TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Ernährungsphysiologie

Structure-activity relationship of selected flavonoids on aging and stress-resistance in *Caenorhabditis elegans*

Gregor Grünz

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. J. J. Hauner

Prüfer der Dissertation:
1. Univ.-Prof. Dr. H. Daniel
2. Univ.-Prof. Dr. M. Klingenspor

Die Dissertation wurde am 17.02.2012 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 16.05.2012 angenommen.

Inmitten der Schwierigkeiten liegt die Möglichkeit!

-Albert Einstein-

Table of content

Table of content....3
1. Introduction....5
1.1. Aging and aging theories....5
1.2. Reactive oxygen species....7
1.2.1 Generation and effects of reactive oxygen species....7
1.2.2 Antioxidant defense mechanisms....9
1.2.3 Physiological effects of ROS....10
1.3. The nematode Caenorhabditis elegans....12
1.3.1 C. elegans as model-organism....12
1.3.2 Genetic control of C. elegans aging and stress-resistance....14
1.4. Flavonoids....20
1.4.1 Classification, chemistry and occurrence....20
1.4.2 Uptake and metabolization....21
1.4.3 Bioactivity of flavonoids 22
2. Aim of the work....24
3. Material and Methods....25
3.1. Material....25
3.1.1 Equipment and Kits....25
3.1.2 Chemicals and media....25
3.1.3 C. elegans and bacteria strains....29
3.2. Methods....30
3.2.1 Basic C. elegans methods 30
3.2.2 Analysis of flavonoid bioavailability....31
3.2.3 Lifespan and stress-resistance analysis....34
3.2.4 Analysis of ROS-generation in mice and C. elegans....35
3.2.5 Analysis of protein-carbonyls....38
3.2.6 Analysis of metabolic parameters....40
3.2.7 Analysis of GFP-reporter strains....41
3.2.8 Data interpretation and statistical analysis 44
4. Results....45
4.1. Bioavailability of flavonoids....45
4.1.1 Visualization of flavonoids by Naturstoff reagent A 45
4.1.2 HPLC/DAD analysis of flavonoids in C. elegans....46
4.2. Influence of flavonoids on C. elegans lifespan....48
4.3. Impact of flavonoids on mitochondrial ROS....52
4.3.1 ROS-generation in isolated mouse mitochondria....52
4.3.2 ROS levels in C. elegans....55
4.4. Impact of flavonoids on protein-carbonyl content....58
4.5. Flavonoids effects on exogenous stress....60
4.5.1 Paraquat-induced ROS in isolated mouse mitochondria....60
4.5.2 Effects on paraquat-induced stress in C. elegans....61
4.5.3 Impact on heat-resistance....65
4.6. Impact of flavonoids on metabolic parameters....66
4.6.1 Effects on the pharynx pumping rate....66
4.6.2 Effects on respiration rate....67
4.7. Impact of flavonoids on the IIS-cascade....70
4.7.1 DAF-16 sub-cellular localization 70

4.7.2 Involvement of DAF-16 linked-genes....71
4.7.3 Contribution of DAF-16 on the beneficial effects....75
5. Discussion....78
6. Summary and conclusions....92
7. Zusammenfassung und Schlussfolgerungen....94
8. Appendix....96
List of figures....99
List of tables....100
List of abbreviations....101
References....103
Danksagung....118
Lebenslauf....119
Erklärung....120

1. Introduction

1.1. Aging and aging theories

> "**aging**, Gradual change in an organism that leads to increased risk of weakness, disease, and death. It takes place in a cell, an organ, or the total organism over the entire adult life span of any living thing. There is a decline in biological functions and in ability to adapt to metabolic stress." [1]

The universal and inevitable nature of aging has always fascinated people, and overcoming this fatal process is a long time dream. Despite this, it is still not fully understood why and how an organism ages but several theories exist that try to elucidate this multifaceted process. Classical theories try to explain why aging occurs in the context of evolution. One of them postulates that aging might limit the size of a population and force reproduction to enhance adaptation to a changing environment. Another prominent theory in this context is the disposable-soma theory of aging postulated by Thomas Kirkwood in 1977 [2]. It states that there is a trade-off of the limited energy resources of an organism between the maintenance of the soma and reproduction. This might explain why species with a higher number of offspring are usually short-lived compared to such with fewer progeny. Although such theories may suggest why aging occurs, they do not elucidate the underlying mechanisms. The basis for mechanistic aging theories was established in 1908 by the work of Max Rubner, describing that smaller animals showed a reduced lifespan but an increased metabolic rate [3]. This inverse relationship between lifespan and metabolic rate was the basis of the rate-of-living theory formulated by Raymond Pearl in 1928 [4]. It states that organisms with higher metabolic rate have a reduced maximum lifespan. On this basis Denham Harman proposed the free radical theory of aging in 1956 [5]. He suggested that reactive oxygen species (ROS) which are a by-product of normal metabolism accumulate and thereby damage macromolecules such as proteins, lipids or DNA which finally results in aging. As mitochondria are thought to be the major source of endogenously produced ROS, this theory was extended to the mitochondrial free radical theory of aging [6]. Especially ROS-induced impairment of DNA plays an important role in this process, as the frequency of mutations increases with age while the ability to repair such damage is decreasing [7]. In accordance with this, telomeres were shown to have an important impact on cellular aging. Telomeres are composed of short repetitive sequences and telomere binding proteins protecting the end of

chromosomes from degradation and/or fusion. With increasing number of cell divisions the length of the telomeres shortens, finally resulting in cell-cycle arrest and apoptosis [8]. Besides the number of cell-division, oxidative stress was shown to reduce the length of telomeres, underlining the strong impact of ROS in this process [9]. The free radical theory of aging attracted much attention in the last decades but it is challenged by more recent work [10] [3]. For example, deletion of mitochondrial antioxidant defense enzymes did not necessarily reduce the lifespan in the nematode *Caenorhabditis elegans* [11]. These results indicate that ROS are not the sole mechanism underlying aging but the deleterious effects of oxidative stress are well-known to contribute also to numerous diseases and senescence.

In 1935 McCay described that a reduction of food supply extended the lifespan in rats by 30% [12]. This finding was initially explained by a reduced metabolic rate and thus a decreased ROS-generation. As it is now generally accepted that dietary or caloric restriction (CR) does not necessarily reduce the metabolic rate, this causal relationship seems questionable [13]. The lifespan-increasing effect of CR, however, has been demonstrated in several other model organisms, ever since, but the precise underlying mechanisms and the possible role of ROS remain to be elucidated [14] [15].

1.2. Reactive oxygen species

Reactive oxygen species are chemically active molecules containing at least on atom of oxygen comprising superoxide anion radical ($O_2^{°-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{°}$), ozone, hypochlorus acid, organic hydroperoxides and their according radicals (ROOH, $ROO^{°}$) and several more. The radicals among them possess unpaired electrons making them highly reactive. Besides ROS there are also reactive nitrogen species which are formed by a reaction of nitric oxide with $O_2^{°-}$ to yield peroxinitrite ($ONOO^-$) which can react to further reactive species such as nitrogen dioxide or dinitrogen trioxide. ROS can be formed by exogenous sources like UV-light or ionizing radiation but also endogenously by enzymes such as those located in the mitochondrial electron transport chain (ETC, see below), NADPH oxidase, xanthin oxidase, aldehyde oxidase, cytochrome P450 and different oxygenases [16]. Nevertheless the major source of endogenously generated ROS and especially superoxide-generation is the ETC.

1.2.1 Generation and effects of reactive oxygen species

The electron transport chain and its role in ROS formation

The mitochondrial ETC, consisting of five complexes situated in the inner mitochondrial membrane, transfers electrons along the complexes and thereby translocates protons from the matrix to the intermembrane space. The established proton gradient drives the F_0F_1 ATP synthase to phosphorylate ADP to ATP, which is serving as ultimate energy source. Briefly, electrons primarily generated from the citric acid cycle or the β-oxidation of fatty acids are shuttled as nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide to the NADH dehydrogenase of complex I. Additionally, succinate from the citric acid cycle can be directly oxidized by the succinate dehydrogenase of complex II. Both complexes directly pass the electrons to ubiquinone (UQ, coenzyme Q) that is reduced to ubiquinol (UQH_2). The electrons are then transferred from ubiquinol to complex III (cytochrome *bc1* complex) which itself passes the electrons to cytochrome c. Finally, the electrons are accepted by the cytochrome c oxidase (complex IV) that transfers them to oxygen to generate water. The driving force of this electron flow is the increase of the standard redox-potentials ranging from complex I to IV (from -320 mV to +380 mV) [17]. Due to this electron flow, protons are translocated from the matrix into the intermembrane

space via complex I, III and IV. As a result a proton gradient is established that is used as a driving force for ATP synthesis by the F_0F_1 ATP synthase (complex V) (Fig. 1).

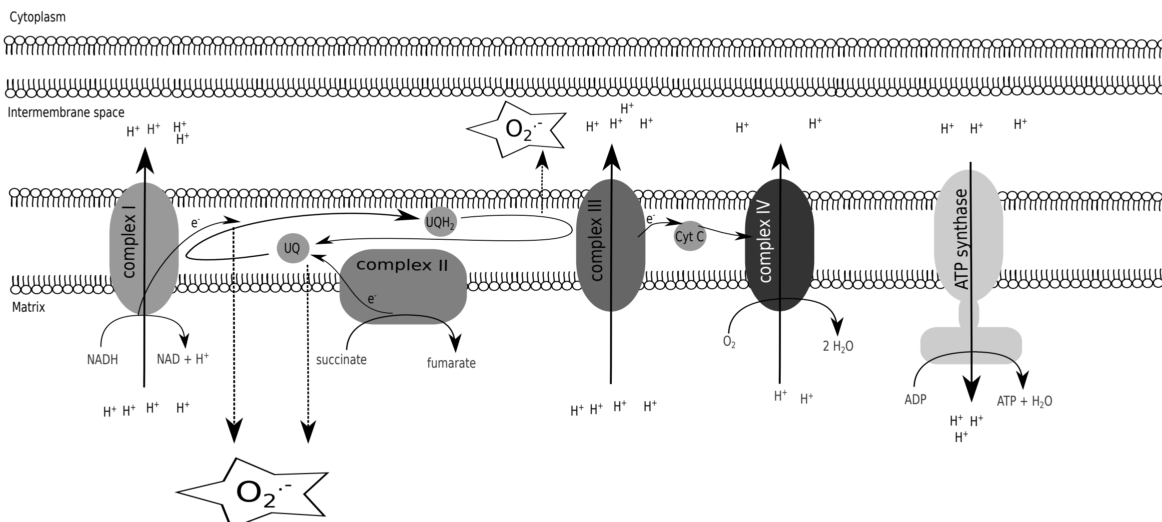


Fig. 1 The mitochondrial electron transport chain
Schematic picture of the mitochondrial electron transport chain with major sites of superoxide anion radical generation. Description see text.

In this process electrons can also be directly passed from the ETC to oxygen resulting in superoxide anion radical generation. Theoretically all electron carriers in this chain could pass electrons to oxygen (redox-potential = +815 mV), due to their lower redox-potentials. Studies, with isolated mitochondria revealed however that this mainly occurs when electrons are transferred from complex I to ubiquinone and from ubiquinone to complex III [18]. Also inside complex II $O_2^{°-}$ can be generated [19]. Superoxide generated at complex I is primarily released into the matrix while that generated by complex III leaks into the intermembrane space (Fig. 1) [3].

Superoxide anion radicals are thought to remain in the compartment where they are generated, as they are considered to be membrane impermeable in their unprotonated form [20]. They are rapidly converted by the superoxide dismutase (SOD) to H_2O_2, a reaction that also occurs spontaneously but at a much lower rate. In contrast to superoxide, H_2O_2 can permeate membranes easily and can be partially reduced to hydroxyl radicals by Fenton chemistry or fully to water by catalase or peroxidases [16].

Damage induced by ROS

Due to their high reactivity, ROS interact with many different molecules. Especially the allylic positions of polyunsaturated fatty acids are a target of ROS. At this position a fatty acid radical is formed that reacts with oxygen to a peroxyl radical (ROO°). This highly reactive radical can abstract a hydrogen atom from another polyunsaturated fatty acid thereby forming a stable lipid peroxide (ROOH) and another fatty acid radical. As fatty acids are highly abundant in membranes, such a chain reaction compromises their fluidity and increases ionic permeability resulting in cell death. Oxidative modification of low-density lipoproteins is discussed to contribute to the pathogenesis of arteriosclerosis [21]. ROS-induced damage of DNA can lead to modifications of nucleotides or strand breaks of the DNA helix. Depending on the site of damage this can result in mutations or genomic instability and thus in cancer or cell death [16]. For example in yeast, mutations are drastically increased after ablation of antioxidant defense mechanisms and reduced when cells are grown under anaerobic conditions [22]. Modification of proteins induced by ROS include sulfoxidation of methionine, nitration of tyrosine and carbonylation of amine residues. Such damage can lead to inactivation of enzymes or enhance protein aggregation, an effect that is discussed as causative in the pathogenesis of neurodegenerative diseases [16].

1.2.2 Antioxidant defense mechanisms

Enzymatic and low-molecular antioxidant defense mechanisms exist to protect cells against the deleterious effects of ROS. The superoxide dismutase, that converts $O_2^{\circ -}$ to H_2O_2, was isolated from erythrocytes by McCord and Fridovich in 1969, demonstrating copper to be required for its activity [23]. In mammals three different SOD isoforms can be found, a cytosolic copper/zinc (Cu/Zn) SOD, a mitochondrial manganese (Mn) SOD, and an extra-cellular Cu/Zn SOD. The mitochondrial SOD seems of special relevance for survival, as mice lacking this SOD but not the Cu/Zn SOD die short time after birth [24] [25]. This is also a clear indication that the ETC plays an important role in ROS formation. The dismutation of superoxide forms H_2O_2 that can be detoxified enzymatically by catalase, glutathione peroxidase and/or peroxiredoxins. Thereby catalases are the more economic way of H_2O_2 detoxification as in contrast both peroxidases finally use NADPH as reducing equivalent. Mutations in the genes encoding peroxiredoxins result in severe

oxidative damage under physiological conditions in yeast and mice, while mutations in genes encoding catalases or glutathione peroxidases do not show any major effects. Therefore it is suggested that peroxiredoxins preferably scavenge physiological levels of H_2O_2 while catalase and glutathione peroxidase handle high levels of H_2O_2 [16].

