internal matrix. With the technical advancement of microscopy, the discovery of fluorescent mitochondrial probes and the development of live cell imaging (Jakobs, 2006) the knowledge of mitochondrial anatomy has progressed considerably. Crista junctions, small junctions separating cristae from the inner boundary membrane, were discovered (fig 5a) (Frey and Mannella, 2000). Importantly, these new developments have led to a three-dimensional view, where mitochondria are visualized as networks of long tubular structures. They vary in length but have relatively constant diameters of 0.5-1.0 µm (fig 5b) (Griparic and van der Bliek, 2001). Also, mitochondria can move along microtubules between different regions of the cell (Benard and Rossignol, 2008). Depending on the tissue studied, the morphology of the mitochondrial networks differs, together with variations in number of cristae and matrix density (Benard et al., 2006). Cristae shape can vary from simple tubular entities to complicated lamellar assemblies, with the potential to form microcompartments that limit diffusion of substrates and ions and permit internal differences in pH between microcompartments (Voet et al., 2006).

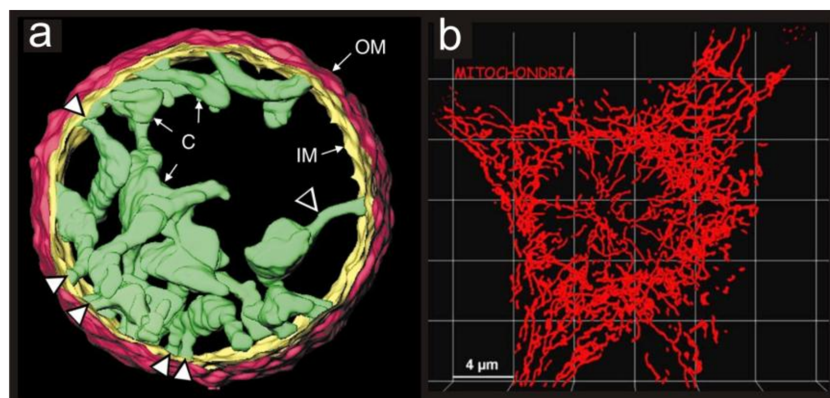


Figure 5a: 3D image of an isolated rat-liver mitochondrion, showing cristae (C), inner boundary membrane (IM) and outer membrane (OM) (Frey and Mannella, 2000). Figure 5b: 3D reconstruction of confocal laser scanning microscopic images of a HeLa cell transiently expressing a mitochondria-targeted red-fluorescent protein (Graier et al., 2007).

1.2.2.3. mtDNA, proteins and supercomplexes

As mentioned, mitochondria contain a genome separate from the nuclear DNA, termed mitochondrial DNA or mtDNA. The mitochondrial genome is circular and double-stranded; its length varies depending on the species (about 16 600bp in humans, 16 300 bp in mice and 13 800 bp in *C. elegans*). It encodes genes for 22 transfer RNAs, 2 ribosomal RNAs and 13 respiratory system subunits (12 subunits in *C. elegans*, fig 6) (Okimoto *et al.*, 1992; Falkenberg *et al.*, 2007). Genes for other respiratory system components and proteins needed for mtDNA replication, transcription and translation are encoded in the nucleus. mtDNA contains no introns and few or no non-coding nucleotides. In somatic mammalian

cells, up to 10 000 copies of mtDNA are present, organized in nucleoid structures (Falkenberg et al., 2007).

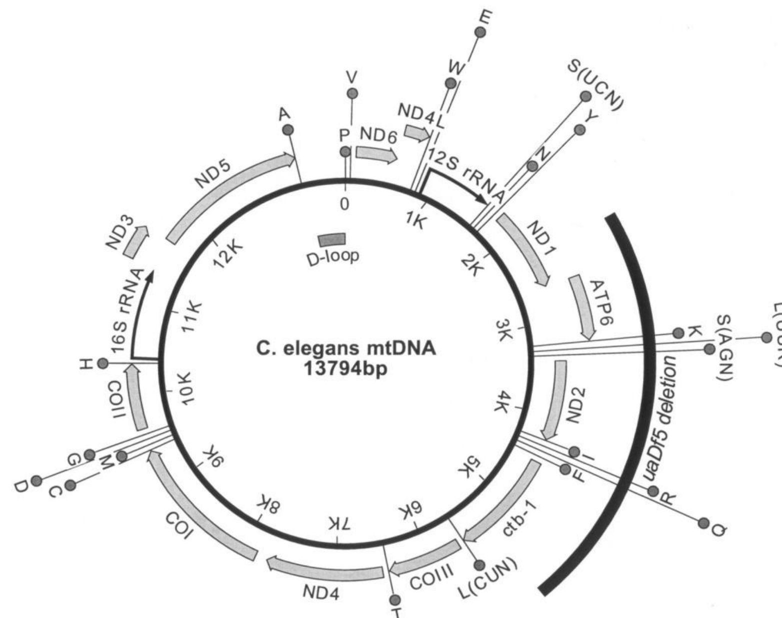


Figure 6: Gene map of the *C. elegans* mtDNA (Lemire, 2005)

Mitochondria contain 1000 to 1500 proteins, functioning in energy metabolism, lipid and amino acid metabolism, transport, signaling, membrane remodeling, protein degradation and programmed cell death (Chacinska et al., 2009). Key proteins for energy metabolism make up the electron transport system and the ATP synthesis machinery (fig 7). These proteins represent 80% of the total protein mass of the inner mitochondrial membranes and cover up to 50% of the total membrane area; ETS proteins preferentially locate at the cristae membranes (Benard and Rossignol, 2008). The electron transport system consists of four enzyme complexes (Complex I-IV) and two intermediary substrates (coenzyme Q and cytochrome *c*). In mammals, Complex I contains 46 subunits with a total mass of about 1 MDa. This L-shaped protein complex has one arm embedded in the inner mitochondrial membrane while the rest of the complex extends into the matrix. Of the 46 subunits, 7 hydrophobic proteins are coded for by the mtDNA (Carroll et al., 2005). Complex II, which is part of the TCA cycle, consists of 4 subunits, all encoded by the nuclear genome. It is the smallest and most hydrophilic of the ETS complexes. Purified as a dimer, the mammalian monomer of complex III contains 11 subunits, 1 of which is mtDNA-encoded, and has a total mass of about 240 kDa (Iwata et al., 1998). Mammalian Complex IV also crystallizes as a dimer and has 13 subunits per monomer; 3 of these are the largest mitochondrial encoded subunits (Hüttemann et al., 2007). Of the two mobile redox components, the lipophilic isoprenoid ubiquinone is found in the inner mitochondrial membrane; depending on the source organism, the number of isoprenoid units connected to the quinone group varies. It is this hydrophobic tail that makes ubiquinone soluble in the inner mitochondrial membrane (Voet et al., 2006). The peripheral membrane protein cytochrome *c* is a heme protein; it was shown to contain 104 amino acids in several mammals (Hüttemann et al., 2007). ATP synthesis takes place at complex V or F_1F_0-ATP synthase. This protein complex has a molecular mass of about 550 kDa. It can be subdivided

in a hydrophobic (F_0) and a hydrophilic (F_1) domain. The F_0 domain is situated in the inner mitochondrial membrane while the F_1 domain, connected to F_0 by a central and a peripheral stalk, is directed towards the matrix. F_0 contains 8 different subunits while F_1 consists of 5 types of subunits. Two subunits of Complex V are encoded by mtDNA (Voet et al., 2006).

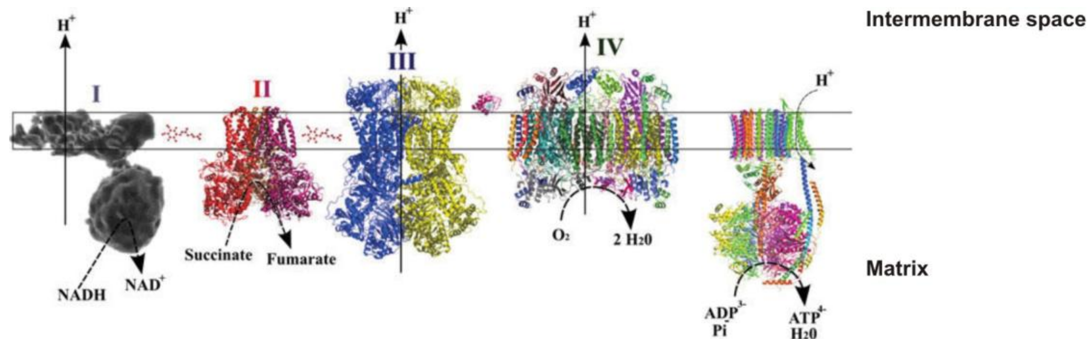


Figure 7: the mammalian respiratory chain (Rigoulet et al., 2011); complexes are not scaled to relative size.

Mitochondrial F_1F_0-ATPsynthase is often isolated in dimeric or oligomeric forms (Wittig and Schagger, 2008). In fact, in recent years, it has become apparent that not only Complex V, but also Complex I, III and IV can be organized in higher order structures. In contrast to the 'random collision model' where ETS complexes were regarded as independent entities (Hackenbrock et al., 1986), sufficient evidence has arisen that respiratory complexes I, III and IV are mutually associated. What's more, derived from their relative abundances it was postulated that they are organized in supercomplexes. These supercomplexes were originally termed respirasomes (Schägger and Pfeiffer, 2000) but may even be connected, forming 'respiratory strings' (fig 8) (Wittig et al., 2006). Proposed functions for respiratory supercomplexes include enhancement of substrate channeling (direct transfer of electrons between two consecutive enzymes by successive reduction and reoxidation of the intermediate without its diffusion in the bulk medium (Lenaz and Genova, 2009) and the assembly and stabilization of Complex I (Krause, 2007). ATP synthase dimerisation/oligomerisation studies have shown a tight relationship with the morphology of the cristae. The angle between the two ATP synthases in a dimer is most likely responsible for membrane bending and curvature of the cristae (Dudkina et al., 2005); in mutant yeast, defects in ATP synthase dimerization/oligomerization were linked to structural anomalies in mitochondria (Velours et al., 2009). Correct ATP synthase assembly is also necessary for stable complex III-IV supercomplex formation and association with the transport machinery of the inner mitochondrial membrane (Saddar *et al.*, 2008). Associations have been found between ATP synthase and other membrane protein structures like the ADP/ATP translocator and the phosphate carrier protein (see section 1.2.4.1.2.); these associations were termed ATP synthasomes (Ko et al., 2003).

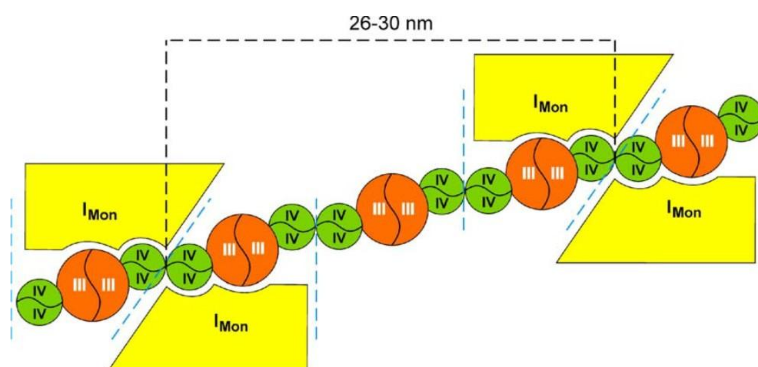


Figure 8: Respiratory string model for mammalian mitochondria, showing assemblies of respiratory chain complexes into supercomplexes. These supercomplexes interact to form larger supramolecular structures. I_{Mon}: complex I monomer (Wittig et al., 2006).

The complexes of the ETS, located in the inner mitochondrial membrane, are set in a phospholipid bilayer; a major component of this bilayer is cardiolipin (fig 9). In eukaryotes, cardiolipin is the only phospholipid that is not synthesized by the endoplasmatic reticulum but in the mitochondrion (Schlame and Haldar, 1993). ETS complexes depend on cardiolipin for their structure and activity, as do many mitochondrial carrier proteins (Haines, 2009). The formation and stabilization of supercomplexes are also reliant on the presence of cardiolipin (Zhang *et al.*, 2005; McKenzie *et al.*, 2006). Cardiolipin is believed to be necessary for coordination of programmed cell death (Schug and Gottlieb, 2009). The structure of cardiolipin is quite different from other phospholipids. It has a double glycerophosphate backbone and four fatty acyl side chains. Its conformation maximizes the pool of protons for proton pumping in oxidative phosphorylation (Haines, 2009).

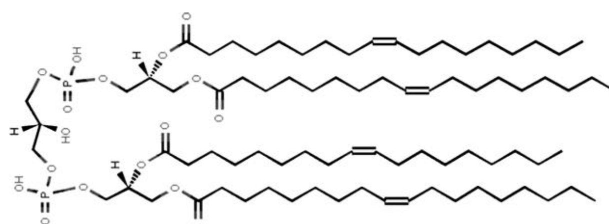


Figure 9: Molecular structure of cardiolipin (Kiebish et al., 2008)

1.2.3. Mitochondrial turnover

For cellular homeostasis, a balance between protein synthesis and degradation, or protein turnover, is needed. Several mechanisms contribute to mitochondrial protein turnover, or the 'quality control' of mitochondria, in order to avoid accumulation of damaged or misfolded mitochondrial proteins. At the molecular level, two types of AAA proteases (ATPases associated with a number of cellular activities) are embedded in the inner mitochondrial membrane. AAA proteases are ATP-dependent proteolytic complexes; m-AAA proteases expose their catalytic site towards the matrix, while i-AAA proteases are directed towards the intermembrane space (Arnold and Langer, 2002). AAA proteases form complexes composed of closely related or identical subunits. At the center of the AAA proteases is a cavity where proteolysis occurs, separated from the rest of the cell; ATP is needed to unfold and to transport proteins to this proteolytic cavity (Koppen and Langer,

2007). These proteolytic systems are highly conserved among eubacteria and eukaryotes. They contribute to cellular homeostasis by removal of misfolded or damaged proteins, and by regulation of expression of some mtDNA-encoded genes via protein processing (Arnold and Langer, 2002). For degradation of proteins from the outer mitochondrial membrane, the cell seems to rely on the cytosolic ubiquitin-proteasome system (Yonashiro et al., 2006).

Mitochondrial quality control at the organellar level is coordinated by a combination of mitochondrial dynamics and autophagy of mitochondria, termed mitophagy. As discussed, mitochondria are present in networks of tubular structures with varying lengths. This structural organization is a dynamic process. Fusion and fission, two opposing processes causing the merging and division of mitochondria, occur continuously and are essential for optimal physiological functioning of these organelles. While the regulatory mechanisms responsible for fusion and fission are largely known (Benard and Karbowski, 2009), their significance in maintenance of cell function is the subject of speculation. Mitochondrial function could benefit from mixing of mtDNA and matrix and membrane proteins (Karbowski and Youle, 2003) or from dilution of defective proteins (Benard and Karbowski, 2009). An attractive model for the role of fusion and fission in cellular homeostasis is proposed by (Twig et al., 2008). In their hypothesis, they incorporate autophagy as a key mechanism which, combined with fusion and fission, can selectively remove damaged mitochondrial components. The autophagic process is responsible for digestion of organelles and cytoplasmic components. To remove mitochondria, the organelles are enclosed by a double membrane vesicle (the autophagosome) which fuses with lysosomes in order to be degraded and recycled (mitophagy) (Kiffin et al., 2006). As it is a way of reusing cellular contents, autophagy is indispensable during nutrient deprivation and fasting (Komatsu et al., 2005). However, it is also capable of removing damaged and unneeded organelles (Lemasters, 2005). Once thought to be an unselective process, recent findings show that autophagy can be selective (Kim et al., 2007). It was suggested that depolarization marks mitochondria for degradation by autophagy (Elmore et al., 2001; Priault et al., 2005). Moreover, fusion is less likely to occur with depolarized mitochondria as it seems to depend on mitochondrial membrane potential (Legros et al., 2002). The proposed model assumes that through fusion and fission, damaged mitochondrial components are sequestered into daughter mitochondria that are selectively degraded by autophagy.

At the cellular level, mitochondria are key regulators of apoptosis. This process of programmed cell death is controlled by a complex signaling machinery. It is essential in development, organ homeostasis and removal of damaged or infected cells (Gulbins et al., 2003). How mitochondria exert their role in apoptosis will be discussed in section 1.2.4.2.3.

1.2.4. Mitochondrial functions

1.2.4.1. Energy supply

A major function of mitochondria is to supply the cell of adenosine triphosphate (ATP), the universal energy carrier. In the mitochondrion, electrons derived from oxidation of intermediates by catabolic processes (glycolysis and the breakdown of lipids and proteins) and carried by reducing equivalents (e.g. NADH and $FADH_2$) are transferred via the components of the electron transport system to oxygen. Coinciding with the transfer of electrons between the redox centers of the ETS, protons are expelled from the mitochondrial matrix towards the intermembrane space. It is the free energy decrease that accompanies electron transfer which is exploited to drive proton translocation and to build up an electrochemical gradient across the mitochondrial membrane. F_1F_0-ATPsynthase can use this proton gradient as an energy source to drive phosphorylation of ADP to ATP. This process is termed oxidative phosphorylation (Voet et al., 2006). The chemiosmotic theory by Mitchell dates back to 1961 (Mitchell, 1961), but many details of the mechanisms involved remain to be elucidated.

1.2.4.1.1. Enzymatic functions of the respiratory complexes

Complex I

The transport of electrons, the pumping of protons, the build-up of the membrane potential and the reduction of oxygen are situated at complex I, II, III and IV and the two mobile redox components Coenzyme Q (or ubiquinone) and cytochrome *c*. The most complicated enzyme of the ETS, Complex I (NADH:Coenzyme Q oxidoreductase), passes electrons from NADH to Coenzyme Q. As redox-active groups, it contains a molecule of flavin mononucleotide (FMN) and 8-9 iron-sulfur clusters ([2Fe-2S] or [4Fe-4S]), which all reside in the peripheral arm of the L-shaped complex (Zickermann *et al.*, 2008). Binding of NADH and FMN, oxidation of NADH and Coenzyme Q reduction take place in the peripheral arm. The site of NADH oxidation is connected to the ubiquinone binding site by a 'wire' of 7 Fe-S clusters, transferring electrons from NADH to ubiquinone (fig 10) (Brandt, 2006).

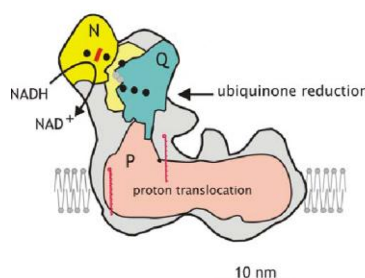


Figure 10: Schematic representation of Complex I. Shown are the sites of NADH oxidation, Coenzyme Q reduction and proton pumping. Adapted from (Zickermann et al., 2008).

The proton translocation site is situated in the membrane arm of the enzyme. Conformational changes are invoked to explain how Complex I overcomes the distance between the regions where redox chemistry takes place and the region containing the

proton translocation machinery in order to transduce energy derived from electron transfer to drive proton pumping (Belogrudov and Hatefi, 1994). Mutations in numerous subunits of the proton translocation site lead to lowered ubiquinone reduction capacity, suggesting tight coupling between redox chemistry and proton pumping (Zickermann *et al.*, 2008).

Complex II

Another place for electrons to enter the ETS is succinate:ubiquinone oxidoreductase or Complex II. This four subunit enzyme catalyzes oxidation of succinate to fumarate, thereby reducing ubiquinone to ubiquinol and connecting the TCA cycle to the ETS. Insight in the structure of Complex II was gained from the X-ray crystal structure of *E. coli* succinate:ubiquinone oxidoreductase. In this trimer, the monomers consist of two cytoplasmic hydrophilic subunits and two hydrophobic integral membrane anchor subunits. In the cytoplasmic domain, succinate oxidation occurs at the flavin adenine dinucleotide (FAD) covalently linked to the SdhA subunit, and electrons are transferred via three Fe-S clusters ([2Fe-2S], [4Fe-4S] and [3Fe-4S]) located at the SdhB subunit to the membrane anchor domain, which binds one heme *b* and one ubiquinone (fig 11). Heme *b* does not take part in electron transfer. Ubiquinone passes electrons on to Complex III. Complex II does not translocate protons from the matrix to the intermembrane space, and consequently, does not contribute to the build-up of the membrane potential (Horsefield *et al.*, 2004; Voet *et al.*, 2006). Complex II can also function in the opposite direction, as a fumarate reductase; this issue will be discussed in section 1.2.4.2.1.

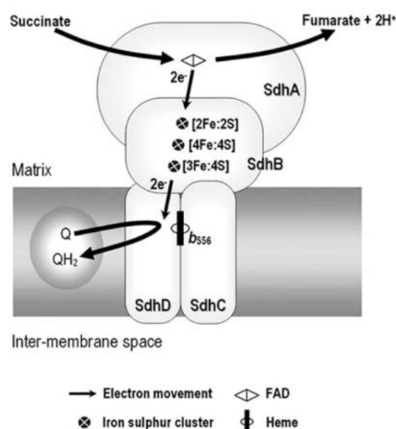


Figure 11: schematic representation of Complex II. Shown are the four subunits of the complex and sites of electron translocation. Adapted from (Horsefield et al., 2004)

Complex III

Electrons originating from Complex I or II are passed on by Coenzyme Q to Coenzyme Q:cytochrome *c* oxidoreductase, also known as the cytochrome bc_1 complex or Complex III. This complex contains up to 11 subunits, 3 of which are known to contribute to the redox chemistry of the enzyme. The cyt *b* subunit contains two b hemes, a lower-potential b_L heme and a higher-potential b_H heme. Another heme, heme c_1 is situated in the cyt c_1 subunit; the third subunit contains a [2Fe-2S] center known as the Rieske center. These 3 subunits are anchored in the inner mitochondrial membrane and extend in the matrix and the intermembrane space (Voet *et al.*, 2006).

A model for electron transfer at the Complex III location, the modified Q cycle, is based on a concept by Mitchell (Mitchell, 1975). At the Q_o-site (located near the intermembrane space), QH_2 is oxidized, and the two electrons derived from this process are each diverted down to a different acceptor. The high-potential chain formed by the Rieske center and heme c_1 of cyt c_1 accepts and transfers one electron to cyt *c*; this electron will consequently be delivered to cytochrome *c* oxidase (Complex IV). The other electron is donated to the low-potential chain of hemes consisting of b_L and b_H, thereby contributing to an electron flux traversing the inner mitochondrial membrane. At the Q_i-site (close to the matrix side of the inner mitochondrial membrane), this electron reduces Q to a semiquinone ($Q^{\cdot-}$) or $Q^{\cdot-}$ to QH_2 (fig 12). Q and QH_2 are expected to be capable of diffusing through the hydrophobic membrane. Reactions occurring at the Q_o-site need to take place twice to fully reduce ubiquinone at the Q_i-site, in such a manner that for oxidation of two molecules of QH_2, one molecule of Q is reduced (Crofts, 2004; Crofts *et al.*, 2008; Rich, 2008).

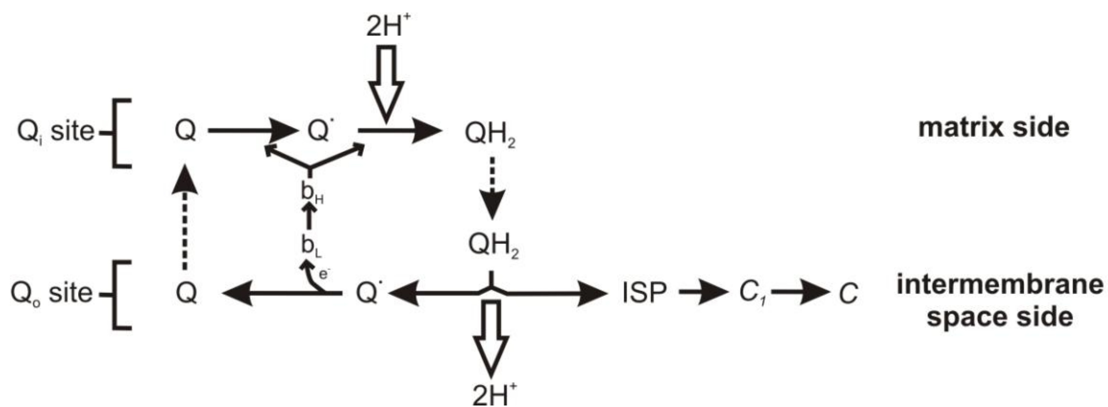
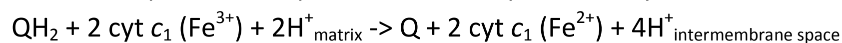


Figure 12: Schematic representation of the protonmotive Q cycle. Adapted from (Brandt and Trumpower, 1994).

The mechanism of proton translocation from the matrix to the intermembrane space by Complex III is remarkable in that it is a redox center, Coenzyme Q, which transports the protons across the inner mitochondrial membrane by diffusion. The two Coenzyme Q processing sites are situated on opposite sides at the border of Complex III's hydrophobic core; protons are taken up from the matrix at the Q_i-site and are carried across the membrane by ubiquinol, to be released to the intermembrane space at the Q_o-site. The net reaction illustrates the proton transport stoichiometry of the Q cycle:



$$QH_2 + 2\text{ cyt } c_1 (Fe^{3+}) + 2H^+_{matrix} \rightarrow Q + 2\text{ cyt } c_1 (Fe^{2+}) + 4H^+_{intermembrane\ space}$$

Cytochrome c

Electron transfer between Complex III and Complex IV is performed by the water-soluble peripheral membrane protein cytochrome *c*. The conformation of the binding site between cytochrome *c* and Complex III is such that it provides optimal circumstances for transient interaction and efficient electron transfer (Solmaz and Hunte, 2008). Cytochrome *c* docks at the Fe-S protein subunit of Complex III to take up an electron; x-ray structures show that only one cytochrome *c* molecule is bound to a Complex III dimer at a time (Lange and Hunte, 2002). Furthermore, it was established that the dimer structure is slightly

asymmetric, with binding of cytochrome *c* coinciding with conformational changes in Complex III, for instance in the ISP-ED domain (Solmaz and Hunte, 2008).

Complex IV

Electrons donated by Complex III to cytochrome *c* are passed on to Complex IV, also termed cytochrome *c* oxidase. The monomers in this homodimer exist of up to 13 subunits, predominantly held in the inner mitochondrial membrane. Mammalian Complex IV monomers, while containing 13 subunits and having a molecular mass of ~200kDa, rely on just 3 mtDNA-encoded subunits for their catalytic activity. Complex IV has 4 redox centers (fig 13), one of which, the redox active copper center Cu_A, is situated in subunit II. It is formed by 2 copper atoms and located just above the inner membrane surface. Subunit I contains the three remaining redox centers: heme *a* and the binuclear redox center composed of heme a_3 and Cu_B. Electrons are rapidly passed on from cytochrome *c* to the Cu_A center, which donates them to the heme *a*. The location of heme *a* is very close to heme a_3, ensuring fast electron transfer to the binuclear center (Belevich and Verkhovsky, 2008). Subunit III contains no redox factors; its function has not yet been established (Brzezinski and Gennis, 2008; Saraste, 1999).

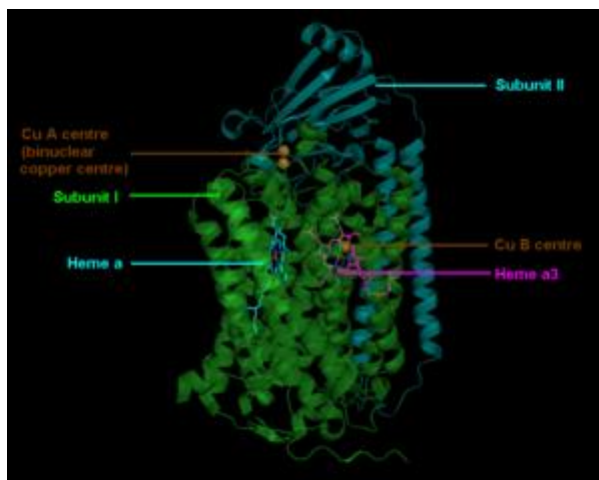
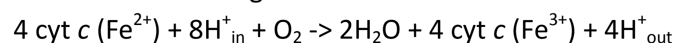


Figure 13: Structure of Complex IV subunits I and II, showing the location of the four redox centers (Wikimedia commons).

At Complex IV, the last enzyme in the electron transport system, the one-electron oxidation of 4 reduced cytochrome *c* molecules is catalyzed, coinciding with the 4-electron reduction of an O_2 molecule. The cytochrome *c* oxidation site is at the intermembrane-space side of Complex IV and protons are taken up from the matrix, while the oxygen redox site is located in the middle of the membrane. This implies that the electrons and protons required to reduce O_2 to H_2O stem from opposite sides of the inner mitochondrial membrane. Importantly, Complex IV qualifies as a proton pump (Wikstrom, 1977): free energy derived from reduction of oxygen is used to drive the pumping of 4 protons from the matrix to the intermembrane space. Thus, Complex IV has two mechanisms of charge separation. This results in the following net reaction:



$$4 \text{ cyt } c\ (Fe^{2+}) + 8H^+_{in} + O_2 \rightarrow 2H_2O + 4 \text{ cyt } c\ (Fe^{3+}) + 4H^+_{out}$$

Evidently, the proton pump mechanism contributes considerably to the membrane potential of the inner mitochondrial membrane: per oxygen molecule to be reduced, 8

charges cross the membrane, and for each electron used in the chemistry, one proton is pumped (Voet *et al.*, 2006). We have to note that the oxygen chemistry is not dependent on the proton pumping: certain Complex IV mutations prevent proton pumping but not the enzymatic activity of the complex (Brzezinski and Gennis, 2008).

Alternative enzymes

Delivery of electrons to the ETS can occur via other pathways besides Complex I and II; for instance, in yeast, electrons stemming from cytosolic NADH reach the ETS through alternative NADH dehydrogenases and glycerol-3-phosphate dehydrogenases (Rigoulet *et al.*, 2004). Similarly, a highly branched ETS has been described for plant mitochondria (Rasmusson *et al.*, 2008); also, in mammals, the glycerol-3-phosphate dehydrogenase shuttle allows delivery of reducing equivalents from cytosolic NADH to the ETS in certain tissues like brown adipose tissue (Houstek *et al.*, 1975). In the systems mentioned, electrons are transferred to Coenzyme Q. In several species of plants, fungi and some protists, and even in some prokaryotes and animal species, a non-proton-pumping alternative oxidase exists that oxidizes ubiquinol and reduces oxygen. McDonald (2008) describes its potential functions; among them are control of ROS generation and balance of carbon metabolism and electron transport.

Complex V

Driven by electron transport, protons are transported across the inner mitochondrial membrane by complexes I, III and IV. The inner mitochondrial membrane separates the low H^+ concentration of the matrix from the high H^+ concentration of the intermembrane space. The electrochemical gradient that is formed (or protonmotive force) can deliver the energy needed to drive ATP synthesis by Complex V, also called F_1F_0-ATPsynthase. The protonmotive force has an electric component, $\Delta\Psi$, and a chemical component, ΔpH. The use of energy supplied by transmembrane proton concentration gradients to synthesize ATP in mitochondria is called oxidative phosphorylation.

Synthesis of ATP from ADP and inorganic phosphate is an intricate process, of which many but not all molecular mechanisms have been uncovered. The roles played by many of the Complex V subunits in this process have been determined; studying the structure of the enzyme in bacteria has contributed a great deal to this knowledge. In bacteria, the enzyme can be found in its simplest form, consisting of 8 different subunits composed as $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1a_1b_2c_{10\text{-}15}$. $\alpha_3\beta_3$ represents the F_1 sphere composed of alternating α and β subunits. The central stalk contains subunits γ and ε while δ and b_2 form the peripheral stalk. Situated in the inner mitochondrial membrane is a ring of 10-15 c subunits in association with the a subunit (fig 14) (Weber and Senior, 1997). To some extent, mitochondrial F_1F_0-ATPsynthase differs from the bacterial enzyme. It contains additional subunits, some of which are needed for the enzyme's supercomplex organization (Wittig and Schagger, 2008). Also, for some subunits, nomenclatures in human and yeast mitochondria and in *Escherichia coli* differ.

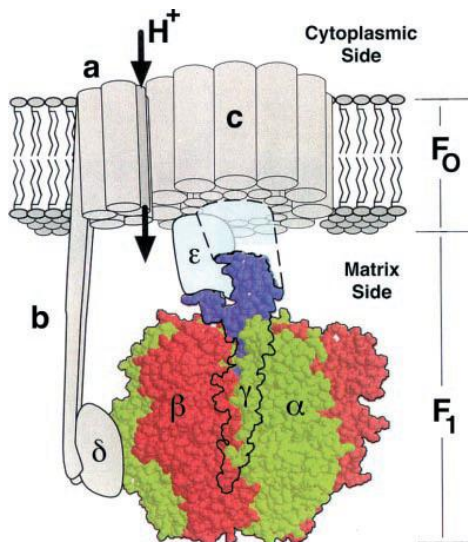


Figure 14: Essential structural features of Complex V (Saraste, 1999).

For Complex V to synthesize and release ATP, it must utilize the protonmotive force to power its rotary motor F_0 which will in turn drive the chemical motor F_1. The system, as it is known at present, consists of proton translocation from the intermembrane space to the matrix through F_0, coupled to the rotation of a subcomplex of the enzyme (the rotor) which induces conformational changes in the F_1 domain that allow ATP synthesis and its release from the enzyme (Saraste, 1999). ATP synthesis and release at the F_1 domain can be explained by the binding change mechanism by Boyer (Boyer, 1993; Menz *et al.*, 2001). The sphere of the F_1 domain is assembled as a hexamer with alternating α and β subunits, around the γ subunit of the center stalk. The β subunits, with some residues contributed by α, are the catalytic sites of the enzyme; they can adopt 3 kinds of conformations, which influence binding of and interaction between the substrates of these subunits. It is most likely the position and rotation of subunit γ which determines the alternating conformation of the β subunits. As there are no hydrogen bonds or ionic interactions at the contact site between γ and $\alpha_3\beta_3$, the γ subunit is allowed to rotate freely. For the mechanism of rotation generated by proton translocation we refer to (Fillingame et al., 2003; Junge et al., 1997; Nakamoto et al., 2008). Rotation of γ is associated with conversion, in one β subunit, of the 'open' to the 'binding' conformation, which binds ADP and inorganic phosphate. The other β subunits synchronously undergo conformation changes: a 'binding' conformation is converted to a 'tight' one, where ATP is synthesized, while an ATP-binding 'tight' conformation is switched to an 'open' conformation, which allows ATP to dissociate. ATP synthesis by the β subunit of F_1 in its 'tight' conformation is an exergonic process; the energy released is used for disrupting the enzyme-ATP interaction (in other words, the shift to the 'open' state) (Voet *et al.*, 2006).

1.2.4.1.2. Proteins associated with energy supply by mitochondria

Though separated from the cytosol by two membranes, the mitochondrion is not an independent entity; import and export between the mitochondrion and the rest of the cell are required for proper mitochondrial functioning. Most metabolites cross the outer mitochondrial membrane via mitochondrial porins, also termed voltage-dependent anion-selective channels (VDAC). In this way, the outer mitochondrial membrane is a semi-

permeable barrier, allowing transport of water soluble metabolites between the cytosol and the intermembrane space; ΔΨ determines the conductance and selectivity of the channels (Colombini, 2004).

The impermeability of the inner mitochondrial membrane is essential in energy supply; only through regulated, selective transport in and out of the mitochondrion can it perform its functions. Processes taking place in mitochondria rely on import and export of amino acids, proteins and metabolites. The nuclear-encoded proteins in enzymes of the TCA cycle, the ETS, oxidative phosphorylation and fatty acid oxidation need to be imported from outside the organelle. Likewise, transporters are required for substrates that fuel these metabolic processes and for the export of their end products. We will describe transport mechanisms needed for oxidative phosphorylation. Other transport mechanisms, e.g. for the TCA cycle, β-oxidation of fatty acids, amino acid transport, cation transport and import of mitochondrial proteins coded for by the nuclear genome, will not be discussed here.

Some transporters are directly involved in ATP production. Import of phosphate (P_i) is performed by the phosphate carrier, either by proton co-transport or in exchange for hydroxyl ions (Laloi, 1999). This electroneutral transport is driven by the ΔpH across the membrane. It is essential for phosphorylation of ADP, for enzyme reactions (succinyl CoA synthetase) in the TCA cycle and for uptake of other metabolites, by acting as the exchange substrate (Palmieri, 2004). Cytosolic ADP is exchanged for mitochondrial ATP by adenine nucleotide translocase (ANT) under conditions of oxidative phosphorylation. In this way, cytosolic use of ATP is linked to mitochondrial ATP synthesis. The driving force is the ΔΨ across the inner membrane (Traba *et al.*, 2009). Next to this electrogenic exchange (ADP^{3-} for ATP^{4-}) there is also an electroneutral way to exchange ATP via the ATP-Mg/P_i carrier which is important for maintenance of the net content of adenine nucleotides (Aprille, 1988). The membrane potential can be dissipated by uncoupling proteins (UCPs). These mitochondrial carriers allow protons to return from the intermembrane space to the matrix without passing through Complex V; the net result of this process is an increase in oxygen consumption by mitochondria and conversion of electrochemical energy in heat (Mozo *et al.*, 2005). UCPs play a role in thermogenesis. As they have also been identified in poikilotherms like *Drosophila* and *C. elegans*, they most likely have additional roles (Fridell *et al.*, 2004; Iser *et al.*, 2005; Krauss *et al.*, 2005).

1.2.4.1.3. Regulation of oxidative phosphorylation

Since bioenergetics concerns the flow and transformation of energy in and between living organisms and between living organisms and their environment, mitochondrial bioenergetics cannot be described based on mitochondrial processes alone. The rate of oxidative phosphorylation is determined by numerous factors at various levels of organization. At the organellar level, oxidative phosphorylation is set by the type and availability of substrates, the presence of ADP and P_i, the activity of the respiratory complexes and the ability of the mitochondrion to maintain ΔΨ. All of these factors can be influenced by crosstalk with other cellular components, by the energetic state of the cell, by signals from surrounding and distant cells, by the type of tissue that the cell is part of, by the health state and energetic needs of the organism and by the environment that the organism

is in; moreover, these variables are interdependent, therefore it is difficult to dissect their effects.

Examples of environmental circumstances that influence the metabolic rate of an organism –and consequently its oxidative phosphorylation- are temperature (Frappell and Butler, 2004), the type and amount of nutrients that are available (Houthoofd *et al.*, 2005), and oxygen availability (Van Ginneken and van den Thillart, 2009). At the organismal level, rates of metabolism are influenced by developmental state and energetic needs; for instance, mitochondria in embryos increase in amount at the time of shift from glycolytic to oxidative metabolism (Alcolea *et al.*, 2007). Likewise, exercise in skeletal muscle elicits mitochondrial biogenesis (Hood, 2001). Within the organism, mitochondrial differences emerge when different tissues are compared (Benard *et al.*, 2006). While tissues may differ in metabolic state, they are also able to influence the metabolism of other tissues in the organism; for instance, a rise in blood glucose levels causes pancreatic β cells to release insulin, which shifts metabolism toward glycolysis and fat storage in other tissues (Moyes and Schulte, 2008). In order to fine-tune metabolism, communication at the cellular level is indispensable. This is illustrated by the abovementioned mitochondrial biogenesis, requiring coordination of expression of the nuclear and mitochondrial genome through crosstalk between the nucleus and the mitochondria (Ryan and Hoogenraad, 2007).

The majority of the parameters that influence oxidative phosphorylation do so by affecting signaling pathways. In this way, even stimuli received from the organism's environment eventually initiate a change in the mitochondrion. The most important kinase signaling pathways can all target the mitochondrion; several protein kinases and phosphatases can localize at the cytoplasmic surface of or inside mitochondria, and all respiratory complexes together with some anion and cation channels and metabolic enzymes can be phosphorylated (Horbinski and Chu, 2005; Hüttemann et al., 2007; Salvi et al., 2005). One of the kinases known to target mitochondria is the serine/threonine kinase Akt. In different types of cell cultures, Akt was found inside mitochondria after exposure to insulin-like growth factor-1 (IGF-1), insulin or heat stress, resulting in phosphorylation of both the β subunit of complex V and glycogen synthase kinase-3β (GSK3β), which, when active, inhibits pyruvate dehydrogenase. Other targets of Akt in mitochondria remain unidentified (Bijur and Jope, 2003).

In addition to phosphorylation/dephosphorylation, there are other mechanisms that affect the activity of respiratory complexes; one of them is allosteric regulation. ATP is capable of binding to both cytochrome *c* and Complex IV, inhibiting the reaction between them and the enzyme activity of Complex IV as a way to adjust energy production to physiological demand. Allosteric inhibition by ATP can be prevented by thyroid hormone T2, which allows a high Complex IV activity even when ATP levels are high (Hüttemann *et al.*, 2007). Competitive inhibition via nitric oxide (NO) is also a way of reversibly inhibiting cytochrome *c* oxidation, when NO competes with oxygen at the binuclear center (Huttemann *et al.*, 2008). Finally, the activity of the ETS is potentially affected by the organization of respiratory complexes in supercomplexes through enhancement of substrate channeling or stabilization of respiratory enzymes (see section 1.2.2.3.).

Oxidative phosphorylation is also influenced by the metabolites entering and exiting the organelle. The type and amount of substrates fueling the ETS are determined by regulation of the processes that supply them, for instance by the enzymes that control

glycolysis and the TCA cycle (see section 1.2.1.), and by the transport proteins that import them. As an example, increased mitochondrial Ca^{2+} uptake stimulates the activity of certain TCA enzymes, leading to increased $NADH/NAD^+$ ratios and an increase in mitochondrial ATP synthesis (Goldenthal and Marin-Garcia, 2004). In contrast, a lowered demand for ATP can slow down the TCA cycle. Consequently, less reducing equivalents are delivered to the ETS. Moreover, the accumulated citrate exits the mitochondria and inhibits PFK, decreasing the rate of glycolysis (Pogson and Randle, 1966). Likewise, levels of ATP, ADP and Pi are regulated by the demand for ATP combined with the proper functioning of the ANT, ATP-Mg/P_i and P_i-transporter (Aprille, 1988). ATP usage provides the substrates for oxidative phosphorylation (ADP and P_i). Increased cytosolic ADP stimulates ADP uptake in exchange for ATP export, which augments the matrix ADP-to-ATP ratio (Brown, 1992).

Since many transporters depend on $\Delta\Psi$ to exert their function, it is evident that the ability to maintain $\Delta\Psi$ is also a determinant of oxidative phosphorylation. Importantly, it is the driving force for ATP synthesis. Consequently, any intervention that impinges on $\Delta\Psi$, changes oxidative phosphorylation. Loss of integrity of the mitochondrial membranes prevents the organelle from maintaining $\Delta\Psi$. When transport of protons back to the matrix is not coupled to ATP synthesis, $\Delta\Psi$ is dissipated and mitochondrial respiration increases (Voet *et al.*, 2006). Uncoupling occurs not only through the presence of UCPs or addition of chemical uncouplers, but also by an inherent basal proton leak caused by a partial permeability of the inner mitochondrial membrane to protons; this process is of some importance under nonphosphorylating conditions, but less so when ADP and P_i are present and ATP synthesis is active (Brookes, 2005). Mitochondria can to some extent resist the dissipation of $\Delta\Psi$: F_1F_0-ATPsynthase is a reversible enzyme that can consume ATP to pump protons to the intermembrane space in order to counteract the loss of $\Delta\Psi$. To prevent necrotic cell death following ATP depletion, the endogenous inhibitor protein IF_1 prevents ATPase activity of Complex V. IF_1 has also been implicated in the formation of F_1F_0 protein complex dimers and the quantity of cristae formed in mitochondria (Campanella *et al.*, 2009); future research will no doubt reveal more about the functions and regulation of IF_1 and its influence on oxidative phosphorylation.

1.2.4.2. Other functions of mitochondria

1.2.4.2.1. Energy supply in the absence of oxygen

As described, mitochondria produce ATP coupled to reduction of oxygen. However, mitochondria from some organisms can function anaerobically, synthesizing ATP through electron transport but using terminal electron acceptors other than O_2. Various electron acceptors exist; here we focus on fumarate and the process of malate dismutation. Phosphoenolpyruvate from glycolysis is converted to oxaloacetate, further reduced to malate and imported into the mitochondria. There, malate is degraded by dismutation: part of it is oxidized, the rest is reduced. Among the products formed by reactions following malate oxidation is succinyl Coenzyme A which contains sufficient energy in its thioester bond for substrate-level phosphorylation of ADP. Fumarate, formed by reduction of malate, is further reduced to succinate by the enzyme fumarate reductase. The electrons needed for this reduction are derived from NADH oxidation by Complex I (which contributes to $\Delta\Psi$) and delivered to fumarate reductase by a rhodoquinone. Succinate is further metabolized to

propionate, which is excreted. This form of energy conversion is common in parasitic helminths (Tielens *et al.*, 2002).

1.2.4.2.2. Other aspects of metabolism

Apart from oxidative phosphorylation and the TCA cycle, other enzymatic processes also take place in mitochondria. Amino acid metabolism and β-oxidation of fatty acids (associated with the synthesis of ketone bodies), as well as the urea cycle and the biogenesis of Fe-S clusters (Palmieri, 2008) are all –at least partially- located in the mitochondrial matrix.

1.2.4.2.3. Signaling

In recent years, it has become apparent that the role that mitochondria play in receiving, integrating and transmitting signals related to a myriad of cellular processes is vital for the functioning of the organism. Based on the integration of signals stemming from cellular demand for energy, nutrient sensing, cell growth, cell death and stimuli and stresses exerted by the environment of the organism, mitochondria mediate the release of signaling molecules directed to the appropriate target process; retrograde signaling is essential for relaying information from the mitochondria to the nucleus. It was established that the signaling molecules targeted to and transmitted from mitochondria include ions, gases, metabolites, phospholipids, protein kinases, hormones and transcription factors, as well as mitochondrial-generated oxidative stress signals and energy-related signaling (Goldenthal and Marin-Garcia, 2004; Pagliarini and Dixon, 2006). However, the characterization of all signaling pathways revolving around mitochondria is still incomplete. It is beyond the scope of this thesis to give a complete picture of retrograde signaling. As an example, we refer to studies concerning expression of genes for mitochondrial biogenesis, centered around the transcriptional coactivator PGC 1α (peroxisome-proliferator-activated receptor γ coactivator-1 α), a coactivator of nuclear receptors. NAD:NADH, AMP:ATP, $Ca^{2+}$and ROS all have the capacity to influence regulation of transcription of genes required for mitochondrial biogenesis via their indirect action on PGC-1α. One of the possible consequences of modulation of PGC-1α is an increase in transcription of genes implicated in oxidative phosphorylation, impacting on oxidation of substrates and energy production (Ljubicic et al., 2010).

ROS are no longer seen only as causal agents of molecular damage; recently, they have emerged as signaling molecules essential in cellular communication (Droge, 2002). The mechanisms of ROS production are described in section 1.1.2.1.2.2 and 1.2.5; as for their role in signaling, the majority of studies focus on extramitochondrial ROS. There is some discussion about whether ROS derived from the ETS contribute to signaling (Groeger *et al.*, 2009). However, some studies suggest a role for mitochondrial ROS in signaling. Nemoto and co-workers (2000) report that generation of H_2O_2 by mitochondria stimulates the c-Jun N-terminal kinase (JNK), a stress-responsive kinase. This results indirectly in reduced input of glucose into oxidative metabolism. Hurd and co-workers (2007) describe how the activity of a small subset of mitochondrial thiol proteins, mostly belonging to β-oxidation and the regulation of pyruvate dehydrogenase, is modulated by mitochondrial ROS production. In

vascular endothelial cells, mitochondrial ROS may be employed as signaling molecules together with nitric oxide to activate AMP-activated protein kinase (AMPK), a kinase important for cellular energy homeostasis which is normally dependent on AMP:ATP ratios (Quintero *et al.*, 2006). In mammals, it has been shown that the increase in ROS levels associated with hypoxia is essential in stabilizing the transcription factor hypoxia inducible factor-1α (HIF-1α), which is needed for adaptation to hypoxic conditions (Poyton *et al.*, 2009). Mitochondrial ROS can activate mild mitochondrial uncoupling pathways that are themselves key regulators of mitochondrial ROS generation (Brand *et al.*, 2004).

A crucial component of signaling by mitochondria is the regulation of calcium uptake and release. When Ca^{2+} crosses the plasma membrane or is released by the endoplasmic reticulum, it is taken up by mitochondria through the Ca^{2+} uniport, depending on ΔΨ. Mitochondria act as calcium sinks and have a buffering role for the cell. They control the release of Ca^{2+} to the cytosol (Szabadkai and Duchen, 2008). As mentioned in section 1.2.4.1.3, Ca^{2+} also regulates the activity of certain TCA cycle enzymes (McCormack *et al.*, 1990).

Ca^{2+} plays an important part role in apoptosis or programmed cell death. This process of cellular self-destruction is essential for development and for the removal of unwanted cells. Multiple cellular events trigger apoptosis; one of them is massive and/or prolonged accumulation of Ca^{2+} in the mitochondria. When Ca^{2+} is accumulated at high concentrations in the mitochondrial matrix, a chain of events causes the mitochondrial permeability transition pore (PTP) to open, leading to matrix swelling. The molecular components of the PTP are not characterized completely, but most likely, ANT, VDAC and cyclophilin D, a molecular chaperone, take part in its formation. Rupture of the outer mitochondrial membrane causes release of cytochrome *c*, which is a signal for the apoptotic machinery of the cell to exert cell elimination (Jeong and Seol, 2008).

1.2.5. ROS production by mitochondria

In this section, we will look into the principal ways of ROS production by mitochondria. Andreyev and colleagues (2005) describe various suspected sites of ROS production in mitochondria. Cytochrome b5 reductase, monoamine oxidases, dihydroorotate dehydrogenase, α-glycerophosphate dehydrogenase, aconitase and the α-ketoglutarate dehydrogenase complex will not be discussed here; these sites did prove to produce ROS under experimental conditions but their contribution to ROS production under physiological conditions is uncertain. We will focus on ROS produced as a side-effect of oxygen reduction in the ETS (fig 15). A portion of the electrons aimed for transfer to oxygen via a sequential four-electron reduction, reduce oxygen only partly; one-electron reduction results in superoxide generation. The actual site of full reduction of oxygen to water, Complex IV, has not been established as a site of ROS production. Leak of electrons to oxygen occurs at preceding sites in the ETS. Andreyev and co-workers (2005) mention Complex II as a potential but unconfirmed site of ROS production. Much more is known about Complex I and Complex III as the probable origin of mitochondrial ROS (Turrens, 2003).

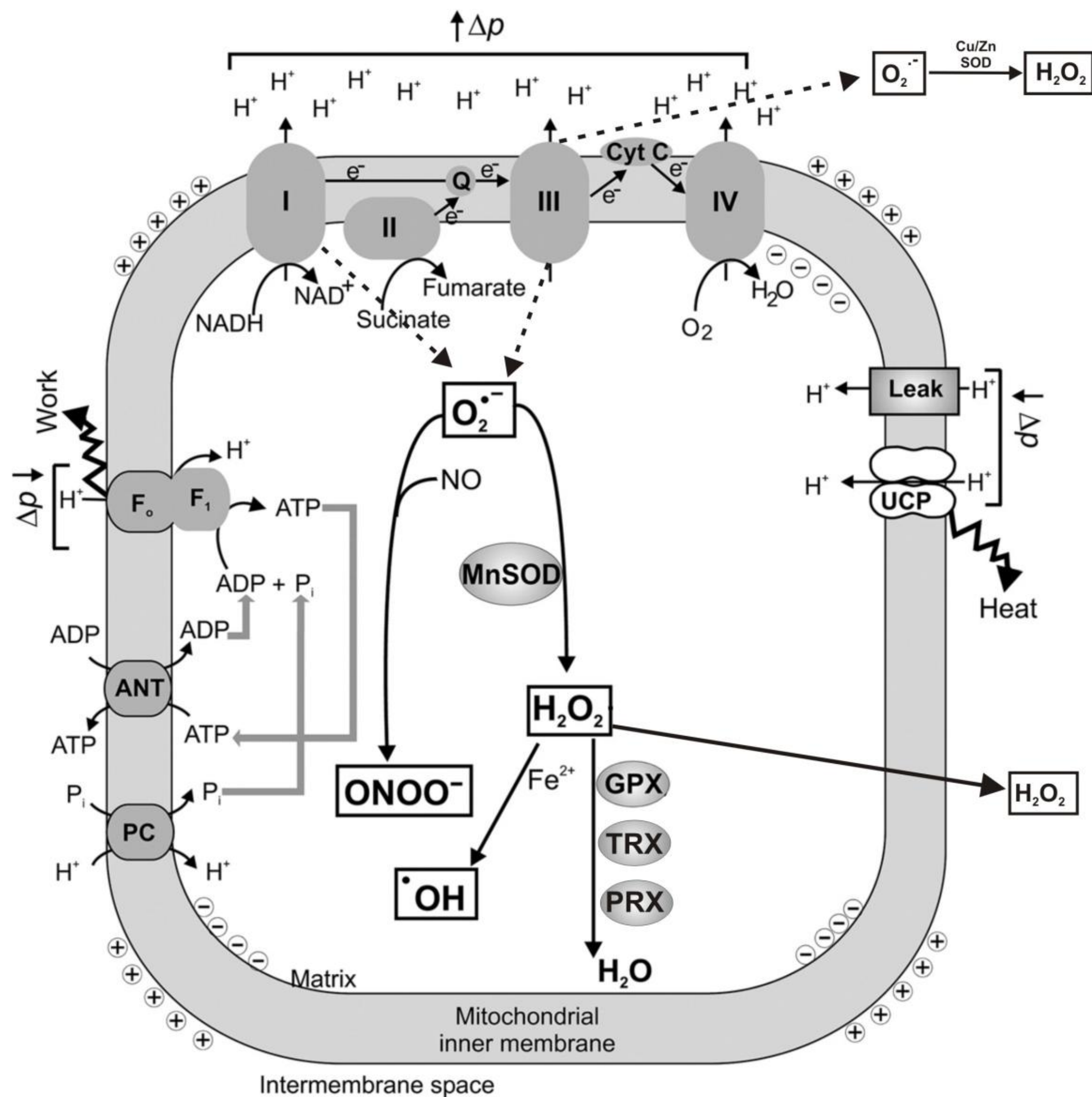


Figure 15: Schematic representation of the mitochondrial electron transport chain. Shown are sites of mitochondrial ROS production, as well as mechanisms of ROS degradation and release, and some of the factors influencing the fate of ROS. Adapted from Echtay (2007).

