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Effects of nutritional components on stress response and aging in the nematode *Caenorhabditis elegans*

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1 Introduction

1.1 Aging

Over the last few centuries of human history life span has dramatically increased. The mean life expectancy of humans in Germany increased by 4.4 years to 79.8 years in 2006 compared to 1990 [1]. Due to the growing percentage of older people in our modern society, aging and aging related diseases more and more become a central topic of political, medical and scientific interest.
The term "aging" describes the passage of time [34][33] whereas the term "senescence" mainly describes processes occurring in the life history phase from full maturity to death. Accumulation of metabolic byproducts and a decreased probability of reproduction and survival are central features of senescence, which as cell inherent processes describe alterations in a variety of basic molecular and physiological processes [10][11][34][33][47]. Life span can be defined as the length of the life of an organism. Life span analysis in *Caenorhabditis elegans* and various other species has been used as an experimental approach to study aging and aging related effects.

1.2 Theories of aging

Various theories to explain aging have been formulated. One of the most popular ones is the free radical theory of aging by Harman [68] which is based on the close link between oxidative stress caused by reactive oxidative species (ROS) and cellular and whole organism aging processes [40]. A theory very closely linked to the free radical theory of aging is the "rate of living hypothesis" in which species with higher metabolic rates are considered to age faster and have a shorter maximum life span [185]. To slow the metabolic rate the worm *Caenorhabditis elegans* or the fruit fly *Drosophila melanogaster* were grown at lower temperatures which slows the metabolic rate down and furthermore results in a life span extension [136]. In yeast life span is extended by reducing glucose content of the medium, which results in caloric restriction and reduced metabolic rate [91]. Another aging theory is related to the telomere length. Telomeres are short repetitive DNA sequences located at

the ends of eukaryotic chromosomes protecting these from degradation, fusion and recombination. In somatic cells the DNA sequences at the telomeric ends of each chromosome are not replicated during cell division [130]. There is a close link between cellular senescence and an increasing reduction in the number of telomeric repeats. In line with that are experimental observations that cultured human cells can be prevented from undergoing senescence by overexpression of the telomerase, an enzyme that prevents telomere shortening in germ cells [20].

1.3 Reactive oxygen species and stress response

Reactive oxygen species (ROS) like superoxide anion and hydroxyl radicals are mainly produced in mitochondria during normal cellular metabolism [206][6]. About 1-3 % of all electrons in the electron transport chain (ETC) lead to generation of superoxide instead of contributing to the reduction of oxygen to water [59][63]. In addition, ROS are also generated in response to different exogenous stimuli like heat, metal ions, UV radiation, chemicals or hyperoxia [28][15][65][66]. ROS play a crucial role in several human diseases like atherosclerosis, neurodegenerative diseases, cancer and metabolic disorders like diabetes mellitus. They can cause a wide range of damage like oxidation of important macromolecules including lipids, proteins and DNA resulting in an impaired function [196].
The ETC is localized in the mitochondria. The mitochondrion has two highly specialized membranes. These two membranes, the inner and the outer one, create separate mitochondrial compartments, the internal matrix space and the intermembrane space [49]. The central function of the mitochondrion is the generation of energy in form of ATP by oxidizing hydrogen, derived from oxidation of organic acids, such as pyruvate and fatty acid, with oxygen to generate water by the process of oxidative phosphorylation. The ETC includes 5 complexes. Electrons are collected by the tricarboxylic acid (TCA) cycle and β-oxidation and transferred either to NAD^+ to generate NADH or FAD to give $FADH_2$. NADH transfers electrons to complex I (NADH dehydrogenase), succinate from the TCA cycle passes electrons to complex II (succinate dehydrogenase) and both complexes give the electrons to ubiquinone (coenzyme Q_{10} or CoQ) to generate ubisemiquinone and then ubiquinol ($CoQH_2$). The electrons are transferred to complex III from $CoQH_2$, then cytochrome c, complex IV (cytochrome c oxidase), and finally to molecular oxygen to give water. The released energy from the electron transport is used to pump pro-

tons out of the mitochondrial inner membrane to create an electrochemical gradient across the intermembrane space. This electrochemical gradient serves as an energy source to drive complex V (ATP synthase) to condense ADP + P_i to give ATP. Finally ATP is exported to the cytosol [207]. Furthermore, mitochondria are essential for several other functions like the biosynthesis of heme, lipids and amino acids, the Krebs cycle, the urea cycle, fatty acid oxidation, and iron homeostasis [177].
ROS can either be released into the mitochondrial matrix to disturb mitochondrial metabolism or are transported out of the mitochondria into the cytoplasm to damage cellular components with which they can interact. Mammalian cell culture studies provide experimental evidence that ROS are a critical determinant of life span. Life span of primary cells in culture can be significantly increased by lowering ambient oxygen concentration, to prevent excessive ROS generation [155].
Oxidative stress is a disequilibrium of pro- and antioxidative molecules in favor of a pro-oxidative state. To prevent damage caused by oxidative stress the organism utilizes different defense mechanisms against free radicals that involve enzymatic and non-enzymatic strategies. Enzymatic antioxidants include mainly superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalse (CAT). Superoxide dismutase converts superoxide anions into hydrogen peroxide. Two different forms of SOD are known. One is manganese (Mn) dependent and is localized in mitochondria (MnSOD) to limit oxidative damage there [50]. The other one uses copper (Cu) and zinc (Zn) (CuZnSOD) as cofactors and appears in the cytosol and in the extracellular space to scavenge ROS outside the mitochondrion. Catalase and different peroxidases can detoxify hydrogen peroxide by degrading it into water. Non-enzymatic antioxidants, which chemically inactivate ROS are represented for example by ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH), carotenoids and flavonoids [111][44][148]. Glutathione is a tripeptide (L-gamma-glutamyl-L-cysteinglycine) synthesized in cells to act there as a redox buffer. It is highly abundant in the cytosol (1-11 mM), nuclei (3-15 mM) and mitochondria (5-11 mM). Glutathione is a cofactor of several ROS-detoxifying enzymes, scavenges a lot of radicals directly and is able to regenerate exogenous antioxidants, such as vitamin C and vitamin E back to their active forms [158]. Since glutathione (2GSH/GSSG) represents the major cellular redox buffer its status can be used as an indicator for the redox environment of a cell [42][176]. Cellular life span can be prolonged via overexpression for example of SOD [181]. Conversely cellular senescence can be induced by knockdown of SOD by RNAi [18].

1.4 The nematode Caenorhabditis elegans

1.4.1 Metabolism and development

Sydney Brenner started in 1965 research on a small free living nematode *Caenorhabditis elegans*. It is characterized by its small size of 1 mm, its rapid life cycle, its genetic heritability, and its transparency so every single cell can be watched during development. A maximum life span of about 30 days in wild type animals makes *C. elegans* a valid model for aging studies. The generation time of the nematode is about three days. The lineage of every cell is completely known. The hermaphrodite, the more abundant and self reproductive gender, has 959 and the male has 1031 somatic cells. The worm has muscle cells, 302 neurons, a reproductive tract and an intestine. The nematode is free living, non parasitic and found commonly in many parts in the soil. In 1998 the whole genome of this organism was sequenced and in 2001 the Nobel price for Physiology and Medicine was awarded to Sydney Brenner and John Sulston for inventing *C. elegans* as a model organism to study basic cellular processes.
For laboratory use *C. elegans* is maintained either on agar dishes seeded with an *Escherichia coli* lawn as a food source or in liquid culture which is axenic (i.e. without other organisms as food source) or contains dead or alive *E. coli* bacteria. The worm is a filter feeder [12] and food, normally bacteria with a diameter up to 0,25 μm are taken up via pumping, peristaltic contractions of muscles in the corpus, the anterior isthmus and the terminal bulb of the pharynx. The particles are ground in the terminal bulb and passed into the intestine. This organ is composed of a one-cell-thick epithelial tube. Microvilli occur in the luminal surface. Studies with fluorescent probes suggest pinocytosis as a major uptake mechanism for larger food components [32]. If the molecules are smaller they are taken up by specific transporter or receptors. After the nutrients are taken up by intestinal cells these cells probably secrete them through the basal surface into the pseudoelomic fluid. This fluid contacts all tissues and serves as a nutrient source for individual cells. Defecation occurs by periodic muscle contraction. The duration between uptake and release is only a few minutes, as shown by tracer studies [12].

The worm is grown at an ambient temperature of 20 °C. The life cycle from an egg to a reproductive adult is about three days. During the development there are four larval stages (from L1 to L4) each separated by molt before they become adult

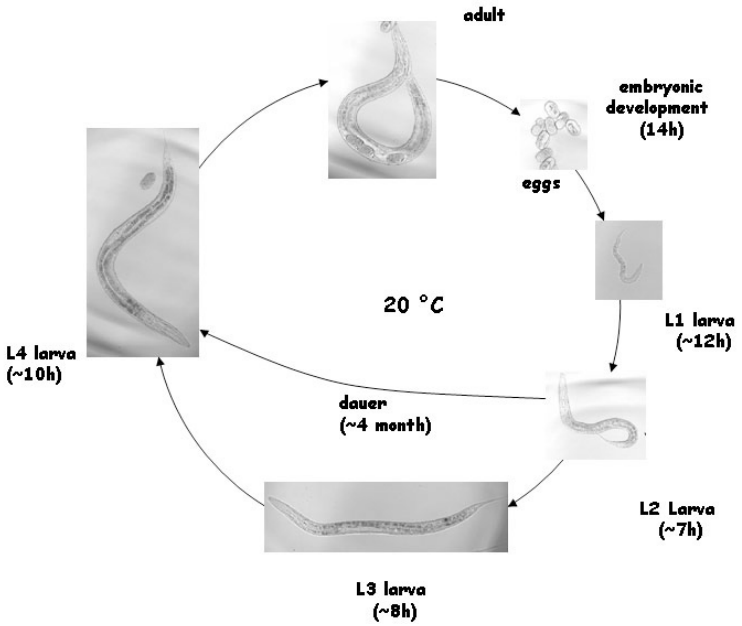

Figure 1.1: **Life cycle of *C. elegans* (modified according to [166]).** Under normal growth conditions, *C. elegans* begins life as an egg. After hatching it goes through four larval stages, each separated by a molt and then final molt into an adult hermaphrodite. Under unfavorable growth conditions like scarce food, overpopulation or elevated temperature the worm has the ability to enter an alternative life cycle in L2 and form dauer larvae at the third larval stage. When conditions become more suitable again worms will enter the life cycle as a L4 and then form a normal reproductive adult with the same adult life span as a worm that has not gone through dauer. Numbers in brackets indicates hours after fertilization at 20 °C.

hermaphrodites. When food supply is limited or population density is too high animals at the L1 stage proceed to the dauer at the L2 stage. Dauers are developmentally arrested, resistant to adverse environmental conditions and are adapted for long term survival [166]. It seems that dauer larvae are not aging since the post-dauer life span is not affected by a prolonged dauer stage of up to two months [103]. When food is available again they molt normal to develop into L4 larva.

Animals can be frozen at - 80 °C or in liquid nitrogen and stored so for several years. There are two sexes among the worm, a hermaphrodite (XX) and a male (XO) which only occasionally appears at a frequency of 0.1 % with a spontaneous X chromosome loss. But males can also be experimentally generated by starvation or heat shock. Together with the pair of sex chromosomes *C. elegans* has a diploid pair of five autosomal chromosomes (I, II, III, IV and V). The hermaphrodite can fertilize itself without mating since it produces both, oocytes and sperms. The sperms are the limiting factor.

Many techniques are applicable in worm research like RNA interference (RNAi) and nearly every knockout strain can be generated and is available from a deletion library. Moreover, it is possible to generate transgenic animals by microinjection. Because of the small size, the rapid life cycle and about 300 progenies by self fertilization of *C. elegans* many assays can be carried out in a 96-well microtiter plate and high throughput technologies can be utilized. The advantage of *C. elegans* over *in vitro* or cellular models is that functional pathways can be studied in context of a whole organism.

1.4.2 Aging and aging pathways in C. elegans

In *C. elegans* the aging process mainly takes place in the post-mitotic stage of development since adult worms do not undergo further cell divisions once the development is complete. Since the whole genome of *C. elegans* was sequenced it is feasible to identify genes and signaling pathways that are involved in aging. Many genes that affect life span in *C. elegans* have already been isolated and successfully shown to affect life span in other model systems as well.

Gene	Biological function	% increase in life span
daf-2	Insulin-like receptor	100
age-1	PI 3-kinase	40
clk-1	Coenzyme Q synthesis	20-90
eat-2	Pharyngeal pumping	50
isp-1	Electron transport (complex III)	65

Table 1.1: **Genes extending life span in *C. elegans*.** Life span measurements were done at 20 °C [9][46][51][107][108].

A long-lived *C. elegans* mutant, *isp-1*, which has a defect in complex III of the respiratory chain that is localized in mitochondria shows a reduction in oxygen consumption that is associated with a low ROS production [46][72]. Coenzyme Q, also known as ubiquinone is an important and well-conserved electron acceptor for electrons transferred from complex I and II of the mitochondrial respiratory chain. A mutant lacking an enzyme, encoded by the *clk-1* gene that is required for the biosynthesis of coenzyme Q has an increased life span compared to wild type N2 worms [107]. These mutants accumulate a precursor of ubiquinone and have lower cytoplasmic ROS levels [184]. Conversely, *mev-1* mutants which have a defect in complex II of the respiratory chain are short lived. These mutants have abnormalities in

mitochondrial structures and an increased ROS generation at complex II [180].
Another way to extend life span is caloric restriction. The *eat-2* gene is required for pharyngeal pumping. Mutations in *eat-2* which result in a slow down of the pumping rate cause an increase in life span. In eat mutants the severity of pharyngeal function correlates with extension of life span [108]. In contrast life span is shortened with enhanced food supply [79][103]. Several mechanisms have been suggested to explain life span enhancement caused by dietary restriction. One was based on a reduced metabolic rate and therefore a decreased ROS production [108]. However, direct measurement of oxygen consumption revealed that respiration rate in *eat-2* mutants is higher than in wild type N2 worms [80].
According to the telomere shortening theory, a reduction in the number of telomeric repeats is closely related to cellular senescence. In line with that finding *C. elegans* life span is increased by overexpression of HRP-1, a telomere binding protein which gradually increases telomeric length. The life span increase of long telomeres was dependent on *daf-16* but appears to be independent of stem cell cycling. These results suggest that life span regulation of an organism will be initialized in post-mitotic cells by telomere length [92]. Life span can also be influenced by the germline in *C. elegans*. Ablation of germ line precursors leads to an increase of life span by 60 % [82].

The insulin-like signaling pathway is the best characterized pathway that regulates life span in *C. elegans*. In the beginning of that pathway are the genes *unc-64* and *unc-31*, encoding two proteins that are thought to affect insulin processing and/or release in producing cells [4]. Loss of function of these genes causes extension of life span. The insulin receptor appears to be encoded by only one gene, *daf-2* whereas a total of 37 insulin family members have been identified in the *C. elegans* genome [61][162]. Activation of *daf-2* by ligand binding results in an activation of PI-3-kinase. Phosphoinositide-3-phosphate (PIP_3) is generated which acts like in mammalian systems as an intracellular second messenger to activate downstream kinases [5][102]. One of the first genes of the insulin signaling pathway that was identified to extend life span was *age-1* [104]. *Age-1* encodes in *C. elegans* the catalytic subunit p110 which forms together with the regulatory p85 subunit *AAP-1* the two subunits of PI-3-kinase [141][216]. Mutations in *age-1* cause a resistance to stress, such as heat, oxidative damage and heavy metals [13][77][123][124][145]. Mutations that inactivate DAF-2 promote dauer constitution like *age-1* mutants, increase life span and increase stress resistance [56][77][101][114][124][145][193]. PDK-1, SGK-1, AKT-

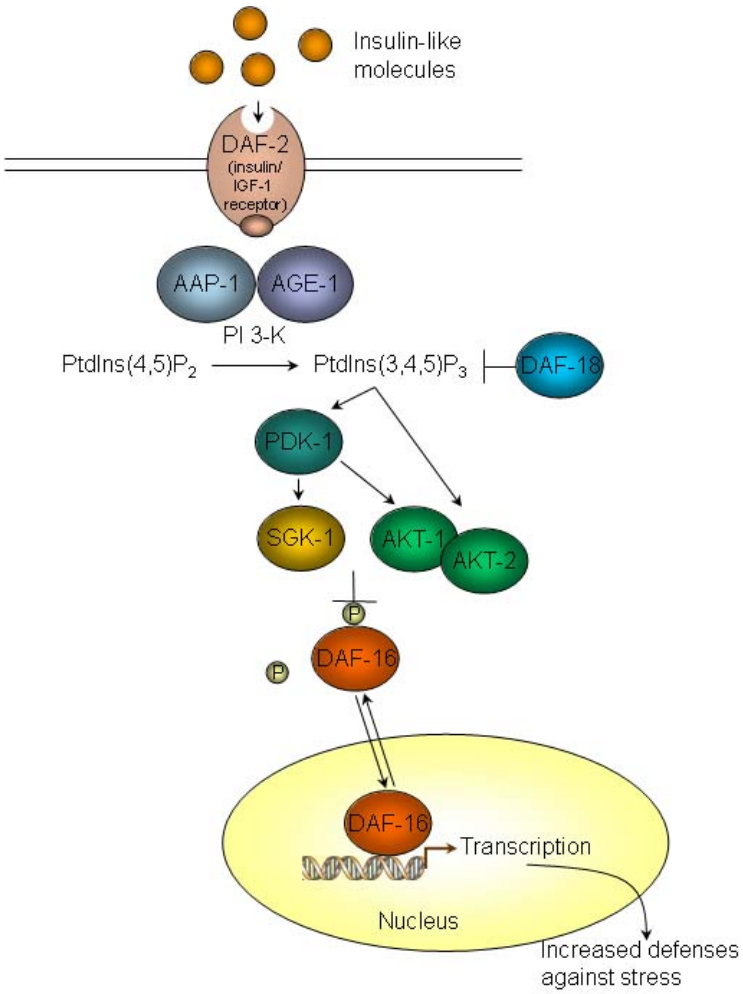

Figure 1.2: ***Daf-2* signaling in *C. elegans*.** The figure is explained in the text (according to [144]).

1 and AKT-2 are kinases downstream of *age-1* and gain-of-function mutants were identified as suppressors of the dauer constitution of *age-1* in *C. elegans* [156][157]. A homologue of the mammalian tumor suppressor PTEN phosphatase is encoded by *daf-18* [58][135][152][171] and regulates the levels of PIP_3 [128]. A life span decreasing effect and suppression of both *daf-2* and *age-1* dauer phenotypes is caused by a loss-of-function mutation in *daf-18* [41][58][114][135][152][171].

The final down-stream target of the *daf-2* signaling pathway is a forkhead transcription factor encoded by *daf-16* [151]. AKT and SGK-1 phosphorylate and thereby retain DAF-16 in the cytosol [75][120][153]. A loss-of-function mutation in *daf-16* decreases life span and suppresses completely all phenotypes in double-mutant combinations with *daf-2* or *age-1* [107][45][108]. Inactivating mutations in one of the

upstream genes of *daf-16* or prevention of ligand binding to the DAF-2 receptor lead to a release of DAF-16 from the phosphorylation-mediated suppression and to a translocation into the nucleus [73][120]. When DAF-16 enters the nucleus it is supposed to bind to and activate numerous genes involved in stress response, life span extension, dauer formation and metabolism. In concordance to this mutations of the upstream genes (*daf-2*, *age-1*, *aap-1*, *agk-1* and *akt-1/2*) that lead to a reduction of phosphorylation state of DAF-16 result in an increase in life span [51][75][99][101][119][147][151][153][162][216].

In 2001 *old-1* a transmembrane tyrosine kinase has been identified by Murakami and Johnson that is required for life span extension in *daf-2* and *age-1* mutants [146]. Further molecular studies and analysis of the expression pattern lead to the suggestion that OLD-1 expression is dependent on *daf-16* [146].

Several *C. elegans* laboratories have identified an insulin supergene family based on sequence homology and structural predictions with 37 members: ins-1 through ins-37. These ligands of the DAF-2 receptor can be sub-classified into four types according to their predicted disulfide bond connections. The gamma type includes mammalian insulin, the insulin like growth factors (IGFs) and most of the other previously identified members of this superfamily [162].

Another regulator of longevity which is evolutionary conserved is SIR2 the silent information regulator 2. Silent information regulators belong to a family of NAD^+ dependent protein deacetylases which respond to energy availability and cellular stress. Under stress conditions and starvation increased dosages of SIR2 can extend life span up to 50 % in yeast, flies and worms [96][169][192]. In *C. elegans sir-2.1*-dependent effects on aging are regulated by the insulin/IGF-1 pathway and the transcription factor DAF-16. SIR-2.1 is thought to downregulate DAF-2 signaling [192]. Sir-2.1 is also required for the life span increasing effect of caloric restriction [210]. The mammalian homolog SIRT1 can interact with transcription factors such as p53, PPARγ and NF-κB as well as transcription cofactors such as p300 and CBP [126][112][190][161][199][222][22]. In mammals forkhead proteins can be deacetylated by SIRT1 which results either in suppression or activation of the transcription of their target genes [27][143][22].

DAF-16 translocation is also regulated by 14-3-3 binding. 14-3-3 proteins are small acidic proteins which are highly conserved. They bind phosphoserine and phosphothreonine residues in a context-specific manner [43]. Key biological processes such as cell cycle, apoptosis and transcription are regulated by 14-3-3 proteins through

interaction with their partners [195]. In *C. elegans* 14-3-3 proteins are encoded by two genes, *par-5* and *ftt-2*, both are concentrated in the embryo and the gonad [209][142]. Within individual cells *C. elegans* 14-3-3 proteins are mostly localized in the cytoplasm [209]. Downregulation of 14-3-3 proteins results in a nuclear localization of DAF-16.

Another pathway that can influence life span in *C. elegans* is the highly conserved target-of-rapamycin (TOR) signaling. The TOR protein kinase controls in response to nutrients and growth factors cell growth. In *C. elegans* DAF-16, also a TOR-interacting protein and let-363 (CeTOR), leads to an extension of life span to 30 % [90].

1.5 Reactive oxygen species and stress response in C. elegans

The forkhead transcription factor DAF-16, the *C. elegans* homolog to the mammalian FOXO is essential for the activation of most of the defense mechanisms against ROS as discussed in the previous chapter. Downstream of DAF-16 are many genes which are involved in oxidative stress response. In *C. elegans* there are five genes coding for superoxide dismutase: *sod-2* and *sod-3* encode mitochondrial MnSODs, *sod-1* and *sod-5* encode cytosolic CuZnSODs and *sod-4* encodes a possibly secreted CuZnSOD [113][57][188][85][52]. Three genes are coding for catalase in the nematode: *ctl-1* encodes a cytosolic and *ctl-2* a peroxisomal catalase [191][86]. Essentially nothing is known about *ctl-3*.

DAF-16 also upregulates heat shock proteins/chaperons [83][131]. Heat shock proteins stabilize protein folding prevent protein damage, and have been shown to play a role in life span determination in *C. elegans* [140][83][147].

In summary, numerous proteins are critically involved in aging processes and one of the most important signaling pathways that links metabolism to life span regulation is the insulin/IGF-1 pathway. It has a central role in energy metabolism and homeostais and determines also free radical generation during mitochondrial respiration. Mitochondrial ROS production is a driving force in aging and has in the past mainly be considered as a toxic side reaction. But more recently it became obvious that mitochondrial ROS are also important as an input trait in several redox-dependent signaling processes [37][149][213].

1.6 Aim of the work

Since ROS are suggested to play a crucial role in acceleration of aging and a variety of aging processes, *C. elegans* was used in this study as a model system to study the impact of ROS on adaptations related to antioxidative defense mechanisms and aging.

A general stress response to ROS was studied by application of juglone, a generator of superoxide anion radicals. In this context enhanced ROS levels led to hormesis, which resulted in longevity. To asses why an increased ROS level leads to longevity, activities of the antioxidative enzymes SOD and catalase as well as cellular glutathione levels were determined. Moreover, to identify the role of individual genes in these adaptative processes either *C. elegans* mutant (as in the case of the *daf-16* knockout strain) or transgenic strains (DAF-16::GFP, *hsp-16.2*p::GFP) were used or alternatively RNAi interference mediated gene knockdown (as in the case of *daf-16*, *sir-2.1*, *ftt-2*, and *par-5*) was performed.

To specify the role of mitochondrial ROS in the context of insulin signaling, worms were exposed to enhanced glucose concentrations in liquid medium to increase electron flow through the respiratory chain. O_2-consumption as a marker for mitochondrial respiratory chain activity and ATP-generation, mitochondrial ROS-production, antioxidative responses, and DAF-16 nuclear translocation as a measure of insulin/IGF-1 signaling activity were measured together with life span determinations.

Since secondary plant ingredients like polyphenols and flavone are considered to have potent antioxidative activity, the effect of selected molecules were tested for their impact on aging and stress response with special emphasis on the *daf-16* and *sir-2.1* gen known to be involved in stress resistance and longevity.

2 Material and methods

2.1 Material

2.1.1 Instruments

instrument	company
centrifuge	Hettich
microscope Leica DM-IL	Leica, Heidelberg
microscope Leica MZ75	Leica, Heidelberg
shaker	Heidolph
lamina flow HeraSafe	Kendro, Langenselbold

2.1.2 Buffers and solutions

The chemicals were purchased from:

- Sigma-Aldrich, Steinheim (D)
- Roth, Karlsruhe (D)
- Merck, Darmstadt (D)
- Biorad, Munich (D)
- Serva, Heidelberg (D)
- Gibco BRL, Karlsruhe (D)
- USB, Cleveland, Ohio (USA)

C. elegans habitation and reproduction (CeHR) medium

chemical	company	final concentration
Glycogen	USB	10 %
Salt solution		
$CaCl_2 \cdot 2H_2O$	Roth	0.136 mM
$MgCl_2$	Roth	2.016 mM
Sodium citrate $\cdot H_2O$	Sigma-Aldrich	0.986 mM
Potassium citrate	Sigma-Aldrich (Fluka)	1.510 mM
$CuCl_2 \cdot 2H_2O$	Sigma-Aldrich	0.041 mM
$MnCl_2 \cdot 4H_2O$	Sigma-Aldrich	0.101 mM
$ZnCl_2$	Roth	0.073 mM
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	Sigma-Aldrich	0.153 mM
Vitamins and growth factors		
N-Acetylglucosamine	Sigma-Aldrich	0.108 mM
DL-Alanine	Sigma-Aldrich	0.269 mM
p-Aminobenzoic acid	Sigma-Aldrich	0.088 mM
Biotin	Sigma-Aldrich	0.025 mM
Cyanocobalamin	Sigma-Aldrich	0.004 mM
Folinate (Ca)	Sigma-Aldrich	0.012 mM
Niacin	Sigma-Aldrich (Fluka)	0.097 mM
Niacinamide	Sigma-Aldrich	0.098 mM
Pantetheine	Sigma-Aldrich	0.011 mM
Pantothenate (Ca)	Sigma-Aldrich	0.050 mM
Pteroylglutamic acid (folic acid)	Sigma-Aldrich	0.027 mM
Pyridoxal 5' -phosphate	Sigma-Aldrich	0.025 mM
Pyridoxamine 2 HCl	Sigma-Aldrich	0.025 mM
Pyridoxine HCl	Sigma-Aldrich	0.058 mM
Riboflavin 5'-PO_4(Na)	Sigma-Aldrich	0.025 mM
Thiamine HCl	Sigma-Aldrich	0.036 mM
DL-6,8-thioctic acid	Sigma-Aldrich	0.029 mM
Nucleic acid mix		
Adenosine 5'-PO_4	Sigma-Aldrich	1.991 mM
Cytidine 5'-PO_4	Sigma-Aldrich	1.272 mM
Guanosine 5'-PO_4	Sigma-Aldrich	1.132 mM
Uridine 5'-PO_4	Sigma-Aldrich	1.200 mM
Thymine	Sigma-Aldrich	1.199 mM

chemical	company	final concentration
Other components		
MEM Amino acids	Gibco BRL	3.5 %
MEM non essential amino acids	Gibco BRL	2.2 %
KH_2PO_4	Roth	9.98 mM
Cholin di-acid citrate	Sigma-Aldrich	3.06 mM
i-Inositol	Sigma-Aldrich	2.62 mM
D-Glucose	Roth	128.02 mM
Lactalbumin hydrolysate	Sigma-Aldrich	0.67 %
Cytochrome C (bovine heart)	Sigma-Aldrich	6.21 mM
Hemin chloride	Sigma-Aldrich	16.63 mM
HEPES	Roth	10.94 mM
Cholesterol	Sigma-Aldrich	42.47 μM
BIS-TRIS (pH 6.4)	Sigma-Aldrich	11.38 μM

For a pH-value of 6.0 to 6.3 10 % KOH (Roth) was used. 1 ml final medium contains:

- 0.5 ml CeHR Medium
- 0.3 ml DYT
- 0.2 ml skim milk (0.3 % fat) (Erlenhof, Germany)
- 0.2 μl Gentamycin (50 mg/ml) (USB)

Other chemicals

chemical	company	final concentration
DYT		
Peptone	Roth	16 %
Bacto yeast extract	Roth	10 %
NaCl	Roth	85.5 mM
NGM agar		
Agar-Agar	Serva	1.7 %
NaCl	Roth	51.3 mM
Peptone	Roth	0.25 %
KH_2PO_4	Roth	25 mM
$CaCl_2$	Roth	1.0 mM
$MgSO_4$	Roth	1.0 mM
Cholesterol	Sigma-Aldrich	12.9 mM
Nystatin (10000U/ml)	Sigma-Aldrich	0.7 %
Saccharose	Roth	0.5 %

Only for the RNAi plates isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma-Aldrich;

1 mM final concentration) and carbenicillin (Sigma-Aldrich; 25 mg/ml final concentration) were added.

chemical	company	final concentration
M9 buffer		
KH_2PO_4	Roth	22.0 mM
Na_2HPO_4	Roth	38.5 mM
NaCl	Roth	85.5 mM
$MgSO_4$	Roth	1.0 mM
Lysis buffer for glutathione measurement		
Tris HCl	Roth	100 mM
NaCl	Roth	200 mM
EDTA	Roth	1 mM
Glycerol	Roth	8 %
Freezing buffer		
K_2HPO_4	Roth	6.45 mM
KH_2PO_4	Roth	43.55 mM
NaCl	Roth	0.1 M
Glycerol	Roth	30 %
Tris Cacodyl buffer (SOD measurement)		
Tris	Roth	5.00 mM
Cacodyl acid	Sigma	5.00 mM
Titriplex V	Merck	1.01 mM
Phosphate buffer (CAT measurement)		
A:KH_2PO_4	Roth	50.04 mM
B:Na_2HPO_4	Roth	62.69 mM
Mix A and B in a 1:1.55 ratio before use		
Phosphate buffer (glutathione measurement)		
A: KH_2PO_4	Roth	124.9 mM
EDTA	Roth	8.0 mM
B:K_2HPO_4	Roth	124.9 mM
EDTA	Roth	8.0 mM
Mix A and B in a ratio 1:1.18 before use		

chemical	company
Other components	
Aceton	Roth
Agarose	Roth
Ascorbic acid	Sigma-Aldrich
Bradford reagens	BioRad
Dimethyl sulfoxide	Sigma-Aldrich (Fluka)
DTNB	Sigma-Aldrich
Ethanol 99.0 %	J. T. Baker, Devender
Fisetin	Sigma-Aldrich
Flavone	Sigma-Aldrich
5-Fluoro-2'-deoxyuridine (FuDR)	Sigma-Aldrich
Glutathione reductase	Sigma-Aldrich
5-Hydroxy-1,4-Naphtochinon (Juglone)	Sigma-Aldrich
Myricetin	Sigma-Aldrich
Pyruvate	Sigma-Aldrich
Quercetin	Sigma-Aldrich
Resveratrol	Sigma-Aldrich
Succinate	Sigma-Aldrich
Tween 80	Sigma-Aldrich
2-Vinylpiridine	Sigma-Aldrich

2.1.3 Caenorhabditis elegans strains

The stocks were purchased from Caenorhabditis Genetics Center (CGC) University of Minnesota, Minneapolis, USA.