Besides these enzymatic mechanisms there are several low-molecular compounds contributing to the antioxidant defense. The tripeptide glutathione, consisting of glutamate, cysteine and glycine, is one of the most abundant antioxidants in cells. It can directly be oxidized, or reduce substrates indirectly via glutaredoxin that itself can restore peroxiredoxins. Furthermore it can function for conjugation of toxic lipophilic substrates via glutathione S-transferases to enhance the compounds hydrophilicity and thereby aid their detoxification. Vitamin E (α-tocopherol) serves as scavenger of ROS in membranes and lipoprotein particles due to its lipid-solubility, whereas vitamin C (ascorbic acid) quenches ROS in the hydrophilic milieu. Additionally, phenolic plant-derived compounds such as flavonoids or carotinoids also exert antioxidant effects. In short, numerous different mechanisms exist to balance the ROS load, and to minimize the deleterious effects of increased ROS thus to prevent “oxidative stress”.

1.2.3 Physiological effects of ROS

Besides their deleterious impact also beneficial and/or physiological effects are well-known for ROS. The respiratory burst of macrophages and neutrophile granulocytes for example represents an important mechanism to protect an organism against microbial infections. Thereby pathogens are killed by the release of huge amounts of ROS generated by the NADPH oxidase [26]. Nitric oxide (NO), which became the “molecule of the year 1992” by “Science” journal, plays an important role in blood pressure regulation. It is generated in endothelial blood vessel cells from arginine by nitric oxide synthase and diffuses to the smooth muscle cell layer where it stimulates the guanylate cyclase finally resulting in muscle relaxation [27]. Cytosolic hydrogen peroxide is thought to mediate the downstream signaling of growth factors (e.g. platelet derived and epidermal growth factor) by inhibition of tyrosine phosphatases that counteract different protein kinases [28]. The tumor suppressor PTEN, for example, that antagonizes the phosphatidide inositol 3(PI 3)-kinase was demonstrated to be inhibited by H_2O_2 consequently resulting in increased insulin-like signaling (IlS) [29]. Also mitochondrial ROS (mtROS) were demonstrated to

be involved in signal transduction. Under low oxygen conditions mitochondria produce more ROS that are thought in turn to stabilize the hypoxia-inducible transcription factor that counteracts the low-oxygen state by regulating the expression of enzymes augmenting oxygen supply [30]. ROS-induced inhibition of phosphatases also plays a role during programmed cell-death. Upon binding of tumor necrosis factor α, mitochondrial ROS are released resulting in inhibition of JNK (c-Jun N-terminal kinases) phosphatase and thus in activation of the pro-apoptotic JNK [31]. In view of these essential roles of ROS, the perception that they are just deleterious by-products of metabolism has to be revised, and it becomes obvious that the tight regulation of ROS-generation and detoxification is the most critical element.

1.3. The nematode *Caenorhabditis elegans*

1.3.1 *C. elegans* as model-organism

The around one mm long nematode *Caenorhabditis elegans* belongs to the family of *rhabditidae* and was first described in 1900 by Maupas [32]. Its natural habitat is the soil where it feeds from bacteria and other microorganisms. The “career” of *C. elegans* in science is based on Sydney Brenner’s seminal work in the early 1970s [33] [34]. Starting as a genetic model for behavioral and neuroscience, *C. elegans* was developed also into a powerful tool to analyze the genetic control of organ development and apoptosis. This work culminated in the awarding of the 2002 Nobel Prize in Physiology or Medicine to Brenner, Horvitz and Sulston for their work on this “Nature’s gift to science” [35]. Today, *C. elegans* is used in almost all fields of biology due to several outstanding characteristics.

There are two sexual forms existing, self-fertilizing hermaphrodites (about 99.9% of a mixed population) with two X chromosomes and males with just one (about 0.01% of a mixed population). The occurrence rate of males can be increased by unfavorable conditions like heat, lack of food and cultivation in liquid culture over several generations. The number of progeny varies greatly among the sexes. A self-fertilizing hermaphrodite can lay 300 to 400 eggs while hermaphrodites that mate with males have up to 1000 offspring. *C. elegans* has a constant number of somatic cell nuclei (959 in hermaphrodites and 1031 in males) in an adult worm (eutelie) and the complete lineage of every cell is known [36] [37]. Studies on the cell morphology and fate are greatly enhanced by its transparent nature. Due to its small size, its huge number of progeny and a short generation time *C. elegans* can be easily grown in bulk quantities. They are usually cultivated on agar plates seeded with *Escherichia coli* as food source at 20°C. The 3 to 4 days lasting life cycle of *C. elegans* starts with the fertilization of the eggs and proceeds over 4 larval stages until adulthood (Fig. 2). If environmental conditions are unfavorable, L1 larvae can enter an alternate development stage called the dauer where development ceases [38]. Dauer larvae are resistant against various stresses and they can survive for several months. If conditions improve dauer larvae re-enter the regular life cycle as L4 larvae. The total lifespan of wild-type *C. elegans* is about 2 to 3 weeks.

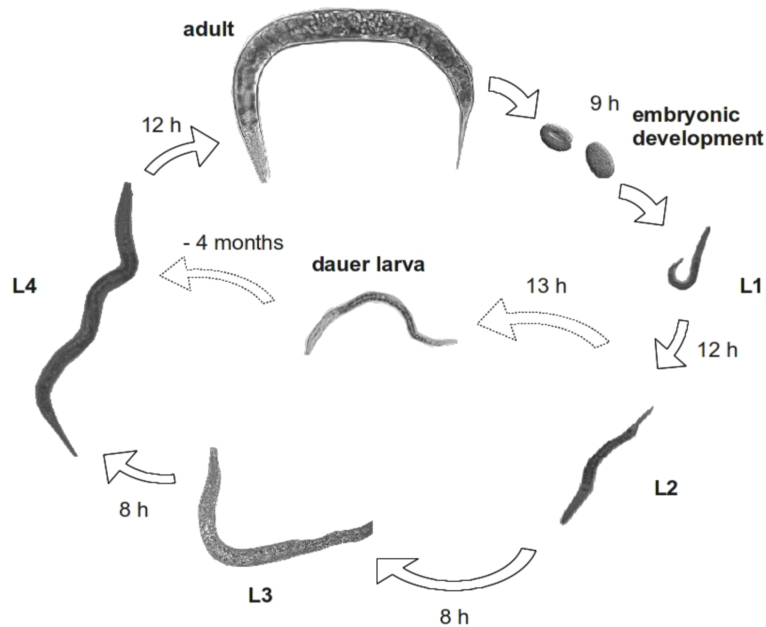


Fig. 2 Life cycle of wild-type *C. elegans*
Scheme of wild-type *C. elegans* development at 20°C. Nine hours after fertilization of eggs, L1 (first larval stage) larvae hatch and develop to L2 for 12 hours. For 8 hours L2 larvae grow to L3 followed by development to L4 larvae (8 hours). After further 12 hours worms reach adulthood and egg-laying phase starts again. Each larval stage is marked by a molt and synthesis of the cuticula. If conditions are harsh (food deprivation, unfavorable temperature or overcrowding) the worms (L1) enter an alternate stage called the dauer. The food intake and movement is reduced and worms can rest in this stage up to four months until environmental conditions improve. [adapted from www.wormatlas.org 2011-03-10 with modifications].

The genome of *C. elegans* is organized in five pairs of autosomes and one pair or a single sex chromosome (see above). In 1998 the 97 megabase genome of *C. elegans* with about 20,000 genes was (except for some gaps) completely sequenced as the first of a multicellular organism [39]. For genetic investigations a huge number of mutant strains exist or can be easily generated. Specific genes can be silenced via RNA-interference, a technique first employed in *C. elegans*. Thereby specific double-stranded (ds)RNA is introduced into the worms leading via a complex mechanism to the degradation of the corresponding mRNA. This can be achieved by injection of dsRNA [40], soaking of worms in dsRNA [41] or simply by feeding of dsRNA expressing bacteria [42]. The discovery of this method was awarded with the Nobel Prize in Physiology or Medicine to Andrew Fire and Craig Mello in 2006. Furthermore, plasmids can be injected directly into the gonad thereby integrating genetic markers into the genome (chromosomal or extra-chromosomal) of the progeny [43]. Such plasmids can carry complementary DNA of the green-fluorescent protein (GFP) coupled to promoters or fused to complete genes of interest. GFP

is a protein of 238 amino acids, that fluoresces green after excitation with UV light and was discovered in jellyfish by Osamu Shimomura in 1962 [44]. Due to *C. elegans* transparent nature, GFP allows to monitor the promoter activity and/or the localization of proteins in the living animals without the need of additional substrates [45]. The discovery of GFP and its establishment as reporter in *C. elegans* was awarded with the "third worm prize" – the Nobel Prize in Chemistry 2008 to Chalfie, Tsien and Shimomura [46].

1.3.2 Genetic control of *C. elegans* aging and stress-resistance

Due to its short lifespan and its well-characterized genome, *C. elegans* is a suitable tool to investigate the genetic mechanisms of aging. The first studies on aging in *C. elegans* were conducted in 1977, showing that a decrease in temperature or a reduction of food supply can increase its longevity [47]. Since then, much effort was made in the search for genes that might regulate lifespan (sometimes called gerontogenes).

The insulin/IGF-like signaling cascade

In 1993 a mutation in the *age-1* gene (aging alteration) was demonstrated to increase lifespan without showing other severe secondary phenotypic changes [48] [49]. Later Cynthia Kenyon demonstrated that a mutation in the *daf-2* (abnormal dauer formation) gene, that was initially discovered to cause constitutive dauer formation [50], increases the lifespan in *C. elegans*, too [51]. These dauer constitutive [52] and lifespan-extending effects [51] of the *daf-2* mutation were abrogated when another gene, *daf-16,* was mutated. Furthermore it was demonstrated that this *daf-16* mutation abolished also the long-lived phenotype of the *age-1* knockout, implying *daf-2*, *age-1* and *daf-16* to be in the same pathway [53]. Later these genes were cloned and it turned out that *daf-2* encoded the sole orthologue of the insulin/Insulin-like growth factor 1 (IGF-1) receptor [54], *age-1* the PI 3-kinase catalytic subunit (p110) [55] and *daf-16* a forkhead-family (FOXO) transcription factor [56] [57]. Hence, the IlS cascade was the first pathway that was shown to regulate lifespan. Further genetic studies elucidated the precise mechanisms of this cascade in more detail.

C. elegans possesses 40 genes that are predicted to encode insulin-like peptides (www.wormbase.org 2011-03-14) that can act either as agonists or antagonists to the DAF-2 receptor. When activated, DAF-2 initiates signaling to PI 3-kinase, consisting of the two

subunits AAP-1 and AGE-1, that phosphorylates phosphoinositide 3,4-di-phosphate (PIP_2) [55] [58]. This results in generation of phosphoinositide 3,4,5-tri-phosphate (PIP_3) which is antagonized by the PTEN tumor suppressor orthologue DAF-18 [59]. PIP_3, however, activates PDK-1, the PI 3 kinase-dependent kinase that transduces the signal to the AKT/PKB kinase orthologues AKT-1/AKT-2 [60] and to SGK-1 (serum- and glucocorticoid inducible kinase), respectively [61]. Recently PPTR-1, an orthologue of a protein phosphatase 2A subunit, was shown to regulate this process by dephosphorylation of AKT-1 [62] Finally, AKT-1/AKT-2 and SGK-1 phosphorylate DAF-16 thereby preventing its translocation into the nucleus [63]. When IlS is reduced, AKT/SGK inhibition of DAF-16 is abolished and it enters into the nucleus where it can regulate target gene expression. DAF-16 itself controls numerous genes affecting stress-response such as superoxide dismutases [64], glutathione S-transferases, heat-shock proteins [65], metallothioneines [66] and catalases [67] [68] (Fig. 3). In accordance with these diverse targets, *C. elegans* with reduced IlS are not just long-lived but also resistant against pathogens [69], UV light [70], heavy metals [66] and oxidative stress [64]. The lifespan-extending effect of reduced IlS, however, is evolutionary conserved as it is also observed in *Drosophila melanogaster* and mice [71] [72]. Besides the impact on aging and stress-resistance, IlS also affects reproduction and development [73] [74]. Taken together, when growth conditions are favorable, insulin-like peptides can bind to DAF-2 and consequently DAF-16 activity is reduced, pushing *C. elegans* towards reproduction and growth. Whereas scarce food availability, reduces IlS and thus increases DAF-16 activity favors a preserving and long-lived phenotype.

Since the IlS cascade affects many different processes, additional regulative mechanisms must exist that specific effects can be provoked. The point of time when a reduction in IlS is induced seems to play an important role for the effects on longevity and reproduction. Silencing of *daf-2* in an early developmental stage induces rather dauer formation and delays the onset of reproduction while RNAi treatment in the early adult stage initiates the longevity phenotype [73].

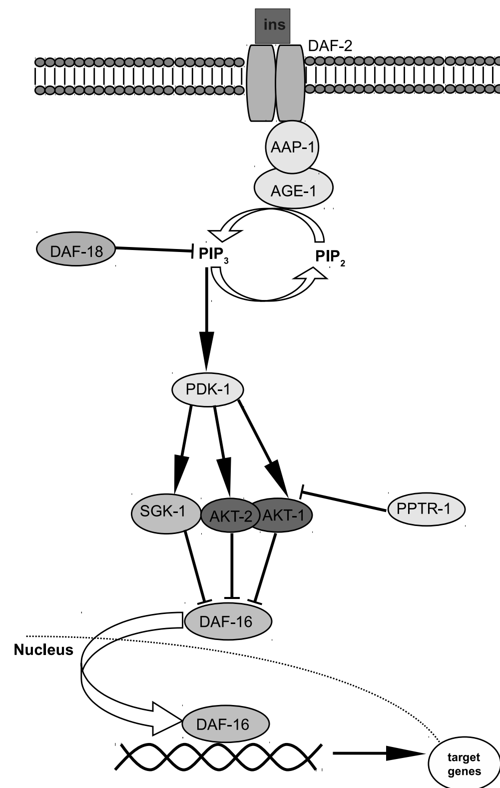


Fig. 3 Simplified scheme of the insulin-like signaling cascade
Description see text.

Furthermore, IlS alterations are tissue-specific. In *daf-2* mutants a restoration of *daf-2* in neurons but not in the intestine re-established the wild-type lifespan [75]. On the contrary, in *daf-2;daf-16* double-mutants the long-lived phenotype was recovered when *daf-16* was reconstituted in intestinal cells only [76]. This contradiction might be explained by non-cell autonomous signaling, as insulin-like peptides that are further targets of DAF-16 might act as hormones and regulate IlS in a feedback manner [67]. Cell autonomous IlS on the other hand regulates expression of target genes such as *sod-3* [76].