1.2.5.1. Complex I and Complex III as sources of ROS

Highest levels of $O_2^{\cdot-}$ production are recorded in situations where reverse electron transfer (RET) from Complex II to Complex I is induced (Adam-Vizi and Chinopoulos, 2006). Addition of rotenone, a Complex I inhibitor, suffices to largely abolish RET-dependent $O_2^{\cdot-}$ production (Murphy, 2009). Of note, addition of succinate at levels needed to cause RET is much higher than its physiological concentration (Adam-Vizi and Chinopoulos, 2006). In different experimental conditions, rotenone can increase $O_2^{\cdot-}$ production. High NADH:NAD$^+$ ratios elicit considerable amounts of $O_2^{\cdot-}$ by keeping the FMN centre in a highly reduced form; addition of rotenone in the presence of a NADH-producing substrate like pyruvate, malate or glutamate leads to backup of electrons onto FMN. Conditions of low ATP demand (and low respiration) are also suspected of increasing the NADH:NAD+ ratio. Murphy (2009)

describes a number of *in vivo* situations where this form of $O_2^{\cdot-}$ production may be of significance.

In contrast, in a normally functioning ETS, it was ascertained that ROS production levels are low (Adam-Vizi and Chinopoulos, 2006; Murphy, 2009). They are stimulated by a high membrane potential (Andreyev et al., 2005; Korshunov et al., 1997), but when ATP is synthesized, or when the proton motive force is consumed for other functions, efflux of ROS from mitochondria is considered negligible under physiological conditions.

ROS production by Complex III originates from the reaction of O_2 with an ubisemiquinone bound to the Q_o site (Turrens, 2003). It is important to state that this form of $O_2^{\cdot-}$ production was registered in the presence of the Complex III inhibitor antimycin, which blocks the Q_i site. In the absence of antimycin, $O_2^{\cdot-}$ release from Complex III is low. Also, formation of this semiquinone has never been detected under physiological conditions (Andreyev *et al.*, 2005). Adam-Vizi and Chinopoulos conclude that Complex III ROS generation is of limited physiological importance (2006). However, Murphy remarks that in physiological conditions, when $O_2^{\cdot-}$ production by RET is low, the contribution of Complex III may become relatively significant (2009). *In vivo*, large stretches of time are spent by mitochondria on ATP synthesis and the $O_2^{\cdot-}$ released in this process may turn out to be of greater biological importance.

1.2.5.2. Factors that influence mitochondrial ROS production

A number of factors have been established that influence ROS production by the ETS. From what is known about the modes of $O_2^{\cdot-}$ production by the ETS, it is evident that interference with its workings will affect the process. Damage, mutation, post-translational modifications and inhibition of the ETS complexes all impinge on ROS production (Murphy, 2009). We have already mentioned the inhibitors rotenone and antimycin. Other inhibitors increase or decrease ROS production as well, depending on experimental circumstances (Andreyev et al., 2005; Murphy, 2009; Turrens, 2003). Addition of certain xenobiotics is also capable of increasing ROS production (Turrens, 2003). Fatty acids can cause mild uncoupling of mitochondria, lowering ROS production. This can be countered by adding a protein like bovine serum albumin (Adam-Vizi and Chinopoulos, 2006). Another way of eliciting mild uncoupling is through the activity of UCP's. This activity is augmented by $O_2^{\cdot-}$, constituting a negative feedback system and attenuating $O_2^{\cdot-}$ production at the cost of a slightly lowered oxidative phosphorylation efficiency (Brand *et al.*, 2004). Alternative oxidases have also been suggested to reduce ROS production by using up excess electrons in the ETS and altering the reduction state of respiratory complexes (McDonald, 2008).

Naturally, the presence of antioxidants influences the net amounts of ROS produced. However, the way in which they do so, is a subject of discussion. The beneficial effects of antioxidant defenses have been described in section 1.1.2.1.3; Turrens (2003) stresses that these antioxidant defenses need to be considered when assessing deleterious effects caused by ROS production. However, MnSOD, the superoxide dismutase present in the mitochondrial matrix, has been shown to increase the flux of electrons from electron donors to H_2O_2 (Murphy, 2009).

According to Turrens (2003), $O_2^{\cdot-}$ production should increase when oxygen concentration available to the ETS increases, yet he also reports studies that suggest that $O_2^{\cdot-}$

release is higher in both hyperoxic and moderately hypoxic conditions. Some putative explanations for this phenomenon are given by Murphy (2009). However, high $O_2^{\cdot-}$ production at low oxygen concentrations was not reported by Hoffman and co-workers (2007); instead they showed that ROS emission is almost unaffected by changes in oxygen tension ranging from ambient oxygen levels to as low as the intracellular range. At the present time, it is unclear what the exact influence of oxygen concentration on $O_2^{\cdot-}$ release is (Kowaltowski *et al.*, 2009). In contrast, differences in ROS release dependent on the organism or tissue under study, its physiological state, its age and its hormonal status are widely reported (Kowaltowski *et al.*, 2009; Murphy, 2009; Tahara *et al.*, 2009).

1.2.5.3. Topology of mitochondrial ROS production

It has been established that ROS produced by mitochondria can be released to various locations in and around the mitochondria, particularly in the matrix, on both sides of the inner mitochondrial membrane and on the outer mitochondrial membrane (Turrens, 2003). The topology of $O_2^{\cdot-}$ production by different sources in the ETS was studied in isolated mitochondria, in the presence of combinations of substrates, ETS inhibitors and/or exogenous SOD (Miwa and Brand, 2005; Miwa et al., 2003; Muller et al., 2004; St-Pierre et al., 2002). It can be concluded that while Complex I releases $O_2^{\cdot-}$ to the matrix, Complex III releases $O_2^{\cdot-}$ to both the intermembrane space and the matrix of the mitochondria. These and other (Nohl *et al.*, 2003) experiments showed that under physiological conditions, very low or even no detectable levels of ROS escape from intact mitochondria.

1.2.5.4. Measuring mitochondrial ROS

Since $O_2^{\cdot-}$ is the primary form of ROS released from the ETS, measuring $O_2^{\cdot-}$ is an evident choice when assaying ROS levels. However, direct $O_2^{\cdot-}$ measurement within mitochondria is challenging because it is not released from the mitochondria but dismutates rapidly in the presence of SOD. As will be discussed later, choosing H_2O_2 as a proxy only partly solves this problem, since intramitochondrial H_2O_2 can be consumed by peroxidases (Murphy, 2009). According to Andreyev and colleagues (2005), ROS removal in intact mitochondria may be sufficient to cope with even the highest intramitochondrial rate of ROS production.

The main issue in assessing ROS levels is the difficulty to emulate the *in vivo* situation in experimental conditions. Most often, reports of ROS levels concern isolated mitochondria, where levels of substrates, oxygen concentration and the surroundings of the mitochondrion are all severely different from the intracellular environment. Likewise, the state of activity of isolated mitochondria and the membrane potential are dependent on exogenously added ADP, while *in vivo*, little is known about the physiological state of mitochondria (Murphy, 2009) which very likely varies spatiotemporally within the mitochondrial network due to uneven distribution of antioxidants and variation in metabolic load (Andreyev *et al.*, 2005). Also, other parameters known to change with the physiological condition of the organism influenced by age, health or hormonal status cannot be mimicked *in vitro* (Murphy, 2009).

It has to be noted that, as several methods used for assessment of ROS levels rely on redox properties of ROS, they are prone to artifacts occurring due to similarities of reactivity or the production of intermediates by the probe (Turrens, 2003). It is no surprise then that the percentages of total oxygen consumption originally proposed to be reduced to $O_2^{\cdot -}$ (~3%) can be viewed as a considerable overestimation. Recent studies suggest that mitochondria convert approximately 0.1%-0.3% of the consumed oxygen to superoxide (Fridovich, 2004; St-Pierre et al., 2002).

Measurement of ROS levels produced by isolated mitochondria is based on spectrophotometric or fluorometric methods, chemiluminescence and electron paramagnetic resonance (EPR), also known as 'spin trapping'. Examples of spectrophotometric assays are the reduction of epinephrine or acetylated cytochrome *c* (Brand *et al.*, 2004). Some commonly used chemiluminescent ROS-detectors are luminol and lucigenin (Turrens, 2003). Dyes sensitive specifically to $O_2^{\cdot -}$ are hydroethidine (HE) and an analog of HE, mitoSOX (Zielonka *et al.*, 2008). Also, the inactivation rate of the ETS enzyme aconitase is said to reflect rates of $O_2^{\cdot -}$ production (Murphy, 2009).

A number of dyes are frequently used for H_2O_2 detection in combination with horseradish peroxidase (HRP) and/or exogenously added SOD. For instance, scopoletin loses fluorescence when it reacts with HRP and H_2O_2. Amplex Red forms the fluorochrome resorufin. Peroxyfluor-1 forms fluorescein as an end product without the use of HRP (Adam-Vizi and Chinopoulos, 2006). Assessing H_2O_2 with Amplex Red on isolated mitochondria in the presence of HRP and exogenously added CuZnSOD reflects most of the $O_2^{\cdot -}$ produced by the ETS as follows. $O_2^{\cdot -}$ produced towards the intermembrane space and the cytosol is converted by the added SOD. $O_2^{\cdot -}$ released in the matrix can be converted by the MnSOD contained in the matrix, after which H_2O_2 can exit the mitochondria and react with the detection agent. However, part of the $O_2^{\cdot -}$ may not be converted to H_2O_2 but instead may react with other electron acceptors or $NO^{\cdot}$. Moreover, not all H_2O_2 formed in the matrix exits the mitochondria; it can be scavenged by intramitochondrial peroxidases (Murphy, 2009). In this way, H_2O_2 measurement is at best a fair estimation of $O_2^{\cdot -}$ production. Detection of ROS in intact cells is hampered even more by cytosolic catalase and peroxidases. In this case, more direct detection assays are used like 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) which can enter the mitochondria and fluoresce upon reaction with ROS (Adam-Vizi and Chinopoulos, 2006).

Attempts have been made to assess release of ROS to the environment by relatively uncomplicated live model organisms like the fungus *Podospora* (Sellem *et al.*, 2005) and the nematode *C. elegans* (Chavez et al., 2007). However, in the case of *C. elegans*, it has to be noted that ROS release in undisturbed circumstances is hardly measurable. Only in the presence of pathogenic bacteria can a clear ROS signal be registered, elicited by immunological defense mechanisms.

1.2.6. Oxidative metabolism, mitochondria and aging in selected model organisms and humans

According to the Free Radical Theory of Aging, mitochondria are at the heart of aging (see section 1.1.2.1), and in most model organisms, changes in mitochondrial composition and function with increasing age have been registered, such as decreased activities of the

ETS complexes, increases in ROS release from mitochondria and increased oxidative damage to mitochondrial proteins, lipids and DNA. These changes inevitably affect the organismal oxidative metabolism to a certain degree. However, in aging organisms, mitochondria may not only be affected by the damaging effects of ROS release. Moreover, mitochondrial loss of function may also be consequence rather than cause of the aging process (Figueiredo *et al.*, 2008), though this distinction is difficult to make. Here we describe how aging affects biochemical, bioenergetic and molecular parameters related to oxidative metabolism in a range of model organisms.

1.2.6.1. Gene expression and proteomics

Decreases in expression of genes related to energy metabolism have been reported for *C. elegans*, *Drosophila*, rodents, non-human primates and humans. Components of the mitochondrial respiratory chain, the ATP synthase complex and/or the TCA cycle are affected (Duce et al., 2008; Girardot et al., 2006; Kayo et al., 2001; Kim et al., 2005; Lee et al., 1999a; Lombardi et al., 2009; McCarroll et al., 2004; Pletcher et al., 2002; Preston et al., 2008; Tollet-Egnell et al., 2001; Welle et al., 2003); it can be predicted that these alterations in gene expression will impinge on energy producing reactions of carbohydrate metabolism and mitochondrial bioenergetics. However, for *Drosophila* and rodents, a high degree of tissue-specific regulation was found (Chakravarti et al., 2008; Chakravarti et al., 2009; Preisser et al., 2004; Zhan et al., 2007). Specifically, for *Drosophila*, very limited overlaps of age-related genes and only small overlaps of age-related pathways were established among tissues[1]. Age-related alteration in gene expression in humans depended on the tissue investigated, as direct comparison in human muscle, kidney and brain did not reveal any significant overlap among these tissues. Only when gene sets were compared, some common signature of aging was found, notably a decreased expression of genes encoding subunits of the mitochondrial electron transport chain (Zahn et al., 2006).

Decreases in mitochondrial protein levels that are potentially relevant to aging have also been found in most model organisms mentioned above (Li et al., 2007; Lombardi et al., 2009; Piec et al., 2005; Short et al., 2005; Sohal et al., 2008; Yan et al., 2004; Yang et al., 2008). In rat, tissue-specificity of these proteomic changes has been reported (Chang *et al.*, 2007), as well as age-dependent increases in certain proteins related to the ETS (O'Connell and Ohlendieck, 2009). In primates, gender differences were apparent (Yan *et al.*, 2004). Importantly, from proteomics studies, it has become evident that mRNA levels are not always reliable indicators of protein expression (Willis, 2007): according to Dubessay and co-workers (2007), the content of certain key ETS subunits in *Drosophila* did not decrease, even though transcription of the corresponding genes did decrease.

The causality of changes in gene expression and the proteome for aging can be questioned; the fact that both assays sometimes fail to match, illustrates this issue. The search for similarities in gene expression profiles of different model organisms is probably

1 In different *Drosophila* tissues, Zhan and co-workers (2007) identified a large amount of age-related genes exhibiting clear transcript level changes with age, but of these changes, less than 10% were in common with any other tissue. Moreover, of the biological processes enriched with age-related genes, less than 20% were in common between any two tissues.

the most relevant approach. de Magalhaes and colleagues (2009) have compared microarrays from aging humans and rodents. Common signatures of aging were upregulation of inflammation genes and immune response genes and of genes associated with lysosome function, and underexpression of collagen genes and of genes associated with energy metabolism, particularly mitochondrial genes, as well as alterations in the expression of genes related to apoptosis, cell cycle and cellular senescence biomarkers. Zahn and co-workers (2006) identified common aging signatures in different human tissues and compared these to microarrays performed on aging mice, *Drosophila* and *C. elegans*. One of the human aging signatures identified, a downregulation of genes related to the electron transport chain, was seen in mice and *Drosophila*, but not in *C. elegans.*

1.2.6.2. Metabolic parameters in live organisms and in isolated mitochondria

A substantial decline in metabolic capacity is often seen as a hallmark of aging. Metabolic parameters related to mitochondrial processes that can be assessed in aging organisms include heat dissipation, oxygen consumption, release of carbon dioxide and determination of ATP content. These parameters have been investigated in model organisms such as *C. elegans* and the fruit fly. In *C. elegans*, all metabolic parameters mentioned above decrease with increasing age from early adulthood onwards, though there is variation in their rates of decline (Braeckman et al., 2002; Van Voorhies and Ward, 1999). In other model organisms, changes with age occur, but these are never as severe as in *C. elegans*. For the largest part of *Drosophila*'s lifespan, age-related trends are negligible when compared to those in *C. elegans* (Hulbert et al., 2004; Promislow and Haselkorn, 2002; Ross, 2000; Vernace et al., 2007). In humans, resting metabolic rate decreases at a rate of only 1-2% per decade after 20 years of age (Elia et al., 2000; Manini, 2010).

With regard to TCA cycle enzyme activities, aconitase stands out as an enzyme affected by age in *Drosophila*, housefly and mice (Das et al., 2001; Figueiredo et al., 2009; Yarian et al., 2005; Yarian and Sohal, 2005; Yarian et al., 2006). Its activity also decreases in aging *C. elegans* (unpublished results, Matthijssens F.). In humans, it is uncertain whether age has an influence on enzyme activities of the TCA cycle. Conflicting results were reported for age-related trends in citrate synthase activity, and in skeletal muscle, various TCA cycle enzymes had invariant activities with increasing age (Lanza et al., 2008; Rasmussen et al., 2003a; Short et al., 2005).

Due to conflicting results and tissue-specificities, it is a difficult task to generalize age-associated trends in activities of ETS complexes. Nonetheless, data suggest that the activities of Complex I, IV and V decrease with age in most model organisms. In contrast, Complex II and III generally show limited changes with aging (Barrientos et al., 1996; Choksi and Papaconstantinou, 2008; Cooper et al., 1992; Dubessay et al., 2007; Ferguson et al., 2005; Kwong and Sohal, 2000; Lombardi et al., 2009; Mansouri et al., 2006; Miro et al., 2000; Miyazawa et al., 2009; Muller-Hocker, 1989; Navarro and Boveris, 2007; Ojaimi et al., 1999; Preston et al., 2008; Rasmussen et al., 2003a; Rooyackers et al., 1996; Schwarze et al., 1998b; Tonkonogi et al., 2003; Yarian et al., 2005; Yasuda et al., 2006). Still, it is highly possible that, when more studies on ETS enzyme activities become available, no common pattern among tissues or model organisms will be found.

Through polarographic studies of oxidative phosphorylation by isolated mitochondria, several parameters related to mitochondrial function can be assessed, such as the respiratory control ratio (RCR) and the ADP:O ratio (fig 16). The RCR reflects the control of oxygen consumption by phosphorylation (Lesnefsky and Hoppel, 2006) and illustrates the coupling between respiration and ATP synthesis. It is seen as a measure of the functional integrity of mitochondria (Magalhaes *et al.*, 2005). The ADP:O ratio assesses the amount of molecular oxygen consumed to phosphorylate a known quantity of exogenously added ADP. It is an index of the efficiency of oxidative phosphorylation (Magalhaes *et al.*, 2005). Other parameters that can be assessed alongside the polarographic measurements are the rate of ATP synthesis and the membrane potential.

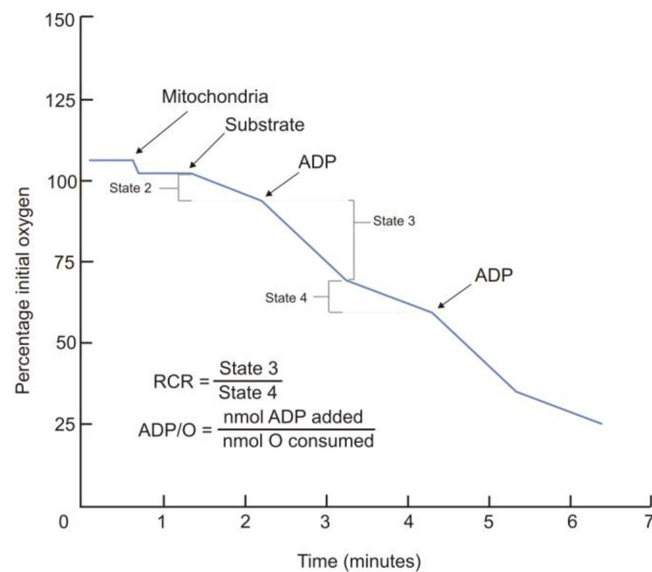


Figure 16: polarographic assessment of oxidative phosphorylation in isolated mitochondria. Mitochondria are supplied with sufficient amounts of substrate (e.g. pyruvate and malate). A known amount of ADP is added, increasing the rate of mitochondrial oxygen consumption (state 3). When the added ADP is used up, the oxygen consumption rate decreases (state 4). The respiratory control ratio (RCR) is calculated by dividing state 3 by state 4. After the amount of oxygen used up during state 3 is calculated, the ADP/O ratio can be determined by dividing the amount of ADP added by the amount of molecular oxygen consumed.

Ignoring some conflicting results and tissue specificities, age-associated decreases in these parameters have been observed for *Drosophila* and (certain tissues of) rodents (Drew et al., 2003; Dubessay et al., 2007; Fannin et al., 1999; Ferguson et al., 2005; Figueiredo et al., 2009; Gouspillou et al., 2010; LaFrance et al., 2005; Mansouri et al., 2006; Meng et al., 2007; Preston et al., 2008; Puche et al., 2008; Tummino and Gafni, 1991; Ventura et al., 2002).

The majority of aging studies on human mitochondria have focused on muscle degeneration. Though some studies reported a decline in mitochondrial function with age in human muscle (Petersen *et al.*, 2003; Short *et al.*, 2005), it has been debated whether this really is the case (Barrientos et al., 1996; Lanza and Nair, 2010; Maklashina and Ackrell, 2004; Tonkonogi et al., 2003). According to Lanza and co-workers (2008), age-related declines in ATP production rates occurred in sedentary but not in trained subjects. Brierley and colleagues (1997a, b) and Rasmussen and colleagues (2003a, b) have shown that mitochondrial function in skeletal muscle of aging individuals was not decreased. What's more, mitochondrial capacities are considered as far in excess of whole-body performance and are related to levels of physical activity, not to age.

1.2.6.3. ROS production, antioxidant defense and oxidative damage

Though increases in ROS production with age, measured as $O_2^{\cdot-}$, H_2O_2 or general ROS, are frequently reported in various model organisms and tissue types (Ali et al., 2006; Cavazzoni et al., 1999; Chabi et al., 2008; Lopez-Torres et al., 2002; Mansouri et al., 2006; Melvin and Ballard, 2006; Miyazawa et al., 2009; Moghaddas et al., 2003; Nabben et al., 2008; Nohl and Hegner, 1978; Ross, 2000; Sastre et al., 1996; Sohal et al., 1994; Sohal and Sohal, 1991), some of these studies contain contradictory findings. We cannot generalize that increase in age is accompanied by increases in ROS production. For example, Yasuda and co-workers (2006) reported no significant age-related change in superoxide anion levels produced by isolated mitochondria of wild-type *C. elegans*, and studies on ROS production in human skeletal muscle reported invariant levels or decreases with age (Hutter et al., 2007; Tonkonogi et al., 2003).

The Vanfleteren & Braeckman group assayed the activity of the antioxidant enzymes catalase and superoxide dismutase rather extensively in the wild type strain (N2) of *C. elegans* grown in various experimental setups (Houthoofd, 2003). Often, no age-related effect on enzyme activity was observed. In some instances, a slight decrease in activity of one or both enzymes was noted, but this decrease was minor compared to the major metabolic changes that are known to occur in aging *C. elegans*. As for other model organisms, no clear increase or decrease of total antioxidant defense with increasing age was apparent. No similarities in age-related trends were obvious when antioxidant enzyme activities were compared among different model organisms; discrepancies were found even among studies on the same tissue of a model organism (Aydin et al., 2010; Bejma and Ji, 1999; Durusoy et al., 1995; Gianni et al., 2004; Hazelton and Lang, 1980; Massie et al., 1980; Meng et al., 2007; Miyazawa et al., 2009; Navarro et al., 2005; Niedzwiecki et al., 1992; Novoselov et al., 2010; Pansarasa et al., 1999; Rao et al., 1990; Sestini et al., 1991; Sohal et al., 1990; Tian et al., 1998; Tonkonogi et al., 2003; Vaanholt et al., 2008).

For all model organisms, reports on increases in oxidative damage, in the form of levels of carbonyls, lipofuscin, 8-OHdG DNA damage and/or mtDNA deletions and point mutations, can be found (Adachi et al., 1998; Nakamura et al., 1999; Yasuda et al., 2006). However, these increases are not always corroborated by analogous studies (Bejma and Ji, 1999; Cocco et al., 2005; Davies et al., 2001; Drew et al., 2003; Figueiredo et al., 2009; Goto et al., 1999; Lopez-Torres et al., 2002; Mansouri et al., 2006; Miyazawa et al., 2009; Navarro and Boveris, 2004; Puche et al., 2008; Schwarze et al., 1998a; Takasawa et al., 1993; Tian et al., 1998; Valls et al., 2005; Yui et al., 2003). Importantly, oxidative modifications do not necessarily lead to changes in the function of the affected proteins or DNA (Choksi and Papaconstantinou, 2008; Musicco et al., 2009; Yarian et al., 2005).

Progressive oxidative damage has been reported in various human tissues (Gianni et al., 2004; Lee et al., 1999b; Michikawa et al., 1999; Miro et al., 2000; Oliver et al., 1987; Pesce et al., 2001; Short et al., 2005). However, for skeletal tissue, ample conflicting results can be found. Hutter and colleagues (2007) found no increase in oxidative modification of proteins in muscle from elderly donors. Likewise, according to Drew and co-workers (2003), 8-OHdG levels were unaffected. As for point mutations in mtDNA, Pallotti and colleagues (1996) found no correlation with age, at least up to 70 years of age. Also, a common

deletion of mtDNA increased with age but its low absolute levels were unlikely to contribute significantly to mitochondrial dysfunction (Cooper *et al.*, 1992).

1.2.6.4. Conclusion

It is evident that for all parameters discussed here, generalization of age-related changes is a difficult task. Very often, different studies assessing the same parameter in a similar model organism lead to conflicting results. Within studies, differences between tissues are repeatedly reported and even when one type of tissue is analyzed, results vary depending on the section or cell type studied. Undoubtedly, these factors are also influenced by different methodological approaches as well as the choice of the biological material used and the extensiveness of the age ranges studied. Naturally, this impedes a comprehensive view of aging trends in energy metabolism, antioxidants and ROS. Moreover, drawing parallels between parameters that are expected to be interrelated and to influence one another is seemingly not feasible. Decreases in gene transcription are not always translated at the level of the proteome, and oxidative changes in protein do not necessarily influence mitochondrial function. Moreover, as will be discussed in section 7.1, interference with mitochondrial function is not always unfavorable for lifespan. Ample evidence of age-related decreases can be found for all parameters related to oxidative metabolism. For instance, expression of genes related to oxidative metabolism is partly downregulated in nearly all model organisms discussed. Similarly, several parameters of mitochondrial function are affected by age and increases in molecular damage are seemingly common phenomena. However, counter-evidence can also be found. In addition, the physiological importance of these phenomena is unclear and conclusions cannot be made without assessing their contribution to the aging process. Our aim is to expand the knowledge of changes in oxidative metabolism in aging *C. elegans* and to evaluate their potential link to the aging process by comparing wild-type profiles with those of a long-lived mutant strain.

1.3. Aging in the model organism *C. elegans*

1.3.1. Introduction: *C. elegans* as a model organism

Model organisms are indispensable scientific tools for understanding complex biological pathways; one of them is *Caenorhabditis elegans*. This free-living roundworm, a member of the Rhabditidae, was chosen as a model organism for genetic studies by Sydney Brenner (Brenner, 2003) based on its short life cycle and generation time, high fecundity, transparency and simple cellular assembly; moreover, genetic alterations are readily introduced using various techniques. It has a fully sequenced genome and is easily maintainable at a reasonable cost. A large number of researchers base their studies on this model organism and their findings on the genetics, genomics and biology of the worm are compiled in an online community database (www.wormbase.org). Moreover, thousands of mutant strains including targeted knockout strains are publicly available to researchers. Large advantages in using *C. elegans* as a model organism are its rapid generation time, its limited number of cells and its hermaphroditism. On the other hand, it has limitations as well: it possesses a subset of genes that have no mammalian orthologs, or that are divergent from mammalian homologs at the sequence level; also, it has a low number of specialized tissues. Importantly, its ease of use as a genetically amenable model isn't always paired with suitability for other experimental studies, such as biochemistry. The reasons for this are the difficulty to obtain large quantities of synchronized individuals, the presence of eggshells and cuticles forming tough barriers and the impossibility of isolating pure tissue in biochemically relevant quantities (Mains and McGhee, 1999).

1.3.1.1. Life cycle and development

A population of this widespread free-living soil nematode consists predominantly of hermaphrodites. Oocytes are produced by the hermaphrodite after the development of sperm has taken place. Self-fertilization leads to a progeny of approximately 300 nematodes. The development from eggs to adult worms occurs through 4 larval stages (L1 to L4) separated by molts (fig 17). The rate of this process is dependent on the temperature of the environment. At 25°C, the life cycle from egg over larval stages and adulthood to egg-laying takes about 2 days. The reproductive phase lasts about 4 days and is followed by a post-reproductive phase of about 2 to 3 weeks.

Higher temperatures, food limitation or overcrowding can lead to the development of an alternative to the L3 stage: the anatomically and behaviorally different, non-feeding dauer stage is capable of enduring adverse environmental conditions for an extended period of time, relying on its fat stores for energy; metabolism and stress resistance are significantly altered in the dauer. Part of the genes controlling the switch to dauer are homologs of the vertebrate insulin-signaling pathway; many play a role in *C. elegans* longevity (see section 1.3.2.1). When conditions improve it molts to the L4 stage and continues its normal development and life cycle.

A rare event in the development of gametes is meiotic non-disjunction. From this, males can originate. Next to 5 sets of autosomes, they contain only one X chromosome and

are recognizable through morphological differences in the tail region. Mating between a male and a hermaphrodite increases progeny size to about a thousand.

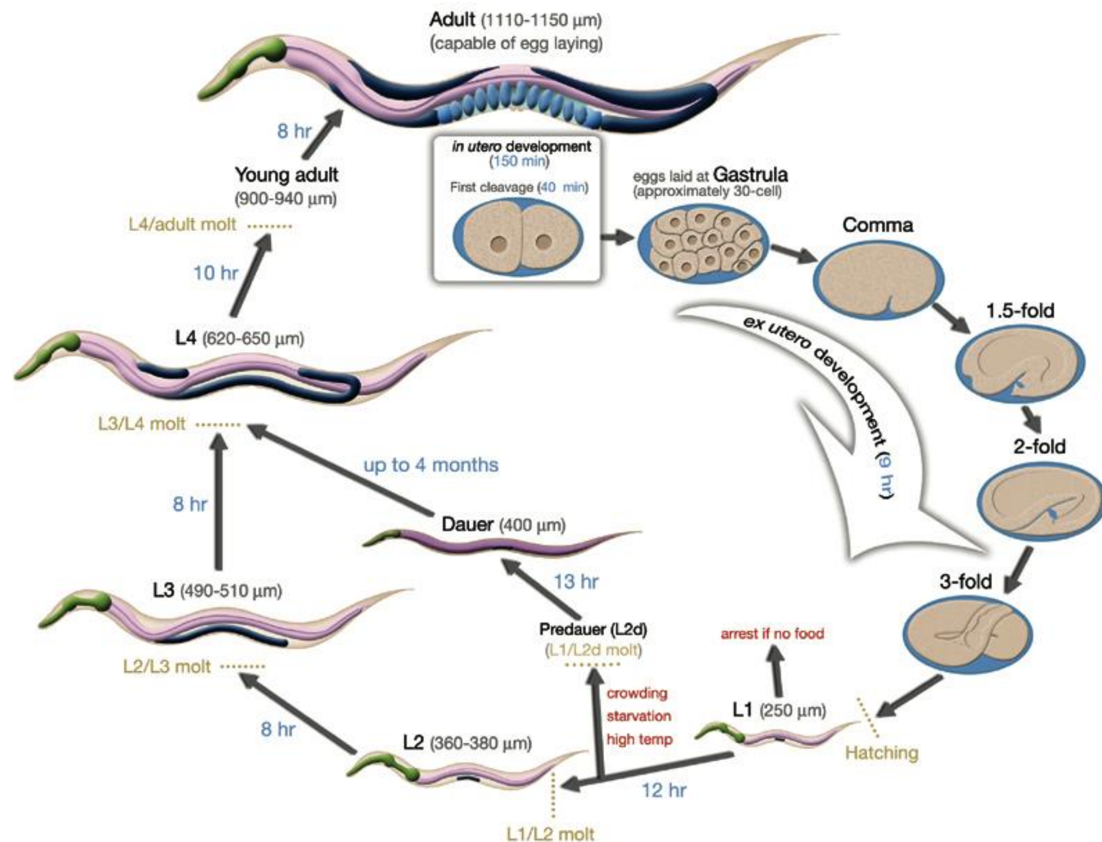


Figure 17: *C. elegans* life cycle. (WormAtlas)

1.3.1.2. Morphology and culturing

C. elegans adults are simple multi-cellular organisms composed of a limited number of somatic, post-mitotic cells; next to germ cells, the adult hermaphrodite contains 959 somatic nuclei; the male has 1031 somatic nuclei. The worm consists of 2 concentric cylinders divided by the pseudocoelomic space. The outer cylinder comprises the cuticle, hypodermis, excretory system, neurons and muscles; the pharynx and intestine are part of the inner cylinder (fig 18). Hydrostatic pressure in the pseudocoelomic cavity, which contains the gonad, maintains the body shape of the nematode.

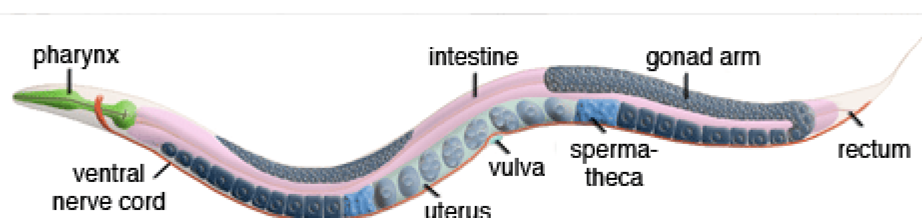


Figure 18: Anatomy of an adult hermaphrodite. Schematic drawing of anatomical structures (WormAtlas).

The body wall consists of a collagenous cuticle secreted by the hypodermis, under which part of the nervous system is situated. A thin basal lamina separates neurons and hypodermis from the muscle system. Body wall muscles, arranged into 4 quadrants, stretch along the length of the nematode and cause its sinusoidal movement. The excretory system, composed of 4 cells at the ventral side of the head and an excretory pore, functions in osmoregulation and waste disposal.

The alimentary system consists of the pharynx, the intestine, rectum and anus. The pharynx, equipped with its own nervous system, muscles and epithelium, passes food from its lumen to the intestinal lumen via the intestinal pharyngeal valve. The gut is connected to the rectum by a rectal valve.

The reproductive system, located in the pseudocoelomic space, comprises the somatic gonad, the germ line and the egg-laying apparatus. The spermathecae connect the uterus to the two U-shaped gonad arms, one orientated anteriorly, the other posteriorly but both consisting of an ovary and oviduct. Oocytes mature while passing through the oviduct; fertilization takes place in the spermatheca, zygotes are stored in the uterus and pass through the vulva located at the center of the animal, at the ventral midline. Gonads in the male consist of only one arm, connected to the cloaca via the vas deferens. The copulatory apparatus at its tail is a fan-shaped structure morphologically shaped for mating.

C. elegans hermaphrodites contain 302 neurons and have two distinct nervous systems: the somatic nervous system, found throughout the body of the nematode, and the pharyngeal nervous system, contained within the pharynx. Sensory organs in the head and tail receive signals from the environment; amphids are the principal sensory organs located on the head, while phasmids are situated in the tail region.

Though *C. elegans* is mostly found as dauers in the soil, it is known that non-dauers feed on bacteria growing on decaying vegetable matter. *C.elegans* is a colonizer of nutrient- and microorganism-rich organic material, though the full range of microorganisms it can feed on in nature is not known (Kiontke and Sudhaus, 2006). *E. coli*, either grown on an agar surface or added to liquid medium is a surrogate food source in laboratory conditions. These bacteria can sustain growth and reproduction of *C. elegans* for an indefinite number of generations. Also, a chemically defined medium can be prepared that sustains the nematodes in the absence of bacteria. This medium needs to contain minerals, glucose, amino acids, vitamins, growth factors and precursors for nucleic acid synthesis (Buecher et al., 1966; Lu and Goetsch, 1993; Sayre et al., 1963; Szewczyk et al., 2003). It can be substituted by a mixture of soy peptone and yeast extract, but for both media, further nutritional requirements include sterols and a source of heme (Hieb and Rothstein, 1968; Hieb et al., 1970; Szewczyk et al., 2003; Vanfleteren, 1974). Moreover, *C. elegans*, preferably juvenile stages, can be frozen, placed in liquid nitrogen for long-term storage and recovered at a later date. In this way, the *Caenorhabditis Genetics Center* or CGC (www.cbs.umn.edu/CGC/) keeps a large stock of strains and mutant alleles available to all, which allows researchers to build their own collection of appropriate strains that can be thawed and cultured when needed. Wormbook offers a review of available protocols for maintenance of *C. elegans* cultures (Stiernagle, 2006).

1.3.1.3. Intermediary metabolism

Most probably, the major metabolic pathways of eukaryotic intermediary metabolism are represented in *C. elegans.* The nematode has orthologs for the majority of key enzymes involved in these pathways (Vastrik *et al.*, 2007), suggesting that the intermediary metabolic network is well conserved among eukaryotes. Despite the fact that *C. elegans* as a model organism is not very suitable for classical biochemical study, several investigators have attempted and succeeded in unveiling the biochemical events that occur in *C. elegans*' life cycle (Bolla, 1980). This has been achieved not only by studying *C. elegans* but also *C. briggsae*, a sibling species with only a few subtle morphological differences and with approximately 62% of the predicted *C. briggsae* genes being one-to-one orthologs of *C. elegans* (a further 33% has one or more clearly detectable *C. elegans* homologs) (Stein *et al.*, 2003).

Under aerobic conditions, *Caenorhabditis* metabolizes energy via the standard metabolic pathways. The energy sources it can employ in laboratory conditions are long-chain fatty acids, ethanol, n-propanol, acetate (Lu *et al.*, 1978) and also glucose, glycogen, trehalose, fructose and sucrose (Lu and Goetsch, 1993); di- and tricarboxylic acids can probably also be transported into gut cells and used as fuel for the TCA cycle (Fei *et al.*, 2004). Storage of energy in free-living nematodes is generally under the form of lipids. One third of dry body mass in *C. elegans* is lipid, half of which is formed by triacyl glyceride as a form of fat storage (Cooper and Van Gundy, 1971; Lee and Atkinson, 1976). Another, less extensive store of energy is carbohydrate, primarily glycogen but also trehalose and glucose (Cooper and Van Gundy, 1970; Foll et al., 1999; Hanover et al., 2005). An enzyme regulating the relative use of these storage macromolecules is O-GlcNac transferase (OGT); knocking out the gene for this enzyme increases trehalose and glycogen levels and decreases triglyceride levels (Hanover *et al.*, 2005). Starvation leads to rapid metabolization of glycogen; in dauers, lipid reserves are the major source of energy for long-term survival (Cooper and Van Gundy, 1970; O'Riordan and Burnell, 1990).

According to (Murfitt *et al.*, 1976), *C. elegans* possesses a system for oxidative metabolism which is quite similar to that of mammalian species. Depending on conditions and nutrient availability, flow of intermediates from glycolysis to the TCA cycle may be altered; a candidate modulator is the enzyme pyruvate dehydrogenase kinase, a negative regulator of pyruvate dehydrogenase. As seen in section 1.2.1, an alternative to glycolysis is the pentose phosphate pathway, breaking down glucose derivatives for anabolic purposes. Evidence for the occurrence of this pathway was shown in *Turbatrix aceti*, another small free-living roundworm member (Panagides and Rothstein, 1973). A partial alternative to the TCA cycle is the glyoxylate pathway, where acetyl-CoA is converted to succinate and malate instead of being oxidized completely to CO_2. This pathway is represented in *C. elegans* but it consists of only one bifunctional glyoxylate cycle protein containing separate domains for both isocitrate lyase (cleaving isocitrate to succinate and glyoxylate) and malate synthase (condenses glyoxylate with acetyl-CoA to form malate) activities on a single polypeptide (Liu *et al.*, 1995).

1.3.1.4. Antioxidant defenses

In *C. elegans*, antioxidant defense mechanisms similar to those of higher organisms are present. In contrast, these nematodes often contain more isoforms than antioxidant enzymes of other eukaryotes. Many of these still lack functional characterization.

Unlike higher animals, which mostly contain only one cytosolic, one mitochondrial and one extracellular SOD, *C. elegans* contains 6 SODs. Both SOD-1 and SOD-5 are cytosolic SODs; the gene *sod-1* is an isoform expressed during normal development and *sod-5* is upregulated in dauers. Two mitochondrial MnSODs are known: SOD-2 and SOD-3. Again, the gene encoding one of these SODs is specifically expressed in reproductive development (*sod-2*), the other during the dauer stage (*sod-3*) (Giglio et al., 1994a; Giglio et al., 1994b; Honda and Honda, 1999; Hunter et al., 1997; Jensen and Culotta, 2005; Suzuki et al., 1996; Wang and Kim, 2003). A fifth gene encoding SOD is *sod-4*. Alternative splicing results in two isoforms of the SOD-4 protein (Fujii et al., 1998). SOD4-1 is homologous to extracellular, secreted mammalian Cu/ZnSOD; SOD4-2 probably remains attached to the cell surface after secretion.

The *C. elegans* genome contains a tandem array of three catalase genes (Petriv and Rachubinski, 2004). *ctl-1* is predicted to encode a cytosolic catalase (Taub et al., 2003; Togo et al., 2000). The protein encoded by *ctl-2* is peroxisomal ; it contributes up to 80% of total catalase activity. *ctl-3* is expressed in muscle and neurons of the pharynx.

At least seven putative glutathione peroxidase (GPX) genes are present in the *C. elegans* genome. However, Vanfleteren (1993) could detect no GPX activity with tert-butyl-hydroperoxide or H_2O_2 as substrates; perhaps these GPX reduce other lipid hydroperoxides.

Likewise, the *C. elegans* genome contains several putative thioredoxin orthologs. For one of them, *trx-1*, it has been established that it is expressed in ASI and ASJ neurons (Jee et al., 2005; Miranda-Vizuete et al., 2006). Thioredoxin reductase has been found in the cytosol and in mitochondria (Gladyshev et al., 1999; Lacey and Hondal, 2006). Three genes are predicted to encode peroxiredoxins: *CePrx-1*, *CePrx-2* and *CePrx-3*. *CePrx-1* codes for a mitochondrial peroxiredoxin, and *CePrx-2* is expressed in distinct pharyngeal neurons (Isermann et al., 2004).

51 putative glutathione-S-transferases (GST) are encoded in the *C. elegans* genome. GST-4 protects against paraquat, and GST-5, GST-6, GST-8, GST-10 and GST-24 increase resistance to HNE (Ayyadevara et al., 2007; Leiers et al., 2003; Tawe et al., 1998).

Other forms of antioxidant defense present in *C. elegans* include metal trafficking proteins, encoded by *mtl-1* and *mtl-2* (metallothionein) and *ftn-1* and *ftn-2* (ferritin heavy chain), the methionine sulfoxide reducing enzyme MsrA (methionine sulfoxide-S- reductase) and the enzyme NNT or mitochondrial nicotinamide nucleotide transhydrogenase. This enzyme reduces NADP+, thus providing NADPH necessary for reduction of glutathione (Arkblad et al., 2005; Freedman et al., 1993; Kim et al., 2004; Lee et al., 2005).

1.3.1.5. Genetics

C. elegans has 5 pairs of autosomes and 1 (males) or 2 (hermaphrodites) X chromosomes. The genome has been fully sequenced (*C. elegans* Sequencing Consortium, 1998). It consists of about 100 000 000 base pairs and this DNA sequence is publicly

accessible (http://www.wormbase.org). The *C. elegans* genome contains approximately 20,000 protein-coding genes. Knowledge of the *C. elegans* genome has allowed establishing the extent of similarity of its genes to those of humans; for instance, approximately 40% of genes that are associated with human disease have homologs in the *C. elegans* genome (Culetto and Sattelle, 2000).

The sexual dimorphism of *C. elegans* advances this species' use for genetics. Homozygous mutations can be maintained by self-fertilization; males can be used for genetic crosses. In order to obtain a larger amount of males than that which is observed in a normal culture (0.1-0.2%), L4s can be heat-shocked; an alternative is the use of mutations or RNA interference (RNAi) that increase the frequency of males in a population (*High Incidence of Males* or *Him*). The process of crossing, which is used not only for obtaining double mutants but also for genetic mapping of mutations, is discussed at length in a WormBook chapter (Fay, 2006).

Much knowledge about the genetics of an organism and the function of genes is derived from studying organisms where gene function is altered. Two complementary approaches are used: forward and reverse genetics. In forward genetic screening, treatment with mutagens induces DNA lesions; mutants with an interesting phenotype are then isolated (Brenner, 1974; Duhon *et al.*, 1996). Reverse genetics studies gene function starting from its sequence. Through RNAi, the function of the chosen gene is altered and the effect of this manipulation on the development or behaviour of the organism is studied (Fire *et al.*, 1998). Gene expression is reduced through RNA silencing elicited by introduction of double-stranded RNA molecules; this RNA is administered by soaking, injection or feeding (Grishok, 2005). Homologous and heterologous expression of genes can also be achieved by injecting DNA into the gonad of a hermaphrodite (Mello *et al.*, 1991) or by microparticle bombardment or biolistic transformation (Berezikov *et al.*, 2004); the presence of multiple copies of the introduced DNA in extrachromosomal arrays or integrated in the offspring's genome can result in overexpression.

Next to gene function, the location, timing and extent of expression of a gene is crucial in understanding the genetics of an organism. Owing to the transparency of *C. elegans*, *gfp* (green fluorescent protein) reporters can be used to examine the expression pattern of a gene (Chalfie *et al.*, 1994). Global gene expression patterns can be studied via microarrays (Hill *et al.*, 2000) or serial analysis of gene expression (SAGE) (Jones *et al.*, 2001). We refer to a comprehensive review on genomics in *C. elegans* by (Hillier *et al.*, 2005).

1.3.2. Lifespan regulation and mechanisms of lifespan extension in *C. elegans*

There are three major ways by which investigators can significantly influence the lifespan of *C. elegans*: via intervention in the Insulin/IGF pathway, via dietary restriction and through disruption of mitochondrial function. These three approaches have also been shown to affect lifespan in one or more other model organisms.

1.3.2.1. Ins/IGF-1 and DAF-16 as regulators of lifespan

In the last 20 years, significant progress has been made in uncovering the genetics of aging in *C. elegans*, triggered by the finding that a single mutation can extend the lifespan of this nematode (Friedman and Johnson, 1988; Kenyon et al., 1993). A mutation in *C. elegans'* sole Ins/IGF-1-like receptor *daf-2* doubles lifespan. The transcription factor DAF-16, a FOXO (Forkhead Box[1] O) family transcription factor, is the main downstream effector of DAF-2 and is required for this lifespan extension (Kenyon et al., 1993; Lin et al., 1997; Ogg et al., 1997). Next to lifespan, this forkhead transcription factor also influences other processes including development and dauer formation (Baugh and Sternberg, 2006; Vowels and Thomas, 1992), thermotolerance (Walker and Lithgow, 2003), resistance to pathogens (Garsin *et al.*, 2003; Jia *et al.*, 2009), metabolism (Kimura *et al.*, 1997; Ogg *et al.*, 1997), autophagy (Hansen *et al.*, 2008; Jia *et al.*, 2009) and stress resistance (Henderson and Johnson, 2001; Murakami and Johnson, 1996). The signaling pathway from DAF-2 to DAF-16 has been unraveled; a large number of studies have attempted to uncover the transcriptional targets of DAF-16, as well as its many co-regulators and co-factors.

1.3.2.1.1. The Ins/IGF-1 pathway

When conditions are beneficial for growth and reproduction, an insulin-like ligand binds to and activates DAF-2, initiating a phosphorylation cascade which inhibits DAF-16. The *C. elegans* genome encodes 40 putative insulin-like ligands (some are agonists of DAF-2, some may inhibit the receptor) (www.wormbase.org) (Duret et al., 1998; Kawano et al., 2000; Li et al., 2003; Murphy et al., 2007; Pierce et al., 2001). A phosphatidylinositol- 3-kinase (PI3K), consisting of a regulatory (AAP-1) and a catalytic (AGE-1) subunit (Morris *et al.*, 1996; Wolkow *et al.*, 2002), is phosphorylated by activated DAF-2. In turn it phosphorylates phosphatidylinositol-(4,5)-bisphosphate (PIP_2) forming phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3). PIP_3 recruits the kinases AKT-1, AKT-2, SGK-1 and the Akt/PKB kinase homolog PDK-1 to the plasma membrane. Activated PDK-1 phosphorylates AKT and SGK-1 (Hertweck et al., 2004; Paradis et al., 1999). DAF-16 is then phosphorylated and inactivated by the AKT-1/AKT-2/SGK-1 complex. While AKT-1 and AKT-2 play a role in the regulation of dauer formation, SGK-1 is crucial for regulation of lifespan and stress resistance (Hertweck et al., 2004; Paradis and Ruvkun, 1998). DAF-16 is sequestered in the cytoplasm (Lin *et al.*, 1997; Ogg *et al.*, 1997), preventing transcriptional activation of its target genes. As a

[1] This transcription factor is termed FOX as it is characterized by a type of DNA-binding domain; this motif is known as the forkhead box. These FOX proteins are classified into subgroups on the basis of sequence similarity. DAF-16 belongs to the O subclass; human FOXO's include FOXO1, FOXO3, FOXO4 and FOXO6.

consequence, reduced Ins/IGF-1 signaling, either through environmental conditions or by a mutation in the Ins/IGF-1 pathway, allows DAF-16 to enter the nucleus and activate expression of these target genes (fig 19).

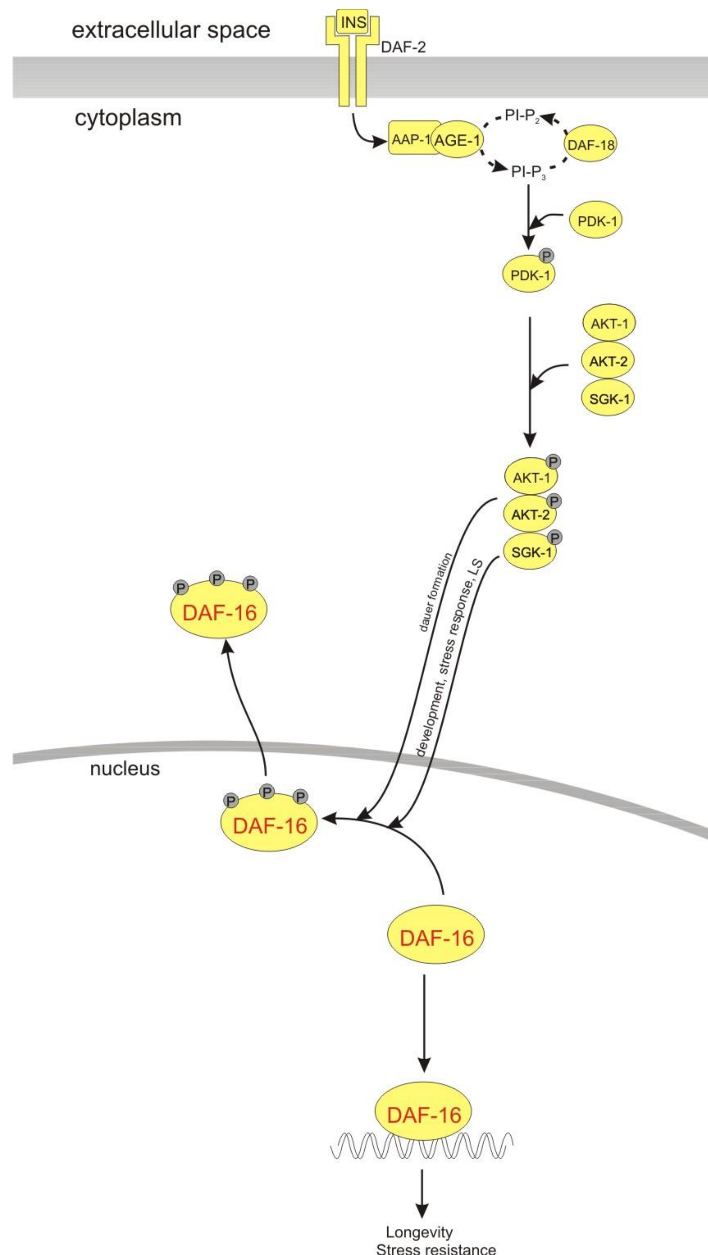


Figure 19: the *daf-2/daf-16* signaling pathway in *C. elegans*. Adapted from Back *et al.*, 2010.

Some inhibitors of this signaling cascade are known: the PIP_3 lipid phosphatase DAF-18 antagonizes the phosphorylation of PIP_2 (Ogg and Ruvkun, 1998), and PPTR-1, a regulatory subunit of protein phosphatase 2A, regulates insulin signaling through AKT-1 dephosphorylation (Padmanabhan *et al.*, 2009). ARR-1, the sole ortholog of the adaptor protein arrestin in *C. elegans*, is a positive regulator of DAF-2 signaling, most likely by negative regulation of DAF-18 (Palmitessa and Benovic, 2010).