Strain	Gen	Reference
N2 var. Bristol	wild type	
CL2070	hsp-16.2p::GFP	[121]
CF1038	*daf-16(mu86)*	[119]
TK22	*mev-1(kn1)*	[76]
TJ356	DAF-16::GFP	[73]
VC199	*sir-2.1(ok434)*	
CF1553	SOD-3::GFP	[118]
MQ876	*daf-2(e1370);isp-1(qm150)*	[46]

2.1.4 Escherichia coli bacterial strains

OP50
RNAi strains:
Y95B8A-84.g as control
R13H8.1 (*daf-16*)
R11A8.4 (*sir-2.1*)

F52D10.3 (*ftt-2*)
M117.2 (*par-5*)

2.1.5 Oligonucleotides

For outcrossing of the strain VC199 (PCR) following oligonucleotides were used:
sense oligonucleotide: TTCCGATGCACCCGAAACAA
antisense oligonucleotide: TGAATCGGCTCGTTGCAAGT
For verification of the *sir-2.1* RNAi experiments and the mRNA levels (real time RT-PCR) following oligonucleotides were used:
sense oligonucleotide: CGTGCTCCTGTAAGGC
antisense oligonucleotide: GGTACGAACTGTCCTGG

2.2 Methods

2.2.1 Preparation of stock solutions

Glucose and ascorbic acid were dissolved in water. Flavonoids, resveratrol and juglone were prepared in 92 % Ethanol (v/v) and 8 % Tween 80 (v/v) as solvent. Controls were always treated with identical amounts of solvent.

2.2.2 Maintenance of C. elegans

Worms were grown on NGM agar plates seeded with *E. coli* strain OP50 according to published protocols [24]. Animals were cultured at 20 °C. Freezing of worms for long term storage and obtaining of synchronous populations of worms was done as described [187]. For all experiments synchronous populations gained by bleaching were used and worms were incubated in indicated substances at the young adult stage.
Before transfer to the liquid medium worms were washed off the NGM agar plates with M9 buffer. After transfer to a 15 ml Falcon tube worms were pelleted by centrifugation (300 x g, 2 min), supernatant was discarded and replaced by fresh M9 buffer. After several similar washing steps until the supernatant has cleared, worms were transferred to 6-well or 12-well plates containing 3 ml or 2 ml CeHR medium, respectively. For life span analysis 3.5 cm plates were used. Worms were cultured at 20 °C in indicated substances under constant shaking conditions (120 rpm), to provide proper oxygenation. After 2 to 3 days the worms were transferred

into new wells or new plates containing fresh medium. Handling of liquid medium was performed under a laminar flow hood and sterile techniques were used.

2.2.3 Life span analysis

For life span analysis 50 to 100 adult hermaphrodites were transferred to fresh NGM agar plates allowed to lay eggs for 3 hours and then removed. Three days later, when the hatched larvae had reached L4 stage they were transferred into individual wells of a 6-well plate containing 3 ml CeHR medium each. After reaching the young adult stage worms were distributed (70 worms per group) into new 3.5 cm plates containing either solvent as control or the compound to be tested in a final volume of 2 ml CeHR medium. Worms were transferred to fresh plates every third day with a glass capillary and scored as dead when they did not respond to a mechanical stimulus or pharynx contraction was not visible anymore.

2.2.4 Exposure experiments

For measurements of the antioxidant enzymes SOD and catalase as well as for GSH levels, HDAC activity and real time RT-PCR nematodes were incubated in indicated substances with 50 μM FuDR to prevent production of progeny for 72 hours.
The strain CL2070 with the hsp-16.2p::GFP construct was treated with or without juglone or in combination with glucose or flavonoids and resveratrol for 4 hours. Incubation time for the strain TJ365 was 3 hours and for CF1553 72 hours with indicated substances.
For experiments with high glucose medium, glucose was diluted from a stock solution prepared in aqua bidest. to the concentrations indicated. Basic CeHR medium has a concentration of 128.02 mM glucose and final CeHR medium has a glucose concentration of 64.01 mM.

2.2.5 Confocal laser scanning microscopy

For confocal laser scanning microscopy worms were washed several times after the incubation period with M9 buffer followed by centrifugation (300 x g, 2 min) as described above, until supernatant has cleared. Thereafter pelleted nematodes were anesthetized with 5 mM levamisol. A drop of the worm pellet was put on a glass slide pre-equipped with a thin layer of 2 % agarose gel, to avoid desiccation of the worms. The drop with the worms was covered with a cover slide. For microscopy a confocal

laser scanning microscope (TCS SP2 Leica Microsystems, Heidelberg, Germany) was used.

The following assays were performed using the confocal laser scanning microscope:

Assaying ROS-production in mitochondria

To assay ROS-production in mitochondria the dye CM-H_2XRos (Invitrogen, Karlsruhe, Germany) was used. This dye enters active respiring cells, accumulates in mitochondria and is oxidized there by ROS. In this form the dye creates a fluorescent conjugate with thiol groups of proteins and peptides which can be detected and quantified with an absorption maximum at 578 nm and emission at 590-650 nm. Thereby emitted fluorescence corresponds to amount of ROS generated in mitochondria [183].

The dye was dissolved in DMSO and used in a final concentration of 0.5 μM. After an incubation time of 24 hours, pictures were taken using the 63 x magnification and fluorescence intensities were determined for the anterior part of the worm until the end of the pharynx with the Leica Confocal Software, version 2.5. For each experiment 10 worms per group were measured.

Since this dye has only been used in cell culture so far, here was verified the dependence of fluorescence upon incubation with CM-H_2XRos on respiratory chain function in life worms. Pharmacological inhibition of the respiratory chain which leads to increased ROS generation in mitochondria [70][127] induces enhanced fluorescence in CM-H_2XRos incubated worms (fig. 2.1 (A)). Whereas *daf-2(e1370);isp-1(qm150)* mutant worms, which have a low respiration that is associated to a low ROS production [46][72], show a tendency to reduced fluorescence (fig. 2.1 (B)).

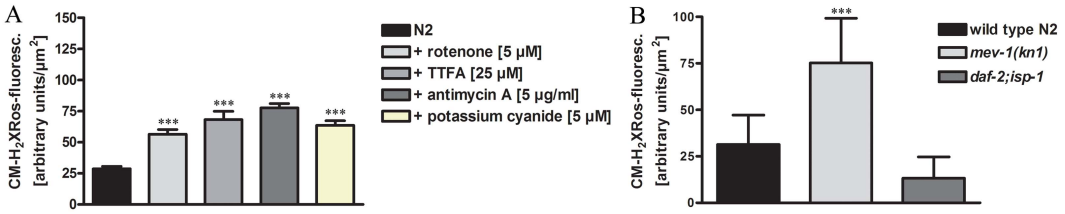

Figure 2.1: **ROS levels in mitochondria.** Fluorescence intensities of the dye CM-H_2XRos in (A) wild type N2 in absence (control) or in presence of inhibitors of the respiratory chain. Rotenone inhibits complex I, TTFA (2-thenoyltrifluoroacetone) inhibits complex II, antimycin A inhibits complex III and potassium cyanide inhibits complex IV. Incubation time of respiratory chain inhibitors was 10 min and for the dye CM-H_2XRos 24 hours. (B) Comparison of ROS levels in mitochondria of wild type N2 worms, *mev-1(kn1)* and *daf-2(e1370);isp-1(qm150)* mutants. Incubation time was 24 hours. Error bars indicate SD. *** P<0.001 versus control or wild type N2, respectively by ANOVA. n=10 from 3 independent experiments. Note the increased basal fluorescence in *mev-1(kn1)* mutant worms versus N2 wild type worms.

Lipofuscin determination

As a result of aging worms accumulate fluorescent pigments [103]. These pigments accumulate in intestinal epithelium cells as secondary lysosomes [32] which can be measured with an excitation at 360 nm and emission at 420 nm. N2 wild type worms were incubated for 1, 5, 10 and 15 days in medium containing respective compounds and 50 μM FuDR to prevent progeny. Pictures were taken with the 20 X objective and fluorescence ratios were determined for the whole worm using Leica Confocal software, version 2.5. For each experiment 10 worms per group were quantified and the experiment was done for at least 3 times.

GFP expression

For the transgenic strains expressing GFP in conjunction with *daf-16* (TJ356) or *sod-3* (CF1553) or under control of *hsp-16.2p* extinction was set at 488 nm and absorption at 543 nm. For experiments with the transgenic strain CL2070 pictures were taken using the 63 X objective. Incubation time was 4 hours and fluorescence intensities were determined for the pharynx until the anterior bulb using the Leica Confocal Software, version 2.5. For each experiment 10 worms per group were used. For heat shock experiments CL2070 worms were incubated for 1.5 hours at 35 °C on agar plates. After heat shock worms were transferred into CeHR medium containing either solvent control or indicated substances for 3.5 hours at 20 °C. Pictures were taken using the 20 X objective.

To measure the translocation of DAF-16 into the nucleus incubation time with the test compounds was set to 3 hours and pictures were taken using the 20 X objective.

2.2.6 Protein extraction

For protein extraction, animals were transferred from wells with liquid medium into 15 ml tubes and washed several times with M9 buffer followed by centrifugation, as described under 2.2.2. After the supernatant had clarified in the last washing step the worm pellet was resuspended either in lysis buffer for the total glutathione (tGSH) measurement or in 5 mM EDTA for enzyme activity measurements and centrifuged for 2 min at 300 x g. The resulting worm pellet was resuspended in worm lysis buffer or 5 mM EDTA to a total volume of 1 ml. The pellet was frozen in liquid nitrogen and stored at -80 °C until use. After thawing on ice, 100 μl of complete mini protease inhibitor stock solution (for the stock solution 1 tablet of complete mini9, Roche, was dissolved in 1 ml water) was added to the thawed worm pellet. The worm pellet was resuspended and dropped into liquid nitrogen in a sterile mortar. The small frozen particles were strongly pounded until they liquefied again. This suspension was immediately transferred into a new 15 ml tube on ice and sonified 3 times for one minute (amplitude 35, cycle 0,5). To obtain the protein the suspension was centrifuged by 5000 x g for 15 min at 4 °C. The supernatant containing the protein was transferred into new 2 ml tubes. Protein concentration was determined with the BioRad Protein Assay according to manufacturers' guidelines.

2.2.7 GSH and GSSG measurement

Worms were treated for measurements of GSH and GSSG as described in 2.2.4. After incubation with either solvent control or indicated compounds worms were washed several times with M9 buffer and the whole lysate was prepared as described above (see section 2.2.6). GSH and GSSG were measured in whole worm lysates by the rate of formation of 5,5'-dithio-(2-nitrobenzoic acid) as described [164]. The dye 2,2'-dinitro-5,5'dithio-dibenzoic acid (DTNB)(Sigma-Aldrich) also called Ellman's reagent reacts with the thiol group of glutathione. This results in formation of a disulfide bond between glutathione and TNB (5-thio-2-nitro-benzoat) and this adduct absorbs at 412 nm. Extinction measurements were performed in a Varioscan (Thermo, Dreieich). For the GSSG determination GSH had to be blocked before by usage of 2-vinylpiridine. Absorption/Extinction was measured after 10 min and the blank, consisting of all necessary chemicals with water instead of protein sample, was subtracted.
For measurement of tGSH- and GSSG-levels a calibration curve was constructed (fig.

2.2). The GSH content was calculated by the difference of the tGHS and the GSSG content.

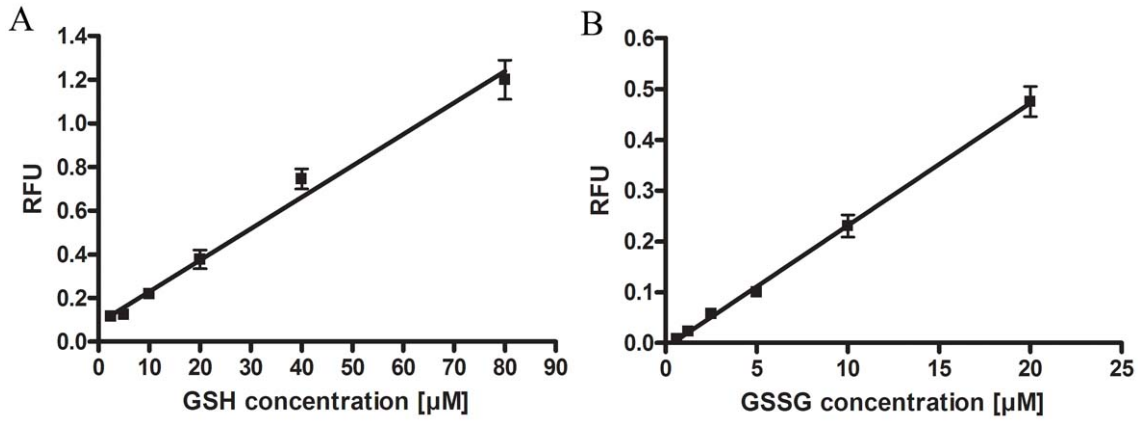

Figure 2.2: **Calibration curves of tGSH and GSSG.** Calibration of (A) tGSH and (B) GSSG.

2.2.8 Superoxide Dismutase activity measurement

Activity of superoxide dismutase (SOD), which catalyses the reaction of superoxide radicals to hydrogen peroxide was determined following the Marklund and Marklund method [129] based on pyrogallol (60 mM) autooxidation. Measurements were performed in a photometer (Uvicon 930, Kontron instruments) at a wavelength of 420 nm. Worms were incubated as described in 2.2.4 and after the incubation time whole worm lysates were prepared (see section 2.2.6) for SOD activity measurement.

2.2.9 Catalase activity measurement

Activity of catalase, which catalyses the reaction of hydrogen peroxide to water was determined by the rate of disappearance of hydrogen peroxide at 240 nm in a photometer (Uvicon 930, Kontron instruments) according to the Aebi method [2]. Worms were treated as described before (see section 2.2.4) and after the incubation period whole worm lysates were prepared (see section 2.2.6) for CAT activity measurement.

2.2.10 Histone deacetylase activity measurement

The Histone deacetylase (HDAC) activity assay from Biomol based on the deacetylation by SIRT1 of a synthetic substrate (Fluor de Lys-SIRT1 substrate) that consists

of four amino acids with one acetylated lysine group (Arg-His-Lys-Lys(Ac)) and a fluorophore. Fluorescence is generated by adding Developer II and was measured in a fluorescent plate reader (Varioscan) after extinction at 360 nm and emission at 460 nm at 25 °C. In brief, whole worm lysates gained as described in section 2.2.6 were incubated for 4 hours at room temperature before the reaction was stopped by adding the Developer according to the Biomol method. Each well contained 8 μg protein. To exclude activity of other histone deacylases trichostatin A (TSA, inhibits class I and II histone deacetylases) was added. The intensity of the fluorescence signal is directly related to the sir-2.1 HDAC activity. Nematodes were incubated as described in 2.2.4.

2.2.11 Oxygen consumption measurement

Presens (Regensburg) OxoPlates were used to measure oxygen consumption of worms. The OxoPlate is a microtiter plate in a 96-well format with a sensor integrated at the bottom of the wells. The sensor contains two different dyes, one is the oxygen indicator, whose phosphorescence is dependent on the oxygen concentration of the sample in the well. The second dye is the reference whose fluorescence intensity is independent of the oxygen concentration. For the oxygen measurement the Fluoroscan Ascent (Thermo, Dreieich) was used with two filter pairs 450/650 nm and 540/650 nm for extinction and emission, respectively. For the measurement about 90 worms were transferred into wells containing CeHR medium supplemented either with solvent control or indicated compounds. Fluorescence reading was performed every 30 seconds for a total time of 30 minutes. The oxygen concentration was calculated according to manufacturers' guidelines. After finishing the measurement worms were counted and only wells with 90 $\pm$ 5 worms were scored.

2.2.12 RNA interference (RNAi) experiments

RNAi experiments were done as previously described using bacteria from the Ahringer deletion library [97]. To induce dsRNA expression by the bacteria plates were incubated for 3 hours at 37 °C. Before use the plates were cooled down to 20 °C. L1 larvae were put onto plates with RNAi-producing bacteria and cultured as described (section 2.2.1). After reaching adulthood synchronous populations were obtained by eggprep and put onto new RNAi plates. After reaching adulthood again worms were washed off the plates with M9 buffer and transferred into liquid medium for further experiments. When embryonic lethal genes were targeted by RNAi, worms were put

onto RNAi plates after hatching and were taken for experiments after reaching L4 stage.

2.2.13 Out-crossing of the strain VC199

The strain VC199, a *sir-2.1* knockout strain which was not out-crossed after receiving from the CGC. To avoid background mutations it is necessary to out-cross worms after deletion of a target gene. For that purpose male N2 wild type worms were used. In the first step L4 knockout hermaphrodites and N2 males were put on a small plate (3,5 cm) in a 1:3 ratio (3 VC199 hermaphrodites and 10 N2 male worms). After 24, 48 and 72 hours males were removed and adult hermaphrodites were transferred to fresh plates. After F1 generation was hatched heterozygous L4 hermaphrodites and heterozygous L4 males were put together on a new plate in a 1:3 ratio and after 24, 48 and 72 hours males were removed and adult hermaphrodites were transferred to fresh plates. Now 40 worms of the F2 progeny were singled and further analyzed by PCR. Only worms homozygous for the deletion in the *sir-2.1* gene were taken for the next step which was similar to step one. Again heterozygous L4 hermaphrodites and heterozygous L4 males were put on new plates in a 1:3 ratio and the progeny was singled. The progeny was analyzed by PCR to get homozygote worms. These worms that have been out-crossed for four generations, were used for life span analysis.

2.2.14 Single worm PCR

A single worm was put into a 200 μl PCR tube containing 3 μl of lysis buffer and proteinase K. Then the tubes were incubated for 1 hour at 65 °C and for 10 minutes at 95 °C. For the PCR reaction 0.5 μl of the worm lysate was used as a template.
25 μl PCR mixture contained: 10-100 ng DNA template (*C. elegans* worm-lysate), 2.5 μl polymerase buffer mix with $MgCl_2$, 2.5 μl dNTP-mix (each dATP, dCTP, dGTP, dTTP), 10 pmol sense oligonucleotide, 10 pmol antisense oligonucleotide, 0.3 μl Taq-polymerase (2.5 U, Fermentas) and 18.8 μl H_2O. For each reaction annealing temperature was chosen according to the melting temperature of the primers. The first step was a denaturation at 93 °C for 2 min. Prior to 30 cycles under following conditions:
57.3 °C, 0:30 primer annealing temperature
72 °C, 2:30 extending step
PCR products were separated on a 1.5 % agarose gel in a TBS buffer system and

visualized by ethidium bromide staining. After only one PCR reaction the resulting bands allowed identification of knockout alleles.

2.2.15 Isolation of total RNA

After incubation as described in 2.2.4 worms were washed several times with M9 buffer by centrifugation. The RNeasy Mini kit (Quiagen, Hilden, Germany) was used for the isolation of total RNA according to the manufactures guidelines. RNA was stored in RNAse free water at - 80 °C. The yield was calculated based on the absorbance at 260 nm in the Nanodrop (Peqlab, Erlangen, Germany). RNA integrity was verified by denaturating agarose gel electrophoresis. Intact total RNA preparations should appear as two bright bands with a ratio of intensities of the 28S and 18S rRNA of approximately 2:1. For the cDNA synthesis 0.5 μg total RNA was reverse transcribed using random hexamer primers yielding a cDNA concentration of 12.5 ng/μl.

2.2.16 Real-time RT-PCR

Quantitative RT-PCR (or real-time RT-PCR) was done with the LightCycler instrument. Primers were designed by the LightCycler Probe Design software (Roche Diagnostics, Mannheim, Germany). Blast searches were done in the published sequence database GenBank (http://www.ncbi.nlm.gov/BLAST/) to reveal that primers are gene-specific and if possible, those primers which span at least one intron were chosen. RT-PCR was performed using the LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. To each PCR reaction 12.5 ng cDNA was added. Authenticity of the amplified products was checked by dissociation analysis and agarose gel electrophoresis. The individual level of initial target mRNA was expressed as the difference in C_T between control and sample (Δ C_T). The relative amount of target mRNA was normalized to a reference gene (housekeeping gene) and calculated according to the following formula [160]:

$$RF(regulation factor) = 2\Delta C_T^{target(control-sample)} / 2\Delta C_T^{reference(control-sample)}$$

For normalization expression of the house keeping gene *ama-1* was used.

2.2.17 Calculations and statistics

Each variable represents data from at least three independent experiments. Data were depicted as the mean ± SD. For statistical analysis Graphpad Prism 4.01 software was used. For comparison of survival curves the Log rank test was applied. A P value of <0.05 indicates statistical significance. For life span analysis Kaplan-Meier survival curves are shown.

3 Results

3.1 Juglone treatment in C. elegans

3.1.1 Influence on life span caused by juglone treatment

Reactive oxygen species (ROS) have been implicated in a wide range of diseases e.g. by oxidation of important macromolecules including lipids, proteins and DNA [196]. In cellular metabolism ROS are generated particularly in mitochondria as a consequence of macronutrient oxidation [125]. To asses whether ROS generation *in vivo* has an impact on live span in *C. elegans* the redox active quinone juglone (5-hydroxy-1,4-naphtoquinone) was used. Juglone is reduced after cellular uptake with NAD(P)H by a diaphorase thereafter it is able to reduce oxygen to superoxide anions [19].

Juglone in a concentration of 40 μM caused a significant increase in life span in N2 wild type worms compared to controls (fig: 3.1 (A)). A concentration of 100 μM juglone had no influence on life span and treatment with 250 μM juglone caused a significant reduction of survival.

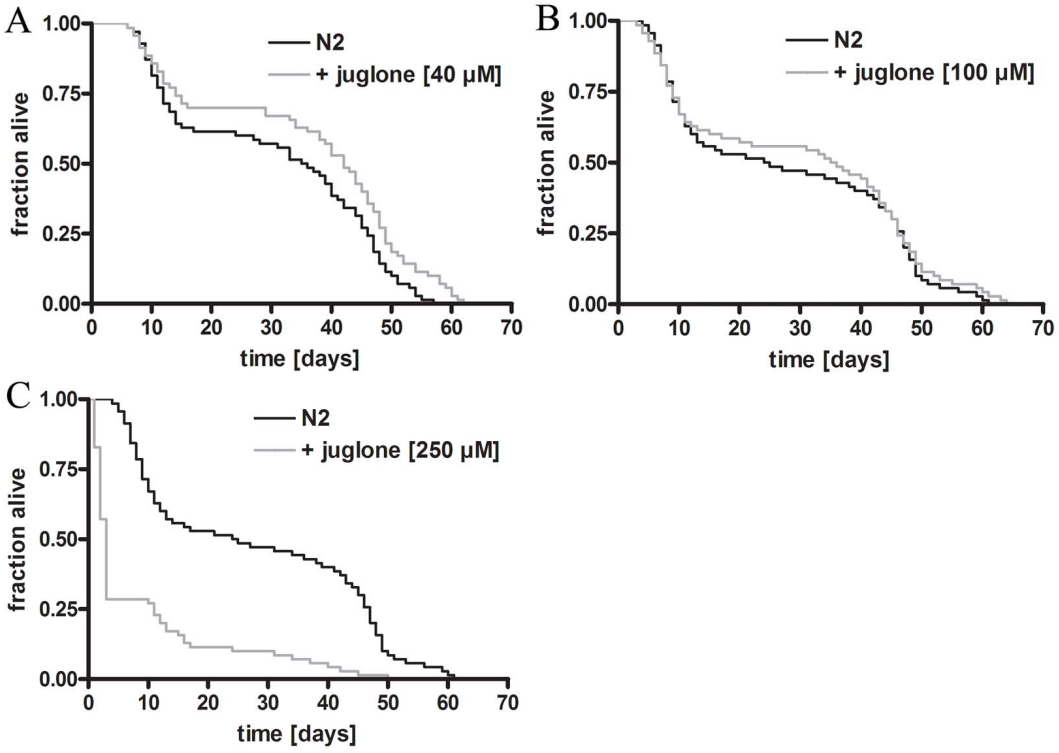

Figure 3.1: **Kaplan-Meier survival curves upon juglone treatment in wild type N2.** Life span measurements in N2 wild type worms in absence (control) or presence of either (A) 40 μM juglone, (B) 100 μM juglone or (C) 250 μM juglone. Significances of differences in life span versus control were as follows: (A) P=0.0142, (B) P=0.4795 and (C) P<0.001 by Logrank test.

DAF-16, a homolog of the mammalian forkhead transcription factor FOXO [27] mediates not only extension in life span observed in mutants of the insulin/IGF-1 signaling cascade [101] but leads also to a higher stress resistance [16]. To determine the role of *daf-16* on oxidative stress induced by juglone treatment a *daf-16* knock out mutant was used. No alteration was seen in life span in the *daf-16(mu86)* mutant compared to control worms if juglone was provided in a concentration of 40 μM. The higher concentration of 100 μM juglone was also tolerated quite well by this mutant. Only 250 μM juglone led in *daf-16* nematodes to a dramatic reduction in life span (fig. 3.2). Moreover, the *daf-16(mu86)* mutants was much more sensitive to ROS-stress induced by 250 μM juglone versus the wild type N2 exposed to the same concentration.

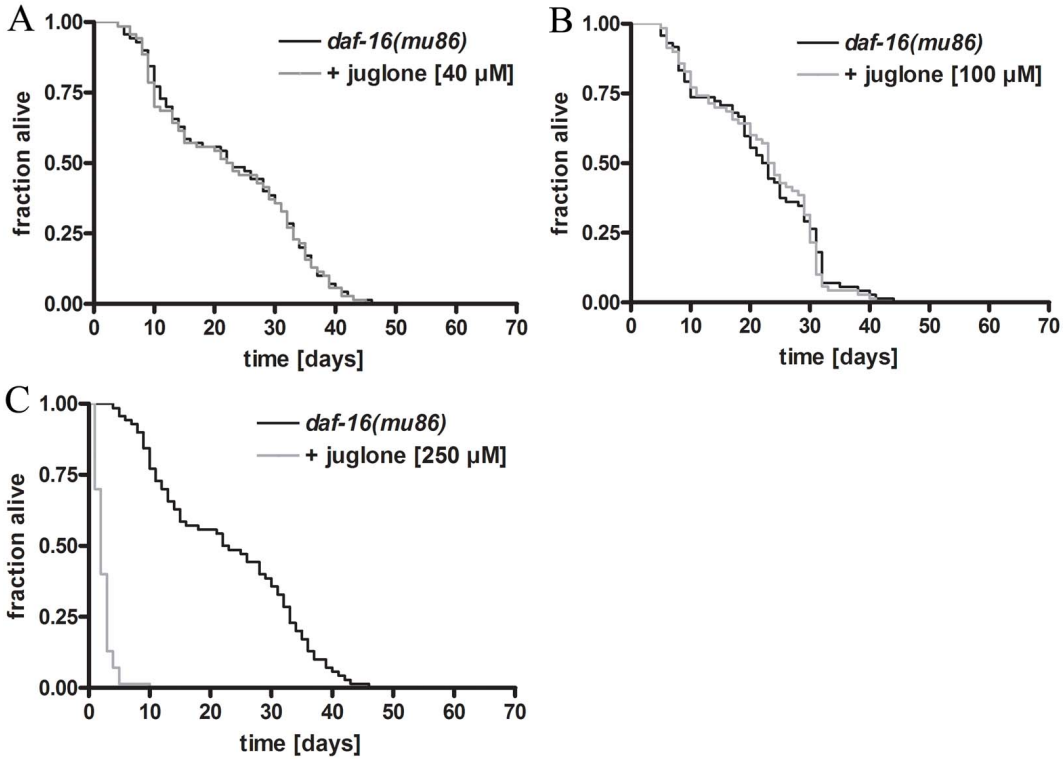

Figure 3.2: **Kaplan-Meier survival curves upon juglone treatment in *daf-16(mu86)* mutants.** Life span measurements in *daf-16(mu86)* mutants in absence (control) or presence of either (A) 40 μM juglone, (B) 100 μM juglone or (C) 250 μM juglone. Significances of differences in life span versus control were as follows: (A) P=0.8243, (B) P=0.6809 and (C) P<0.001 by Logrank test.

These results suggests that *daf-16* is involved in the longevity effect induced by low dose juglone treatment in the wild type since there is no effect detectable after deletion of *daf-16*.

3.1.2 ROS generation upon juglone treatment

To visualize the effect of ROS generation on expression of the small heat shock protein HSP-16.2 the transgenic strain CL2070 was used, that expresses GFP under the control of the *hsp-16.2* promotor ([121] and fig. 3.3). All tested juglone concentrations led to an alteration in expression of HSP-16.2 with a maximum GFP-fluorescence at 40 μM and 100 μM whereas response at 250 μM was lower (fig. 3.4).

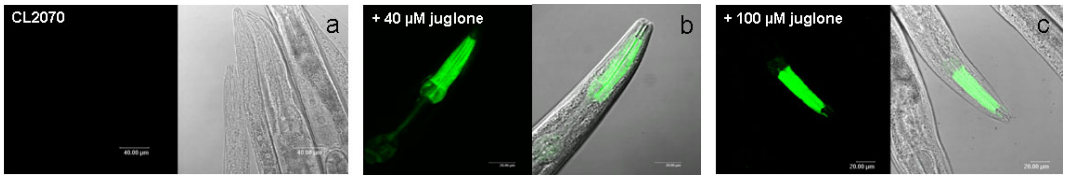

Figure 3.3: **Visualization of HSP-16.2 expression in CL2070 upon juglone treatment.** GFP expression in CL2070 in (a) absence (control) or presence of either (b) 40 μM juglone or (c) 100 μM juglone. Incubation time was 4 hours. Left panels show fluorescence pictures and right panels show overlays of fluorescence and light microscopic pictures. Pictures represent typical results obtained from 3 independent experiments.

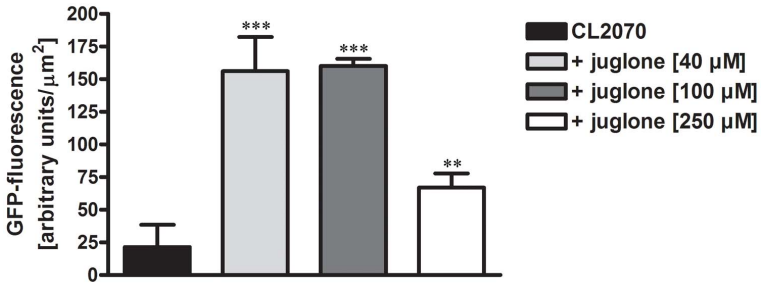

Figure 3.4: **HSP-16.2 expression in CL2070 upon juglone treatment.** Intensities of GFP expression in CL2070 in absence (control) or presence of juglone. Incubation time was 4 hours. Data are given as mean ± SD. ** P<0.01 and *** P<0.001 versus control by ANOVA. Results are from 3 independent experiments.