Interestingly, nuclear localization of DAF-16 alone, induced by mutation at the AKT-phosphorylation site, is not sufficient to extend lifespan in *C. elegans* [77]. This implies that further factors are needed for IlS-mediated longevity. Many of such DAF-16 regulators have been described, as reviewed by Landis *et al.* [78]. Among these factors is *jnk-1* a c-Jun N-terminal kinase orthologue that is highly conserved and induced under stress conditions [79]. The heat-shock factor-1 orthologue *hsf-1* was shown to increase heat-resistance and lifespan in a DAF-16 dependent manner [65]. HSF-1 is a well-conserved transcription factor, which is necessary for the expression of heat-shock proteins

which prevent aggregation of misfolded, oxidized or otherwise damaged proteins [80]. Additionally, over-expression of *sir-2.1* also increased lifespan and stress-resistance dependent on DAF-16 [81]. *sir-2.1* is orthologue to yeast the SIR2 sirtuin, a protein that shows NAD^+-dependent de-acetylase activity. Thereby it de-acetylates acetylated lysine residues of different proteins, such as histones or transcription factors such as FOXO [82]. The lifespan-extending effect of caloric restriction is dependent on sirtuins since SIR mutants in yeast and *Drosophila* do not show elevated CR-induced longevity [14] [83]. In contrast to that, mutations in the gene *ftt-2* increased DAF-16 nuclear localization and longevity [84]. *ftt-2* and *par-5* encode members of the 14-3-3 proteins that are highly conserved and mediate protein-protein interactions [85], implying a role in DAF-16 cytosolic retention. Another factor interacting with IlS is the SKN-1 transcription factor that is orthologue to Nrf (nuclear respiratory factor) an inducer of phase II enzyme expression in response to oxidative stress [86]. IlS was shown to oppose SKN-1 in parallel to DAF-16 demonstrating the close linkage of these two transcription factors [87]. Taken together these observations imply that DAF-16 and IlS are not acting alone to affect longevity and stress-resistance but work in a complex orchestra of signaling cascades.

Caloric restriction mediated pathways

Caloric restriction is a well-known intervention to induce longevity in various species from yeast to rodents [14] [12]. In *C. elegans* food deprivation and mutations in *eat* genes that reduce the pharyngeal pumping rate and thus ingestion of food [88], mostly result in an elevated lifespan [47] [89] [90]. The underlying genetic mechanisms, however, remain mainly elusive. Although CR-mediated longevity was demonstrated not to be exclusively dependent on *daf-16* [91], pathways closely linked to IlS seem to have some impact. Especially cascades involved in nutrient sensing are good candidates to mediate CR-induced lifespan extension. The AMP-activated kinase, for example, senses the cellular energy status by monitoring the AMP:ATP ratio and induces catabolic pathways to generate ATP [92]. Its catalytic subunit orthologue *aak-2* was shown to be necessary for DAF-16-dependent gene expression while mutations abolished the increased lifespan in food-deprived *C. elegans* [93]. Another mechanism regulating basic cellular processes such as translation, ribosome biogenesis and autophagy in response to amino acids and energy status is the highly-conserved TOR pathway [94]. In *C. elegans* the active TOR 1 complex is encoded by *let-363* (orthologue to mTOR) and the associated regulatory protein (Raptor)

orthologue *daf-15* [95]. *daf-15* and *let-363* mutants are known to be long-lived and *daf-15* expression is regulated by IlS [95] [96]. Despite this close interaction of nutrient sensing and longevity via TOR, it is still not clear whether CR-mediated longevity is dependent on TOR [96] [97]. Furthermore, *trx-1* encoding a thioredoxin in neuronal cells and *pha-4*, an orthologue of mammalian FOXA transcription factor, were also shown to be necessary for CR-induced longevity [98] [99]. Bishop and Guarente demonstrated that SKN-1 activity in sensory neurons is necessary for CR-mediated longevity possibly by endocrine signaling [100]. Additionally, a mutation in *sir-2.1*, the yeast sirtuin orthologue, was also shown to suppress the CR-induced longevity in *C. elegans* [101]. Hence, the genetic mechanisms, regulating CR-induced longevity are diverse and it seems plausible that they may act all together in a complex network responding to energy deprivation.

Mitochondria and ROS defense in aging

According to the "free radical theory of aging", mitochondria as the primary source of endogenously generated ROS are also a major determinant of lifespan. Several *C. elegans* mitochondrial mutants with altered lifespan and stress-resistance are known. In 1990 a mutation in the gene *mev-1* was found not just to increase the sensitivity against the ROS-generating compound methyl viologen (paraquat) but also to decrease the lifespan in *C. elegans* [102]. This gene was later identified to encode a subunit of the succinate dehydrogenase of complex II of the respiratory chain, implying a link between mitochondria and aging processes [103]. Since then several other mitochondrial genes were discovered in which mutations result in decreased lifespan and ROS-resistance. Among them are *gas-1* and *nuo-1*, both encoding complex I subunits and *sdhb-1* that encodes a succinate dehydrogenase subunit [104] [105] [106]. Conversely, the first mitochondrial mutant with an increased lifespan was found to have a genetic alteration in *clk-1* [107]. *clk-1* encodes a hydroxylase that is necessary for the synthesis of ubiquinone which transfers electrons from complex I and II to complex III in the respiratory chain. Despite the decreased respiration rate in *clk-1* mutants, suggesting reduced ROS-generation [108], these animals are not resistant against oxidative stress [64]. Another mutation that increases lifespan is found in the gene *isp-1,* encoding the Rieseke iron sulfur protein of complex III [109]. These worms show slow development rates that can be rescued by an additional mutation in *ctb-1*, the cytochrome b subunit of complex III. These worms are also resistant against paraquat, implying a reduced ROS-generation contributing

to the longevity effect [109]. However, ROS levels are not just influenced by their generation rate but also by their detoxification rates determined partially by antioxidant enzymes.

In contrast to most other organisms, *C. elegans* possesses 5 genes encoding superoxide dismutases. *sod-1* and *sod-5* encode cytosolic Cu/Zn SOD, *sod-2* and *sod-3* encode mitochondrial manganese SOD [110] [111] [112] while *sod-4* encodes a membrane-bound extracellular Cu/Zn-SOD [113]. Thereby *sod-1* and *sod-2* account for almost 95% of total *sod* mRNA [114]. Findings on their role on lifespan regulation are rather controversial and need further elucidation. Although interventions that increase longevity, such as reduced IlS and mitochondrial inhibition were shown to increase SOD expression [64] [115] [109], deletion of single or multiple SOD had only minor or no effects on normal lifespan [114] [11]. *C. elegans* possesses 3 genes encoding catalases: *ctl-1* a cytosolic catalase, *ctl-2* found in the peroxisome and *ctl-3* of which only little is known [116]. As with SOD, increased mRNA levels and enzyme activity of catalase is found in IlS reduced, long-lived strains [117] [110]. Mutations in *ctl-2* but not *ctl-1* reduced the lifespan in *C. elegans* [118]. Although altered ROS levels are associated with aging, controversy exists whether there is a "causal or casual relationship" between ROS and aging as reviewed by Raamsdonk and Hekimi [3].

Other mechanisms

The length of telomeres might also influence the lifespan in *C. elegans*. Over-expression of *hrp-1*, a gene encoding a telomere binding protein increased telomere length and lifespan and heat-stress-resistance in a DAF-16-dependent manner [119]. Also the loss of germ cells was shown to increase longevity by elevated translocation of DAF-16 into the nucleus [120], suggesting a close link between lifespan and reproduction.

1.4. Flavonoids

1.4.1 Classification, chemistry and occurrence

(Poly)phenolic substances are one of the most diverse groups of secondary compounds abundant in plant kingdom and are characterized by the presence of one or more phenol rings. According to the basic chemical structure they can be classified by their carbon backbone: C_6-C_1 backbone (mainly hydroxybenzoic acids), a C_6-C_3 backbone (hydroxycinnamic acids and hydroxycumarins), C_6-C_2-C_6 backbone (stilbenes) and those with a C_6-C_3-C_6 structure (flavonoids).

The flavonoids represent a major class of polyphenols, all having a flavan backbone as basic structure in common (Fig. 4). They can be classified into several subgroups according to additional structural features including (without any claim of completeness) flavones, flavonols, flavanones, flavan-3-ols (catechins) and isoflavones (Fig. 4). Each sub-groups comprises numerous members differing in the attached moieties. Flavonoids are synthesized in vascular plants to protect against UV-light, as signaling molecules, to attract symbionts or to ward competitors [121]. Consequently they are found in many plant-derived foodstuffs such as in onions (quercetin), grapefruits (naringenin), soy (genistein), tea (catechins) and wine (anthocyanins). Their concentration is rather low and ranges from 10 - 30 mg * kg^{-1} fresh weight with highest concentrations in outer tissues. Except for catechins, they are present frequently in glycosylated form mostly conjugated with glucose, rhamnose or disaccharides [122].

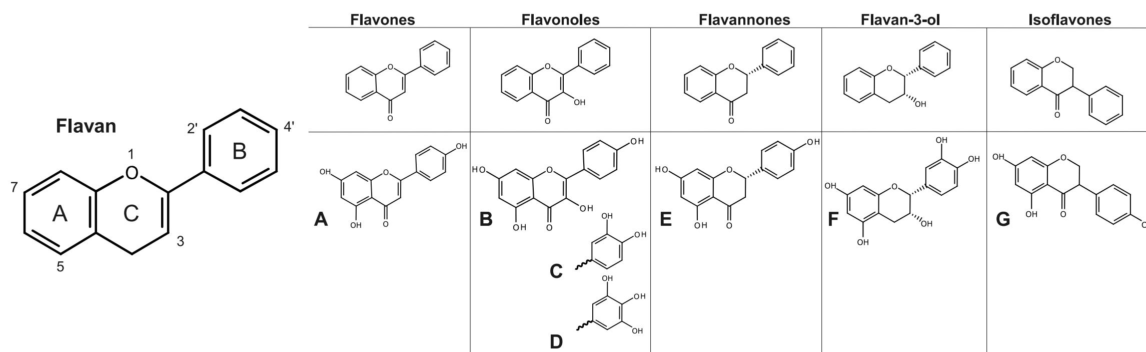


Fig. 4 Structures of selected flavonoids
Structures of flavan and flavonoid classes flavones, flavonoles, flavanones, flavan-3-ol, isoflavones and stilbenes with selected examples. A apigenin, B kaempferol, C B-ring of quercetin, D B-ring of myricetin, E naringenin, F catechin, G genistein.

1.4.2 Uptake and metabolization

The daily intake of flavonoids varies greatly among regions and individual eating habits but was estimated between 5-125 mg per day [122]. The absorption process of dietary flavonoids in the gut remains poorly understood but active and passive transport mechanisms are discussed. In their conjugated form the flavonols and isoflavones are substrates of the lactase phlorizin hydrolase in the brush border membrane of intestinal epithelial cells, rapidly cleaving the sugar moiety off to release the aglycon [123]. The remaining aglycon then may be entering the enterocyte by passive diffusion. Additionally the sodium-dependent glucose transporter (SGLT-1) was proposed to transport quercetin-glycosides into the enterocyte where a cytosolic ß-glucosidase is thought to hydrolyze the conjugate [124]. This proposed active transport mechanism, however, was challenged as in *Xenopus laevis* oocytes expressing hSGLT-1 no transport of different flavonols/glycosides (among them quercetin and naringenin and some of their glycosides) was detected [125]. In the enterocyte, however, the flavonoid aglycones are again conjugated comprising glucuronidation, sulfatation and methylation reactions prior to transport into plasma or followed by re-excretion into the gut lumen [126]. In plasma, the majority of the flavonoids is bound to albumin reaching concentrations in the low micromolar range with highest levels detected for isoflavones [127]. Further conjugation reactions can also occur in the liver and finally the flavonoids are excreted via bile and urine, depending on their size and solubility. Those that escape small intestinal absorption or are excreted with bile reach the colon where they are extensively metabolized by the microflora. Besides

hydrolysis, the microbiota can split the heterocyclic ring thereby forming phenolic acids that are further converted to benzoic acid derivatives [122]. These metabolites are re-absorbed and again conjugated prior to excretion.

1.4.3 Bioactivity of flavonoids

In the 1930s, Rusznyak and Szent-Györgyi were the first who discovered the biological activity of flavonoids in mammals. By demonstrating an influence on the activity of vitamin C they suggested flavonoids to be essential, subsequently terming them vitamin P [128]. Although the essentially of flavonoids is dismissed today, a huge range of health-beneficial effects including anti-oxidant, anti-inflammatory, anti-carcinogenic and anti-viral action are attributed to them. The positive effects of a plant-based diet on chronic diseases such as cardiovascular diseases, asthma or cancer were partially attributed to the high abundance of flavonoids in such foods [129] [130] [131]. Especially their high *in vitro* antioxidant potential led to the assumption that flavonoids exert their effects by scavenging of ROS directly or by chelation of transition metals and thereby reducing oxidative damage [132] [133]. Although the structural features determining their antioxidant properties *in vitro* are known, such as a catechol function at the B-ring (3', 4'-OH) and hydroxylation (3-OH) and a double bond at the heterocyclic C-ring, their effects on ROS *in vivo* are rather elusive [134] [135].

Besides their interactions with ROS, recent *in vitro* studies suggest that flavonoids might also exert numerous effects by influencing molecular signaling cascades. These proposed effects are almost as numerous as the diversity of flavonoid structures. Soy isoflavones and especially one of their bacterial degradation product equol, for example, are well-known to exert estrogenic properties due to their similarity to estradiol [136]. Whether this implies a role in the suppression or progression of sex-specific cancers is controversially discussed [137]. Tea catechins such as (-)epigallocatechin-3-gallate are in the focus of research due to an attributed anti-cancer activity. They are thought to unfold such effects partly by the suppression of the anti-apoptotic nuclear factor-kappa B (NFκB) and the proliferation-promoting activator protein-1 (AP-1) [138] [139]. Flavonols and flavanones were shown to reduce IlS by inhibition of the PI 3-kinase, implying that they could have an impact in diabetes and cell-proliferation [140] [141]. Additionally anti-cancer properties are attributed to these two flavonoid sub-classes. The inhibition of

cytochrome P450 (CYP)1A1, implies an inhibitory effect on the toxification of pro-carcinogens, suggesting interaction with phase I metabolism [142]. A similar effect on the CYP isoform 1A2, is well-known for the grapefruit flavanone naringenin. Its suppression can reduce the metabolism of xenobiotics and drugs, which can severely disturb their clearance [143]. A role of the aryl hydrocarbon receptor, regulating CYP expression upon xenobiotic binding, might be a possible mechanism of action since certain flavonols were shown to suppress its xenobiotic-induced activity [144]. Quercetin was also shown to affect cellular xenobiotic excretion by the inhibition of ABC family transporters. The suggested modes of action include competitive inhibition, interactions at the ABC transporters ATP-binding site or depletion of intracellular glutathione, necessary for the efflux [145] [146].