1.3.2.1.2. Ins/IGF signaling, dauer formation and lifespan extension

Lifespan extension by reduction of Ins/IGF signaling can be regarded as a secondary consequence of a genetic program designed to adapt to environmental conditions. This pathway allows the nematode to enter the dauer stage when food is scarce. It is most likely not evolutionarily selected to influence lifespan of adults (Antebi, 2007b). A multitude of genes involved in regulation of dauer development have been identified; they belong to 3 major pathways which converge on the nuclear hormone receptor DAF-12: the cGMP signaling pathway, the TGF-β (Transforming Growth Factor- β) signaling pathway and the Ins/IGF pathway (Inoue and Thomas, 2000; Thomas et al., 1993). Mutations in any of the genes partaking in these 3 pathways can elicit inappropriate dauer formation; mutations in the Ins/IGF and TGF-β pathway can cause lifespan extension[2] .

Three important phenotypes caused by mutation in *daf-2* are constitutive dauer formation (Daf-c), increased thermotolerance and increased adult longevity; additional allele-dependent phenotypes were studied in-depth by Gems et al. (Gems *et al.*, 1998). In worms carrying mutations, it is difficult to evaluate the timing with which the mutation exerts its effects on the phenotype. In contrast, RNAi can be initiated and stopped at any stage in the life of the nematode. Logically, Ins/IGF signaling regulates dauer formation during the first 2 larval stadia (Golden and Riddle, 1984). With RNAi it was discovered that *daf-2* RNAi caused lifespan extention regardless of whether it was initiated in the first juvenile stage or in young adults; lifespan regulation apparently requires *daf-2* in the adult stages only while control of stress resistance occurs at both juvenile and adult stages. Moreover, *daf-2* controls reproduction and lifespan independently (Dillin *et al.*, 2002a).

Next to timing of gene expression, location of gene expression plays a role in determining the nematode's phenotype. By creating genetic mosaic worms, it was established that *daf-2* can act cell-nonautonomously to control lifespan, reproduction and dauer formation (Apfeld and Kenyon, 1998). Tissue-specific promotors allow investigators to restore *daf-2* pathway function in desired cell types such as neurons, intestine or muscle. Originally, it was found that restoring DAF-2 and AGE-1 activity in neurons could shorten lifespan in *daf-2* and *age-1* mutants; restoration in the intestine could not (Wolkow *et al.*, 2000). However, more recently, the same group found that the lifespan of an *age-1* mutant could be shortened by restoring AGE-1 in the intestine (Iser *et al.*, 2007).
DAF-16 activity promotes dauer formation in *daf-16;daf-2* mutants when restored in the neurons, and it partially restores lifespan extension when restored in the intestine (Libina et al., 2003). *age-1; daf-16* mutants with DAF-16 activity restored in either neurons or intestine alone had little effect on lifespan; combined expression in both tissues could considerably lengthen lifespan of the double mutants, suggesting that DAF-16 acts incrementally and in multiple tissues to control lifespan.

[2] Longevity through mutation in the TGF-β pathway is masked by an egg-laying defect, preventing extended lifespan; it is only after chemical prevention of this effect that the lifespan-extending phenotype in TGF-β pathway mutants becomes visible (Larsen *et al.*, 1995; Shaw *et al.*, 2007).

1.3.2.1.3. DAF-16 is central to lifespan regulation

Other pathways besides the Ins/IGF-1 signaling pathway regulate DAF-16. In wild type, amphid and phasmid neurons on the nematode's exterior pass on environmental signals that determine the state of the whole organism. Several mutants with defects in cilia structure of these sensory organs were reported to be long-lived; this lifespan extension was partially dependent on DAF-16 (Apfeld and Kenyon, 1999). A number of sensory neurons express insulin-like peptides, potentially linking these neurons to the Ins/IGF pathway (Pierce et al., 2001), and supporting the role of sensory perception in lifespan.

C. elegans lifespan is also under the control of the reproductive system. Removal of the germline extends lifespan in a DAF-16-dependent way; therefore in wild type, the germline acts to shorten lifespan. No such lifespan extension is noted when both the somatic gonad and the germline are ablated, implying that a signal from the somatic gonad counterbalances the germline signal (Hsin and Kenyon, 1999). Ablation of germ cells causes nuclear localization of DAF-16 in intestinal cells (Lin et al., 2001) through endocrine signaling from the reproductive system to the intestine; for DAF-16 to accumulate in the intestinal nuclei, the activity of KRI-1, an intestinal ankyrin repeat protein, is required together with expression of *daf-9* (cytochrome P450) and *daf-12* (a nuclear hormone receptor) (Berman and Kenyon, 2006). In germline-defective worms, DAF-18 (see section 1.3.2.1.1) and SMK-1 (see below) are also required for longevity and nuclear localization of DAF-16 (Berman and Kenyon, 2006; Wolff et al., 2006). In contrast, the lipophilic signaling pathway and *kri-1* are not required for nuclear localization of DAF-16 in *daf-2* mutants; inhibiting the development of the germline in a *daf-2* mutant extends its lifespan further regardless of the presence of the somatic gonad, suggesting the possibility that the somatic gonad signals through DAF-2 to counteract the germline (Berman and Kenyon, 2006; Lin et al., 2001; Mukhopadhyay et al., 2006). Interestingly, Ins/IGF-1 signaling is required for robust larval germline proliferation via the putative insulin-like ligands INS-3 and INS-33, the Ins/IGF-1 pathway kinases and inhibition of DAF-16. However, this inhibition of DAF-16 is germline-specific: it does not occur in neurons nor in the intestine; distinct insulin-like ligands contribute to different phenotypes by acting on Ins/IGF signaling in different tissues (Michaelson *et al.*, 2010).

DAF-16 activity is regulated by various stress-dependent pathways that act in parallel to the Ins/IGF-1 pathway. In response to environmental cues, cytoplasmic DAF-16 is phosphorylated by a molecular sensor for various stresses: JNK-1 (c-Jun NH_2-terminal kinase), a member of the MAPK (mitogen-activated protein kinases) superfamily. This phosphorylation enhances nuclear translocation of DAF-16. JNK physically interacts with DAF-16 at sites different from those of AKT phosphorylation; it is a positive regulator of lifespan as its overexpression leads to increased stress resistance and an extended lifespan. For this effect, DAF-16 is required but not DAF-2 or AKT, implying that the JNK signaling pathway controls lifespan in parallel to Ins/IGF but converging on DAF-16 (Oh *et al.*, 2005).

Another positive regulator of lifespan is SIR-2.1, a member of the sirtuins or NAD+-dependent deacetylases; overexpression of sir-2.1 extends lifespan and increases thermal and oxidative stress resistance in a DAF-16-dependent way (Tissenbaum and Guarente, 2001). Besides DAF-16, the scaffolding/adaptor proteins 14-3-3 are also needed for the positive effect of sir-2.1 expression on lifespan. Reduced expression of *ftt-2* (one of two 14-

3-3 genes encoded in the *C. elegans* genome) promotes dauer formation and nuclear localization of DAF-16 (Li et al., 2007). Berdichevsky and co-workers (2006) propose the following model: under normal conditions, DAF-16 is inactive and retained in the cytoplasm by binding of 14-3-3 proteins at the Akt phosphorylation sites. Following heat stress, DAF-16, still bound to the 14-3-3 proteins, becomes phosphorylated (potentially by JNK-1) at a site different from the Akt phosphorylation sites and enters the nucleus. There, the 14-3-3 proteins bridge the interaction between DAF-16 and SIR-2.1 (Berdichevsky et al., 2006). This pathway thus acts in parallel to the insulin-like pathway to activate DAF-16 and extend life span under conditions of stress. Lifespan prolongation by SIR-2.1 is under debate as recently, conflicting results have been reported by Valentini and co-workers (2010).

CST-1 is yet another DAF-16-dependent positive regulator of lifespan which activates DAF-16 in response to oxidative stress. Its mammalian ortholog, MST1, can induce phosphorylation of DAF-16 in *C. elegans*. This phosphorylation disrupts the interaction of DAF-16 with 14-3-3 proteins, promoting translocation of DAF-16 to the nucleus and longevity (Lehtinen *et al.*, 2006), in a pathway parallel to Ins/IGF-1. In the nucleus, phosphorylated DAF-16 can be bound and activated by the *C. elegans* B-catenin homolog BAR-1. *bar-1* is needed for expression of *sod-3* under conditions of oxidative stress (Essers *et al.*, 2005).

DAF-16 activity can be influenced in other ways as well. The transcription factor HSF-1 (Heat Shock Factor-1) functions together with DAF-16 to activate expression of a specific subset of stress defense genes, including the small heat shock proteins (shsp, e.g. hsp-16.1 and hsp-16.2); inactivation of HSF-1 does not affect nuclear localization of DAF-16 in *daf-2* mutants, and loss of DAF-16 does not diminish the induction of several HSF-1 targets under conditions of heat stress (Hsu *et al.*, 2003).

The nuclear co-regulator SMK-1 (originally identified as suppressor of MEK in the fungus *Dictyostelium discoideum*) co-localizes with DAF-16 to modulate part of DAF-16's activities. It is needed for longevity of *daf-2* and germ-line ablation, and for immune, UV and oxidative stress response (Wolff *et al.*, 2006); it affects expression of certain oxidative stress (*sod-3* & *ctl-1*) and pathogen (*lys-8*) response genes.

HCF-1 (host cell factor 1) is a negative regulator of DAF-16 (Li *et al.*, 2008). It physically associates with DAF-16 and co-regulates the transcription of a subset of DAF-16 target genes; loss of HCF-1 extends lifespan and increases resistance to oxidative and heavy metal stress.

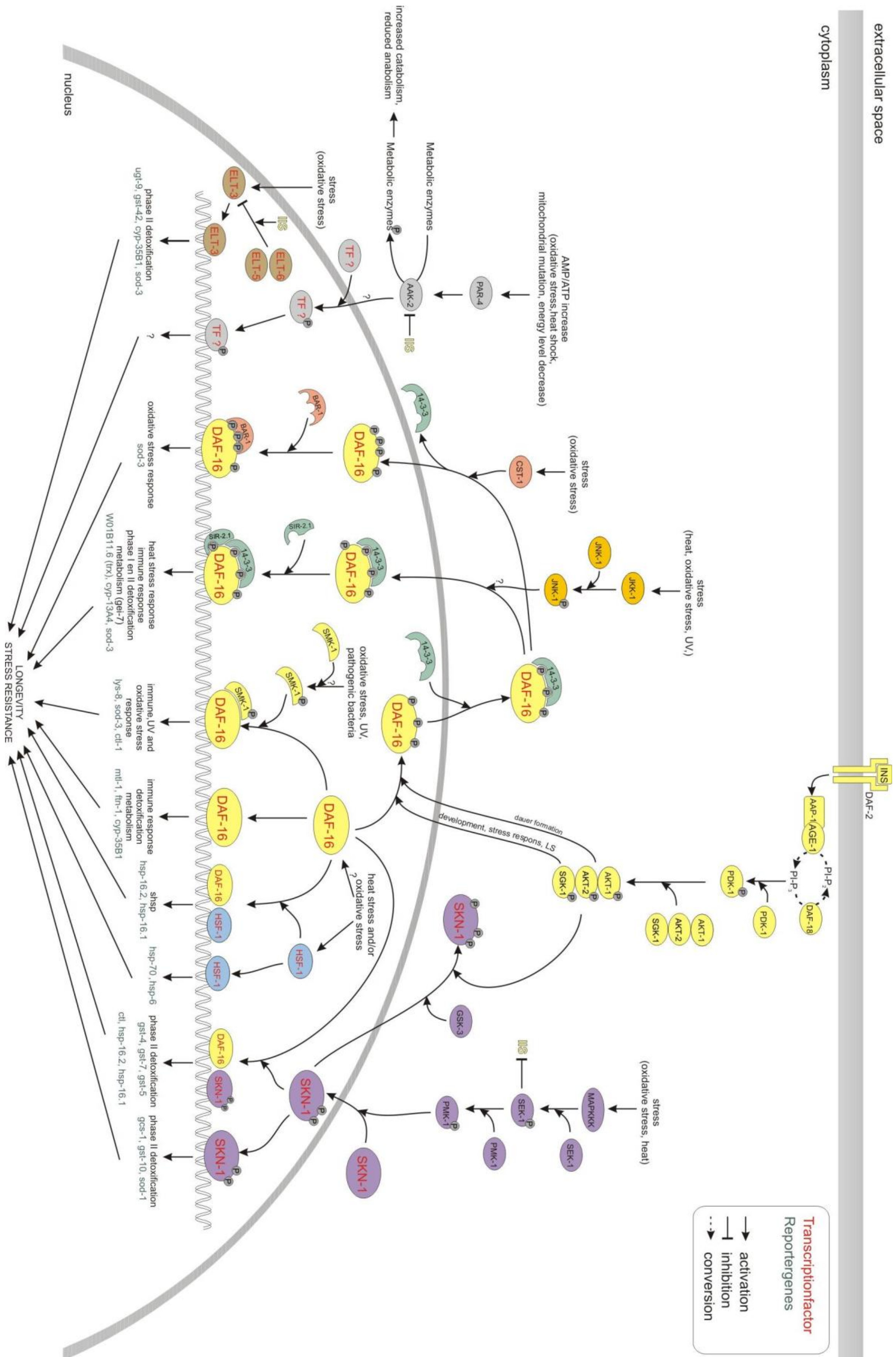


Figure 20: Schematic overview of stress-related pathways associated with DAF-16. Adapted from Back *et al.*, 2010.

Besides DAF-16, Ins/IGF signaling targets another transcription factor related to both stress resistance and lifespan. The transcription factor SKN-1 is expressed in the intestine and activates the phase II detoxification system[3] in response to oxidative stress and is controlled by the p38 mitogen-activated protein kinase (MAPK) cascade (An and Blackwell, 2003; Inoue et al., 2005). In addition, it upregulates expression of genes for detoxification and cellular repair, and downregulates genes for reduction of stress resistance and lifespan, in non-stressed conditions (Oliveira *et al.*, 2009). In the absence of stress, it is located in the cytoplasm and is phosphorylated by glycogen synthase kinase-3 (GSK-3) (An *et al.*, 2005). Moreover, like DAF-16, it is phosphorylated and inhibited by the Ins/IGF-1 pathway kinases. Under conditions of reduced insulin signaling, it accumulates in the nuclei of the intestine and is needed for longevity and stress resistance (Tullet *et al.*, 2008). According to Tullet *et al.*, SKN-1 increases life span independently of DAF-16 when located constitutively in the intestinal nuclei. There are indications that SKN-1 and DAF-16 act together for induction of a subset of target genes (Tullet *et al.*, 2008). The workings of this potential interaction are as yet unknown.

Recently, a novel signaling arm in Ins/IGF signaling has been described that diverges from PDK-1, functions in parallel to DAF-16 and acts on the protein WWP-1 (WW domain Protein-1) to regulate innate immunity. It is also assumed to positively influence lifespan as *wwp-1* mutants are short-lived; this short lifespan is not due to hypersensitivity of the mutant (Chen *et al.*, 2010).

1.3.2.1.4. Downstream transcriptional targets of DAF-16

Because of the role of Ins/IGF-1 signaling in lifespan extension, investigators have attempted to uncover its downstream targets. In initial studies, particular genes were investigated, often involved in stress response; *sod-3* (MnSOD) (Honda and Honda, 1999), *mtl-1* (metallothionein) (Barsyte *et al.*, 2001), small heat-shock proteins (Walker *et al.*, 2001) and the transmembrane tyrosine kinase *old-1* (Murakami and Johnson, 2001) were found to act downstream of DAF-16.

The discovery of specific DNA sequences able to bind DAF-16 or DBE's (D*af-16 family protein* B*inding* E*lements*) (Furuyama *et al.*, 2000) allowed researchers to find potential DAF-16 targets based on gene sequence. *scl-1*, a gene encoding a cystein-rich secretory protein was identified in this way (Ookuma *et al.*, 2003).

Other methods that aim to reveal targets of DAF-16 are based on transcriptional profiling: large amounts of putative DAF-16 targets were revealed by comparing the expression profiles of strains containing a mutation in *daf-2* with WT or with strains containing mutations in both *daf-2* and *daf-16* [4], via SAGE (Serial Analysis of Gene Expression) and microarraying. These genes were categorized according to their involvement in stress resistance, resistance against pathogens, and metabolism; a large number of genes with unknown function was also reported (Golden and Melov, 2004;

[3] The phase II detoxification system is part of a mechanism to dispose of toxic endobiotic or xenobiotic compounds; it consists of a set of reactions which increase solubility to toxins, aiding excretion.

[4] Often a *glp-4* or *fer-15* mutation is also included in these strains to avoid development of eggs; treatments with DAF-2 versus DAF-2 and DAF-16 RNAi were also compared.

Halaschek-Wiener et al., 2005; McElwee et al., 2003; McElwee et al., 2004; Murphy et al., 2003). Next to *sod-3*, other stress resistance genes found to be targeted are *sod-5*, *ctl-1* and *ctl-2* (catalase genes) and *gst-4* (glutathione S-transferase). Also inducible by DAF-16 are heat shock proteins *hsp-70*, *hsp-90*, *hsp-12* and *hsp-16*, and *mtl-1* (Halaschek-Wiener et al., 2005; McElwee et al., 2003; Murphy et al., 2003), and genes encoding antimicrobial agents like lysosymes *lys-7* and *lys-8* (Murphy *et al.*, 2003). Metabolic genes identified through microarrays are involved in amino acid metabolism, the glyoxylate pathway and ubiquitin-mediated protein degradation, but no indication was found that genes involved in protein turnover and mitochondrial function are DAF-16 targets (Halaschek-Wiener et al., 2005; McElwee et al., 2003; Murphy et al., 2003). Some genes identified also play a role in dauer formation, including small *hsp*s, cytochrome P450, short-chain dehydrogenase/reductase, UDP-glucuronosyltransferase and *gst*'s. Other overlapping gene classes between dauers and Ins/IGF mutants are implicated in gluconeogenesis, the glyoxylate pathway and biosynthesis of trehalose (McElwee *et al.*, 2006).

Lastly, in another method to find DAF-16 targets, ChIP or chromatin immunoprecipitation, antibodies pull down a transcription factor along with crosslinked DNA; it has the advantage that the target genes recognized are direct targets of DAF-16 (Oh *et al.*, 2006). In a recent study, DBE analysis showed that MsrA (Methionine sulfoxide reductase A) is a putative DAF-16 target; this was confirmed by ChIP analysis (Minniti et al., 2009).

A search for common motifs in upstream sequences of genes controlled by DAF-16 resulted in identification of the GATA motif; a transcription factor potentially responsible for regulation of these genes is *elt-3* (Budovskaya et al., 2008). RNAi of the GATA transcription factor *elt-3* suppresses *daf-2* mutant longevity, showing that *elt-3* promotes lifespan. This gene shows a tissue-specific decrease in expression late in life, which affects expression of its target genes (e.g. *sod-3*). Budovskaya *et al*. found that the insulin-like signaling pathway exerts a constant level of regulation on *elt-3* expression throughout life; however this regulation is not connected to the decreased expression of *elt-3* late in life. Regulation of *elt-3* by Ins/IGF is either independent of DAF-16 or indirectly regulated via DAF-16.

1.3.2.1.5. Ins/IGF-1 pathway mediated lifespan regulation is evolutionarily conserved

From reviews by Kenyon (2005) and Tatar and colleagues (2003) it is evident that insulin-like signaling is an evolutionarily conserved mechanism for regulation of aging rate; Insulin and IGF have been implicated in lifespan of worms, flies and mice.

In *Drosophila*, lifespan is increased by reduction-of-function mutations in the Ins/IGF-1 receptor or mutation in the receptor substrate CHICO (Clancy *et al.*, 2001; Tatar *et al.*, 2001; Tu *et al.*, 2002). There are indications that the *Drosophila* FOXO transcription factor (a transcription factor homologous to DAF-16) also regulates lifespan: lifespan is increased following overexpression of dFOXO (Giannakou *et al.*, 2004; Hwangbo *et al.*, 2004).

In vertebrates, there are separate receptors for insulin and IGF-1. For both receptors, reduced activity leads to lifespan extension in the mouse (Bluher *et al.*, 2003; Holzenberger *et al.*, 2003). Lifespan is also extended by reduction in growth hormone most likely by reducing levels of circulating IGF-1 and Ins (Brown-Borg et al., 1996; Coschigano et al., 2003; Flurkey et al., 2002).

Until recently, the involvement of FOXO transcription factors in human lifespan was uncertain, but some studies on human centenarians identify both FOXO and Ins/IGF-1 receptors as genes related to extreme longevity (Flachsbart et al., 2009; Suh et al., 2008; Willcox et al., 2008).

1.3.2.2. Dietary restriction (DR)

First described in the 1930's (McCay *et al.*, 1935), dietary restriction or the reduction of food intake without malnutrition is a lifespan-extending intervention applicable to a wide range of model organisms (McDonald and Ramsey, 2010). Several protocols were developed to subject *C. elegans* to DR, including reduction of bacterial densities (Hosono et al., 1989; Houthoofd et al., 2002b; Kaeberlein et al., 2006; Klass, 1977; Lee et al., 2006), use of mutants defective in pharyngeal pumping (Houthoofd et al., 2002b; Lakowski and Hekimi, 1998; Wang and Tissenbaum, 2006), and growth in a defined medium without bacteria (Houthoofd et al., 2002a; Houthoofd et al., 2005; Vanfleteren, 1978). Importantly, Greer and Brunet (2009) have established that different DR regimens do not induce the same molecular processes to extend lifespan.

Various mechanisms have been suggested as mediators of DR-related lifespan extension in *C. elegans*. The concept of decreased ROS production through slowed metabolism, the role of reduced Ins/IGF-1 signaling as well as the role of *sir-2.1* in DR have been challenged (Hansen et al., 2007; Houthoofd et al., 2002a, b; Houthoofd et al., 2005; Kaeberlein et al., 2006; Lakowski and Hekimi, 1998; Wang and Tissenbaum, 2006). In contrast, the TOR (Target Of Rapamycin) pathway (Wullschleger *et al.*, 2006), a signaling pathway with a crucial role in nutrient sensing, appears to be implicated in lifespan extension by DR (Walker *et al.*, 2005) (see also section 1.3.2.4.1).

Two transcription factors with a verified role in lifespan specific for certain DR regimens are PHA-4 and SKN-1 (fig 21). PHA-4, which plays a role in pharynx development of the embryo, regulates the response of adult *C. elegans* to DR (Panowski *et al.*, 2007). *pha-4* RNAi suppresses the long lifespan of *eat-2* mutants and inhibits the effect of bacterial dilution on wild-type lifespan. Under DR circumstances, levels of expression of *sod-1*, *sod-2*, *sod-4* and *sod-5* are dependent on PHA-4. In contrast, PHA-4 has a negligible effect on *daf-2* mutants, confirming earlier findings that IIS and DR act through independent pathways to extend lifespan in *C. elegans*.

The second transcription factor found to specifically regulate response to DR is SKN-1 (Bishop and Guarente, 2007). As mentioned in section 1.3.2.1.3, an isoform of SKN-1 expressed in the intestine activates the phase II detoxification system in response to oxidative stress; it is another isoform, expressed in the ASI neurons, which is needed for lifespan extension by DR. Bishop and Guarente (2007) found that *skn-1* is activated in these neurons as a consequence of DR, which causes increases in metabolic activity in peripheral tissues, illustrating the importance of cell non-autonomous signaling in lifespan determination.

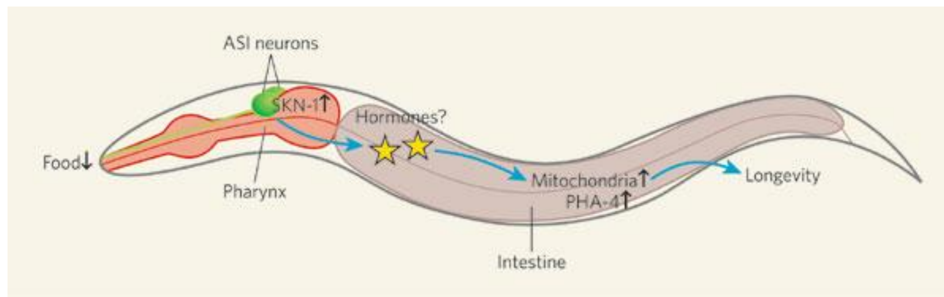


Figure 21: putative mechanism of DR-induced longevity (Antebi, 2007a).

Other genes that have recently been shown to partake in lifespan extension through DR are *nlp-7*, *cup-4* and *wwp-1*. *nlp-7* encodes a neuropeptide-like protein expressed in neurons, and *cup-4* encodes an ion channel functioning in endocytosis by coelomocytes. Both are downstream targets of SKN-1 (Park et al., 2010). *wwp-1* encodes a ligase with a role in protein ubiquitination (Carrano et al., 2009).

1.3.2.3. Mutations or RNAi of mitochondrial components – the Mit phenotype

The first mitochondrial mutation identified in *C. elegans*, *mev-1*(Ishii et al., 1990), is a missense mutation in the cytochrome *b* subunit of Complex II (Ishii *et al.*, 1998). This mutation causes an 80% reduction in Complex II activity along with a high sensitivity to increased oxygen levels and a shortened lifespan (Honda et al., 1993; Ishii et al., 1998; Ishii et al., 1990; Kayser et al., 2004a). Likewise, a point mutation in the gene *gas-1* coding for the 49kDa subunit of Complex I (Kayser *et al.*, 1999) shortens lifespan, slows development, renders nematodes sensitive to elevated oxygen concentrations and oxidative stress and decreases Complex I activity while doubling Complex II activity (Hartman *et al.*, 2001; Kayser *et al.*, 2001). For both *mev-1* and *gas-1*, an increase in ROS production is reported (Kondo et al., 2005; Senoo-Matsuda et al., 2001). Like *daf-2*, *mev-1* mutants show translocation of DAF-16 to the nucleus. According to (Kondo *et al.*, 2005), normal stress response activated by translocation of DAF-16 can be overwhelmed; high levels of ROS production are assumed to be causal to these mutants' short lifespan.

Remarkably, interference with the ETS can also have beneficial effects on lifespan. A mutation in the gene *clk-1*, which codes for an enzyme required for the biosynthesis of the *C. elegans* ubiquinone CoQ9, extends lifespan and slows several temporal processes in the nematode (Felkai et al., 1999; Hekimi et al., 1995; Jonassen et al., 2001; Lakowski and Hekimi, 1996; Miyadera et al., 2001; Stepanyan et al., 2006). The mutation causes accumulation of the CoQ9 precursor DMQ9 (demethoxyubiquinone 9)(Jonassen *et al.*, 2001); the mutant requires bacterial CoQ8 for development (which, remarkably, has been proven to shorten lifespan in adult *clk-1* and WT (Larsen and Clarke, 2002)). Lifespan extension in *clk-1* was originally attributed to slowed metabolic rate (Ewbank et al., 1997; Lakowski and Hekimi, 1996). CoQ shuttles electrons from Complex I or II to Complex III. Complex I activity is considerably decreased in *clk-1*, comparable to *gas-1*; however, Complex II activity is normal (Kayser *et al.*, 2004b). Moreover, *clk-1* whole-worm respiration rates are similar to those of WT (Braeckman *et al.*, 1999). Consequently, use of alternative metabolic pathways, possibly entailing less damage (Rea and Johnson, 2003) is precluded as a cause for lifespan

extension. According to Yang and co-workers (2009), ROS production of *clk-1* is not reduced but DMQ9 helps the mutant to scavenge ROS more effectively. CLK-1 also has mtDNA-binding properties; potentially, CLK-1 plays a role in regulation of mtDNA replication (Gorbunova and Seluanov, 2002).

Another mutant affecting mitochondrial function is *lrs-2*, which carries a mutation in a gene coding for a leucyl-tRNA synthetase, affecting translation of genes encoded by the mitochondrial genome, and consequently, ETS activity (Lee *et al.*, 2003b).

(Feng *et al.*, 2001) describe a mutation in the Fe-S protein of Complex III, *isp-1*. This mutant exhibits a longer lifespan, slow physiological rates, reduced oxygen consumption, increased resistance to exogenously produced ROS (following addition of paraquat) and high levels of *sod-3* expression. The authors interpreted these data as follows: low rates of respiration imply low ROS production, which is underpinned by the mutant's resistance to paraquat. Low endogenously produced ROS levels should allow the organism to use its defense mechanisms to eliminate exogenously produced ROS. Increased *sod-3* expression may reduce ROS even further. Low rates of molecular damage accumulation would then explain lifespan extension in *isp-1*. However, no effort was made to actually measure ROS production in this mutant.

RNAi against certain genes acting in the ETS or involved in its assembly is also capable of extending lifespan. Inactivation of *nuo-2* (a component of Complex I), *cyc-1* (a Complex III subunit), *cco-1* (a Complex IV component) and *atp-3* (a F0F1 ATP synthase subunit) all resulted in lifespan extension, slowed development and low ATP production. In a systematic RNAi screen for lifespan-extension, Lee and co-workers (2003b) found a clear over-representation of mitochondrial function genes affecting lifespan, including genes for mitochondrial carriers, electron-transport chain components and a mitochondrial ribosomal subunit. RNAi causing mitochondrial defects often resulted in lower oxygen consumption, lower ATP levels and altered mitochondrial morphology. A higher sensitivity to paraquat occurred in some RNAi-treatments, while resistance to H_2O_2 was normal or enhanced. This was also seen in worms treated with RNAi against *frh-1*, a gene coding for the mitochondrial protein frataxin which is required for assembly of Fe-S clusters. These nematodes were resistant to H_2O_2 but sensitive to juglone, a superoxide-generating compound (Ventura *et al.*, 2005).

In initial experiments, lifespan extension through RNAi of mitochondrial genes was only observed when applied during development: RNAi administered only during adulthood did not increase lifespan, and restoring mRNA levels to normal during adulthood did not rescue lifespan back to WT. As a possible explanation, it was suggested that a regulatory mechanism determines the aging rate according to mitochondrial function during development (Dillin *et al.*, 2002b). However, recent research shows that this doesn't always need to be the case: lifespan can also be increased through late inactivation (L4 stage) of mitochondrial genes in a strain that is more susceptible to RNAi (Curran and Ruvkun, 2007). Also, effects of RNAi on lifespan can be altered through RNAi dilution: depending on the level of RNAi dilution and RNAi-mediated disruption, lifespan is increased up to a point where mitochondrial function becomes a limiting factor and lifespan decreases (Rea *et al.*, 2007). Lifespan extension is only observed under conditions of partial ETS disruption; the authors term this the threshold effect. Increase in lifespan correlates with reduction in rates

of postembryonic development, fecundity, fertility and adult size. Thus, the authors state that initiation of RNAi must occur in the late L3/ early L4 stage at the latest, when mtDNA increases extensively (Tsang and Lemire, 2002). Under conditions of mitochondrial disruption, oxidative stress and damage are often regarded as key factors in lifespan; however, in this study the authors found little correlation between oxidative stress and lifespan.

The overall conclusion to be drawn from these experiments is that the cause of lifespan extension through mitochondrial disruption is elusive. It is not dependent on DAF-16 and acts independently from IIS signaling (Dillin et al., 2002b; Feng et al., 2001; Lee et al., 2003a; Lee et al., 2003b; Wong et al., 1995). Suggested causes of lifespan extension such as low metabolic rates, low levels of ROS production and alternative metabolic pathways cannot be generalized for all Mit mutants and RNAi treatments; moreover, there are some caveats to consider when comparing worm models with mitochondrial disruptions. Added interventions to different mutants can have divergent effects on lifespan, as is illustrated by Van Raamsdonk and Hekimi (2009): *sod-2* deletion extends the lifespan of *clk-1* and shortens the *isp-1* lifespan. Not only can RNAi results be influenced by RNAi dilution and timing of administration; also, RNAi of a single subunit in the ETS may affect multiple enzymatic steps of electron transfer; e.g. the activity of Complex I is reduced by RNAi of a Complex IV subunit, most likely because the normal supercomplex I:III:IV configuration is precluded (Suthammarak *et al.*, 2009). Importantly, Yang and Hekimi (2010b) have shown that Mit RNAi and Mit mutant worms have considerable differences in their phenotypes, represent two different types of mitochondrial dysfunction and prolong lifespan by distinct mechanisms.

In spite of the issues mentioned, some advances in understanding lifespan extension in the Mit phenotype have recently been made. Lee and co-workers (2010) reported that the hypoxia-inducible factor HIF-1 is needed for lifespan extension of *isp-1* and *clk-1* mutants. They noted an increase in ROS levels for *isp-1* and *clk-1* and proposed a mechanism for lifespan extension in these mutants: possibly, longevity is promoted by activation of HIF-1 through mild increases in ROS. Durieux and co-workers (2011) have established that the mitochondrial unfolded protein response (UPR^{mt}) is needed for longevity in Mit mutants. When mitochondrial defects occur, this stress response mechanism induces expression of mitochondrial associated protein chaperones by the nucleus (Zhao et al., 2002).

1.3.2.4. Other ways of affecting lifespan

1.3.2.4.1. The TOR pathway

Cell growth and metabolism in response to cellular amino acid availability is under the regulation of the conserved TOR (Target Of Rapamycin) signaling pathway. Under conditions beneficial for growth, TOR is active, leading to activation of one of its targets, S6 kinase (S6K), and inhibition of another, the translation initiation factor 4E-BP (not present in the *C. elegans* genome), with increased protein synthesis as a result. When the pathway is inhibited, proteins are degraded, and recycling of cellular components through autophagy increases (Hay and Sonenberg, 2004; Wullschleger et al., 2006).

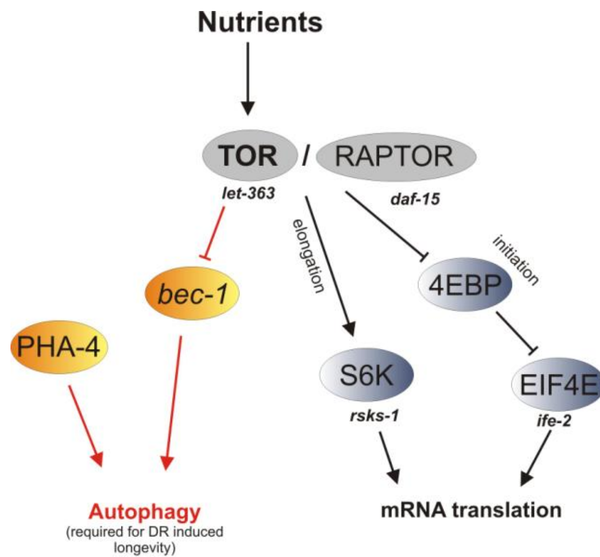


Figure 22: Schematic overview of the TOR pathway.

In *C. elegans*, TOR deficiency affects lifespan significantly. While absence of TOR/LET-363 activity causes larval arrest at L3 through inhibition of global mRNA translation (Long *et al.*, 2002), RNAi of *let-363* more than doubles lifespan (Vellai *et al.*, 2003). In *C. elegans* also, TOR inhibition reduces mRNA translation and increases autophagy (fig 22). In addition, knockdown of genes responsible for mRNA translation, encoding ribosomal proteins and translation initiation factors such as the S6K homolog RSKS-1 or the eIF4E homolog IFE-2, lowers protein synthesis and extends lifespan (Curran and Ruvkun, 2007; Hansen et al., 2007; Pan et al., 2007; Syntichaki et al., 2007). This extension of lifespan through inhibition of mRNA translation is independent of Ins/IGF-1 signaling, DR, the Mit phenotype and the *sir-2.1* pathway. For instance, mutation in *daf-16* does not suppress longevity of *let-363* RNAi nematodes (Vellai et al., 2003).

Administering dsRNA for *let-363* or *daf-15* (which encodes the regulatory associated protein of TOR, RAPTOR) in early adulthood also has a lifespan-increasing effect (Jia et al., 2004; Vellai et al., 2003). Importantly, this type of lifespan extension is not additive to the effect of DR on lifespan (Hansen *et al.*, 2007), suggesting interaction of both pathways. mRNA translation is not the only cellular process influenced by the TOR pathway; autophagy is markedly increased in TOR-deficient worms. In a study by Sheaffer and colleagues (2008), it was shown that the forkhead transcription factor PHA4 is needed for extension of adult lifespan in response to reduced TOR signaling. Moreover, autophagy is required for DR-induced lifespan extension, and the autophagic response to DR requires both TOR and PHA-4 (Hansen *et al.*, 2008). Thus, the induction of autophagy upon nutrient starvation is involved in the regulation of *C. elegans* longevity (Hansen et al., 2008; Jia and Levine, 2007; Toth et al., 2008).

An interaction between TOR and the Ins/IGF-1 pathway has also been revealed. Vellai and colleagues (2003) noticed similarities between worms treated with *let-363* dsRNA and *daf-2* mutants: both affect lifespan during adulthood, and both show lipid accumulation in intestinal cells and reduced fertility, as well as increased embryonic or larval arrest. Moreover, though *daf-16* is not capable of rescuing the lifespan of *let-363* RNAi worms, treatment with *let-363* RNAi cannot prolong the lifespan of *daf-2(e1370)*. From this it was concluded that the TOR and Ins/IGF-1 pathway act in parallel or converge downstream of DAF-16 to extend lifespan.

Mutation of *daf-15* (RAPTOR) results in increased *C. elegans* life span, but this lifespan extension is dependent on functional DAF-16; in other words, DAF-16 negatively regulates *daf-15* transcription (Jia et al., 2004). The following model is proposed: reduction of Ins/IGF-1 signaling activates DAF-16 which represses *daf-15*; as a consequence, TOR

activity is decreased and longevity is enhanced. The authors of this study underpin the importance of autophagy in both TOR- and Ins/IGF-1-related lifespan extension. Autophagy is increased in *daf-2* mutants and required for their longevity phenotype; whether it is sufficient for lifespan extension, has not been verified so far (Hansen et al., 2008; Melendez et al., 2003).

Discerning the effects of *let-363* RNAi on lifespan is complicated by confounding factors. The operon that *let-363* is part of, also contains a mitochondrial ribosomal subunit downstream of *let-363* (Mair and Dilin, 2008). This gene sequence could also become inhibited by *let-363* RNAi. Suppression of the mitochondrial ribosomal subunit extends lifespan independently of DAF-16 (Lee et al., 2003a; Mair and Dillin, 2008). Unraveling the genetic pathway(s) downstream of *let-363*/TOR may be impeded by these confounding factors.

1.3.2.4.2. aak-2

The conserved AMP-activated protein kinase (AMPK) is a sensor of low energy levels (Hardie and Hawley, 2001); under conditions of high AMP to ATP ratios, AMPK is activated. In *C. elegans*, the α subunit AAK-2 of AMPK is a determinant of lifespan: mutation in *aak-2* leads to lifespan shortening, and *aak-2* overexpression increases lifespan (Apfeld *et al.*, 2004).

AAK-2 is needed for lifespan extension and dauer formation in Ins/IGF-1 mutants: mutation in *aak-2* (at least partially) reduces lifespan and inhibits dauer formation of *daf-2* mutants (Apfeld *et al.*, 2004). Since mutation in *aak-2* further reduces the lifespan of a *daf-16* null mutant, it was concluded that *aak-2* is able to influence lifespan in a *daf-16*-independent manner, and that *daf-16* and *aak-2* act in parallel to influence lifespan. Lifespan extension related to hormesis (see section 1.3.2.4.3), *sir-2.1* overexpression, and *isp-1* and *clk-1* mutation is fully or partially dependent on active AAK-2 (Apfeld *et al.*, 2004; Curtis *et al.*, 2006; Schulz *et al.*, 2007). Though germline- and DR-related longevity were reported to be independent of AAK-2, certain DR regimens supposedly need AAK-2 for lifespan extension (Greer and Brunet, 2009; Greer et al., 2007).
From this, it can be assumed that AAK-2 represents a node in a network of pathways influencing *C. elegans* lifespan (Curtis *et al.*, 2006).

1.3.2.4.3. Hormesis

In various model organisms, lifespan can be extended through exposure to a variety of mild stresses; this process is termed hormesis (reviewed by Minois, 2000). Exposure of *C. elegans* early in life with high temperature, high oxygen pressure or a sublethal dose of juglone not only allows nematodes to cope better with subsequent treatments, but also extends lifespan (Cypser and Johnson, 2002).

Being a poikilotherm, *C. elegans'* lifespan is inversely related to temperature (Hosono et al., 1982; Klass, 1977). Recently it was shown that 'thermocycling' WT, and *daf-2*, *eat-2* and *clk- 1* mutant animals between 12°C and 25°C resulted in lifespans similar to those at a constant temperature of 12°C; however, the same treatment on *daf-16* mutant worms could not prolong lifespan. It was shown that incubation at higher temperatures for

short time intervals caused DAF-16-dependent induction of expression of stress-response genes, particularly HSPs, that lead to a significant extension of life span (Galbadage and Hartman, 2008).

Treatment with juglone can also cause hormesis and lifespan extension. This effect is associated with increased expression of HSP-16.2 and enhanced glutathione levels; it was shown to be dependent on both DAF-16 and SIR-2.1 (Heidler *et al.*, 2010).

Another form of stress that can elicit longevity is reduced glucose availability (Schulz *et al.*, 2007). According to the authors, this treatment increased oxidative stress resistance by promoting formation of ROS; in evidence, administering antioxidants prevented this 'mitohormesis'. From this, Ristow and Zarse (2010) deduce that ROS are essential signaling molecules required to promote health and longevity.

1.3.2.4.4. A common metabolic signature for longevity?

As just described, several interventions can indeed successfully extend life span of *C. elegans*, but the mechanisms underpinning this effect are not understood. Moreover, DR and mitochondrial disruption can lead to widely divergent (or even opposite) effects on life span depending on the experimental conditions. Evidently, it is difficult to find a unifying mechanism of lifespan extension from these studies.

Very recently, Fuchs and co-workers (2010) have studied metabolomic profiles of aging Ins/IGF-1 signaling mutants, translation-defective animals, and WT dauer larvae. The authors reported a common metabolic signature, characterized by upregulation of gluconeogenesis and the glyoxylate shunt, and changes in amino acid catabolism. Similar results were obtained with mitochondrial mutants and mitochondrial RNAi-treated nematodes (Falk *et al.*, 2008). Based on these studies, Gallo and Riddle (2010) proposed a prominent role for protein metabolism in determining life span. An upregulation of certain branched-chain amino acids observed in both studies suggests a link to the TOR pathway: for instance, in *daf-2* mutations, DAF-16 would inhibit RAPTOR, decreasing the function of TOR which would result in decreased translation and consequent accumulation of amino acid pools.

1.4. Aims and outline of the thesis

The use of simple model organisms may contribute to our knowledge of a complex process such as human aging. Several mechanisms influencing aging have been identified in a variety of model organisms, indicating that these mechanisms are conserved in evolution. In *Caenorhabditis elegans*, it has been shown that mechanisms modulating the energy metabolism of the nematode can alter lifespan. Dietary restriction, the Clock phenotype, the Ins/IGF-1 pathway and dauer diapause all affect the worm's metabolism. Questions that arise are: are the metabolic changes comparable among these longevity mechanisms? Are these metabolic changes causal to longevity? And if so, through which molecular mechanism do they act to prolong lifespan?

A generally accepted theory of aging is the Free Radical Theory of Aging by Harman (1956). It postulates that reactive oxygen species (ROS) are causal to aging. How could this theory be fitted in to the mechanisms that prolong life in *C. elegans*? The metabolic alterations found in *C. elegans* would have to be linked to either a decrease in the production of ROS, or better defense mechanisms against ROS, or both. Pearl's Rate-of-Living theory (1928) is a potential explanation for decreased ROS production in organisms with decreased metabolic rates: if it is assumed that lower metabolism implies lower ROS production, this low metabolism could be causal to lifespan extension. Studying antioxidant defense mechanisms could also contribute to our knowledge of aging: if increased antioxidant defenses can be unambiguously linked to longevity, this could also strengthen the value of the Free Radical Theory of Aging.

In this study, we aimed to (1) evaluate the validity of the Rate-of-Living and Free Radical theories of aging, and (2) to examine the putative role of energy metabolism in *C. elegans* lifespan.

Through assessment of energy metabolism and antioxidant defense in several long-lived *C. elegans* models, we investigated whether a slower rate of metabolism or an increased antioxidant defense are likely factors in *C. elegans* longevity (chapter 2). The models examined were dauer diapause, Clock mutations and dietary restriction.

These studies were extended to the long-lived Ins/IGF-1 mutant *daf-2(e1370)* in chapter 3. In this chapter we confirmed that *daf-2(e1370)* has a distinct whole-worm energy metabolism, pointing to a higher metabolic efficiency in this mutant.

These studies did not unambiguously support the Rate-of-Living and Free Radical theories of aging. They also showed that energy metabolism was not similar among the mutants and treatments tested, at least not on all levels. However, they did not exclude the possibility that alterations in energy metabolism could affect aging. Especially for the Ins/IGF-1 mutant *daf-2(e1370)*, a study of its mitochondrial characteristics was called for in order to assess the effect of its putative elevated metabolic efficiency on aging. Available studies on mitochondrial gene expression, and an assessment of mitochondrial protein abundances could hint at mitochondrial adaptations in the long-lived mutant. As for energy metabolism, our aim was to evaluate whether the increase in metabolic efficiency *in vivo* could be confirmed in isolated mitochondria. Also, an assessment of mitochondrial ROS production capacity would be a necessary addition to the evaluation of the role of ROS in aging.

To explore this further, we developed a suitable protocol to isolate functional mitochondria from *C. elegans*, and studied mitochondrial characteristics at the protein level (chapter 4). Particularly, we assessed the age-dependent profiles of key mitochondrial proteins and electron transport chain complexes in wild-type and *daf-2(e1370)* adult animals.

Next we examined whether *daf-2(e1370)* mutation alters mitochondrial aerobic energy production (chapter 5). This was done by evaluating bioenergetic competence and membrane potential of mitochondria isolated from wild-type and *daf-2(e1370)* animals.

In order to assess whether ROS levels are determinants of lifespan, we measured ROS production by isolated mitochondria and determined concurrent *in vivo* damage to mitochondrial protein and DNA (chapter 6).

A general discussion of the results is provided in chapter 7, with the emphasis on the potential role of metabolism and ROS in aging and longevity, along with some perspectives for future research.

PART II: RESULTS

Chapter 2

Longevity, metabolism and stress defense in *C. elegans*: dauer diapause, Clk mutants and dietary restriction

Redrafted from:

Houthoofd *et al* (2002c). Ageing is reversed, and metabolism is reset to young levels in recovering dauer larvae of *C. elegans*. Exp Gerontol **37**, 1015-1021

Braeckman *et al* (2002a). No reduction of energy metabolism in Clk mutants. Mech Ageing Dev **123**, 1447-1456

Houthoofd *et al* (2002b). No reduction of metabolic rate in food restricted *Caenorhabditis elegans*. Exp Gerontol **37**, 1357-1367

Houthoofd *et al* (2002a). Axenic growth up-regulates mass-specific metabolic rate, stress resistance, and extends life span in *Caenorhabditis elegans*. Exp Gerontol **37**, 1369-1376

Personal contribution:

Nematode culturing, adaptation and execution of catalase activity assays

Contribution to the general discussion

ABSTRACT

A decrease in metabolic rate and limited ROS damage through antioxidant defense are generally viewed as causal to extended longevity. Three processes known to extend lifespan in *C. elegans* affect metabolism: Clock (Clk) mutations, dietary restriction and the dauer diapause stage. In the following chapter we examine whether metabolic alterations and high antioxidant activity are causal to extended lifespan. Our results show that slow metabolic rate is most likely not the cause of longevity, and that antioxidant defense is not uniformly upregulated in all three cases, questioning its role in lifespan extension.

2.1 Introduction

Based on studies in a wide range of model organisms, several mechanisms of lifespan extension have been described; some of these are believed to be conserved throughout evolution. Dietary restriction (DR) has been shown to extend lifespan in yeast, *Podospora*, *Caenorhabditis elegans*, *Drosophila* and rodents; studies on Rhesus monkeys are ongoing (Anderson et al., 2009; Chippindale et al., 1993; Jiang et al., 2000; Kemnitz et al., 1993; Klass, 1977; Maas et al., 2004; McCay et al., 1935). Lifespans of these model organisms can also be influenced by alterations in mitochondrial functioning, such as induction of the retrograde response (*S. cerevisiae*, (Kirchman *et al.*, 1999)), use of an alternative oxidase (*Podospora*, (Borghouts et al., 2001; Schulte et al., 1988)) and mutations/RNAi causing mitochondrial dysfunction (*C. elegans*, (Feng et al., 2001; Lakowski and Hekimi, 1996; Lee et al., 2003); *Drosophila*, (Copeland *et al.*, 2009); mice, (Dell'agnello et al., 2007; Liu et al., 2005)). The molecular mechanisms underlying longevity through dietary restriction and mitochondrial dysfunction are as yet unknown. However, both interventions affect metabolism, and in both cases, a slowing-down of metabolic processes, reductions in ROS levels and/or increased antioxidant defense have been suggested as possible explanations for lifespan extension (Beckman and Ames, 1998; Feng et al., 2001; Lakowski and Hekimi, 1996; Youngman et al., 1992).

The following section addresses DR and defective mitochondrial functioning in *C. elegans*. In the nematode, DR can be imposed in several ways, either through dilution of the bacterial food source, use of food-uptake-deficient mutants or growth in a semi-defined medium in the absence of *E. coli*. Mitochondrial dysfunction can be caused by mutations in a range of genes related to the ETS; here we focus on the Clk phenotype. Mutations in any of four Clk genes inflict a slow phenotype, slowing down development and behavioral activity, and extending adult lifespan.

Interestingly, *C. elegans* can alter its metabolism and extend lifespan in unfavourable environmental conditions: the nematode is capable of arresting development and entering a diapause state, the dauer larva. Dauers can survive several times the normal lifespan, apparently unaffected by the aging process.

In this chapter, we examine physiological changes associated with these 3 mechanisms of lifespan extension. To evaluate the Rate-of-Living (Pearl, 1928) and Free Radical (Harman, 1956) theories of aging in elucidating causes of lifespan extension, we focus on metabolic parameters and stress resistance.

Our results show that metabolic activities are downregulated in dauers relative to young adults, and are relatively stable. An observed decline in ATP levels can be seen as an indication of an aging process in dauers; however, it is reversible at dauer exit. In dauers, antioxidant capacities are increased, possibly facilitating the survival of the dauer, but it is most likely not the only factor in dauer longevity.

Not one of the four Clk mutants described shows reduced metabolic rates when compared to wild-type. Moreover, Clk mutants show no consistent upregulation of antioxidant activities. Antioxidant capacities are increased by DR, albeit to different degrees depending on the treatment. Metabolic activities vary with the method of DR applied, but there is no indication for lower metabolic rates in DR nematodes.

2.2 Metabolic rate and antioxidant defense during the extreme lifespan of dauer

The most striking physiological characteristic of the dauer to post-dauer transition is that the duration of the dauer state has no effect on post-dauer lifespan. Klass and Hirsh (1976) interpreted dauers to be non-aging, and depletion of stored nutrients to be the cause of their eventual death. To test this hypothesis, we examined several parameters of metabolic activity, including respiration, heat production and ATP content in juveniles, adults, dauers and post-dauer nematodes. Also, levels of SOD and catalase activity were determined to assess antioxidant capacity during the dauer and post-dauer stages.

2.2.1. Materials and methods

2.2.1.1. Strains and culture conditions

The wild type strain N2 was used and maintained at 24°C throughout this study. Synchronous populations were initiated from eggs prepared as follows: gravid worms were dissolved in a mixture of sodium hydroxide and bleach (Sulston and Hodgkin, 1988; Vanfleteren and De Vreese, 1996). The eggs were allowed to hatch overnight in S buffer (0.1 M NaCl, 0.05 M potassium phosphate buffer, pH 6.0). The first stage larvae (L1) were grown on nutrient agar plates containing a lawn of freshly grown *E. coli* 9001 cells. Samples were taken for the various biochemical assays after 0 (unfed L1), 6 (L1), 12 (L1), 18 (L2), 24 (L2), 30 (L3), 36 (L4), 42 (L4) and 48 (young adult) hours. At age 38 h, the worms were rinsed off from a portion of the plates and suspended in S buffer (pH 6.0) containing 10µg/ml cholesterol, at densities not exceeding 2000/ml. Worm suspension cultures were grown in 250 ml portions in Fernbach flasks, shaken at 120 rpm. The worms were fed frozen *E. coli* cells; bacterial density was checked daily by measuring turbidity (550 nm) and kept at a relatively constant concentration of 3 x 10^9 cells/ml. The cells were added from a 1:1 v/v suspension in S buffer, pH 6.0 that was dripped in liquid nitrogen and stored at -75°C. When the worms reached the fourth juvenile stage, FUdR was added at 50 (suspension cultures) or 400 µM (plate cultures) final concentration to prevent progeny production.

Dauers were grown by spreading 150,000 eggs, 10^{10} heat-killed *E. coli* cells and 1 mg haemoglobin (from a 5% stock solution in 0.1N KOH, autoclaved for 10 min) on 10 cm agar (made up with 25 mM potassium phosphate buffer, pH 7.0 and 12.9 µM cholesterol) plates. These conditions induce almost 100% dauer formation. Immediately after dauers had formed, a portion of the plates was harvested and these dauers were transferred into fernbach flasks containing 200 ml S buffer (pH 7.0), and shaken at 120 cycles per minute at 24°C.

Dauers occasionally recovered on the plates with time. Plates containing less than 99% dauers were discarded. Forced recovery after diapause periods lasting for 6 and 27 days was achieved by transferring washed dauers onto nutrient agar plates with freshly grown *E. coli* cells. Dauer survival was near 100% up to 28 days. Metabolism during recovery was monitored by sampling every 3h, for a total of 12h. For investigating post-dauer metabolism, post-dauer L4 juveniles were further grown in liquid culture as described.