3.1.3 Responses of antioxidative defense mechanisms upon juglone treatment

The antioxidative defense systems are affected by cytosolic ROS generation. First glutathione as a classical antioxidant defense mechanism was measured. An increase was detectable for the reduced form of glutathione (GSH) in wild type N2 worms after juglone treatment in a concentration of 40 μM and 100 μM. The oxidized form of glutathione (GSSG) showed nearly no alteration compared to control (fig. 3.5). This indicates that cytosolic generation of oxidative stress leads to a de novo synthesis of this non-enzymatic antioxidant system.

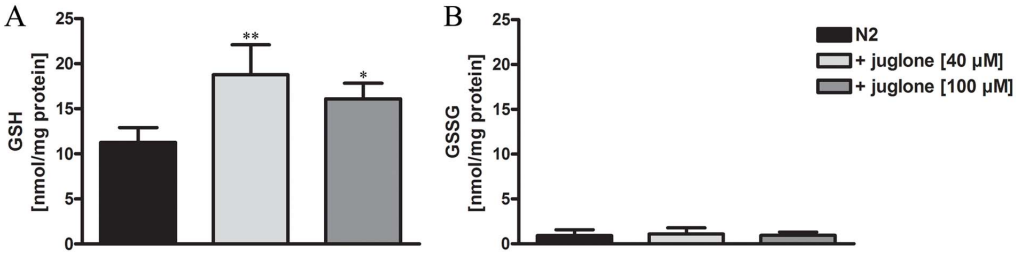

Figure 3.5: **Glutathione levels in wild type N2 after juglone incubation.** Levels of (A) GSH and (B) GSSG in N2 wild type worms after 72 hours incubation in absence (control) or presence of either 40 μM juglone or 100 μM juglone. Glutathione was measured in whole worm lysates. Data are given as mean ± SD. * P<0.05 and ** P<0.01 versus control by ANOVA. Results are from 3 independent experiments.

Next superoxide dismutase activity, an antioxidative enzyme that converts superoxide radicals into hydrogen peroxide, was determined. Enzyme activity increased in animals exposed to 40 μM juglone (fig. 3.6 (A)) but no difference was found when a higher juglone concentration was used (100 μM). As second antioxidant enzyme catalase which converts hydrogen peroxide into oxygen and water was investigated. Catalase activity was also increased at a juglone concentration of 40 μM, while only a slight yet not significant increase was detectable at a concentration of 100 μM (fig. 3.6 (B)).

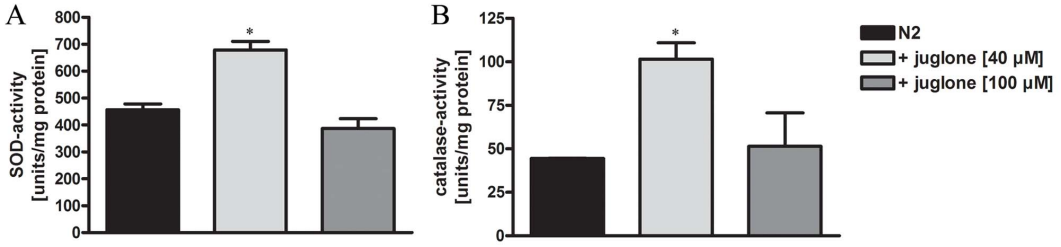

Figure 3.6: **SOD and catalase activity in wild type N2 worms after juglone incubation.** Activity of (A) SOD and (B) catalase in N2 wild type worms after 72 hours incubation in absence (control) or presence of either 40 μM juglone or 100 μM juglone. Enzyme activities were measured in whole worm lysates. Data are given as mean ± SD. * P<0.05 versus control by ANOVA. Results are from 3 independent experiments.

These findings suggest that oxidative stress induced by juglone treatment leads to a compensatory increase in antioxidative defense mechanisms in particular at lower concentration of juglone (40 μM) that also prolong life span.

3.1.4 Impact of juglone on DAF-16 and downstream targets

DAF-16, a forkhead transcription factor, upregulates many genes which are involved in oxidative stress response such as catalases CTL-1 and CTL-2 and, the superoxide dismutase SOD-3 [118] [147]. Also heat shock proteins/chaperons are upregulated by *daf-16* signaling [83] [131]. Both mechanisms can prolong life span in *C. elegans* [140] [83] [147]. To test if juglone is able to promote DAF-16 translocation into the nucleus a DAF-16::GFP transgenic strain was used. This strain contains a DAF-16 fusion construct to allow visualization of DAF-16 translocation into the nucleus by GFP [73]. DAF-16 remaining in the cytosol results in a diffuse fluorescence as shown in fig. 3.7 (a). To study the impact of juglone incubation on DAF-16 nuclear localization the DAF-16::GFP strain TJ356 was used. As shown in fig. 3.7 juglone exposure led to a translocation of DAF-16 into the nucleus visible by intense staining (fig. 3.7 (b) and (c)).

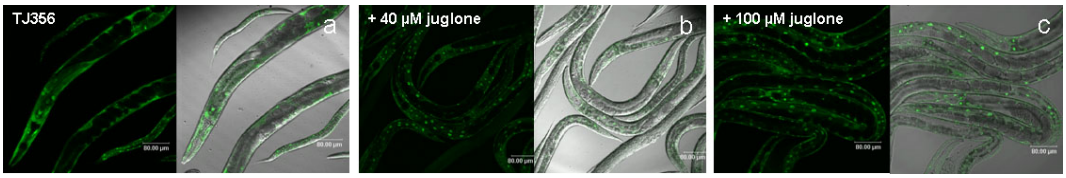

Figure 3.7: **DAF-16 translocation into the nucleus upon juglone treatment.** Translocation of DAF-16 into the nucleus after 3 hours incubation in (a) absence (control) or presence of either (b) 40 μM juglone or (c) 100 μM juglone. Translocation of DAF-16 into the nucleus is visible by intense staining. Left panels show fluorescence pictures and right panels show overlays of fluorescence and light microscopic pictures. Pictures represent typical results obtained from 3 independent experiments.

Juglone treatment was obviously able to promote DAF-16 translocation into the nucleus which should lead to increased expression of genes involved in stress defense and longevity. To directly asses this a *muIs84* mutant worm carrying a sod-3::GFP transgene was used. As shown in fig. 3.8 GFP expression indeed increased upon juglone treatment of these animals in support of the hypothesis that juglone may cause the upregulating of downstream targets of DAF-16.

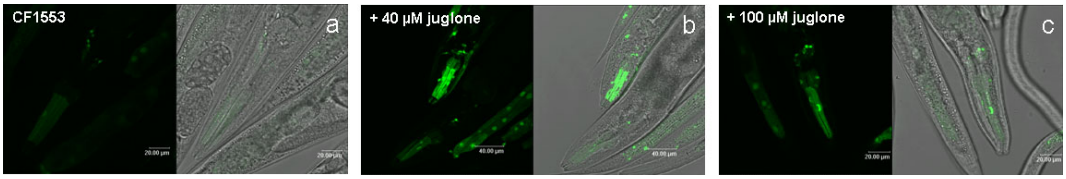

Figure 3.8: **GFP expression upon juglone treatment in *muIs84* mutants carrying a sod-3::GFP transgene.** *MuIs84* mutants carrying a sod-3::GFP transgene after 72 hours incubation in (a) absence (control) or presence of either (b) 40 μM juglone or (c) 100 μM juglone. Left panels show fluorescence pictures and right panels show overlays of fluorescence and light microscopic pictures. Pictures represent typical results obtained from 3 independent experiments.

To asses whether selected genes downstream of DAF-16 are involved in the juglone-induced stress response, selective RNAi experiments were performed for putative DAF-16 targets. From L1 until the young adult stage CL2070 worms were fed with bacteria producing siRNA directed against *daf-16* (R13H8.1), *sir-2.1* (R11A8.4), *ftt-2* (F52D10.3) and *par-5* (M117.5) (in brackets Ahringer library clones). Bacteria with empty vector were used as control (Ahringer library clone Y95B8A-84.g). After reaching the young adult stage CL2070 nematodes were incubated with 40 μM juglone for 4 hours in CeHR medium. GFP expression was detectable after treatment with 40 μM juglone in worms feeding control bacteria (fig. 3.9 (a)). However, no GFP expression was seen in the 40 μM juglone treated CL2070 worms fed with *daf-16*, *sir-2.1*, *ftt-2* or *par-5* siRNA producing bacteria (fig. 3.9 (b) to (e)).

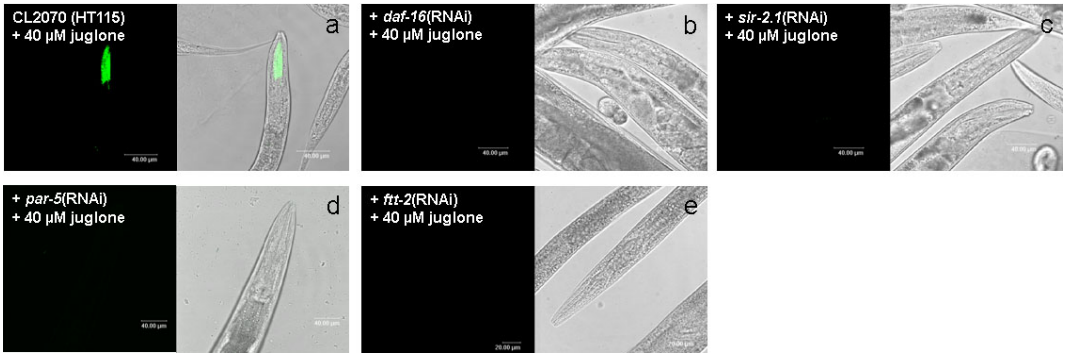

Figure 3.9: **GFP expression in CL2070 upon 40 μM juglone incubation.** CL2070 were fed with dsRNA producing bacteria (a) HT115 (control bacteria), (b) *daf-16*(RNAi), (c) *sir-2.1*(RNAi), (d) *par-5*(RNAi) and (d) *ftt-2*(RNAi) and incubated for 4 hours in the presence of 40 μM juglone. Left panels show fluorescence pictures and right panels show overlays of fluorescence and light microscopic pictures. Pictures represent typical results obtained from 3 independent experiments.

Ftt-2 and *par-5* are encoding 14-3-3 proteins in *C. elegans* [142] [209]. 14-3-3

proteins are highly conserved and regulate through interactions with their partners key biological processes, such as cell cycle, apoptosis and transcription [195]. The RNAi data indicated that not only DAF-16, but also histone deacetylase SIR-2.1 and the 14-3-3 proteins are involved in *hsp-16.2* activation after ROS generation.
To analyze if SIR-2.1 is also involved in translocation of DAF-16 into the nucleus as shown in mammalian cells [53] the strain TJ356 was fed with bacteria producing siRNA directed against *sir-2.1*. Worms were incubated for 3 hours in medium alone (control) or with 40 μM juglone. As shown in fig. 3.10 DAF-16 translocation was again visible in juglone-treated worms but was not affected by silencing of *sir-2.1*.

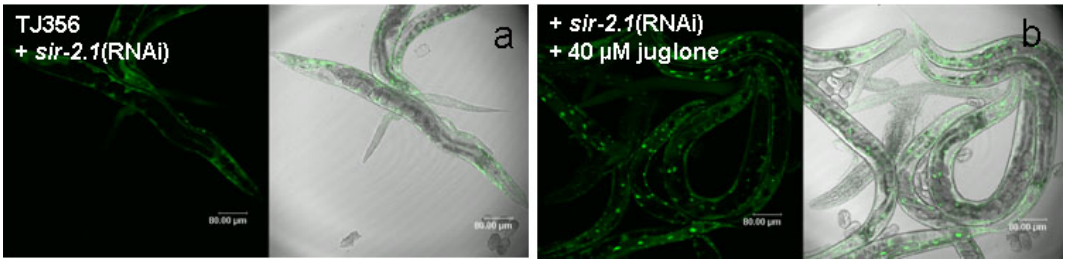

Figure 3.10: **DAF-16 translocation into the nucleus upon juglone incubation after *sir-2.1* siRNA treatment.** Translocation of DAF-16 into the nucleus, which is visible by intense staining after *sir-2.1* siRNA treatment either in (a) absence (control) or presence of (b) 40 μM juglone. Incubation time was 3 hours. Left panels show fluorescence pictures and right panels show overlays of fluorescence and light microscopic pictures. Pictures represent typical results obtained from 3 independent experiments.

The life span increasing effect of low dose (40 μM) juglone treatment on N2 wild type animals therefore seems to depend critically on *daf-16*. Moreover, juglone upregulates DAF-16 expression and similarly effects downstream targets like GSH, SOD, CAT and the heat shock protein HSP-16.2 that showed enhanced levels. The histone deacetylase SIR-2.1 and the 14-3-3 proteins are as well involved in the stress response and *hsp-16.2* expression.

3.2 C. elegans exposed to high glucose loads

3.2.1 ROS generation induced by higher glucose concentrations

Break down of glucose via glycolysis is one major energy source for the respiratory chain localized in mitochondria. To identify whether *C. elegans* exposed to high glucose concentrations can increase ROS levels in mitochondria wild type N2 and *mev-1(kn1)* mutants were kept in liquid media in which on top of the normal glucose concentration additional 100 mM and 250 mM of glucose were added.

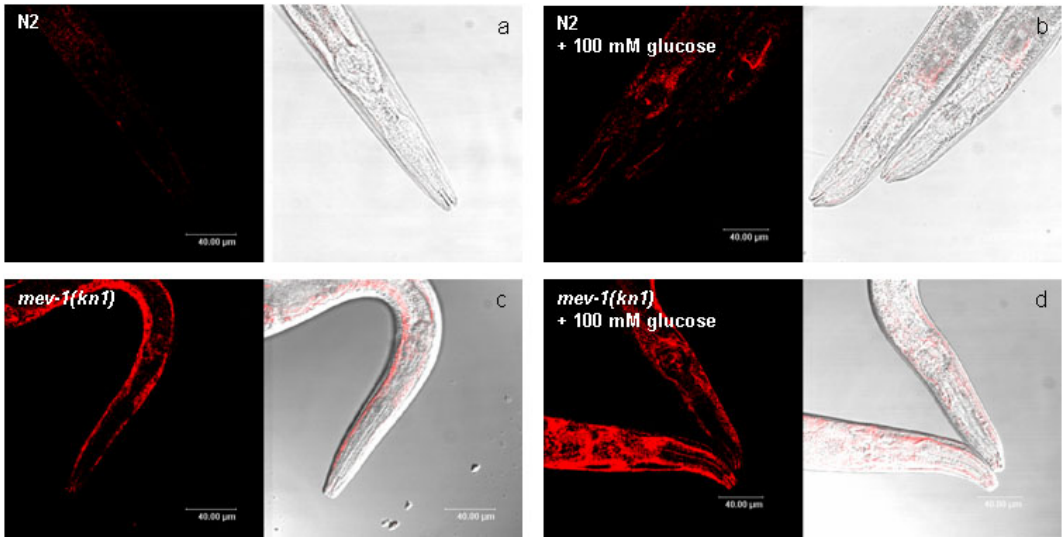

Figure 3.11: **Mitochondrial ROS-production determined by CM-H_2XRos fluorescence in N2 wild type and *mev-1(kn1)* mutant worms.** Fluorescence of the dye CM-H_2XRos in (a) N2 wild type worms in absence (control) or (b) in presence of 100 mM additional glucose and in (c) *mev-1(kn1)* mutants in absence (control) or (d) in presence of 100 mM additional glucose. Incubation time was 24 hours. Left panels show fluorescence pictures and right panels show overlays of fluorescence and light microscopic pictures. Pictures represent typical results obtained from 3 independent experiments.

As shown in fig. 3.12 exposure of animals to high glucose concentrations in the medium increased ROS levels in mitochondria significantly. In addition *mev-1(kn1)* mutant worms which accumulate higher ROS levels in mitochondria due to a defect in complex II of the respiratory chain [180] showed intrinsically higher ROS-induced fluorescence which was also further increased significantly by glucose exposure. Similar effects were obtained when succinate or pyruvate were used as substrates for oxidative metabolism (Data not shown). In all cases an increase of CM-H_2XRos-fluorescence could be detected, reflecting increased ROS generation (fig. 3.11).

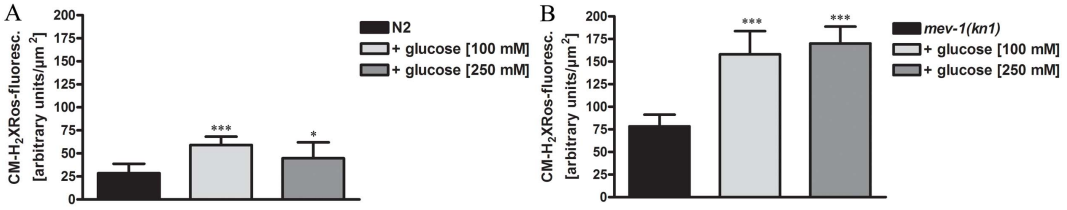

Figure 3.12: **ROS levels in mitochondria after incubation with higher glucose concentrations.** Fluorescence intensities of the dye CM-H_2XRos in (A) N2 wild type worms and (B) mev-1(kn1) mutants in absence (control) or presence of additional glucose concentrations. Incubation time was 24 hours. Data are given as mean ± SD. * P<0.05 and *** P<0.001 versus control by ANOVA. Results are from 3 independent experiments.

3.2.2 Oxygen consumption rates under high glucose load

Enhanced ROS production in mitochondria upon exposure of animals to high glucose levels was associated with a higher oxygen consumption rate in wild type N2 worms in a concentration dependent manner (fig. 3.13).

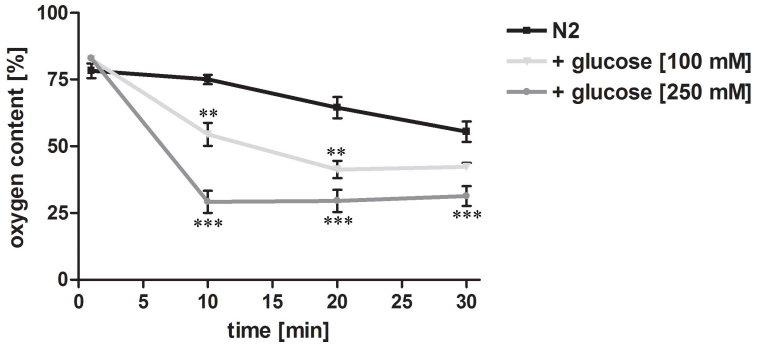

Figure 3.13: **Oxygen consumption in wild type N2 with higher glucose concentrations.** Oxygen content in the medium with N2 wild type worms incubated in absence (control) or presence of additional glucose. Incubation time was 30 minutes. Data are given as mean ± SD. ** P<0.01 and *** P<0.001 versus control by ANOVA. n=90 ± 5 worms from 3 independent experiments.

3.2.3 Impact of glucose-induced ROS generation on aging markers and life span

Lipofuscin, a heteromeric molecule formed by oxidized and cross linked molecules like carbohydrates, lipids and peptides [32] is used as a surrogate aging marker and lipofuscin content in *C. elegans* increases with age [103]. Lipofuscin shows autofluorescence in intestinal granula, which can easily be detected and quantified. However, despite an increased ROS generation in *mev-1(kn1)* mutants, lipofuscin

accumulation did not increase in animals exposed to extra 100 mM and 250 mM glucose when compared to controls (fig. 3.14).

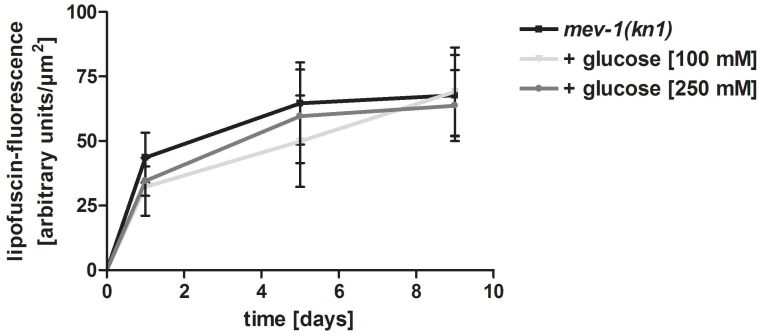

Figure 3.14: **Lipofuscin accumulation in *mev-1(kn1)* mutants upon treatment with higher glucose concentrations.** Intensities of lipofuscin fluorescence in *mev-1(kn1)* mutants in absence (control) or presence of additional glucose. Incubation time was for 1, 5 and, 9 days. Data are given as mean ± SD. Results are from 3 independent experiments.

The effects of high glucose load on life span were assessed in wild type N2, *mev-1(kn1)*, and *daf-16(mu86)* animals. Higher glucose concentrations (100 mM) did not alter life span in wild type N2 worms and *daf-16(mu86)* mutants compared to the control. However, in *mev-1(kn1)* mutants a significant decrease of life span versus the control group was seen as shown in fig. 3.15.

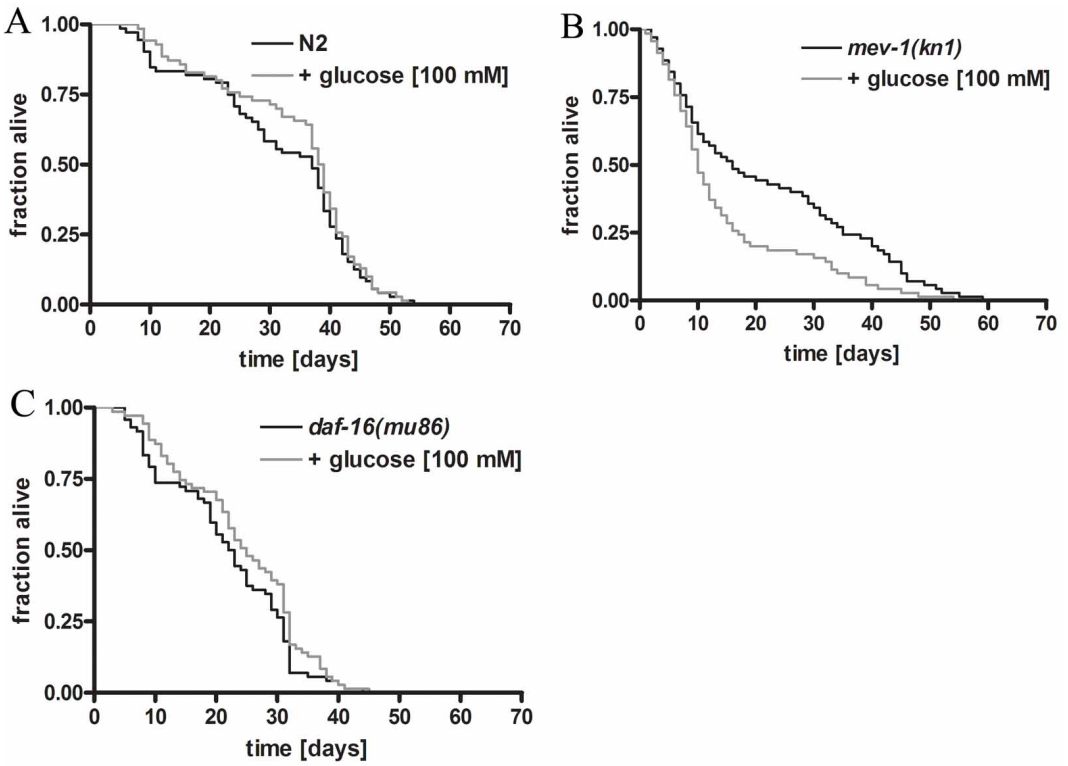

Figure 3.15: **Kaplan-Meier survival curves upon treatment with 100 mM glucose.** Life span measurements in (A) N2 wild type worms, in (B) *mev-1(kn1)* mutants, and in (C) *daf-16(mu86)* mutants in absence (control) or presence of 100 mM glucose. Significances of differences in life span versus control were as follows: (A) P=0.4495, (B) P=0.0046 and (C) P=0.1354 by Logrank test.

When animals were exposed to an extra of 250 mM glucose there was again no difference in *daf-16(mu86)* mutants versus control detectable. In wild type N2 worms there was even an increase of life span (fig. 3.16) whereas the *mev-1(kn1)* mutant line again displayed a significant reduction in life span.

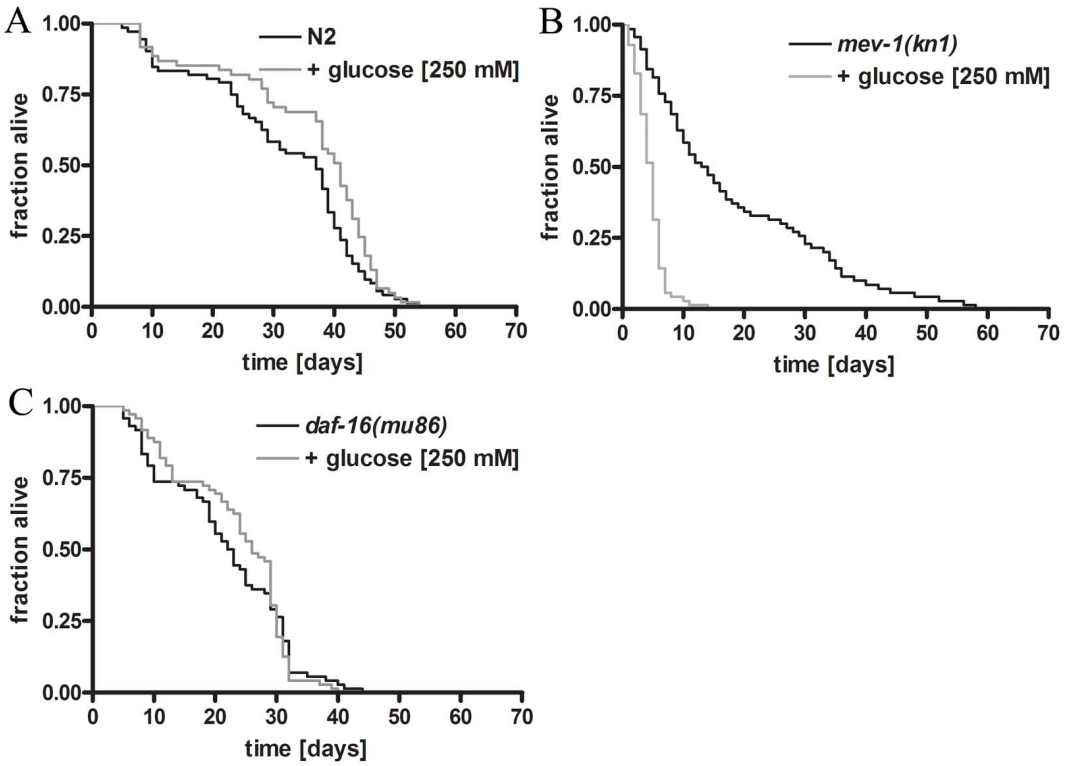

Figure 3.16: **Kaplan-Meier survival curves upon treatment with 250 mM glucose.** Life span measurements in (A) N2 wild type worms, in (B) *mev-1(kn1)* mutants, and in (C) *daf-16(mu86)* mutants in absence (control) or presence of 250 mM glucose. Significances of differences in life span versus control were as follows: (A) P=0.0495, (B) P<0.0001 and (C) P=0.9512 by Logrank test.

In summary, exposure of worms to a high glucose load has no effect or prolongs life span modestly in wild type N2 and *daf-16(mu86)* mutant worms. Only in *mev-1(kn1)* mutants where ROS generation in mitochondria is already elevated under normal conditions, a concentration dependent reduction on life span by glucose was detected.

3.2.4 Antioxidant defense mechanism upon glucose incubation

To asses if animals increase their antioxidant defense system to prevent additional ROS production in mitochondria upon high glucose load, enzymatic and non-enzymatic antioxidative mechanisms were analyzed.

The glutathione system (GSH/GSSG) is the major non-enzymatic defense mechanism in cellular metabolism, which inactivates ROS by conversion of GSH to GSSG [7]. Neither in wild type N2 worms (shown in fig. 3.17 (A)) nor in *mev-1(kn1)* mutants (shown in fig. 3.18 (A)) GSH levels were altered after extra glucose exposure. However, an increase in GSSG content was observed (shown in fig. 3.17 (B) and 3.18 (B)). This underlines stronger occupation of this antioxidant system in treated worms. It is possible that the increase in total amount of glutathione is caused by de novo synthesis but also a reduced capacity for reduction is seen.

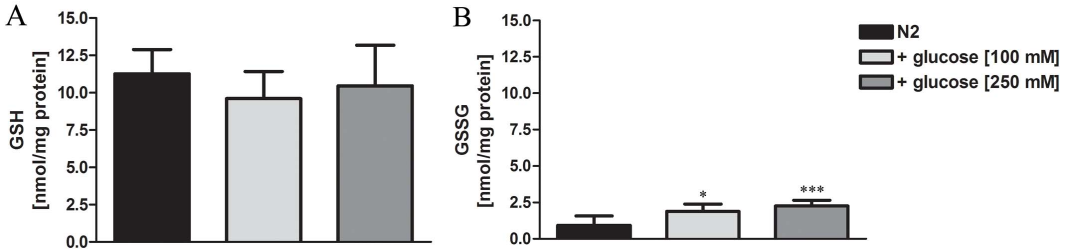

Figure 3.17: **Glutathione levels in wild type N2 worms upon treatment with higher glucose concentrations.** Levels of (A) GSH and (B) GSSG in wild type N2 worms after 72 hours incubation in absence (control) or presence of either 100 mM or 250 mM additional glucose. Glutathione was measured in whole worm lysates. Data are given as mean ± SD. * P<0.05 and *** P<0.001 versus control by ANOVA. Results are from 3 independent experiments.

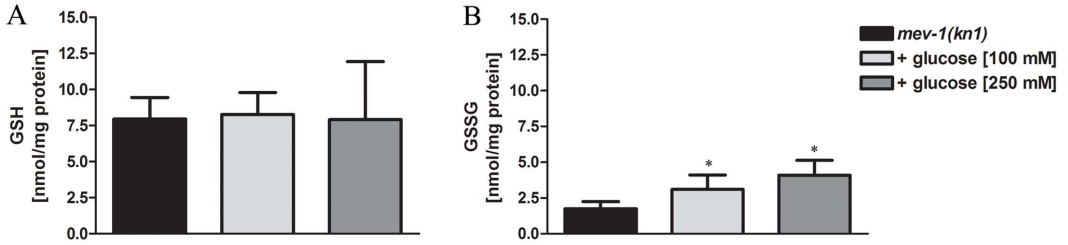

Figure 3.18: **Glutathione levels in *mev-1(kn1)* mutants upon treatment with higher glucose concentrations.** Levels of (A) GSH and (B) GSSG in *mev-1(kn1)* mutants after 72 hours incubation in absence (control) or presence of either 100 mM or 250 mM additional glucose. Glutathione was measured in whole worm lysates. Data are given as mean ± SD. * P<0.05 versus control by ANOVA. Results are from 3 independent experiments.

In wild type N2 worms (fig. 3.19 (A)) and in *mev-1(kn1)* mutants a decrease in SOD activity was observed in animals exposed to extra 250 mM glucose. However, when 100 mM extra glucose was applied no significant alteration of SOD activity was detectable in both worm strains.