However, despite the vast number of putative molecular actions of flavonoids, great care is required when extrapolating these data mainly obtained in cell culture studies to complex organisms. The numerous modifications that flavonoids can undergo in metabolism, and the complexity of pharmacokinetics in complex systems may result in *in vivo* effects that are distinct from observations obtained *in vitro*.

2. Aim of the work

As flavonoids possess a magnificent diversity, their different structural properties consequently determine their biochemical functions especially with regard to beneficial effects on aging and stress-resistance. Knowledge on structure-activity relationship is provided essentially only from *in vitro* models whereas their influence *in vivo* remains largely elusive. Therefore *C. elegans* was used to determine the impact of four structurally related flavonoids on longevity and stress-resistance as well as to identify their putative modes of action *in vivo*. To investigate structural features necessary for beneficial effects the flavonols myricetin, quercetin and kaempferol, differing from each other in the number of OH-groups attached to the B-ring and additionally the flavanone naringenin were employed. In accordance with these structural features determining antioxidant action *in vitro*, the following order of antioxidant capacities has been described: quercetin > myricetin >> kaempferol > naringenin [132] [133] [147]. To elucidate whether this comes also into effect *in vivo* in *C. elegans* and in isolated mouse mitochondria, the flavonoid effects on ROS-generation was assessed. *C. elegans* mutants with initially altered ROS status were used to further examine the role of antioxidant action on lifespan. Bioavailability of flavonoids, a prerequisite for their effects *in vivo* was demonstrated by different analytic methods. To investigate putative metabolic mechanisms, oxygen consumption and food intake were assessed in the nematodes. Additionally, all compounds were tested for their ability to cause nuclear translocation of DAF-16, the prime target of IIS, and changes in target gene expression.

3. Material and Methods

3.1. Material

3.1.1 Equipment and Kits

Table 1. Equipment and Instruments

Apparatus/Kit	Company
Confocal laser scanning microscope system (DM IRBE; TCS SP2)	Leica Microsystems, Wetzlar, Germany
Stereomicroscope System (Leica MZ7.5; KL 2500)	Leica Microsystems, Wetzlar, Germany
Tecan Infinite 200 well reader	Tecan, Männedorf, Switzerland
Fast prep FP120 BIO101	Thermo Savant Corporation, München, Germany
Oxygen sensing system Apollo 4000 equipped with iso-oxy-2 electrode and ISO-TEMP-2 temperature sensor	World Precision Instruments, Sarasota, FL, USA
Varioskan	Thermo Elektron Corporation, München, Germany
OxyBlot Protein Oxidation Detection Kit	Millipore, Billerica, MA, USA
Centrifuge universal 32R	Hettich, Tuttlingen, Germany

3.1.2 Chemicals and media

Chemicals used for this work

Table 2. Chemical and Supplier information

Chemical	Supplier
2-aminoethyl diphenyl borate (Naturstoff reagent A)	Roth, Karlsruhe, Germany
5-Fluoro-2'-deoxyuridine	Sigma-Aldrich, Steinheim, Germany
Acrylamide (30%)	Roth, Karlsruhe, Germany

Chemical	Supplier
Agar-agar (high strength)	Serva, Heidelberg, Germany
Agarose	Roth, Karlsruhe, Germany
Ammonium acetate	Merck, Darmstadt, Germany
Amplex Red	Invitrogen, Darmstadt, Germany
APS (ammonium persulfate)	Serva, Heidelberg, Germany
ATP (Adenosine-5'-triphosphate)	Sigma-Aldrich, Steinheim, Germany
Bacto yeast extract	Roth, Karlsruhe, Germany
Bio-Rad protein assay	Bio-Rad, Munich, Germany
BSA (bovine serum albumin)	Sigma-Aldrich, Steinheim, Germany
$CaCl_2$	Roth, Karlsruhe, Germany
Cholesterol	Sigma-Aldrich, Steinheim, Germany
Coomassie blue R	Sigma-Aldrich, Steinheim, Germany
DMSO (dimethyl sulfoxide)	Sigma-Aldrich, Steinheim, Germany
DTT	Roth, Karlsruhe, Germany
EDTA (ethylene diamine tetraacetic acid)	Sigma-Aldrich, Steinheim, Germany
EGTA (ethylene glycol tetraacetic acid)	Roth, Karlsruhe, Germany
Ethanol	Merck, Darmstadt, Germany
Glycerol	Roth, Karlsruhe, Germany
Glycine	Merck, Darmstadt, Germany
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Roth, Karlsruhe, Germany
HisPic	Sigma-Aldrich, Steinheim, Germany
HRP (horseradish peroxidase)	Sigma-Aldrich, Steinheim, Germany
Juglone (5-hydroxy-1,4-naphthalenedione)	Sigma-Aldrich, Steinheim, Germany
K_2HPO_4	Roth, Karlsruhe, Germany
Kaempferol	Sigma-Aldrich, Steinheim, Germany
KCl	Roth, Karlsruhe, Germany
KH_2PO_4	Roth, Karlsruhe, Germany
KOH	Roth, Karlsruhe, Germany
Levamisole	Sigma-Aldrich, Steinheim, Germany
Methanol	Merck, Darmstadt, Germany
$MgCl_2$	Roth, Karlsruhe, Germany
$MgSO_4$	Sigma-Aldrich, Steinheim, Germany
Mitotracker Red CM-H_2XRos	Invitrogen, Darmstadt, Germany
Myricetin	Sigma-Aldrich, Steinheim, Germany
Na_2HPO_4 dihydrate	Roth, Karlsruhe, Germany
NaCl	Roth, Karlsruhe, Germany
NaOCl (12%)	Roth, Karlsruhe, Germany
Naringenin	Sigma-Aldrich, Steinheim, Germany
Nystatin dihydrate	Roth, Karlsruhe, Germany
Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride dihydrate)	Sigma-Aldrich, Steinheim, Germany
Peptone	Roth, Karlsruhe, Germany
Quercetin	Sigma-Aldrich, Steinheim, Germany
SDS (sodium dodecyl sulfate)	Roth, Karlsruhe, Germany
SOD (superoxide dismutase)	Sigma-Aldrich, Steinheim, Germany
Subtilisin A	Sigma-Aldrich, Steinheim, Germany
Succinate	Sigma-Aldrich, Steinheim, Germany
SuperSignal West Pico Chemiluminescent substrate	Pierce, Rockford Il, USA
TEMED (Tetramethylethylenediamine)	Sigma-Aldrich, Steinheim, Germany
Tris (2-Amino-2-hydroxymethyl-propane-1,3-diol)	Roth, Karlsruhe, Germany
TWEEN 20	Sigma-Aldrich, Steinheim, Germany
TWEEN 80	Sigma-Aldrich, Steinheim, Germany

Preparation of stock solutions

All flavonoid and the juglone stock solutions were prepared with ethanol : Tween 80 (92:8; v:v) in a concentration of 10 mM. Tween 80 interfered with the detection of H_2O_2 in isolated mouse mitochondria, therefore the flavonoids were dissolved in ethanol for these experiments. Levamisole and paraquat stock solutions were prepared with dH_2O to a concentration of 5 mM and 50 mM, respectively. Fifty micrograms of Mitotracker Red CM-H_2XRos were dissolved in one milliliter of DMSO. 2-aminoethyl diphenyl borate (Naturstoff reagent A) was dissolved in ethanol at a concentration of 5% (w:v).

Buffers and media used for mouse mitochondria isolation and H_2O_2-detection

Table 3. Buffers and media for mouse mitochondria isolation

Chemical	Concentration	Chemical	Concentration
Chappell–Perry buffer 1 (CP1) pH 7.4		Chappell–Perry buffer 2 (CP2)	
KCl	100 mM	ATP	1 mM
Tris/HCl	50 mM	$MgCl_2$	5 mM
EGTA	2 mM	BSA	0.55 (w:v)
		Subtilisin A	1 U/mL
KHE buffer		all in CP1 buffer	
KCl	120 mM		
HEPES	3 mM		
EGTA	1 mM		
KH_2PO_4	5 mM		

C. elegans and OP50 culture buffers and media

Table 4. Buffers and media for *C. elegans* and OP50 culture

Chemical	Concentration	Chemical	Concentration
Potassium phosphate buffer		M9	
KH_2PO_4	108.53 g/L	KH_2PO_4	3 g/L
K_2HPO_4	35.28 g/L	Na_2HPO_4 dihydrate	6.4 g/L
pH 6 adjusted with KOH (5 M)		NaCl	5 g/L
		$MgSO_4$ (1 M)	0.1% (v:v)
Nystatin			
Nystatin dihydrate	12.5 g/L	Bleach solution	
Ammonium acetate (578.1 g/L)	50% (v:v)	NaOCl (12%)	5% (v:v)
Ethanol	50% (v:v)	KOH (5 M)	6.25% (v:v)

Chemical	Concentration
Nematode growth medium (NGM)	
Agar-agar (high strength)	17 g/L
NaCl	3 g/L
Peptone	2.5 g/L
Cholesterol (5 g/L ethanol)	0.1% (v:v)
$CaCl_2$ (1 M)	0.05% (v:v)
$MgSO_4$ (1 M)	0.1% (v:v)
Potassium phosphate buffer (pH6)	2.5% (v:v)
Nystatin	1.3% (v:v)

Chemical	Concentration
DYT	
Peptone	16 g/L
Bacto yeast extract	10 g/L
NaCl	5 g/L
Worm lysis buffer (WLB1)	
Tris/HCl (pH 8)	100 mM
NaCl	200 mM
Glycerol	8 % (w:v)
EDTA	1 mM

Buffers for protein-carbonyl detection

Table 5. Solutions for oxyblot analysis

Chemical	Concentration
Stacking gel buffer	
Tris/HCl (pH 6.8)	0.139 M
SDS	0.11% (v:v)
Resolving gel buffer (3x)	
Tris/HCl (pH 8.8)	1.126 M
SDS	0.3%
Stacking gel	
Stacking gel buffer	82% (v:v)
Acrylamide (30%)	15% (v:v)
TEMED	0.07% (v:v)
APS (10%)	2.4% (v:v)
Resolving gel (12.5%)	
Resolving gel buffer (3x)	33% (v:v)
dH_2O	25% (v:v)
Acrylamide (30%)	41% (v:v)
TEMED	0.06% (v:v)
APS (10%)	1.1% (v:v)
Probe buffer	
Tris/HCl (pH 8)	100 mM
NaCl	200 mM
Glycerol	8 % (w:v)
EDTA	1 mM
DTT (1 M)	0.2% (v:v)
HisPic	0.4% (v:v)

Chemical	Concentration
SDS running buffer	
Tris	25 mM
SDS	0.35 mM
Glycine	192 mM
Transfer buffer	
Tris	2 mM
Glycine	150 mM
SDS	0.7 mM
Methanol	20% (v:v)
PBS-T	
NaCl	0.8% (w:v)
KCl	0.02% (w:v)
Na_2HPO_4	0.144% (w:v)
KH_2PO_4	0.024% (w:v)
pH 6 adjusted with NaOH	
TWEEN 20	0.1% (v:v)
Blocking buffer	
BSA	10% (w:v)
in PBS-T	
Coomassie staining solution	
Coomassie blue R	0.5% (w:v)
Acetic acid	10% (v:v)
iso-propanol	20% (v:v)

3.1.3 *C. elegans* and bacteria strains

All *C. elegans* strains and OP50 feeding *E. coli* were obtained from Caenorhabditis Genetics Center (CGC), University of Minnesota, USA. Except the *hsp-70::gfp* strain (AM446) which was a generous gift from Dr. Richard I. Morimoto Laboratory, Northwestern University, IL, USA.

Table 6. *C. elegans* strains used

Strain	Genotype	Description	Reference
N2 *var Bristol*	wild-type		
TK22	*mev-1(kn1)III*	paraquat sensitive, short lifespan	[102] [148]
TJ356	*zIs356 IV[Pdaf-16::daf-16-gfp; rol-6]*	*daf-16::gfp* reporter	[149]
CF1553	*muIs84[pAD76(sod-3::gfp)]*	*sod-3::gfp* reporter	[76]
MQ989	*isp-1(qm150) IV; ctb-1(qm189)*	ROS-resistant, long-lived	[109]
CL2070	*dvIs70[hsp-16.2::gfp; rol-6(su1006)]*	*phsp-16.2* reporter	[150]
AM446	*rmls223[pC12C8.1::gfp; rol-6(su1006)]*	*phsp-70::gfp* reporter	[151]
CF1038	*daf-16(mu86)I*	short-lived	[56]
MT6308	*eat-4(ky5)III*	reduced pharyngeal pumping	[88]

3.2. Methods

3.2.1 Basic *C. elegans* methods

C. elegans and OP50 culture

All strains were maintained at 20 °C on nematode growth medium (NGM) plates seeded with OP50 feeding bacteria as described previously [34]. OP50 *E. coli* which are auxotrophic for uracil to prevent overgrowing of the worms, were grown overnight in DYT medium at 37 °C.

Inactivation of OP50 bacteria

To prevent metabolism of the flavonoids by the bacteria, OP50 *E. coli* were grown overnight in DYT, concentrated 5x by centrifugation and heat-killed at 65 °C for 30 min according to Gruber *et al.* [152]. Proper inactivation of OP50 was confirmed by streaking the bacteria out on NGM plates and incubation at 37 °C overnight.

Preparation of C. elegans flavonoid containing plates

To prepare treatment plates, flavonoid stock solutions were added on the top of the NGM agar together with the heat-killed OP50 bacteria feeding solution to a final concentration of 100 µM, unless stated otherwise. An equal amount of solvent was used on control plates. The plates were allowed to dry overnight to ensure proper distribution of the flavonoids.

Synchronization of C. elegans cultures

Eggs were obtained by treatment of gravid hermaphrodites with bleach solution and allowed to hatch in M9 buffer overnight. The first stage larvae (L1) were transferred to NGM plates spotted with alive OP50 and grown until fourth larval stage (L4).