Samples were taken at regular time intervals for the various assays. Dead worms, owing to their slightly lower gravity, were removed by centrifugation through 36% (v/v) Percoll in S buffer at 180 g for 2 min (Fabian and Johnson, 1994). Live worms were recovered from the sediment and debris and remaining bacteria were removed by floatation on 40% sucrose (Braeckman et al., 1999; Sulston and Hodgkin, 1988), followed by three washes with S buffer to remove the sucrose. Harvesting was discontinued when the percentage of dead worms in the cleaned sample began to exceed 5%.

For respiration and heat production assays, cleaned, live worms were used immediately after harvesting; the rest of the sampled worm suspension was aliquoted and stored at -75°C for assays that can be performed using frozen worms.

2.2.1.2. Assays

Oxygen consumption

Oxygen consumption was monitored as described in (Braeckman et al., 2002b) with a six-channel Strathkelvin (Glasgow, Scotland) thermostatted respirometer equipped with Clark-type electrodes. Briefly, 1 ml aliquots of nematodes suspended in basal axenic medium (for composition, see 2.4.1) were delivered in the cells of the respirometer; oxygen concentration was registered for 10-30 min and straight sections of the plots of oxygen concentration against time were used to derive the oxygen consumption rate.

Heat production

Heat production was measured with a thermal activity monitor (TA Instruments, New Castle, DE, USA), which accommodates four measuring units. Each unit received a control ampoule (containing assay medium, but no worms) and a test ampoule (medium and worms). The measuring units were sunk into a very precisely regulated water bath and heat flows were monitored. Adults grown on plates were also assayed on an agar surface containing a lawn of autoclaved *E. coli* cells; those grown in liquid culture were suspended in axenic medium, which supports sustained energy expenditure. Antibiotics (250 U/ml penicillin and 0.25 mg/ml streptomycin) were added to prevent interference by bacterial growth for many hours. Dauers were assayed in S buffer or on agar made up in S buffer, because the axenic medium strongly promotes dauer exit.

ATP content

ATP content can be measured by monitoring the amount of light emitted when luciferin reacts with oxygen in the presence of luciferase. This reaction is driven by ATP present in the sample. A frozen aliquot containing a nematode sample (100µl) was taken from the freezer (-75°C) and immediately submersed in a boiling water bath for 15 min to destroy ATPase activity and to release ATP into the medium. All subsequent manipulations were done in a flow bench and sterile solutions were used. After adding 1 ml twice distilled water, the worm corpses were sedimented by centrifugation at 20,800 g for 5 min, and 200µl supernatant was transferred into a microcentrifuge tube and used for preparing four

1/100 dilutions. Portions containing 50µl of an ATP dilution series (within a range of 10 pM to 1 µM) or diluted sample were pipetted into the wells of a white 96 well microtiter plate. Equal volumes of luciferin/luciferase reagent (Roche Diagnostics, Mannheim, Germany) previously reconstituted according to the manufacturer's instructions were added and light emission was monitored by the WallacVictor^2 Multilabel Counter (Perkin-Elmer, Waltham, MA, USA). The average light intensity measured over 30 min, after an initial 10 min period was used to calculate the ATP content.

Worm homogenates

Worm homogenates were prepared to assess SOD and catalase enzyme activities. 100 µl nematode samples were removed from the freezer and homogenized in a Mini-beadbeater (Biospec Products, Bartlesville, OK, USA), in the presence of 100 µl 50 mM Na/K phosphate buffer, pH 7.8 and 100 mg glass beads (0.249-0.318 µm), for 1 min at 5000 rpm. 1% chaps (final concentration) was added and samples were incubated on ice for 15 min. The samples were centrifuged for 8 min at 20,800 g and 4°C; the supernatants were used for enzyme activity assessment.

Superoxide dismutase activity

Superoxide dismutase (SOD) was assayed essentially according to an existing protocol (Corbisier et al., 1987), adapted for high-throughput analysis (Lenaerts et al., 2002). Aliquots of 6.7 µl were taken from a sample dilution series, made from worm homogenate, and added in duplicate to the wells of a white microtiter plate. Next, 20 µl aliquots of xanthine oxidase reagent (xanthine oxidase diluted in double distilled water such that the blank reaction containing 6.7 µl water, 20 µl XO dilution and 174 µl reaction mixture yielded approx. 1.2 x 10^5 counts/s) and 174 µl of reaction mixture (5.2 ml 0.1 M glycine, 1 mM EDTA, adjusted to pH 9.0 with NaOH; 10 ml 0.108 mM xanthine; 2.1ml 1mM lucigenin; 1.2 ml water for a total of 18.5 ml) were added quickly by using a multichannel pipette. Luminescence was measured for 0.1 s during the time span required for 25 consecutive plate measurements at 25°C using the Victor^2 Multilabel Counter. One unit of SOD activity is defined as the amount of SOD able to reduce the luminescence intensity by 50%. The homogenate fraction (dilution) reducing luminescence by 50% was derived mathematically from plots of the luminescence intensities measured as a function of the homogenate fraction.

Catalase activity

Catalase activity was assayed at 25°C according to the method of Aebi (1984), adapted for use in microtiter plate format. Worm homogenates were prepared as described previously and diluted as needed to obtain absorption levels at 240nm between 0.6 and 0.55 at the start of the reaction. 6.9 µl sample volumes were added to the wells of a 96 well flat bottom UV transparent microtiter plate. The reaction was started by adding 200µl substrate (11.4 mM hydrogen peroxide in 50 mM Na_2HPO_4 : KH_2PO_4 (Sørensen) buffer, pH 7.0) using a multichannel micropipette. The decrease in absorbance was monitored at 240 nm

(Spectramax 190, Molecular Devices, Sunnyvale, CA, USA) for 25 reads (12 s interval, total measuring time: 4 min, 17 s). The amount of peroxide decomposed was calculated using a molar coefficient of $\varepsilon_{240nm, 1cm} = 39.4$. The enzyme activity decomposing 1 µmole of hydrogen peroxide per min equals 1 unit of catalase activity.

Protein content

In this experiment, the bicinchoninic acid (BCA; Thermo Scientific, Rockford, IL, USA) method was used to assay protein content as follows: 100µl nematode aliquots were dried overnight in a Savant Speed Vac Concentrator, and 180 µl 1 N NaOH was added to the dry pellets. Fat was degraded by heating at 70°C for 25 min. The concentration of the base was lowered to 0.1 N by adding 1,620 µl distilled water. After vortexing, the tubes were centrifuged at 20,800 g for 5 min and 10 µl portions of the supernatant were pipetted into the wells of a 96 well microtiter plate. A dilution series of bovine serum albumin was included as a standard. Next, 200µl BCA reagent prepared according to the manufacturer's instructions was added. The microtiter plate was covered with sealing tape and incubated at 37°C for 1 h. Absorbance was measured in the Victor^2 Multilabel Counter at 560 nm.

Scaling to biomass

The experimental data were scaled to total protein amount to account for differences in biomass[1]. The metabolic measurements were mostly repeated at least three times to reduce assay variation. The source populations consisting of age-synchronized worms were grown 3-6 times at varying time intervals to reduce inadvertent environmental fluctuation.

2.2.1.3. Statistics

Statistical analyses were performed using algorithms implied in Excel and SPSS.
For further details, we refer to (Houthoofd et al., 2002a).

2.2.2. Results and discussion

Figure 1a represents age-dependent oxygen consumption profiles for juvenile stages, adults, post-dauer adults after 6 or 27 days of diapause, and dauers; cultures on solid and in liquid medium are shown. Oxygen consumption was relatively high in juveniles and was followed by an exponential decline (lack-of-fit test for linearity after log transformation, solid culture, $P = 0.544$; liquid culture, $P = 0.502$) during adulthood. Dauer larvae consumed substantially less oxygen and maintained constant respiration rates over time. Remarkably, respiratory activity rose steeply as the dauers exited their diapause stage; levels of post-dauer oxygen consumption were hardly affected by the duration of the dauer stage (two-

[1] Parameters were also scaled to body volume; normalizing to volume instead of protein content would increase all dauer activities by some 50% →20%, relative to young adult → senescent worms (results not shown). We have to note that allometric scaling as described by Braeckman et al (2002c) has not been performed on this data set.

way ANOVA, *P* = 0.921). Differences in age-specific declines in respiration rate could even be ignored between post-dauers and adults that bypassed the dauer stage (F-test for equality of slopes of several regression lines, *P* = 0.448).

Release of energy as heat can be recorded with a calorimeter. Though both catabolic and anabolic pathways dissipate heat, contribution by anabolism is negligible (Kemp and Guan, 1997). As glycolysis is low at normoxia (Foll et al., 1999), heat dissipation profiles were similar to respiration profiles; for instance: as in respiration, a steep rise was noted when dauers exited diapause. There is one exception: dauers raised on plates, and assayed on an agar surface, dissipated more heat than those in suspension culture (fig 1b). A possible explanation is a higher oxygen supply associated with exposure to a gaseous environment. Respiration did not reveal this effect, likely because it can only be measured in liquid medium. Even so, we have to remark that, while in dauers solid-surface and liquid-state heat dissipation differed greatly, this difference was negligible in adults.

For adult stages, ATP profiles were similar to those of oxygen consumption and heat dissipation[2], yet in dauers, ATP content did not follow respiration and heat production trends. Dauer ATP concentrations decreased gradually with age (fig 1c). This decrease could be interpreted as a result of progressive exhaustion of fat and carbohydrate stores. However, there are indications that this is not the case. If depletion of energy-rich stores were a cause of ATP content decrease, we would expect an initial phase where these stores are not limiting and ATP levels are unchanged, followed by a decrease in ATP when these stores become depleted. Here we show that ATP levels decrease exponentially with time (lack-of-fit test for linearity after log transformation, solid culture,*P* = 0.787; liquid culture, *P* = 0.447) from the first measurement onwards. Moreover, respiration and heat dissipation remain unchanged over the entire time span, confirming that exhaustion of combustible stores seems unlikely. An alternative explanation for decreasing ATP content is the occurrence of declines in mitochondrial function. Importantly, it is not known whether or not this is a regulated process. However, ATP content increased sharply at dauer exit, suggesting that if mitochondrial function decreases over time in dauers, this decrease is readily reversible.

Catalase (fig 1d) and SOD (fig 1e)[3] activities were substantially upregulated in dauers; they declined sharply after exposure to food to stabilize at adult levels. Solid-surface-grown adults had generally higher enzyme activities relative to those in liquid culture. Again, this could be due to a higher oxygen supply, yet this trend cannot be generalized to dauers.

The long life of dauers is often seen in light of the Rate-of-Living theory (Pearl, 1928), where it is assumed that organisms can only spend a predetermined amount of energy during their lifetime, and as a consequence, lifespan is believed to be inversely proportional to metabolic rate. By assuming that molecular damage, caused by free radicals, is lower in organisms with a slow metabolism, the link to the Oxidative Damage theory of Aging

[2] Potential ATP, respiration and heat dissipation profile differences between transitions from juvenile to adult stages can be explained by the fact that metabolic parameters of juveniles and adults were assessed in separate experiments (see legend of fig. 1).

[3] At the time the dauer experiments were done, catalase and SOD activity protocols were the sole antioxidant activity protocols available to the lab. This was also the case for experiments on Clk mutants (section 2.3) and dietary restricted worms (section 2.4).

(Harman, 1956) is easily made. However, based on our findings we can exclude 'rate of living' as the cause of lifespan extension in dauers: oxygen consumption profiles observed for adult worms that bypassed, and those that entered and exited the dauer stage were similar. Therefore potential lifespan is not determined by metabolic rate, and other mechanisms of lifespan extension must be involved. Long lifespan in dauers is associated with enhanced resistance to oxidative stress. Elevated SOD and catalase activities could be essential for dauer survival; however, our findings do not provide strict proof. It must be noted that juvenile stages exhibited high metabolic rates (fig 1 a&b) and low antioxidant activity (fig 1 d&e); this suggests that oxidative stress is most likely not the only cause of aging, and/or that high metabolic rates do not necessarily implicate high ROS production.

Stable oxygen consumption and heat output rates suggest that dauers are non-aging. However, gradual declines of ATP content imply that dauers cannot completely defy aging. Possibly, diminished mitochondrial function is the cause of decreasing ATP stores. Perhaps signs of decreases in mitochondrial efficiency can manifest themselves in decreasing ATP while oxygen consumption and heat production remain unaltered. An indication supporting the fact that not every age-related change is immediately reflected in parameters related to this change, can be found in measured activities of aconitase. The enzyme aconitase is part of the TCA cycle responsible for supplying reducing equivalents to the electron transport system. Though this enzyme's activity decreases in dauers (results not shown), this decrease has no apparent effect on oxygen consumption. However, decreasing ATP concentration is not readily reconciled with stable respiration and heat dissipation. Another potential explanation is a general decrease in nucleotide content with increasing duration of dauer diapause. This decrease could affect standing ATP levels while the available nucleotide pool could still be sufficient to saturate the oxidative phosphorylation of the dauer mitochondrion. An assessment of the nucleotide pool at different time points in dauer diapause would evaluate this possibility. To conclude, mitochondrial aging might occur at the dauer stage, but at dauer exit, aging-related changes disappear promptly and with no apparent consequences on post-dauer life. Therefore we suggest that in recovering dauer larvae, aging is reversed and the 'aging clock' is reset.

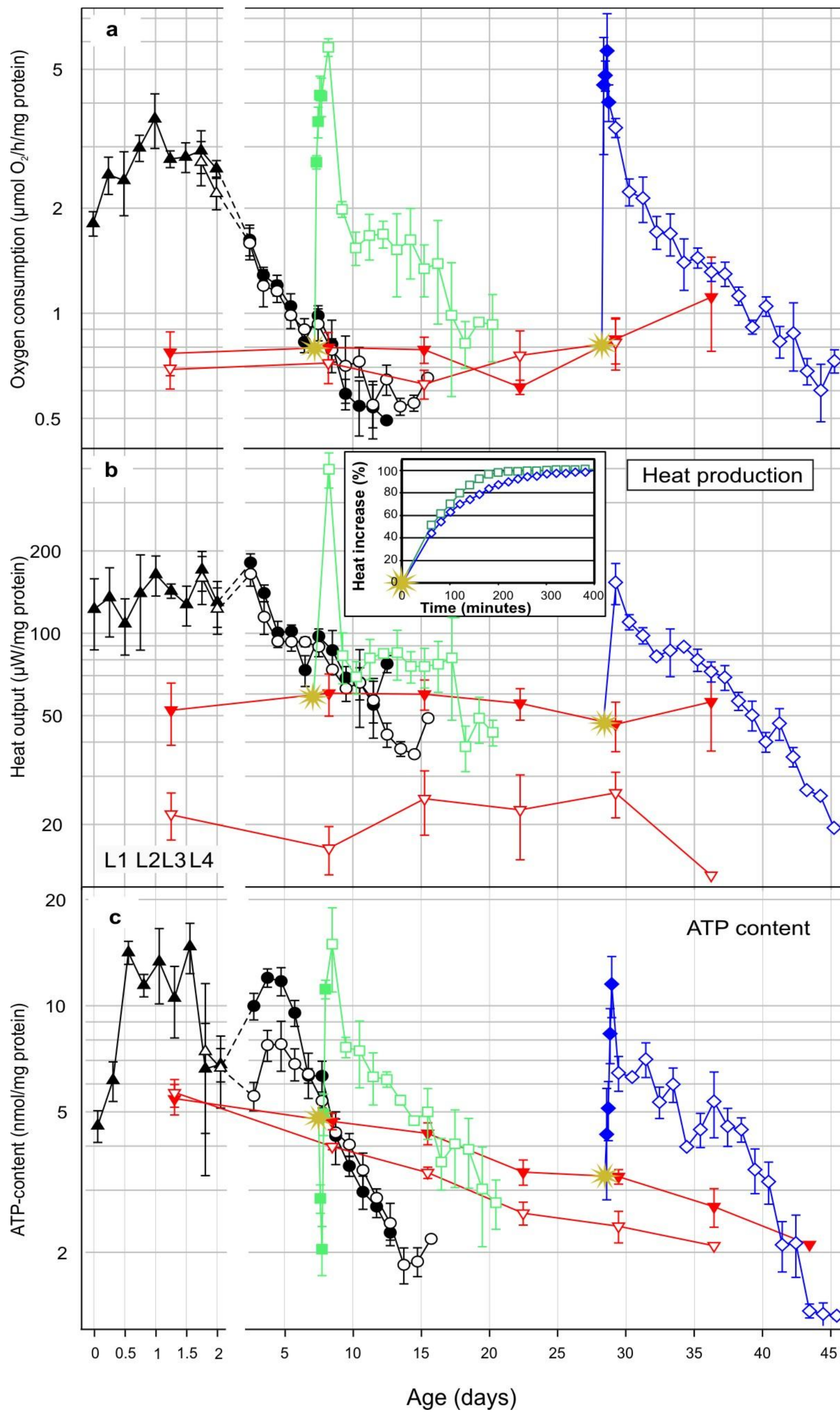


Figure 1: for legend: see next page.

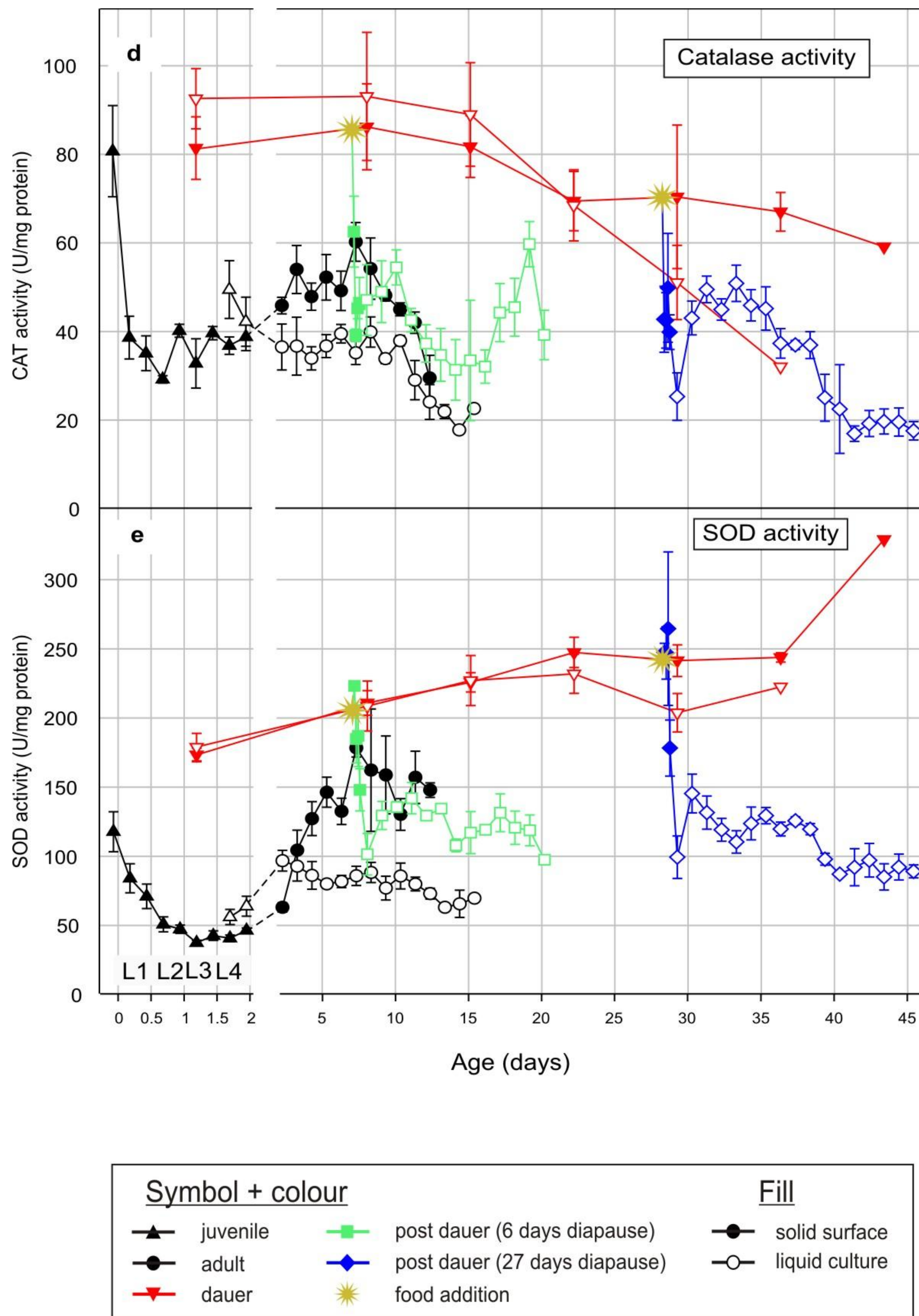


Figure 1 continued: Metabolic and antioxidant enzyme activity profiles as a function of development and ageing in wild-type. a, respiration rate; b, heat production; c, ATP content; d, catalase activity; e, SOD activity. Full lines indicate consecutive sampling of source populations; dotted lines connecting symbols indicate that the data correspond to separate experimental populations. Real time heat increase during dauer recovery is shown as an insert to (b). The relatively long time for stabilisation (30–60 min) and the fast rise of heat production during recovery precludes monitoring of heat production by means of consecutive sampling. L1, L2, L3, L4: first, second, third and fourth larval stage. Data represent the mean ± S.E.M. of three to six independent experiments.

2.3 Clk mutations extend lifespan without major changes in metabolic rate and antioxidant defense

The Clk genes *clk-1*, *clk-2*, *clk-3* and *gro-1* control the timing of a variety of physiological processes. The proteins encoded by these genes seem unrelated; yet for all four genes, mutation causes similar phenotypes: an increase in adult lifespan and a slowing down of development, pharyngeal pumping, defecating, egg laying and moving.

Some of the Clk genes have been molecularly characterized, others await further elucidation. The molecular identity of *clk-3* is unknown. The mitochondrial protein CLK-1 is a putative hydroxylase in the biosynthesis of nonaprenylated ubiquinone (UQ9) (Branicky et al., 2001; Hekimi et al., 2001; Jonassen et al., 2001; Miyadera et al., 2001); however, respiration in *clk-1* mutants is influenced only slightly (Braeckman et al., 1999; Felkai et al., 1999) as DMQ9, a biosynthetic intermediate, accumulates in the mitochondria and can function as an electron carrier. Another putative function of CLK-1 is its involvement in ADP-dependent regulation of mtDNA replication (Gorbunova and Seluanov, 2002). The essential functions of CLK-2 remain ill-defined. At one point thought to be implicated in telomere length regulation (Benard et al., 2001), Ahmed and co-workers (2001) have uncovered a function in DNA damage response signaling for CLK-2. *gro-1* encodes a tRNA-modifying enzyme (isopentenylpyrophosphate:tRNA transferase), required for efficient translation (Lemieux *et al.*, 2001). It can be translated into two different proteins by alternative translation initiation, one of which contains a mitochondrial targeting signal; failure of this particular mitochondrial protein results in the *gro-1* mutant phenotype.

If the assumption is made that slowing of the rate of living is linked to reduction in energy consumption and metabolic rate, and that reduction of metabolic rate would lower ROS production, then the Clk mutants would support rate-of-living and oxidative damage as causes of aging. However, as reported in previous studies, respiration rates and ATP levels were influenced only slightly by mutation in *clk-1* or *gro-1* (Braeckman et al., 1999; Felkai et al., 1999).

Here we assessed changes in energy metabolism (respiration, heat production and ATP content) by mutation in any of the four Clk genes. As in dauers, oxidative stress resistance was evaluated by determining levels of SOD and catalase activity.

2.3.1. Materials and methods

2.3.1.1. Strains and culture conditions

The wild type strain N2 was used as well as Clk mutants *clk-1(e2519)*, *clk-2(qm37)*, *clk-3(qm38)* and *gro-1(e2400)*. The worms were grown at 17-18°C on nutrient agar plates seeded with *E. coli* and synchronous cultures were established as in (2.2.1.1.). Fourth stage larvae were rinsed off the plates, transferred to Fernbach flasks and maintained at 24°C as described in (2.2.1.1.). Sampling was performed as in (2.2.1.1.).

2.3.1.2. Assays

The assays for this experiment (oxygen consumption, heat production, ATP content, SOD and catalase activity and protein content) were executed as described in (2.2.1.2.).

All data were normalized to protein content to correct for size differences[4]. For Clk mutants, source populations were grown in triplicate; wild-type series were collected six times.

2.3.1.3. Statistics

Statistical analyses were performed on log transformed data using SPSS. Strains were compared by using two-way analysis of variance (ANOVA), and pairwise comparisons were made with Scheffé post-hoc tests.

For further details, we refer to (Braeckman et al., 2002a).

2.3.2. Results and discussion

Metabolic rate, as assessed by oxygen consumption (fig 2a) and heat production (fig 2b) rates, was not lower in any of the Clk mutants relative to wild-type: *clk-1(e2519)* and *gro-1(e2400)* mutants had higher respiration and heat dissipation rates ($P < 0.001$) while *clk-2(qm37)* and *clk-3(qm38)* generally matched wild-type. ATP content (Fig 2c) was higher in *clk-1(e2519), clk-3(qm38)* and *gro-1(e2400)* ($P < 0.001$) but similar to wild-type in *clk-2(qm37)* over almost the entire adult lifespan. For the *clk-1* mutant, we have to note that our measurements may even slightly underestimate its true metabolic rate as this mutant is unable to adapt to temperature shifts (Branicky et al., 2001; Wong et al., 1995); after the temperature shift from 17 to 24°C at the L4 stage, *clk-1* metabolic rates may persist at lower levels than those normally expected after a temperature raise.

Antioxidant enzyme activities in Clk mutants were generally different from those of wild-type, but a consistent pattern was lacking. Catalase activity (Fig 2d) was generally elevated (*clk-1*, *clk-3*, *gro-1* $P < 0.01$) but SOD activity (fig 2e) was lower (*clk-1*, *clk-2*, *clk-3* $P < 0.001$) than or similar (*gro-1*) to wild-type.

These results are in conflict with the aforementioned Rate-of-Living and Free Radical theories of aging. First of all, as suggested by respiration, heat dissipation and ATP content, not one of the four Clk mutants showed reduced metabolic rates when compared to wild-type. Secondly, we saw no consistent upregulation of antioxidant activities in these mutants: while catalase levels were generally higher in mutant than in wild-type, SOD activity was lower in all but one Clk mutant. These results suggest that extended longevity in Clk mutants is not attributable to decreased metabolic rate nor to a catalase- or SOD-related increased resistance to oxidative stress.

[4] Data were also normalized to worm volume; these results can be found in Braeckman et al. (2002a). We have to note that allometric scaling as described by Braeckman and colleagues (2002c) has not been performed on this data set. Data on worm volume in the different Clk mutants can be found in the addendum to chapter 3 (section 3.3).

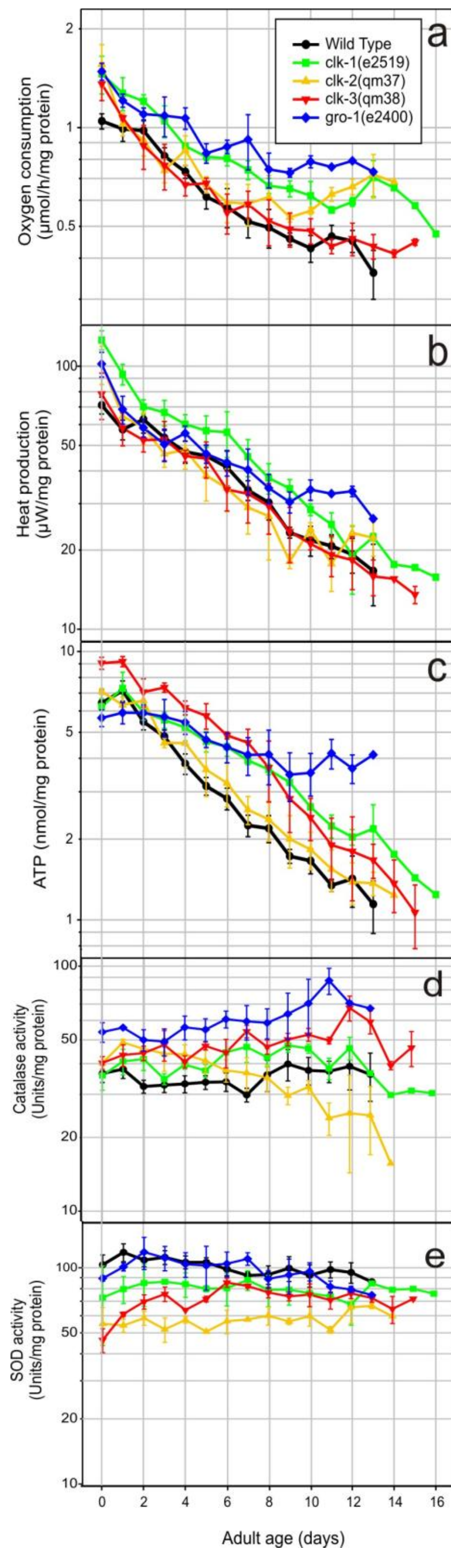


Figure 2:
Longitudinal study of energy metabolism and antioxidant defense in Clk mutants: a, oxygen consumption ; b, heat production; c, ATP levels; d, catalase activity; e, SOD activity. Data were scaled to protein. Data represent the mean ± S.E.M. of three (Clk's) or six (wild type) independent experiments.

Even though according to our data metabolic rate is not downregulated in Clk mutants, mitochondrial function, and electron transport in particular, may be indirectly involved in the slow phenotype. *clk-1* mutants have to rely on DMQ_9, the precursor of UQ_9, or dietary UQ_8 to supply Complex III with electrons (Larsen and Clarke, 2002; Miyadera et al., 2001). *gro-1* mutation compromises translational fidelity and efficiency (Lemieux et al., 2001) of the 12 mitochondrially encoded proteins, all involved in electron transport. At least for these two Clk mutants, there is a strong indication that a slight alteration of the mitochondrial electron transport suffices to result in the Clk phenotype through an unknown mechanism that is not directly associated with metabolic rate.

2.4 Dietary restriction does not decrease metabolic rate but enhances antioxidant defense

The mechanism of lifespan extension by dietary restriction (DR), though effective in invertebrates and vertebrates, has not been elucidated entirely. One leading hypothesis assumes that DR acts by decreasing oxidative stress (Sohal and Weindruch, 1996), supposing that the beneficial effect of DR is associated with a hypometabolic state (Lakowski and Hekimi, 1998; Lee et al., 1999a).

Dietary restriction can be imposed on *C. elegans* in different ways, including bacterial dilution, mutation affecting pharyngeal pumping and growth in synthetic media in the absence of bacteria. For each of these culture conditions, the effect of age on metabolic rate (respiration, heat production and ATP content) was determined, together with antioxidant activity levels (SOD and catalase).

2.4.1. Materials and methods

2.4.1.1. Strains and culture conditions

The wild type strain N2 was used as well as two Eat mutants, *eat-2(ad465)* and *eat-2(ad1113)*. The latter has a more severe eating defect, and has a longer lifespan than the former (Lakowski and Hekimi, 1998). *glp-4(bn2*ts*)* mutants essentially lack a germline when raised at the restrictive temperature; in this way, potentially confounding effects resulting from differences in the number of eggs carried by gravid worms could be ruled out. Monoxenic age-synchronous cultures were established as described in (2.2.1.1.). For axenic dietary restriction (ADR), axenic cultures were obtained by two consecutive cycles of hypochlorite treatment to achieve sterility. L1s were inoculated into axenic culture medium. Axenic culture medium contained 3% (w/v) soy peptone and 3% (w/v) dry yeast extract and was sterilized under standard conditions. After cooling, haemoglobin stock solution (prepared by dissolving 5 g haemoglobin in 100 ml 0.1N KOH and autoclaving for 10 min) was diluted 100-fold into the basal medium. To improve synchronous development, heat-killed *E. coli* cells were added at 3×10^9 cells/ml. When the worms reached the fourth larval stage, they were washed with sterile S buffer and suspended into Fernbach flasks containing 250 ml axenic medium (without heat-killed bacteria) and FUdR was added at 50µM final concentration. Culture conditions were the same as for monoxenic cultures; all aging cohorts were maintained at 24°C. Sampling was performed as in (2.2.1.1.).

2.4.1.2. Assays

The assays for this experiment (oxygen consumption, heat production, ATP content, SOD and catalase activity and protein content) were executed as described in (2.2.1.2.).

Data were normalized to protein content. Oxygen consumption and heat production data were also corrected for differences in volume: the rate of energy expenditure per unit mass varies with body size and results in the negative allometric relationship between mass and metabolic rate. Allometric scaling of data was performed as in (Braeckman et al., 2002c)(see section 3.2.2.2.). Section 3.3. (addendum to chapter 3) elaborates on the effect of feeding conditions on worm size and its consequences for normalization.

2.4.1.3. Statistics

Statistical analyses were performed using SPSS. Strains were compared by using two-way analysis of variance (ANOVA), and pairwise comparisons were made with LSD post-hoc tests. Additionally, F-tests for linear regression were performed in excel.
For further details, we refer to (Houthoofd et al., 2002a) and (Houthoofd et al., 2002b).

2.4.2. Results and discussion

In this experiment, a number of approaches to assess food restriction were investigated. Firstly, the *eat-2* mutation brings about defects in pharyngeal pumping, causing a reduced rate of feeding (Raizen et al., 1995). Metabolic and antioxidant parameters of two alleles of this mutant were assayed after incubation in monoxenic feeding conditions: *eat-2(ad465)* and *eat-2(ad1113)* adult worms were kept in liquid bacterial suspension, containing non-restrictive amounts of *E. coli*. Secondly, wild-type adult worms were subjected to incubation in liquid medium supplemented with reduced concentrations of bacteria. Klass (1977) showed that reduction of *E. coli* as food supply in liquid suspension from 10^9 to 10^8 cells/ml resulted in 60% lifespan extension. As standard conditions, ad-libitum *E. coli* concentrations are kept at 3 x 10^9 cells/ml. By reducing this concentration, DR can be imposed on *C. elegans*. A range from 0.2 x 10^9 to 6 x 10^9 cells/ml was chosen to investigate the effect of bacterial dilution on metabolic rate and antioxidant capacity in the wild-type; 2-day-old adults were tested. Also, bacterial dilution was implemented on the *glp-4 (bn2*ts) mutant, which was used to control for potentially confounding effects resulting from differences in the number of eggs carried by gravid worms. Thirdly, wild-type worms were subjected to incubation in axenic medium (ADR or axenic dietary restriction), which is a synthetic medium containing soy peptone as a source of amino acids, yeast extract for vitamins and growth factors, hemoglobin as a heme source and sufficient amounts of sterols as contaminating compounds of the former constituents (Vanfleteren, 1980). It causes slow growth and reduced fecundity but also extends adult lifespan (Braeckman et al., 2000; Vanfleteren and Braeckman, 1999). Lastly, *eat-2* mutants were incubated in axenic medium to assess the combined effect of these DR conditions.

Metabolic rate, as measured by respiration (fig 3a) and heat production (fig 4a) rates, was elevated in *eat-2* mutants when kept in standard *E. coli*-supplemented liquid

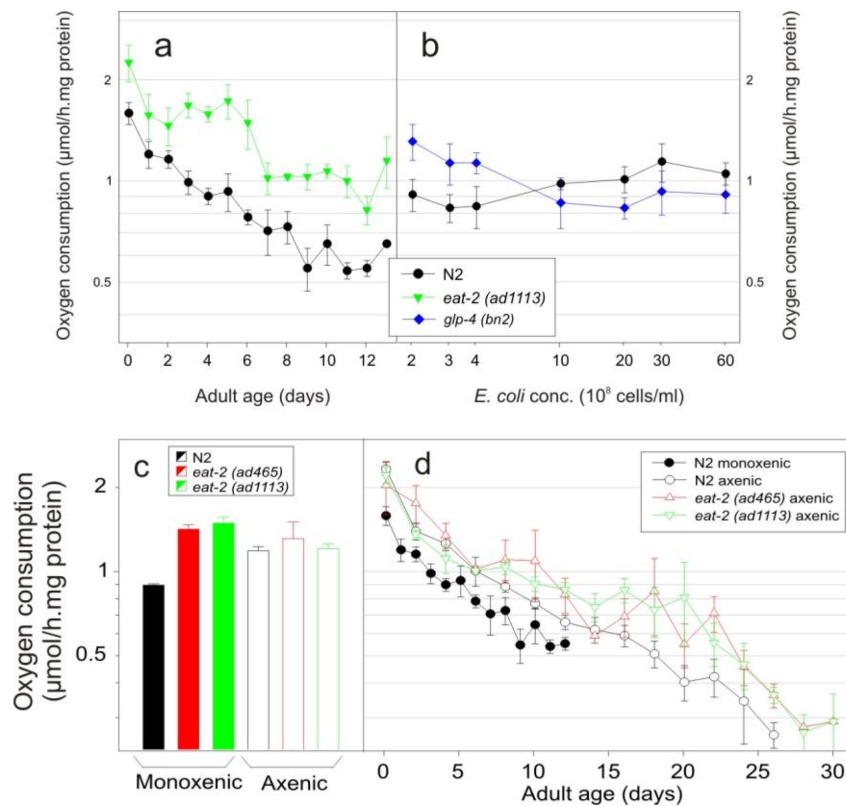


Figure 3: Effects of food restriction on respiration rate. a, WT and *eat-2(ad1113)* in monoxenic liquid culture with a bacterial density of 3 x 10^9 cells/ml; b, WT and *glp-4(bn2)* 2-day-old adults, raised in liquid culture containing increasing *E. coli* densities (log scale) ; c, WT and *eat-2* mutants, monoxenic (bacterial density 3 x 10^9 cells/ml) or axenic liquid culture, average measures for the first 13 days of adult life; d, WT and *eat-2* mutants, axenic liquid culture, shown during the adult life trajectory, and with WT monoxenic shown as a reference. Data are corrected for size-dependent changes; data represent the mean ± S.E.M. of three to five independent experiments.

medium (only the *ad1113* allele is shown). Dietary restriction through bacterial dilution had no effect on wild-type oxygen consumption rates (fig 3b) (F-test for regression: *P* = NS), while it did affect heat dissipation (fig 4b): heat production rates decreased with increasing bacterial concentrations (F-test for regression: *P* < 0.001). Trends were similar for *glp-4(bn2)* (F-test for regression: oxygen consumption, *P* = NS; heat dissipation, *P* < 0.001), indicating that the observed effects were not caused by varying egg production. Wild-type worms had higher oxygen consumption (fig 3c & d) and heat production (fig 4c & d) rates in axenic medium than in monoxenic liquid culture (oxygen consumption: *P* < 0.01; heat dissipation: *P* < 0.05); stimulation of metabolic rate by axenic medium was far less outspoken in *eat-2* than in wild-type (fig 3c & 4c).

While the *eat-2* mutation had little effect on ATP concentration in monoxenic liquid culture, at least during the first week of adult life (fig 5a), differences in ATP content associated with bacterial dilution were remarkable (fig 5b). Here, ATP concentrations increased with increasing amounts of food, even more so for *glp-4* than for the WT (F-test for linear regression, N2: *P* = 0.009; glp-4: *P* < 0.001). Young adults in axenic culture had lower ATP concentrations relative to worms raised in bacterial culture (fig 5c & d) (two-way ANOVA on data from day 0, *P* = 0.001). All three strains showed reduced rates of age-

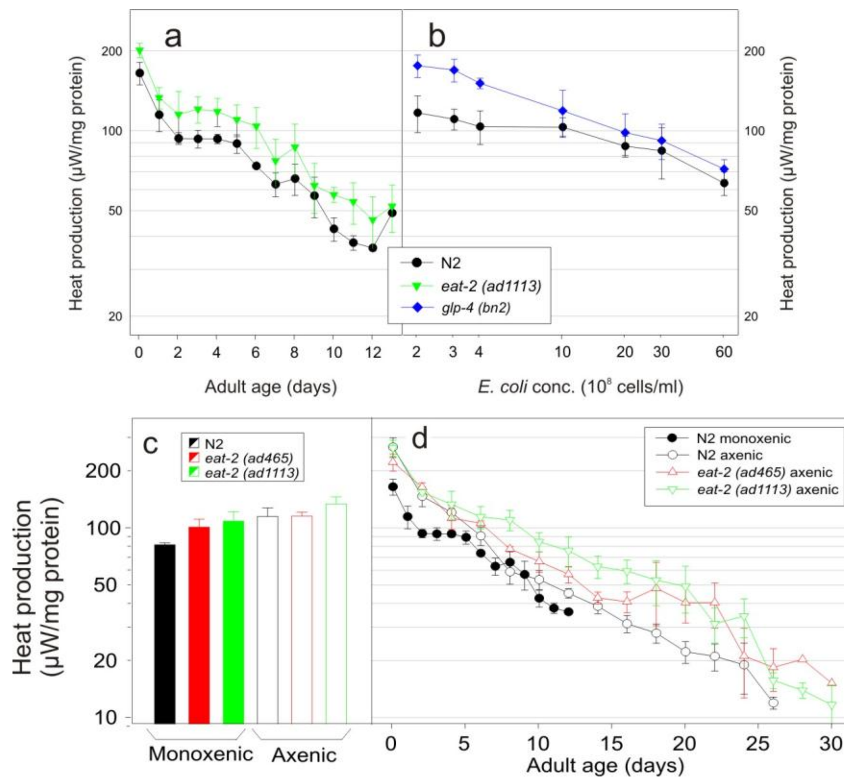


Figure 4: Effects of food restriction on heat dissipation; legend as in fig. 3.

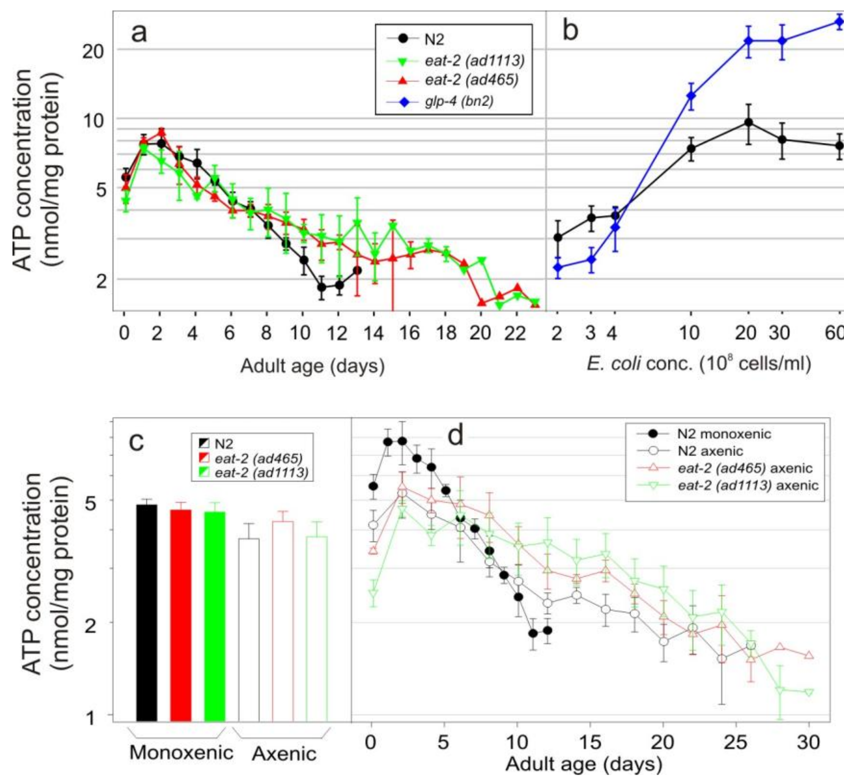


Figure 5: Effects of food restriction on ATP content; legend as in fig. 3 without correction for size-dependent changes.

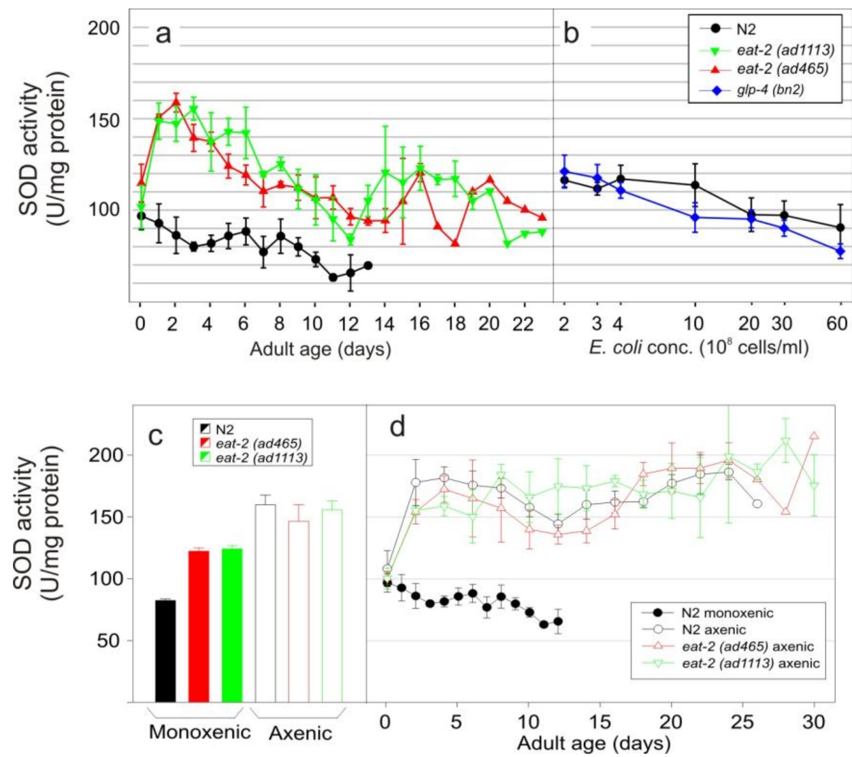


Figure 6: Effects of food restriction on SOD activity, legend as in fig. 5.

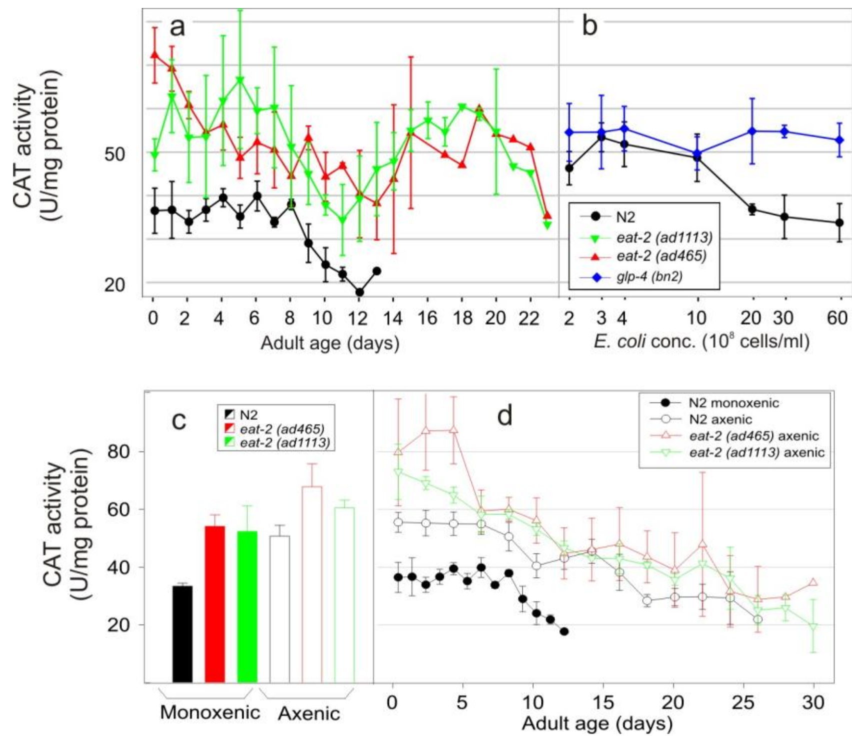


Figure 7: Effects of food restriction on catalase activity, legend as in fig. 5.

specific ATP content decline in axenic versus bacterial medium (fig 5d) (F-test for equality of slopes of several regression lines, *P* < 0.01).

eat-2 mutants reached over 40% higher SOD activity levels relative to wild-type in monoxenic liquid culture; this difference persisted over the entire adult life trajectory (fig 6a) (two-way ANOVA and post-hoc LSD test, *P* < 0.001 for both *eat-2* alleles). In wild-type and *glp-4* subjected to bacterial dilution, SOD activity tended to decrease weakly with increasing bacterial concentration (fig 6b) (F-test for linear regression, N2: *P* = 0.007; *glp-4*: *P* < 0.001). In axenic medium, SOD activities doubled in the wild-type and increased to those wild-type levels for both Eat mutants (fig 6c & d). In monoxenic suspension culture, catalase activity was higher in *eat-2* than in wild-type (fig 7a) (two-way ANOVA and post-hoc LSD test, *eat-2 (ad465)*: *P* = 0.013; *eat-2(ad1113)*: *P* = 0.047). In wild-type, catalase activity decreased with increasing availability of bacteria (F-test for linear regression, *P* = 0.001), but no DR-related catalase trends were seen in *glp-4* (*P* = NS) (fig 7b). Axenic culture increased catalase activity of the wild-type when compared to monoxenic conditions (*P* = 0.014) (fig 7c & d), but the effect of axenic culture on Eat mutants had no statistical significance (*P* = NS for both *eat-2* alleles).

From SOD and catalase activity decreases through bacterial dilution, it could be suggested that antioxidant activity levels negatively correlate with food intake. This could also be construed from experiments related to dauer antioxidant activity: the non-feeding dauer has elevated antioxidant defenses. However, some findings obscure this possibility. First of all, catalase activity is invariant with decreasing bacterial dilution in *glp-4*, showing that at least in this mutant, a negative correlation between food intake and antioxidant activities is not a general trend. Secondly, antioxidant activities are higher for N2 maintained on plates than for N2 kept in liquid monoxenic medium (Houthoofd et al., 2002b). It is known that for *C. elegans*, feeding is facilitated when the worms are kept on a lawn of bacteria, so we can assume that the worm's feeding rate will be higher on plates than in liquid culture. In these conditions, increased antioxidant activities could also be caused by higher oxygen availability. This experiment shows that other factors besides nutrient levels can influence antioxidant enzyme activities, making it difficult to discern the effect of feeding on antioxidant defense. Moreover, since *E. coli* is mildly pathogenic (Darby, 2005) and is an unnatural food source for *C. elegans* (Kiontke and Sudhaus, 2006), we would expect that antioxidant defenses would be beneficial especially in fully-fed conditions.

These experiments suggest that DR is not associated with a hypometabolic state. Bacterial dilution did not cause a decrease in metabolic rate; food restriction through pharyngeal defects and axenic medium seemed to upregulate metabolic rate. While DR by *eat-2* mutation did not show lower ATP content than the wild-type, bacterial dilution lowered ATP content substantially. Perhaps ATP consumption was enhanced in these worms to support de novo synthesis of biomolecules otherwise supplied with food, resulting in low ATP content in conditions of low bacterial supply. ATP content was also lower in young adults when subjected to axenic medium, but decreased at a slower rate with increasing age.

In *eat-2* and in axenic medium, lifespan extension seemed to be correlated with elevated antioxidant capacity. However, for bacterial dilution, results were more ambiguous: while SOD activity increased with decreasing bacterial supply, the increases were small. The same could be said for catalase in the wild-type, and in *glp-4*, DR effects on

catalase were absent. Moreover, worms restricted by bacterial dilution acquired no elevated protection against paraquat and hydrogen peroxide (results not shown). The role of antioxidant defense in DR-mediated lifespan extension has yet to be clarified. Most importantly, we have shown that, though physiological effects imparted by DR through bacterial dilution, axenic medium and *eat-2* mutation are not identical, they all oppose the notion that DR-induced lifespan extension might be caused by lower metabolic rate.

2.5 General discussion and conclusions

The link between low metabolism and the three lifespan-extending manipulations described in this chapter is easily made. Dauers are non-feeding, Clk mutants have a slow life rythm and dietary restriction entails uptake of less nutrients. As less 'fuel' is available to drive the ETS, a lower rate of metabolism could be expected. If the assumption is made that low metabolic rate leads to low ROS production, a plausible explanation for postponed aging at the molecular level is found. Hence the importance of assessment of metabolic parameters and antioxidant levels: they can contribute to the evaluation of the most important and generally accepted theory of aging.

In the mid 90s, Lakowski and Hekimi (1998) constructed the *eat-2;clk-1* double mutant in order to study the interaction between DR and metabolic rate. The lifespans of the single mutants were not additive, from which the authors concluded that *eat-2* and *clk-1* influence aging through a common process; they suggested reduced metabolic rate and lower ROS production.