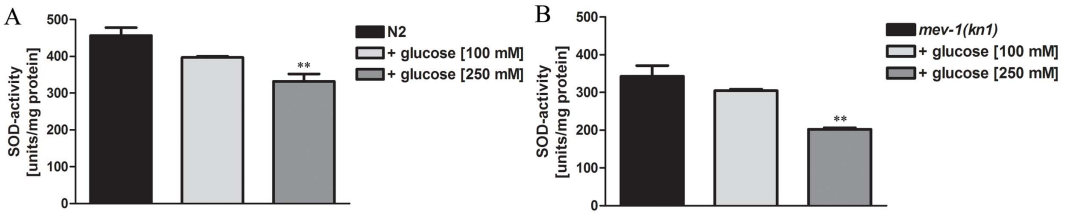

Figure 3.19: **SOD activity in wild type N2 worms and *mev-1(kn1)* mutants after glucose treatment.** Activity of SOD in (A) wild type N2 and (B) *mev-1(kn1)* mutants after 72 hours incubation in absence (control) or presence of either 100 mM or 250 mM glucose. Enzyme activity was measured in whole worm lysates. Data are given as mean ± SD. ** P<0.01 versus control by ANOVA. Results are from 3 independent experiments.

Another enzymatic defense mechanism is represented by catalase, which converts the reaction product of SOD, hydrogen peroxide, to oxygen and water. Measurement of CAT activity revealed a significant increase in the high glucose concentration in N2 wild type (fig.3.20 (A)) as well as in the *mev-1(kn1)* mutant worms (fig.3.20 (B)). Again no alteration of CAT activity was seen at 100 mM extra glucose incubation.

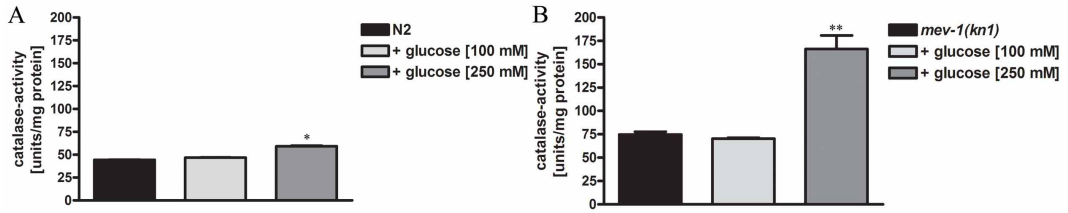

Figure 3.20: **Catalase activity in wild type N2 worms and *mev-1(kn1)* mutants after glucose treatment.** Activity of catalase in (A) wild type N2 and (B) *mev-1(kn1)* mutants after 72 hours incubation in absence (control) or presence of either 100 mM or 250 mM glucose. Enzyme activity was measured in whole worm lysates. Data are given as mean ± SD. * P<0.05 and ** P<0.01 versus control by ANOVA. Results are from 3 independent experiments.

Increased ROS generation in mitochondria upon high glucose load in different concentrations led obviously to a higher demand of the GSH/GSSG system. In contrast the two enzymatic defense mechanisms were only affected upon treatment with the highest glucose concentration and in particular catalase activity was increased in the 250 mM glucose group.

3.2.5 DAF-16 signaling upon high glucose load

The DAF-2 receptor activates in *C. elegans* the insulin like signaling via a PI3-kinase upon ligand binding. In *C. elegans* more than 37 ligands of the DAF-2 receptor have been identified based on sequence homology and structural predictions [162]. Receptor activation leads to phosphorylation and downregulation of the transcription factor DAF-16. Certain mutations in DAF-2 cause an inhibition of DAF-16 translocation and other downstream signaling genes and results in increased dauer formation, increased stress resistance and longer life span in worms (reviewed in [62]). To asses the effects of high glucose loads the daf-16::GFP transgenic strain was used. Translocation can be induced by 100 μM juglone incubation after 3 hours (shown in fig. 3.21). The translocation of DAF-16 into the nucleus induced by juglone could almost completely be blocked by treatment of animals with an extra of 100 mM glucose (shown in fig.3.21). This provides further evidence for a negative effect of high glucose exposure on *daf-16* signaling, most likely via activation of the *daf-2* signaling pathway.

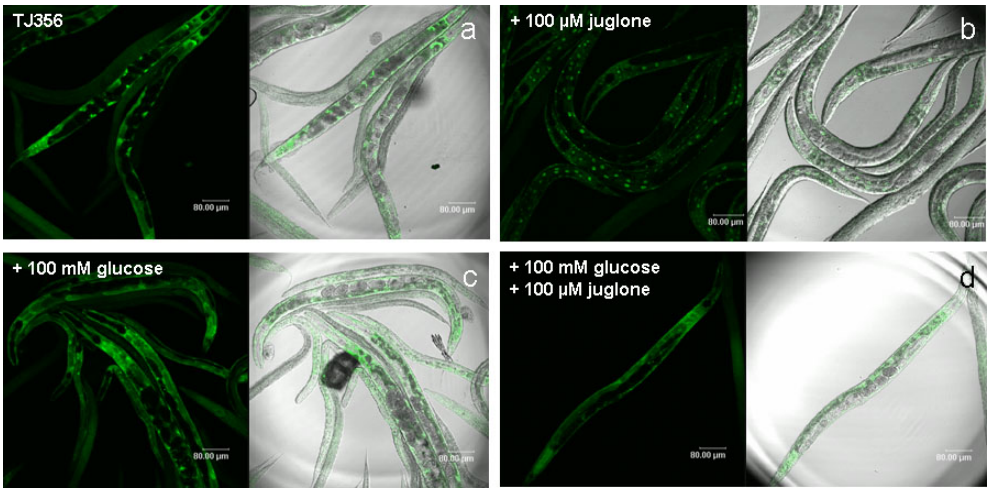

Figure 3.21: **DAF-16 translocation in TJ356.** Translocation of DAF-16 into the nucleus after 3 hours incubation in (a) absence (control) or presence of either (b) 100 μM juglone, (c) 100 mM glucose or (d) co-incubation of 100 mM glucose and 100 μM juglone. Translocation of DAF-16 into the nucleus is visible by intense staining. Left panels show fluorescence pictures and right panels show overlays of fluorescence and light microscopic pictures. Pictures represent typical results obtained from 3 independent experiments.

To study the potential impact of extra glucose on *daf-2* signaling, the transgenic strain CL2070 that expresses GFP under the control of the promotor of the small heat shock protein hsp-16.2 [121] was used. The chemical ROS generator juglone

[19] indeed strongly induced GFP expression in the pharynx of CL2070 (fig. 3.22). Interestingly this ROS-induced GFP expression could strongly be suppressed by co-incubation with high glucose in a concentration dependent manner (fig. 3.22). This also suggests a negative effect of a high glucose load on *daf-16* mediated processes that control chaperon status and protein folding.

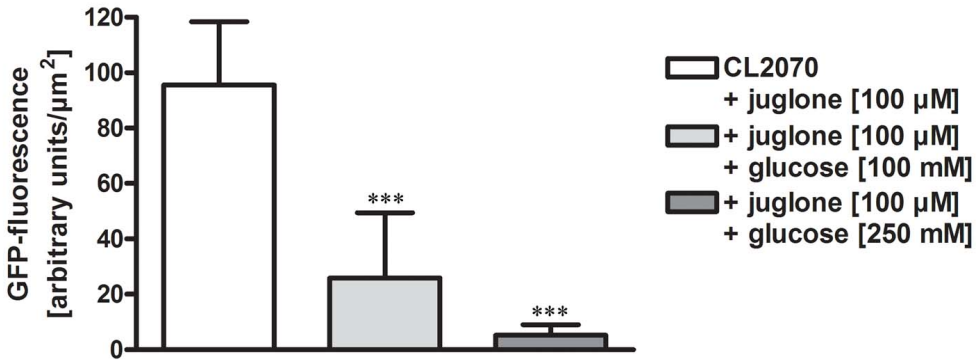

Figure 3.22: **HSP-16.2 expression in CL2070 upon juglone and glucose incubation.** Intensities of GFP expression in CL2070 in absence (control) or presence of either 100 μM juglone or co-incubation of 100 μM juglone together with 100 mM or 250 mM extra glucose. Incubation time was 4 hours. Data are given as mean ± SD. *** $P<0.001$ versus control by ANOVA. Results are from 3 independent experiments.

3.2.6 Glucose and ascorbate treatment in mev-1(kn1) mutants

As described above, in *mev-1(kn1)* mutants a decrease in life span induced by higher glucose intake in a concentration dependent manner was detectable. Since this negative effect of ROS on life span seemed to depend on a certain threshold amount of ROS generated in mitochondria, it was tested if quenching of ROS in mitochondria by the classical antioxidant ascorbic acid can counteract this phenomenon.
Indeed co-incubation with 100 μM ascorbate could rescue the effect of 100 mM extra glucose on life span in this mutant as shown in fig. 3.23 (A). Also in 250 mM extra glucose treated *mev-1(kn1)* mutants ascorbate was able to rescue the life span reducing effect of 250 mM glucose alone (fig 3.23 (B)).

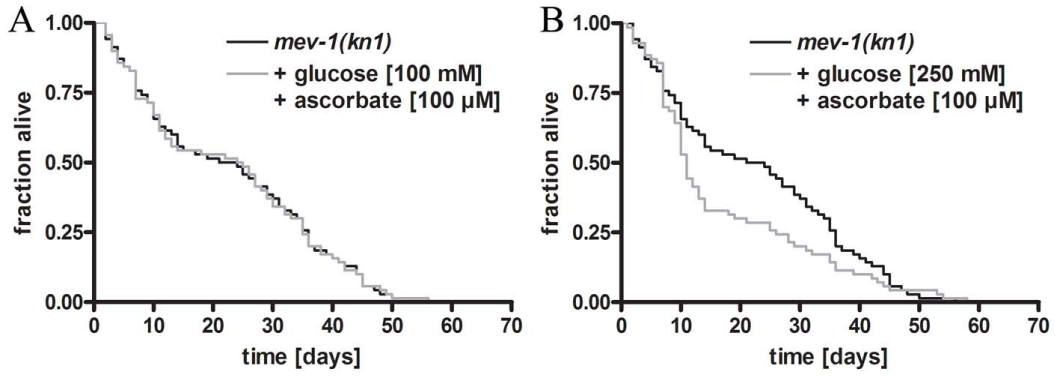

Figure 3.23: **Kaplan-Meier survival curves upon co-incubation of glucose and ascorbate in *mev-1(kn1)* mutants.** Life span measurements in *mev-1(kn1)* mutants either in absence (control) or presence of co-incubation of 100 μM ascorbate together with (A) 100 mM or (B) 250 mM glucose. Significances of differences in life span versus control were as follows: (A) P=0.9921 and (B) P=0.1218 by Logrank test.

ROS levels in mitochondria were significantly reduced by glucose upon co-incubation with ascorbate (fig. 3.24). Although concentrations of 1 mM vitamin C scavenged ROS in the mitochondria as efficiently as 100 μM vitamin C in the presence of both 100 and 250 mM glucose, the effects of 1 mM vitamin C on life span rescue were significantly smaller than those shown for 100 μM vitamin C (Data not shown). This suggests the pro-oxidant activities of 1 mM of ascorbate which probably exerts its effects under these conditions mainly in the cytosol.

For co-incubation of 100 mM glucose and 100 μM ascorbate also glutathione levels were measured and a significant increase in GSH levels and no alteration in GSSG levels (fig. 3.25) were seen, reflecting a lower requirement of the GSH/GSSG system and a higher GSH de novo synthesis.

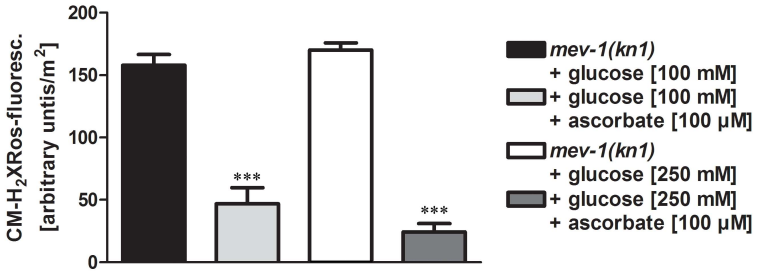

Figure 3.24: **ROS levels in mitochondria after co-incubation of glucose and ascorbate.** Fluorescence intensities of the dye CM-H_2XRos in *mev-1(kn1)* mutants after glucose (100 mM or 250 mM) alone or in combination with 100 μM ascorbate. Incubation time was 24 hours. Data are given as mean ± SD. *** P<0.001 versus glucose (100 mM respectively 250 mM) by ANOVA. Results are from 3 independent experiments.

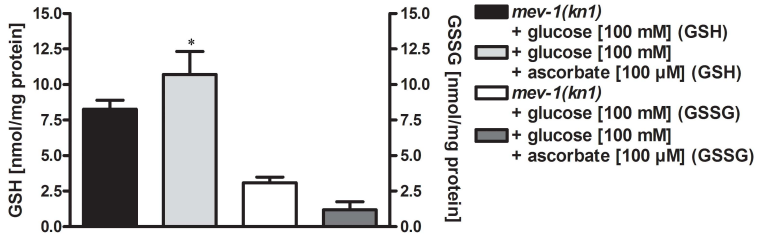

Figure 3.25: **Glutathione levels in *mev-1(kn1)* mutants after co-incubation of glucose and ascorbate.** GSH and GSSG levels in *mev-1(kn1)* mutants after glucose (100 mM or 250 mM) incubation alone or together with 100 μM ascorbate. Incubation time was 72 hours. Glutathione was measured in whole worm lysates. Data are given as mean ± SD. * P<0.05 versus 100 mM glucose by ANOVA. Results are from 3 independent experiments.

In summary, it could be shown that ROS levels in mitochondria were decreased upon ascorbic acid treatment when animals were subjected to a high glucose load and this coincided with significantly elevated GSH levels. These observations may also explain the rescue effect of ascorbic acid on glucose-induced life span reduction in *mev-1(kn1)* mutant worms.

3.3 Flavonoid and resveratrol effects on aging processes in C. elegans

In the previous chapters it was shown that food components such as glucose were able to increase ROS generation in mitochondria. However, this can lead to a life span reduction only when mitochondrial ROS levels are already elevated, as is the case in *mev-1(kn1)* animals. On the other hand, juglone treatment leads to an increase in antioxidant defense mechanisms and thereby can prolong life span. This hormesis effect is only observed at low juglone concentrations whereas high concentrations lead to premature death since adaptative mechanisms appear to be saturable. Glucose caused oxidative stress in mitochondria which induce hormesis only in N2 wild type animals but not in *mev-1(kn1)* mutants and therefore the effects of glucose are dependent on the genetic background and on the basal ROS levels in mitochondria and/or mitochondrial functionality.

Flavonoids and resveratrol are secondary metabolites of plants (also named secondary plant ingredients) and contribute in plants to defense against ultraviolet radiation or act as phytoalexins (a class of antibiotics of plant origin). Polyphenolic compounds are very abundant in our diet, have antioxidant properties and suggested roles in the prevention of various diseases which are associated with oxidative stress, like cancer, cardiovascular and neurodegenerative diseases [175]. Since polyphenols are able to scavenge ROS it was tested if selected compounds including resveratrol have effects on aging processes in worms.

Resveratrol (3,4',5-trihydroxy-trans-stilbene) is a plant derived polyphenolic compound which has been observed to have therapeutic benefits including cancer prevention [3], cardio protection [84] [173], and antifungal effects (reviewed in [159]). There is substantial evidence that resveratrol is able to extend live span in a SIR2-dependent manner in a wide variety of model organisms like *S. cerevisiae*, *C. elegans* and *D. melanogaster* [81] [217] [202] [89]. Resveratrol is synthesized in response to environmental stress or pathogenic attacks. It is classified as a phytoalexin for this reason. It occurs in red wine, grapes, peanuts and mulberries [182].

Therefore it was tested if secondary plant ingredients are able to influence stress response and aging in *C. elegans*. First the impact of resveratrol treatment was analyzed dependent on the concentration. In a concentration of 10 μM no increase in life span in N2 upon resveratrol incubation was detectable (fig. 3.26 (A)). After incubation in 100 μM resveratrol life span was significantly prolonged (fig. 3.26 (B)).

Also flavone, myricetin, quercetin and fisetin were tested. None of the flavonoids was able to alter life span in this experimental setting in a concentration of 10 μM. No influence on life span was detectable when treatment was started from L1 stage, as well as when N2 or *mev-1(kn1)* mutants were exposed to flavonoids only during development (from L1 until young adult stage) (Data not shown).

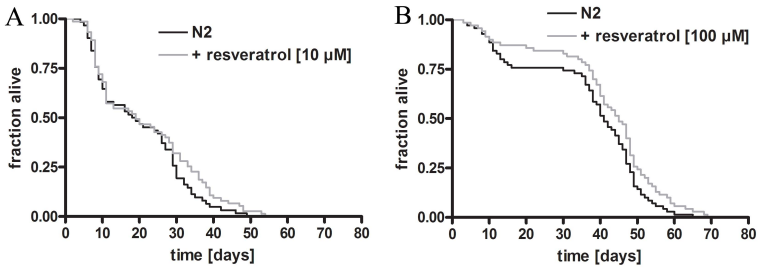

Figure 3.26: **Kaplan-Meier survival curves in wild type N2 upon resveratrol treatment.** Life span measurements in N2 wild type worms in absence (control) or presence of (A) 10 μM or (worms were incubated as L1) (B) 100 μM resveratrol (worms were incubated as young adults). Significances of differences in life span versus control were as follows: (A) P=0.0924 and (B) P=0.0492 by Logrank test.

As a consequence of these results concentrations of resveratrol and flavonoids were increased to 100 μM and worms were incubated from young adult stage on. To exclude starvation of worms upon flavonoid and resveratrol treatment worms were incubated as L4 larvae and brood size was measured. In N2 the number of progeny was not altered upon flavonoid and resveratrol treatment (fig. 3.27).

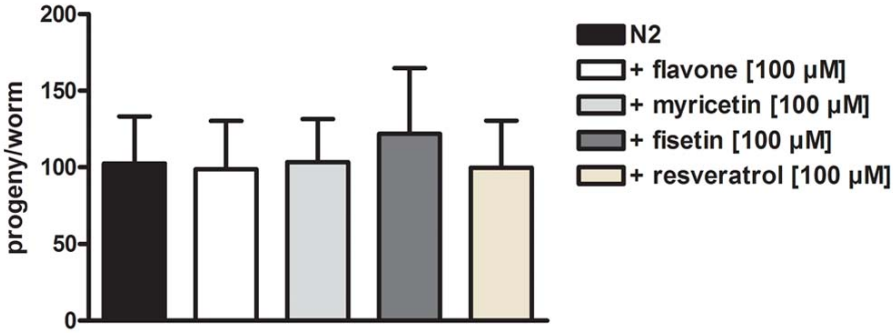

Figure 3.27: **Progeny after flavonoid and resveratrol treatment.** N2 wild type worms were incubated either in absence (control) or presence of 100 μM flavone, 100 μM myricetin, 100 μM fisetin, and 100 μM resveratrol after reaching the L4 stage. n=12 from 3 independent experiments.

3.3.1 Flavone and resveratrol treatment

Flavone (2-phenyl-4H-1-benzopyran-4-one) represents the core structure of the flavone subgroup. It occurs in many cereal grains and in dill weed [134]. Previous studies have shown that flavone is able to enhance the activity of mitochondrial lactate and citrate transporters allowing an increased delivery of oxidizable substrates to mitochondria and in turn increase the rate of production for oxygen radicals [212].
Life span analysis in *C. elegans* upon exposure of animals to 100 μM flavone in wild type N2 as well as in *daf-16(mu86)* knockout mutants is shown in fig 3.28 (A and B). In the *sir-2.1(ok434)* line only a slight, but not significant decrease in life span was detectable (fig. 3.28 C). The life span reducing effect of flavone is therefore independent of *daf-16* activity but may be modulated by *sir-2.1*.

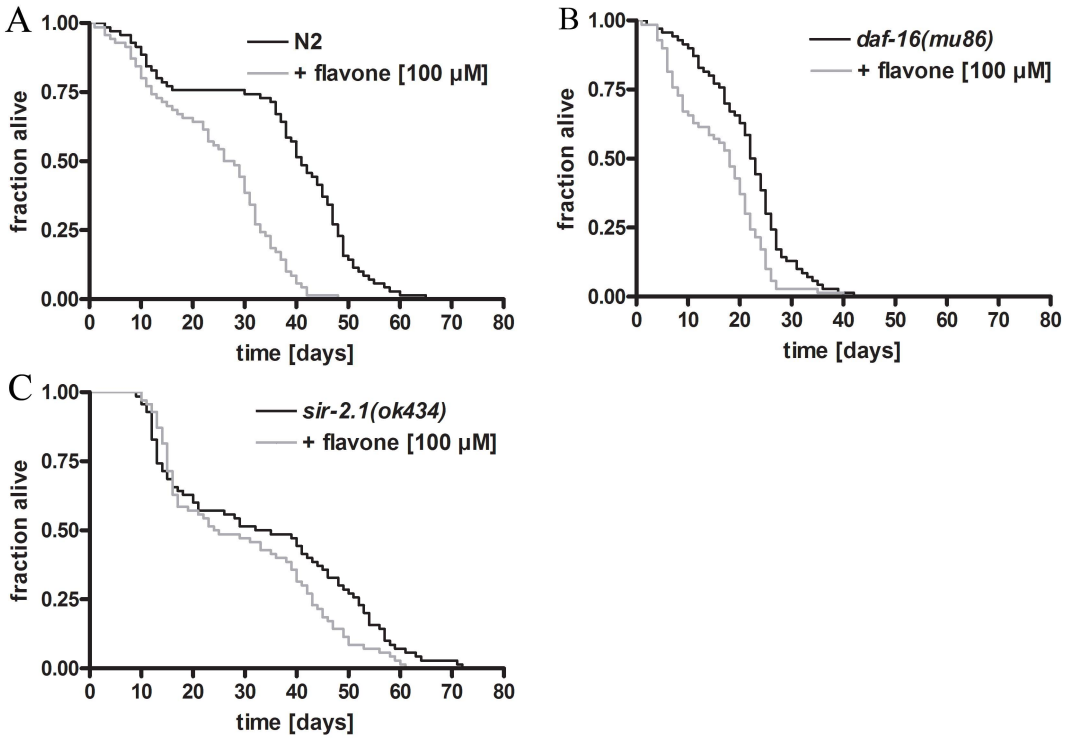

Figure 3.28: **Kaplan-Meier survival curves upon flavone treatment.** Life span measurements in (A) N2 wild type worms, (B) *daf-16(mu86)* mutants, and (C) *sir-2.1(ok434)* mutants in absence (control) or presence of 100 μM flavone. Significances of differences in life span versus control were as follows: (A) P<0.0001, (B) P=0.0004 and (C) P=0.0517 by Logrank test.

In case of resveratrol treatment in N2 a life span increasing effect was detectable (fig. 3.29) but there was no effect in *daf-16(mu86)* and *sir-2.1(ok434)* lines. This suggests that resveratrol effects need a proper function of *daf-16* and *sir-2.1* to exert

alterations of life span.

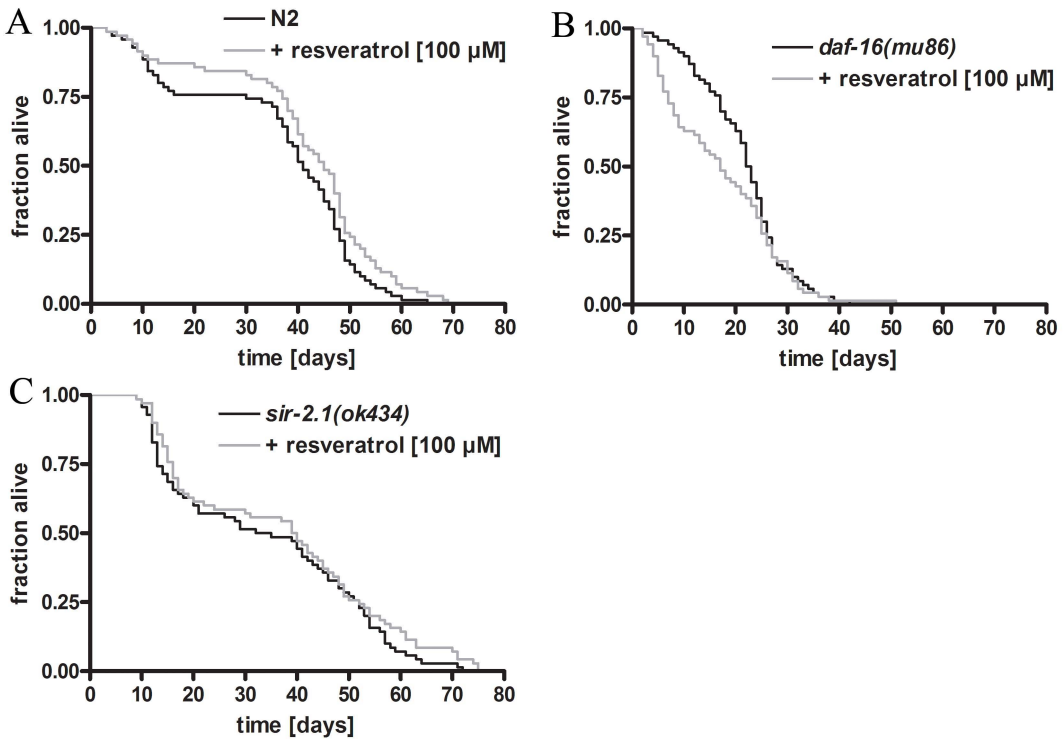

Figure 3.29: **Kaplan-Meier survival curves upon resveratrol treatment.** Life span measurements in (A) N2 wild type worms, (B) *daf-16(mu86)* mutants, and (C) *sir-2.1(ok434)* mutants in absence (control) or presence of 100 μM resveratrol. Significances of differences in life span versus control were as follows: (A) P=0.0492, (B) P=0.1998 and (C) P=0.2352 by Logrank test.

Since flavone was able to reduce life span whereas resveratrol prolonged life span in a *sir-2.1* dependent manner mRNA levels of *sir-2.1* were analyzed under treatment conditions. Flavone decreased and resveratrol increased significantly mRNA levels versus the control as shown in fig. 3.30.

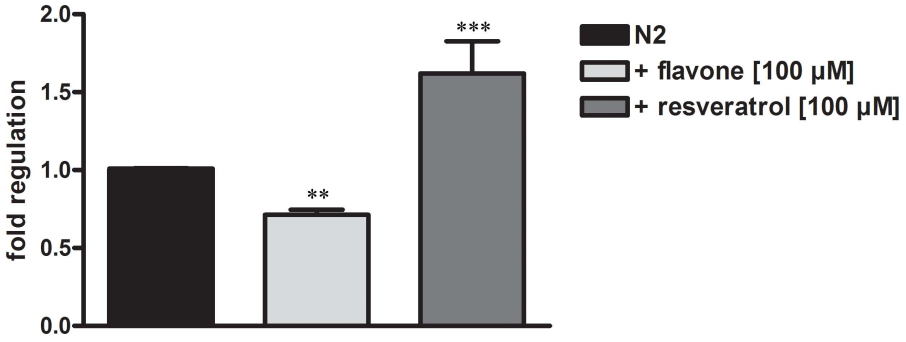

Figure 3.30: **Regulation of mRNA levels of *sir-2.1* upon flavone and resveratrol incubation.** Regulation of mRNA levels of *sir-2.1* in N2 wild type worms in absence (control) or presence of 100 μM flavone or 100 μM resveratrol treatment after 72 hours incubation time. Data are given as mean ± SD. ** P<0.01 and *** P<0.001 versus control by ANOVA. Results are from 3 independent experiments.

To asses whether flavone or resveratrol also alter the SIR-2.1 deacetylase activity, enzyme activity was measured in worm homogenates. As shown in fig. 3.31, flavone was able to decrease activity of SIR-2.1 whereas resveratrol increased sir-2.1 activity significantly (fig. 3.31).

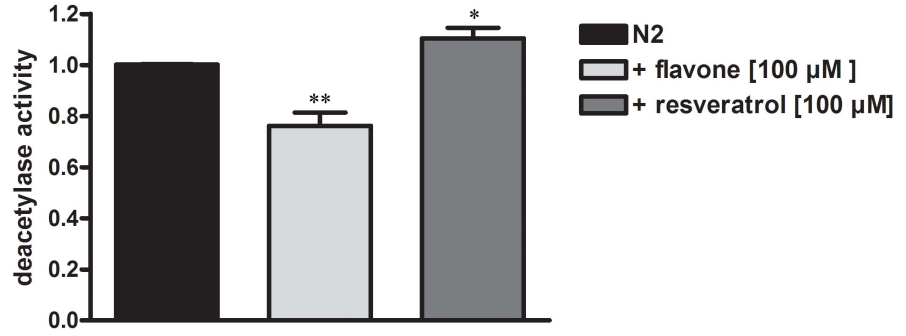

Figure 3.31: **Deacetylase activity of SIR-2.1 upon flavone and resveratrol treatment.** Activity of SIR-2.1 in N2 wild type worms either in absence (control) or presence of 100 μM flavone or 100 μM resveratrol. Incubation time was 72 hours. * P<0.05 and ** P<0.01 versus control by ANOVA.

Since in *sir-2.1* knockdown animals the life span decreasing effect was not as strong as in N2, it may be hypothesized that flavone can cause an inhibition of SIR-2.1. As shown, flavone and resveratrol had opposite effects on life span. Therefore the question occurs if there are also different effects on central mechanisms in life span regulation.

Next the role of *daf-16* was assessed since life span and aging critically depends on *daf-16*. As shown in fig. 3.32, flavone as well as resveratrol led to a translocation of

DAF-16 into the nucleus by the punctuate appearance of GFP in the strain TJ356.

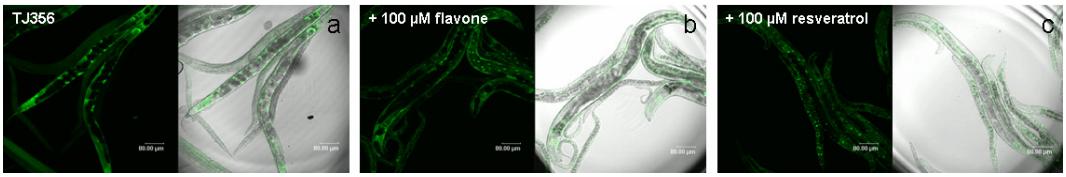

Figure 3.32: **DAF-16 translocation into the nucleus upon flavone and resveratrol treatment.** Translocation of DAF-16 into the nucleus after 3 hours incubation in (a) absence (control) or presence of either (b) 100 μM flavone or (c) 100 μM resveratrol. Translocation of DAF-16 into the nucleus is visible by intense staining. Left panels show fluorescence pictures and right panels show overlays of fluorescence and light microscopic pictures. Pictures represent typical results obtained from 3 independent experiments.

These findings may indicate that flavone as well as resveratrol can prevent phosphorylation of DAF-16 that is required for its retrieval in the cytosol and this seems to be independent of *sir-2.1* as siRNA treatment had no effect on translocation of DAF-16 into the nucleus after flavone or resveratrol exposure (fig. 3.33).