Determination of protein content

Worms were washed-off the NGM plates with M9 buffer, pelleted by centrifugation (977 x *g*, 1.5 min) and washed with fresh M9 buffer 2-3 times. Thereafter worm lysis buffer I (WLBI) and an equal amount of glass beads (0.5 mm, Roth, Germany) were added.

Samples were macerated in Fast prep FP120 BIO101 four times for 30 s. Worm samples were cooled on ice between each step. The homogenates were transferred to 15 mL tubes and centrifuged at 8800 x *g* at 4 °C for 2.5 min. Protein concentration was determined in the supernatant using Bio-Rad protein assay according to suppliers instructions.

3.2.2 Analysis of flavonoid bioavailability

As demonstrated in human studies, plasma concentrations of polyphenols after oral administration is in the low micromolar range [127], hence their bioavailability is regarded as rather low. To assess their biological effects *in vivo* it is therefore essential to determine absorption and fate of the compounds in *C. elegans*.

In vivo visualization of flavonoid accumulation in C. elegans with 2-aminoethyl diphenyl borate

Detection of flavonoids in *C. elegans* usually requires analytic techniques such as high-performance liquid chromatography (HPLC), rather large amounts of worm material but gives no information on where the compounds may accumulate [153]. To overcome this, an *in vivo* method by using 2-aminoethyl diphenyl borate (Naturstoff reagent A, NSRA) was established to detect flavonoids in individual nematodes. 2-aminoethyl diphenyl borate is employed in thin layer chromatography, plant histology and cell culture to enhance auto-fluorescence of polyphenols [154] [155]. It forms a chelate with the polyphenolic compounds, leading to a shift of the spectral band (bathochromic shift) and thus to an intensified signal. The effectiveness of NSRA and the appropriate excitation and emission wavelengths of the flavonoids were determined in 96-well plates in a Varioskan plate well-reader. Therefore the flavonoid stock solution was diluted in M9 buffer at a concentration of 100 µM with or without addition of 0.2% (v:v) NSRA and screened for the adequate wavelengths.

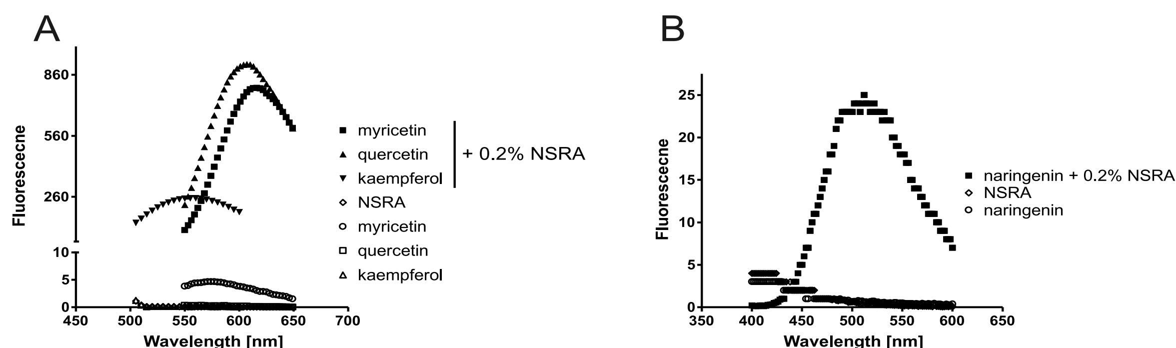


Fig. 5. Emission wavelengths of flavonoids with or without NSRA
Emission wavelengths of 100 µM of indicated flavonoids with or without 0.2% (v:v) NSRA or NSRA alone were determined in 96-well plates in a Varioskan plate reader. Excitation wavelength for myricetin, quercetin and kaempferol was 488 nm (A) and for naringenin 350 nm (B).

Analysis revealed wavelengths for excitation at 488 nm and for emission at 590-620 nm for myricetin and quercetin and at 540-560 nm for kaempferol. The flavanone naringenin displayed best wavelengths for excitation at 350 nm and for emission at 520 nm (Fig. 5). Thereby it was demonstrated that NSRA selectively enhances auto-fluorescence of the flavonoids as fluorescence of the flavonoids and/or NSRA alone was considerably lower.

To analyze the flavonoid uptake in *C. elegans*, synchronized wild-type L4 larvae were incubated on NGM plates containing myricetin, quercetin, kaempferol or naringenin in increasing concentrations (0, 10, 50 or 100 µM) or the solvent for 48 hours. Thereafter, the worms were incubated in M9 buffer containing 10% heat-killed OP50 bacteria and 0.2% 2-aminoethyl diphenyl borate for 2 hours. Subsequently, fluorescence was monitored using a confocal laser scanning microscope with the above-determined wavelengths at a constant laser intensity.

HPLC/DAD analysis

To validate these results and to get insight into metabolism in nematodes, a HPLC-based analysis with diode array detection (HPLC/DAD) of the flavonoids in *C. elegans* was conducted by the group of Prof. Kulling (Max Rubner-Institut, Karlsruhe, Germany). Wild-type *C. elegans* were grown on standard NGM plates containing 100 µM of myricetin, quercetin, kaempferol or naringenin for 48 hours. To remove all adhered flavonoids, *C. elegans* were subsequently washed 4-5 times with 0.1% (w:v) bovine serum albumin in dH_2O and frozen in liquid nitrogen. Worm samples were thawed, homogenized

and protein content was determined as described above. Fifty microliters of the resuspended homogenate were spiked with 3 µL of the internal standard (0.5 mM apigenin stock solution in DMSO) and incubated for 15 min at room temperature. The total flavonoid content was determined as aglycon equivalents after hydrolysis of the samples. Therefore the samples were incubated in 850 µL of an ammonium acetate buffer (0.1 M, pH 5.0) containing 1,4-dithiothreitol (100 µM), 2000 U ß-glucuronidase (*Helix pomatia*, Typ-H-1) and 75 U arylsulfatase (*Helix pomatia*, Typ H-1) at 37°C for 2 hours. After acidification with 300 µL of 10% (w:v) citric acid solution, the samples were extracted four times with ethyl acetate. Thereafter the four extracts were combined, evaporated to dryness under a gentle nitrogen stream and the residue was redissolved in 150 µL methanol:water (1:1) prior analysis by HPLC/DAD. For quantification of the flavonoid aglycon content of the samples, the same protocol was used without addition of the enzymes ß-glucuronidase and arylsulfatase.

A Shimadzu LC system equipped with a controller (CBM-20A), a degasser (DGU-20A3), two pumps (LC-20AD), an autosampler (SIL-20AC HT), a column oven (CTO-20AC) and a diode array detector (SPD-M20A) was used for HPLC/DAD analysis. The LC system was controlled by the software LCsolution 1.24. Separation and detection of the aglycones was performed under following conditions:

Table 7. HPLC/DAD Analysis

Column	YMC Pack Hydrosphere C18 column (3.0 mm internal diameter, 150 mm length, 3 µm particle size)
Column temperature	40 °C
Solvent A	25 mM ammonium formate buffer (pH 3.0)
Solvent B	acetonitrile
Flow rate	0.8 mL/min
Injection volume	20 µL
LC-gradient	0 min 10% solvent B, 90% solvent A 29 min 40% solvent B, 60% solvent A 30 min - 34 min 95% solvent B, 5% solvent A 35 min – 46 min 10% solvent B, 90% solvent A
Detection wavelengths	290 nm naringenin 340 nm apigenin 370 nm myricetin, quercetin, kaempferol

The identity of each compound was confirmed by the retention time and the UV-Vis spectra. The limits of quantification for each flavonoid were as follows: 2.10 pmol for

apigenin, 2.94 pmol for naringenin, 1.14 pmol for kaempferol, 1.80 pmol for quercetin and 1.62 pmol for myricetin. The flavonoid content was calculated relative to the protein content in pmol*mg^{-1} protein. The average recovery rates of the flavonoids under the described experimental protocol were determined to be 87.5 ± 5.8 % for myricetin, 83.1 ± 5.2 % for quercetin, 78.0 ± 5.0 % for kaempferol, 94.9 ± 6.1 % for naringenin, and 95.3 ± 6.2 % for apigenin.

3.2.3 Lifespan and stress-resistance analysis

Lifespan analysis

L4 larvae were randomly picked from NGM plates and transferred onto plates containing the flavonoids or the solvent control. Due to differences in the duration of development among the strains, treatment was always started with animals in L4 stage. Surviving and dead animals were counted daily while animals that crawled up the side of the petri dish and desiccated or suffered from internal hatching were censored. Worms were scored as dead when they failed to respond to a gentle touch with a platinum wire. To prevent overcrowding by progeny, animals were transferred to new plates every second day until aging did not allow further handling of the worms.

Resistance against oxidative stress

To induce chronic oxidative stress the redox-cycling agent paraquat (Fig. 6) dissolved in water, was used. Paraquat is widely employed to increase ROS-production, possibly by stimulating superoxide anion radical-generation in the mitochondria via complex I [156]. NGM plates were prepared with dead OP50 bacteria and flavonoids as mentioned above. Thereafter the plates were spotted with paraquat at a final concentration of 1.6 mM and allowed to dry. L4 larvae were randomly picked and transferred onto these treatment plates. Surviving worms were counted daily and transferred ever second day to new plates to ensure full activity of paraquat. Worms that escaped from the plates or died from internal hatching were censored.

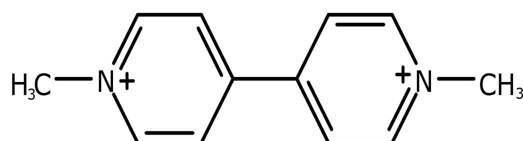


Fig. 6 Chemical structure of paraquat

To determine the impact of acute paraquat-stress the wild-type L4 *C. elegans* were incubated with the flavonoids on NGM plates for 24 or 48 hours as described above. Subsequently the worms were transferred to 3.5 cm petri dishes with M9 buffer containing paraquat in a final concentration of 100 mM. This medium was supplemented with 10% (v:v) heat-killed OP50 bacteria to prevent starvation and the plates were constantly shaken at 120 rpm and 20 °C. The surviving animals were counted after 24 hours.

Resistance against heat-stress

To analyze the impact of thermal stress, L4 *C. elegans* were incubated on flavonoid-containing NGM plates for 48 hours and subsequently transferred to 34 °C. Surviving worms were counted hourly and animals were scored as dead when they failed to respond to a touch with a platinum wire.

3.2.4 Analysis of ROS-generation in mice and *C. elegans*

H_2O_2-generation in isolated mouse mitochondria

As mitochondria are thought to be the major source of endogenously generated ROS, the impact of the flavonoids on superoxide anion radical production was analyzed in isolated mouse skeletal muscle mitochondria. This model was chosen due to its relevance in mammals and humans. Additionally, isolated mitochondria provide a well-established tool to measure mitochondrial function [157] and could therefore serve as a valid *in vitro* system to analyze the characteristics of the flavonoids. Superoxide anion radicals show low diffusion rates across membranes [158], but are rapidly converted to H_2O_2 which permeates membranes more easily. Elucidation of mitochondrial H_2O_2-production was conducted using the Amplex Red Assay that determines the H_2O_2 dependent conversion of Amplex Red to the fluorescent resorufin by the horseradish peroxidase (HRP). The isolation of

mitochondria and H_2O_2 measurement was done by Kerstin Haas (Technische Universität München, Freising, Germany).

Four male C57BL/6J mice (8 weeks of age) were sacrificed for the isolation of skeletal muscle mitochondria according to the method of Talbot and Brand with minor modifications [159]. Briefly, skeletal muscle tissue was collected from the hind limbs, quickly freed from visible fat and connective tissue and finely scissored and rinsed in ice cold CP1 buffer. The minced tissue was stirred in CP2 buffer for 3 min, further homogenized using a Polytron PT 3100 (3 x 4 s, 1800 rpm) and finally stirred for another 3 min. The cell fragments were pelleted at 490 x *g* for 10 min and the supernatant was filtered through a gaze. The supernatant was cleaned-up twice by centrifugation at 10400 x *g* for 10 min and the pellet was resuspended after each centrifugation in CP1. To obtain the mitochondrial fraction centrifugation at 3800 x g for 10 min was carried out followed by resuspending the pellet in a minimum volume of CP1 buffer. Finally the protein concentration was determined by use of the Bradford assay. All centrifuge steps were performed at 4 °C.

H_2O_2-generation by isolated mitochondria was monitored using Tecan Infinite 200 M plate-reader (Tecan, Switzerland). Mitochondria (20 µg protein per well) were incubated in 180 µL KHE buffer supplied with 0.3% (w:v) BSA. Additionally 6 U/mL HRP, 30 U/mL SOD, 50 µM Amplex Red, and 100 µM of the different flavonoids and/or solvent as control were added per well. Additionally, 2 µM rotenone, 1 mM ADP and 5 µM paraquat were added, respectively, depending on the design of the experiment. Fluorescence of Amplex Red was measured using excitation/emission wavelength of 560 and 590 nm at 37°C for 45 min. Succinate dissolved in KHE buffer was injected to a final concentration of 10 mM to fuel mitochondria. The H_2O_2-production rate was calculated using a standard curve which plotted the relative fluorescence of resorufin to known amounts of H_2O_2 (Fig. 7).

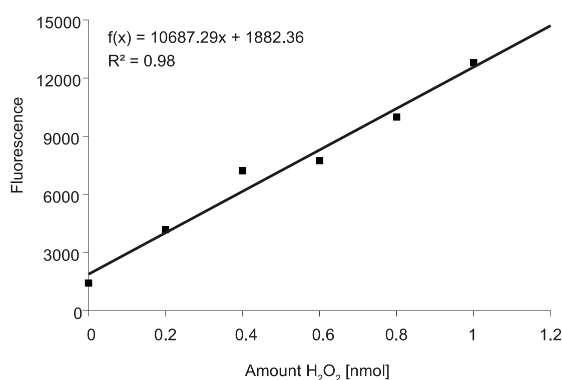


Fig. 7 H_2O_2 standard curve
Standard curve showing fluorescence of resorufin induced by a known amount of H_2O_2. Slope and correlation coefficient is given.