As proven by previous (Braeckman *et al.*, 1999) and these studies, there is little difference in oxygen consumption between *clk-1* and WT, undermining the hypothesis of slow metabolism as a way to extend lifespan. However, other researchers come to quite different conclusions; according to Van Raamsdonk and colleagues (2010), oxygen consumption is lower in *clk* mutants relative to WT. In agreement with the Braeckman study, higher levels of ATP were recorded in *clk* mutants, but these were attributed to decreased energy expenditure. The Hekimi group concluded that *clk* mutants illustrate the value of some aspects of the Rate-of-Living theory. Though the divergence in oxygen consumption results could be explained by differences in experimental setups, the fact that in our hands, *clk* mutants are capable of maintaining higher metabolic rates and longer lifespans (personal communication, B.P. Braeckman) cannot be ignored and is a strong indication against the Rate-of-Living theory. Moreover, in all but one of the lifespan-extending situations described here, metabolic rate is not lowered, and often it is enhanced: in DR-treated *C. elegans*, oxygen consumption and heat production rates are generally higher than in fully fed conditions. In the dauer, metabolism is low but stable; the rise of metabolic rates after subjection to food is instant. Likewise, in other model organisms, long lifespans are not necessarily associated with slow metabolism. Within-species comparisons in *Drosophila*, mice and dogs show that metabolic rate and longevity are not inversely related (Speakman et al., 2004; Speakman et al., 2003; Van Voorhies et al., 2003). DR results in other model organisms underpin the increased rates of metabolism found in *C. elegans*: in rats submitted to long-term DR, no decrease of metabolic rate is recorded (Lambert et al., 2004; McCarter et al., 1985; McCarter and Palmer, 1992). The same is true for DR

Drosophila (Hulbert *et al.*, 2004). Lin and co-workers (2002) showed that DR in *S. cerevisiae* results in increased respiration. Together, these data reject the notion that lifespan extension can be attributed to low metabolic rate.

Though antioxidant activities are not convincingly upregulated in the case of DR through bacterial dilution, they are increased, in one form or another, in other cases described here. This could suggest that antioxidants play a role in lifespan extension. Since metabolic rates are seemingly not decreased in mutations or conditions that lead to lifespan extension, high antioxidant activity could reduce levels of ROS and ROS damage and consequently, result in a long lifespan. However, several findings refute this notion. Firstly, supplying wild-type *C. elegans* with mimetics for the antioxidant enzyme SOD does not prolong their lifespan (Keaney et al., 2004). According to Van Raamsdonk and co-workers (2010), most *clk* mutants show increased sensitivity to juglone and paraquat; however, expression of *sod-1* and *sod-2* is generally similar or higher relative to WT. It is suggested by these authors that increased sensitivity to oxidative stress is caused by increased ROS production, not by decreased antioxidant defense. In contrast, *clk-2* was found to be more resistant to juglone (Johnson *et al.*, 2001) though it has lower SOD activity than WT. In *clk* mutants, increased oxidative damage is also recorded despite their longer life span (Van Raamsdonk *et al.*, 2010); these findings demonstrate that ROS do not negatively regulate lifespan. The Hekimi group agrees that this contests the Free Radical theory of aging; long life is neither the result of increased resistance to oxidative stress, nor of lowered oxidative damage. Others have confirmed this by knocking down *sod* genes, and potentially increasing oxidative damage; these treatments do not necessarily shorten lifespan, regardless of whether they are applied to wild-type, DR-treated worms, *clk-1* or the Ins/IGF-1 mutant *daf-2* (Doonan et al., 2008; Honda et al., 2008; Van Raamsdonk and Hekimi, 2009; Yang et al., 2007; Yen et al., 2009). Though the data presented here are insufficient to verify the importance of high antioxidant levels in lifespan extension for dauers, Clk mutants and DR, other studies suggest that for several long-lived *C. elegans*, longevity is not dependent on antioxidant defense. In conclusion, the three cases of *C. elegans* longevity described in this chapter not only cast doubt on the putative causal relationship between antioxidant defense and lifespan extension, they also refute the Rate-of-Living theory as an explanation for longevity.

PART II: RESULTS

Chapter 3

Longevity, metabolism and stress defense in *C. elegans*: effects of Ins/IGF-1 signaling

Redrafted from:

Brys *et al* (2010). Disruption of insulin signaling preserves bioenergetic competence of mitochondria in ageing *Caenorhabditis elegans*. BMC Biology **8**, 91

Brys *et al* (2007). Testing the rate-of-living/oxidative damage theory of aging in the nematode model *Caenorhabditis elegans*. Exp. Gerontol. **42**, 845-851

Complementary sources:

Houthoofd *et al* (2005)a. DAF-2 pathway mutations and food restriction in aging *Caenorhabditis elegans* differentially affect metabolism. Neurobiol Aging **26**, 689-696

Houthoofd *et al* (2005)b. Metabolism, physiology and stress defense in three aging Ins/IGF-1 mutants of the nematode *Caenorhabditis elegans*. Aging Cell **4**, 87-95

Personal contribution:

Brys *et al* (2010): all nematode cultures and metabolic assays, some qPCR experiments, writing of manuscript

Brys *et al* (2007): Nematode culturing, adaptation and execution of catalase and GSH activity assays, writing of manuscript

Houthoofd *et al* (2005)a: nematode culturing, contribution to the general discussion

Houthoofd *et al* (2005)b: nematode culturing, adaptation and execution of catalase activity assay, contribution to the general discussion

ABSTRACT

Lifespan extension by disruption of the Insulin/IGF-1 pathway has been studied extensively in *C. elegans*. A prominent characteristic of Ins/IGF-1 mutants is their elevated antioxidant defense. Also, their metabolism is altered; it has been reported that their metabolic rate is reduced when compared to the wild-type (Van Voorhies and Ward, 1999). Reduced metabolism and high antioxidant defense have been interpreted as support for the Rate-of-Living and Free Radical theories of aging. We have assessed metabolic rates and antioxidant activity in the *daf-2(e1370)* mutant. Our results confirm the elevated antioxidant defense of this mutant, but we show that this Ins/IGF-1 mutant is not hypometabolic. It exhibits a shift in metabolism, possibly indicating a higher metabolic efficiency. The potential link between *daf-2(e1370)*'s metabolism and lifespan extension is discussed in this chapter.

3.1 Introduction

The life span of *C. elegans* is regulated by multiple signaling pathways that converge on a battery of downstream target genes. Among these, insulin/IGF-1 like signaling (IIS) is currently best understood. Activated Insulin/IGF-1-like receptor encoded by the gene *daf-2* triggers downstream kinases to phosphorylate a FOXO transcription factor encoded by *daf-16.* Phosphorylated DAF-16 protein is sequestered in the cytoplasm and inactive. Reduction of IIS signaling in the absence of ligand or via reduction- or loss-of-function mutation in the *daf-2* gene relocates DAF-16 to the nucleus and triggers a genetic program for lifespan extension (Gems et al., 1998; Henderson and Johnson, 2001; Kenyon et al., 1993; Kimura et al., 1997; Lee et al., 2001; Lin et al., 2001; Ogg et al., 1997).

Mutation in *daf-2* also enhances resistance to oxidative stress (Honda and Honda, 1999). The concurrent features of longevity and resistance to oxidative stress have been interpreted as supporting the Free Radical Theory of Aging which proposes a central role for oxygen free radicals and derived reactive oxygen species (ROS) in causing the aging process (Balaban et al., 2005; Beckman and Ames, 1998; Finkel and Holbrook, 2000; Harman, 1956, 1972; Viña et al., 2007). We studied oxidative metabolism and antioxidant capacity in both the long-lived *daf-2(e1370)* mutant and the null mutant *daf-16(mgDf50)* in order to validate the Free Radical Theory of Aging as well as the Rate-of-Living Theory by Pearl (1928). Results show that the long-lived *daf-2* mutant is not hypometabolic. Remarkably, it displays a low calorimetric-to-respirometric ratio, high ATP levels and an upregulated antioxidant defense. Possible explanations for these phenomena and their potential role in lifespan extension are discussed.

3.2 Whole worm oxidative metabolism of *daf-2*

3.2.1. Materials and methods

3.2.1.1. Strains and culture conditions

The wild type strain was the Bristol N2 male stock; the Ins/IGF-1 mutants used were *daf-2(e1370)* and *daf-16(mgDf50)*. The worms were grown at 17°C on nutrient agar plates seeded with *E. coli* and synchronous cultures were established as in (2.2.1.1.). Fourth stage larvae were rinsed off the plates, transferred to Fernbach flasks and maintained at 24°C as described in (2.2.1.1.). Sampling was performed as in (2.2.1.1.).

3.2.1.2. Assays

Oxygen consumption and heat production were executed as described in (2.2.1.2.), as were SOD and catalase activity and protein content. The calorimetric-to-respirometric ratio (C/R) was calculated by dividing heat dissipation by oxygen consumption.

ATP and ADP

ATP and ADP were extracted from frozen worm tissue with perchloric acid as follows: to 100 µl of nematode suspension in S-buffer, 400 µl of $HClO_4$ 8% (v/v) and 200 mg of glass beads were added. The samples were homogenized using a Mini-Beadbeater (Biospec Products, Bartlesville, OK, USA), operated at 5000 strokes/min for 1 min. Next, 450 µl (3 consecutive portions of 150 µl to avoid excessive formation of bubbles) of 1.33 M $KHCO_3$ and 150 µl H_2O were added. After leaving for 15 min at room temperature the sample was degassed in a Savant Speed Vac Concentrator for 10 min and cleared by centrifugation at 20,800 g for 8 min. Aliquots of the supernatant were used for ATP determination using the ATP Bioluminescence Assay Kit CLS II (Roche Diagnostics, Mannheim, Germany) and a Wallac Victor² Multilabel Counter (Perkin-Elmer, Waltham, MA, USA) as previously described (Braeckman et al., 2002b). ADP was measured as excess ATP detected after conversion of all ADP to ATP. This was achieved in a coupled reaction in which 64 µM phosphoenolpyruvate was converted to pyruvate in 40 mM potassium phosphate, pH 7.6 in the presence of 4 mM $MgSO_4$ and 1 U/ml pyruvate kinase. After leaving for 10 min at room temperature, the reaction was stopped by heating the samples for 8 min at 99°C, and the supernatant was cleared by centrifugation at 20,800 g for 8 min. Total ATP determination was performed as described in (2.2.1.2.), and ADP content was calculated as the difference between total ATP and ATP content before conversion of ADP.

Reduced glutathione (GSH) levels

Levels of reduced glutathione were measured as follows: in the presence of the catalyst glutathione-S-transferase (GST), GSH binds rapidly and specifically to the fluorochrome monochlorobimane (MCB) (Ublacker et al., 1991). In a black microtiterplate, 10µl of worm homogenate (prepared as described in (2.2.1.2.)) was added to 90µl of reagent solution containing 41.7 mM glycylglycine buffer pH 8.0, 0.133U/ml GST and 55,6 µM MCB. For conversion of fluorescence units to nmoles of GSH, a GSH standard curve was included in each assay. After a 50 minute incubation period at 25°C, fluorescence was measured in the Victor² Multilabel counter at excitation and emission wavelengths of 360 and 460nm, respectively.

Body volume & allometry

Body volume was determined by measuring the length and thickness of a subsample of nematodes, fixed in 4% formaldehyde, using the RapidVue particle analyzer (Beckman Coulter, Fullerton, CA, USA) and using a cylindrical model for volume calculation. All metabolic parameters were correlated to protein content to account for differences in body mass. Respiration rate and heat production were also corrected for size-specific differences according to the Brody-Kleiber equation (Braeckman et al., 2002c) (see results and discussion section).

Quantitative PCR

Quantitative PCR was performed as described in (Hoogewijs et al., 2008) on 2- and 8-day-old adults of WT and *daf-2(e1370)* mutants; primersets used were: *sod-3*: (forward primer: AGAACCTTCAAAGGAGCTGATG; reverse: CCGCAATAGTGATGTCAGAAAG); *mai-1*: (forward primer: ATATGACGCAAAGCCAACAG; reverse: GTTCCGTGTCCTTTCTCGAT); *mai-2*: (forward primer: CGCGAGGACGAGTACTTCTA; reverse: GTTCGAGCACCTTCTTGTGA).

3.2.1.3. Statistics

Regression analysis of age-related changes was performed using the mixed linear regression model PROC MIXED in SAS statistical software; data were log-transformed when needed to allow the best possible fit and tests of fixed effects provided *P* values for strain, age and age*strain. When $P_{age*strain}$ was <0.05, age-related changes (slopes) differed significantly between the strains compared. When $P_{age*strain}$ was not significant, further conclusions were drawn based on P_{age}, P_{strain} or both. As an auxiliary analysis, a Student's t-test was used to compare differences between strains or ages at specific time points. Error bars depict standard error of the mean.

3.2.2. Results

3.2.2.1. Replication of previous experiments

In *C. elegans*, metabolic rate can be determined by several complementary assays. Two of them, respirometry and microcalorimetry, can be combined to assess metabolic efficiency. By dividing heat output by oxygen consumption, the calorimetric-to-respirometric (C/R) ratio is obtained; it represents the amount of heat produced per mole of oxygen used.

Metabolic rate of live *daf-2(e1370)* has been studied at length previously in our lab. However, there were some convincing grounds to repeat these studies. In Braeckman et al., 2002c, reviewed in Brys et al., 2007, *daf-2(e1370)* oxygen consumption was very similar to the wild-type (Appendix 1, fig 1a), but heat dissipation was lower than in wild-type for most of the *daf-2* lifespan (App. 1, fig 1b). Consequently, the calorimetric-to-respirometric ratio was generally lower in the long-lived mutant (App. 1, fig 1c), indicative of a higher catabolic efficiency (Kemp and Guan, 1997), as less heat is lost for the same amount of oxygen consumed. In Houthoofd et al., 2005b the same conclusions were drawn regarding the C/R ratio (App. 2, fig 2c), but respiration and heat dissipation showed quite different profiles: in this study, oxygen consumption declined steadily with age in N2 but much less so in *daf-2(e1370)* (App. 2, fig 2a). *daf-2(e1370)* released less heat early on in adulthood but the difference disappeared as the worms aged (App. 2, fig 2b). For these discrepancies, no logical explanation was found; replication of these measurements was essential for a full understanding of metabolic rate in the long-lived Ins/IGF-1 mutant *daf-2(e1370)*, central to this study. Also, a different method was used to extract the energy carrier ATP from frozen nematodes, along with a technique to assay ADP content in samples. This allowed for more accurate measurement of nucleotide content than reported in earlier studies. The third

reason to re-assess metabolic rate of Ins/IGF-1 mutants is based on results obtained previously from the *daf-16(m26)* mutant (Houthoofd et al., 2005a). The *daf-16* gene encodes a forkhead transcription factor and is negatively regulated by DAF-2 signaling. Down-regulation of DAF-2 causes nuclear localization of DAF-16 and transcriptional regulation of genes whose activities shape the phenotype of the *daf-2* mutant. A considerable number of *daf-2*'s phenotypic characteristics are suppressed by mutation in *daf-16*, indicating that mutations in *daf-2* require intact activity of *daf-16* for their expression. Consequently, phenotypes exhibited by *daf-2* and *daf-16* mutant animals are expected to be opposite, and those of the double mutant *daf-2;daf-16* to be similar to *daf-16*. In the mentioned study, the *daf-16* allele *m26* was chosen; it is believed to cause loss of function with respect to the age phenotype (Lee et al., 2001; Lin et al., 2001). Oxygen consumption in this mutant was similar to the wild-type (App. 3, fig 1b). Unexpectedly, *daf-16* mutants displayed reduced heat dissipation when compared to wild-type, reminiscent of *daf-2* heat production profiles (App. 3, fig 2b). This led to reduced C/R ratios for a short-lived *C. elegans* mutant (App. 3, fig 3b), a phenotype difficult to reconcile with that of *daf-2*. In contrast, ATP content was lowest in *daf-16* and intermediate in *daf-2;daf-16* (App. 3, fig 4c); these unexpected results led us to include a *daf-16* mutant in our setup. This time, the *mgDf50* allele was chosen, a molecular null allele denoting a large deficiency that deletes nearly the entire *daf-16* coding region (Ogg et al., 1997). This mutation is expected to suppress all Daf-2 phenotypes that rely on intact DAF-16.

3.2.2.2. Allometry

Figure 1 represents the volumes of the strains used in this experiment. Wild-type *C. elegans* volumes increased in young adult stages but stabilized at later ages. In contrast, *daf-2(e1370)* volumes decreased with increasing age (fig 1a), while *daf-16(mgDf50)* reached volumes considerably higher than the wild-type (fig 1b). An age-dependent shortening of the *daf-2* mutant worms was the main cause of their decreasing volume, most likely through shrinkage of the cells. *daf-16* worms had an increased thickness when compared to the wild-type. As before, all data were normalized to protein content. Additionally, because strain and age effects on worm volume are not negligible, we also corrected respiration and heat production data for differences in body size. Metabolic rate per unit mass varies with body size; small organisms have higher mass-specific metabolic rates than larger organisms. This even holds true for subcellular organelles (West et al., 2002).

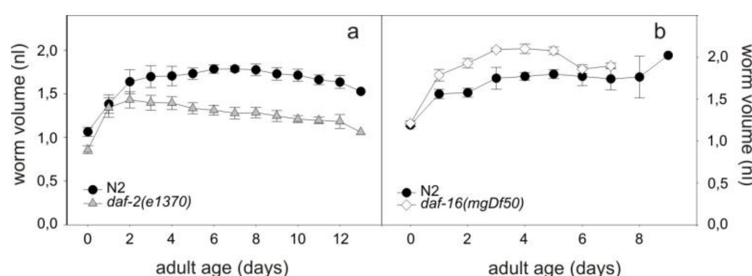


Figure 1: Worm volumes as a function of age; a, WT and *daf-2(e1370)*, b, WT & *daf-16(mgDf50)*. Data represent the mean of a minimum of three replicate cultures.

We chose to correct for this allometric effect by using the Brody-Kleiber equation, originally designed for interspecies comparisons (Brody, 1945; Kleiber, 1947), but compatible with within-species comparisons: $P = aM^b$, where P equals metabolic rate, M stands for body mass, a is the scalar mass constant and b is the mass exponential constant. The equation as it is used to adjust the metabolic rate of a strain to a reference strain is the following: $P_{corr} = P(M_{ref}/M)^{b-1}$, where P_{corr} is the corrected metabolic rate and M_{ref} is the mass of the reference strain. The experimental value for b ranges from 0.67 to 0.80 for multicellular organisms and was determined to be 0.72 ±0.09 for nematodes (Finch, 1990; Klekowski et al., 1972; Peters, 1983).

3.2.2.3. Oxidative metabolism

We obtained respiration and heat production rates from wild-type (N2) worms, *daf-2(e1370)* and *daf-16(mgDf50)* animals. Respiration declined with age in all three strains. The rate of decrease was smaller in *daf-2(e1370)* animals [$P_{age*strain}$ (d0-d7) = 0.0002], but overall, respiration rates were grossly similar in all three strains (fig. 2 a&b), confirming results in (Appendix 1, fig 1a). Heat dissipation also decreased with age in N2 and *daf-16(mgDf50)* but was markedly lower in *daf-2(e1370)* animals (fig 2 c&d), much like in (Appendix 1, fig 1b). These findings seem to comply more with the study by Braeckman and co-workers (2002c) than with the (Houthoofd et al., 2005b) study. Calorimetric-to-respirometric (C/R) ratios were considerably lower in *daf-2 (e1370)* than in wild-type, for most of its lifespan (fig 2e). Since the C/R ratio provides an indication of catabolic efficiency (Kemp and Guan, 1997) these results indicate that the efficiency of aerobic energy production is upregulated in *daf-2(e1370)* animals during the first 7-9 days of their adult life span. Interestingly, loss of *daf-16* function also caused lower C/R ratios than wild-type after the third day of adult life (fig 2f). As mentioned, a similar observation was previously reported for the reduction-of-function allele *daf-16(m26)* (Houthoofd *et al.*, 2005a) (Appendix 3, fig 3b).

Next we asked if this putative upregulation of catabolic efficiency in *daf-2(e1370)* would be observed in the standing levels of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) and we found that this was indeed the case (fig 2g&i) [*daf-2* versus N2, both ATP and ADP: $P_{age*strain} < 0.0001$]. Thus, unlike wild-type and *daf-16(0)* worms, *daf-2(e1370)* animals are able to attenuate the age-specific depletion of the instantly utilizable energy source ATP.

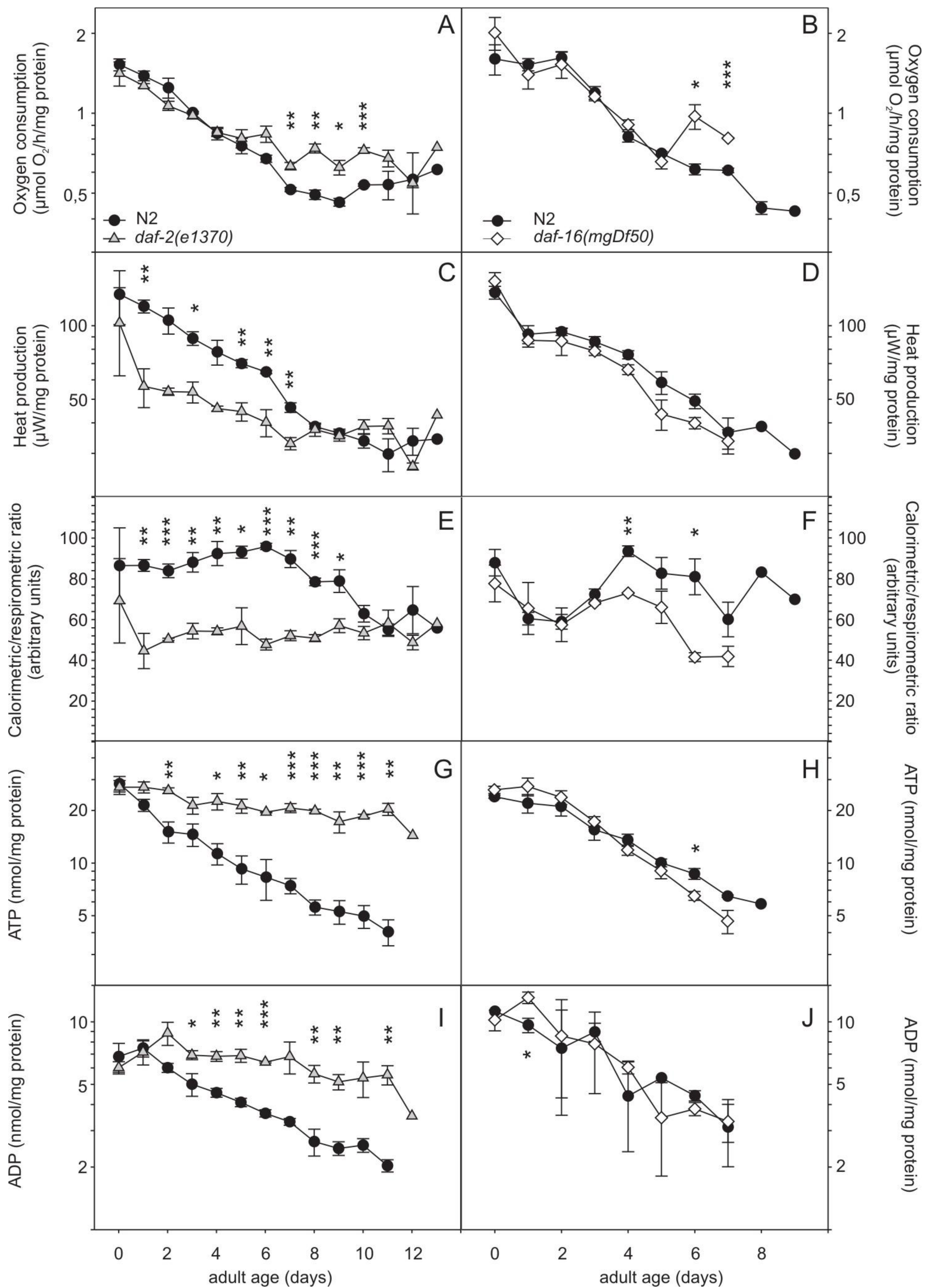


Figure 2: Ins/IGF-1 signaling controls age-related changes in aerobic energy production**.** Left panels: WT versus *daf-2(e1370)*, right panels: WT versus *daf-16(mgDf50)*. (A-B) Respiration rate. (C-D) Metabolic heat production. (E-F) Calorimetric to respirometric ratio. (G-H) Adenosine triphosphate content. (I-J) Adenosine diphosphate content. Displayed values are means ± standard error of mean for three replicate cultures; *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$ (student's t-test).

3.2.2.4. Antioxidant capacity

Previously, estimations of antioxidant defense in *daf-2(e1370)* were performed by assaying superoxide dismutase (SOD) and catalase activity. Both Braeckman and colleagues (2003) (reviewed in Brys et al. (2007)) (fig 3 a&b) and Houthoofd and co-workers (2005b) (results not shown) found increased SOD and catalase activity in the long-lived mutant. Additionally, we measured its levels of reduced glutathione (fig 3c) which decreased exponentially with age, but at a slower rate in *daf-2* than in the wild-type, leading to relatively high GSH levels at advanced age and confirming the elevated antioxidant defense phenotype of *daf-2(e1370)* ($P_{age*strain}$ = 0.0186).

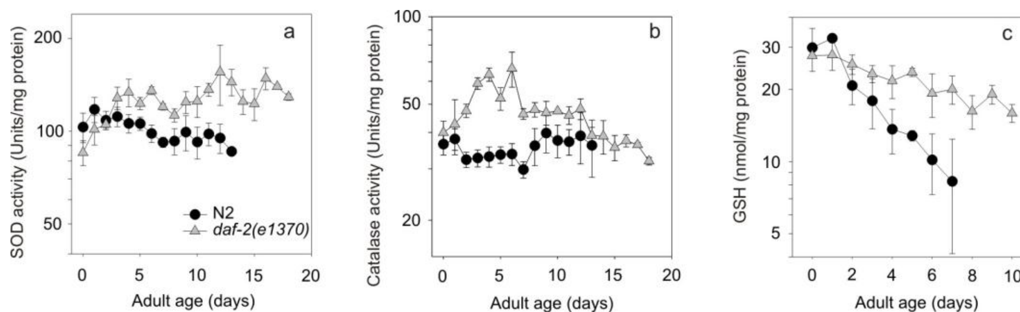


Figure 3: Antioxidants in wild type and *daf-2(e1370)* adults. (a) SOD activity, (b) catalase activity, (c) GSH content. Data represent means and standard errors for a minimum of three replicate cultures.

3.2.3. Discussion

3.2.3.1. Low *daf-2(e1370)* C/R ratios

We have confirmed that *daf-2(e1370)* animals consume similar amounts of O_2 but dissipate substantially less heat than wild type animals; this discrepancy is best illustrated by lower C/R ratios for most of the *daf-2* lifespan. Several physiological phenomena could be responsible for differences in C/R ratios.

Firstly, one of the phenotypical characteristics of *daf-2(e1370)* is its low fertility: brood size of *daf-2(e1370)* is about 70% lower than in the wild-type (Houthoofd et al., 2005b). The lower C/R ratio in *daf-2* could be ascribed to the reduced number of eggs in the gravid hermaphrodites. However, this suggestion is not tenable: Houthoofd and co-workers (2005b) determined C/R ratios in the sterile *glp-4(bn2)* mutant and found no decrease in this parameter relative to wild-type.

Secondly, differences in reactions to experimental conditions may be causal. C/R is calculated by dividing heat measurements by oxygen consumption measurements. Polarographic measurement of oxygen consumption can only be done in liquid media and requires constant stirring of the suspended worms to avoid settling and formation of an oxygen concentration gradient. The respiration assay is generally completed within 30 minutes. In contrast, the microcalorimetric approach to measuring heat production entails suspension of nematodes in undisturbed assay medium. Stable heat signals are acquired 1 or 2 hours after the start of the experiment. Conceivably, the animals could be excited in one experimental environment and at rest in another. In the latter condition, an oxygen concentration gradient can form. *daf-2(e1370)* is known to be resistant to hypoxia (Scott et

al., 2002). It cannot be ruled out that its low heat dissipation is a metabolic adaptation to conditions of low oxygen, not observed in the wild-type. To test this possibility, we measured heat production of wild-type and *daf-2(e1370)* exposed to normoxic oxygen concentrations. For this, we let the nematodes crawl on Whatman filter paper saturated with axenic medium; we calculated C/R ratios in normoxia and compared them to the C/Rs of nematodes immersed in axenic medium. We found that initially, heat dissipation is higher in normoxia for both strains, leading to elevated C/R ratios and suggesting that these higher oxygen concentrations have an effect on metabolism in both strains. This difference diminished with increasing age; eventually at day 4 of adulthood, C/R from normoxic conditions was equal to C/R of worms immersed in axenic medium in both wild-type and *daf-2* (fig 4). We cannot readily explain why the *daf-2(e1370)* mutant had a higher $C/R_{normoxia}$-to-$C/R_{immersed}$ ratio than the wild-type at day 0 of adulthood. This difference may be linked to the slower transition of L4-to-adult metabolism. From this experiment, we feel that the comparison of C/R ratios obtained via the standard method is appropriate and that low C/R in *daf-2* is most likely not caused by a metabolic adaptation to low oxygen.

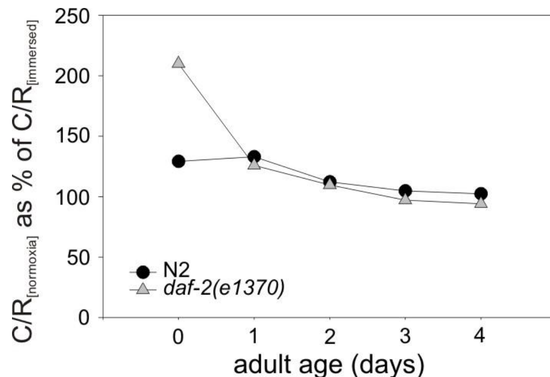


Figure 4: Relative calorimetric to respirometric (C/R) ratio in normoxia expressed as percentage of C/R measured in standard conditions; WT versus *daf-2(e1370)*, measured from day 0 to day 4 of adulthood.

A different explanation for low C/R in *daf-2* can be found in the alternative way of ATP-synthesis in nematodes. ATP synthesis is usually driven by aerobic pathways, but it can be supported by anaerobic metabolism as well. When anaerobic support increases, the C/R ratio will also increase since anaerobic metabolism produces heat without consuming oxygen. This would suggest that the proportion of anaerobic metabolism in wild-type worms is higher compared to the *daf-2* strain. This seems unlikely, firstly because *daf-2(e1370)* mutants are more resistant to hypoxia (Scott et al., 2002), suggesting that wild-type worms rely more on oxygen. Secondly, it has been speculated that a shift to glycolysis and fermentation along with aerobic respiration would be more likely associated with *daf-2(e1370)* adult animals, as this mutant has similarities with the dauer stage (Rea and Johnson, 2003). If so, this would increase the *daf-2(e1370)* C/R ratio, a prediction refuted by our results. We reasoned that a shift towards anaerobic metabolism cannot be responsible for differences in C/R ratios of the two strains.

Potentially, the use of different fuel types can partly be accountable for low C/R ratios in *daf-2*. Depending on whether an animal burns carbohydrates, proteins or fat, its heat production per consumed unit of oxygen will vary. It has been noticed that the *daf-2(e1370)* strain stores large amounts of fat in its gut (Kimura et al., 1997; McElwee et al., 2006) and tends to stop eating after a few days of adulthood (unpublished observation). This may lead to a predominant burning of fat in adult *daf-2(e1370)* mutants. Burning fat releases less heat per consumed unit of oxygen than burning carbohydrate or protein. This

should lead to a lower C/R ratio. However, a switch from carbohydrate to fat breakdown can only be responsible for a C/R decrease of 6-7% (carbohydrate: 21.1 J/ml O_2, fats: 19.8 J/ml O_2). Thus, the difference in C/R ratio between wild-type and *daf-2* is too large to be explained exclusively by use of different fuel types.

One other possible explanation is that C/R ratios in *daf-2(e1370),* having a reduced food uptake, are low because this mutant is forced to use its available energy efficiently. Low C/R might then reflect a dietary restriction effect only. Further studies, including the non-Eat *daf-2(m41)* allele, could verify this possibility. However, low C/R in *daf-2(e1370)* is apparent from day 1 of adulthood, before the mutant stops eating, weakening this argumentation. Moreover, WT worms submitted to DR do not have C/R ratios reduced to levels of fully fed *daf-2(e1370)* (see (Houthoofd *et al.*, 2005a)).

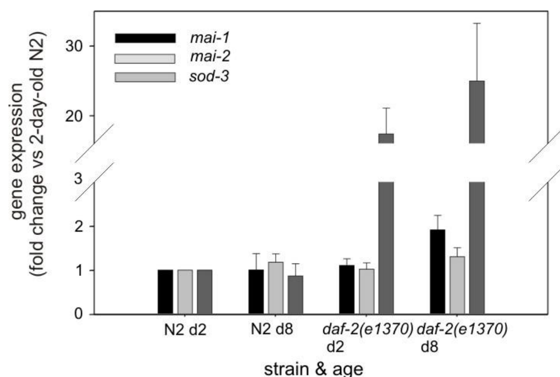


Figure 5: Normalized mRNA expression levels for *mai-1* and *mai-2*; WT versus *daf-2(e1370)*, harvested at day 2 and day 8 of adulthood. The relative expression ratios are the average values from 3 replicate cultures; expression of *sod-3* is added as a positive control.

We considered the possibility that the *daf-2(e1370)* mitochondria might contain more ATP synthase inhibitor protein, IF_1. This protein has the capacity to inhibit the intrinsic F_1-ATPase activity (Lebowitz and Pedersen, 1996). Since futile ATP hydrolysis is expected to generate heat we reasoned that more IF_1 might contribute to the reduced heat production in *daf-2(e1370)* animals. McElwee and colleagues (2006) reported that one of both *C. elegans* IF_1 encoding genes, *mai-1* is expressed at a higher level in *daf-2.* However, *mai-1* lacks a mitochondrial import signal (www.wormbase.org) casting doubt as to its mitochondrial action. We compared the expression of *mai-1* and *mai-2* (which has a mitochondrial import signal) in 2- and 8-day-old adults using qPCR and found no difference between the wild-type and *daf-2* mutant strains nor between the age classes (fig 5). We reasoned that IF_1 is most likely not causal to low C/R ratios in *daf-2*. However, a recent proteomics study, performed in collaboration with our lab, does point to higher concentrations of MAI-2 in a *daf-2* mutant background (personal communication, G. Depuydt). Higher levels of IF_1 in *daf-2(e1370)* remain a possible explanation for lower C/R ratios.

Another possible but far from verified explanation could be either a difference in levels of uncoupling protein (UCP-4) or a difference in 'futile cycling' of fructose-6-phosphate. Mutant animals lacking UCP-4 reportedly contain elevated ATP levels and are sensitive to cold stress (Iser *et al.*, 2005). The *ucp-4* gene is expressed at equal levels in wild-type and *daf-2(e1370)* (McElwee *et al.*, 2006), yet the activity of the protein may be regulated differently in the mutant. Futile cycling of fructose-6-phosphate by phosphofructokinase and fructose bisphosphatase results in the net hydrolysis of ATP and thermogenesis (Voet *et al.*, 2006). This pathway is active in vertebrate species and has not yet been extensively studied in *C. elegans*. Phosphofructokinase is upregulated in *daf-2(e1370)* adult animals and both phosphofructokinase and fructose-1,6-bisphosphatase are upregulated in wild-type dauers (McElwee et al., 2006; Wang and Kim, 2003). Since dauers

and *daf-2* adults predominantly use fat stores for energy production, these changes most likely indicate that cells expressing glycolytic activity are different from those that are active in gluconeogenesis (Wang and Kim, 2003). Thermogenesis by cycling of fructose-6-phosphate (i) would involve opposing reactions of phosphofructokinase and fructose-1,6-bisphosphatase in the same tissue (Voet *et al.*, 2006), (ii) is more likely under allosteric control and (iii) may be reduced *in daf-2* adults relative to the WT animals.

Finally, the decrease in *daf-2* C/R ratio can also be explained by mitochondrial coupling efficiency. When mitochondria of *daf-2(e1370)* are coupled more tightly compared to wild-type, it is expected that they will be able to produce more ATP and less heat per oxygen molecule that is consumed. While standing ATP levels decline exponentially over the course of adulthood in wild-type, these levels tend to decline more slowly in *daf-2* mutants. We view this explanation as the most likely cause of low C/R in *daf-2*: reduction in C/R ratio reflects more efficient energy production since less energy is lost as heat (Kemp and Guan, 1997). However, the relationship between mitochondrial efficiency and standing ATP levels may be more complex, as we will discuss next.

3.2.3.2. ATP and ADP

We (Braeckman et al., 1999; Braeckman et al., 2002c; Houthoofd et al., 2005b) and others (Dillin et al., 2002) found repeatedly that impairment of Ins/IGF-1 signaling resulted in much higher standing levels of ATP than normal. ATP levels result from a dynamic equilibrium between the rates of production and consumption. Changes of ATP content caused by mutation indicate that the balance between ATP production and consumption is altered. Does this change result from altered production, or consumption, or both? Higher ATP levels might derive from reduced ATP consumption rates for anabolic reactions: these mutants retain higher fat stores and produce fewer offspring, suggesting that they have a reduced energetic demand for anabolic reactions, including the production of yolk protein (Gems et al., 1998; Murphy et al., 2003). Then again, there are arguments against this idea: Houthoofd and co-workers (2005b) found similar levels of ATP in three Ins/IGF-1 mutants, which have variably reduced levels of fertility: the reduction in fecundity ranged from 71% to 3% relative to the WT's brood size, while their standing ATP levels were similar. Also, the suggestion that the consumption of synthesized ATP happens at slower rates and thereby releases heat more slowly, is in disagreement with Kemp and Guan (1997), who state that produced heat is almost completely due to catabolic reactions, and cannot be used to explain low C/R ratios in *daf-2(e1370)*. Lower ATP consumption in *daf-2* cannot be confirmed nor disproven by our data.

From standing ATP levels, it could be assumed that ATP production is higher in *daf-2* mutants. In contrast, it is possible that the WT produces more ATP, and uses more of it, resulting in lower ATP concentrations. Since oxygen consumption is relatively similar in the two strains, we would have to assume that this higher ATP production in the WT would in part stem from anaerobic metabolism. In agreement with anaerobic energy metabolism in many cells with high proliferative activity, this extra ATP production could be related to the higher fecundity of the WT. If this is the case, then the assumption that *daf-2* mutants are energetically more efficient would not stand. However, some of the arguments cited in support of *daf-2*'s energetic efficiency also contest a role for anaerobic metabolism in

fecundity. First of all, as mentioned, reduced fecundity had no effect on ATP concentrations in three Ins/IGF-1 mutants (Houthoofd et al., 2005b). This would imply that for each of these mutants taking part in the same signaling pathway, their anaerobic metabolism would only be as high as their respective energetic input in their progeny, which seems unlikely. Secondly, WT worms are less resistant to hypoxia than *daf-2* mutants (Scott et al., 2002), pointing instead to *daf-2* as a candidate for higher anaerobic metabolism (but see section 3.2.3.1). 24 hours of hypoxia has a negligible effect on mortality, but it reduces total progeny numbers considerably in the WT, regardless of whether the hypoxia treatment is started before or after the nematode has reached reproductive maturity (Mehta et al., 2009). This is not surprising, as the process of oocyte maturation is energetically very costly (Mendenhall et al., 2009). Since the egg-laying period is limited (at 20°C, egg-laying is concentrated in about 3 days of the adult's life trajectory), we would expect to see some change in the ATP profiles of the WT after its reproductive period if its energy provision is derived from both aerobic and anaerobic processes. This is not the case, leading us to believe that ATP production from anaerobic metabolism is negligible. This was confirmed by Föll and co-workers (1999), who found only very limited amounts of end-products of anaerobic processes in the worm.

Enhanced ATP production is consistent with the lower C/R ratios in *daf-2* mutant animals relative to wild-type, despite their similar rates of oxygen consumption, but it seems to violate common biochemical wisdom that "The activities of the pathways that produce ATP are under strict coordinated control so that ATP is never produced more rapidly than necessary" (quoted from Voet et al. (2006)). It is not clear which alterations cause this apparent uncoupling of ATP production and consumption in *daf-2* mutants. The activity of complex V is controlled by the flux of protons and the concentration of ADP in the matrix. Normally, the concentrations of ATP and ADP are in equilibrium: synthesis of ATP is expected to lower the concentration of ADP, in turn lowering the rate of ATP synthesis by Complex V. Also, any decrease of the ADP/ATP ratio in the cytosol is expected to result in reduced import of ADP into the matrix tending to maintain the ratio constant. In *daf-2* mutants both ATP and ADP concentrations are elevated, complicating interpretation of their possible role in controlling energy production. It should be noted that metabolic control systems normally allow for very little variation in the cellular ATP/ADP/AMP ratios. Thus, it is possible that variations in ADP and ATP inversely correlate with the variations in AMP content between strains or age. AMP content was not assessed in this study. However, because of the large ATP and ADP difference between the strains at old age, this seems unlikely. We suspect that aging is associated with a significant loss of total nucleotide content in the wild-type.

Another potential site of control is the cytochrome *c* oxidase (Complex IV) reaction which is irreversible. In mammals, ATP is known to bind and inhibit Complex IV (Arnold and Kadenbach, 1999) allosterically, thereby adjusting ATP production to energetic demand. 3,5-diiodothyronine can release the allosteric inhibition of complex IV by ATP, allowing high ATP production in the presence of high concentrations of ATP (Arnold et al., 1998). Possibly altered IIS signaling in *daf-2* animals affects an analogous worm control mechanism.

If low heat production in *daf-2(e1370)* is indicative of a higher mitochondrial coupling efficiency, then possibly, DAF-2 signaling controls mitochondrial bioenergetics by regulating the heat-producing proton leak pathway. If and how this is done, remains to be

elucidated. Studies using isolated mitochondria are required to further unravel the role of Ins/IGF like signaling in the control of mitochondrial bioenergetics. Based on data presented here, it is unclear whether or not this effect on mitochondrial bioenergetics is directly involved in the lifespan extension of *daf-2(e1370)*. Some aspects of whole worm oxidative metabolism require our attention when we attempt to link mitochondrial data to the *in vivo* results described in this chapter. For instance, the largest difference in C/R ratio between the two strains can be found around middle age, while ATP content discrepancies are largest at old age. The question to be answered is whether mitochondrial data will allow us to resolve these discrepancies. If not, then other factors must play a role in regulating oxidative metabolism as well.

We cannot state with certainty that the oxidative metabolism profiles measured *in vivo* are indicative of essential, lifespan-determining differences between WT and the long-lived mutant. Although the ATP phenotype of *daf-2(e1370)* is very pronounced, it is not necessarily correlated to its longevity phenotype. RNAi against several mitochondrial genes reportedly lowered ATP substantially but extended life span (Dillin et al., 2002); also, *isp-1* mutants (defect in an iron sulfur protein of mitochondrial complex III) that are hypometabolic and are therefore expected to have low ATP, show a dramatic 60% life span extension (Feng et al., 2001). On the other hand, the *ucp-4(0)* mutant (UCP-4 is the only uncoupling-protein-like protein encoded in the *C. elegans* genome) contains elevated ATP levels yet is not long-lived (Iser et al., 2005). Also, a causal connection between the low C/R ratio and high ATP levels has not been established; we have to note that the *daf-16* mutants have rather low C/R ratios (at least for part of their lifespan) but normal ATP levels ((Houthoofd et al., 2005a) and results in this section).

3.2.3.3. Antioxidant capacity

The elevated antioxidant capacity of *daf-2(e1370)* in the form of higher SOD and catalase activity confirms an earlier study (Vanfleteren and De Vreese, 1995), and in this section, it is also illustrated by assessment of levels of reduced glutathione. Moreover, high *daf-2(e1370)* activity of SOD and catalase can be suppressed by mutation in *daf-16(m26)* (Houthoofd, 2003; Houthoofd et al., 2004), indicating that antioxidant levels are regulated by the Ins/IGF-1 pathway. The *daf-2(e1370)* mutant is extremely resistant to oxidative stress (Honda and Honda, 1999). Its increased stress resistance can most likely be attributed to elevated antioxidant activity, caused by downregulation of in the Ins/IGF-1 pathway, but this does not provide a causal connection between increased stress resistance and lifespan extension. Moreover, a number of recent studies in *C. elegans* provide evidence that oxidative stress is not causal to aging (by knocking-down genes with antioxidant effects and recording lifespan (Doonan et al., 2008; Van Raamsdonk and Hekimi, 2009; Yang et al., 2007)). Also, in evaluating the role of antioxidant capacity in lifespan, we need to take into account the amount of ROS produced and the damage afflicted by these ROS. We will elaborate on these aspects in chapter 6.

In analogy to the potential food intake related justification for higher antioxidant defenses in dauers and DR-treated worms (see section 2.4.2), discrepancies in antioxidant enzyme activities between the WT and *daf-2* could be explained by differences in food uptake. The time point when antioxidant defenses start to differ between the two strains

seemingly matches the time point when the *daf-2* mutant starts eating less (in other words, after day 2 of adulthood; unpublished observation). This is especially apparent in catalase activity profiles, which show a sharp increase from day 3 onwards. However, this does not explain the decrease seen in catalase activity after day 5 of adulthood. Moreover, when these experiments were repeated, antioxidant defense activities in the two strains were dissimilar from day 0 of adulthood onwards, showing no such obvious peak in *daf-2* catalase activity at day 3. Increases in SOD activity did occur in *daf-2*, but only from day 5 of adulthood onwards (Houthoofd et al., 2005b). To confirm the potential inverse relationship between antioxidant defenses and food intake, additional testing is required; for instance, antioxidant defenses could be assessed in WT that are shifted from fully-fed to restricted feeding conditions or vice versa in order to observe potential changes in catalase or SOD enzyme activities.

3.2.4. Conclusion

Oxidative damage is commonly held responsible for cellular aging, as postulated by the Free Radical Theory of Aging (Harman, 1956, 1972). By assuming that the generation of reactive oxygen species is proportional to metabolic rate, the Free Radical Theory seems to be compatible with the Rate-of-Living Theory (Pearl, 1928). We used the long-lived Ins/IGF-1 mutant *daf-2(e1370)* to test the validity of these theories in explaining the aging process. It is clear that Ins/IGF-1 signaling influences aerobic energy production. However, metabolic rates in *daf-2(e1370)* refute the Rate-of-Living theory. In *daf-2(e1370)*, oxygen consumption is similar to respiration in the WT, while heat production is reduced in the mutant. Since direct calorimetry is a measure of total metabolic activity, it could be stated that oxygen dependent metabolism is not reduced but total metabolism is lower in *daf-2*. However, as it has been shown that the contribution by anabolism to heat dissipation is negligible (Kemp and Guan, 1997) and that anaerobic processes are negligible in *C. elegans*, we conclude that metabolism is not reduced in *daf-2(e1370)*. Rather, in this mutant, metabolism seems to be shifted towards a higher efficiency, generating less heat and more ATP for the same amount of oxygen consumed. A study on mitochondrial bioenergetics may verify if this is the case, as well as elucidate the balance between metabolic rate, antioxidant capacity and the production of and damage by ROS. Lastly, we remark that Ins/IGF-1 affects both oxidative metabolism and antioxidant capacity, but whether these aspects of *daf-2(e1370)* play a causal role in its extended lifespan, remains unclear.

3.3. Addendum to chapter 3: Normalization of data

3.3.1. Introduction

A major methodological question that can be raised is how to normalize experimental data on metabolic rates in aging studies. Ideally, a factor independent of age would be used for scaling. The most appropriate basis for normalizing metabolic data is unit metabolically active mass. However, this parameter is usually not known. Volume, wet weight, dry weight and protein content are commonly used proxies. In this section, we will clarify which normalization method has been performed for which dataset of chapters 2 and 3, and we will elaborate on why these choices were made.

3.3.2. Normalization to worm number

It has been argued that metabolic data of aging studies need to be normalized to worm number, in other words: metabolic parameters should be expressed per individual (Van Voorhies and Ward, 1999). If the size of the organism tested is invariant to strain, age and culture conditions, changes could be normalized to worm number. For some of the nematode strains used in this thesis, age-related volumes have been recorded (fig 6-9). Eat mutants are smaller in size than WT for the largest part of their lifespan. Generally, Clk mutants are slightly smaller relative to WT over the entire age trajectory; in contrast, *clk-3* is considerably larger than WT. *daf-2*(*e1370)* worms appear to shrink as they age. For WT and *glp-4*, volume was very dependent on bacterial concentration.

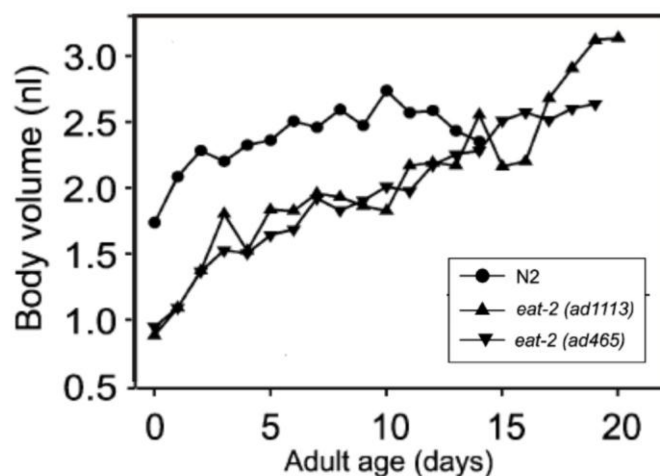


Figure 6: Body volume of N2, *eat-2(ad1113)* and *eat-2(ad465)* in monoxenic liquid culture. Source: Houthoofd et al. (2002b).

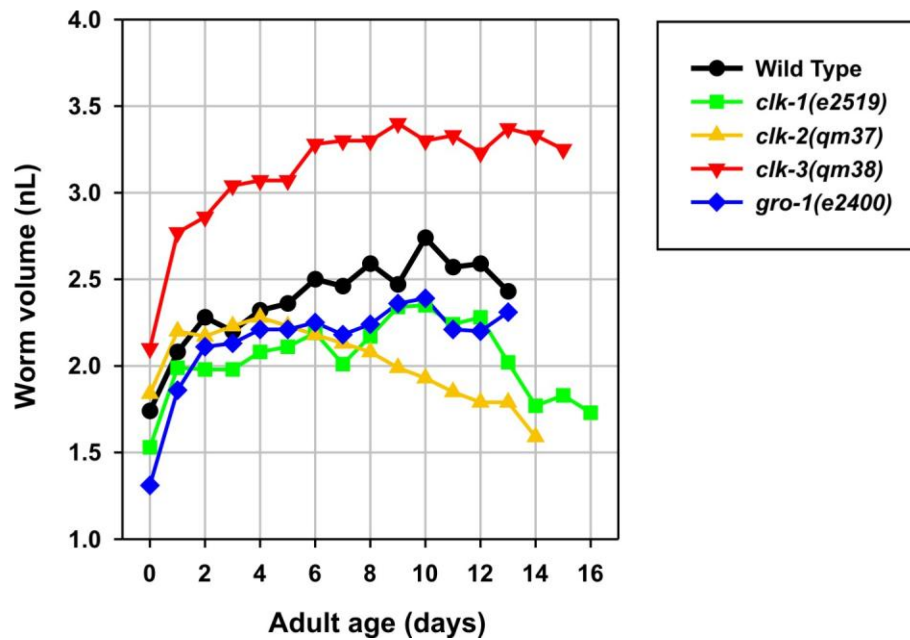


Figure 7: Body volume of N2 and 4 Clk mutants in monoxenic liquid culture. Source: Braeckman et al. (2002a).

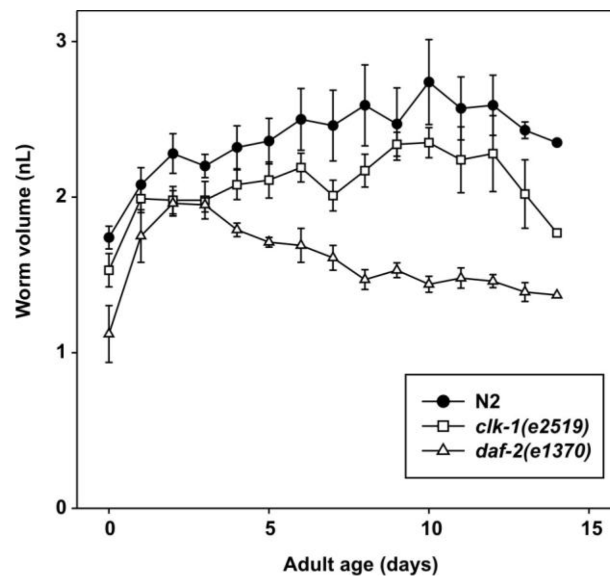


Figure 8: Body volume of N2, *clk-1* & *daf-2* in monoxenic liquid culture. Source: Braeckman et al. (2002c).

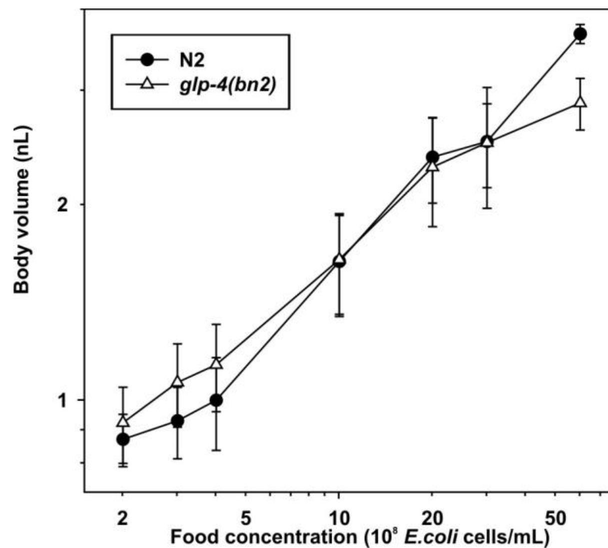


Figure 9: Body volume of N2 and *glp-4* in a gradient of bacterial dilutions (monoxenic liquid culture). Source: Braeckman et al. (2002c).