Figure 3.33: **DAF-16 translocation into the nucleus upon flavone and resveratrol incubation after *sir-2.1* siRNA treatment.** Translocation of DAF-16 into the nucleus after *sir-2.1* siRNA treatment in (a) absence (control) or presence of either (b) 100 μM flavone or (c) 100 μM resveratrol incubation for 3 hours. Translocation of DAF-16 into the nucleus is visible by intense staining. Left panels show fluorescence pictures and right panels show overlays of fluorescence and light microscopic pictures. Pictures represent typical results obtained from 3 independent experiments.

To test the effect of flavone and resveratrol on the antioxidant capacity in worms exposed to these test compounds the transgenic strain CL2070 was used. This strain expresses GFP under control of the hsp-16.2 promotor to serve as a tool for activation of a *daf-16* downstream target. Flavone and also resveratrol (100 μM) were similar able to reduce GFP-expression induced by juglone treatment (fig. 3.34). Similar findings were also observed when worms first were incubated for 2 hours with 100 μM juglone and then 100 μM flavone was added for another 3 hours in the presence of juglone. These data favors that there is a direct effect of flavone on ROS levels generated upon juglone treatment, rather than an interaction of juglone and flavone in the medium. Flavone treatment alone had no effect on GFP expression in CL2070

(data not shown) but flavone as well as resveratrol inhibited activation of the *daf-16* downstream target HSP-16.2 - however, without an effect on life span.

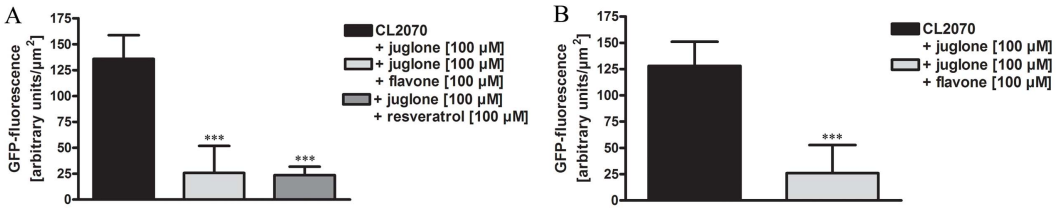

Figure 3.34: **HSP-16.2 expression in CL2070 upon flavone and resveratrol treatment.** (A) Intensities of GFP expression in CL2070 after incubation in 100 μM juglone alone or in combination with 100 μM flavone or 100 μM resveratrol. (B) Intensities of GFP expression in CL2070 that were incubated with 100 μM juglone for 5 hours. 100 μM flavone was added after 2 hours for 3 hours. *** P<0.001 versus 100 μM juglone by ANOVA. n=10 from 3 independent experiments.

GFP expression in CL2070 can also be induced by heat shock. For that worms were incubated at 35 °C for 1.5 hours followed by an incubation in CeHR medium alone or in addition with 100 μM flavone or 100 μM resveratrol for 3.5 hours at 20 °C respectively. In contrast to inhibition of *hsp-16.2* promotor activity that was induced by juglone flavone and also resveratrol were not able to decrease HSP-16.2 induction caused by heat stress. However, the spatial distribution of GFP expression induced by heat stress was also different from that after juglone treatment. Juglone induced GFP expression mainly in the pharynx, which is also the first organ getting into contact with orally delivered compounds including flavone and resveratrol. These data suggest that flavone and also resveratrol have a potential antioxidative effect, which is, however, limited to certain stress inducing conditions, as exerted by juglone.

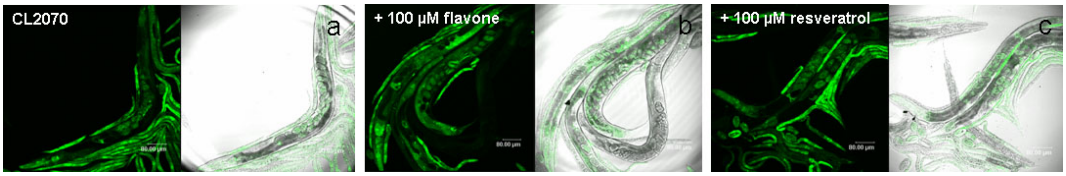

Figure 3.35: **GFP expression in CL2070 induced by heat shock.** GFP expression in CL2070 after incubation for 1.5 hours at 35 °C, then following incubation in CeHR medium for 3.5 hours in (a) absence (control) or presence of (b) 100 μM flavone or (c) 100 μM resveratrol at 20 °C. Pictures represent typical results obtained from 3 independent experiments.

To directly visualize the effect of flavone and resveratrol treatment on a downstream target of DAF-16, the *muls84* mutant carrying a SOD-3::GFP transgene was used. Exposure of animals to 100 μM flavone or 100 μM resveratrol led to an increase in GFP fluorescence as seen in fig. 3.36.

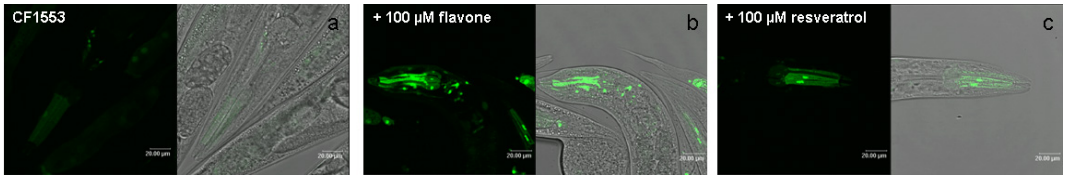

Figure 3.36: **GFP expression upon resveratrol and flavone treatment in *muIs84* mutants carrying a sod-3::GFP transgene.** *MuIs84* mutants carrying a sod-3::GFP transgene after 72 hours incubation in (a) absence (control) or presence of either (b) 100 μM flavone or (c) 100 μM resveratrol. Left panels show fluorescence pictures and right panels show overlays of fluorescence and light microscopic pictures. Pictures represent typical results obtained from 3 independent experiments.

Next SOD activity was analyzed, as one downstream target of DAF-16. In N2 SOD activity was reduced after 100 μM flavone exposure whereas in *daf-16(mu86)* and after knockdown of *sir-2.1* SOD activity was increased. This suggests that decrease of SOD activity caused by flavone treatment is dependent on *daf-16* and *sir-2.*1. Resveratrol also decreased activity of SOD in wild type N2 in a *daf-16* and *sir-2.1* dependent manner with even stronger impact than flavone.

Also CAT activity was measured as another DAF-16 downstream target. In wild type N2 as well as in the *daf-16(mu86)* mutant and in N2 after *sir-2.1* siRNA treatment an increase of CAT activity after flavone incubation could be observed (fig. 3.38). In contrast, resveratrol treatment decreased catalase activity in N2 wild type worms. The reduction appeared to depend on *daf-16* and on *sir-2.1* since activity was slightly elevated after *daf-16* knockout and *sir-2.1* knockdown. Resveratrol treatment thus seemed to decrease endogenous antioxidative defense mechanisms in

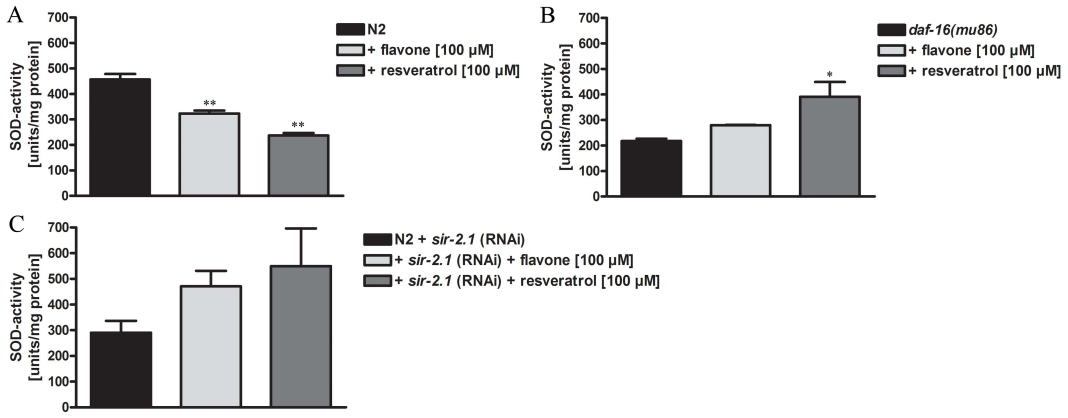

Figure 3.37: **SOD activity upon flavone and resveratrol treatment.** Activity of SOD in (A) N2 wild type worms, (B) *daf-16(mu86)* mutants, and (C) N2 after *sir-2.1* siRNA treatment after 72 hours incubation in absence (control) or presence of either 100 μM flavone or 100 μM resveratrol. Enzyme activities were measured in whole worm lysates. Data are given as mean ± SD. * P<0.05 and ** P<0.01 versus control by ANOVA. Results are from 3 independent experiments.

a *daf-16* and *sir-2.1* dependent manner, despite the enhanced ROS levels in mitochondria. Flavone in contrast was able to increase GSH levels and catalase activity dependent on *daf-16* and *sir-2.1*.

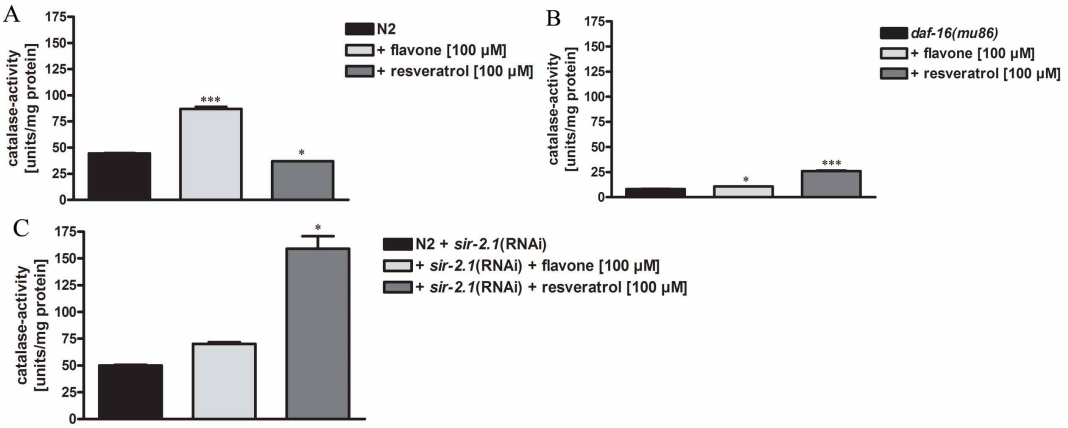

Figure 3.38: **Catalase activity upon flavone and resveratrol treatment.** Activity of catalase in (A) N2 wild type worms, (B) *daf-16(mu86)* mutants, and (C) N2 after *sir-2.1* siRNA treatment after 72 hours incubation in absence (control) or presence of either 100 μM flavone or 100 μM resveratrol. Enzyme activity was measured in whole worm lysates. Data are given as mean ± SD. * P<0.05 and *** P<0.001 versus control by ANOVA. Results are from 3 independent experiments.

As the most important cellular antioxidant glutathione was measured. An increase of the reduced form of glutathione (GSH) in wild type N2 worms (fig. 3.39) was detectable after exposure to flavone whereas upon resveratrol treatment GSH

levels were significantly decreased in the wild type N2. The lowered GSH levels had obviously no relevance for life span extension caused by resveratrol. No alterations were seen in *daf-16(mu86)* mutants and after *sir-2.1* RNAi treatment in N2 wild type worms upon flavone and also resveratrol incubation. However, in *daf-16(mu86)* mutants an increase in GSSG levels could be detected after flavone treatment whereas in N2 worms GSSG levels were not altered. Oxidative stress in mitochondria induced by flavone led only in N2 worms to an upregulation of the glutathione system in a *daf-16* and *sir-2.1* dependent manner. SIR-2.1 seems to be required for GSH de novo synthesis since after *sir-2.1* knockdown GSH levels were not altered. DAF-16 seems to be necessary on one hand for synthesis of glutathione since after knockout of *daf-16* GSH levels were decreased compared to N2 wild type worms and on the other hand led flavone to enhanced GSSG synthesis after *daf-16* knockout suggesting it altered the redox-state.

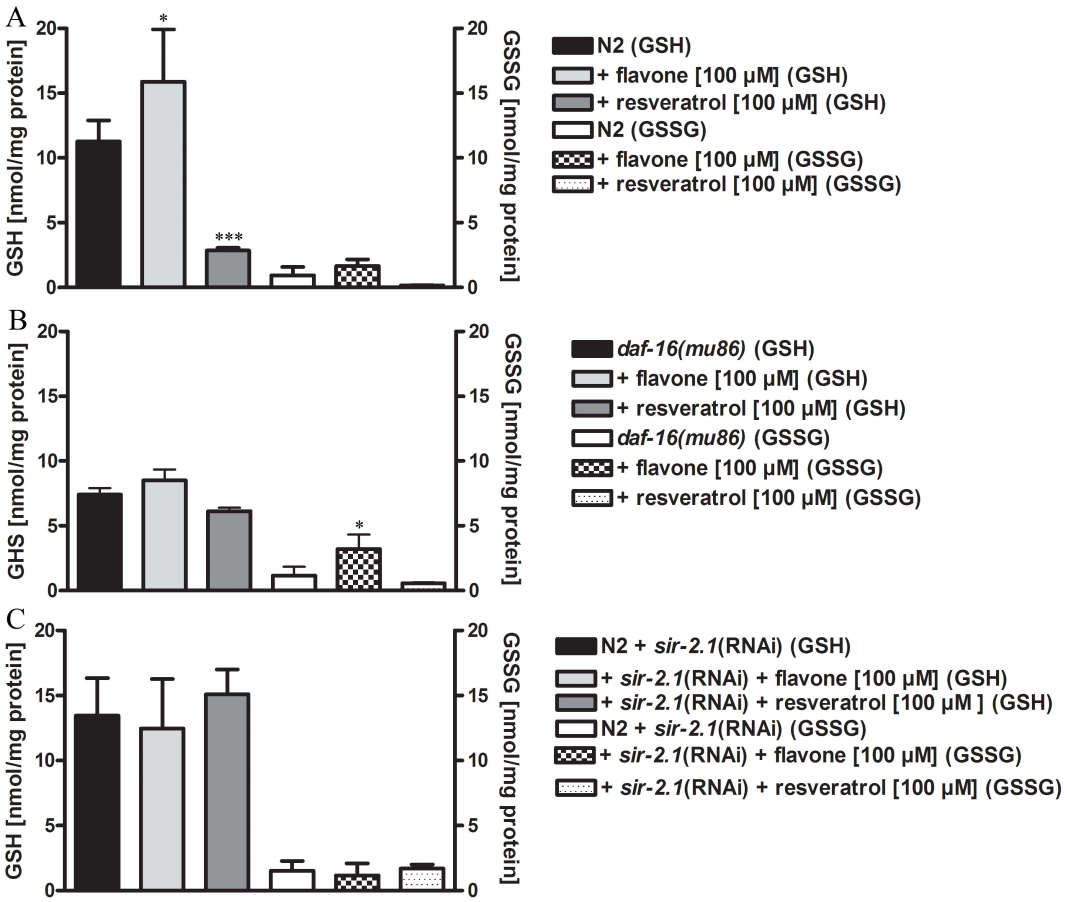

Figure 3.39: **Glutathione levels upon flavone and resveratrol treatment.** Levels of GSH and GSSG in absence (control) or presence of either 100 μM flavone or 100 μM resveratrol in (A) N2 wild type worms, (B) *daf-16(mu86)* mutants, and (C) N2 after *sir-2.1* siRNA treatment. Incubation time was 72 hours. Glutathione was measured in whole worm lysates. Data are given as mean ± SD. * $P<0.05$ and ** $P<0.01$ versus control by ANOVA. Results are from 3 independent experiments.

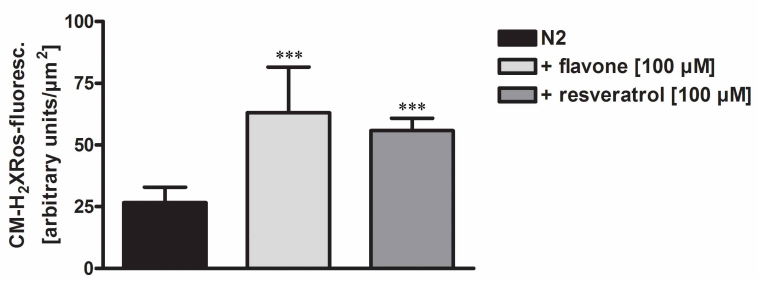

Figure 3.40: **ROS levels in mitochondria upon flavone and resveratrol treatment.** Fluorescence intensities of the dye CM-H_2XRos in N2 wild type worms in absence (control) or presence of 100 μM flavone or 100 μM resveratrol. Incubation time was 24 hours. Data are given as mean ± SD. *** P<0.001 versus control by ANOVA. Results are from 3 independent experiments.

Besides the potential antioxidative capacity, the pro-oxidative potential of flavone inside the mitochondria has been clearly delineated previously [212]. Flavone indeed increased ROS levels in mitochondria of N2 wild type. The same effect, however, was detectable after resveratrol treatment (fig. 3.40).

As flavone as well as resveratrol increased mitochondrial ROS levels in N2 also lipofuscin was measured as a surrogate marker for aging. An increase in lipofuscin content in wild type N2 worms after treatment with flavone and also with resveratrol, but not as strong as in case of flavone was seen (fig. 3.41). This suggests that enhanced ROS generation caused by flavone and resveratrol treatment results in an increased content of oxidized molecules. In contrary glucose was despite the enhanced mitochondrial ROS production not able to increase lipofuscin accumulation in *mev-1(kn1)* mutants suggests additional effects of flavonoids.

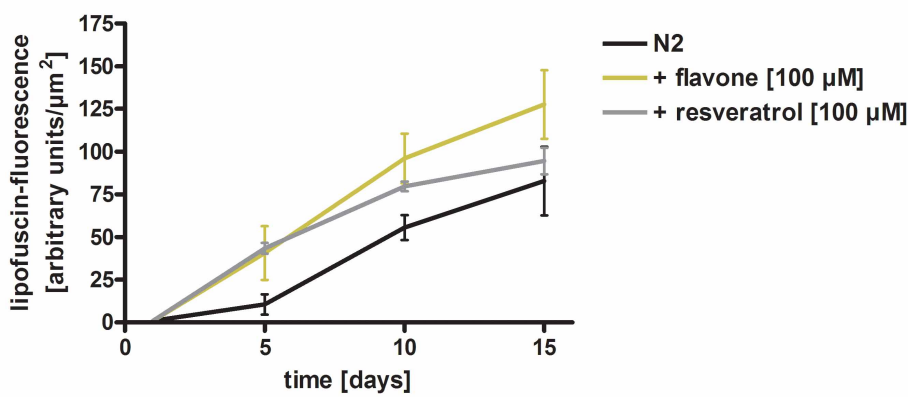

Figure 3.41: **Lipofuscin accumulation upon flavone and resveratrol treatment.** Intensities of lipofuscin accumulation in wild type N2 worms in absence (control) or presence of 100 μM flavone or 100 μM resveratrol. Incubation time was 1, 5, 10, and 15 days. Data are given as mean ± SD. Results are from 3 independent experiments.

Oxygen consumption was measured to assess whether elevated ROS generation goes in parallel with an enhanced respiration rate. An increase in oxygen consumption in N2 wild type worms after flavone treatment was observed in line with the enhanced ROS generation in mitochondria(fig. 3.42).

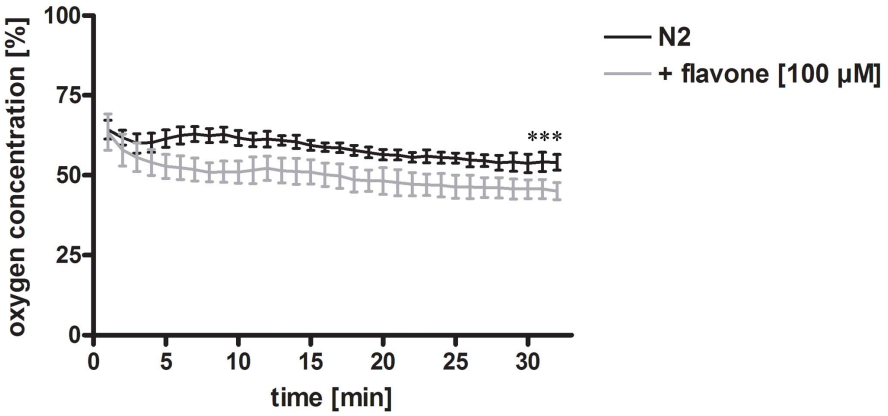

Figure 3.42: **Oxygen consumption in wild type N2 upon flavone treatment.** Oxygen measurements in N2 wild type worms in absence (control) or presence of 100 μM flavone. Incubation time was 30 minutes. Data are given as mean ± SD. *** P<0.001 versus control by ANOVA. n=90 ± 5 worms from 3 independent experiments.

In contrast resveratrol treatment had no effect on oxygen consumption in wild type N2 (fig. 3.43).

Although resveratrol increased ROS levels in mitochondria but did not lead to an enhancement of any of the tested antioxidative mechanisms in N2 nematodes, it increased life span significantly in a *sir-2.1* dependent manner.

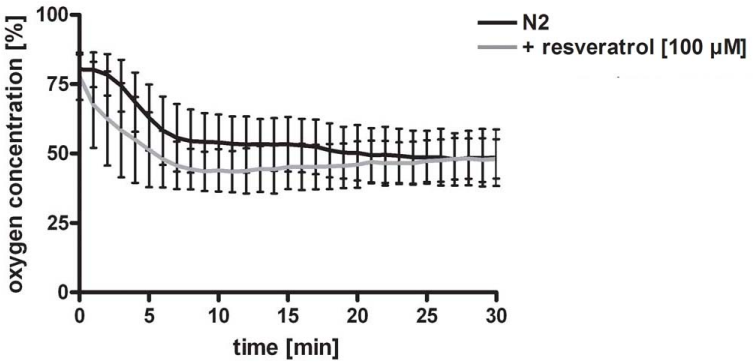

Figure 3.43: **Oxygen consumption in wild type N2 upon resveratrol treatment.** Oxygen measurements in N2 wild type worms in absence (control) or presence of 100 μM resveratrol. Incubation time was 30 minutes. Data are given as mean ± SD. n=90 ± 5 worms from 3 independent experiments.

In summary, resveratrol effects on life span are independent on the antioxidative enzymatic defense systems and GSH/GSSG whereas flavone increased GSH levels and activity of catalase but reduced life span. The simplest explanation based on these findings is that *sir-2.1* has the most prominent role for life span regulation and the response of antioxidative mechanisms is secondary to this.

3.3.2 Myricetin

Myricetin is a flavonol with a widespread occurrence in plants which include berries, tea plants, fruits, and vegetables [74]. It has antioxidant properties and was found to scavenge effectively superoxide radicals generated by the phenazine methosulfate-NADH system. Also hydroxyl radicals generated by ultraviolet photolysis of hydrogen peroxide can be quenched by myricetin [167].
For assessing the impact of myricetin on life span in *C. elegans*, nematodes were exposed to 100 μM myricetin but no impact was observed for N2 and *sir-2.1(ok434)* mutants. A decrease in life span in *daf-16(mu86)* mutants as shown in fig. 3.44 was detectable, suggesting that *daf-16* seems to protect wild type N2 worms of the enhanced ROS production in mitochondria that is induced upon myricetin treatment which is shown in fig. 3.47.

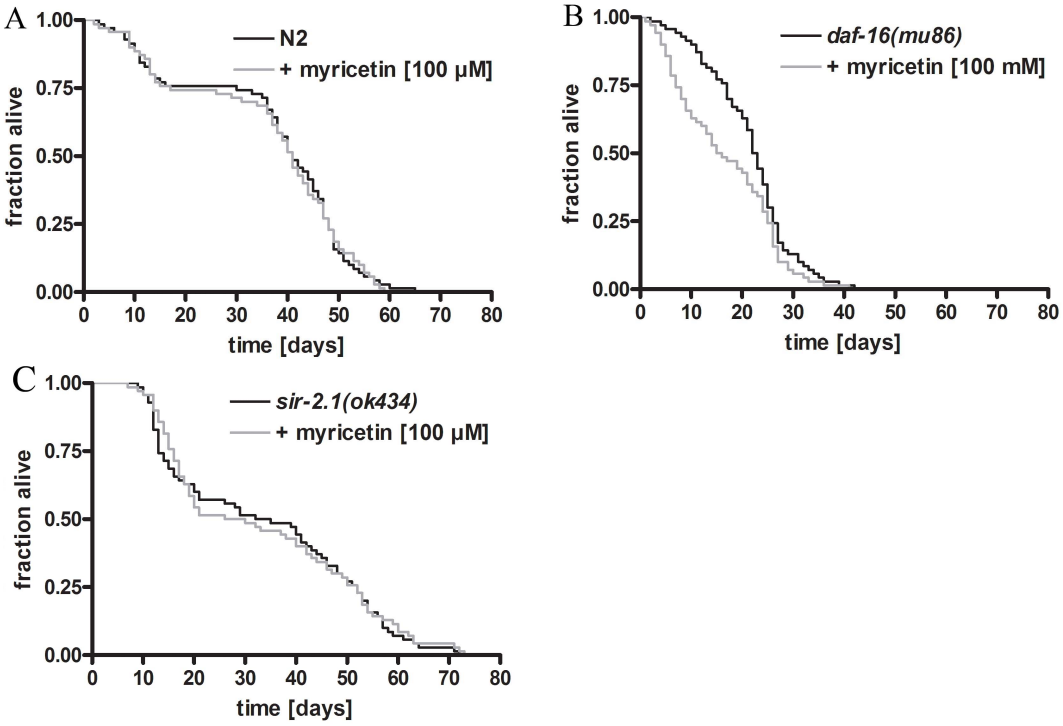

Figure 3.44: **Kaplan-Meier survival curves upon myricetin treatment.** Life span measurements in (A) N2 wild type worms, (B) *daf-16(mu86)* mutants, and (C) *sir-2.1(ok434)* mutants in absence (control) or presence of 100 μM myricetin. Significances of differences in life span versus control were as follows: (A) P=0.8388, (B) P=0.0338, and (C) P=0.7568 by Logrank test.

To test if myricetin is able to reduce GFP expression induced by juglone in CL2070, worms were co-incubated with 100 μM myricetin and 100 μM juglone (fig. 3.45).

Myricetin was able to decrease GFP expression, even in the time delayed incubation (after 2 hours 100 μM juglone treatment 100 μM myricetin was added for 3 hours in the presence of juglone) but not as potent as in the co-incubation. No GFP expression was detectable after myricetin exposure in the absence of juglone (data not shown). This suggests that myricetin has a profound antioxidative capacity, first by activation of antioxidant mechanisms and second by scavenging of ROS.

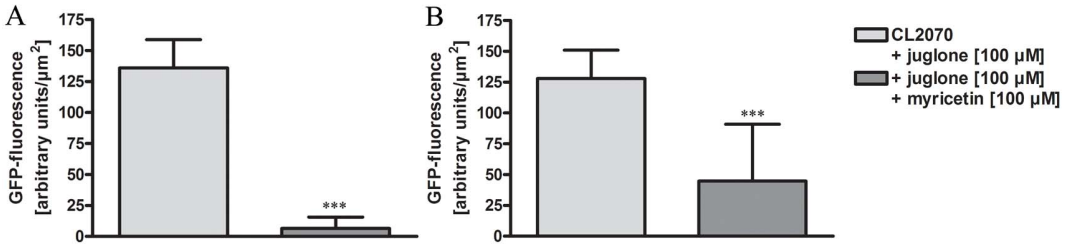

Figure 3.45: **HSP-16.2 expression in CL2070 upon myricetin treatment.** (A) Intensities of GFP expression in CL2070 after incubation in 100 μM juglone alone or in combination with 100 μM myricetin. (B) Intensities of GFP expression in CL2070 after time delayed incubation of 100 μM juglone for 5 hours, 100 μM myricetin was added after 2 hours for 3 hours. *** P<0.0001 versus 100 μM juglone by ANOVA. n=10 from 3 independent experiments.

When CL2070 worms were stressed by heat shock treatment myricetin failed to decrease GFP expression (fig. 3.46) similar to the lack of effect of flavone and resveratrol after heat stress. This underlines that the stress reducing effect of myricetin is limited to specific stressors, such as high ROS levels in the cytosol.

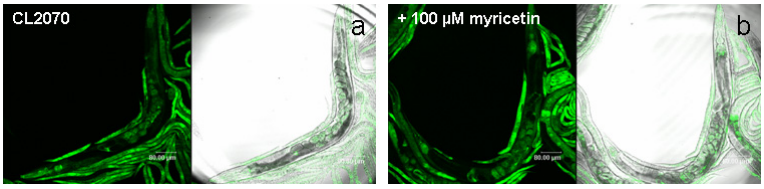

Figure 3.46: **GFP expression in CL2070 induced by heat shock.** GFP expression in CL2070 after incubation for 1.5 hours at 35 °C, then following incubation in CeHR medium for 3.5 hours in (a) absence (control) or presence of (b) 100 μM myricetin at 20 °C. Pictures represent typical results obtained from 3 independent experiments.

To analyze if myricetin is also able to act as a prooxidant like flavone and resveratrol did, mitochondrial ROS levels were measured and proved that myricetin was able to increase levels of mitochondrial ROS in N2 wild type worms as shown in fig. 3.47.

Despite enhanced production of ROS in mitochondria in the presence of myricetin, accumulation of the aging marker lipofuscin was even decreased compared to con-

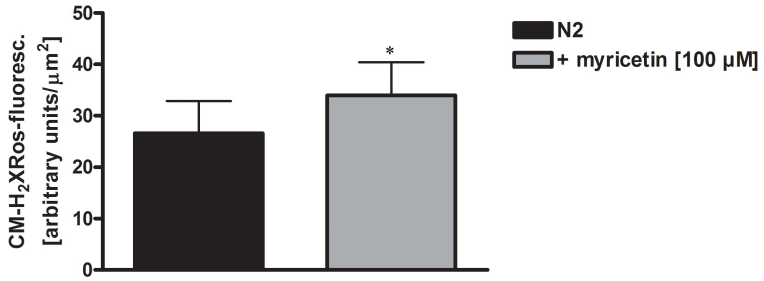

Figure 3.47: **ROS levels in mitochondria upon myricetin treatment.** Fluorescence intensities of the dye CM-H_2XRos in N2 wild type worms in absence (control) or presence of 100 μM myricetin. Incubation time was 24 hours. Data are given as mean ± SD. * P<0.05 versus control by ANOVA. Results are from 3 independent experiments.

trol after 10 to 15 days of myricetin treatment in wild type N2 suggesting that it can protect prone biomolecules from oxidation by effecting endogenous antioxidant defense systems.

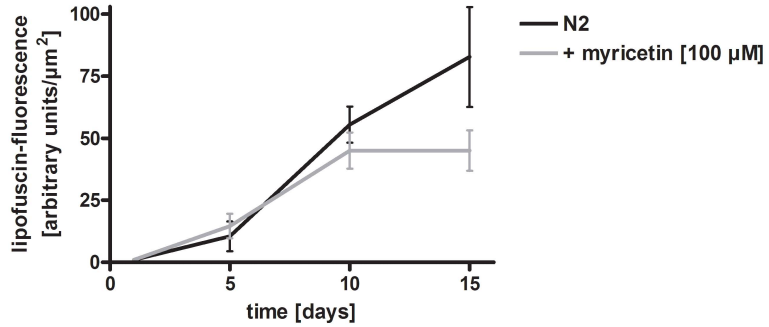

Figure 3.48: **Lipofuscin accumulation upon myricetin treatment.** Intensities of lipofuscin accumulation in wild type N2 worms in absence (control) or presence of 100 μM myricetin. Incubation time was for 1, 5, 10, and 15 days. Data are given as mean ± SD. Results are from 3 independent experiments.