ROS levels in C. elegans

To measure mtROS in *C. elegans*, the dye Mitotracker Red CM-H_2XRos was used. CM-H_2XRos enters mitochondria dependent on the membrane potential, where it is oxidized to its fluorescent form by ROS and sequestered in the matrix [160]. The dye was dissolved in DMSO and spotted together with the heat-killed OP50 bacteria on top of the agar plates at a final concentration of 0.5 µM. The synchronized L4 nematodes were incubated on NGM plates containing the flavonoids or the solvent for 2, 6, 24 or 48 hours. Twenty-four hours prior measurement, these worms were then transferred onto plates containing 0.5 µM CM-H_2XRos and the flavonoids or solvent, respectively. Subsequently, the living nematodes were washed with M9 buffer, anesthetized with 5 mM levamisole, placed on agarose pads (2%) on object slides and analyzed by confocal laser scanning microscopy with excitation at 578 nm and emission at 590-650 nm. The fluorescence intensity of the pharyngeal area of at least ten worms per group was examined by overlaying the images of 10 z-slices. To validate this method, worms with initially altered ROS levels were analyzed (Fig. 8).

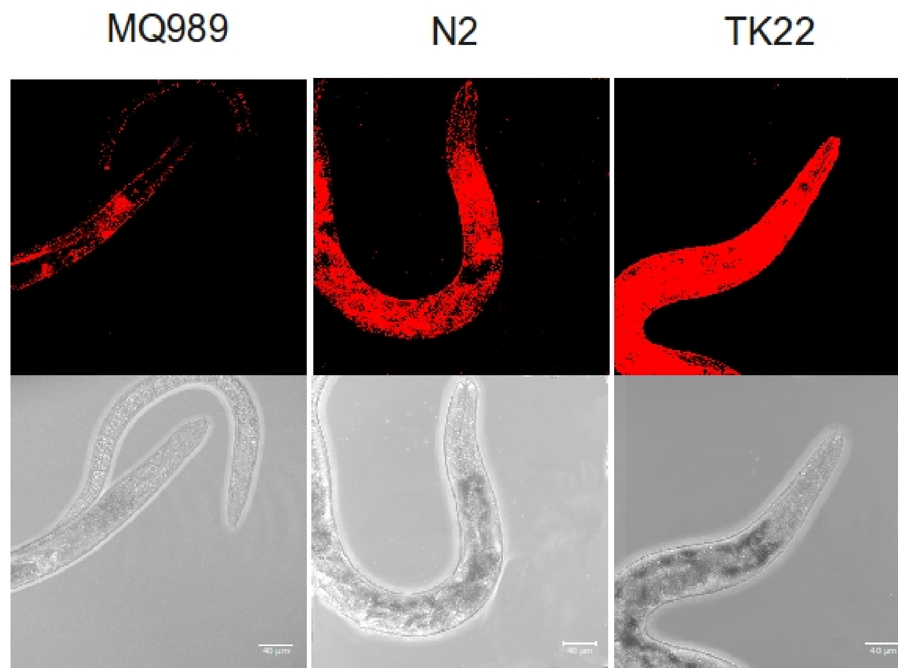


Fig. 8 Fluorescence of CM-H_2XRos in *isp-1(qm150);ctb-1(qm189)* (MQ989), wild-type (N2) and *mev-1(kn1)* (TK22) *C. elegans*
Representative images of *isp-1(qm150);ctb-1(qm189),* wild-type *and mev-1(kn1) C. elegans* after 24 hours on 0.5 µM CM-H_2XRos. Upper panel shows fluorescence pictures and lower panel shows transmitted-light pictures. The anterior end of the nematodes is located to the top.

isp-1(qm150);ctb-1(qm189) mutants carry two genetic defects in complex III of the ETC namely in *isp-1* (a gene encoding a Rieske iron sulfur protein subunit) and *ctb-1* (a gene encoding cytochrome b). These mutations make the worms long-lived and more resistant against oxidative stress, suggesting a reduced mitochondrial ROS-generation [109]. By contrast, *mev-1(kn1)* mutants carry a mutation in a gene encoding a succinate dehydrogenase subunit C (complex II of ETC) making them short-lived and sensitive against oxidative stress [102] [103]. Furthermore, this strain was shown to possess elevated levels of superoxide anions [19] [161]. As fluorescence was reduced in *isp-1(qm150);ctb-1(qm189)* and increased in *mev-1(kn1)* mutants, CM-H_2XRos fluorescence was verified as marker for mtROS load.

3.2.5 Analysis of protein-carbonyls

Increased concentrations of ROS can lead to oxidative damage of macromolecules including proteins, resulting in various modifications of the amino acid backbone consequently damaging the protein structure. Introduction of a carbonyl function to amino acids, called carbonylation, marks these proteins for degradation [162] and protein carbonyl levels correlate with ROS and aging [163]. Therefore the protein-carbonyl content (PCC) was determined in *C. elegans* after treatment with flavonoids using the OxyBlot Protein Oxidation Detection Kit. Here the protein-carbonyl groups are derivatized by 2,4-dinitrophenol hydrazine (DNPH) to 2,4- dinitrophenol hydrazone (Fig. 9) that is detectable with immunological methods [164].

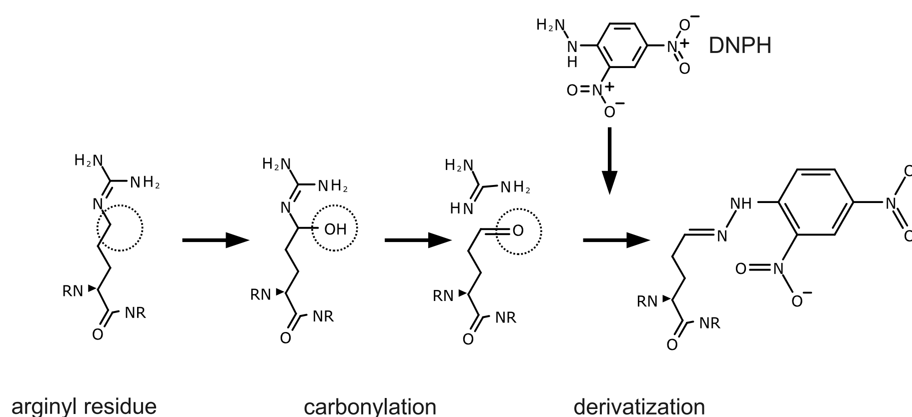


Fig. 9 Carbonylation and derivatization of an arginyl residue
Schematic representation showing the oxidative carbonylation of an arginyl residue and its derivatization by 2,4- dinitrophenol hydrazine (DNPH) as described in [164].

Briefly, worms were synchronized and allowed to grow until L4 stage, as described above. Flavonoid treatment plates and plates for 11-day-old worms were prepared as described under 3.2.1, except that 50 µM of 5-Fluoro-2'-deoxyuridine was added to the NGM to prevent hatching of progeny. After incubation, nematodes were washed-off the plates and the protein content was determined as described above except that probe buffer was used instead of WLB1 to prevent protein degradation. All steps were carried out on ice. The samples were diluted with probe buffer to 1 µg/µL and 10 µL aliquots were denatured with 10 µL of SDS (12%). For derivatization, 20 µL of 1x DNPH was added and samples were incubated at room temperature for 15 min. Derivatization was stopped by addition of 15 µL of neutralization solution and subsequently the samples were stored at 4 °C overnight.

Two identical SDS polyacrylamide gels were prepared, each consisting of a stacking gel and a 12.5% resolving gel, one of which serving as loading control. Equal amounts of each sample were loaded onto each SDS gel and the gels were run at 160 V in SDS running buffer for approximately 40 min. The loading control gel was stained for 3-5 hours in Coomassie staining solution and proper loading of the samples was analyzed after destaining with acetic acid (10%). This method to control the correct protein-loading was used since determination by a “housekeeping” protein (e. g. actin) failed and just non-derivatized samples showed a signal on the blots (data not shown).

The proteins of the second gel were electro-blotted on a nitrocellulose membrane in transfer buffer at 0.36 A for 20 min. After confirmation of proper protein transfer by

staining with Ponceau S, the membrane was blocked with blocking buffer solution for one hour. Thereafter, the membrane was incubated with 1° antibody (rabbit anti-DNPH, 1:150 (v:v), in blocking buffer) for one hour, followed by three washing steps with PBS-T each lasting 10 min. The 2° antibody (goat anti-rabbit horseradish-peroxidase conjugated, 1:300 (v:v), in blocking buffer) was added to the membrane and incubated for one hour. After three washing steps with PBS-T (each lasting 10 min), SuperSignal West Pico Chemiluminescent substrate was added and chemiluminescence was detected on radiographic film. Films were scanned and analyzed using ImageJ gel analysis software (http://rsbweb.nih.gov/ij/index.html 2011-03-01) [165].

3.2.6 Analysis of metabolic parameters

Determination of pharynx pumping rate

The analysis of the pharynx pumping rate is a useful tool to analyze alterations in the food intake, which – when reduced – can increase the lifespan in *C. elegans* [89]. Synchronous L4 larvae were incubated with 100 µM flavonoids or solvent control plates for 48 hours, as described above. Pharyngeal pumping was counted for 15 seconds of 20-30 individuals of every group and trial. As positive control the strain MT6308 (*eat-4(ky5)III*) was employed, showing reduced pharyngeal pumping [88] due to a genetic defect in a gene encoding a vesicular glutamate transporter [166].

Determination of oxygen consumption

Respiration rate of *C. elegans* was determined by the use of oxygen sensing system Apollo 4000 equipped with iso-oxy-2 electrode. The electrode was calibrated by exposure to nitrogen (defined as 0% oxygen) and to compressed air (defined as 21% oxygen). Temperature of the oxygen-sensing chamber was kept constant at 20 °C by an external circulating bath and controlled with the equipped ISO-TEMP-2 temperature sensor. Synchronized L4 *C. elegans* were grown on NGM plates containing 100 µM of the tested flavonoids or solvent control (see above) and 50 µM of 5-Fluoro-2'-deoxyuridine to prevent hatching of progeny. The worms were washed-off the plates and washed with M9 buffer 3-4 times thoroughly. The pellet of one plate was resuspended in 1 mL M9 buffer and transferred into the closed oxygen-sensing chamber. After measurement of the respiration for at least 10 minutes, worms were carefully recovered and immediately frozen

in liquid nitrogen. Protein content was determined as described under 3.2.1. The rate of respiration was calculated from the decline of the oxygen concentration (Fig. 10) in relation to the amount of protein in the samples. Assuming an oxygen saturation at 20 °C and normal atmospheric pressure (21% O_2) of 9.1 mg/L (=0.56875 mM), the respiration rate was calculated in [nmol*μg^{-1}*mL^{-1}*min^{-1}].

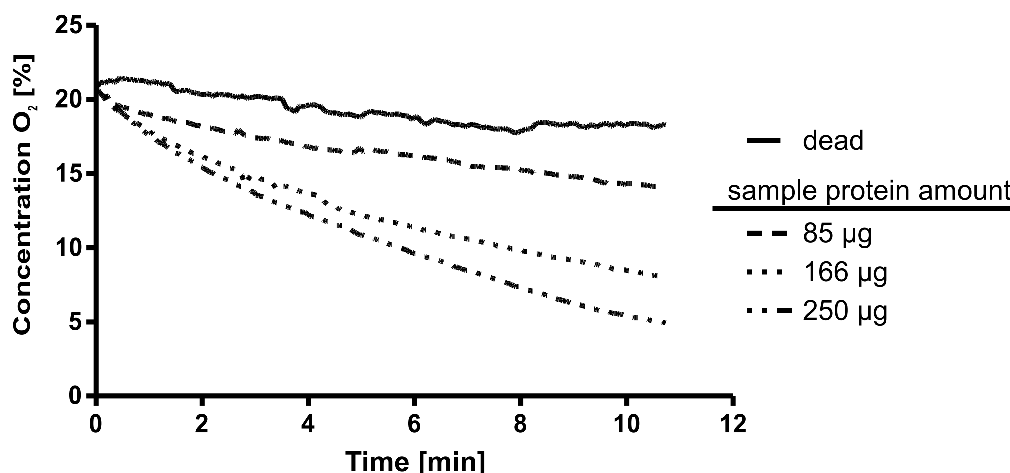


Fig. 10 Oxygen consumption of different sample size and killed *C. elegans*
Representative diagram showing oxygen concentration changes over time in an oxygen-sensing chamber. Results of *C. elegans* of different samples size (85 µg, 166 µg and 250 µg protein) and freeze-killed animals are shown.

3.2.7 Analysis of GFP-reporter strains

Analysis of DAF-16-distribution and sod3-promoter activity

To visualize the distribution of the DAF-16 transcription factor the *daf-16::gfp* reporter strain TJ356 was used [149]. Synchronous L4 larvae were incubated on 100 µM flavonoid or solvent control plates for 48 hours, as described above. Subsequent to this treatment, the living worms were washed-off the plates, anesthetized with 5 mM levamisole solution, placed on agarose pads (2%) and analyzed by confocal laser scanning microscopy with excitation at 488 nm and emission at 543 nm. At least 100 worms were analyzed per group and trial and classified with respect to the majority of the DAF-16::GFP fusion protein localization into the categories nuclear, intermediate and cytosolic (Fig. 11).

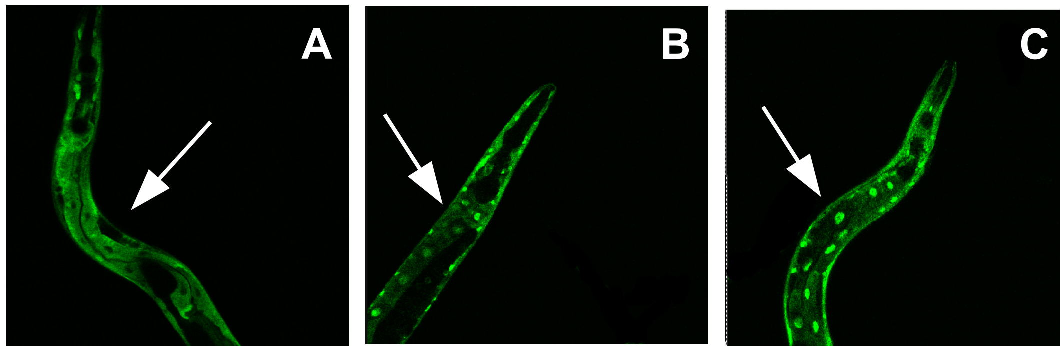


Fig. 11 DAF-16::GFP distribution
Representative microscopical images at 40-fold magnification of *daf-16::gfp* reporter worms showing nuclear (A), intermediate (B) and cytosolic (C) distribution of the DAF-16::GFP reporter. The gut is indicated by arrows.