As is clear from these measurements, worm volume is not an invariant parameter: worm size depends on age, strain and/or feeding conditions. Scaling to worm number ignores the size differences between ages and strains. In our case, it would be a suboptimal choice to scale to worm number, as we cannot limit our comparisons to worms of equivalent size and development stage: equivalence for size and development cannot be obtained at the same time when one strain develops slower and is smaller than the other at all ages. If everything except size is kept constant, the largest individuals will always yield the largest scores in metabolic parameters. Moreover, data obtained through scaling per worm number are not comparable with measurements of enzymatic activity. As a consequence, worm volume was generally not used as a normalization method. This issue has been discussed at length previously (in: Braeckman et al. (2002c,d), Van Voorhies (2002a,b)).

The reasoning behind the use of worm number as a normalization factor is that aging is viewed as a single organismal trait. We do not adhere to this theorem. Rather, it is at the (sub)cellular level that age-dependent changes of metabolic rate occur: aging is a cellular trait. This is illustrated by the fact that not all cells of *C. elegans* age at the same rate; neurons are still in good condition in very old worms while their muscle cells suffer from sarcopenia (Herndon et al., 2002).

3.3.3. Age- and strain-related differences in protein density

If scaling to worm number is not an option due to differences between ages, strains and culture conditions, then we have to choose an acceptable proxy of biomass. A practical approach to this is to use either volume or protein content of worms. If protein density (protein content/volume of worm) shows little strain- and age-dependent variation, both scaling to either protein or volume is a good choice. Protein density has been assessed in several strains (fig 10-12). This parameter changes during the adult life trajectory of most strains: for instance, in the WT, age-specific rises are followed by a tendency to reach a plateau at very old age. Protein density is similar to WT and increases with age in the *eat-2* mutant. All four Clk mutants have similar protein densities at comparable ages, but contain

less protein at mid-age than the WT. In *daf-2* also, protein density is lower than in WT, especially at mid-age.

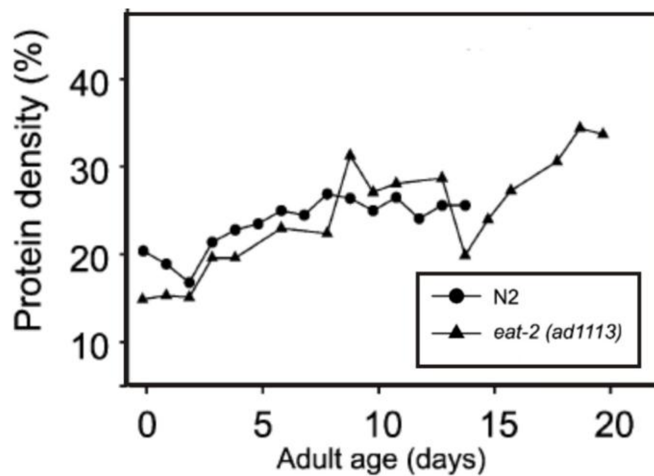


Figure 10: Protein density of N2 and *eat-2 (ad1113).* Source: Houthoofd et al. (2002b).

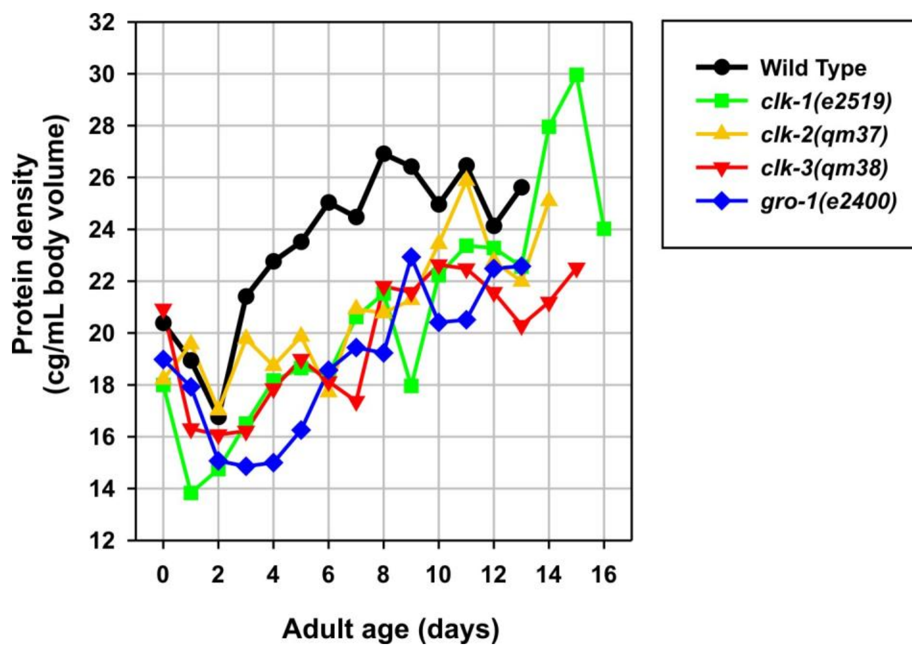


Figure 11: Protein density of N2 and 4 Clk mutants in monoxenic liquid culture. Source: Braeckman et al. (2002a).

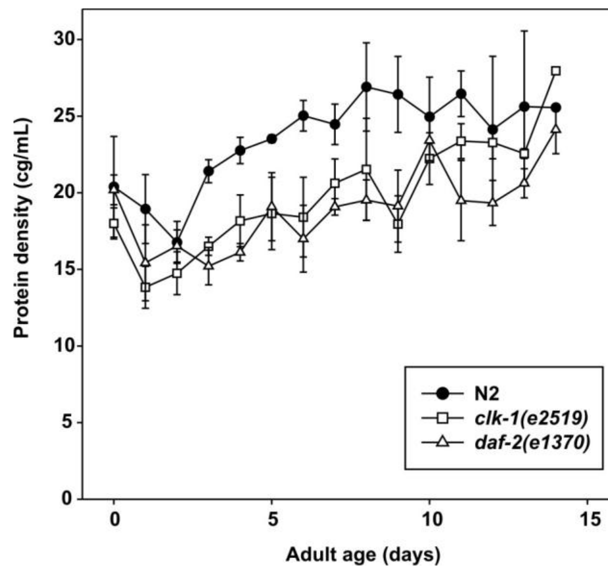


Figure 12: Protein density of N2, *clk-1* and *daf-2(e1370)* in monoxenic liquid culture. Source: Braeckman et al. (2002c).

Normalizing to protein is based on the implicit assumption that all protein is metabolically active. Protein can be used as a proxy for metabolically active biomass, though their correlation is not perfect: for example, in aging *C. elegans*, the contribution of cuticular to total mass increases. In other words: the increase in protein may be partially due to metabolically inactive protein.

The alternative, scaling to volume, is also compromised due to age- and strain-related differences. Here also, changes in volume will not likely be perfectly matched to changes in metabolically active biomass. As moderate to substantial differences occur in size and protein density among WT and mutant worms of comparable age, scaling to protein and volume will produce slightly different outcomes.

3.3.4. Normalization to protein versus volume

It is not always clear which scaling factor best approximates the metabolically active mass.

For the dauer (section 2.2) and Clk (section 2.3) datasets, both normalizations have been performed, but only results for normalization to protein have been reported in this thesis. For dauers, normalization according to volume instead of protein increases metabolic values with 50% -> 20% relative to young -> old adults due to age-related changes in protein density. For Clks, results expressed per volume can be found in Braeckman et al. (2002a). The reason for using both scaling factors in this study is to avoid over-interpretation of small differences between mutants and WT. Scaling to volume reduces differences between Clk mutants and the WT but does not alter the final conclusion of the article. When the most conservative interpretation of data was made, it was concluded that Clk mutants generally do not have a lower metabolic rate than WT, and that antioxidant defenses were generally not upregulated in these mutants.

3.3.5. Normalization to protein and allometric scaling

The use of mass-specific units for scaling assumes that metabolic rate varies isometrically with body mass. As this is not always the case, and as protein content or worm volume are used as proxies for metabolically active biomass, expressing the data in an appropriate allometric relationship is required. When the body sizes compared differ substantially among individuals from different strains, age groups and treatment conditions, the rate of energy expenditure shows a negative allometric relationship with body mass. Correction for size differences has been applied to some of the data in this thesis, using the Brody-Kleiber equation (see section 3.2.2.2).

Metabolic parameters for dietary restriction (section 2.4) and Ins/IGF pathway mutants (Chapter 3) were scaled to protein to correct for differences in biomass. Additionally, respiration and heat dissipation were allometrically scaled, taking into account the fact that the rate of energy expenditure per unit mass varies with body size. Allometric scaling ensures that the size-specific component is subtracted to obtain physiological changes that result from the treatment (in this case, food restriction) or the mutation (e.g. *daf-2*).

3.3.6. Conclusion

Metabolic activities generally change with age, among strains and among treatments. In addition, protein density generally increases with age in reproductive adults, and adult worms may show large differences in size between strains. Consequently, for quantitative comparisons between age groups, all experimental data should be normalized appropriately.

The question arises which factor for normalization is most appropriate. Due to considerable differences in size among strains, ages and treatments, scaling to worm number would lead to inexact conclusions. The standard scaling factor in biochemistry is protein content, as it can be readily and accurately determined. Normalization to volume is less common and more labour-intensive. For dauer and Clk datasets, we have normalized the data to both volume and protein content. Consequences for dauer profiles were summarized, and for Clk data, a conservative interpretation of the results did not alter the key conclusions of the study.

For dietary restriction and Ins/IGF pathway mutants, data were scaled to protein. Additionally, metabolic rates (respiration rate and heat production) were allometrically scaled to correct for differences in body volume caused by different culture conditions, age and mutation, according to the Brody–Kleiber equation. In our view, this correction for size differences using the Brody–Kleiber allometric equation is the most appropriate approach to scaling metabolic rates to biomass.

PART II: RESULTS

Chapter 4

Ins/IGF-1 and mitochondrial function: gene expression, proteomics and structural organization

In part redrafted from:
Brys *et al* (2010). Disruption of insulin signaling preserves bioenergetic competence of mitochondria in ageing *Caenorhabditis elegans*. BMC Biology **8**, 91

Personal contribution:
Brys *et al* (2010) all nematode cultures, Western blots and enzyme activities, writing of manuscript
Contribution to work by Proteosys and Dencher labs: preparation of *C. elegans* mitochondrial samples

Proteosys and Dencher labs, and Vanfleteren lab took part in MiMage, an Integrated Project within the 6th Framework Programme of the European Commission focusing on the role of mitochondria in conserved mechanisms of aging.

ABSTRACT

Though metabolic rates decrease with age in both wild-type *C. elegans* and the *daf-2(e1370)* mutant, the *e1370* mutation alters *in vivo* oxidative metabolism substantially. So far, the causes of these metabolic differences have not been fully elucidated by gene expression or proteomic studies. In this chapter we report effects of the *e1370* mutation on mitochondrial characteristics at the proteome level. In wild-type worms, the abundance of key mitochondrial proteins declines with age, although the mitochondrial mass, inferred from the mitochondrial DNA copy number, remains unaltered. In contrast, the age-dependent decrease of key mitochondrial proteins and electron transport chain complexes is considerably attenuated in *daf-2(e1370)* adult animals.

4.1 General introduction

In the previous chapter, we have ascertained that both age and Ins/IGF-1 mutation affect *C. elegans* oxidative metabolism. Metabolic rates decrease with increasing age in both wild-type and *daf-2(e1370)*. In *daf-2(e1370)*, metabolism is seemingly shifted towards a higher efficiency, producing less heat and more ATP for the same amount of oxygen consumed. In this chapter, the influence of age and Ins/IGF-1 pathway disruption on oxidative metabolism are assessed at the level of mitochondrial gene expression and proteome.

For an in-depth study of oxidative metabolism, a suitable protocol for isolation of mitochondria followed by assessment of potential damage to the outer and inner mitochondrial membranes is required. Next, determining mitochondrial DNA copy number will tell us if age-related decreases in metabolic rate are linked to decreases in mitochondrial mass. To define effects of genetic interventions on the aging process, genome-wide scans for transcript abundance are often the chosen tool. Several research groups have performed these arrays to characterize the *C. elegans* transcriptome; their data sets allow us to assess changes in gene expression of ETC complex subunits under the influence of defective Ins/IGF-1 signaling. The effect of age and mutation on the abundance of proteins related to oxidative metabolism can be assessed through proteomics analysis of mitochondrial samples, and Western blotting on both mitochondrial material and whole worm lysates. Finally, we study the mitochondrial proteome of wild-type and *daf-2* mutant worms through assessment of the mitochondrial structural organization in supercomplexes.

A useful protocol for isolation of mitochondria from *C. elegans* has been created, resulting in largely intact isolated mitochondria. Assessment of mitochondrial DNA copy number shows that mitochondrial mass remains unaltered with increasing age. Though there are no manifest differences in the expression of genes related to the electron transport chain between wild-type and the *daf-2* mutant, at the proteome level, we demonstrate that the age-dependent decrease of key mitochondrial proteins and electron transport chain complexes is attenuated in the long-lived mutant.

4.2 Isolation of mitochondrial suspensions

4.2.1. Introduction

In the late 1940's, the group of Pallade and co-workers (Hogeboom et al., 1948) pioneered in the use of differential centrifugation, or the separation of the constituents of the cell based on their different sedimentation properties following mechanical homogenization of the tissue, to isolate mitochondria from rat liver. Differential centrifugation utilizes the difference in weight between the organelle of interest, in this case the mitochondrion, and the biological material that needs to be removed from the preparation (nuclei, intact liver cells, red blood cells…). By centrifugation at low speed (where the pellet is considered as waste and needs to be removed) followed by centrifugation of the supernatant at high speed, (Hogeboom et al., 1948) obtained mitochondrial suspensions with hardly any contamination.

Isolation of functional, purified and intact mitochondria is relatively easy when it is performed on homogenous tissues like mouse liver, or on a cell culture like fibroblasts; brain and heart mitochondrial isolation require extended protocols (Frezza et al., 2007).

The *C. elegans* body consists of two concentric tubes separated by the pseudocoelome which contains the gonad. The inner tube is the intestine; the outer tube consists of muscles, neurons, hypodermis and a tough cuticle, composed of collagens, cuticulins, lipids and glycoproteins (www.wormatlas.org). To isolate mitochondria from *C. elegans*, the cuticle needs to be broken, taking care to limit damage to the underlying tissues; consequently, the method of homogenization will be crucial. Also, we need to carry out repeated mitochondrial preparation from an aging cohort of a very small organism; relatively large daily sample sizes of approximately 300,000 worms amount to just 1 ml of dense worm suspension, yielding only about 1 mg of mitochondrial protein. Since these mitochondria will ultimately be needed to evaluate oxidative phosphorylation, they have to be functionally intact; hence the procedure for isolation is kept as short as possible.

4.2.2. Materials and methods

4.2.2.1. Growth and harvest

Wild-type animals were cultured as in (2.2.1.1.), yet at a much larger scale. When the total population size needed for all successive harvests was reached (approximately 5 million worms), the nematodes were rinsed off the plates shortly after the molt to the fourth larval stage, transferred to Fernbach flasks in S buffer and supplemented with *E. coli* and FUdR as described in (2.2.1.1.). For such large-scale culturing, substantial numbers of Fernbach flasks were needed, taking into account that the number of live worms dropped considerably with increasing age. Feeding and maintenance of the cultures was done as in (2.2.1.1.). Harvesting started approximately 24 hours after the worms were shifted from 17 to 24°C. We defined this time point as day 0 of adulthood. Samples containing approximately 300,000 live worms were harvested at regular intervals and freed from dead worms, bacteria and debris as described in (2.2.1.1.). The cleaned worms were suspended in 15 ml S-buffer and aliquots of 0.1 ml were pipetted into microcentrifuge tubes and stored at – 75 °C for assays that can be performed using frozen worms (citrate synthase activity, cytochrome *c* Western Blot, protein determination). The bulk of live animals were used for the isolation of mitochondria.

4.2.2.2. Isolation of mitochondria

Mitochondria were isolated essentially following the method described by (Kayser et al., 2001), with alterations to reduce the amount of nematodes needed per mitochondrial isolation. Briefly, approximately 300,000 age-synchronized animals were harvested from the monoxenic cultures at regular time intervals, cleaned and washed with distilled water to remove the S buffer, and finally suspended in mitochondrial isolation buffer consisting of 220 mM mannitol, 70 mM sucrose, 5 mM MOPS and 2 mM EDTA, pH 7.4 (MSME). All subsequent treatments were performed at 4 °C. One ml of concentrated worm suspension was transferred to a microcentrifuge tube and chopped for 40 s using an IKA Ultraturrax

rotor-stator mixer (IKA Werke, Staufen, Germany) operated at the maximum speed of 25,000 rpm. Next, 1 ml of MSME containing 0.4 % BSA was added and the suspension was thoroughly mixed by inversion, and centrifuged for 5 min at 380 g to remove large debris and nuclei. The supernatant was transferred to a fresh centrifuge tube and centrifugation was repeated. The resulting supernatant containing crude mitochondria was centrifuged for 5 min at 4,500 g and the resulting mitochondrial pellet was resuspended in MSME. Aliquots were used for instant measurement of mitochondrial respiration, ATP synthesis, membrane potential and production of H_2O_2. Results of these assays will be described in the next chapters. The remainder was frozen at -75 °C in 10 µl aliquots for quantification of citrate synthase activity, cytochrome *c* and protein content, as well as carbonylation levels (see chapter 6). On average, 300,000 nematodes yielded ~1mg of mitochondrial protein. For proteomic analysis and OXPHOS supercomplexes studies, BSA was left out of the isolation buffer and the entire yield was flash-frozen in liquid nitrogen. Before freezing, samples for proteomics were spun down at 20,800 g for 5 min and the supernatant was removed.

4.2.3. Results and discussion

To obtain a protocol for isolation of functional mitochondria with an acceptable degree of purity, several ways of nematode disruption and mitochondrial purification were attempted. Among the former attempts, we mention the use of a discontinuous sucrose gradient and ultracentrifugation. However, the centrifugation steps in this protocol not only took too much time, but also delivered no clear and replicable banding of fractions. Homogenization of nematode suspensions with a Teflon or glass pestle in a Potter/Elvehjem tissue grinder resulted in insufficient disruption of the cuticle and consequently, low yield. Conversely, manual homogenization applying a douncer with a rough glass surface did disrupt the cuticle sufficiently, but the duration of the process was difficult to standardize. Finally, we assembled a protocol derived from Kayser and co-workers (2001), where a mechanically driven rotor-stator mixer[1] for small volumes was used on a highly concentrated worm suspension. We chose to decrease the duration and speed of the centrifugation step that pellets the mitochondria, because we found that after higher speed centrifugation, the mitochondria were not easily resuspended. The omission of certain steps in the Kayser assay (a proteinase treatment, extra disruption of tissue with a Teflon pestle in a Potter/Elvehjem tissue grinder and filtration through a gauze before final centrifugation) and the use of centrifugation speeds as set out by Murfitt and co-workers (1976) provided us with a fast and easily repeatable protocol that delivers active mitochondria isolated from only 1 ml of dense worm suspension. More details on the justification of the protocol steps used can be found in section 4.7, table 2 (addendum to chapter 4). We could not rule out the presence of minor contaminants; e.g. mitochondrial isolates tested positive for the presence of Histone H4, even after treatment with proteinase K, indicating that some nuclear material is still present in the suspension. The performance of mitochondrial suspensions prepared in this way will be discussed in chapter 5.

[1] A rotor-stator mixer consists of a slotted rotor operating inside a slotted stator. The rotor turning at high speeds functions as a centrifugal pump, drawing materials in from above and below. As the rotor blades pass the stator, they mechanically shear the contents.

4.3. Cytochrome *c* abundance, citrate synthase activity and mitochondrial genome copy number

4.3.1. Introduction

Whatever age- or mutation-related mitochondrial differences we may find, it is essential that we interpret them correctly, taking into consideration potential artifacts generated by the isolation procedure and the assay conditions. Also, we need to ascertain whether or not these differences, as well as the whole-worm metabolic rates determined in chapter 3 are linked to age-related changes in mitochondrial mass.

4.3.2. Materials and methods

4.3.2.1. strains & culture conditions

Strains used were the wild-type N2 (male stock provided by the CGC), and Ins/IGF-1 mutants *daf-2(e1370)* and *daf-16(mgDf50)*. For culture conditions and isolation of mitochondria, we refer to (4.2.2.).

4.3.2.2. assays

Protein determination and cytochrome *c* quantification

Protein was generally determined using the BCA (bicinchoninic acid) method as described in (2.2.1.2.), but prior degradation with alkali was only applied for estimating whole worm protein content. For quantification of cytochrome *c* in whole worm extract and in mitochondrial preparations, frozen samples with known protein concentration were mixed with Laemmli buffer, heated at 99°C for 5 min and equal amounts of protein were loaded on gels. Western blotting was performed as described by (Matthijssens et al., 2008). Primary antibodies against cytochrome *c* were purchased from Mitosciences (OR, USA). Secondary antibody was horseradish peroxidase (HRP)-conjugated anti-mouse antibody from Sigma.

Citrate synthase activity

Citrate synthase activity in crude extract and mitochondria was determined as follows: mitochondrial preparations were made 1% in CHAPS and the resulting solution was clarified by centrifugation at 20,800 g for 10 min. Whole-worm extract was prepared as described in (2.2.1.2.). Citrate synthase was assayed by monitoring the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) at 412nm (ε_{412} = 13.6 mM^{-1} cm^{-1}) coupled to the reduction of Coenzyme A by the citrate synthase reaction in the presence of oxaloacetate. The protocol described by (Trounce et al., 1996) was adapted for use with microtitre plates. Briefly, 0.1 M Tris-HCl (pH 8.0), 0.3mM acetyl-CoA, 0.1mM DTNB, and samples of the

mitochondrial preparation containing approximately 5 µg protein or whole-worm extract containing approximately 40 µg protein were incubated for 10 minutes at 24°C. The reaction was initiated by the addition of 0.75 mM oxaloacetate (final concentration), and the rise in absorbance at 412 nm was monitored for 3 minutes in a Spectramax 190 (Molecular Devices, CA, USA) plate reader.

Determination of mtDNA copy number

For mtDNA copy number assessment, age-synchronized N2 and *daf-2(e1370)* L1 larvae were grown at 17 °C until they reached the third juvenile stage to avoid induction of dauer formation at higher temperature by the mutation in *daf-2*. Next they were shifted to 24 °C. For nucleic acid extraction, 1-, 3-, 6-, 9- and 12-day-old adult worms were transferred to individual microcentrifuge tubes containing 25 µl of worm lysis buffer (50 mM KCl, 2.5 mM $MgCl_2$, 0.15% Nonidet P40 (octylphenoxypolyethoxyethanol), 0.15% Tween 20, 10 mM Tris.HCl pH 8.3) and frozen at -75 °C for 10 min. Next, the samples were thawed, 1 µl of proteinase K was added at a final concentration of 100 µg/µl and protein was digested for 1 h at 65 °C, followed by 10 min at 95 °C to inactivate the enzyme.

mtDNA copy number was assayed according to (Koekemoer et al., 1998). A standard curve for determining the mitochondrial genome content was obtained as follows. Staged worms were lysed as described and PCR was performed to amplify 3 mitochondrial genes: ND5 (forward primer: CCACACCGGTGAGGTCTTTGGTTCATAGTAG; reverse: GTGAAAGTGTCCTCAAGGCTACCACCTTC), COII (forward: TCGTTGTGTTATTCCTTGTGATACT, reverse: ACAAATCTCTGAACATTGACCATAA) and COIII (forward: TACAGTAACTTGAGCACATCACAGA, reverse: ATACTCCGTCTGCAATAGAAAATCT)
The PCR product was purified with the QIAquick PCR purification kit (Qiagen, Venlo, the Netherlands) and the concentration of the resulting templates was determined using the NanoDrop ND-1000 diode array spectrophotometer (NanoDrop Technologies, DE, USA). The copy number was then calculated from the weight in Daltons and Avogadro's number and a serial dilution was used to generate a standard curve for quantitative PCR.

Increasing amounts of template (10-10^8 copies per reaction) were amplified and the standard curve was constructed by plotting the cycle threshold (Ct) values versus the logarithm of the initial template copy number. For assessing the average mitochondrial genome content per worm we grew parallel cultures of N2 and *daf-2(e1370)* and harvested staged worms of increasing age as described above. This scheme was repeated 3 times to account for environmental variation. DNA samples were prepared from 48 single worms per time point, and pooled to dampen variation in individual mitochondrial genome content. QPCR amplification was carried out using the Qiagen Rotor-Gene real-time cycler with Invitrogen Platinum SYBR Green qPCR SuperMix-UDG. The cycling conditions were as follows: 50°C for 2 min, initial denaturation at 95°C for 2 min followed by 45 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Following the final cycle, melting curve analysis was performed to examine the specificity in each reaction tube (absence of primer dimers and other non-specific products). We first determined the mtDNA content as a function of age and strain differences and found that all three amplicons yielded moderately diverging estimates of the mitochondrial copy number but with equal age- and strain-related trends. Absolute copy numbers were therefore normalized as follows: for each primer pair, wild-

type values from all ages were averaged and this value was set to 100. All original values were then rescaled relative to this reference value and pooled to obtain an estimate of mtDNA content per animal relative to the reference value.

Statistics

For statistics, we refer to (3.2.1.3.).

4.3.3. Results and discussion

The isolation of mitochondria from nematode tissue requires harsh treatments to break the tough cuticle. If old worms contain more fragile mitochondria, the isolation process might, by itself, yield a larger portion of damaged mitochondria. In order to ascertain that mitochondrial isolation does not lead to disproportionate amounts of damaged organelles with progressing age and strain differences, we compared the activity level of citrate synthase, a key citric acid cycle enzyme of the matrix and the abundance of cytochrome *c*, an essential component of the electron transport chain which is present in the intermembrane space. We reasoned that, if the relative abundance of these proteins in isolated mitochondria and in whole worm extracts is identical, this would indicate that the preparation procedure caused no harm to the mitochondria or, at least, that possible damage to the mitochondrial inner or outer membrane inflicted by the isolation process was proportionate at all ages.

Western blots showed that cytochrome *c* protein levels declined with age in all three strains and at similar rates in both mitochondrial preparations and whole worm extracts (fig. 1 B). Remarkably, this decline was much slower in the long-lived *daf-2(e1370)* indicating that these mutant animals can attenuate age-dependent reduction in cytochrome *c* content. As this effect was also seen in whole worm extracts, it is not due to higher resilience of the *daf-2* mitochondrial outer membrane to disruption during isolation. Cytochrome *c* is also known as an important factor in apoptosis. We considered the possibility that loss of cytochrome *c* could be related to apoptotic processes. However, in *C. elegans*, apoptosis chiefly plays a role in development and in the gonad. No apoptotic cells could be observed in somatic cells, and apoptosis had no effect on lifespan (Garigan et al., 2002). From this we conclude that apoptosis is most likely not causal to differences in cytochrome *c* content among strains.

Similarly, mitochondrial preparations and whole worm extract yielded identical age-specific activity profiles of citrate synthase (fig. 1 C&D). Much like cytochrome *c* content, citrate synthase activity declined more gradually with age in *daf-2(e1370)* than in wild-type and *daf-16(0)* animals [*daf-2* versus N2: $P_{age*strain}$ in worms = 0.0074; in mitochondria = 0.0485; *daf-16* versus N2, for both worm and mitochondria: P_{strain} = NS, P_{age} <0.0001]. These results dispel the notion that mitochondrial preparations from wild-type worms might contain a higher proportion of disrupted organelles and concomitant loss of mitochondrial proteins.

Having established that whole-worm energy production declines dramatically with age, we asked whether this could be caused by age-related loss of mitochondria. We used quantitative real-time polymerase chain reaction (qPCR) to assay the copies of mtDNA in *daf-2(e1370)* and WT animals. We quantitated three mitochondrial genes in staged worms

from three independent replicate cultures to enforce the robustness of the observations. Counter to our expectation, we did not detect any age-related changes in mitochondrial DNA (mtDNA) content (P_{age} NS) (fig. 1 A). Hence the age-specific decrease in energy production is not caused by systematic loss of mitochondrial genome copy number. Interestingly, wild-type animals had about double the number of mtDNA copies compared to *daf-2(e1370)* worms, with *P* values bordering on significance (P_{strain} = 0.0585). This difference is likely caused by differences in germline proliferation.

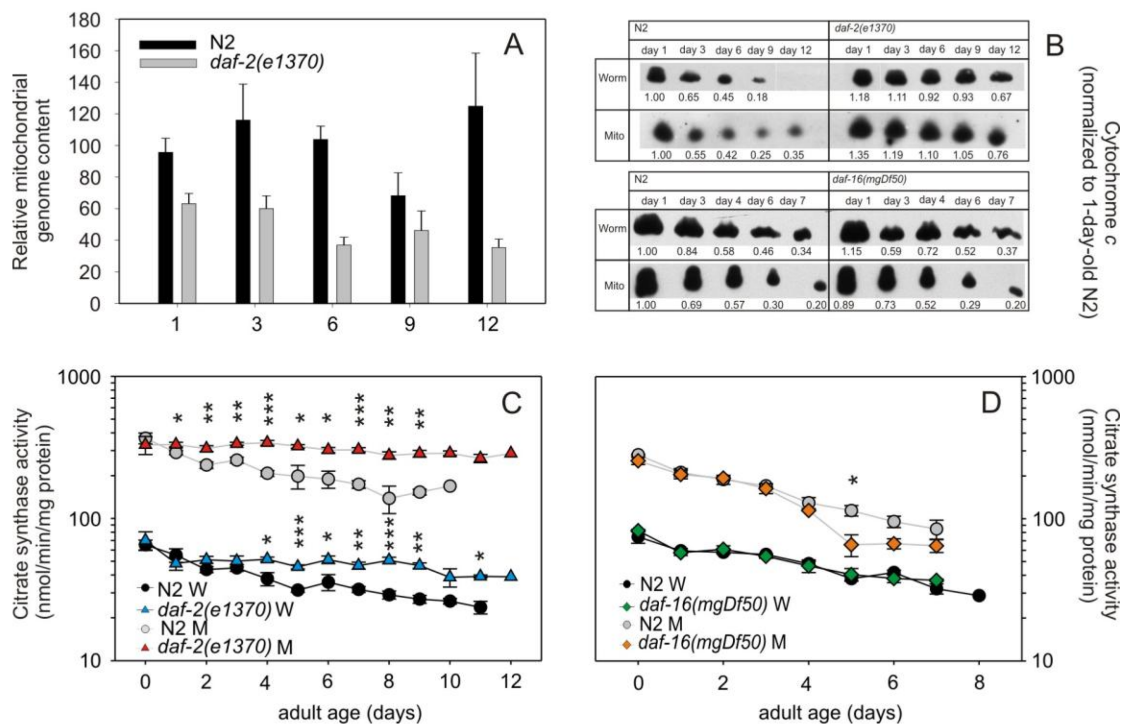


Figure 1: Effect of age on mitochondrial genome and cytochrome ***c*** content and citrate synthase activity**.** (A) Age-specific mitochondrial genome content of wild-type and *daf-2(e1370)* animals. Three mitochondrial genes were quantified; the results were normalized to obtain the relative mitochondrial genome content per strain and age cohort. The error bars indicate ± standard error of mean for three mitochondrial genes and three replicate ageing cohorts. (B) Western blots showing age-related changes of cytochrome *c* abundance in crude worm extract and isolated mitochondria. Results from one representative experiment are shown. The numeric values below each spot denote the abundance of cytochrome *c* in that spot normalized to the corresponding spot from 1-day-old wild-type (WT) adults. (C-D) Activity levels of citrate synthase in crude worm extract (indicated as 'W') and in isolated mitochondria (indicated as 'M'). Left panel: WT versus *daf-2(e1370)*, right panel: WT versus *daf-16(mgDf50)*. Data represent means ± standard error of mean (bars) for at least three replicate cultures; *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ (Student's t-test).

4.4. Changes in gene expression and protein abundance due to mutation in *daf-2*: mRNA transcript abundance, proteomics analysis and Western blotting

4.4.1. Introduction

To gain insight into mechanisms regulated by Ins/IGF-1 signaling which control oxidative metabolism, a starting point is to study differential expression of genes coding for proteins involved in the electron transport chain (ETC), the TCA cycle and other biochemical processes related to metabolism. In this section, we compiled results from whole-worm genome-wide scans for mRNA transcript abundance performed by other research groups to assess changes in expression of genes that are of interest to our research. Next, proteomics analysis and Western blotting were performed to establish whether the *daf-2* mutation influences the abundance of mitochondrial proteins.

Gene expression & age

We would like to point out that even the impact of age alone on ETC transcript levels seems to generate ambiguous results. Hill and colleagues (2000) found a significant decrease in gene expression of over a hundred genes, among which the ATPase-encoding genes and genes related to carbohydrate metabolism and energy generation were highly represented, from 60h adults to 2-week-old worms. According to McCarroll and colleagues (2004), aging represses genes encoding many components of the mitochondrial respiratory chain, the ATP synthase complex and the TCA cycle. Yet the majority of this repression seemingly occurs between 0h and 16h of adulthood and little change is recorded between 16h- and 6 day-old adults. Despite assaying an extensive age range, (Lund et al., 2002) observed no consistent alteration in expression of genes that encode mitochondrial proteins and genes involved in resistance to oxidative stress. (Zahn et al., 2006) compared the Lund dataset to data from humans, mice and flies, and found age regulation in the ETC pathway for these 3 organisms, but not for *C. elegans*.

Gene expression & daf-2

From a literature search into the effect of *daf-2* mutation on gene transcript levels, we could not deduce a unanimous view on differential expression of genes coding for ETC complex subunits. Some researchers found no major differences in expression of genes related to oxidative metabolism between a reference strain and a strain with a *daf-2* mutation (McElwee *et al.*, 2003; Murphy *et al.*, 2003; McElwee *et al.*, 2004; McElwee *et al.*, 2006) while others did find some ETC complex subunit genes to be differentially expressed in *daf-2*, but never a systematic up- or downregulation of various ETC genes (Ruzanov et al., 2007), even when the effect of age was also taken into account (Golden & Melov, 2004; Halaschek-Wiener *et al.*, 2005). These inconclusive results do not seem to point to a significant effect of *daf-2* on ETC gene expression. However, regulation of cellular processes consists not only of expression of genes measured as mRNA levels, but also of post-transcriptional variation such as synthesis, processing and modification of proteins (Nie et al., 2007). While expression of mitochondrial genes in *daf-2* may not be markedly different to wild-type, translation of transcripts into proteins may differ.

Proteomics analysis and Western blotting

Proteome analysis in whole worm young adult wild-type and *daf-2* has first been published by (Dong et al., 2007). They reported higher amounts of aconitase, malate dehydrogenase and enzymes of the glyoxylate cycle in *daf-2* compared to wild-type. However, they made no mention of differences in ETC protein content between young adult wild-type and *daf-2*. Seemingly, transcriptomics and proteomics do not allow us to discern if aging and/or Ins/IGF-1 signaling affect oxidative metabolism.

In the study by (Dong et al., 2007), only nematodes in their first day of adulthood were used for proteome analysis; the assay was executed on whole worm lysates. We set out to assess the effect of age and Ins/IGF-1 signaling on protein abundances in isolated mitochondria, isolated from wild-type and *daf-2(e1370)* at day 2 and 8 of adulthood. Isolated mitochondria were supplied by us. They were subjected to proteomics analysis by Mimage partner Proteosys.

4.4.2. Materials and methods

4.4.2.1. Strains & culture conditions

The strains used were wild-type N2 (CGC male stock) and *daf-2(e1370)*. Nematode culture and mitochondrial isolation were performed as in (4.2.2.).

4.4.2.2. Assays

Differential proteomic profiling was performed as described in Groebe et al. (2007). Proteosys were provided with aliquots of mitochondrial samples isolated from wild-type and *daf-2(e1370)* at two time points: at day 2 and 8 of adulthood. This setup was performed three times separately, so for every strain/age combination, three aliquots were prepared, which amounted to twelve in total. Briefly, each sample containing mitochondrial proteins was iodinated with either ^{125}I or ^{131}I, with identical chemical iodine concentrations. Aliquots of day-2-adult[2] wild-type and *daf-2* radiolabelled samples were mixed in equal amounts and separated by 2D-PAGE covering a pH range of 4-9. Through high sensitivity radio imaging, discrimination between ^{125}I and ^{131}I in one 2D-PAGE gel was possible and a quantitative multicolour differential display of proteins from separate samples labeled with different iodine isotopes was generated. For each pair of samples, reverse replicate gels were prepared where the labels (^{125}I and ^{131}I) were inverted. Gel image analysis, spot quantification and statistical identification were performed as in Groebe et al. (2007) and

[2] As the pooling scheme described in (4.4.2.2.) can only be applied to two samples, young and old wild-type, as well as young and old *daf-2* were pooled and each pool regarded as one sample by Proteosys; from this they attempted to make a comparison between the two strains. Next, young wild-type and *daf-2*, as well as old wild-type and *daf-2* were pooled to assess the effect of age on protein abundance. Because chronological age differs from physiological age when comparing strains with different average lifespans, such pooling is meaningless and we could not use the resulting data set. Thanks to high-throughput technological advances, future proteomics analyses will no longer be limited to one-to-one comparisons.

spots meeting significance criteria were subjected to MALDI-TOF peptide mass fingerprinting as in Vogt et al. (2005).

For Western blotting, live wild-type and *daf-2(e1370)* worms were lysed directly with Laemmli buffer and stored at -80°C. Prior to Western blotting, samples were thawed, protein concentration was determined using the Pierce 660 nm protein assay (Thermo Scientific, IL, USA) and equal amounts of protein were loaded and run. Western blotting was performed as described by (Matthijssens et al., 2008). Primary antibodies against Complex I NDUFS3 subunit, pyruvate dehydrogenase subunit E1alpha, Complex IV subunit I, Complex V subunits alpha and beta and adenine nucleotide transferase were purchased from Mitosciences (OR, USA). Secondary antibody was HRP-conjugated anti-mouse antibody from Sigma.

4.4.3. Results and discussion

Previously, we have ascertained that whole worm oxidative metabolism is altered when insulin signaling is disrupted. To assess the influence of the *daf-2* mutation on mitochondrial age-related alterations at the proteome level, we aimed to perform proteomics analysis on isolated mitochondria from young (day 2 of adulthood) and old (day 8 of adulthood) wild-type and *daf-2(e1370)*. Unfortunately, comparisons of aged samples were not performed as originally planned and could not be included in this section. We will focus on differential proteomic profiling of wild-type and *daf-2(e1370)* at 2 days of adult age.

First, we would like to put into perspective the differences we can expect between young wild-type and *daf-2*. (Dillin et al., 2002) have established that Ins/IGF-1 signaling controls diapause and lifespan at different stages in the life of *C. elegans*: the insulin pathway regulates diapause during development, while lifespan is regulated during adulthood. Moreover, in the case of *daf-2* RNAi, RNAi implemented during adulthood alone suffices to extend the lifespan of wild-type *C. elegans*. Therefore young adult nematodes with the same initial mitochondrial characteristics as those treated with RNAi vector bacteria can be induced to live longer lifespans. As *daf-2* mutation exerts its lifespan-extending influence from adulthood onwards, initial similarities in mitochondrial proteome composition between the 2 strains would not be unexpected. Alternatively, since we chose to sample 2-day-old adults, the effect of the *daf-2* mutation may already be apparent, even before differences in metabolic parameters become significant. Taking these findings into consideration, we evaluate proteomics results from our mitochondrial isolates. We have to note that the interpretation of these results is complicated by the presence of the yolk protein vitellogenin in our mitochondrial preparations and by partial proteolysis of certain proteins.

A number of TCA cycle enzyme components were found to be significantly more abundant in mitochondria isolated from 2-day-old *daf-2* than from the wild-type (table 1), for instance, the α subunit of isocitrate dehydrogenase and the β subunit of succinyl-CoA synthase. Dihydrolipoamide dehydrogenase, a component of pyruvate dehydrogenase, has comparable mutation-related patterns, in two isoforms. One isoform has an experimental mass close to the expected molecular mass; another isoform is heavier than the theoretical mass but both are significantly more abundant in *daf-2* than in the wild-type. We cannot

state for certain what kind of translational modification may be responsible for the difference in isoforms.

Likewise, the ETC protein cytochrome c oxidase subunit Va/COX6, part of complex IV, was not only more abundant in *daf-2*, but also present in three isoforms with equal (yet lower than theoretical) experimental mass; the only difference responsible for this result was the isoelectric point of the isoforms, pointing to secondary modifications of this protein.

daf-2 **(2d,Test) versus N2 (2d,Ref)**

Gene	Protein	Accession Number	Mass	PI	Exp. mass	Exp. PI	Daf-2 %	N2 %	p-value	**Spot-ID**
	ETC components									
cco-2 Y37D8A.14	Cytochrome c oxidase subunit Va/COX6	gi|17555666	20212	6,1	16000	5,3	60	40	<0.0001	N2_daf2_d2_5-6_1267 bzw. N2_daf2_5-6_1267_1
cco-2 Y37D8A.14	Cytochrome c oxidase subunit Va/COX6	gi|17555666	20212	6,1	16000	5,35	63,2	36,8	<0.0001	N2_daf2_d2_5-6_1268 bzw. N2_daf2_5-6_1268_1
cco-2 Y37D8A.14	Cytochrome c oxidase subunit Va/COX6	gi|17555666	20212	6,1	16000	5,1	59,8	40,2	<0.0001	N2_daf2_d2_5-6_1257 bzw. N2_daf2_5-6_1257_1
	Krebs cycle enzymes									
F23B12.5	Dihydrolipoamide dehydrogenase (component of pyruvate dehydrogenase)	gi|17560088	53719	8,4	57000	5,9	67	33	<0.001	N2_daf2_d2_5-6_547 bzw. N2_daf2_5-6_547_1
F23B12.5	Dihydrolipoamide dehydrogenase (component of pyruvate dehydrogenase)	gi|17560088	53719	8,4	53500	6	73,9	26,1	<0.0001	N2_daf2_d2_5-6_566 bzw. N2_daf2_5-6_566_1
F43G9.1	Isocitrate dehydrogenase, Alpha subunit	gi|71986051	38898	7,4	40500	6	68	32	<0.0001	N2_daf2_d2_5-6_741 bzw. N2_daf2_5-6_741_1
ZK669.4	Dihydrolipoamide branched chain transacylase (E2 component of pyruvate dehydrogenase	gi|17537937	49945	8,8	58000	7	56,5	43,5	<0.0001	N2_daf2_d2_6-9_787 bzw. N2_daf2_6-9_787_1
F47B10.1	Succinyl-CoA synthase, beta subunit	gi|17567829	47845	6,3	50000	5,45	61,5	38,5	<0.0001	N2_daf2_d2_5-6_585 bzw. N2_daf2_5-6_585_1
mdh-1 F20H11.3	NAD dependent malate dehydrogenase	gi|17554310	35155	10	34000	8,2	65,9	34,1	0,0001	N2_daf2_d2_6-9_991 bzw. N2_daf2_6-9_991_1
	Other mitochondrial functions									
ucr-1 F56D2.1	Ubiquinol-cytochrome c oxidoreductase complex / mitochondrial processing peptidase	gi|17553678	51704	6,5	54000	5,9	68,4	31,6	0,0003	N2_daf2_d2_5-6_626 bzw. N2_daf2_5-6_626_1
ucr-1 F56D2.1	Ubiquinol-cytochrome c oxidoreductase complex / mitochondrial processing peptidase	gi|17553678	51704	6,5	56000	5,7	62,4	37,6	<0.0001	N2_daf2_d2_5-6_604 bzw. N2_daf2_5-6_604_1
ucr-1 F56D2.1	Ubiquinol-cytochrome c oxidoreductase complex / mitochondrial processing peptidase	gi|17570205	42799	8,4	44000	6,4	66,5	33,5	<0.0001	N2_daf2_d2_6-9_895 bzw. N2_daf2_6-9_895_1
ucr-1 F56D2.1	Ubiquinol-cytochrome c oxidoreductase complex / mitochondrial processing peptidase	gi|32566323	44465	9,2	44000	6,4	66,4	33,6	<0.0001	N2_daf2_d2_6-9_895 bzw. N2_daf2_6-9_895_2
Y25C1A.13	Enoyl-CoA isomerase	gi|17536985	33297	7,4	30000	5,65	59,6	40,4	<0.0001	N2_daf2_d2_5-6_930 bzw. N2_daf2_5-6_930_1
B0272.3	3-hydroxyacyl-CoA dehydrogenase	gi|17549919	33530	8,1	34000	6,9	60,3	39,7	<0.0001	N2_daf2_d2_6-9_1002 bzw. N2_daf2_6-9_1002_1
F59A2.3	Acidic protein of the mitochondrial matrix	gi|17553758	26508	4,7	27000	4,4	64,2	35,8	<0.0001	N2_daf2_d2_4-5_1125 bzw. d2_d8_4-5_1125_1

Table 1: Mitochondrial proteins showing divergent relative abundances between 2-day-old WT and *daf-2(e1370)*, identified by differential proteomic profiling, and their patterns of isoforms. Equal amounts of mitochondrial protein were loaded on the gels. Protein spots were detected and identified via MALDI-TOF Peptide Mass Fingerprinting after differential iodine radiolabeling and separation through 2D-PAGE as described in (Groebe et al., 2007). Only selected mitochondrial proteins with clearly elevated abundances in *daf-2* mutants are shown. Due to the experimental difficulties encountered (see sections 4.4.2.2 and 4.4.3), info on the total number of proteins identified and quantified is not given.

Generally, proteomics analysis is an excellent source of information, not only on differential protein abundance, but also on the presence of secondary modifications, which could contribute considerably to our understanding of aging (Groebe et al., 2007). Unfortunately, here it has contributed only a very limited dataset to elucidate the

modification of oxidative metabolism by Ins/IGF-1 pathway disruption. In addition, we are well aware that partial proteolysis and strain-specific differences in contaminating yolk protein complicate correct interpretation of the results.

However, we may be able to make the following prediction. As mentioned before, *daf-2* mutation exerts its influence on lifespan from adulthood onwards. It is remarkable that some of the effects of the *daf-2* mutation at the proteome level were apparent even in 2-day-old adults; this may implicate that the impact of Ins/IGF-1 signaling on oxidative phosphorylation of isolated mitochondria will also be measurable before morphological aging becomes apparent. Data from a recent proteomics study, performed in collaboration with our lab, support this prediction: the study, performed on whole worm homogenates, suggests that for a considerable amount of proteins related to the TCA cycle and the electron transport chain, protein abundances are significantly higher in 2-day-adult *daf-2* mutants than in same-age controls (personal communication G. Depuydt).

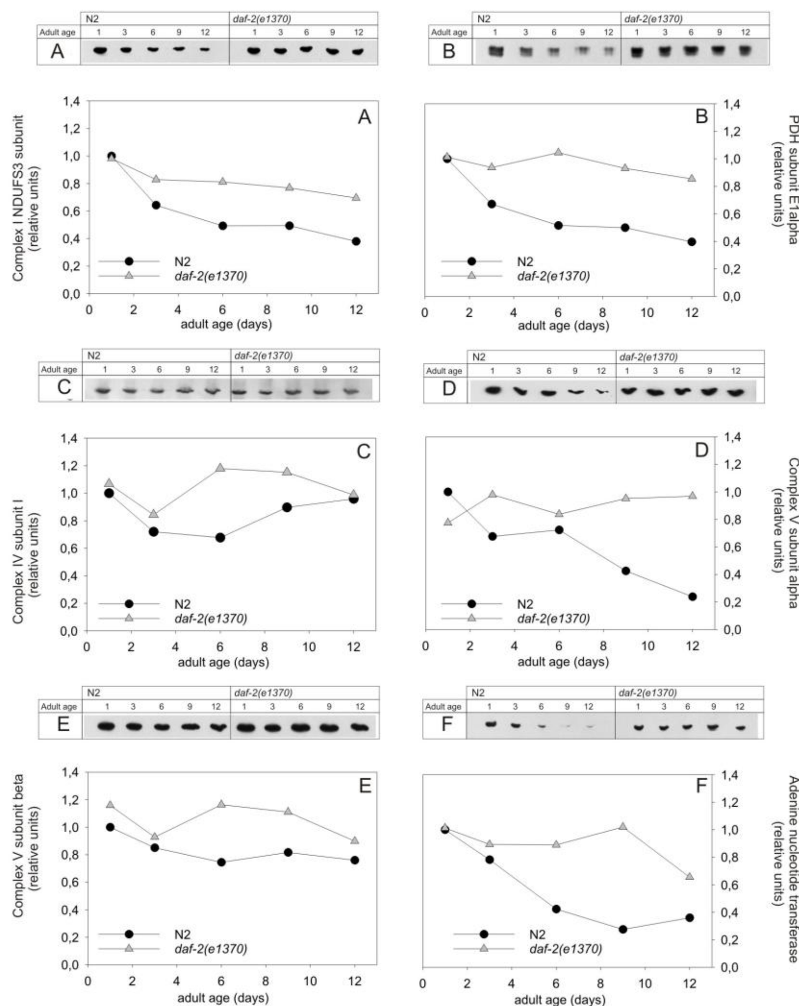


Figure 2: The *daf-2(e1370)* allele attenuates the age-specific decline in abundance of key mitochondrial proteins. (A-F) Western blots showing age-related changes in the abundance of important mitochondrial proteins in crude worm extract. The numeric values for each protein denote its abundance in 1-,3-,6-,9- and 12-day-old adults normalized to the abundance in 1-day-old wild-type adults and are plotted in the corresponding graphs. (A) Complex I NDUFS3 subunit. (B) Pyruvate dehydrogenase subunit E1 alpha. (C) Complex IV subunit I. (D) Complex V subunit alpha. (E) Complex V subunit beta. (F) Adenine nucleotide transferase.

In the previous experiment, results were potentially obscured by proteolysis of certain proteins. By immediate lysis of live nematodes (through heating of samples in the presence of Laemmli buffer) and subsequent Western blotting of whole worm lysates, we were able to overcome this problem. We quantified the abundance of the Complex I NDUFS3 subunit, the pyruvate dehydrogenase subunit E1α, Complex IV subunit I, Complex V subunits α and ß and adenine nucleotide transferase on Western blots of whole worm extracts (fig. 2). For the Complex I NDUFS3 subunit, pyruvate dehydrogenase subunit E1α, adenine nucleotide transferase and Complex V subunit α, a clear decrease in protein content with age was observed in WT worms, whereas only minor decreases were detected in long-lived *daf-2(e1370)* animals (fig. 2 A, B, F & D). For Complex V subunit ß, we observed a slight decrease with age in both strains, but the *daf-2* signal was generally higher than that of WT (fig. 2 E). Only Complex IV subunit I content did not decrease with increasing age (fig. 2 C). Taken together with data from cytochrome *c* abundance and citrate synthase activity (see 4.3.3.), these results suggest that the *daf-2(e1370)* allele attenuates the age-specific decline in abundance of key mitochondrial proteins.

Validation of our limited proteomics dataset has not yet been performed. This could be done by Western blotting. Since the availability of antibodies specific for *C. elegans* is low, custom antibodies will have to be designed. Quantitative PCR could be used in order to assess whether differences in protein abundances are transcriptionally regulated. Additional RNAi and overexpression experiments could reveal the importance of mitochondrial protein abundances for lifespan.

4.5. OXPHOS supercomplexes

4.5.1. Introduction

The electron transport chain consists of 5 ETC complexes, each made up of multiple subunits arranged in a complex 3D structure. Originally it was thought that the ETC complexes floated freely in the inner mitochondrial membrane. A more recent view, underpinned by sufficient experimental evidence, is that individual respiratory chain complexes assemble into supercomplexes. This supercomplex architecture suggests a kinetic advantage that increases the efficiency of the ETC and stabilizes the complexes (reviewed in Vonck & Schafer, 2009). Mitochondria from wild-type worms and *daf-2(e1370)* animals were subjected to Blue Native (BN)-PAGE and 2D SDS-PAGE to assess the effect of age and Ins/IGF-1 signaling on supercomplex formation in *C. elegans*.

Isolated mitochondria were supplied by us. They were subjected to analysis by the Dencher lab, under the supervision of F. Krause and N.A.D. Dencher.

4.5.2. Materials and methods

4.5.2.1. Strains & culture conditions

Strains used were the wild-type N2 (male stock provided by the CGC) and the Ins/IGF-1 mutant *daf-2(e1370).* For culture conditions and isolation of mitochondria, we refer to (4.2.2.). Mitochondria were prepared on day 2 and day 8 of adulthood.

4.5.2.2. Assays

Native electrophoresis of isolated mitochondria was carried out as follows:
solubilization and blue-native electrophoresis were performed as described in (Krause and Seelert, 2008; Maas et al., 2009; Marques et al., 2007). In detail, mitochondria were thawed on ice and centrifuged at 20,800*g* for 8 min. The pellet was suspended in the solubilization buffer containing 50 mM NaCl, 50 mM imidazole/HCl (pH 7.0), 10% glycerol and 5 mM 6-aminocaproic acid (final concentration). Mitochondria containing 150 µg protein were solubilized with digitonin (AppliChem, A1905) using a detergent/protein ratio of 4 g/g at a final detergent concentration of 1% by adding a freshly prepared 10% detergent solution. The samples were incubated for 30 min at 4 °C with slight agitation followed by centrifugation at 20,800*g* for 10 min. The extracts were directly loaded onto native gels. For BN-PAGE (Blue Native polyacrylamide gel electrophoresis), linear 3–13% gradient gels overlaid with a 3% stacking gel were used in a Hoefer SE 600 system ($18 \times 16 \times 0.15$ cm^3) with electrophoresis conditions as described in (Krause and Seelert, 2008; Marques et al., 2007). The apparent molecular masses of the OXPHOS complexes and their supercomplexes were calibrated by digitonin-solubilized bovine heart mitochondria applied to the same first-dimension BN gel as described by (Maas et al, 2009; Marques et al., 2007). Lanes from the first-dimension BN-PAGE were then excised and used for a second-dimension 13% SDS-PAGE (Maas et al., 2009; Marques et al., 2007) with subsequent silver staining. The supercomplexes were assigned according to their characteristic subunit compositions

revealed in 2D SDS-PAGE and apparent molecular masses. Additionally, some of the subunits of the *C. elegans* OXPHOS complexes were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as described (Marques et al., 2007).