In the strain TJ356 effects on *daf-16* signaling were assessed and demonstrated that myricetin induced a translocation of DAF-16 into the nucleus (fig. 3.49) but this proved to be independent of *sir-2.1* (fig. 3.50).

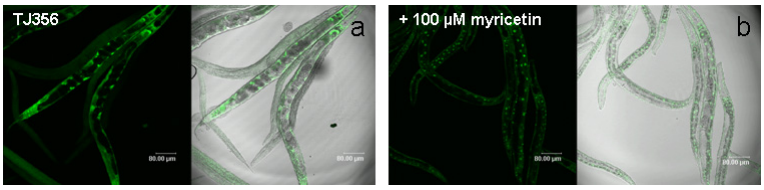

Figure 3.49: **DAF-16 translocation into the nucleus upon myricetin treatment.** Translocation of DAF-16 into the nucleus after 72 hours incubation in (a) absence (control) or presence of (b) 100 μM myricetin. Translocation of DAF-16 into the nucleus is visible by intense staining. Left panels show fluorescence pictures and right panels show overlays of fluorescence and light microscopic pictures. Pictures represent typical results obtained from 3 independent experiments.

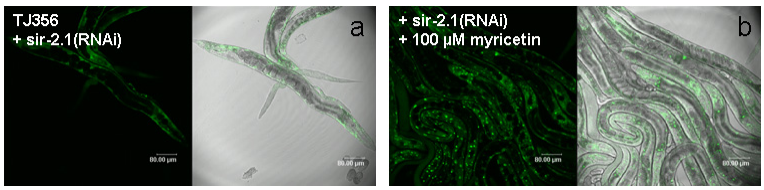

Figure 3.50: **DAF-16 translocation into the nucleus upon myricetin incubation after *sir-2.1* siRNA treatment.** Translocation of DAF-16 into the nucleus after *sir-2.1* siRNA treatment in (a) absence (control) or presence of (b) 100 μM myricetin. Translocation of DAF-16 into the nucleus is visible by intense staining. Left panels show fluorescence pictures and right panels show overlays of fluorescence and light microscopic pictures. Pictures represent typical results obtained from 3 independent experiments.

As a possible protector against the mitochondrial stress induced by myricetin incubation glutathione levels were measured. In wild type N2 and in *daf-16(mu86)* mutants a decrease was detectable in GSH levels upon myricetin treatment that appeared to depend on *sir-2.1* (fig. 3.51). Also GSSG levels were decreased upon myricetin incubation in a *sir-2.1* dependent fashion suggesting that the compound can inhibit GSH synthesis.

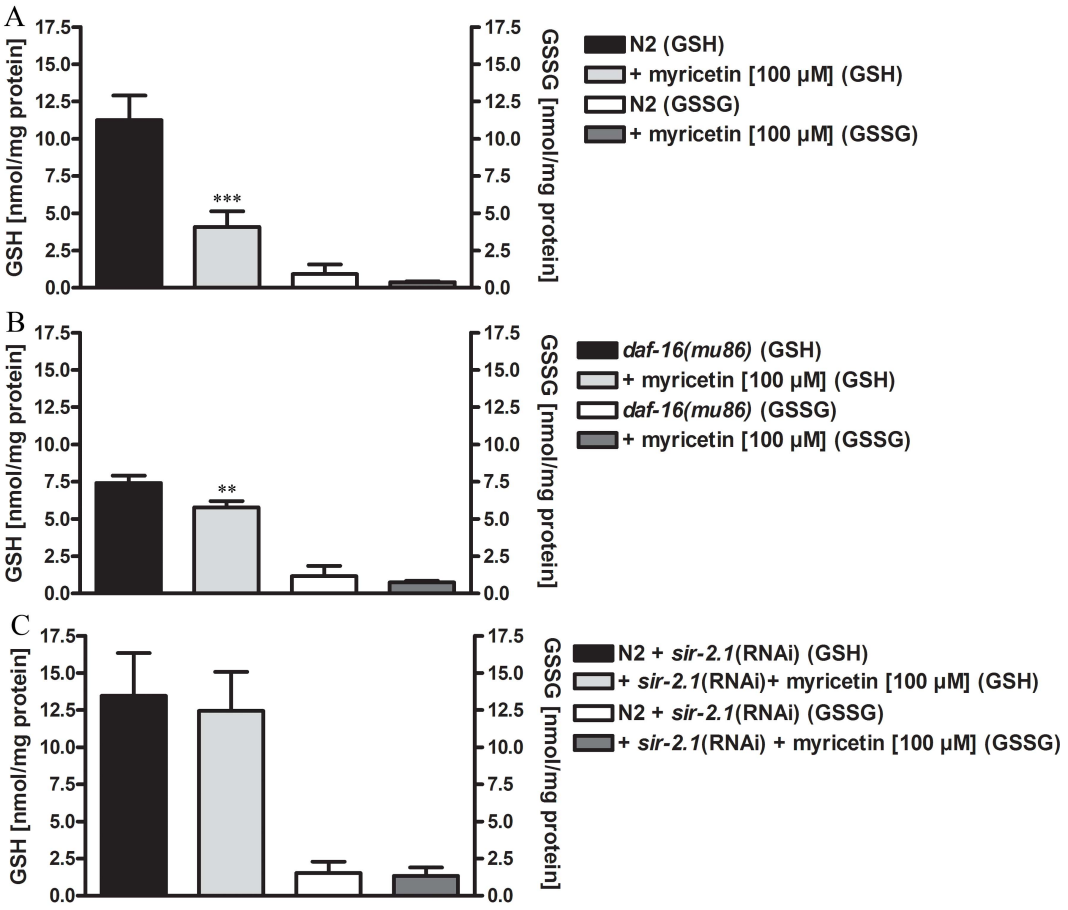

Figure 3.51: **Glutathione levels upon myricetin treatment.** Levels of GSH and GSSG in absence (control) or presence of 100 μM myricetin in (A) wild type N2 worms, (B) *daf-16(mu86)* mutants, and (C) N2 after *sir-2.1* siRNA treatment. Incubation time was 72 hours. Glutathione was measured in whole worm lysates. Data are given as mean ± SD. ** P<0.01 and *** P<0.001 versus control by ANOVA. Results are from 3 independent experiments.

In the transgenic *C. elegans* line expressing a GFP-SOD-reporter protein, 100 μM myricetin activated the DAF-16 downstream target SOD-3 as shown in fig. 3.52 but decreased SOD activity in wild type N2 worms in dependence on *daf-16* and *sir-2.1* (fig. 3.53).

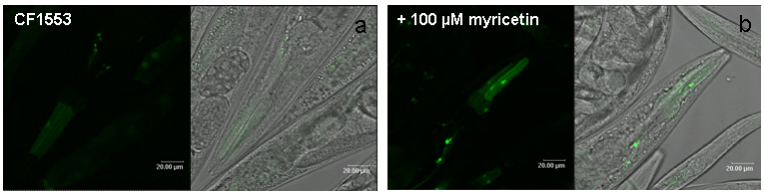

Figure 3.52: **GFP expression upon myricetin treatment in *muIs84* mutants carrying a sod-3::GFP transgene.** *MuIs84* mutants carrying a sod-3::GFP transgene after 72 hours incubation in (a) absence (control) or presence of (b) 100 μM myricetin. Left panels show fluorescence pictures and right panels show overlays of fluorescence and light microscopic pictures. Pictures represent typical results obtained from 3 independent experiments.

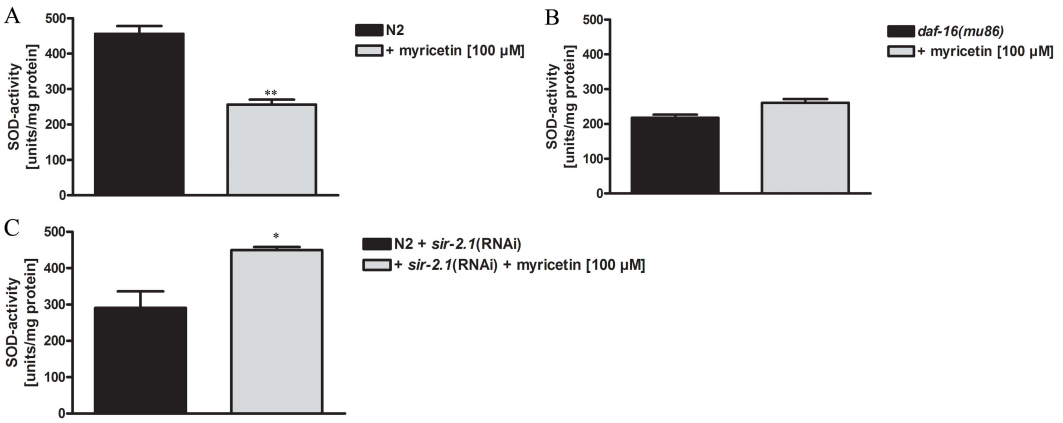

Figure 3.53: **SOD activity upon myricetin treatment.** Activity of SOD in (A) wild type N2 worms, (B) *daf-16(mu86)* mutants, and (C) N2 after *sir-2.1* siRNA treatment after 72 hours incubation in absence (control) or presence of 100 μM myricetin. Enzyme activities were measured in whole worm lysates. Data are given as mean $\pm$ SD. * $P<0.05$ and ** $P<0.01$ versus control by ANOVA. Results are from 3 independent experiments.

No alteration in CAT activity was detectable after myricetin treatment (fig. 3.54). Only in *daf-16(mu86)* mutants and after *sir-2.1* siRNA treatment in N2 wild type animals catalase activity was increased upon myricetin incubation.

In summary, myricetin inhibited activity of SOD dependent on *daf-16* and *sir-2.1* and *sir-2.1* was also important for transmitting the effect on GSH synthesis. Treatment with myricetin was able to enhance catalase activity in the *daf-16(mu86)*

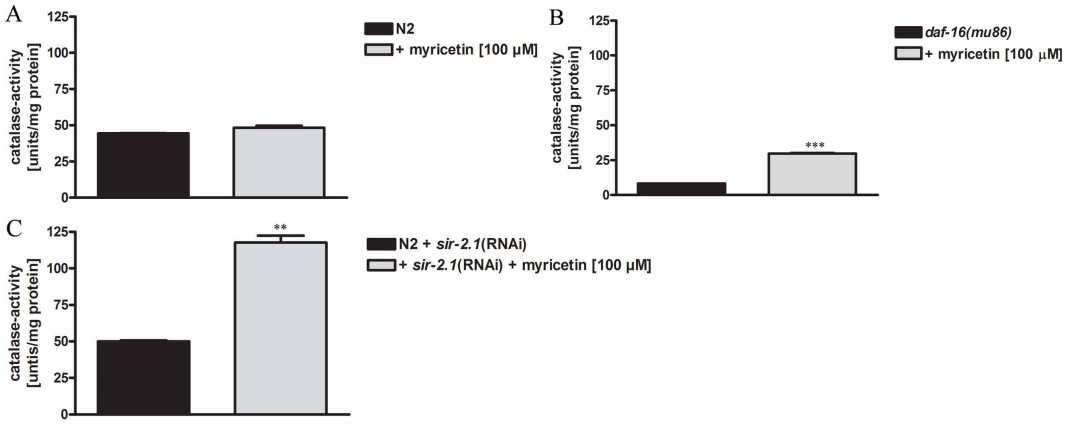

Figure 3.54: **Catalase activity upon myricetin treatment.** Activity of catalase in (A) wild type N2, (B) *daf-16(mu86)* mutants and (C) N2 after *sir-2.1* siRNA treatment after 72 hours incubation in absence (control) or presence of 100 μM myricetin. Enzyme activity was measured in whole worm lysates. Data are given as mean ± SD. ** P<0.01 and *** P<0.001 versus control by ANOVA. Results are from 3 independent experiments.

mutant and in animals treated with *sir-2.1* siRNA. Since myricetin was able to induce increased ROS generation in mitochondria the downregulation of antioxidant mechanisms involving GSH and SOD might cause these effects. However, this seems not to play an effect for life span which only was affected in animals lacking *daf-16*.

3.3.3 Quercetin

Quercetin is one of the most abundant natural flavonoids present in several fruits and vegetables. The daily intake in humans is about 25 mg [35]. Various beneficial effects on human health have been attributed to quercetin including decrease of capillary fragility, a protection against diabetic cataracts [71], antiviral and anti-allergic activities [203], and anti-inflammatory potential [23].
To test direct impact of quercetin on aging life span was measured. Life span remained unaffected upon quercetin treatment in N2, *daf-16(mu86)* and *sir-2.1(ok434)* mutants (fig. 3.55).

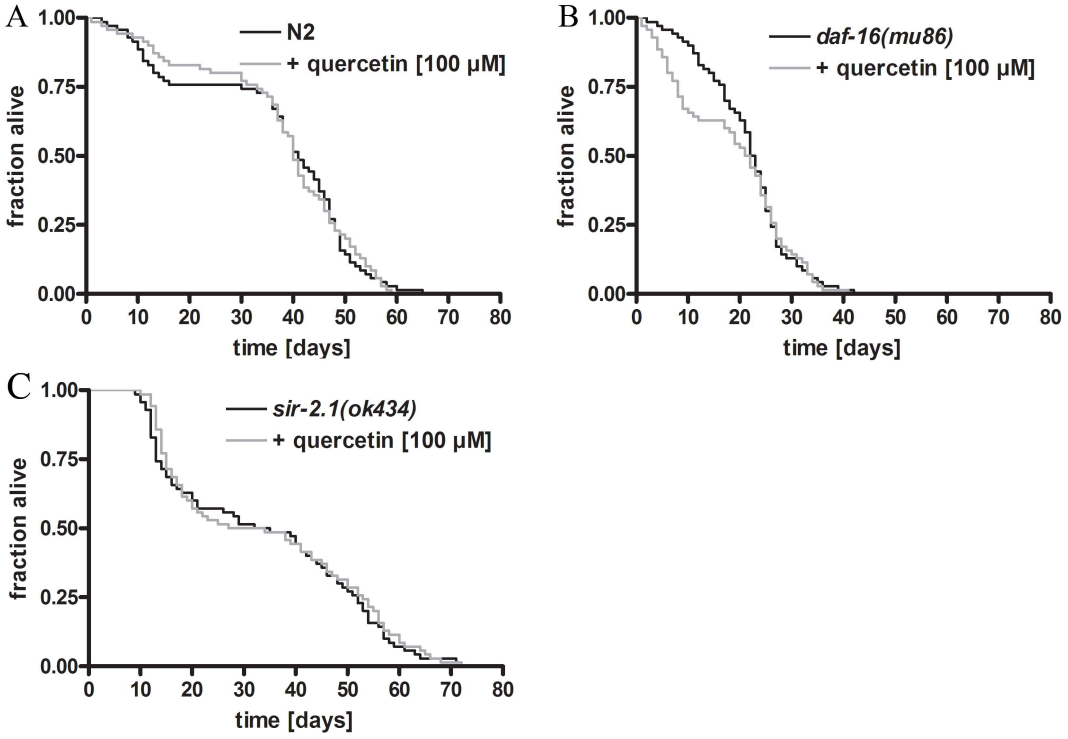

Figure 3.55: **Kaplan-Meier survival curves upon quercetin treatment.** Life span measurements in (A) N2 wild type worms, (B) *daf-16(mu86)* mutants, and (C) *sir-2.1(ok434)* mutants in absence (control) or presence of 100 μM quercetin. Significances of differences in life span versus control were as follows: (A) P=0.9445, (B) P=0.5278 and (C) P=0.6320 by Logrank test.

In CL2070 worms quercetin was able to decrease GFP expression induced by juglone exposure. Upon co-incubation of 100 μM juglone and 100 μM quercetin a reduction in GFP expression was detectable (fig. 3.56 (A)). Even in the time delayed incubation (after 2 hours 100 μM juglone treatment followed for 3 hours 100 μM quercetin treatment in the presence of juglone) quercetin was able to decrease

GFP expression (fig. 3.56 (B)) as potent as when juglone and quercetin were applied simultaneously.

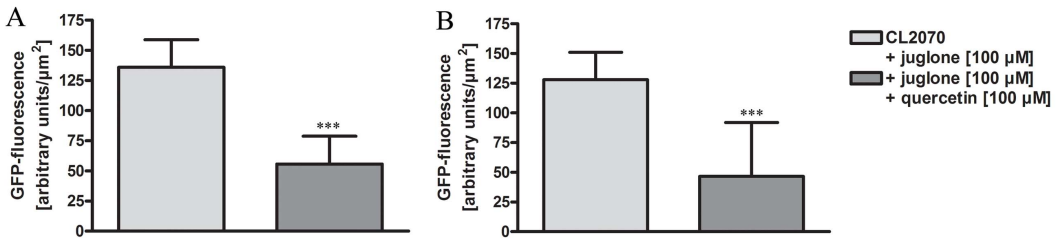

Figure 3.56: **HSP-16.2 expression in CL2070 upon quercetin treatment.** (A) Intensities of GFP expression in CL2070 after incubation in 100 μM juglone alone or in combination with 100 μM quercetin. (B) Intensities of GFP expression in CL2070 after time delayed incubation of 100 μM juglone for 5 hours, 100 μM quercetin was added after 2 hours for 3 hours. *** P<0.0001 versus 100 μM juglone by ANOVA. n=10 from 3 independent experiments.

In analogy to the studies with the other tested secondary plant ingredients, quercetin failed to reduce GFP expression in animals submitted to a heat shock (fig. 3.57) demonstrating that a decrease of HSP-16.2 expression by quercetin is only observed upon ROS-mediated stress employing for example juglone.

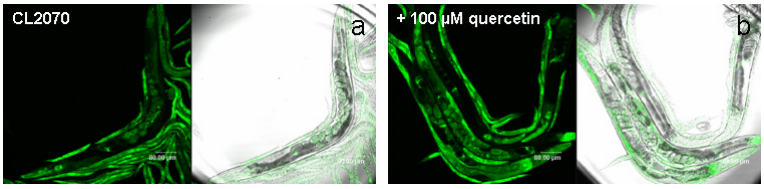

Figure 3.57: **GFP expression in CL2070 induced by heat shock.** GFP expression in CL2070 after incubation for 1.5 hours at 35 °C, then following incubation in CeHR medium for 3.5 hours in (a) absence (control) or presence of (b) 100 μM quercetin at 20 °C. Pictures represent typical results obtained from 3 independent experiments.

ROS-levels in mitochondria determined by the mitochondrial specific ROS-sensitive dye CM-H_2XRos in worms exposed to quercetin did not change (fig. 3.58).

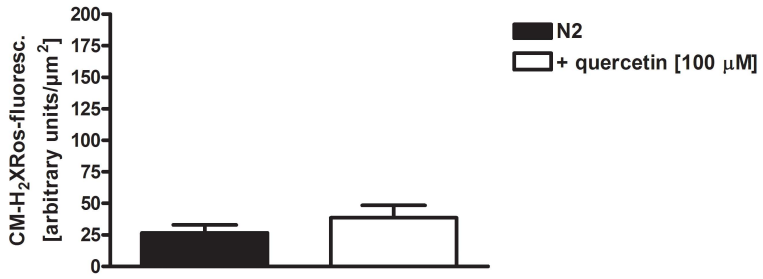

Figure 3.58: **ROS levels in mitochondria upon quercetin treatment.** Fluorescence intensities of the dye CM-H_2XRos in N2 wild type worms in absence (control) or presence of 100 μM quercetin. Incubation time was 24 hours. Data are given as mean ± SD. Results are from 3 independent experiments.

The aging marker lipofuscin was not influenced upon quercetin treatment (fig. 3.59). However, since quercetin did not lead to an increase in ROS production in the mitochondria no accumulation of lipofuscin was expected.

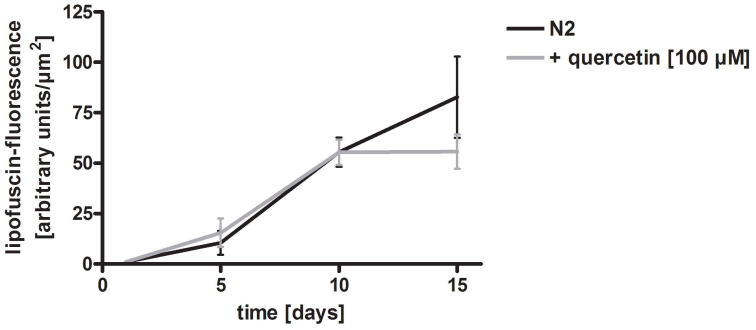

Figure 3.59: **Lipofuscin accumulation upon quercetin treatment.** Intensities of lipofuscin accumulation in wild type N2 worms in absence (control) or presence of 100 μM quercetin. Incubation time was 1, 5, 10, and 15 days. Data are given as mean ± SD. Results are from 3 independent experiments.

As shown in fig. 3.60 quercetin led to a translocation of DAF-16 into the nucleus as indicated by dense nuclear staining but this effect was independent of *sir-2.1* as shown in fig. 3.61.

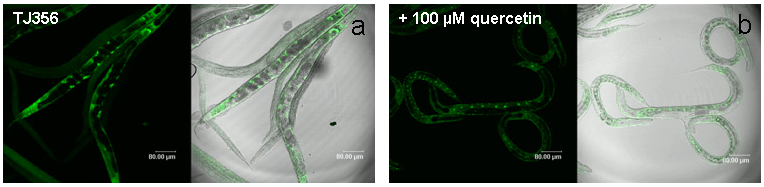

Figure 3.60: **DAF-16 translocation into the nucleus upon quercetin treatment.** Translocation of DAF-16 into the nucleus after 72 hours incubation in (a) absence (control) or presence of (b) 100 μM quercetin. Translocation of DAF-16 into the nucleus is visible by intense staining. Left panels show fluorescence pictures and right panels show overlays of fluorescence and light microscopic pictures. Pictures represent typical results obtained from 3 independent experiments.

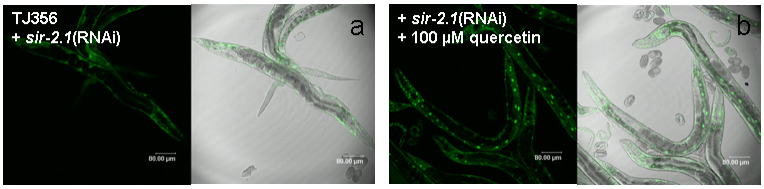

Figure 3.61: **DAF-16 translocation into the nucleus upon quercetin incubation after *sir-2.1* siRNA treatment.** Translocation of DAF-16 into the nucleus after *sir-2.1* siRNA treatment in (a) absence (control) or presence of (b) 100 μM quercetin. Translocation of DAF-16 into the nucleus is visible by intense staining. Left panels show fluorescence pictures and right panels show overlays of fluorescence and light microscopic pictures. Pictures represent typical results obtained from 3 independent experiments.

GSH levels in N2 animals treated with quercetin were significantly reduced and this was proved to dependent on *daf-16* and *sir-2.1* (fig. 3.62). GSSG levels also were reduced suggesting an altered redox state in the animals.

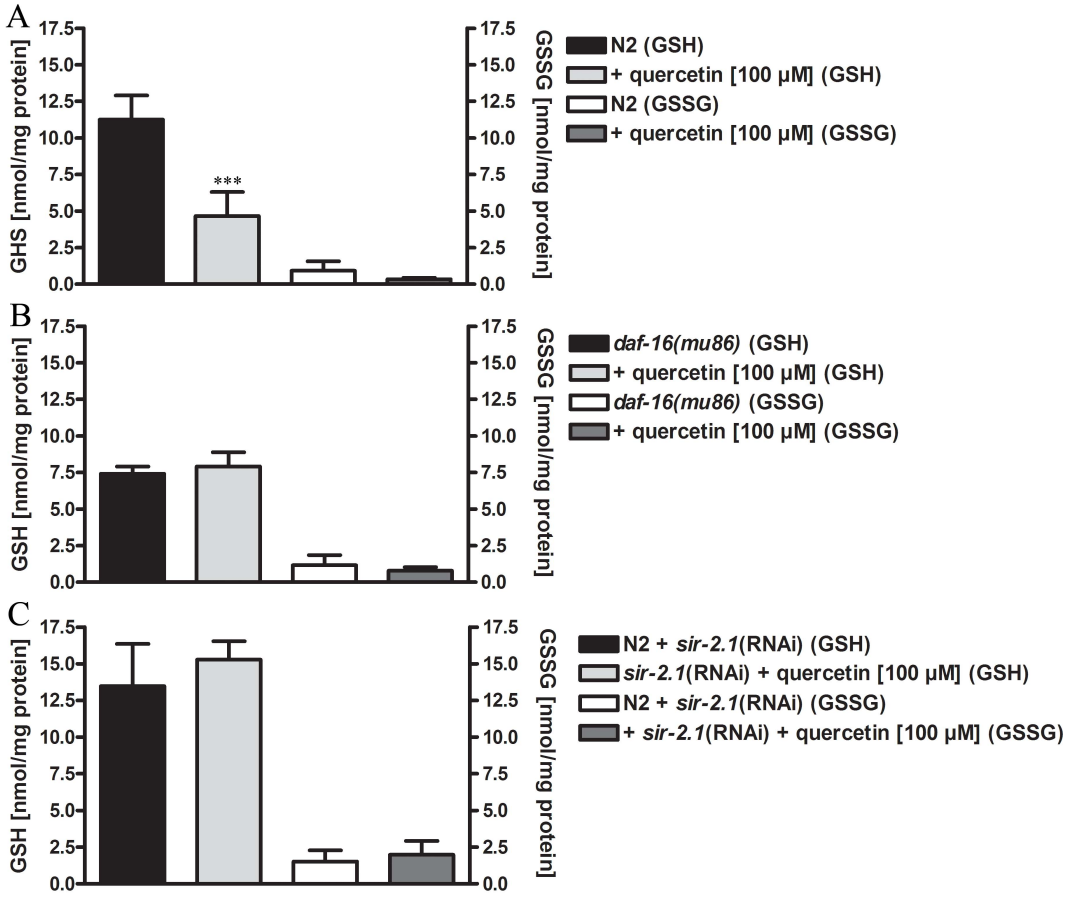

Figure 3.62: **Glutathione levels upon quercetin treatment.** Levels of GSH and GSSG in absence (control) or presence of 100 μM quercetin in (A) wild type N2 worms, (B) *daf-16(mu86)* mutants, and (C) N2 after *sir-2.1* siRNA treatment. Incubation time was 72 hours. Glutathione was measured in whole worm lysates. Data are given as mean ± SD. *** $P<0.001$ versus control by ANOVA. Results are from 3 independent experiments.

Quercetin was also able to increase the SOD-3 reporter protein expression as shown in fig. 3.63.

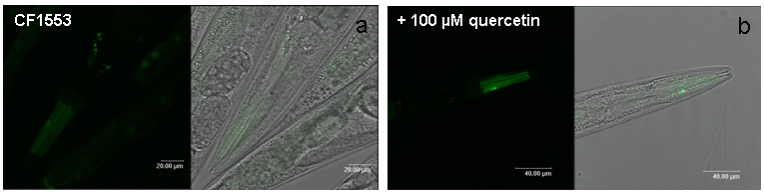

Figure 3.63: **GFP expression upon quercetin treatment in *muIs84* mutants carrying a sod-3::GFP transgene.** *MuIs84* mutants carrying a sod-3::GFP transgene after 72 hours incubation in (a) absence (control) or presence of (b) 100 μM quercetin. Left panels show fluorescence pictures and right panels show overlays of fluorescence and light microscopic pictures. Pictures represent typical results obtained from 3 independent experiments.

Activities of the two tested antioxidative enzymes, superoxide dismutase and catalase, were not altered in wild type animals upon quercetin treatment (fig. 3.64 and fig. 3.65). However, maintaining SOD and CAT activities obviously requires *daf-16* and *sir-2.1* since after *daf-16* knockout and *sir-2.1* knockdown catalytic activity of both enzymes were elevated.

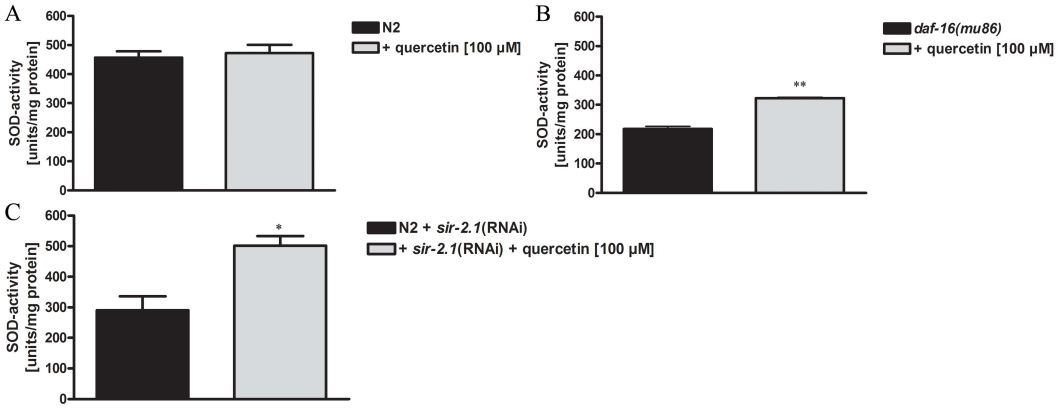

Figure 3.64: **SOD activity upon quercetin treatment.** Activity of SOD and in (A) wild type N2 worms, (B) *daf-16(mu86)* mutants and (C) N2 after *sir-2.1* siRNA treatment after 72 hours incubation in absence (control) or presence of 100 μM quercetin. Enzyme activities were measured in whole worm lysates. Data are given as mean ± SD. * $P<0.05$ and ** $P<0.01$ versus control by ANOVA. Results are from 3 independent experiments.

DAF-16 is important for baseline levels of GSH but this seems not to translate into longevity effects since in N2 animals life span remains unchanged. Activation of SOD induced by quercetin was obviously not able to prevent the slight life span reducing effect in the *daf-16(mu86)* mutant. Induction of *sir-2.1* on the other side

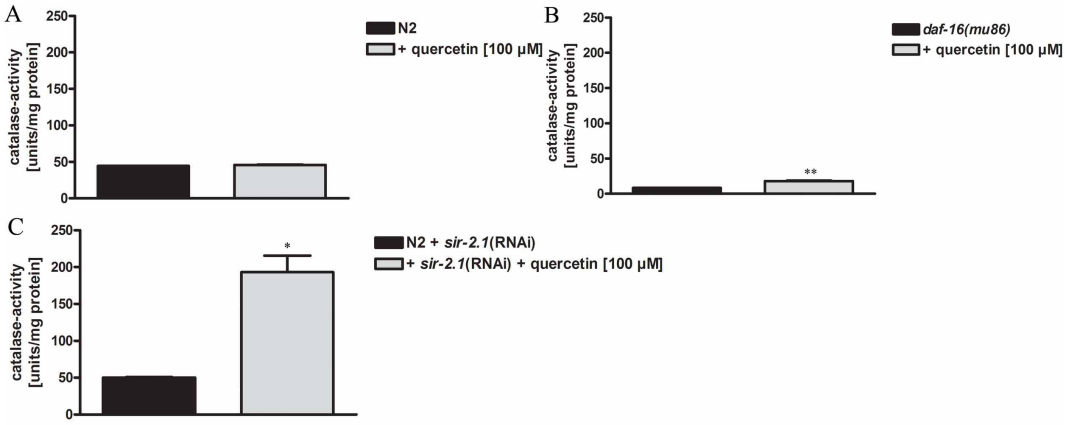

Figure 3.65: **Catalase activity upon quercetin treatment.** Activity of catalase in (A) wild type N2, (B) *daf-16(mu86)* mutants, and (C) N2 after *sir-2.1* siRNA treatment after 72 hours incubation in absence (control) or presence of 100 μM quercetin. Enzyme activity was measured in whole worm lysates. Data are given as mean ± SD. * $P<0.05$ and ** $P<0.01$ versus control by ANOVA. Results are from 3 independent experiments.

was able to prevent the modest life span reduction that observed in *daf-16(mu86)* and siRNA treatment increased both, the activities of SOD and catalase in quercetin exposed animals.