SOD-3 is a mitochondrial manganese superoxide dismutase (SOD) and its encoding gene is a well-known target of the DAF-16 transcription factor [64] [167]. To visualize *sod-3* promoter activity, *sod-3::gfp* reporter worms, expressing GFP under control of the promoter of *sod-3* were used. Synchronous L4 worms were treated as described for the *daf-16::gfp* strain above, except that one group of worms were incubated on agar-plates containing 150 µM of the redox-cycling agent juglone for 3 hours prior analysis. Fluorescence of whole worms of at least 10 animals per group and experiment were determined by overlaying the images of 10 z-slices obtained by use of confocal laser scanning microscopy with excitation at 488 nm and emission at 543 nm.

Analysis of heat-shock protein promoter activity

Heat-shock proteins are a group of proteins that is known to be expressed at high levels under stress conditions such as heat- or ROS-stress. They thereby can act as molecular chaperones, help to clear misfolded proteins, cure stress-induced nucleic acid modifications or regulate transcription [168]. C12C8.1 encodes a *C. elegans* HSP-70 protein belonging to the group of large heat-shock proteins serving as chaperone that was shown to be, at least partly, necessary for IlS-associated longevity [151]. HSP-16.2 belongs to the class of small heat-shock proteins which are likely to serve as molecular chaperones [169]. Its expression in *C. elegans* also depends on DAF-16 transcription factor [170] and its promoter activity was shown to be an indicator of various stresses [150]. The promoter activities of *hsp-70* and *hsp-16.2* were analyzed using the promoter::GFP reporter strains AM446 and CL2070, respectively. Synchronized L4 larvae were grown on flavonoid

treatment and/or control plates for 48 hours, as described above. Fifty worms were randomly picked and transferred into one well (containing 60 µL of M9 buffer) of a black 384-well plate. For each treatment condition four replicates per experiment were performed. Fluorescence was determined in a Varioskan well-reader (Thermo Elektron, USA) with excitation at 488 nm and emission at 543 nm under constant shaking every 10 min for a total of 330 min at 20 °C. As no fluorescence was detectable under stress-free conditions (Fig. 12), heat-stress was induced by incubation at 34 °C for 2 x 30 min prior Varioskan analysis.

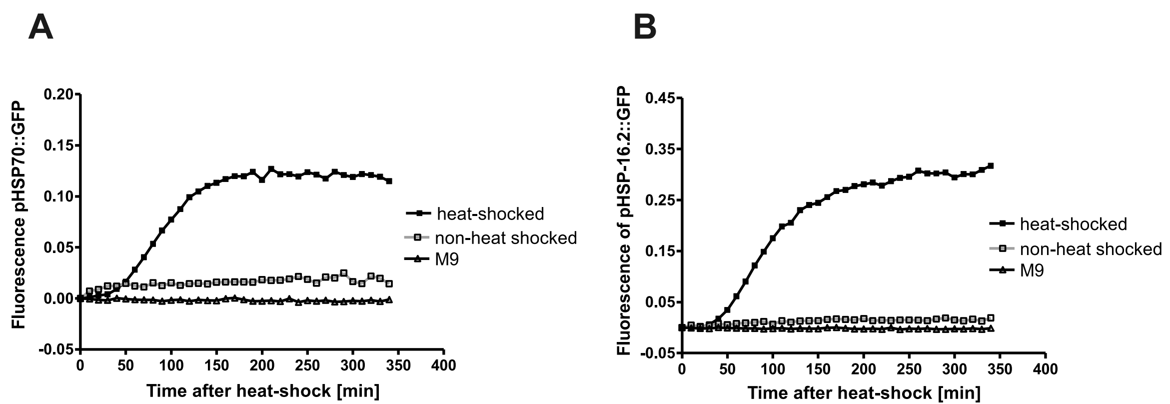


Fig. 12 Induction of HSP::GFP fluorescence after heat shock
Representative diagram showing fluorescence of GFP in C12C8.1*::gfp* (A) and *hsp-16.2::gfp* (B) *C. elegans* over time. Curves represent animals that were incubated at 34 °C for 2x 30 min (heat-shocked), at 20 °C (non-heat shocked) or empty wells, just containing the buffer (M9). Values are present means of 50 animals per well and 4 wells per group.

hsp-16.2 has also been shown to be a good ROS-stress sensor as its promoter activity was induced after feeding the ROS-generator juglone [171]. Furthermore, the juglone-induced promoter activity of *hsp-16.2* was demonstrated to be diminished by a plant-derived extract [172]. To assess the impact of the flavonoids on *hsp-16.2* promoter activity, synchronous L4 *hsp-16.2::gfp C. elegans* were incubated on 100 µM flavonoid or solvent control plates for 48 hours, as described above. Subsequent to this treatment, the living worms were washed-off the plates and incubated in M9 buffer containing 5% heat-killed OP50 bacteria, 100 µM juglone or solvent for 4 hours. Fluorescence of the pharyngeal area of at least 10 animals per group and experiment were determined by overlaying the images of 10 z-slices obtained by use of confocal laser scanning microscopy with excitation at 488 nm and emission at 543 nm.

3.2.8 Data interpretation and statistical analysis

All data were collected and analyzed using GraphPad Prism 4.03 for Windows. Lifespan and stress-resistance data were compared by Log rank test and plotted as Kaplan-Meier survival curves. Maximum lifespan was calculated as the median survival of the longest living 10% of a population. Data between groups were compared, after elimination of outliers by Grubb's test, by one-way ANOVA with Dunnett's post-hoc test and means were considered significant different at $P < 0.05$, $P < 0.01$ and $P < 0.001$ (see figures).

4. Results

4.1. Bioavailability of flavonoids

4.1.1 Visualization of flavonoids by Naturstoff reagent A

Detection and semi-quantification of flavonoids was conducted by derivatization with 2-aminoethyl diphenyl borate (see 3.2.2). Following a 48-hour exposure, the auto-fluorescence of myricetin, quercetin and kaempferol was increased by NSRA in a dose-dependent manner demonstrating their availability to the nematodes. In contrast to the flavonols, no fluorescence was detected in the worms treated with naringenin at any concentration (Fig. 13).

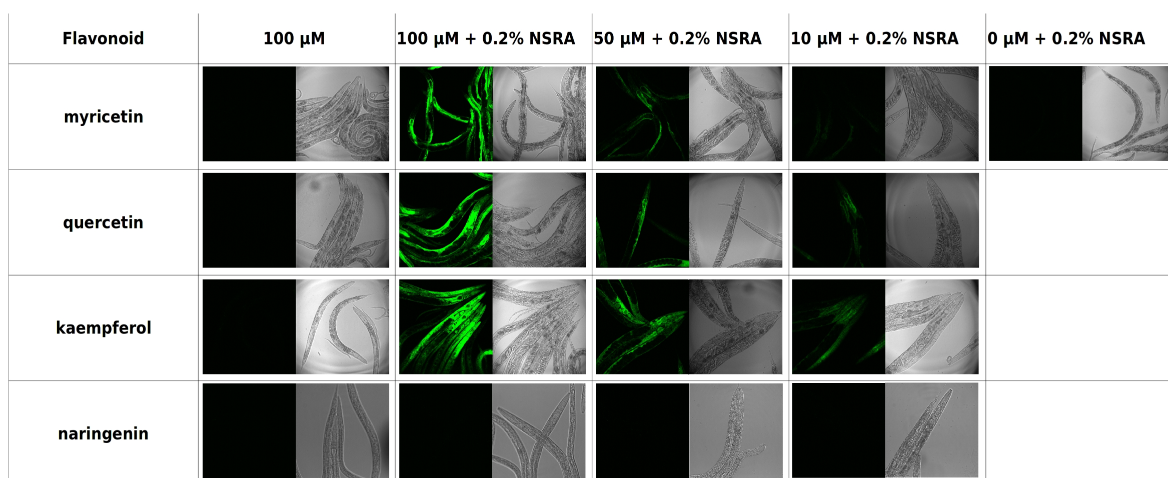


Fig. 13 Enhanced auto-fluorescence of flavonoids in wild-type *C. elegans*.
Representative images of wild-type *C. elegans* treated with 0 µM, 10 µM, 50 µM or 100 µM of indicated flavonoids or solvent control for 48 hours. After flavonoid-exposure worms were incubated with or without 0.2% 2-aminoethyl diphenyl borate (Naturstoff reagent A, NSRA) for 2 hours to enhance auto-fluorescence of the flavonoids.

Although this might suggest that naringenin is not taken up by the worms, it has to be taken into account that NSRA enhanced auto-fluorescence of the flavonols *in vitro* by up to ~900 fold whereas auto-fluorescence of naringenin was increased just ~25 fold (see 3.2.2 Fig. 5). So, in these animals, naringenin detection is limited by the sensitivity of the assay. Nevertheless, the data show that the flavonols are available to *C. elegans* depending on the administered dose. For all flavonols, highest fluorescence was always obtained in gut epithelial cells while lower signals were found in other cells (Fig. 14). Taken together,

NSRA seems to be a suitable tool to visualize and semi-quantify flavonols *in vivo* in *C. elegans*.

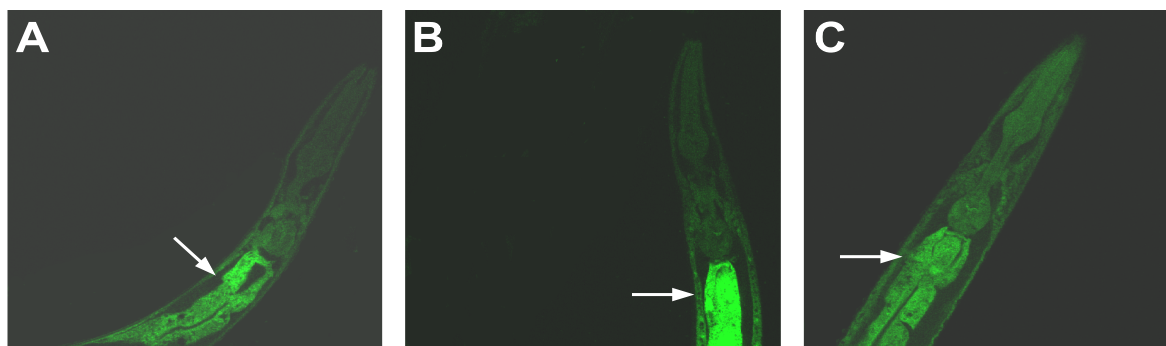


Fig. 14 Closeup view of NSRA-enhanced auto-fluorescence of flavonols in *C. elegans*
Representative pictures of the cranial region of adult wild-type *C. elegans* at 40-fold magnification. Nematodes were treated with 100 µM of myricetin (A), quercetin (B) or kaempferol (C) for 48 hours followed by incubation with 0.2% NSRA. The gut is indicated by arrows.

4.1.2 HPLC/DAD analysis of flavonoids in *C. elegans*

To validate the results obtained by staining with 2-aminoethyl diphenyl borate, the flavonoid content and metabolites in wild-type *C. elegans* was analyzed by HPLC/DAD. All flavonoids were detected in the nematodes with highest concentrations found for naringenin followed by myricetin, quercetin and kaempferol. Essentially all compounds were only detected as conjugates (>95%) with kaempferol showing the lowest conjugation rate followed by naringenin, myricetin and quercetin (Table 8). Although naringenin was not detectable by 2-aminoethyl diphenyl borate *in vivo,* these data clearly show that this flavanone is taken up efficiently by *C. elegans*. The high rate of conjugation observed, strongly suggests the nematodes to possess a large capacity for glucuronidation and sulfatation of flavonoids.

Taken together, chemical analysis and visualization data demonstrate that the flavonoids are efficiently taken up by *C. elegans* in a dose-dependent manner but obviously undergo extensive metabolism by conjugation.

Table 8. Concentration of conjugated and free flavonoids in *C. elegans*

	Flavonoid content [nmol*mg^{-1} protein ± SD]		Free aglycon [%]
Flavonoid	conjugated	free	
Myricetin	23.6 ± 0.46	0.36 ± 0.13	1.5 ± 0.6
Quercetin	23.1 ± 3.51	0.26 ± 0.02	1.1 ± 0.6
Kaempferol	17.6 ± 1.13	1.47 ± 0.07	7.7 ± 0.6
Naringenin	35.4 ± 4.64	1.57 ± 0.26	4.2 ± 0.6

4.2. Influence of flavonoids on *C. elegans* lifespan

Based on their putative effects, the flavonoids could impact on lifespan in *C. elegans.* The nematodes were therefore exposed to 100 µM of the flavonoids, a concentration that has been demonstrated for other plant-derived compounds to be sufficient to induce longevity [173] [174].

Flavonols increase lifespan in wild-type C. elegans

In wild-type *C. elegans* myricetin increased mean adult lifespan of ~12.8 days by 18% followed by quercetin (5.8%) and kaempferol (5.6%). Solely the flavanone naringenin failed to alter lifespan of wild-type nematodes. The maximum lifespan was extended by all flavonoids with strongest effects seen by myricetin and quercetin (Fig. 15; Table 10 appendix). The differences between the compounds suggest that the structural properties at the C-ring are crucial for lifespan extension which obviously increases with the number of hydroxyl groups attached to the B-ring.