4.5.3. Results and discussion

Compelling evidence indicates the existence of specific respiratory supercomplexes (respirasomes) of the proton-pumping complexes I, III and IV as well as of ATP synthase dimers/oligomers in mitochondria of most eukaryotes (reviewed in (Schägger, 2002), (Krause, 2007)) including *C. elegans* (Suthammarak et al., 2009). We found an exceptionally high proportion of preserved OXPHOS supercomplexes in *C. elegans* mitochondria indicating a particularly high detergent-stability of the supercomplexes (fig. 3), even better than in mitochondria isolated from fresh bovine heart (Krause *et al.*, 2005; Krause, 2006; Krause, 2007). Complex I was completely recovered as part of I-III-IV supercomplexes and ATP synthase is mainly present as dimers in young adults from N2 and *daf-2* (fig. 3 a & c). Strikingly, in aged wild type the total amount of OXPHOS complexes decreased significantly, in line with the immunoblot results of representative OXPHOS subunits (fig. 3 b), but with a similar proportion of preserved supercomplexes as in young worms. In contrast, no decline of OXPHOS supercomplexes was observed in 8d *daf-2* mitochondria displaying very similar 2D BN/SDS gels (fig. 3 d).

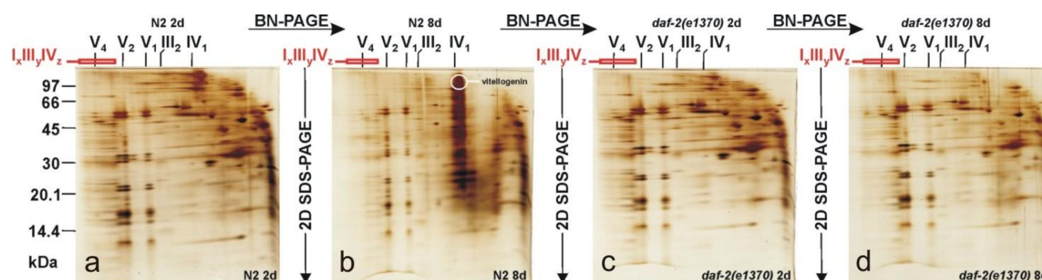


Figure 3: OXPHOS supercomplexes are better preserved during aging in mitochondria from *daf-2(e1370)* animals. 2D BN/SDS-PAGE of digitonin-solubilized mitochondria from young (2d) (fig 3 a & c) and aged (8d) (fig 3 b & d) wild-type N2 and mutant *daf-2(e1370)* worms. OXPHOS complexes and supercomplexes were assigned according to their characteristic subunit compositions and apparent molecular masses. Additionally, some subunits were identified by mass spectrometry. ATP synthase monomers (V_1), dimers (V_2) and tetramers (V_4), the individual respiratory complexes III (III_2) and IV (IV_1) as well as the respiratory supercomplexes $I_xIII_yIV_z$ are indicated. Complex I is completely preserved as part of supercomplexes $I_xIII_yIV_z$.

However, as in proteomics analysis, differing amounts of vitellogenin, particularly in 8-day-old wild-type, complicate this picture. At first sight, this may suggest that the decrease in the amount of supercomplexes in aging WT is an artefact: it could be put forward that the 8-day-old WT sample contains a disproportionately high amount of vitellogenin and consequently, lower amounts of supercomplexes. However, the degree of citrate synthase (CS) activity of the worm as a percentage of the CS activity in mitochondria (fig.4, see also fig. 1c) seems to suggest that the isolation procedure does not enrich vitellogenin in mitochondria prepared from aged worms. Therefore we can state that the vitellogenin showing up on the WT day-8 sample does not affect our conclusions.

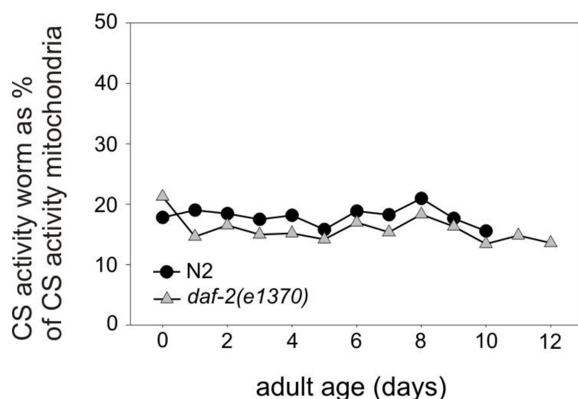


Figure 4: Relative citrate synthase (CS) activity in crude worm extract, expressed as percentage of CS activity in mitochondria, is close to invariable with increasing age in both WT and *daf-2(e1370)*. Data derived from fig 1(c).

4.6. General discussion and conclusions

A standardized method for isolation of mitochondria from *C. elegans* was developed to assess mitochondrial characteristics that could play a role in the effect of Ins/IGF-1 signaling on whole worm metabolism. Mitochondrial genome content was assayed using a quantitative real time PCR approach. It was found to be unaltered by age in both strains tested. Hence reduction of energy production with age could not be caused by systematic loss of mitochondria. As gene expression and proteomics studies on whole nematodes gave few clues as to how the *daf-2* mutation could affect oxidative metabolism, we performed proteomics analyses on isolated mitochondria from 2-day-old adult wild-type and *daf-2(e1370)* and found that the abundance of certain ETC, TCA and other mitochondrial proteins differed between the two strains. Similar to the gene expression studies, the results of the proteomics analysis made only a limited contribution to our knowledge about the influence of Ins/IGF-1 signaling on oxidative metabolism, due to the presence of vitellogenin in the samples, along with partial proteolysis, and because errors in the experimental setup prevented a more comprehensive comparison between mitochondria from young and old nematodes. However, through Western blotting we did find an age-related change in the abundance of proteins involved in mitochondrial function. We found that the abundance of several ETC components decreased with age in both wild-type and *daf-2*, but the decreases were faster in wild-type. Also, age-dependent reduction in citrate synthase activity, a key enzyme of the citric acid cycle, was substantially slower in these mutants.

From data on mitochondrial genome copy number, enzyme activity, Western blotting and proteomics, it could be suggested that differences in mitochondrial compositions between WT and *daf-2* must occur. On a per worm basis, the long-lived mutant contains less mtDNA. On the other hand, cytochrome *c* content and citrate synthase activity expressed per mg worm protein are higher for most of the mutant’s life trajectory. The same conclusion can be drawn for the abundance of most of the other mitochondrial proteins for which antibodies were available. Potential compositional differences between the two strains also appear from the proteomics data. Perhaps mitochondrial density is higher in the WT than in *daf-2*, while in contrast, certain mitochondrial proteins could be present in higher abundances in the long-lived mutant. This tentative conclusion requires

additional experimental testing. Further proteomic experiments comparing protein abundances between controls and *daf-2* are underway; these experiments may confirm or refute mitochondrial compositional differences between the two strains.

Finally, the abundance of OXPHOS complexes which were mainly found as supercomplexes declined with age in wild-type and this decline was again attenuated in the mutant worms. There is mounting evidence that the organization of the ETC in these higher order structures, supercomplexes or respirasomes, controls the bioenergetic competence of mitochondria (Bornhövd et al., 2006; Rosca et al., 2008; Suthammarak et al., 2009). In particular, Bornhövd et al. (2006) proposed a model of microdomain organization of OXPHOS (super)complexes in the mitochondrial inner membrane. Disruption of these microdomains would affect metabolite/substrate channeling and/or efficient cooperation of these complexes, ultimately leading to a reduced flux through the respiratory chain and a lower membrane potential.

4.7 Addendum to chapter 4: justification for final protocol for isolation of mitochondria.

Table 2

Isolation steps	Issues	Conclusion
Murfitt et al. (1976)		
Amount of worms used:	35 g wet weight	
fractionate worms with sea sand in mortar 6-7 min, dissolve in buffer	Not possible to recuperate enough worm tissue from 1 ml of worm	Substitute with rough surface douncer/ alternative
380g 10’ & resuspend pellet	Not using supernatant is too large a loss of tissue	Use supernatant
Nagarse treatment 5’	Replaced by digitonin* treatment (0.05% and 0.01%) but suboptimal reaction to ADP	No chemical purification step
dounce 7x & resuspend	Smooth surface douncer had little effect on tissue fractionation	Omit step
380g 10’, keep 380g pellet supernatant 4500g 5’ & resuspend = first mitochondrial pellet	Centrifugation speeds give good yield, remove larger fractions and pellet is easily resuspendable	Centrifugation speeds ok
resuspend 380 g pellet repeat previous steps on this pellet and pool	Extra yield of second series of centrifugation steps is negligible	Omit steps
Kayser et al. (2001)		
Amount of worms used:	Not specified	Kayser et al. (2004a): “2 to 3 grams”
suspend worms in isolation buffer fractionate with Polytron 20 s 14000 rpm	Replace by rough surface douncer	Fragmentation ok but timing hard to reproduce and loss of tissue during transfer to centrifuge tube
	Replace by IKA rotor/stator mixer 40 sec max speed 25000 rpm Visual evaluation of fractionation	Fragmentation ok Negligible loss of tissue
add proteinase type XXVII, stir 10’	See remarks digitonin*	Omit step
homogenize in Potter/Elvehjem tissue grinder with Teflon pestle	Considerable loss of tissue	Omit step
add 1 volume of isolation buffer made 0.4% in BSA		copied
centrifuge300g 10’	See below**	
filter supernatant through gauze	Cannot be performed on small amount of tissue	Omit step
centrifuge 7000g 10’ resuspend in isolation buffer repeat twice	**combinations tested in order of resulting RCR and ADP/O: 380/380/4500g ≈ 800/800/10000g >380/800/4500g	800/800/10000g combination: issue with removal of larger worm fractions and resuspension of mitochondrial pellet Selected combination: 380/380/4500g

Protocol used		
Amount of worms used:	Harvest and clean approx. 300000 worms Suspend in 1ml of isolation buffer	
Fractionation	Chop 40 sec at 25000 rpm with rotor-stator mixer Add 1 ml of isolation buffer made 0.4% in BSA	
Removal of debris	Centrifuge 380g 5' Transfer supernatant Centrifuge 380g 5'	
Collection of mitochondrial suspension	Centrifuge 4500g 5' Discard supernatant Resuspend mitochondrial pellet	

PART II: RESULTS

Chapter 5
Ins/IGF-1 and mitochondrial function: oxidative phosphorylation and bioenergetic competence

Redrafted from:
Brys *et al* (2010). Disruption of insulin signaling preserves bioenergetic competence of mitochondria in ageing *Caenorhabditis elegans*. BMC Biology **8**, 91

Personal contribution:
Brys *et al* (2010) all nematode cultures, development and execution of mitochondrial assays, writing of manuscript

ABSTRACT

The Ins/IGF-1 reduction-of-function mutation *daf-2(e1370)* induces several whole-worm metabolic alterations. In this chapter we show that it also alters mitochondrial aerobic energy production. In the wild-type, age-related proteomic changes are accompanied by a dramatic decrease in energy production. In contrast, in *daf-2* animals, not only the decrease in abundance of key mitochondrial proteins but also in their bioenergetic competence is considerably attenuated, and is associated with a higher membrane potential. Our findings suggest a higher energetic efficiency of *daf-2(e1370)* animals.

5.1 Introduction

In Chapter 3, we demonstrated that *daf-2(e1370)* worms dissipate less heat than wild-type animals per mole of oxygen utilized. This difference is manifested by a considerable reduction of the calorimetric-to-respirometric ratio, possibly pointing to a higher efficiency of mitochondrial metabolism (also referred to as bioenergetic efficiency) of *daf-2(e1370)* animals. To address the cause of altered energy metabolism in whole worm *daf-2*, we set out to examine energy production by mitochondria. In this chapter, we study the effect of age and of mutation in the Ins/IGF-1 pathway on the bioenergetic competence of isolated mitochondria. Mitochondrial bioenergetic competence is assessed through measurement of mitochondrial oxygen consumption at different states of activity and the capacity of isolated mitochondria to synthesize ATP. Mitochondrial efficiency is also estimated by measuring the mitochondrial calorimetric-to-respirometric ratio and the mitochondrial membrane potential.

Results show that the age-dependent decrease of bioenergetic competence is considerably attenuated in *daf-2(e1370)* adult animals. *daf-2(e1370)* mitochondria are able to better maintain their capacity for oxidative phosphorylation and have a higher membrane potential. However, the lower calorimetric-to-respirometric ratio observed in live *daf-2(e1370)* worms is not recapitulated in isolated mitochondria, suggesting that other targets of Ins/insulin growth factor-1-like signaling act in concert with the mitochondria to control organismal metabolic rate.

5.2. Materials and methods

5.2.1. Strains & culture conditions, isolation of mitochondria

Strains used were N2 wild-type (CGC male stock) and *daf-2(e1370)*, as well as *daf-16(mgDf50)* in some experiments. Culturing of nematode cohorts and isolation of mitochondria were performed as in (4.2.2.). For all experiments except Complex II + III enzyme activity, freshly isolated mitochondria were used.

5.2.2. Assays

Oxygen consumption by isolated mitochondria

Oxygen consumption by isolated mitochondria was monitored polarographically using a Clark type electrode mounted in a respirometer cell and connected to an oxygen meter (Strathkelvin Mitocell MT200A and 782 Single/dual channel oxygen meter, Strathkelvin Instruments, Glasgow, Scotland). An aliquot of isolated mitochondria containing approximately 300 µg of protein was added to 500 µl of air saturated incubation medium (Kayser et al., 2001) at 24°C. Respiration was activated by adding 5 mM pyruvate and 5 mM malate (final concentrations) for assaying Complex-I-dependent respiration or 10 mM succinate and 4 µg/ml rotenone (Complex-II-dependent respiration), followed by

sequential additions of 50 nmol ADP[1]. State 3 and state 4 oxygen consumption, respiratory control ratio (RCR) and ADP/O were calculated according to (Estabrook, 1967) (see also section 1.2.6.2).

Activity of Complex II + III

The Complex II + III activity assay was adapted from a protocol by (Trounce et al., 1996). Frozen mitochondria were freeze-thawed 3 times in liquid nitrogen after a 7-fold dilution in hypotonic medium (Birch-Machin and Turnbull, 2001) consisting of 25mM potassium phosphate, pH 7.2 and 5 mM $MgCl_2$ to fragment mitochondrial membranes. In the wells of a translucent microtiterplate, a reaction mixture consisting of 40mM potassium phosphate buffer, pH 7.4, 5 mM succinate, pH 7.4, 500µM EDTA, pH 8, 2mM KCN and finally 10µl of diluted mitochondrial suspension equaling approximately 3 µg of mitochondrial protein was incubated at 24°C for 10 min. The reaction was initiated by adding 120 µM of cytochrome *c*; the change in absorbance, or the reduction of cytochrome *c* by complex III coupled to succinate oxidation through complex II, at 550 minus 540 nm was monitored for 6 min in a Spectramax 190 (Molecular Devices, CA, USA). Absolute concentrations were calculated using the extinction coefficient 19.0 $mM^{-1}cm^{-1}$.

Quantification of ATP synthesis

ATP synthesis was determined using the Roche ATP Bioluminescence Assay Kit CLS II. Approximately 5 ng of freshly isolated mitochondria were added to the wells of a white microtiter plate containing 96 µl of incubation medium (Kayser et al., 2001), 50µl of luciferase reagent (1 bottle was dissolved in 5 ml of sterile high-performance liquid chromatography (HPLC) water), and 50 µl of substrate/ADP mix (final concentrations in the well: pyruvate 1 mM and malate 1 mM or succinate 5mM with rotenone 2 µg/ml, ADP 100µM). The emitted light was measured for 45 minutes in a Wallac Victor² Multilabel Counter. For determination of background light emission, 2 µg/ml oligomycin, an inhibitor of complex V, was added.

Mitochondrial heat dissipation and calorimetric-to-respirometric (C/R) ratio

For this assay, mitochondria were isolated in the presence of a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) at the concentration recommended by the manufacturer. Heat dissipation was registered by the Thermal Activity Monitor (TAM, TA Instruments, DE, USA) as follows: 560µl of phosphate-enriched incubation medium (100 mM KCl, 50 mM MOPS, 1 mM EGTA, 100 mM potassium phosphate, 1 mg/ml defatted BSA, pH7.4) was transferred to a glass ampoule and made 27,4 mM each in pyruvate and malate from pH 7 stock solutions. Next, ADP and penicillin/streptomycin mixture were added at 16.4 mM and 200 U/200 µg, respectively. Protease inhibitor cocktail was added as needed to meet the recommended concentration. Finally, 250-500 µg of mitochondria were added

[1] Addition of 50 nmol ADP does not lead to the maximum attainable state 3 oxygen consumption rate; this small, non-saturating amount was added to derive multiple readings from one experiment.

and the ampoule was sealed and inserted into the TAM. An identical sample was taken concurrently for registration of a state 3 oxygen consumption rate. Heat dissipation was recorded for 10 min after approx. 40 min of equilibration. Immediately following termination of the recording, the contents of the ampoule were transferred to the cell of the respirometer and a state 3 oxygen consumption rate was determined. This second reading was used for obtaining the mitochondrial C/R ratio.

Determination of the membrane potential

For this assay, mitochondria were isolated in the presence of a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) at the concentration recommended by the manufacturer. The fluorescent probe DASPMI (dimethylaminostyrylmethylpyridinium-iodine) was kindly provided by Prof. Jürgen Bereiter-Hahn and used as an indicator of mitochondrial membrane potential, essentially following the protocol by (Bereiter-Hahn, 1976), with minor changes. Protein concentration in the mitochondrial preparations was determined according to (Bradford, 1976), using a Sigma kit following the manufacturer's instructions, and adjusted as needed to obtain a ratio of approx. 2.9 nmol DASPMI/mg mitochondrial protein in the sample wells. Briefly, to a well of a black microtiter plate, 234µl of incubation medium was added, followed by 3µl of a 1M succinate stock[2], 3µl of a 400µg/ml rotenone solution in DMSO and 15µl of a 96µM DASPMI solution in HPLC-grade water. Next, 30 µl of mitochondrial suspension was added and fluorescence was recorded (Wallac Victor² Multilabel Counter (Perkin-Elmer, MA, USA), excitation at 450 nm, emission at 590 nm) for 4 min to obtain a stable mitochondrial membrane potential signal. Next, 4µl of a 200mM ADP stock solution was added and DASPMI fluorescence in energized mitochondria was recorded for 2 min. Finally, the membrane potential of uncoupled mitochondria was registered for 2 min after addition of 10µl of a 1 mM FCCP solution. Final concentrations of reagents in the well for this last measurement are: succinate, 10 mM, rotenone, 4 µg/ml, DASPMI, 4,8 µM, ADP, 2,7 mM, and FCCP, 33 µM. Fluorescence signals were corrected for small differences in protein content using the BCA (bicinchoninic acid) method (see (4.3.2.2.)) which is more sensitive than the Bradford assay.

Uncoupled respiration

Mitochondrial oxygen consumption was also assessed when respiratory control by the membrane potential was absent. First, state 3 and state 4 oxygen consumption via Complex I were monitored, adding ADP to the mitochondria only once. Next, 5 µM FCCP[3] was added to register oxygen consumption when the ETC was uncoupled.

[2] Membrane potential of mitochondria in the active state was not determined in the presence of substrates fueling Complex I; (Bereiter-Hahn, 1976) found that DASPMI exerts an inhibitory effect at the site of NADH oxidation.

[3] FCCP was titrated to assess the concentration needed for optimal uncoupling; this titration was performed on mitochondria isolated from the WT. Titration on *daf-2* mitochondria may have resulted in a different optimal FCCP concentration.

Protein determination

Oxygen consumption in state 3 and 4, ATP synthesis, Complex II + III activity, membrane potential and uncoupled respiration were all corrected for protein content by the BCA method (see 2.2.1.2. and 4.3.2.2.) without prior degradation of the samples with alkali.

Statistics

For statistics, we refer to (3.2.1.3.).

5.3. Results and discussion

Mitochondria can shift between several energetic states depending on the presence of combustible substrate and ADP. Freshly purified mitochondria lack sufficient amounts of both and consume very little amounts of oxygen, called state I respiration. The addition of metabolizable substrate (state 2 respiration) is not helpful as long as there is no ADP to unlock complex V and permit protons to flow into the mitochondrial matrix. The addition of sufficient amounts of substrate and ADP maximizes proton flow through Complex V and, consequently, electron transport and the reduction of oxygen to water at Complex IV (state 3 respiration), coupled to the conversion of ADP to ATP (oxidative phosphorylation). When ADP is depleted, the mitochondria return to the resting state 4 respiration.

First, we discuss oxidative phosphorylation by mitochondria fueled via Complex I (with pyruvate and malate as substrates). We found that increasing age had little, if any, effect on state 3 respiration. ADP-stimulated oxygen consumption was higher (P_{strain} = 0.0002) in *daf-2(e1370)* mitochondria (fig 1a). In all strains, state 3 respiration remained stable (P_{age} = NS) over the entire life time studied (fig 1a & b). State 4 respiration increased with age very gradually in N2 and *daf-2(e1370)* animals [P_{age} = 0.0002 and P_{strain} = 0.0039] (fig 1c) and faster in *daf-16(mgDf50)* worms [$P_{age*strain}$ = 0.027] (fig. 1d). The limitation placed on electron transport by the chemiosmotic gradient, or respiratory control, can be derived from these data. The ratio of substrate driven oxygen consumption in the presence of ADP (state 3) to that in its absence (state 4), or respiratory control ratio (RCR) decreased with age in all three strains. The rate of this decrease was lowest in *daf-2(e1370)* (fig. 1e) and highest in *daf-16(mgDf50)* (fig. 1f) [N2 vs *daf-2*, $P_{age*strain}$ <0.0001 and N2 vs *daf-16*, $P_{age*strain}$ = 0.0157]. The passage of electrons through the proton translocating complexes I, III and IV is associated with the release of free energy that is recovered when protons flow back inside at complex V, and ADP is converted to ATP. Based on the change in free energy under standard conditions, the theoretical ratio of ADP molecules that can be phosphorylated per atom oxygen that is reduced to water, or ADP/O ratio, is more than 7 (Voet et al., 2006), but maximum attainable ratios are less because of various losses in this process and are ~3 for oxidation of NADH by complex I. We observed an age-related decline of ADP/O in all three strains, more gradual in *daf-2(e1370)* (fig. 1g) and steepest in *daf-16(mgDf50)* (fig. 1h) [N2 vs *daf-2*, $P_{age*strain}$ = 0.0001 and N2 vs *daf-16*, $P_{age*strain}$ = 0.0067]. We have to note that the absolute figures for wild-type mitochondrial oxygen consumption differ between experiments, and that seemingly, state 3 oxygen consumption levels are similar between

daf-2(e1370) and *daf-16(mgDf50)*. However, this is most likely due to variation in replicate cultures; this is corroborated by the fact that RCR and ADP/O ratios are identical for wild-types of both data sets.

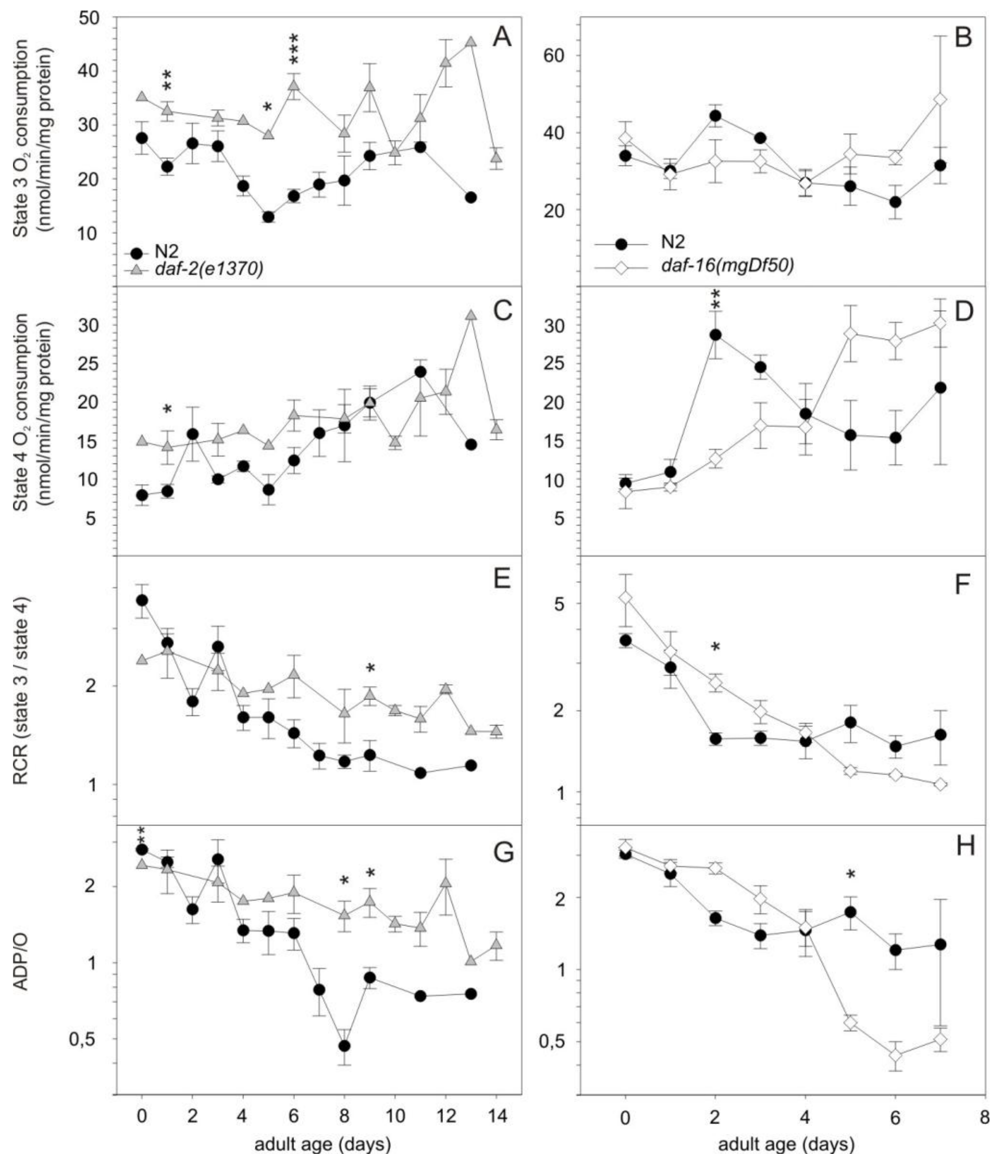
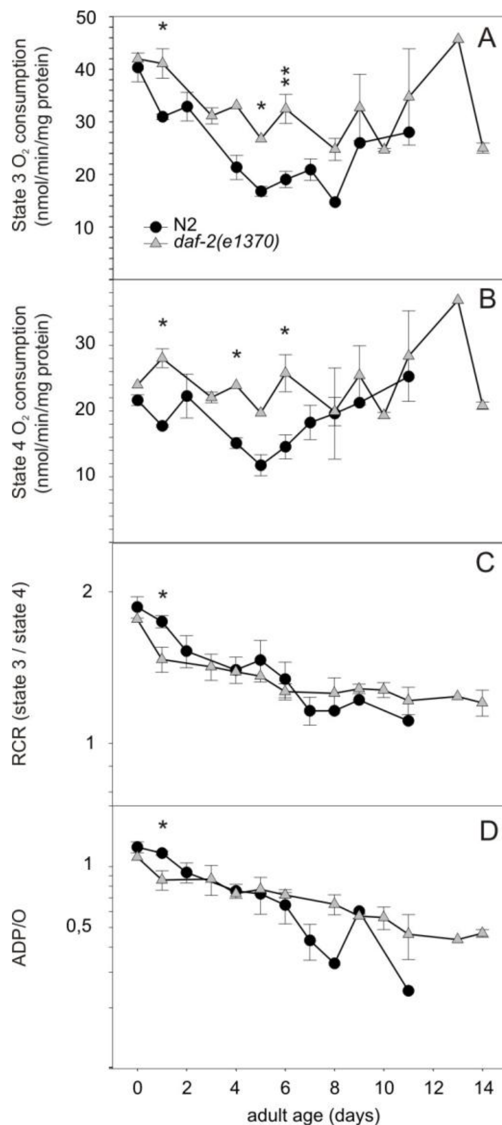


Figure 1: The *daf-2(e1370)* allele preserves mitochondrial bioenergetic competence throughout the adult life trajectory**.** The mitochondria are fueled with pyruvate and malate to activate Complex-I-dependent respiration. Left panels: WT versus *daf-2(e1370)*, right panels: WT versus *daf-16(mgDf50)*. (A-B) State 3 oxygen consumption. (C-D) State 4 oxygen consumption. (E-F) Respiratory control ratio. (G-H) Adenosine diphosphate (ADP)/O ratio. Data represent means ± SEM (bars) for mitochondria isolated from 6 *(daf-2(e1370)*) or 3 (*daf-16(mgDf50)*) replicate cultures; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ (Student's t-test).

Differences between wild-type and *daf-2(e1370)* were less obvious when mitochondria were fueled via Complex II (with succinate as a substrate and rotenone to inhibit activity of Complex I; no *daf-16* dataset is available for this setup): for state 3 oxygen consumption, rates seemed to decrease during the first few days of adulthood, only to increase at later stages in the wild-type [$P_{age*strain}$ for complete dataset = 0.0320] (fig 2a). From day 3 to day 9 of adulthood, statistics did comply with trends observed in mitochondria fueled via Complex I [P_{age} NS, P_{strain} = 0.0017]. Contrary to mitochondria fueled via Complex I, state 4 oxygen consumption fueled with succinate showed no clear increase with age [P_{age} = NS], while between-strain differences were statistically significant [P_{strain} = 0.0235] (fig 2b). A statistically significant age-related decrease in RCR and ADP/O was found; this decrease was slightly less steep in the long-lived mutant *daf-2(e1370)* [$P_{age*strain}$ <0.0001 and = 0.0006, respectively] (fig 2 c&d). Expected ADP/O of mitochondria fueled via Complex II is ~2; mitochondria from both strains did not reach this ADP/O rate, not even at young adult stages. That is why, though these data indicated a functional difference between aging WT and *daf-2(e1370)* mitochondria, we sought confirmation in an alternative experiment.

An alternative way to illustrate the effect of the *daf-2* mutation on the activity of mitochondria fueled via Complex II is by assaying the enzyme activity of complex II and III combined. Sufficient amounts of succinate and oxidized cytochrome *c* were supplied to fragmented mitochondria and complex IV activity was blocked by addition of KCN. In this way, electrons flowed from complex II via Ubiquinone over Complex III, finally reducing cytochrome *c*. Complex II+III activity showed a slight decrease with age in wild-type mitochondria, particularly at older ages, while in the *daf-2*, no age-related decrease was evident; rather, Complex II+III activity seemed to increase with age (fig 3) [$P_{age*strain}$ = 0.0007]. This assay estimates maximal activity of part of the ETC and proves that at least this part of the ETC can perform at higher rates in the long-lived mutant than in the wild-type; it cannot replace oxidative phosphorylation measurements of intact, active mitochondria, but we argue that in the case of succinate-driven oxidative phosphorylation, it is a valuable assay underpinning the difference between wild-type and *daf-2* mitochondria.



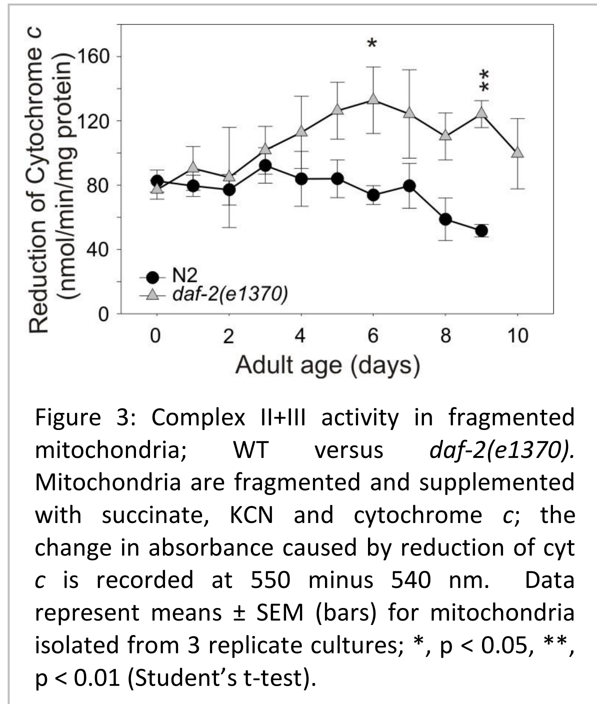


Figure 3: Complex II+III activity in fragmented mitochondria; WT versus *daf-2(e1370).* Mitochondria are fragmented and supplemented with succinate, KCN and cytochrome *c*; the change in absorbance caused by reduction of cyt *c* is recorded at 550 minus 540 nm. Data represent means ± SEM (bars) for mitochondria isolated from 3 replicate cultures; *, p < 0.05, **, p < 0.01 (Student's t-test).

Figure 2: Oxidative phosphorylation in mitochondria fueled via Complex II (with succinate and rotenone); WT versus *daf-2(e1370)*. (A) State 3 oxygen consumption. (B) State 4 oxygen consumption. (C) Respiratory control ratio. (D) Adenosine diphosphate (ADP)/O ratio. Data represent means ± SEM (bars) for mitochondria isolated from 3 replicate cultures; *, p < 0.05, **, p < 0.01 (Student's t-test).

Next we measured the rate of ATP synthesis by isolated mitochondria of all three strains in the presence of non-limiting supply of substrate and ADP. The results reflected the ADP/O profiles. Complex-I-dependent ATP synthesis capacity of *daf-2(e1370)* mitochondria was hardly affected by the aging process, whereas a gradual and a steeper decline was observed for mitochondria prepared from wild type and *daf-16(mgDf50)* animals, respectively (fig.4 a&b) [N2 vs *daf-2*, $P_{age*strain}$ = 0.0222 and N2 vs *daf-16*, $P_{age*strain}$ = 0.0054]. Similar ATP synthesis results were obtained when the mitochondria were fueled with Complex II substrate [N2 vs *daf-2*, $P_{age*strain}$ = 0.0146 and N2 vs *daf-16*, $P_{age*strain}$ = 0.0025] (fig 4 c&d).

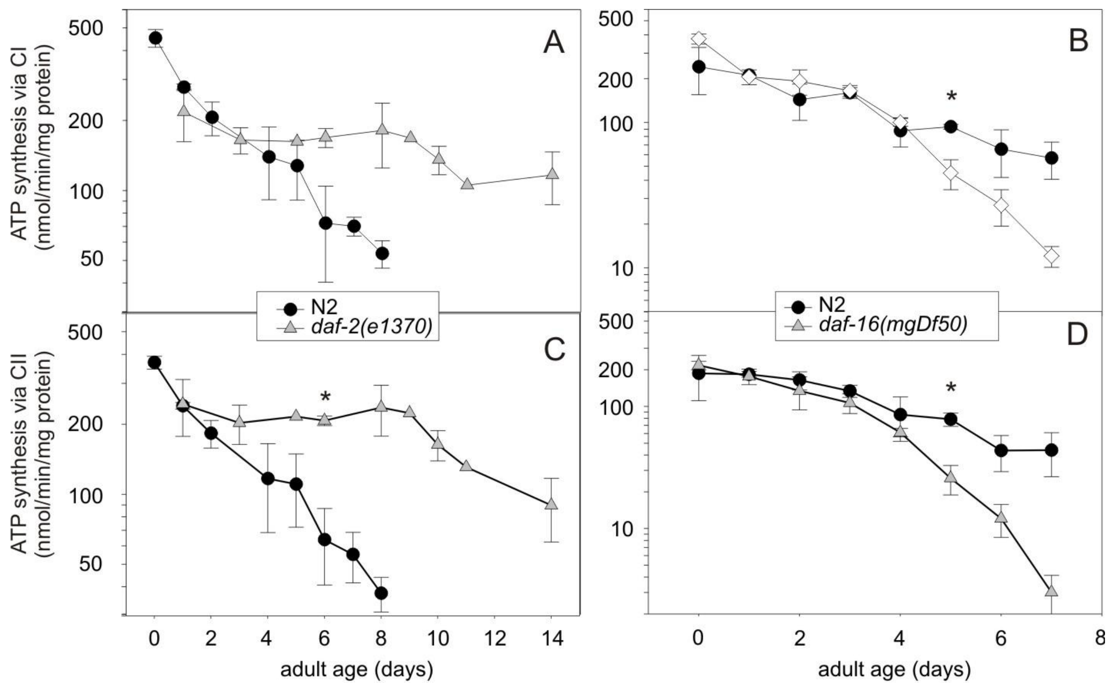


Figure 4: The *daf-2(e1370)* allele preserves mitochondrial ATP synthesis capacity throughout the adult life trajectory. The mitochondria are fueled with pyruvate and malate (A-B) or with succinate and rotenone (C-D). Left panels: WT versus *daf-2(e1370)*, right panels: WT versus *daf-16(mgDf50)*. Data represent means ± SEM (bars) for mitochondria isolated from 3 replicate cultures; *, $p < 0.05$ (Student's t-test).

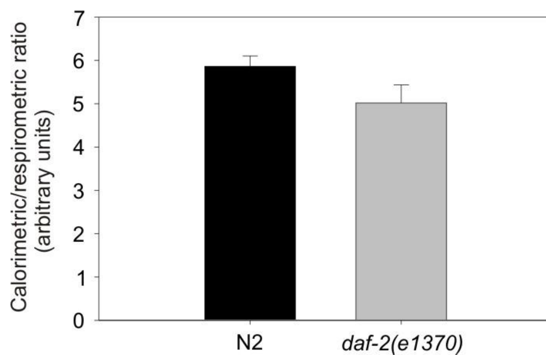


Figure 5: C/R ratios of mitochondria isolated from wild-type and *daf-2(e1370)* mutant worms**.** Mitochondria are isolated from 2-day-old adults and fuelled with Complex I substrates and adenosine diphosphate to activate complex-I-dependent respiration for at least 1 h. The oxygen consumption rates measured after completion of calorimetry are used for calculating the mitochondrial calorimetric to respirometric ratio. Data represent means ± standard error of mean for mitochondria isolated from eight replicate cultures.

A portion of the protons pumped into the intermembrane space by ETC complexes I, III and IV is not used to drive ADP phosphorylation by complex V, but leaks back to the matrix and represents a loss of energy as heat. Since heat released by live *daf-2(e1370)* animals was abnormally low relative to wild-type worms, we asked whether isolated mitochondria would yield similar results. A disadvantage of our thermal activity monitoring method is that it requires prolonged (~40 min) temperature equilibration of the samples in the instrument prior to effective data collection (~10 min). We observed that the oxygen consumption by *daf-2(e1370)* mitochondria was reduced by 15% at the end of the experiment compared to the initial respiration rate (results not shown). Remarkably, wild-type mitochondria lost very little activity under these conditions. To minimize experimental bias, we only used the respiration rates measured after completion of heat measurement to obtain the C/R ratio. Mitochondria respiring in state 3

fueled by non-limiting amounts of ADP and Complex I substrate were used and this experiment was repeated eight times. Overall, we found no significant difference in C/R between the mutant and wild-type mitochondria. (fig. 5).

We considered the possibility that microcalorimetry of isolated mitochondria lacked the necessary resolving power to detect small differences in mitochondrial heat dissipation. Since heat production is inversely proportional with electron transport chain efficiency, we asked if *daf-2(e1370)* mitochondria operate at a higher membrane potential. We used the cationic fluorescent dye DASPMI to probe $\Delta\Psi_{mit}$ of mitochondria prepared from wild-type and *daf-2* animals. The positively charged DASPMI ion readily penetrates the mitochondria and is distributed between the external space and the matrix compartment in accordance to the Nernst equation. The increase of emission of mitochondrial fluorescence is due to enhancement in quantum yield in the more proteinaceous and apolar microenvironment inside the mitochondria and is strictly linear up to ~3 nmol dye/mg mitochondrial protein (Bereiter-Hahn, 1976; Mewes and Rafael, 1981). We compared the fluorescence intensities of DASPMI in the activated OXPHOS state (state 3) and after addition of uncoupler (FCCP) which results in collapse of the membrane potential and equal distribution of the probe inside and outside the mitochondria (fig.6a). The fluorescence intensities of uncoupled wild-type and *daf-2* mitochondria were essentially identical (P_{strain} NS) and invariant with age (P_{age} NS). However, activated *daf-2* mitochondria emitted more fluorescence than wild-type mitochondria (P_{strain} 0.0343) and the fluorescence intensities decreased with age in both strains (P_{age} 0.0157). An age-related decrease (P_{age} 0.0035) was also seen when membrane potential was assessed on state 2 mitochondria. State 2 is similar but not identical to state 4; state 2 does give an indication of near maximal membrane potential. Strain differences for state 2 were not significant; *daf-2* mitochondria in state 3 operated at membrane potentials closer to their maximum than WT mitochondria. The transition of state 2 to state 3 caused an average drop of 5% of the DASPMI signal in *daf-2*. In the WT, an average drop of 14% was observed, with larger differences between mitochondria from young and old nematodes. Adding ADP to *daf-2* mitochondria shows that the mutant can better maintain its membrane potential under conditions of oxidative phosphorylation.

We conclude that the membrane potential in fast-respiring mitochondria decreases with age and that *daf-2* mitochondria most likely operate at higher $\Delta\Psi_{mit}$ values. Very recently, another study reported, using *in vivo* monitoring of the carbocyanine dye 'DiS-C_3(3)' that knock-down of lifespan-limiting genes by mutation or RNAi results in a lower $\Delta\Psi_{mit}$ and a drop in the λ_{max} of the emitted fluorescence (Lemire et al., 2009). These observations are difficult to interpret because they are heavily biased by strain-specific differences in feeding rate, and, of consequence, dye accumulation (Gaskova et al., 2007).

Estimates of mitochondrial membrane potentials can be important for interpretation of mitochondrial respiration data. Fueled with either succinate or pyruvate and malate, mitochondria isolated from *daf-2* have higher state 4 respiration rates than WT mitochondria. Since state 4 is controlled mainly by proton leak (the current of protons that leaks back from the intermembrane space to the matrix without driving complex V), it would seem that *daf-2* mitochondria have a higher proton leak than WT mitochondria, refuting our interpretation that *daf-2* is better coupled than WT. However, proton leak rate is voltage-dependent (Nicholls, 1977, Brand and Nicholls, 2011), with increasing state 4 respiration but no change in proton leak when mitochondrial membrane potentials are elevated. A

tentative interpretation would be that since membrane potential is higher in *daf-2* mitochondria, higher state 4 respiration rates are not necessarily indicative of higher proton leak in this strain. An increase with age of state 4 in both strains was observed when mitochondria were fueled by pyruvate and malate, not by succinate. Higher state 4 respiration at old age may indicate that proton leak increases with age.

Since addition of uncoupler resulted in equal $\Delta\Psi_{mit}$ in both strains, we asked what the consequences of uncoupling would be on mitochondrial oxygen consumption. Addition of the uncoupler FCCP increased respiration to the level of maximum electron transport system capacity. The uncoupled rates were slightly higher than the respective state 3 rates, indicating that the mitochondria from both strains respired close to their maximum capacity. However, both uncoupled and state 3 rates were substantially higher for *daf-2(e1370)* relative to N2 indicating that this capacity is enhanced in *daf-2(e1370)* animals (fig. 6b).

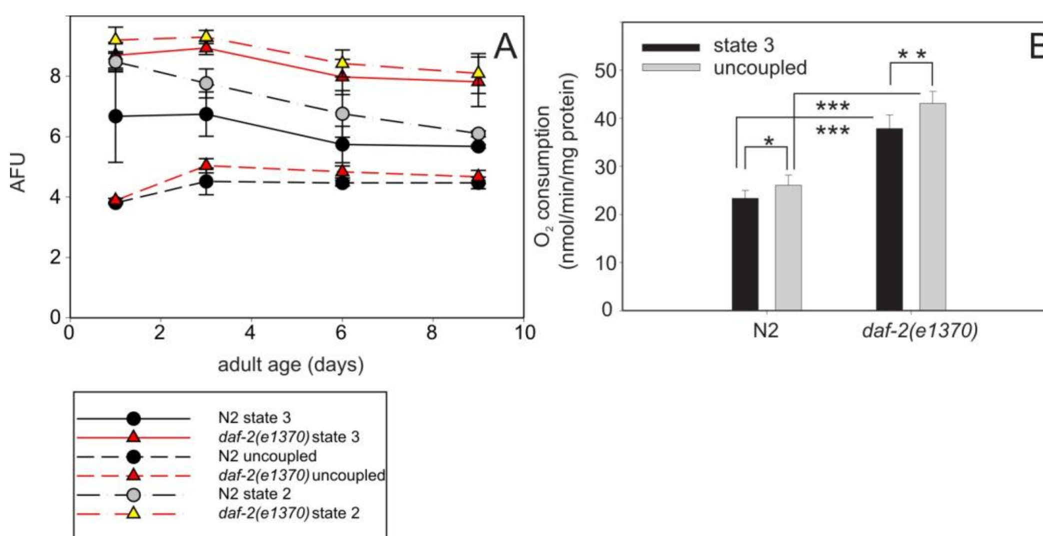


Figure 6: The *daf-2(e1370)* allele causes a higher mitochondrial membrane potential and enhanced respiratory capacity**.** (A) Increased accumulation of the fluorescent probe DASPMI inside energized *daf-2(e1370)* mitochondria. Fluorescence intensity of DASPMI in energized and uncoupled isolated mitochondria of wild-type (WT) and *daf-2(e1370)*. Fluorescence emission over the 2 min interval was averaged for each energetic state. Displayed values are means ± standard error of mean for three replicate cultures. The increase of DASPMI fluorescence is proportional to the amount of dye taken up by the mitochondria which itself is proportional to the membrane potential (Mewes and Rafael, 1981). (B) Higher oxygen consumption in the presence of adenosine diphosphate or uncoupler by *daf-2(e1370)* mitochondria. The assay was performed on two replicate cultures; since no age-dependent differences were noticed, mitochondrial respiration rates were averaged per strain over a 9-day time span. Paired *t*-tests were performed in within-strain comparisons, unpaired *t*-tests were performed in between-strain comparisons; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

5.4 Conclusions

In live nematodes, oxygen consumption decreases with increasing age in both wild-type and *daf-2(e1370)* (see chapter 3). As we found no evidence for a systematic age-related loss of mitochondria (chapter 4), we investigated whether isolated mitochondria show a comparable age-dependent reduction in respiration rate. This was clearly not the case. State 3 respiration was essentially unaffected by age in all three strains tested. State 3

respiration represents maximum performance under conditions of unlimited fuel supply, non-physiologically high oxygen concentration and absence of any cellular control. These results indicate that the ability of the mitochondria to reduce oxygen is not affected by the aging process and that the decline of oxygen consumption and heat output by intact animals during the first week of adulthood is regulated by aspects of mitochondrial function not studied here or by extra-mitochondrial control. In contrast, the mitochondrial coupling efficiency, illustrated by the ADP/O ratio and ATP synthesis under state 3 conditions, and the dependence of respiration on the available ADP, illustrated by the RCR, declined with age in all three strains though more weakly in *daf-2(e1370)* mitochondria. These results suggest that the mitochondrial bioenergetic competence is bound to decline with age but that this decline is attenuated by mutation in *daf-2,* suggesting modulation by Ins/insulin-like growth factor (IGF-1) signaling.

How could the bioenergetic competence of the mitochondria be altered? We found that several components involved in mitochondrial function decreased with age both in N2 and *daf-2* animals though faster in N2 (see chapter 4). However, this observation cannot explain the fairly constant state 3 rates measured over the life trajectories. One possible explanation is that the bioenergetic competence is largely dictated by a higher order structure of the ETC complexes. Bornhövd et al (2006) proposed a model of microdomain organization of OXPHOS (super)complexes in the mitochondrial inner membrane and they argued that disruption of these microdomains would affect metabolite/substrate channeling and/or efficient cooperation of these complexes, ultimately leading to a reduced flux through the respiratory chain and a lower membrane potential. Our measurements of ADP/O, RCR, ATP synthesis and mitochondrial membrane potential in *daf-2* worms versus wild type mitochondria are consistent with such a model.

It is not yet clear how and to what degree this control over bioenergetic competence is exerted *in vivo*. An explanation for decreasing oxygen consumption in aging nematodes is still lacking. In live worms, we found lower C/R ratios in the long-lived mutant than in the wild-type. We assumed that reduction in C/R ratio reflects more efficient energy production since less energy is lost as heat (Kemp and Guan, 1997) and expected to find enhanced coupling of ATP synthesis to the oxidation of NADH and $FADH_2$. However, we could not reproduce the large difference in C/R ratios of live young adult *daf-2(e1370)* and wild-type animals by assaying their isolated mitochondria. Also, the largest difference in worm C/R ratio was observed for a cohort of animals up to 7-9 days of adulthood, but this pattern was not reproduced by their mitochondrial ADP/O ratios. Conversely, we found that the ADP/O ratios for *daf-2(e1370)* mitochondria were generally higher than those obtained for wild-type worms when older animals were assayed, whereas the difference in worm C/R ratios between both strains faded away at advancing age. Thus it appears that the aberrant C/R ratio of *daf-2(e1370)* animals cannot be merely ascribed to an intrinsic property of their mitochondria and other mechanisms must be considered.

PART II: RESULTS

Chapter 6
Ins/IGF-1 and mitochondrial function: production of and damage by ROS

Redrafted from:
Brys *et al* (2010). Disruption of insulin signaling preserves bioenergetic competence of mitochondria in ageing *Caenorhabditis elegans*. BMC Biology **8**, 91

Personal contribution:
Brys et al (2010) nematode culturing, development and execution of H_2O_2 assay, execution of carbonylation assay, writing of manuscript

ABSTRACT

In the Ins/IGF-1 mutant *daf-2(e1370)*, the increase in mitochondrial competence is associated with a higher membrane potential. In line with this finding, we show that reactive oxygen (ROS) production is increased in *daf-2*; in contrast, little damage to mitochondrial protein or DNA occurs. We discuss the role of ROS production and oxidative damage in lifespan determination and conclude that mitochondrial ROS production does not limit the lifespan of *daf-2(e1370)*.

6.1 Introduction

Mitochondria convert approximately 0.1%-0.3% of the consumed oxygen to superoxide which can further react to generate other ROS (St-Pierre *et al.*, 2002; Fridovich, 2004). Hence, a widely held view is that aging initiates in, and spreads from, the mitochondrial compartment (Sastre *et al.*, 2003; Fridovich, 2004; Lenaz *et al.*, 2006; Lesnefsky & Hoppel, 2006). Since ROS production is expected to be related to the mitochondrial membrane potential (Korshunov *et al.*, 1997), we ask whether the higher membrane potential in *daf-2* mitochondria will be mirrored in the production of mitochondrial ROS and in the damage inflicted by these ROS on protein and DNA. We assess the effect of age and disruption of insulin signaling on mitochondrial ROS production in the form of H_2O_2 levels, measured in the presence of exogenous SOD. Next, we estimate the damage inflicted to mitochondrial proteins by ROS produced *in vivo*, and the occurrence of mtDNA deletions in aging controls and *daf-2(e1370)* mutants. We evaluate the role of ROS levels and damage by ROS in *daf-2* longevity, taking into account what is known about the mutant's antioxidant defense.

Results show that higher mitochondrial bioenergetic competence and membrane potential are associated with increased ROS production, but with little, if any, damage to mitochondrial protein or DNA, suggesting that oxidative damage is not a key determinant of aging under normal environmental conditions.

6.2 Materials and methods

6.2.1. Strains & culture conditions, isolation of mitochondria

Strains used were the wild-type N2 (Bristol male stock provided by the CGC) and Ins/IGF-1 mutants *daf-2(e1370)* and *daf-16(mgDf50)*. The double mutants *glp-4(bn2);daf-2(e1370)* and *glp-4(bn2) daf-16(mgDf50)* were used in assessment of mtDNA deletions. For culture conditions and isolation of mitochondria, we refer to (4.2.2.).