3.3.4 Fisetin

The flavonoid fisetin (3,3',4',7-tetrahydroxyflavone) is widely distributed in food of plant origin including onions, apples, grapes and many more herbal edibles in a concentration of 2 - 160 μg/g [8]. Recently it was shown that fisetin may also be useful to protect from ultraviolet radiation induced cataractogenesis by inhibition of induced oxidative stress and activation of NF-κB and MAPK signaling [220].

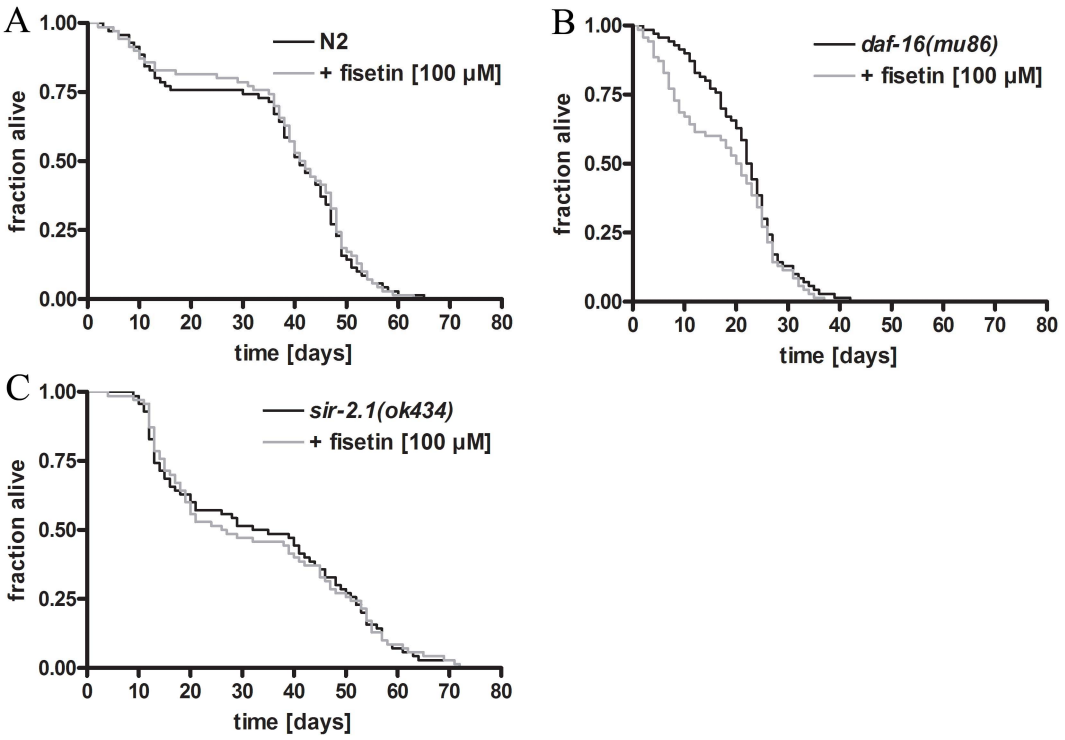

Figure 3.66: **Kaplan-Meier survival curves upon fisetin treatment.** Life span measurements in (A) N2 wild type worms, (B) *daf-16(mu86)* mutants and (C) *sir-2.1(ok434)* mutants in absence (control) or presence of 100 μM fisetin. Significances of differences in life span versus control were as follows: (A) P=0.8225, (B) P=0.1667 and (C) P=0.9995 by Logrank test.

When effects on life span upon fisetin exposure were assessed in all tested worm strains it failed to show an effect (fig. 3.66).
Fisetin treatment was able to reduce *hsp-16.2* reporter expression induced by juglone treatment in the CL2070 line as shown in fig. 3.67 but like the other tested flavonoids and resveratrol it failed to reduce GFP fluorescence when it was induced by heat stress (fig. 3.68) showing that it only works on a ROS-driven response.

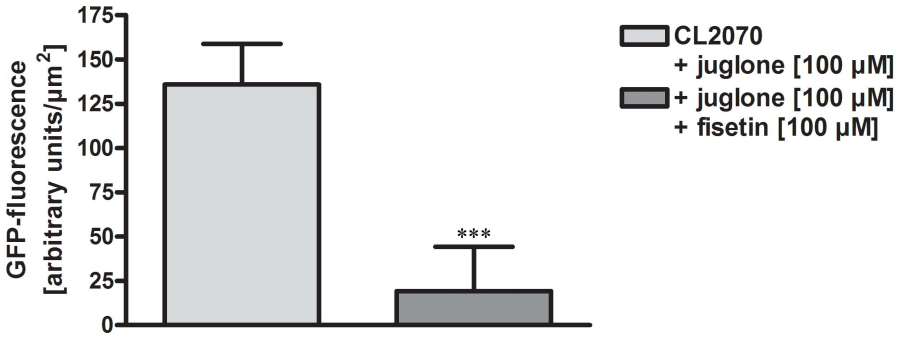

Figure 3.67: **HSP-16.2 expression in CL2070 upon fisetin treatment.** (A) Intensities of GFP expression in CL2070 after incubation in 100 μM juglone alone or in combination with 100 μM fisetin. *** P<0.0001 versus 100 μM juglone by ANOVA. n=10 from 3 independent experiments.

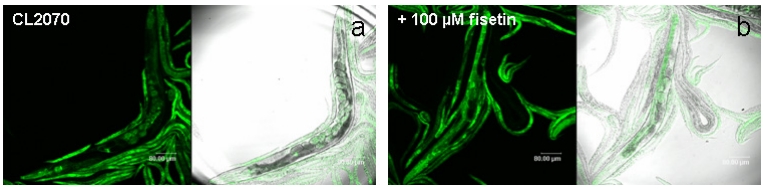

Figure 3.68: **GFP expression in CL2070 induced by heat shock.** GFP expression in CL2070 after incubation for 1.5 hours at 35 °C, then following incubation in CeHR medium for 3.5 hours in (a) absence (control) or presence of (b) 100 μM fisetin at 20 °C. Pictures represent typical results obtained from 3 independent experiments.

N2 animals exposed to fisetin displayed increased ROS levels in mitochondria as demonstrated in fig. 3.69.

Lipofuscin content in fisetin treated wild type animals increased initially above that in controls and decreased slightly towards the end of the experiment (fig. 3.70).

After fisetin treatment a detection of DAF-16 in the nucleus was visible (fig. 3.71) and this effect was independent of *sir-2.1* as demonstrated by lack of an effect on translocation when siRNA for *sir-2.1* was performed (fig. 3.72).

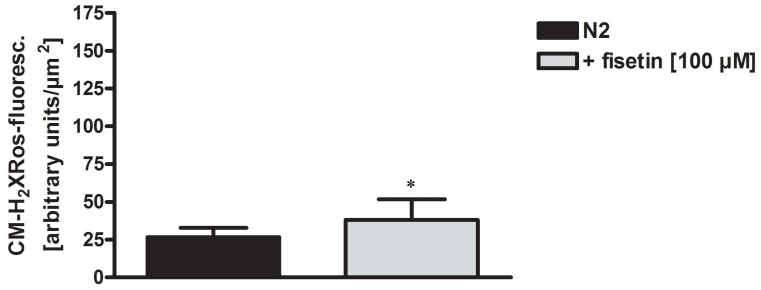

Figure 3.69: **ROS levels in mitochondria upon fisetin treatment.** Fluorescence intensities of the dye CM-H_2XRos in N2 wild type worms in absence (control) or presence of 100 μM fisetin. Incubation time was 24 hours. Data are given as mean ± SD. * P<0.05 versus control by ANOVA. Results are from 3 independent experiments.

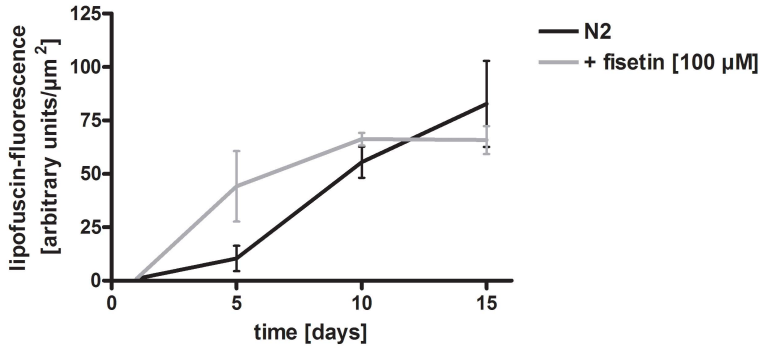

Figure 3.70: **Lipofuscin accumulation upon fisetin treatment.** Intensities of lipofuscin accumulation in wild type N2 worms in absence (control) or presence of 100 μM fisetin. Incubation time was for 1, 5, 10, and 15 days. Data are given as mean ± SD. Results are from 3 independent experiments.

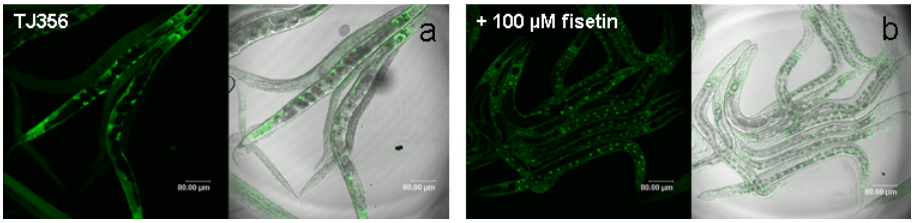

Figure 3.71: **DAF-16 translocation into the nucleus upon fisetin treatment.** Translocation of DAF-16 into the nucleus after 72 hours incubation in (a) absence (control) or presence of (b) 100 μM fisetin. Translocation of DAF-16 into the nucleus is visible by intense staining. Left panels show fluorescence pictures and right panels show overlays of fluorescence and light microscopic pictures. Pictures represent typical results obtained from 3 independent experiments.

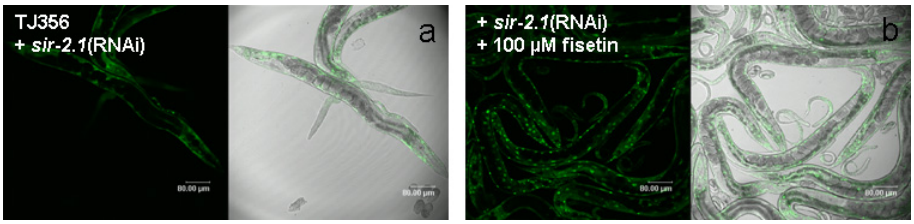

Figure 3.72: **DAF-16 translocation into the nucleus upon fisetin incubation after *sir-2.1* siRNA treatment.** Translocation of DAF-16 into the nucleus after *sir-2.1* siRNA treatment in (a) absence (control) or presence of (b) 100 μM fisetin. Translocation of DAF-16 into the nucleus is visible by intense staining. Left panels show fluorescence pictures and right panels show overlays of fluorescence and light microscopic pictures. Pictures represent typical results obtained from 3 independent experiments.

GSH levels were decreased in wild type N2 and *daf-16(mu86)* after exposure of animals to fisetin and these effects were dependent on *sir-2.1* like in the case of resveratrol, myricetin and quercetin treatment. GSSG levels were only reduced in wild type N2 and *daf-16(mu86)* mutants after fisetin incubation (fig. 3.73).

SOD-3 reporter protein was also increased upon fisetin treatment (fig. 3.74) whereas activities of SOD in all tested worm strains remained unaltered by fisetin exposure (fig. 3.75).

Catalase activity in N2 did not change after fisetin exposure whereas in *daf-16(mu86)* and in N2 after *sir-2.1* silencing an increase was observed (fig. 3.76).

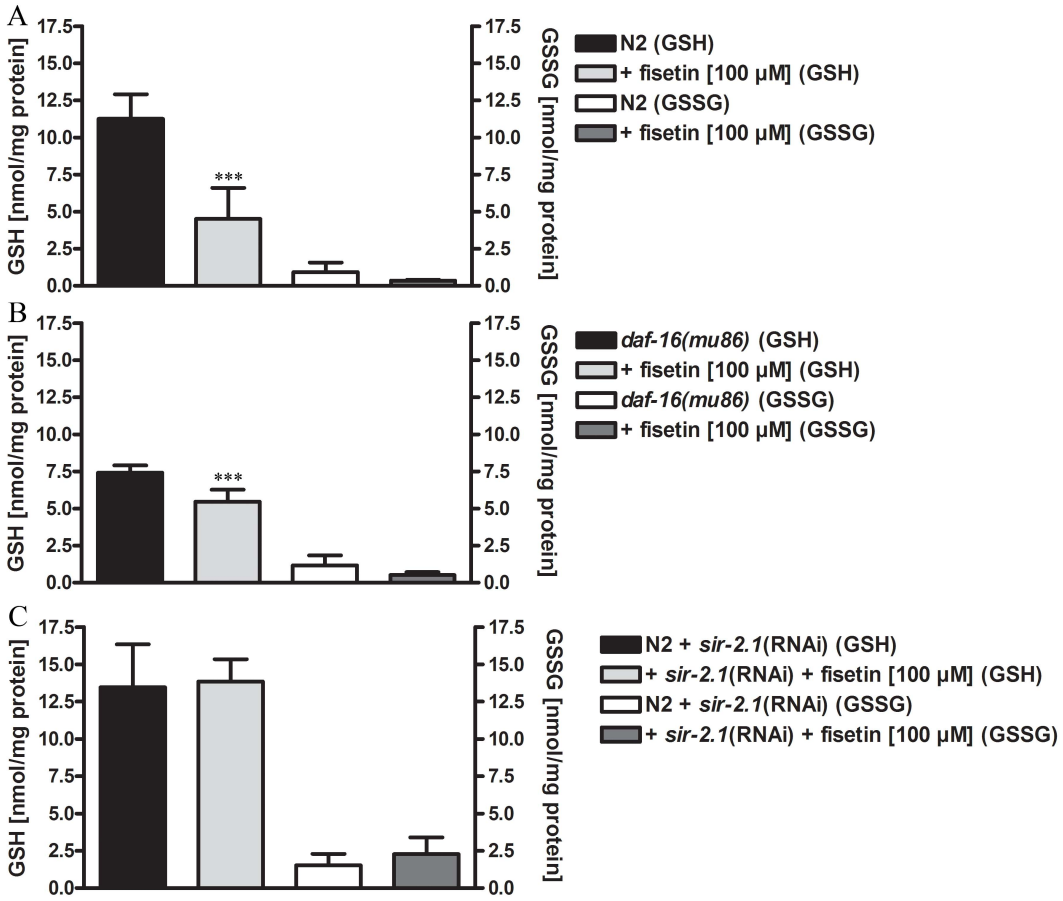

Figure 3.73: **Glutathione levels upon fisetin treatment.** Levels of GSH and GSSG in absence (control) or presence of 100 μM fisetin in (A) wild type N2 worms, (B) *daf-16(mu86)* mutants, and (C) N2 after *sir-2.1* siRNA treatment. Incubation time was 72 hours. Glutathione was measured in whole worm lysates. Data are given as mean ± SD. *** P<0.001 versus control by ANOVA. Results are from 3 independent experiments.

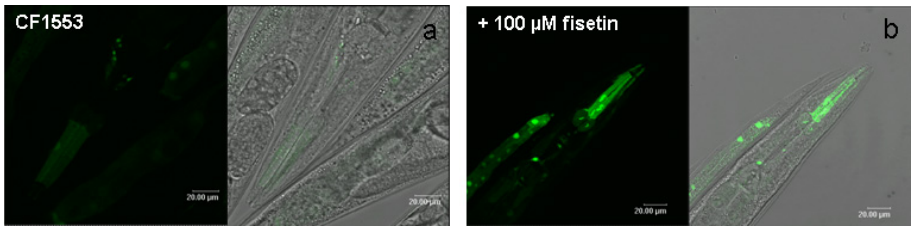

Figure 3.74: **GFP expression upon fisetin treatment in *muIs84* mutants carrying a sod-3::GFP transgene.** *MuIs84* mutants carrying a sod-3::GFP transgene after 72 hours incubation in (a) absence (control) or presence of (b) 100 μM fisetin. Left panels show fluorescence pictures and right panels show overlays of fluorescence and light microscopic pictures. Pictures represent typical results obtained from 3 independent experiments.

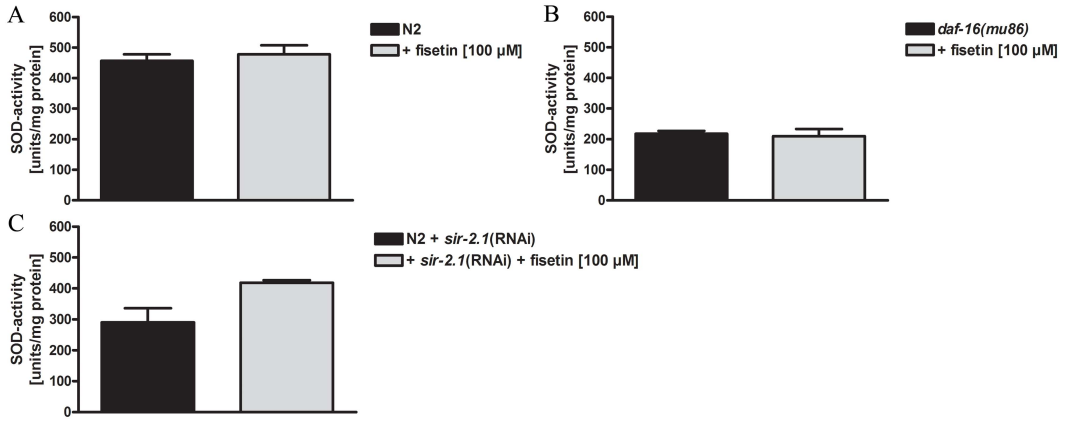

Figure 3.75: **SOD activity upon fisetin treatment.** Activity of SOD in (A) wild type N2 worms, (B) *daf-16(mu86)* mutants, and (C) N2 after *sir-2.1* siRNA treatment after 72 hours incubation in absence (control) or presence of 100 μM fisetin. Enzyme activities were measured in whole worm lysates. Data are given as mean ± SD. Results are from 3 independent experiments.

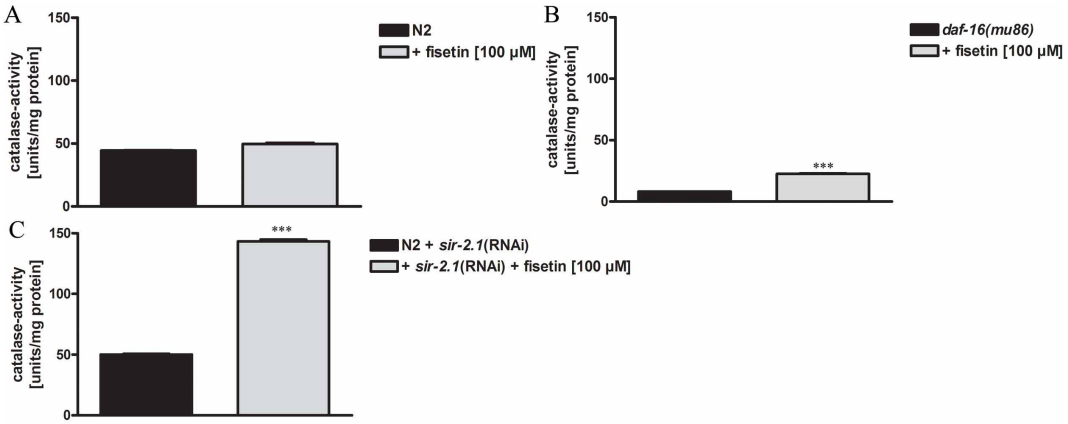

Figure 3.76: **Catalase activity upon fisetin treatment.** Activity of catalase in (A) wild type N2, (B) *daf-16(mu86)* mutants, and (C) N2 after *sir-2.1* siRNA treatment after 72 hours incubation in absence (control) or presence of 100 μM fisetin. Enzyme activity was measured in whole worm lysates. Data are given as mean ± SD. *** $P<0.001$ versus control by ANOVA. Results are from 3 independent experiments.

Fisetin had no effect on life span like myricetin and quercetin. However, it was able to increase mitochondrial ROS levels similar to flavone, resveratrol, and myricetin. GSH-levels and SOD-reporter levels after fisetin treatment changed but SOD activity remained unchanged. The other antioxidative enzyme, catalase, was enhanced by fisetin but only when functions of *daf-16* and *sir-2.1* were suppressed.

In summary, the four flavonoids and resveratrol tested in a comparative approach uniquely showed antioxidative as well as prooxidative capacities. In conclusion it was shown that the here tested polyphenols and flavone had common but also distinct effects. All test compounds were able to decrease hsp-16.2 reporter protein expression when it was induced by the chemical ROS generator juglone. GFP expression under control for the small heat shock protein hsp-16.2 in the CL2070 worm line can be taken as a marker for oxidative stress. Only when ROS levels are increased - but not after a heat stress - compounds were active.
As prooxidants only flavone, resveratrol, myricetin and fisetin were able to increase ROS level of mitochondria in N2 wild type worms. However, only after flavone treatment an increase in GSH content of animals was detectable. With regard to activation of the antioxidative enzyme SOD in N2 animals either no alteration or a decrease was observed in the case of flavone, resveratrol or myricetin. In contrast flavone was able to cause an increase in catalase activity whereas resveratrol reduced CAT activity. All test compounds were able to initiate a nuclear translocation of DAF-16 independent of *sir-2.1*. Lipofuscin accumulation as aging marker was increased by flavone, resveratrol and fisetin treatment in accordance with the enhanced ROS formation in mitochondria. However, all these alterations did not lead to major alterations in life span in N2. Only flavone decreased lifespan and resveratrol increased life span in N2 and these actions were found to depend on *sir-2.1*.

4 Discussion

It is of great interest to understand the aging process and shed more light on the regulation and interactions of signaling pathways to provide a basis for future therapeutic intervention. The model organism *C. elegans* has been instrumental to identify a variety of genes that critically contribute to animals life span. Here different influencing factors on aging like ROS, glucose, and flavonoids were investigated and with focus on two genes, *daf-16* and *sir-2.1*, involved in different or common signaling pathways for aging in *C. elegans*.
An axenic liquid medium for cultivation of worms was used in order to control concentrations of the individual nutritional components and to avoid their metabolic breakdown by bacteria usually used as a food source. However, using liquid media with dead bacteria can result in an increase of life span in *C. elegans* up to 16 % - 40 % [54] [55] and liquid medium without bacteria even caused a two-fold increase in life span [80]. Furthermore, studies documented slow development, increased life span, reduced fecundity, decrease of lipid and protein stores, and gene expression changes in worms cultivated in liquid medium relative to worms on conventional bacterial diet [189] [80]. The axenic liquid medium that was used here is obviously not associated with dietary restriction, reflected by identical median life span of wild type *C. elegans* compared to nematodes that were grown on agar plates with *E. coli*. However, maximum life span is increased in liquid culture conditions, most likely because the proliferation of bacteria and associated bacterial infections cause premature death in older animals [54].

A longevity phenotype in worms correlates in many studies with enhanced resistance against oxidative and other stress [48] [122]. Stress resistance and life span in *C. elegans* can be increased by alterations in insulin/IGF-1 signaling [51] [60] [113], caloric intake [80], mitochondrial respiration [40] [107] [116], and germ line function [9]. Accelerated aging and stress can be caused by free radicals such as reactive oxygen species as was shown here *in vivo* with the ROS generator juglone. Exposure of animals to a high glucose load with an increase in oxygen consumption and likewise enhanced glucose oxidation rates led to an increased delivery of reduction

equivalents to the respiratory chain and consequently to enhanced mitochondrial ROS production. This caused only in *mev-1(kn1)* mutants that already have increased ROS levels by a defect of complex II in the respiratory chain a premature death. Furthermore secondary plant ingredients like flavonoids and resveratrol were investigated in the present study since they are said to be good antioxidants if they can influence life span and how.

4.1 Impact of ROS on aging processes and stress response in C. elegans

Under unfavorable conditions it is essential for an organism to adapt to environmental stressors to secure survival. Those adaptative processes, however, are limited and premature death will occur if protective capacities are overloaded [186]. Otherwise resistance of an organism can be increased by low doses of harmful agents and can also initiate adaptive responses to a consecutive similar challenge. This phenomenon is called hormesis [36] [124]. Among numerous other genes, *daf-16* has been identified in *C. elegans* to play a crucial role in response to various stressors [110] [119]. As key targets of DAF-16, genes coding for small heat shock proteins were identified [83]. Another gene, *sir-2.1*, is also involved in stress response and longevity [16]. Life span extension caused by overexpression of *sir-2.1* was completely dependent on DAF-16, demonstrating a close interaction of those genes. It was deduced that SIR-2.1 has to act upstream of DAF-16 in the *daf-2* signaling pathway or in a parallel pathway that converges to DAF-16 to enhance stress resistance and life span extension. This proposed mode was based on findings demonstrating that a *sir-2.1* deletion did not alter life span in the long lived *daf-2* mutant [210].
Here it was investigated how different concentrations of the ROS generator juglone can influence life span if they were constantly given for the whole life. The adaptations observed in worms exposed to 40 μM juglone were associated with an increased life span in wild type N2 which was fully dependent on *daf-16* since this effect was totally blocked after loss of functional *daf-16*. A moderate juglone concentration of 100 μM had no effect on life span whereas the high concentration of 250 μM caused premature death in wild type N2 and a *daf-16(mu86)* mutant line.
Small heat shock proteins (sHSPs) like HSP-16.2 show generally expression under stress conditions [117] and it was demonstrated in a study of GuhaThakurta et al.

that heat shock factor (HSF) and possibly other transcription factors control *hsp-16.2* induction in response to heat stress [64]. In *C. elegans* the 16 kDa (HSP-16.1, HSP-16.2, HSP-16.41 and HSP-16.48) HSPs are the major forms and are only expressed under stress conditions [117]. Murphy et al. showed that *hsp-16.2* is the transcriptional target of *daf-16* [147] and induction of sHSPs expression causes protection against stress by binding to altered proteins to prevent their aggregation, catalyzation of renaturation and/or supporting repair processes [168]. This all characterize sHSPs as molecular chaperons [78]. Studies of Rea et al. showed that increased expression levels in *hsp-16.2* on the first day of adult life correlates with increased life span as much as fourfold variation [165]. In the present work juglone was able to increase expression of *hsp-16.2* and also to increase life span in the lower concentration of 40 μM. sHSPs are able to modulate intracellular glutathione levels in murine cell culture [132] and in worms [205], similar to what in the present work was shown after 40 μM and 100 μM juglone treatment since expression of *hsp-16.2* and GSH levels were increased. Moreover, the results in the present work further substantiate the crucial role of *daf-16* for the induction of small heat shock proteins which in turn promote longevity and that the stress response to ROS follows the same pathways as shown for stress caused by heat [83] or hypertonic stress [110].
In the present study juglone was able to enhance activity of the antioxidative enzymes, superoxide dismutase and catalase, which also contribute to prolonging life span since also in long lived *daf-2* mutants elevated levels of superoxide dismutase and catalase were measured [113] [198]. An enhanced stress tolerance is detectable after pre-treatment with juglone or heat stress and in addition both conditions provide cross-protection against one another [36]. By using synthetic superoxide dismutase or catalase mimetics an increased longevity with a concomitant reduction of ROS generation is observed [133]. Castro and colleagues showed that most *C. elegans* mutants which were selected for their resistance to juglone were resistant against other stressors as well and also long lived with mean increases in life span ranging from 11 % to 35 % [29].
In the study here juglone translocated DAF-16 into the nucleus to promote expression of downstream target genes. To underline the impact of different genes on the downstream target HSP-16.2 RNAi experiments were performed in the present study. Knockdown of DAF-16 by RNAi treatment caused a reduction of GFP expression in CL2070 induced by juglone incubation. This indicates that DAF-16 is involved in the juglone induced stress response. But not only *daf-16* was necessary for upregula-

tion of *hsp-16.2* but also *sir-2.1* and the 14-3-3 proteins since after RNAi treatment expression was totally repressed after stress induction. The fact that juglone triggers translocation of DAF-16 into the nucleus emphasizes this result. DAF-16 seems to play a crucial role in expression of small heat shock proteins.

Knockdown of *sir-2.1* by RNAi treatment demonstrated here that DAF-16 translocation into the nucleus induced by the ROS-generator juglone was independent of SIR-2.1 in *C. elegans*. Experiments with the strain CL2070 showed indirectly that juglone treatment leads to elevated ROS generation *in vivo*. Enhanced ROS generation has been demonstrated to cause damage in several macromolecules [196] and there is a relationship between the ability to prevent or repair chronic damage of macromolecules and longevity [214] [221].

In analogy to a heat stress with temperatures too high, animals died when high juglone concentration of 250 μM were applied suggesting a limited capacity for adaptive changes [93] [154]. Olsen et al. showed that a mild stress induction early in life is repeated at later stages will result in a beneficial response. If the heat treatment is only later in life the beneficial effect will not be induced. This suggests that early in life enhanced chaperon levels are sufficient to impact survival [154]. Dillin et al. showed that loss of insulin signaling results in the largest increase of life span when it occurs during early adulthood [40]. Here it was shown that juglone treatment was started when worms were in the young adult stage. This led only in the low concentration to an increase in life span.

In conclusion it was demonstrated that not only a short acute stress can induce hormesis in *C. elegans* but also a life long exposure to a low concentration of a ROS generator. The life span prolonging effect is associated with an increased expression level of selected DAF-16 downstream targets such as *hsp-16.2* which in turn controls GSH levels and requires SIR-2.1 and 14-3-3 proteins for proper function. Activities of antioxidative enzymes such as catalase and superoxide dismutase were also elevated upon low dose juglone treatment. The beneficial effect of juglone exposure is only detectable with low to moderate concentrations. However, the stress response capacity is limited and leads to premature death by a ROS overload.

4.2 Effects of high glucose load on aging and stress response in C. elegans

Caloric restriction has proven to prolong live span in *C. elegans* [80]. In contrary, a hyperglycemic state is considered to induce an increase in electron transfer donors (NADH and $FADH_2$) which leads to a higher electron flux through the mitochondrial electron transport chain resulting in hyperpolarization of the mitochondrial membrane potential and partial inhibition of the electron transport in complex III which leads to an accumulation of electrons to coenzyme Q. This provokes the partial reduction of oxygen to generate superoxide anions [150] [25].

In the present work it was consequently tried to simulate such a metabolic condition in *C. elegans* by exposing animals in the liquid medium to high glucose concentrations (high glucose loads). With this approach it was demonstrated that glucose concentrations of 100 mM and 250 mM led in the wild type N2 to an increase in mitochondrial ROS generation. The enhanced ROS production caused by higher glucose intake was paralleled by a higher oxygen consumption rate in N2 worms. Since wild type N2 showed no alteration in life span after incubation in 100 mM glucose and even an increase after 250 mM also a *mev-1(kn1)* mutant was used in this work to provide a good system to test the effects of elevated oxidative stress by higher energy intake induced by glucose treatment. *Mev-1(kn1)* mutant worms show a reduced life span along with other well documented phenotypes that are linked to elevated ROS generation in mitochondria [87] [180]. Mitochondrial ROS levels are increased in *mev-1(kn1)* mutants and were further increased as in the wild type by the high glucose load. But in contrast to wild type life span was decreased in this mutant after exposure to 100 mM and even stronger to 250 mM glucose. These findings also suggest that a certain threshold level in ROS production needs to be reached until life span reducing activities can be detected. In this respect all antixodative response pathways become important with their capacity to maintain this sub critical ROS-level.
Lipofuscin a heterogenic molecule accumulates with age and is therefore considered to serve as a surrogate aging marker [103]. Despite the enhanced ROS levels in mitochondria in *mev-1(kn1)* mutants exposed to high glucose loads lipofuscin levels did not change for all tested glucose concentrations.
Despite enhanced oxidative stress the tested antioxidant mechanisms behaved in an

inhomogeneous manner with the most striking finding that superoxide dismutase activity was even lower at glucose concentration of 250 mM in wild type N2 animals with a modest increase in life span and in *mev-1(kn1)* mutants in which life span was reduced by high glucose. GSSG levels were increased upon glucose incubation leading to a reduction in GSH/GSSG ratio compared to controls in both worm strains, N2 and *mev-1(kn1)* mutants demonstrating altered redox-states. Catalase levels were elevated after treatment with high (250 mM) concentrations of glucose in N2 and *mev-1(kn1)* mutants. Since an enhanced activity of catalase can prolong life span in worms [179], the increased activity levels at 250 mM glucose may provide the basis for the small increase in life span of N2 animals. However, in *mev-1(kn1)* animals, elevated catalase activity was not able to prevent live span decreasing effect of 250 mM glucose exposure although the response was stronger in this mutant line as in wild type animals. Yanase reported that the mRNA levels of *sod-1*, *sod-2*, *sod-3* and *sod-4* were reduced while *ctl-1* and *ctl-2* levels were increased in *mev-1(kn1)* mutants compared to wild type N2 animals [219]. Corresponding to these observations, here was also found a reduced activity of superoxide dismutase while catalase activity was increased in *mev-1(kn1)* in comparison to activities in wild-type animals.

One of the most interesting findings on the effects of high glucose load was obtained in the strain CL2070. Juglone clearly induced *hsp16.2*-driven GFP expression in CL2070 as a surrogate response to increased ROS levels and this response was decreased by glucose treatment. Activation of the *daf-2* system in *C. elegans* causes a retainment of DAF-16 in a cytosolic protein complex and this reduces or prevents the activation of gene transcription of target genes that are essential for stress response and longevity. Translocation of DAF-16 into the nucleus promotes expression of several stress response genes including *hsp-16.2*, *sod-3*, *ctl-1* and *ctl-2* [118] [147]. In the present work was shown that a high glucose load inhibits translocation of DAF-16 into the nucleus even in the presence of juglone. This counterbalancing effect of DAF-2 activation may also explain there was a blunted antioxidative response despite the increased mitochondrial ROS levels observed. Only catalase activity showed an increase after 250 mM glucose treatment while glucose inhibits at 100 mM already the adaptative response to the increased mitochondrial ROS levels but this is without an effect on life span. Only in *mev-1(kn1)* mutants a life span reduction is observed upon high glucose loads and higher glucose concentrations (250 mM) lead to an increased catalase activity but reduced activity of superoxide dismutase. Whereas this caused premature death in *mev-1(kn1)* animals it prolonged modestly

life span in wild type animals. It seems that the higher tested glucose concentration of 250 mM induced additional stress which is not based on mitochondrial ROS production since 250 mM enhanced ROS levels in mitochondria to a similar level as 100 mM. This caused life span prolonging effects in N2 which are not or only in an insufficient amount available in *mev-1(kn1)* mutants.

Since normal life span in *mev-1(kn1)* is reduced compared to wild type worms Ishii et al. applied the antioxidant vitamin E to rescue life span reducing effect caused by the mutation in the *mev-1* gene. However, the reduced life span can not be prolonged by treatment with vitamin E [88]. In contrast, as shown in this study vitamin C, that was not able to prolong life span in *C. elegans* wild type worms [69], was able to rescue the life span decreasing effect caused by glucose in *mev-1(kn1)* mutants. According to the "Mitochondrial Threshold Effect Theory" [170] the cell can cope with a certain degree of mitochondrial dysfunction, most likely through activation of compensatory mechanism. Once beyond the threshold, pathology and life span decrease like in the case of elevated glucose concentrations in the *mev-1(kn1)* mutant ensues.

That ROS generation in mitochondria are responsible for -or at least contributing as a major driver - to aging processes can also be observed in mammalian systems. The role of mitochondrial ROS production and scavenging was addressed by Schriner et al. demonstrating in mice that animals that overexpress human catalase show an increased life span only if the enzyme is targeted to mitochondria. If overexpression of catalase was targeted to the peroxisomes or to the nucleus transgenic mice showed only a slight increase in median life span [178]. The mechanisms by which the mitochondrial ROS status translates into alterations of the aging phenotype are not yet understood. Mitochondrial ROS can cause dysfunctions in mitochondrial proteins and DNA and this can secondarily further increase ROS production which will eventually lead to nuclear DNA damage and senescence [30].

How exogenous antioxidants could affect the ROS-induced damage to macromolecules and the life span has been assessed in a variety of models. In *C. elegans* Keaney et al. applied the synthetic catalytic antioxidant EUK-8 and demonstrated that it was only able to prolong life span in the presence of the superoxide generator paraquat while it was without any effect on life span in wild type N2 worms when EUK-8 was given in the same doses in absence of paraquat [100]. Some antioxidants show a beneficial effect on life span only after induction of oxidative stress, but not under normal culturing conditions. One explanation for that phenomenon could be

that under normal conditions ROS levels are maintained at an optimal set point. A reduction below this point may be deleterious because ROS also function in intracellular signaling or immune defense [138]. Glutathione and vitamin C show a strong functional interdependence in vivo. In the study of Montecinos et al. vitamin C is able to recover the glutathione content [139]. Here it was also shown that ascorbate was able to increase glutathione levels in glucose treated *mev-1(kn1)* mutants. Glucose incubation alone decreased GSH/GSSG ratio in comparison to control worms. Since glucose completely suppressed DAF-16 translocation into the nucleus no beneficial effects of this transcription factor like increased longevity were seen in *mev-1(kn1)* mutants. Neither glutathione levels nor superoxide dismutase or catalase activity were upregulated. By adding ascorbic acid to the extra glucose group the GSH/GSSG ratio is markedly increased, thus rescuing the deleterious effect on life span. In this respect these findings may provide an interesting link to alterations in ROS-status and signaling in the diabetic state.

Mutations in genes which regulate mitochondrial biogenesis have been associated with type 2 diabetes [208]. In diabetes mitochondrial proteins are not directly affected but there is a secondary dysregulation of mitochondrial function responsible for the phenotypic outcome [200]. In diabetes the primary site of superoxide generation is altered so that complex II of the respiratory chain in mitochondria becomes the primary source of electrons and not as under normal conditions complex I and III. This contributes to superoxide formation under diabetic conditions [150]. Yamagishi et al. addressed the high ROS formation under diabetic conditions in their study where they used the complex II inhibitor 2-thenoyltrifluoroacetone and an uncoupler of oxidative phosphorylation, carbonyl cyanide m-chlorophenyldihydrazone. This led to a decrease in ROS generation in several cells exposed to high glucose concentrations [218]. In diabetic patients vitamin C levels are found to be decreased [197]. Koo et al. showed a benefit of antioxidant therapy together with insulin treatment in diabetic rats [105]. This suggests an imbalance of pro- and antioxidant actions in the disease state and the present work simulates such a situation in worms. In the *mev-1(kn1)* mutant *C. elegans* line ROS-levels are increased by a malfunction of complex II [87] and as it was shown here, ascorbic acid under these conditions is beneficial by reducing ROS-levels in mitochondria and by increasing GSH levels.

In conclusion it could be shown that enhanced supply of glucose caused increased

mitochondrial ROS production in association with increased respiration but without alterations in life span in the lower concentration (100 mM) and a modestly increase in the higher concentration (250 mM) in the wild type N2 of *C. elegans*. In the *mev-1(kn1)* mutant line where endogenous ROS levels in mitochondria are already elevated under normal conditions life span was reduced by both tested glucose concentrations. The concomitant activation of the insulin/IGF-1 pathway by glucose is blunted and inhibits the beneficial response mechanisms mainly mediated by DAF-16 which normally increases ROS scavenging capacity. Under these conditions an exogenous antioxidant such as ascorbic acid can rescue the effects of a high glucose load on life span shortening in *mev-1(kn1)* animals. These data here all support the notion that a "critical ROS level" needs to be maintained for proper organismic function and that there is a high "endogenous buffering" capacity for ROS and only when these processes are overruled by extensive ROS generation, deleterious effects leading to a life span reduction is observed.

4.3 Effects of flavonoids and resveratrol on aging and stress response in C. elegans

Flavonoids and especially resveratrol have been intensively investigated in mammalian cell models *in vitro* in recent years and were shown to alter numerous biological processes depending on the substance, its concentration and the cell type if they bear positive or negative effects [215] [211] [137]. They are polyphenolic secondary plant ingredients and are involved in defense processes against ultraviolet radiation or act as phytoalexins (a class of antibiotics of plant origin). Polyphenolic compounds have antioxidant properties and are therefore considered to prevent various diseases which are associated with oxidative stress, like cancer, cardiovascular and neurodegenerative diseases [175]. Numerous compounds have also been studied with respect to aging. Flavonoids and also resveratrol are able to stabilize free electrons obtained from free radicals because of their chemical structure in *in vitro* systems [67] [163]. Since ROS are considered to accelerate physiological aging as well it was plausible to conclude that flavonoids and resveratrol might affect aging and life span. All flavonoids and resveratrol tested here displayed antioxidative capacity *in vivo* in *C. elegans* by their capability to reduce ROS-driven HSP-16.2 expression. Another antioxidative mechanism by flavonoids is the inhibition of superoxide anion production by xanthine oxidase [67]. Interestingly, the flavonoids and resveratrol were only

able to act as antioxidants in CL2070 when the stress was induced by the ROS generator juglone but not by heat. The same effect was shown by feeding artificial antioxidant Euk-8 and Euk-134 in a study of Sampayo et al., only when ROS status was increased by juglone, the antioxidants were active [172].
But polyphenols and flavone have also a prooxidant capacity. Flavonoids for example can also serve as prooxidants to induce apoptosis since they are able to bind to the antiapoptotic proteins BCL-2 and BCL-xL and e.g. epigallocatechin-3-gallate was also shown to produce H_2O_2 by autooxidation when it was added to a cell culture system (reviewed in [109]). For the compounds assessed here, except for quercetin all were able to exert a kind of stress in the nematodes by increasing ROS levels in mitochondria.
The ROS-production in turn activates a pleiotropic program to increase the endogenous stress resistance and DAF-16 here plays a prominent role [77]. DAF-16 is able to respond rapidly to a variety of environmental stressors such as starvation or oxidative stress by moving from the cytoplasm to the nucleus followed by activation of transcription of numerous target genes [73]. As a matter of fact DAF-16 was translocated into the nucleus after polyphenole and flavone treatment for only three hours in the strain TJ356. This is supposed to provide enhanced stress resistance as genes such as superoxide dismutase, catalase and heat shock proteins are transcriptional targets of DAF-16 [147].
Recent reports provide evidence that resveratrol also promotes beneficial effects in mammals via similar mechanisms. It increases life span and can protect from obesity in mice given a high-caloric diet by increasing insulin sensitivity, enhancing AMP-activated protein kinase and PGC-1α activity, increasing the number of mitochondria, and enhancing aerobic capacity [14] [106]. However, these effects can only be detected when pharmacological doses of the compound were provided. Since resveratrol is the "ultimate" sirtuin activator, this pathway has obtained dominant attention recently.
A downstream target of SIRT1 is the tumor suppressor protein TP53, a transcription factor that regulates the expression of genes including p21 and Bax to initiate cell-cycle arrest, apoptosis or senescence under conditions of chronic stress (reviewed in [174]). In addition SIRT1 deacetylates a huge number of cellular targets, including E2F1, NF-κB, FOXO proteins, PGC-1α, Ku70, and the HIV transcription factor Tat. Their activation results in modulation of cellular responses to a variety of stresses including fasting, DNA damage, and infection to promote enhanced stress

resistance and longevity (reviewed in [201]).
In the present study the close functional link of SIR-2.1 and DAF-16 was further substantiated. It was demonstrated here that a *sir-2.1* knockout has no effect on life span but basal activity levels of superoxide dismutase were fully dependent on *daf-16* and *sir-2.1* with a concomitant decline in activity after *daf-16* knockout or *sir-2.1* knockdown. For basal catalase activity only *daf-16* seems essential as catalase activity was not altered after *sir-2.1* knockdown. Basal GSH levels, however, were influenced by both investigated genes in an opposite direction since after *daf-16* knockout levels were decreased and after *sir-2.1* knockdown levels were increased.

DAF-16 and SIR-2.1 do not only play a role for basal superoxide dismutase, catalase and GSH levels but also for life span affecting interventions. Whereas this is of no importance for almost all of the flavonoids investigated, as they did not affect life span, it is important for the life span reducing response to flavone. Flavone reduced life span depending on *sir.2.1* inhibition but independent of *daf-16* because life span reductions were observed by flavone in the *daf-16* mutant strain CF1038 but not after knockout of *sir-2.1*. The prooxidant character of flavone was delineated by enhanced ROS levels in mitochondria and associated with increased oxygen consumption and increased content of the aging marker lipofuscin. In common to juglone, where increased ROS production was answered by increased GSH levels flavone was also able to enhance levels of the reduced form of glutathione. Whereas in N2 wild type nematodes GSH levels were increased by flavone these alterations were prevented after *daf-16* knockout and *sir-2.1* knockdown. Superoxide dismutase activity was decreased by flavone with an interference of *daf-16* and *sir-2.1* as far as levels in *daf-16(mu86)* mutants were not altered and after *sir-2.1* RNAi treatment were increased. Catalase activity appeared upregulated independent of *sir-2.1* but dependent on *daf-16* and this is line with the effects of flavone on activation of *daf-16*. This suggests that flavone can influence both *daf-16* and *sir-2.1* by activating the first and inhibiting down stream *sir-2.1*.

Most of the flavonoids investigated had no influence on life span and myricetin e.g. reduced life span only in a *daf-16* mutant strain, suggesting that its life shortening effects were compensated by activation of DAF-16. Amongst those that did not affect life span was also quercetin that had been described by Kampkötter et al. to extend life span under stress [98]. Interestingly, quercetin was the only flavonoid

tested that did not increase mitochondrial ROS levels but decreased GSH levels and had no influence on activities of superoxide dismutase and catalase. Therefore, overall antioxidant status showed to be decreased or not influenced and lipofuscin levels were slightly decreased upon quercetin exposure. Quercetin was demonstrated to inhibit a number of kinases like CK2, AMPK and PI3K and to reduce the kinase activity to approximately 20 % of control values [39]. The inhibitory activity of quercetin and likewise myricetin is associated with its capability to compete with bindings of ATP to the nucleotide binding site on the kinases. Studies of quercetin binding to phosphoinositide 3-kinase have revealed that quercetin, myricetin and related kinase inhibitors are able to bind in the ATP-binding pocket of the kinase [204]. Taken to the worm model, this may suggest that quercetin and myricetin are able to influence activation of DAF-16 and probably also SIR-2.1 since activity of superoxide dismutase and catalase, and GSH levels were not altered or slightly decreased in dependence on *sir-2.1* and/or *daf-16* in the present study.
Kampkötter et al. showed that fisetin was able to cause translocation of DAF-16 into the nucleus as was shown here. It was able to reduce lipofuscin content after 72 hours incubation time like it did here [98]. In the fruit fly *D. melanogaster* fisetin as well as resveratrol were able to increase life span when provided in a concentration of 100 μM [217] whereas in the present study fisetin failed to increase life span in *C. elegans* whereas resveratrol did.

Resveratrol treatment of *C. elegans* led to a life span extension and this was proven to depend on *daf-16* and *sir-2.1* since in *daf-16(mu86)* and *sir-2.1(ok343)* mutants life span was not altered when they were exposed to resveratrol. Interestingly, its action was obviously prevailed via a prooxidant mechanism with enhanced mitochondrial ROS levels and also a higher lipofuscin content observed in *C. elegans*. However, GSH levels were decreased by resveratrol treatment but remained unaltered when *daf-16* was knocked out or *sir-2.1* was knocked down. Furthermore, superoxide dismutase activity was inhibited by resveratrol which was not observed in *daf-16(mu86)* mutants and catalase activities were reduced as well. Flavone and resveratrol had in part similar capacity with regard to influencing the antioxidative systems but had different properties on life span. The measured antioxidative mechanisms were not important for life span. In contrast to these findings *sir-2.1* activity seems to be crucial for life span extension by resveratrol whereas its inhibition by flavone seems to be necessary for life span reduction.

For resveratrol was previously shown that is also able to activate another transcription factor which is involved in antioxidant response mechanisms, *nrf2*, the homolog of the *C. elegans skn-1* gen. Nrf2 is localized in the cytoplasm by binding to an inhibitory protein, KEAP1 [115]. Resveratrol can activate Nrf2 signaling via mechanisms that include activation of upstream kinases like ERK1/2 and Akt [31].

The fact that *daf-16* is essential for life span extension in worms has been shown previously by *sir-2.1* overexpression [192]. It was reported that *C. elegans* SIR-2.1 forms a complex with a conserved protein family 14-3-3 encoded by *par-5* and *ftt-2*. In mammalian cells 14-3-3 proteins are suggested to interact with FOXO the mammalian DAF-16 homolog which results in a retention of FOXO in the cytoplasm in an inactive state [26]. In a study of Berdichevsky it was shown that *daf-16* RNAi fully suppressed life span extension of *daf-2* mutants. However, *par-5* and *ftt-2* in worms failed to contribute to the activation process suggesting that sir-2.1 and the 14-3-3 proteins act upstream or in parallel to the insulin/IGF-1 signaling pathway [17]. It is not surprising so far that life span extension by flavone is not achieved in spite of nuclear translocation of DAF-16. The importance of such downstream regulations for the function of DAF-16 but also SIR-2.1 was shown for the small adaptor proteins of the 14-3-3 class.
There are, however, also hints that *sir* family proteins are not required for the life span extension caused by dietary restriction in some *S. cerevisiae* strains [94] [194], whereas in other studies resveratrol is able to stimulate SIR2 and to extend life span [81]. Another group reported recently that resveratrol does not in fact activate *sir2 in vivo* [21] [95]. Resveratrol is able to activate independent of *sir2* AMP kinase and has neuroprotective effects in Neuro2a cells and primary neurons in vitro as well as in the brain [38]. This indicates that resveratrol is able to exert some of its biological effects through pathways which are unrelated to SIR2.
Nevertheless in this work it was shown that the life span prolonging effect of resveratrol was fully dependent on *sir-2.1*. This becomes even more evident since mRNA levels and SIR-2.1 activity were increased under resveratrol treatment.

5 Summary

The free radical theory of aging was already formulated in 1956 by Harman [68]. Free radicals like reactive oxygen species (ROS) are generated as by-products of mitochondrial metabolism and respiration. Mitochondrial ROS formation, however, has also been suggested to cause hormetic extension of life span by upregulating distinct stress response genes. In the present study the model organism *Caenorhabditis elegans* was used to investigate whether juglone at low or high concentrations provided orally in a liquid axenic medium affects life span. High juglone concentrations (250 μM) led to premature death, low concentrations (100 μM respectively 40 μM) were well tolerated and even increased life span. Life span extension under moderate oxidative stress was associated with enhanced expression of the small heat shock protein HSP-16.2 that probably accounts for enhanced levels of reduced glutathione observed. Juglone exposure further caused nuclear translocation of the transcription factor DAF-16 and knockdown or knockout of *daf-16* resulted in loss of juglone induced HSP-16.2 expression and concomitant life span extension. *Sir-2.1* RNA-interference did not affect nuclear accumulation of DAF-16 under juglone treatment but prevented increased HSP-16.2 expression.

To enhance mitochondrial respiration and associated ROS production rates high glucose loads were provided to wild type N2 and the ROS sensitive mutant *mev-1(kn1)*. A high glucose load in wild type N2 was associated with enhanced oxygen consumption and generation of mitochondrial ROS but lack of effect on life span or at high glucose levels associated with a modest increased life span. Only in the stress sensitive mutant *mev-1(kn1)* life span was decreased. This seemed to be caused by ROS since scavenging of ROS by ascorbic acid blunted the effects of glucose on life span reduction in *mev-1(kn1)* completely. Strikingly, the high glucose load seemed to prevented certain ROS driven compensatory mechanisms, such as a reduced insulin/IGF-1 signaling with increased nuclear DAF-16 translocation, HSP-16.2 expression and glutathione synthesis, to be activated suggesting a feed-forward stress response. In *mev-1(kn1)* mutants higher ROS levels were detectable even upon compensation like increased catalase activity. But this compensation was not sufficient like in N2. Here it was shown that elevated mitochondrial ROS-levels are able

to reduce life span only when the endogenous ROS levels are already increased as is the case in *mev-1(kn1)*.

Since most polyphenols are able to scavenge ROS, it was tempting to assess how selected flavonoids and resveratrol can influence life span and stress response in *C. elegans*. Flavone reduced life span in N2 and resveratrol prolonged life span whereas the other compounds (myricetin, quercetin and fisetin) failed to affect life span in N2 wild type animals. Despite lack on life span, these compounds displayed various effects on antioxidative systems such as a reduction of GSH levels or decreased SOD activity for example caused by myricetin. In case of resveratrol the tested endogenous antioxidative response systems (GSH levels, superoxide dismutase and catalase activity) were found to be reduced but life span was increased. In contrast flavone was able to enhance GSH levels and activity of catalase but life span was reduced. The most important factor in mediating these effects seemed *sir-2.1* since its activation by resveratrol resulted in longevity whereas inhibition caused by flavone reduced life span.

Interestingly, most compounds increased ROS levels and these can cause adaptive life span increase as demonstrated by exposure of animals to moderate juglone concentrations or resveratrol treatment. But if endogenous ROS levels are already elevated as in the case of *mev-1(kn1)* mutants additional ROS production induced for example by high glucose loads led to a reduction in life span. The secondary plant ingredients that altered *C. elegans* life span in the present study seemed to mediate their effects mainly via *sir-2.1* as the most consistent findings.

6 Zusammenfassung

Die "free radical theory of aging" wurde bereits 1956 von Harman formuliert [68]. Radikale, wie reaktive Sauerstoffspezies (ROS) werden u. a. als Nebenprodukte des mitochondrialen Metabolismus und der Atmungskette gebildet. Mitochondriale ROS Bildung wird ursächlich auch mit der sog. Hormesis, einer adaptiven Verlängerung der Lebensspanne, verursacht durch Hochregulation bestimmter Stress-Antwort-Gene, in Verbindung gebracht. In der vorliegenden Arbeit wurde der Modellorganismus *Caenorhabditis elegans* gewählt, um Effekte von Juglon in hohen und niedrigen Konzentrationen, verabreicht in einem axenischen Flüssigmedium, auf die Lebensspanne zu untersuchen. Hohe Juglon Konzentrationen (250 μM) führten zu frühzeitigem Sterben, niedrige (100 μM und 40 μM) Konzentrationen wurden gut toleriert bzw. führten zur Verlängerung der Lebensspanne. Langlebigkeit unter moderatem oxidativen Stress war assoziiert mit einer gesteigerten Expression des kleinen Hitzeschockproteins HSP16.2, welches wahrscheinlich zu erhöhten Spiegeln von reduziertem Glutathion führt. Juglon bedingte eine nukleäre Translokation des Transkriptionsfaktors DAF-16 und "knockdown" oder "knockout" von *daf-16* führte zum Verlust von Juglon-bedingter HSP-16.2 Expression und Lebensspanne Verlängerung. Eine Reduktion der *sir-2.1* Expression hatte keinen Einfluss auf die nukleäre Akkumulation von DAF-16 nach Juglon Gabe, verhinderte aber die erhöhte HSP-16.2 Expression.

Um die mitochondriale Respirationsrate und damit assoziierte ROS-Produktion zu erhöhen, wurden Wild Typ Tiere (N2) und die ROS-sensitive Mutante *mev-1(kn1)* hohen Glukosekonzentrationen im Medium ausgesetzt. In N2 war dies mit erhöhten O_2-Verbrauch und gesteigerter mitochondrialer ROS Bildung assoziiert, führte aber zu keinen Effekten auf die Lebensspanne, nur bei der höchsten Glukosekonzentration war sie durch Adaptationsmechanismen sogar moderat verlängert. In der stresssensitiven Mutante *mev-1(kn1)* kam es zur Verkürzung der Lebensspanne. Dieser Effekt der Glukose in *mev-1(kn1)* scheint durch erhöhte ROS Spiegel bedingt zu sein, da ein Abfangen der ROS durch Ascorbat die Verkürzung der Lebensspanne völlig aufhob. Exposition der Tiere gegenüber hohen Glukosekonzentration blokkierte offenbar ROS-vermittelte Kompensationsmechanismen, wie die verminderte

Insulin/IGF-1 Antwort mit erhöhter nukleärer DAF-16 Translokation, HSP-16.2 Expression und Glutathion Synthese. In *mev-1(kn1)* wurden trotz Kompensation duch erhöhte Katalase Aktivität, erhöhte mitochondriale ROS Spiegel nach Glukoseexposition gemessen. Im Gegensatz zum Wildtyp waren in der *mev-1(kn1)* Mutante diese Kompensationsmechanismen nicht hinreichend. Hier wurde gezeigt, dass erhöhte mitochondriale ROS-Spiegel nur zu einer Verkürzung der Lebensspanne führen, wenn bereits die endogenen Spiegel erhöht sind, wie in *mev-1(kn1)*.
Verschiedenste Polyphenole sind in der Lage, antioxidativ zu wirken. Deswegen wurden ausgewählte Flavonoide und Resveratrol in ihrer Wirkung auf die Lebensspanne und Stressantwort in *C. elegans* charakterisiert. Flavon reduzierte die Lebensspanne in N2 und Resveratrol führte zu Langlebigkeit, während die anderen gestesteten Flavonoide (Myricetin, Quercetin und Fisetin) keinen Einfluss in N2 auf die Lebensspanne zeigten, wenngleich sie antioxidative Mechanismen zu beeinflussen vermochten. Beispielsweise Myricetin verringerte sowohl die GSH Spiegel, als auch die Superoxiddismutase Aktivität. Im Fall von Resveratrol waren alle getesteten antioxidativen Abwehrmechanismen (GSH, SOD und CAT Aktivität) reduziert, aber die Lebensspanne verlängert. Flavon Exposition dagegen erhöhte die GSH Spiegel und Katalase Aktivität, reduzierte aber die Lebensspanne. Gemeinsam scheint im Mittelpunkt dieser Wirkungen der Polyphenole und Flavon das Gen *sir-2.1* zu stehen, da auf der einen Seite die Aktivierung von *sir-2.1* durch Resveratrol zu Langlebigkeit führt und auf der anderen Seite seine Inhibierung durch Flavon die Lebensspanne verkürzt.
Interessanterweise haben die meisten Substanzen die ROS Spiegel erhöht und dies konnte zu einer Verlängerung der Lebensspanne führen, wie im Fall von moderaten Juglone oder Resveratrol Gaben gezeigt wurde. Falls aber bereits die endogenen ROS Spiegel erhöht sind, wie im Fall der *mev-1(kn1)* Mutante, führte zusätzliche ROS Produktion, wie etwa verursacht durch erhöhte Glukose Konzentrationen zur Lebenszeitverkürzung. Die sekundären Pflanzeninhaltsstoffe, welche einen Effekt auf die Lebensspanne in der vorliegenden Arbeit hatten, scheinen diesen durch *sir-2.1* zu vermitteln.

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Abbreviations

°C	degree celcius
μg	microgram
μl	microlitre
μM	micromolar
age	aging abnormal (*C. elegans* mutant phenotype)
CAT	catalase
cDNA	complementary (to mRNA) DNA
CeHR	*Caenorhabditis elegans* habitation and reproduction medium
C. elegans	*Caenorhabditis elegans*
bzw.	beziehungsweise
daf	abnormal dauer defective (*C. elegans* mutant phenotype)
D. melanogaster	*Drosophila melanogaster*
dsRNA	double stranded RNA
eat	eating abnormal (*C. elegans* mutant phenotype)
EDTA	ethylendiaminetetraacetic acid
E. coli	*Escherichia coli*
e.g.	for example (lat. exempli gratia)
GFP	green fluorescent protein
GSH	reduced form of glutathione
GSSG	oxidized form of glutathione
HDAC	histone deacetylase
HSF	heat shock factor
HSP	heat shock protein
IGF	insulin-like growth factor
isp	iron-sulfur protein (*C. elegans* mutant phenotype)
mev	abnormal methyl viologen sensitivity (*C. elegans* mutant phenotype)
ml	milliliter
mM	millimolar
mRNA	messenger ribonucleic acid
NGM	nematode growth media
OP50	*Escherichia coli* strain, food source of *C. elegans*
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
S. cerevisiae	*Sacharomyces cerevisiae*
sir	silent information regulator (*C. elegans* mutant phenotype)
siRNA	small interference RNA

SOD	superoxide dismutase
sog.	sogenannt
TOR	target of rapamycin
u.a.	unter anderem

Publications:

Parts of this work have already been published:

Poster presentation at the DGE-congress in Kiel, Germany 2005:
"Sauerstoff-Stress führt im Fadenwurm *Caenorhabditis elegans* zu Verkürzung der Lebensspanne" Poster award 2005

Poster presentation at the NuGO introduction course in Marseille, France 2005:
"Oxidative stress reduces life span of the nematode *Caenorhabditis elegans*"'

Poster presentation at the NuGO week in Oxford, UK 2006:
"Mitochondrial ROS generation by high glucose load is tolerated in wild type but not in mev-1 *Caenorhabditis elegans*"

Poster presentation at the European worm meeting in Sevilla, Spain 2008:
"A high glucose load in *mev-1 Caenorhabditis elegans* causes life span reduction by increased production of mitochondrial reactive oxygen species"

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit

Effects of nutritional components on stress response and aging in the nematode *Caenorhabditis elegans*

selbständig verfasst und keine anderen als die angegeben Quellen und Hilfsmittel benutzt habe. Die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen sind als solche kenntlich gemacht.

Die Arbeit hat in gleicher oder ähnlicher Form noch keiner anderen Prüfungsbehörde vorgelegen.

Freising, den 22. Januar 2009

Tanja Heidler