6.2.2. Assays

Quantification of mitochondrial H_2O_2 formation

Mitochondrial H_2O_2 production was measured according to standard procedures by the horseradish-peroxidase-mediated oxidation of Amplex Red (Invitrogen, CA, USA) to the fluorescent compound resorufin. First, the specificity of the assay for H_2O_2 was determined by recording the effect of inhibitors and/or uncouplers of the ETC on H_2O_2 production by mitochondria from day-0-adult wild-type. Next, the effect of age and Ins/IGF-1 pathway disruption was assessed in energized mitochondria. The protocol used on aging cohorts was performed in the following manner. Freshly isolated mitochondria were incubated with the appropriate substrates and ADP and the rate of H_2O_2 production was measured with Amplex red

as follows: aliquots of 96 µl incubation medium containing 4 mM ADP, 10 mM of pyruvate, 10 mM of malate (for Complex-I-dependent respiration) or 20 mM of succinate and 0.8 µg of rotenone (Complex-II-dependent respiration) and 10 U of Cu/ZnSOD (from bovine erythrocytes) were added to the wells of a black microtitre plate. The final assay medium was obtained by adding 100 µl of a mixture containing 100 µM Amplex Red and 4 U/ml horseradish peroxidase. Next, approximately 20 µg of freshly isolated mitochondria were added and the emitted fluorescence was measured for 35 min in the Wallac Victor² Multilabel Counter, at excitation and emission wavelengths of 550 and 590 nm, respectively. Final concentrations of reagents in the well were: ADP, 1.9 mM, pyruvate and malate, 4.8 mM each, or succinate, 9.6 mM, rotenone, 4 µg/ml, Cu/ZnSOD, 24 U/ml, Amplex Red, 50 µM, and HRP, 2 U/ml. The intensity of fluorescence was converted to picomoles of H_2O_2 by running an internal H_2O_2 standard curve to account for quenching caused by mitochondrial constituents. Assessment of specificity of the protocol was performed as follows: mitochondria were incubated with the appropriate combination of succinate and rotenone or pyruvate and malate, together with Cu/ZnSOD, in concentrations reported above. Antimycin A or CCCP were added in final concentrations of 3 and 10µM, respectively. H_2O_2 formation was recorded as described but without an internal H_2O_2 standard curve; rates of H_2O_2 production were expressed as percentages of signals without inhibitors.

Carbonylation assay

The carbonyl load of mitochondrial protein was measured using a Western immunoblot assay after protein derivatization with DNPH, as previously described (Matthijssens et al., 2007). Sample containing 4mg/ml protein was mixed with an equal volume of 12 % SDS. Next, 2 volumes of 20mM DNPH in 10 % TFA were added and the mixture was left for 15 minutes at room temperature. Derivatization was stopped by adding 1.5 volumes of neutralization solution (2 M Tris, 30 % glycerol) and the resulting mixture was loaded into the slots of an SDS-PAGE gel (18% acrylamide). Shortly after the proteins entered the separation gel, electrophoresis was discontinued and the proteins were transferred onto a nitrocellulose membrane by semi-dry blotting. To ascertain loading of equal protein amounts, the protein content in each slot-dot was stained with the reversible dye Memcode (Pierce, Thermo Scientific, IL, USA) and quantified using Image J software. Next, the membrane was destained and blocked with milk-PBS-Tween, incubated with rabbit anti-DNP primary antibody from Sigma followed by anti-rabbit antibody conjugated with HRP (Chemicon, Millipore, MA, USA) and finally with Supersignal chemiluminescent substrate from Pierce. This treatment produced a chemiluminescent signal that was detected on autoradiography film. This signal was quantified using Image J software.

DNA isolation and amplification

Mutation in *glp-4* causes a germline defective phenotype at the restrictive temperature of 24°C; we scanned the long-lived strain *daf-2(e1370); glp-4(bn2)* and the control strain *daf-16(mgDf50) glp-4(bn2)* for possible deletions in mtDNA, thereby avoiding differences in

germline development. Growth conditions were the same as in (4.3.1.2.), as well as the experimental setup for nucleic acid extraction of 3-, 7-, and 10-day-old adult *daf-16(mgDf50) glp-4(bn2)* worms and 3-, 7-, 10-, 14- and 26-day-old *daf-2(e1370); glp-4(bn2)* worms. Samples were used for nested PCR without any further purification. One µl sample and 0.25 µl TaKaRa LA Taq (TaKaRa Bio Inc, Shiga, Japan) were added to PCR tubes containing 24 µl of PCR components and the outer pair of primers (Forward: CTTGTTCCAGAATAATCGGCTAGACTTGTTAAAGCTTGTAC, reverse: CCTAAGCCCTAGGCCCAAAGTAACTATTGAAAAACC), and subjected to 25 cycles (30 s at 94 °C, 30 s at 60 °C and 12 min at 68 °C) of PCR to generate a fragment of 11,492 bp. Next 1 µl of this mixture was transferred to a tube containing 24 µl fresh reaction components and the inner pair of nested primers (forward: GGAGGCTGAGTAGTAACTGAGAACCCTC, reverse: GTGAAAGTGTCCTCAAGGCTACCACCTTC) to generate a final PCR fragment 11,211 bp long. As a control experiment, we also used the Melov primers for the second PCR reaction, generating a 6294 bp long fragment (Melov et al., 1995). The amplified DNA fragments were analyzed on 0.7% agarose gel. We found that fragments larger than ~7 kb would not be resolved from the full-length (~11 kb) amplicons. As a positive control, *him-8(e1489);uaDf5/+*, a strain heteroplasmic for a 3.1-kb deletion in mtDNA (Tsang & Lemire, 2002) did result in both 6kb amplicons and shorter amplicons of ~3kb when the second set of primers were used.

Protein

Mitochondrial protein content was measured with the BCA method (see 2.2.1.2.) without prior degradation of the samples with alkali.

6.3. Results and discussion

The Amplex Red method is a suitable method for measuring H_2O_2 production in isolated mitochondria

The Amplex Red protocol is based on horseradish peroxidase-catalyzed oxidation of the reductant substrate Amplex Red by hydrogen peroxide. The resulting resorufin is highly fluorescent. It has been used for studying the topology of ROS production (Chen et al., 2003; Muller et al., 2004; Tahara et al., 2009) employing inhibitors and uncouplers to alter ROS levels produced by specific complexes of the ETC. We have recorded resorufin fluorescence in the presence of antimycin A (an inhibitor of Complex III) or CCCP (an uncoupler) and calculated the change in H_2O_2 production caused by these manipulations (fig. 1). H_2O_2 signals measured in mitochondria from young-adult wild-type, supplied with exogenous SOD and substrates to fuel the ETC, were used as a reference. As substrates, either a mix of pyruvate and malate or succinate together with rotenone was chosen. Rotenone prevents reverse electron transfer via Complex I. First, addition of uncoupler decreased resorufin fluorescence with a minimum of 16 %, in agreement with its capacity to lower the proton gradient, causing the ETC to become less reduced. This decrease was moderate; we assume that the 'native' ROS production of the ETC (ROS production in the absence of inhibitors) is quite low. Complex III is often seen as an

important site of ROS production by the ETC (reviewed by (Turrens, 2003)); adding antimycin A to mitochondria increases H_2O_2 production rates considerably as it blocks the electron flow through Complex III, inducing the opposite effect of an uncoupler. The relative increase of H_2O_2 production caused by antimycin was highest in the presence of succinate and rotenone. This is not surprising since the activity of Complex II is driven directly by the added substrate and not dependent on other intermediates of the Krebs cycle.

The ability to record increased ROS production in the presence of antimycin and the observation of the opposite effect in the presence of uncoupler indicate that the Amplex protocol is specific for mitochondrially produced H_2O_2. (Tahara *et al.*, 2009) found that the formation of resorufin can largely be prevented by the addition of catalase, strengthening the validity of this protocol for measuring ROS production.

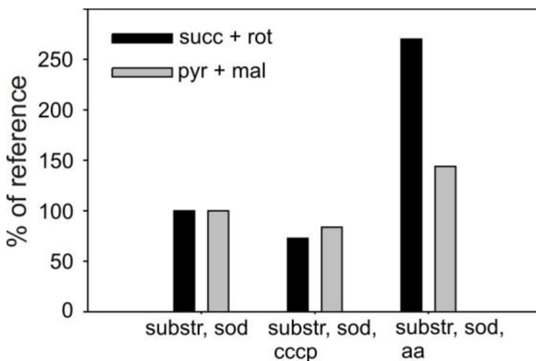


Figure 1: Amplex Red is suitable as an indicator of mitochondrial H_2O_2 production in the presence of exogenous SOD. WT mitochondria fueled via Complex I or Complex II show a decrease or increase in H_2O_2 production after addition of the uncoupler CCCP or the Complex III inhibitor Antimycin A, respectively. Data are expressed as % of reference measurements without addition of uncoupler or inhibitor.

daf-2(e1370) **mitochondria generate more H_2O_2 *in vitro*, but do not reveal higher oxidative damage accumulation in live worms**

In isolated mitochondria, the rate of ROS production is dependent on the mitochondrial membrane potential (Korshunov et al., 1997). As reported, $\Delta\Psi_{mit}$ of energized mitochondria is higher in *daf-2(e1370)* than in the wild-type. This raises questions about levels of ROS production by these mitochondria. We measured the H_2O_2 production capacity of phosphorylating mitochondria, in the presence of exogenous SOD to guarantee that all superoxide would be converted to H_2O_2. We found that H_2O_2 formation declined with age in all three strains, and that *daf-2(e1370)* mitochondria produced higher amounts at all ages tested (fig.2a) [P_{strain} = 0.0045; P_{age} < 0.0001], in line with their higher membrane potential. In contrast, wild-type animals and *daf-16(mgDf50)* mutants produced essentially identical amounts of H_2O_2 during their adult life trajectories (fig. 2b). We have to note that ROS production capacity was not measured in the absence of ADP; from mitochondrial membrane potential assessment it can be concluded that this would result in even higher H_2O_2 production rates. *In vivo*, mitochondria are expected to function in a respiratory state between state 3 and state 4. Likely, H_2O_2 production rates intermediate between state 3 and state 4 would better represent *in vivo* H_2O_2 production. Moreover, this intermediate state could differ between the WT and the long-lived mutant, potentially influencing ROS production differently in the two strains.

We asked if this elevated production of ROS *in vitro* would be reflected in enhanced damage to mitochondrial macromolecules *in vivo*. Firstly, we assayed carbonyl groups on Western blots of mitochondrial samples. The extent of carbonylation was fairly identical in wild-type worms and *daf-2(e1370)* animals during the first week of their adult lives, but mitochondrial protein from old *daf-2(e1370)* animals carried substantially less carbonyl load relative to wild type worms (fig. 3a). No differences were observed in the carbonyl load of mitochondrial protein prepared from wild-type and *daf-16(mgDf50)* animals (fig. 3b).

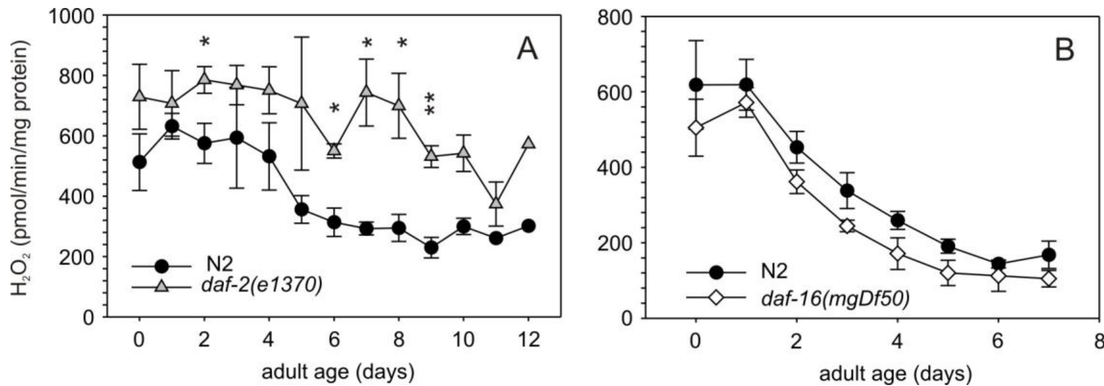


Figure 2: *daf-2(e1370)* mitochondria generate more H_2O_2 while loss of DAF-16 activity does not affect H_2O_2 production. H_2O_2 generation by isolated mitochondria from (A) wild-type and *daf-2(e1370),* (B) wild-type and *daf-16(mgDf50)*. Mitochondria were fuelled with pyruvate, malate and adenosine diphosphate. Cu/Zn SOD from erythrocytes was added to achieve maximal conversion of $O_2^{\cdot -}$ to H_2O_2. Data represent means ± standard error of mean (SEM; bars) for mitochondria isolated from four (*daf-2(e1370)*) or three (*daf-16(mgDf50)*) replicate cultures; * $P < 0.05$ and ** $P < 0.01$ (Student's t-test).

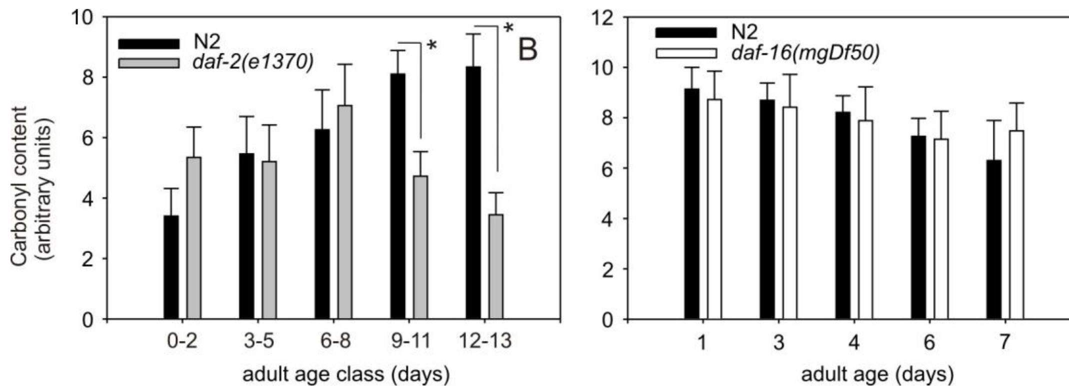


Figure 3: *daf-2(e1370)* mitochondria do not reveal increased carbonyl load and loss of DAF-16 activity does not affect the carbonyl content of mitochondrial protein**.** Carbonyl content of (A) wild-type and *daf-2(e1370),* (B) wild-type and *daf-16(mgDf50)* mitochondrial protein derivatized with diphenylhydrazine and detected by Western blotting and a diphenylhydrazone specific antibody. Data represent means ± SEM. For panel (A), six replicate cultures of each strain were grown but these were sampled at different time intervals, occasionally reducing the number of replicate samples for each time interval to 3; in panel (B), data represent means ± standard error of mean for mitochondria isolated from three replicate cultures;* $P < 0.05$ (Student's t-test).

ROS can also inflict damage to DNA. We monitored the occurrence of mitochondrial deletions with progressing age using long range nested PCR. For this experiment we used the long-lived double mutant strain *daf-2(e1370); glp-4(bn2)* and *daf-16(mgDf50) glp-4(bn2)* as a control. The *glp-4* genetic background was chosen because it is defective in germline development at the restrictive temperature. In total we examined 624 *daf-2(e1370); glp-4(bn-2)* worms picked from 3-, 7-, 10-, 14-, and 26-day-old adult cohorts, and 432 *daf-16(mgDf50) glp-4(bn2)* worms picked from 3-, 7- and 10-day-old adult cohorts. Faint bands representing curtailed fragments were observed incidentally, irrespective of strain or age. However, when the original DNA was assayed again these bands disappeared and novel bands incidentally arose, suggesting that these shortened fragments were generated artefactually during PCR amplification (fig 4; gels from 2 separate PCR experiments on *daf-16(mgDf50) glp-4(bn2)* DNA are shown, as well as 2 *him-8;uaDf5/+* reference samples). Thus we found no evidence for the occurrence of deletions in mtDNA linked to strain differences or progressing age.

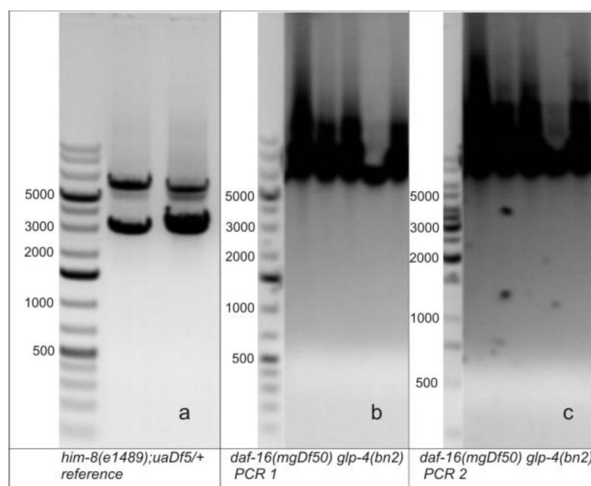


Figure 4: No evidence for occurrence of age- or strain-related deletions in mtDNA. Nested PCR was performed twice, starting from the same DNA extracted from individual worms of *daf-2(e1370);glp-4(bn2)* and *daf-16(mgDf50) glp-4(bn2)*. These worms were sampled at various ages and *him-8(e1489);uaDf5/+* was tested as a reference. Agarose gels from 2 separate PCR experiments (b & c) on 5 age-matched DNA samples (day 7 of adulthood) of *daf-16(mgDf50) glp-4(bn2)* are shown, as well as 2 *him-8;uaDf5/+* reference samples (a). While curtailed fragments smaller than 6kb are present in the reference samples, short fragments occurring in the *daf-16 glp-4* samples are considered artifacts as they are not observed in both PCR experiments.

6.4. Conclusions

The observed increase in *daf-2(e1370)* mitochondrial competence is associated with a higher membrane potential (Chapter 5). In line with these observations, we detected higher mitochondrial ROS production capacity in this long-lived mutant. Corresponding results were reported by Yang & Hekimi (2010a) who found higher levels of mitochondrial superoxide in *daf-2(e1370)* compared to wild-type. Although we cannot prove that *daf-2(e1370)* mitochondria

also generate more ROS *in vivo*, the expectation is that they do so. At first glance, this appears to be at odds with the common belief that a reduction of ROS underlies lifespan extension because it is predicted to slow down oxidative damage accrual (Reviewed by (Muller et al., 2007)). However, this mutant does show increased SOD and catalase activities and levels of reduced glutathione, and resistance to oxidative stress (see chapter 3) (Larsen, 1993; Vanfleteren, 1993; Honda & Honda, 1999; Brys *et al.*, 2007). Microarray analysis revealed that impairment of DAF-2 signaling enhanced DAF-16-dependent expression of *sod-3*, *hsp-16*, *gst-1*, *gst-4*, *mtl-1*, *ctl-1* and *ctl-2* (Murphy *et al.*, 2003; Halaschek-Wiener *et al.*, 2005). Conceivably, the activation of such a generalized defense could be mediated by a process called mitohormesis, where increases in mitochondrial ROS production cause an overcompensating induction of the antioxidant machinery resulting in extension of lifespan, as illustrated by (Schulz et al., 2007). However, for antioxidant defense to double life span of *daf-2(e1370)* relative to wild-type animals we would expect to detect substantial decreases of oxidative damage relative to wild-type worms. We did observe lower levels of carbonylated mitochondrial protein, but only in very old animals. Also, we could not confirm the expected decrease of mtDNA deletion events in the long-lived mutants (Melov et al., 1995). In fact we found no evidence of any such deletions in 624 long-lived and 432 control animals. Moreover, adding the antioxidant N-acetyl-cysteine did not extend but slightly shortened the lifespan of *daf-2(e1370)* (Yang & Hekimi, 2010), and deletion of all mitochondrial SOD activity by null alleles of both MnSOD encoding genes (*sod-2* and *sod-3*) failed to shorten (Doonan et al., 2008; Honda et al., 2008) or even extended (Van Raamsdonk & Hekimi, 2009) life span in an otherwise wild-type background and failed to abolish longevity of *daf-2(m577)* animals (Doonan et al., 2008). In all, these and our findings suggest that oxidative damage is not likely a major determinant of the lifespan of *C. elegans* under normal environmental conditions.

PART III: DISCUSSION

PART III: DISCUSSION

Chapter 7
General discussion and conclusions

7.1 Role of metabolism and reactive oxygen species in aging

In 1956, Denham Harman proposed that free radicals, inevitable byproducts of aerobic energy metabolism, cause molecular damage. Accumulation of damaged molecules would lead to a decrease in cellular activity, a hallmark of aging, and ultimately to the death of the organism (Harman, 1956). This theory predicts that species that produce plenty of free radicals should suffer more molecular damage which in turn should lead to a short life span. Because some reactive oxygen species that do not belong to the class of free radicals can also cause molecular damage, the Free Radical theory has been expanded to the modern Oxidative Damage theory. The Oxidative Damage theory provides a molecular basis for the aging process.

Three decades before Harman's theory was published, Pearl launched the Rate-of-Living theory, stating that lifespan and metabolic rate are inversely correlated (Pearl, 1928) and thereby predicting that long-lived species or individuals should have a lower basal metabolic rate compared to their shorter-lived counterparts. By assuming that the generation of reactive oxygen species is proportional to metabolic rate, the Oxidative Damage theory seemed to be compatible with the appealing Rate-of-Living theory. In this way, long lifespan could be attributable to a reduced metabolic rate that would lead to lower production rates of reactive oxygen species (ROS). These lower ROS levels would in turn cause less molecular damage, and a concurrent retardation of the aging process. Finally, the decreased aging rate would result in an increased lifespan.

In view of the many exceptions to its rule (the extreme longevity of bats and some eusocial animals such as naked mole rats and ant queens), the Rate-of Living theory has rightfully been questioned. Arguments for and against the Free Radical/Oxidative Damage theory of aging in model organisms other than *C. elegans* are described in chapter 1 (section 1.1.2.1.5) but are insufficient to confirm or refute the theory.

Longevity mutants of the invertebrate model system *Caenorhabditis elegans* provide an excellent tool for testing the predictions mentioned above. In this thesis, we have assessed energy metabolism and antioxidant defense of dauers, Clock mutants, DR-treated worms and the Ins/IGF-1 mutant *daf-2(e1370)*. For the latter, the study was broadened to incorporate measurements of mitochondrial ROS production and molecular damage. We have also incorporated existing evidence from the literature: data on metabolism, ROS production, ROS defense and/or damage by ROS that are available for many mitochondrial mutants and worms treated with RNAi to disrupt mitochondrial function, generally referred to as the Mit phenotype. Together, these findings allow experimental verification of the predictions of the Rate-of Living and Free Radical/Oxidative Damage theories of aging.

7.1.1 Slow rate of aging

First evidence that *C. elegans* Ins/IGF mutants age slowly came from demographical studies (Johnson, 1990; Johnson *et al.*, 2001) where it was shown that *age-1* mutants displayed a decreased acceleration of mortality with chronological age.

Movement has been used as a physiological biomarker of senescence and it was found that *age-1* mutants (belonging to the class of Ins/IGF longevity mutants like *daf-2*) showed enhanced

mobility later in life suggesting increased health compared to wild type (Duhon & Johnson, 1995). Later on, it was found that these mutants also showed decreased sarcopenia at advanced age, probably explaining their vitality. However, yolk accumulation in the body cavity, another marker of cellular aging, was not delayed in *age-1* mutants (Herndon *et al.*, 2002). In another study, two aging markers, tissue decline (observed with Nomarski microscopy) and lipofuscin accumulation, were found to be slowed down in *daf-2(e1370)* mutants (Garigan *et al.*, 2002).

Clk mutants have a longer mobility span or voluntary directional movement than WT (Van Raamsdonk *et al.*, 2010). Mit mutants exhibit slower rates of multiple physiological parameters, making it more difficult to evaluate the significance of differences between strains for aging.

7.1.2 Energy metabolism

The conflation of the Rate-of-Living theory with the Oxidative Damage theory of aging predicts that long-lived worms should show a low metabolic rate. We have quantified several parameters of energy metabolism including oxygen consumption, heat production and ATP concentration. Contrary to the prediction, mutants and treatments studied in this thesis did not show a generally decreased energy metabolism: no hypometabolism was recorded for the Clk mutants, *daf-2(e1370)* or DR-treated worms. Dauers did have low energy metabolism but were capable of reaching normal energy levels at dauer exit and post-dauer lifespan, regardless of how long they were kept in the diapause stage.

Several studies have shown that mitochondrial defects, whether they are caused by mutations or imposed by RNAi-treatment, can lead to lifespan extension (Lakowski & Hekimi, 1996; Feng *et al.*, 2001; Dillin *et al.*, 2002; Lee *et al.*, 2003; Hamilton *et al.*, 2005; Hansen *et al.*, 2005). Though the association between long lifespan and disruption of mitochondrial functioning can be perceived as counter-intuitive, it would fit into the Rate-of-Living theory if these mutants have lower metabolic rates. The majority of the long-lived strains tested in this way confirm anticipated low metabolic rates associated with these mutations or RNAi treatments. (Feng *et al.*, 2001) measured oxygen consumption in L1 juveniles of the *isp-1(qm150)* mutant, a mutant with a defect in the Iron-Sulphur Protein of complex III. *isp-1* oxygen consumption is reduced approximately 2-fold when compared to the wild-type. *isp-1*'s low oxygen consumption rate was confirmed in 1st-day-adults, and was accompanied by lower ATP levels (Yang & Hekimi, 2010a). These authors also measured low O_2 consumption in *nuo-6(qm200)*, a strain that carries a mutation in a conserved subunit of complex I (NUDFB4). (Lee *et al.*, 2003) reported a reduction in oxygen consumption rates in strains fed Mit RNAi bacteria –among others, affecting subunits of Complex, I, III or IV- accompanied by decreases in ATP content. A reduction in ATP levels was also observed in worms subjected to RNAi for *cyc-1* (complex III), *atp-3* (ATP synthase) *nuo-2* (complex I) or *cco-1* (complex IV) (Dillin *et al.*, 2002). Low pumping rates were observed in 12 mitochondrial-RNAi-treated strains (Hansen *et al.*, 2005). All these findings seem to confirm the Rate-of-Living theory.

Though oxygen consumption and other activities related to metabolic rate may be low in these Mit phenotypes, there are some observations that put these findings into perspective with respect to the causality of low metabolism in longevity: for instance, the short-lived *mev-1* mutant has a defect in a subunit of Complex II (Ishii *et al.*, 1998), also leading to lower oxygen consumption (Braeckman *et al.*, 2003). The double mutant *isp-1(qm150); ctb-1(qm189)*, which partly suppresses the slow metabolism of the *isp-1* single mutant, has higher oxygen consumption rates but its lifespan is hardly shorter than that of the single mutant (Feng *et al.*, 2001). While ATP levels in the *isp-1*

mutant are lower than in controls, the mutation in *nuo-6* results in ATP levels that are considerably higher (Yang & Hekimi, 2010a). Remarkably, disruption by RNAi results in reduced ATP levels for both genes. In contrast to the *nuo-6* mutant, *nuo-6* RNAi-treated worms do not have lower oxygen consumption rates when compared to WT (Yang & Hekimi, 2010b).

A direct molecular link can be drawn between Mit mutants/RNAi and alterations in mitochondrial metabolism. This link is not evidenced in *daf-2* mutants. Even so, the metabolic profiles of Mit phenotype worms are too divergent to confirm the correlation of longevity with low metabolic rate. Based on our *C. elegans* data and on various studies concerning Mit phenotype worms, we cannot support the prediction that life expectancy is determined by the rate of living.

7.1.3 Role of ROS

According to the Oxidative Damage theory of aging, extended lifespan should be accompanied by low levels of ROS. A technically feasible procedure to accurately measure *in vivo* ROS production rates in *C. elegans* has not yet been published[1]. For dauers, Clk mutants, DR-treated worms and initially for *daf-2(e1370)* as well, we have resorted to the measurement of antioxidant capacity as a proxy for the rate of ROS degradation. We found that in each of these strains, some aspect of antioxidant defense was altered when compared to the WT, but a general increase of antioxidant enzyme activities in all tested longevity strains was not observed.

Next, we developed a protocol for measurement of mitochondrial H_2O_2 production; we have isolated mitochondrial fractions and measured H_2O_2 formation in the presence of exogenous Cu/ZnSOD (converting all superoxide to H_2O_2) and adequate amounts of mitochondrial substrate. Counter to the prediction, we found ROS production to be significantly increased in the long-lived *daf-2(e1370)* strain in this *in vitro* assay. Although these results must be interpreted with care since they may not reflect ROS production levels *in vivo*, the difference found between *daf-2* and wild type ROS production was consistent for the largest part of the age range tested. This finding is compatible with better coupling of *daf-2(e1370)* mitochondria isolated from age-matched nematodes as assessed in chapter 5. In both wild type and *daf-2(e1370)* mitochondria, ROS production rate, like metabolic rate, was found to decrease over age. Data collected by Yasuda and colleagues (2006) on superoxide production in isolated wild type mitochondria showed a 15% decrease over age, but this minor effect was not found to be significant due to large experimental variation. It must be noted that this group measured superoxide production specifically while our data reflect the sum of superoxide and hydrogen peroxide production. ROS production capacity by isolated mitochondria was recently also assessed by the Hekimi group (Yang & Hekimi, 2010a). *daf-2* mutants were found to have elevated superoxide production but no significant increase in overall ROS. Mitochondria from long-lived Mit mutants showed either increases in overall ROS (*clk-1*), increases in superoxide (*isp-1* and *nuo-6*), or no significant differences in overall ROS or superoxide (*sod-2* mutant) when compared to the WT. Increased ROS production in *clk-1* and *isp-1* was also observed by (Lee *et al.*, 2010). For these mutants also, there seems to be no clear-cut inverse relation between ROS production and lifespan.

[1] The fluorescent probe MitoSOX Red, a lipophilic derivative of hydroethidine, can be administered to live worms where it accumulates in mitochondria; however, a number of disadvantages are connected to the use of MitoSOX as a fluorescent probe for *in vivo* measurement of ROS production. For instance, its uptake in the worm is influenced by feeding rate, it accumulates preferentially in *C. elegans*' pharyngeal bulb and its signal can be influenced by non-specific binding (Dingley et al., 2010).

Another approach to investigate whether low ROS production is crucial for longevity is through interference with antioxidant levels. Ubiquitous overexpression of the Cu/Zn *sod* gene *sod-1* only modestly increased lifespan (Doonan *et al.*, 2008). Moreover, supplying worms with the SOD mimetics EUK-8 and EUK-134 elevated SOD activity but did not result in lifespan extension (Keaney & Gems, 2003; Keaney *et al.*, 2004). Knocking out genes encoding antioxidants did not or only weakly shorten life span when implemented on WT, *clk-1*, *nuo-6*, *mev-1* or *daf-2* (Leiers *et al.*, 2003; Yang *et al.*, 2007; Doonan *et al.*, 2008; Honda *et al.*, 2008; Van Raamsdonk & Hekimi, 2009; Yen *et al.*, 2009; Yang & Hekimi, 2010a; Yang & Hekimi, 2010b). *isp-1* lifespan was either shortened or unaltered by knocking down *sod-2* through mutation or RNAi, respectively (Yang *et al.*, 2007; Van Raamsdonk & Hekimi, 2009). The antioxidant N-acetyl-cysteine had no effect on WT and *clk-1* but decreased lifespan in *isp-1*, *nuo-6*, *sod-2* and *daf-2* (Yang & Hekimi, 2010a). These interferences with antioxidant levels also contest the importance of ROS in aging.

It is widely assumed that damage accumulation is a key factor in the molecular aging process and that this type of damage is mainly caused by ROS, inflicting a diversity of modifications to DNA, lipids and proteins. Mitochondria are a major source of intracellular ROS generation and they are therefore considered to be prone to oxidative damage. According to the predictions of the Oxidative Damage theory of aging, long-lived strains should be less affected by ROS and, consequently, show less molecular damage. We have monitored the age-related accumulation of carbonylated proteins in wild type and *daf-2(e1370)* mitochondria and we found increased carbonyl levels but only in mitochondria of very old worms. This is in contrast to the findings of Adachi *et al.* (1998), Yasuda *et al.* (1999), and Ishii *et al.* (2002), who assayed whole *C. elegans* homogenates and found that long-lived mutants such as *daf-2(e1370)* and *age-1* accumulated less protein carbonyls over time compared to the control strain. Melov and colleagues (1995) claimed that age-related increases in damage to mtDNA are attenuated in the Ins/IGF-1 pathway mutant *age-1*; we have not been able to confirm this finding in *daf-2*.

For the short-lived Mit mutants *mev-1* and *gas-1*, it is plausible that the defect in the ETC function caused by the mutation leads to increases in oxidative damage (Adachi *et al.*, 1998; Kayser *et al.*, 2004; Dingley *et al.*, 2010). Possibly their antioxidant defense cannot cope with the increased amounts of ROS (Sedensky & Morgan, 2006). The long-lived Mit mutants, which show no overall signs of decreased ROS production, could be capable of neutralizing ROS and could consequently show lower levels of oxidative damage. However, in many cases, the prediction that longevity is correlated to low oxidative damage cannot be confirmed. No evidence could be found of significant decreases in oxidative damage in Clk mutants or *isp-1* measured as levels of carbonylated proteins (Yang *et al.*, 2007; Van Raamsdonk *et al.*, 2010), though *clk-1* mutant worms did seem to have lower levels of proteins modified by 4-hydroxy-2-nonenal (Kayser *et al.*, 2004; Yang *et al.*, 2009). The *sod-2* mutation confers an extended lifespan and increased carbonyl levels (Van Raamsdonk & Hekimi, 2009). (Dingley *et al.*, 2010) assessed lipid peroxidation in isolated mitochondria from several respiratory chain mutants and *daf-2*, but could not link this parameter to longevity. In a range of RNAi dilutions against the gene *atp-3*, no correlation was found between the degree of lifespan extension and the level of protein oxidative damage (Rea *et al.*, 2007). (Yang *et al.*, 2007) studied the effects of *sod-1* and *sod-2* RNAi on oxidative damage and lifespan in WT, *isp-1*, *clk-1* and *mev-1*. Though decreased expression of *sod-1* or *sod-2* did not always result in significantly increased damage in these strains, lifespan was mostly unaffected, or even increased, in the instances where oxidative damage was clearly elevated.

Although the evidence presented above does not support the Oxidative Damage theory of aging, the possibility remains that forms of damage important for aging are being overlooked. In a recent study, (Morcos *et al.*, 2008) described a novel mechanism impacting on *C. elegans* lifespan which does seem to fit into the Oxidative Damage theory of aging. With increasing age, the modification of mitochondrial proteins by methylglyoxal, a highly reactive dicarbonyl, increases while the activity of its detoxifying enzyme glyoxalase-1 decreases. Overexpression of the gene coding for glyoxalase-1 reduces both mitochondrial ROS production and methylglyoxal-related protein modifications and, importantly, it increases lifespan. Knockdown of this gene exhibits reverse effects. Possibly, this and other types of oxidative damage that have not been studied extensively are decisive for aging. Study of this process in the longevity mutants discussed here may clarify this issue.

Other than by causing molecular damage, ROS could also play a role as intracellular messengers in aging and longevity, e.g. in a protective signaling cascade that is beneficial to longevity. Though the molecular details of a mitochondrial-ROS-related protective mechanism have not been fully elucidated, there are indications that such mechanisms exist (Boneh, 2006; Storz, 2007). In line with this, Yang and Hekimi (2010a) suggest that lifespan extension of the *isp-1* and *nuo-6* mutants could be mediated by superoxide signaling. As lifespan extension in these mutants is not dependent on higher expression of superoxide dismutase genes, these authors argue that protective signaling by superoxide is distinct from a hormesis effect.

In all, from this short review and from studies on other model organisms it is apparent that no conclusive evidence has so far been presented that irrevocably provides proof for the Rate-of-Living and Oxidative Damage theories of aging. Pinpointing ROS as the sole cause of aging would imply ignoring the possibility that aging is, like so many other physiological processes, influenced by multiple molecular mechanisms, many of which remain to be elucidated.

7.2 Are alterations in metabolism causal to longevity?

It is clear that the longevity of Clk, Mit and Ins/IGF-1 mutant animals and DR-treated worms cannot be ascribed to lowered oxidative stress resulting from a slowing of metabolic rate. However, each of these manipulations leads to an alteration in energy metabolism. Could this altered metabolism define lifespan by a mechanism that does not implicate oxidative stress? If this is the case, we can expect that the mechanism involved will differ between the various manipulations as assessing their combined effects on lifespan shows additivity in some cases and non-additivity in others. For instance, when compared to the single mutants, the *daf-2; isp-1* or *daf-2; sod-2* double mutants show no further lifespan extension. In contrast, treatment of *daf-2* worms with Mit RNAi prolongs the *daf-2* lifespan up to 93% (Feng *et al.*, 2001; Hansen *et al.*, 2005; Van Raamsdonk & Hekimi, 2009). Synergistic effects on life span are also observed in *daf-2; clk-1* and *daf-2; clk-2* double mutants (Lakowski & Hekimi, 1996; Van Raamsdonk *et al.*, 2010). Importantly, it was recently reported that the metabolic effects of ETC disruption are not causal to lifespan extension (Yang & Hekimi, 2010a; Zuryn *et al.*, 2010).

The mechanism by which the *daf-2(e1370)* mutation extends lifespan is unknown. Potentially, *daf-2* longevity relies on the expression of dauer-like metabolic characteristics during the adult lifespan. *daf-2(e1370)* mutant animals inappropriately activate parts of the dauer programme at temperatures (20°-25°C) that allow uninterrupted development of WT worms. Since dauers can survive several times the normal lifespan and *daf-2(e1370)* adults live about twice as long as the WT it is reasonable to expect that they share some common mechanisms for extended lifespan. Whole genome transcription profiling identified a cohort of genes that are upregulated in both dauer larvae and *daf-2(e1370)* adult animals, including genes involved in certain aspects of metabolism, oxidoreductase activity, small heat shock proteins, anti-ROS defense and detoxification systems (Murphy *et al.*, 2003; McElwee *et al.*, 2004; McElwee *et al.*, 2006). Many of these changes may foster prolonged survival. However, energy metabolism is quite different in dauers and *daf-2(e1370)* adult worms as it is downregulated in dauers and normal in *daf-2(e1370)* adults. Anaerobic metabolism is upregulated and mitochondrial energy production may be partially shifted to anaerobic functioning during dauer diapause, as typically occurs in many parasitic species (Burnell *et al.*, 2005). However, oxygen consumption is not suppressed in *daf-2(e1370)* adult animals, making it unlikely that a dauer-like energy metabolism is causal to *daf-2* longevity.

Ins/IGF-1 pathway disruption has a distinct effect on metabolism. Oxygen consumption of wild type and *daf-2(e1370)* follows a strikingly similar pattern over the entire adult lifespan. Heat production, however, is lowered in *daf-2(e1370)*, especially during mid-life. These results indicate that, during a large proportion of their adult life, the long-lived *daf-2* mutants produce less heat per oxygen molecule that is consumed. A plausible explanation for this is that the efficiency of aerobic energy production is upregulated in *daf-2(e1370)* animals, evidenced by elevated ATP levels. Possibly, the reason for *daf-2*'s longevity lies in this shift in oxidative metabolism. This would imply a higher mitochondrial coupling efficiency.

Some of our findings confirm that the long-lived *daf-2(e1370)* is able to better preserve its mitochondrial function than the wild-type. In the wild-type, the abundance of key mitochondrial proteins declines with age, although the mitochondrial mass, inferred from the mitochondrial DNA copy number, remains unaltered. These age-related proteomic changes are accompanied by a dramatic decrease in energy production. In *daf-2(e1370)* adult animals, the age-dependent decrease of key mitochondrial proteins and electron transport chain complexes is considerably attenuated, as is the decrease in their bioenergetic competence. This ability to maintain mitochondrial function is associated with a higher membrane potential and increased ROS production; in contrast, little damage to mitochondrial protein or DNA occurs.

The question remains whether this shift in metabolism, which is associated with an attenuation in the decrease of mitochondrial function, is causal to the lifespan extension of the *daf-2* mutant. The metabolic profiles of ageing cohorts of N2, *daf-2(e1370)* and *daf-16(mgDf50)* animals described in chapter 3 point to a complex regulation of energy metabolism, where two pathways emanate from DAF-2, a predominant one that is DAF-16 independent whereas the other requires DAF-16. Given the common view that DAF-16 is a master regulator of longevity, this would implicate that the metabolic changes imparted by *daf-2(e1370)* are auxiliary, rather than essential, mechanisms of lifespan extension. This view is strengthened by several observations. We have demonstrated that the fall of metabolic rate with age is attenuated in *daf-2(e1370)* animals. Yet, while several aspects of *daf-2(e1370)* mitochondrial function are higher or better preserved with age, state 3 respiration of both WT and *daf-2(e1370)* mitochondria shows no such age-specific fall, suggesting that mitochondrial

malfunction is unlikely a primary cause of ageing. Also, the increased energetic efficiency of *daf-2* animals inferred from the C/R ratio is not recapitulated in isolated mitochondria suggesting that in control of whole-worm metabolism, extra-mitochondrial regulatory mechanisms are important. The higher standing levels of ATP cannot be essential either, because RNAi against several mitochondrial genes reportedly lowered ATP substantially but extended lifespan (Dillin *et al.*, 2002). Also, the *ucp-4(0)* mutant (UCP-4 is the only UCP-like protein encoded in the *C. elegans* genome) contains elevated ATP levels yet is not long-lived (Iser *et al.*, 2005). The overproduction of antioxidant enzymes by the *daf-2* mutant will certainly enhance survival under unfavorable conditions that are associated with oxidative stress, but they appear to be hardly effective in extending lifespan under normal conditions, as discussed previously. Combined, these observations suggest that the *daf-2(e1370)* mitochondrial phenotypes are not likely primary mechanisms of *daf-2(e1370)* longevity, and that low *daf-2* function alters the overall rate of aging by a yet unidentified mechanism, with an indirect protective effect on mitochondrial function.

7.3 A candidate mechanism for maintaining mitochondrial function in *daf-2*

To date, no direct molecular link has been established between the *daf-2* mutation and mitochondrial function. Oxidative phosphorylation is regulated in part by cell signaling. Though recent research has shown that all components of the electron transport chain can be phosphorylated (as reviewed in (Hüttemann *et al.*, 2007), it is not yet known whether mutation in *daf-2* influences mitochondrial function directly by altering the phosphorylation of ETC components.

Even if Ins/IGF-1 pathway disruption affects mitochondria only indirectly, it is still possible that the altered function of *daf-2(e1370)* mitochondria contributes to shifts in the metabolic network, not detected by the present approach and impinging on longevity assurance mechanisms. It was recently shown that WT dauer larvae and the long-lived insulin-like signaling (*daf-2*) and translation (*ife-2*) mutants display a common metabolic signature dominated by shifts in carbohydrate and amino acid signature (Fuchs *et al.*, 2010). Many of these metabolites are related to the citric acid cycle, glycolysis, gluconeogenesis and the glyoxylate shunt, metabolic activities that are differently regulated in dauers and adult *daf-2* mutant worms. Fuchs *et al*. (2010) found a general elevation of amino acid pool sizes in both mutant classes, and a striking upregulation of the branched amino acids isoleucine, leucine and valine, possibly resulting from downregulation of breakdown by mitochondrial BCKD[2] enzyme complex. The upregulation of gluconeogenesis and the glyoxylate shunt and downregulation of amino acids catabolism may serve a longevity assurance mechanism that is based on recycling of cellular components.

Multiple pathways and signals control life span of *C. elegans* and evidence is mounting that they converge on autophagy genes (Toth *et al.*, 2008). Autophagic events are approximately 8 times more frequent in *daf-2(e1370)* than in wild-type animals (Hansen *et al*., 2008) and (Melendez *et al*., 2003) demonstrated that *bec-1*, the worm ortholog of yeast and mammalian autophagy genes VPS/beclin 1, was essential for life span extension in *daf-2(e1370)*. However, autophagy alone is not sufficient for lifespan extension as it also occurs in double *daf-2;daf-16* mutants that are not long-lived (Hansen *et al*., 2008). A likely explanation is that both reduction in Ins/IGF-1 signaling and active

[2] branched-chain α-keto acid dehydrogenase

autophagy are required for *daf-2* longevity. Clearly, mitophagy is a candidate mechanism for preserving general mitochondrial competence. We assume that dysfunctional mitochondria are more rapidly degraded by autophagic processes in *daf-2(e1370)* mutant animals. The higher standing levels of ATP and the superior bioenergetic competence of their mitochondria would provide the necessary energy for subsequent mitogenesis ensuring sustained presence of competent mitochondria.

7.4 Technical advancement may aid progression in mitochondrial knowledge

Many of the questions on aging that we attempted to answer in this thesis will benefit from technical progress made in recent years. For instance, attempts are being made to further purify *C. elegans* mitochondria without compromising their functioning. Analyses of mitochondrial respiration will most certainly be more accurate in the future, thanks to the acquisition of an Oroboros Oxygraph for high-resolution respirometry. Moreover, mitochondrial respiration and membrane potentials will be measurable simultaneously. The field of study will be broadened to include other longevity mutants and treatments.

Proteome analyses are being performed on longevity-related mutants and conditions. They are performed on whole worm homogenates instead of on mitochondrial suspensions. Not only mutants but also RNAi-treated worms can be subjected to these analyses. Another advantage is that a high-throughput procedure is being used, which is not limited to one-on-one comparisons. Future proteomics datasets can be validated by Western blotting. Gene expression studies will assess whether observed age- or strain-related differences in protein abundance are transcriptionally regulated. RNAi and overexpression studies may reveal the importance of mitochondrial protein abundances for longevity. There are also indications that extensive proteome comparisons will reveal differences in mitochondrial composition between the strains discussed in chapter 4.

Finally, studies on OXPHOS supercomplexes are ongoing. The cooperation between the Dencher lab, executing the supercomplex analysis, and our lab, providing the mitochondrial samples, will continue as *C. elegans* mitochondria have proven to contain protein complexes with high stability.

7.5 General conclusion and perspectives for future research

In summary, none of the longevity strains and treatments presented in this study support the Rate-of-Living or Oxidative damage theories of aging. ROS are most likely not causal to aging under normal conditions. We have presented evidence that the age-dependent decrease in abundance of key proteins and in bioenergetic competence is considerably attenuated in mitochondria of the long-lived *daf-2(e1370)* mutant animals, and that these changes are associated with a higher membrane potential and increased ROS production. We also showed that the ultimate mechanism by which the *daf-2(e1370)* mutation extends life span cannot be ascribed to the higher standing levels of ATP or reduced oxidative damage. Though the mechanism by which the *e(1370)* mutation extends life span remains largely enigmatic, there are indications that mitophagy represents the indirect molecular link between Ins/IGF-1 disruption and maintained bioenergetic efficiency. This aspect of mitochondrial function requires further investigation; related fields of research that may elucidate the potential importance of this factor in *daf-2* longevity are protein metabolism studies, particularly mitochondrial protein turnover.

LIST OF STRAINS - Short description of the genes/mutants mentioned or used; for mutants used, alleles have been added.

gene	allele	protein	Phenotype
N2			Wild-type CGC Bristol male stock
age-1		P110 subunit of PIP_3 kinase	Dauer formation constitutive Aging abnormal; prolonged lifespan First known long-lived mutant
atp-3		mitochondrial ATP synthase subunit	ETS dysfunction Aging abnormal RNAi: Long-lived
ctl-1		Cytosolic catalase	
daf-2	*e1370*	predicted receptor tyrosine kinase, insulin/IGF receptor ortholog	abnormal DAuer Formation, Aging abnormal; prolonged lifespan 2 known classes: class I and class II
daf-16	*m26* *mgDf50*	Forkhead transcription factor	the sole *C. elegans* forkhead box O (FOXO) homolog Dauer formation defective Suppression of *daf-2*, *age-1*, *aap-1* phenotypes
cco-1		Cytochrome *c* oxidase, subunit Vb/COX4	ETS dysfunction Aging abnormal Growth rate abnormal RNAi: Long-lived
clk-1	*e2519*	Hydroxylase (DMQ -> 5-OH-ubiquinone)	Clock; slow-down of temporal processes Aging abnormal; prolonged lifespan Growth rate abnormal Maternal effect
clk-2	*qm37*	Homologous to Tel2p	Regulates telomere length DNA damage response Clock; slow-down of temporal processes Aging abnormal; prolonged lifespan Growth rate abnormal Maternal effect
clk-3	*qm38*	Unknown	Clock; slow-down of temporal processes Aging abnormal; prolonged lifespan Growth rate abnormal Maternal effect
ctb-1		Cytochrome *b* of mitochondrial complex III	mutation of *ctb-1* suppresses the slow embryonic development of *isp-1* mutants
cyc-1		Cytochrome *c* of mitochondrial complex III	ETS dysfunction Aging abnormal Growth rate abnormal RNAi: Long-lived
eat-2	*ad465* *ad1113*	ligand-gated ion channel subunit	Eating abnormal Abnormal, slower pharyngeal pumping Aging abnormal; prolonged lifespan
fer-15			Fertilization defective sterile
gas-1		49 kDa subunit of mitochondrial complex I	ETS dysfunction General Anaesthetic Sensitivity abnormal Short-lived
glp-4	*bn2*		Germ line proliferation defective sterile
gro-1	*e2400*	Isopentenylpyrophosphate:tRNA-transferase	Clock; slow-down of temporal processes Aging abnormal; prolonged lifespan Growth rate abnormal

ife-2		translation initiation factor 4F, cap-binding subunit (eIF-4E)	Aging abnormal Translation abnormal Long-lived
isp-1		Rieske iron sulphur protein (ISP) subunit of the mitochondrial complex III	ETS dysfunction Aging abnormal Growth rate abnormal Long-lived
mev-1		Cytochrome b_{560}	ETS dysfunction Methyl viologen resistance abnormal: abnormal resistance against paraquat (sensitivity) Growth rate abnormal Short-lived
nuo-2		*C. elegans* ortholog of NDUFS3/30 kDa subunit of complex I	ETS dysfunction Aging abnormal Growth rate abnormal RNAi: Long-lived
nuo-6		*C. elegans* ortholog of NDUFB4/B15 subunit of complex I	ETS dysfunction Aging abnormal Long-lived
sod-1		Copper/zinc superoxide dismutase	Slightly short lifespan or no significant effects on aging
sod-2		Mitochondrial iron/manganese superoxide dismutase	
ucp-4		Uncoupling protein	

LIFESPAN OVERVIEW - Overview of lifespans for mutants used.

gene	allele	culture		Lifespan (days)		Source
		medium	Temp °C	mean	% N2 control	
N2		Monox sol	24	14.4		Houthoofd et al. (2002a)
		Monox liq	24	8.6		Braeckman, unpublished data
		Ax liquid	24	30.9		Braeckman et al. (2000)
		Monox bact dil $1x10^6$ bact/ml $1X10^8$ bact/ml $1x10^{10}$ bact/ml	20	 5 25.9 15.0		Klass (1977) Caveat: age = juvenile + adult!
		Dauer			>4-8x normal lifespan	Klass and Hirsh (1976)
daf-2	*e1370*	Monox liq	24	16.1	187%	Braeckman, unpublished data
		Monox sol	24	24.3	169%	Houthoofd et al. (2003a)
daf-16	*m26*	Monox liq	24	7.9	92%	Braeckman, unpublished data
		Monox sol	24	12.9	90%	Houthoofd et al. (2003a)
daf-16	*mg Df50*	Monox liq	24			
		Monox sol	20		Slightly short-lived	Saul et al. (2008)
clk-1	*e2519*	Monox liq	24	12.8	149%	Braeckman, unpublished data
		Monox sol	20	26.0	140%	Wong et al. (1995)
clk-2	*qm37*	Monox liq	24	10.9	127%	Braeckman, unpublished data
		Monox sol	20	30.4	147%	Harris et al. (2006) Genetics
clk-3	*qm38*	Monox liq	24	12.6	147%	Braeckman, unpublished data
		Monox sol	20		Moderate increase	Van Raamsdonk et al. (2010)
eat-2	*ad465*	Monox liq	24			
		Monox sol	20	25.1	129%	Lakowski and Hekimi (1998)
		Ax liq	24	35.7	138%	Braeckman et al. (2000)
eat-2	*ad 1113*	Monox liq	24			
		Monox sol	20	28.4	146%	Lakowski and Hekimi (1998)
		Ax liq	24			
glp-4	*bn2*	Monox bact dil	24			
		Monox sol	24		Moderate increase	Houthoofd (2003)
		Monox sol vector L4440	25	11.6	-	Tohyama et al. (2008)
gro-1	*e2400*	Monox liq	24			
		Monox sol	25	15.6	170%	Kirkwood (1987)
		Monox sol	20		Moderate increase	Van Raamsdonk et al. (2010) s

Abbreviations:

Monox: monoxenic; Ax.: axenic; Liq.: liquid; Sol.: solid; Bact. dil.: bacterial dilution

List of publications

Publications that have not been discussed in this PhD thesis are marked in *italic*.

Brys, K; Castelein, N; Matthijssens, F; Vanfleteren, JR; Braeckman, BP
Disruption of insulin signalling preserves bioenergetic competence of mitochondria in ageing *Caenorhabditis elegans*
BMC BIOLOGY. Jun 2010; 8:Art No. 91

Brys, K; Vanfleteren, JR; Braeckman, BP
Testing the rate-of-living/oxidative damage theory of aging in the nematode model *Caenorhabditis elegans*
EXPERIMENTAL GERONTOLOGY. Sep 2007; 42(9):845-851

Houthoofd, K; Braeckman, BP; Lenaerts, I; **Brys, K**; Matthijssens, F; de Vreese, A; van Eygen, S; Vanfleteren, JR
DAF-2 pathway mutations and food restriction in aging *Caenorhabditis elegans* differentially affect metabolism
NEUROBIOLOGY OF AGING. May 2005; 26(5):689-696

Houthoofd, K; Fidalgo, MA; Hoogewijs, D; Braeckman, BP; Lenaerts, I; **Brys, K**; Matthijssens, F; De Vreese, A; Van Eygen, S; Munoz, MJ; Vanfleteren, JR
Metabolism, physiology and stress defense in three aging Ins/IGF-1 mutants of the nematode *Caenorhabditis elegans*
AGING CELL. Apr 2005; 4(2):87-95

Houthoofd, K; Braeckman, BP; De Vreese, A; Van Eygen, S; Lenaerts, I; ***Brys, K****; Matthijssens, F; Vanfleteren, JR*
Caloric restriction, Ins/IGF-1 signalling and longevity in the nematode Caenorhabditis elegans
BELGIAN JOURNAL OF ZOOLOGY. Jul 2004; 134(2):79-84

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