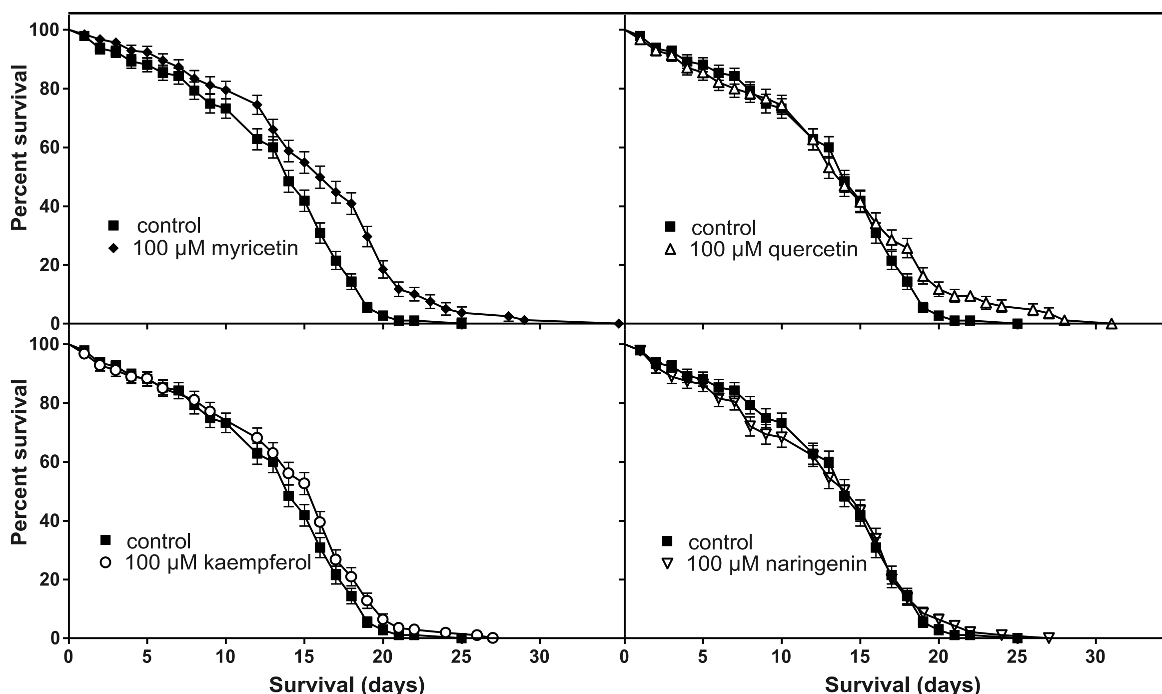


Fig. 15 Effects of flavonoids on adult lifespan in wild-type *C. elegans*
Effects of 100 µM of the selected flavonoids on adult lifespan in wild-type *C. elegans*. Data is plotted as Kaplan-Meier survival curves with SEM based on two individual experiments with at least 90 individuals per group. Details see Table 10, appendix.

To test whether a lower concentration of the flavonoids is also capable to induce a prolonged lifespan, *C. elegans* were exposed to 10 µM myricetin, the flavonol showing the strongest lifespan-extending effect at 100 µM. As no effect on lifespan was observed, this concentrations seems not sufficient to induce life-span extension.

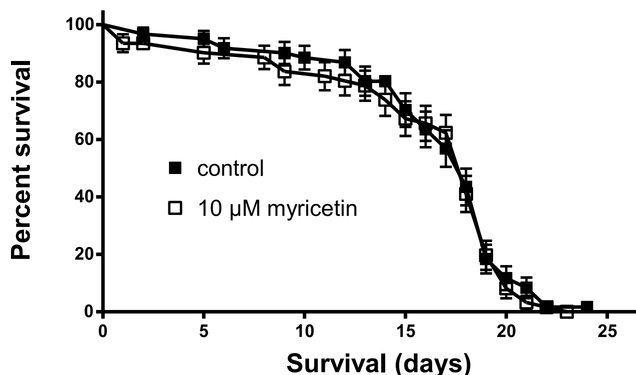


Fig. 16 Effect of 10 µM myricetin on adult lifespan in wild-type *C. elegans*
Effect of 10 µM myricetin on adult lifespan in wild-type *C. elegans* under normal culture conditions at 20 °C. Data is plotted as Kaplan-Meier survival curves with SEM based on at least 60 individuals per group.

Myricetin increases lifespan in mev-1(kn1) mutants

mev-1(kn1)III C. elegans that carry a genetic defect in complex II of the respiratory chain were shown to be short-lived and to possess elevated levels of mtROS [19] [175] [102] [117]. A mean adult lifespan of 5.3 days was observed in this *C. elegans* strain, confirming their reduced lifespan. Myricetin extended the mean lifespan in *mev-1(kn1)* mutants (by ~16%) whereas none of the other flavonoids showed any effect demonstrating again a high number of hydroxyl groups at the B-ring to be necessary for lifespan extension in this strain. Maximum lifespan, however, was elevated by all tested flavonoids by more than 20% when compared to control (Fig. 17; Table 11 appendix).

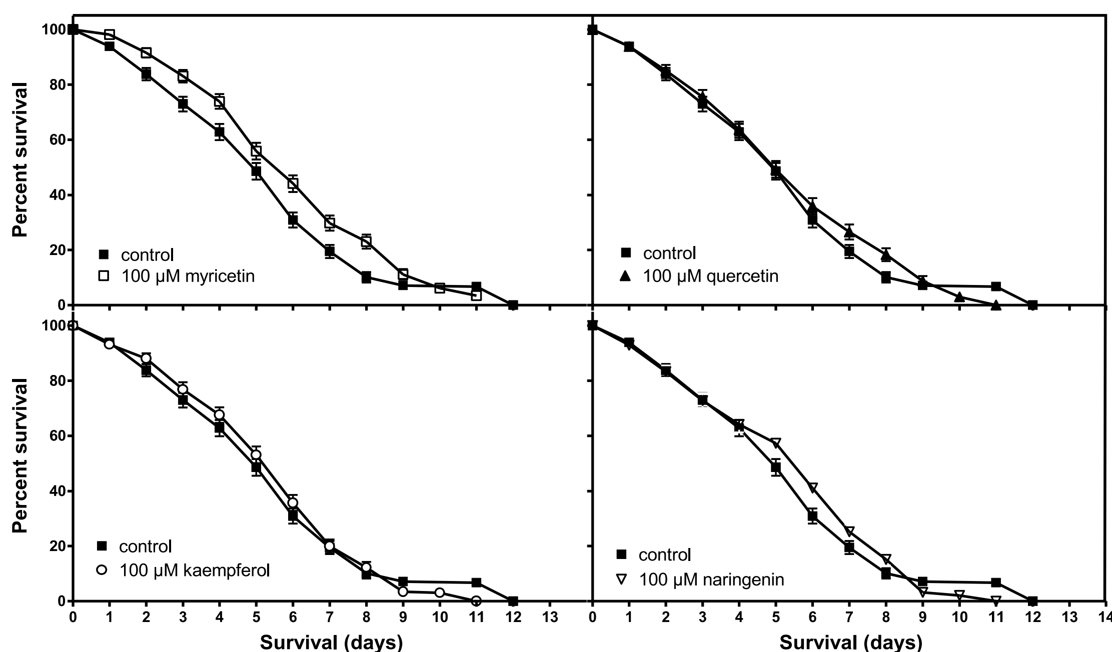


Fig. 17 Effects of flavonoids on adult lifespan in *mev-1(kn1) C. elegans*
Effects of 100 µM of the selected flavonoids on adult lifespan in *mev-1(kn1) C. elegans* under normal culture conditions at 20 °C. Data is plotted as Kaplan-Meier survival curves with SEM based on three individual experiments with at least 90 individuals per group. Details see Table 11 appendix.

Myricetin increases lifespan in isp-1(qm150);ctb-1(qm189) C. elegans

isp-1(qm150);ctb-1(qm189) mutants possess two genetic defects in complex III of the ETC, are described to be long-lived and suggested to have reduced mtROS levels [109]. Their mean adult lifespan was determined with ~11.5 d which is therefore shorter than that of wild-type. However, considering the delayed larval development of *isp-1(qm150);ctb-1(qm189)* nematodes, namely four days longer to reach L4 stage, the total lifespan is longer. As in wild-type and *mev-1(kn1)* mutants, treatment with myricetin increased mean adult lifespan by ~14% whereas quercetin, kaempferol and naringenin had no significant effect. However, maximum lifespan was elevated again modestly by all flavonoids (Fig. 18; Table 12 appendix).

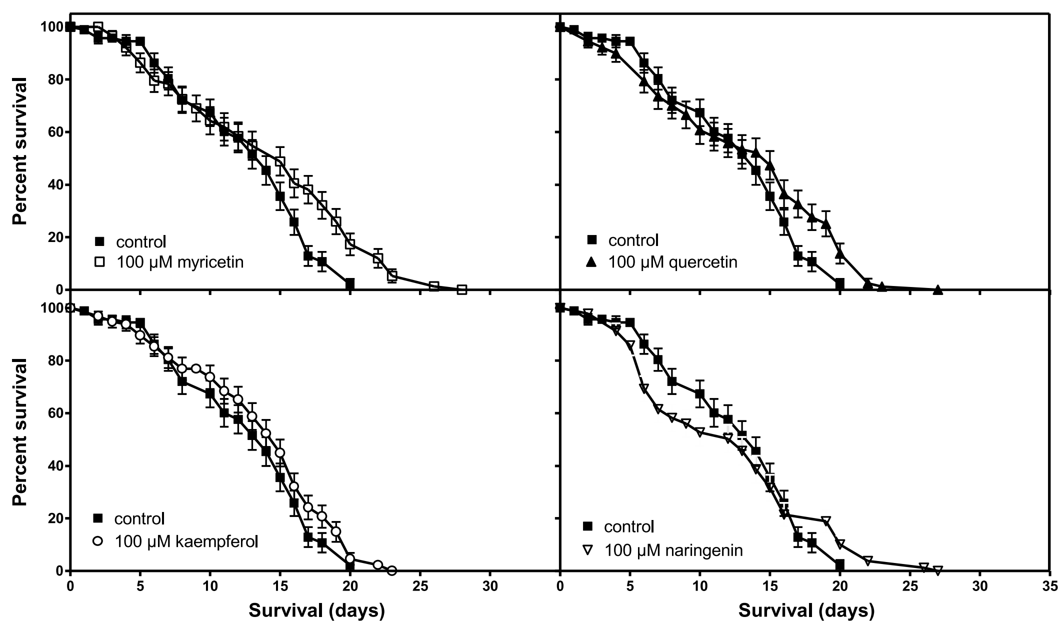


Fig. 18 Effects of flavonoids on adult lifespan in *isp-1(qm150);ctb-1(qm189) C. elegans*
Effects of 100 µM of the selected flavonoids on adult lifespan in *isp-1(qm150);ctb-1(qm189) C. elegans* under normal culture conditions at 20 °C. Data is plotted as Kaplan-Meier survival curves with SEM based on a minimum of 90 individuals per group. Details see Table 12 appendix.

Taken together, the results suggest that the longevity-effects of the compounds depend on the structural properties at the C-ring and an increasing number of hydroxyl groups attached to the B-ring, all structural properties also known to affect the antioxidant potential *in vitro* [176].

4.3. Impact of flavonoids on mitochondrial ROS

The antioxidant action is by far the most recognized property of flavonoids [176]. Despite structural prerequisites and mechanisms being well-characterized *in vitro*, their actions in more complex systems remain largely unknown [147] [132]. Since the flavonoids with high *in vitro* antioxidant capacity increased lifespan in *C. elegans*, an antioxidant mechanism seemed plausible. The compounds putative antioxidant action was therefore analyzed in isolated mouse mitochondria (done in collaboration with Kerstin Haas, AG Klingenspor) and *C. elegans*.

4.3.1 ROS-generation in isolated mouse mitochondria

Mitochondria are thought to be the primary site of endogenous ROS-generation. Therefore, skeletal muscle mitochondria were isolated from mice and ROS-generation was determined *ex vivo* by means of the Amplex Red assay.

ROS are predominantly generated at complex I in isolated mouse mitochondria

To get insights into the mechanisms underlying ROS-generation in this system, the site of ROS-generation was further specified. When supplied with the complex II-linked substrate succinate, H_2O_2-generation drastically increased under state 4 respiratory condition (absence of ADP) from 0.05 to 0.94 nmol*mg^{-1}*min^{-1}. This increase was abolished when ADP was provided (state 3 respiration). As substrate of the F_0F_1 ATP synthase, ADP depletes the proton gradient which is functionally linked to the electron transport from complex I to IV. Thus it is likely that superoxide anion radicals are formed in state 4 respiration by an reverse electron flow from complex II to I rather than through electron flow from complex II to III. To confirm this, rotenone was added which blocks electron transport from complex I to ubiquinone. This as well reduced ROS-generation drastically implying complex I as major site of ROS-generation in this model system (Fig. 19).

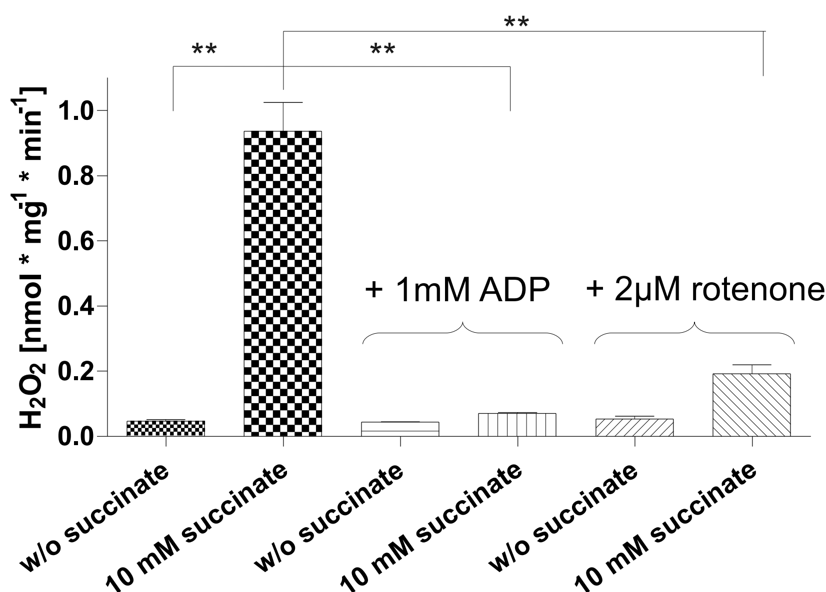


Fig. 19 H_2O_2-generation in isolated mouse mitochondria under different conditions
H_2O_2-generation was analyzed by Amplex Red assay in isolated mouse skeletal muscle mitochondria in the presence or absence of succinate, ADP and/or rotenone. Each bar represents mean ± SEM of 4 individual mice analyzed in duplicates. Statistical significance of differences versus succinate fed, state 4 respiration group was determined by one-way ANOVA with Dunett's post-hoc test (**$P<0.01$).

Flavonoids decrease ROS-generation in mouse mitochondria

To evaluate the antioxidant activity of the flavonoids *ex vivo*, H_2O_2-generation was determined in state 4 respiring mitochondria treated with 100 µM of the flavonoids. The flavonols quercetin and kaempferol reduced the rate of H_2O_2-generation strongest (to 0.13 and 0.14 $nmol*mg^{-1}*min^{-1}$) followed by myricetin (0.24 $nmol*mg^{-1}*min^{-1}$) when compared to controls (0.89 $nmol*mg^{-1}*min^{-1}$). The flavanone naringenin showed the lowest activity and decreased H_2O_2-production to only 0.55 $nmol*mg^{-1}*min^{-1}$ (Fig. 20). As in contrast to naringenin the three flavonols possess the 3-OH group and double bond at the C-ring, these structural features seems to be the major determinant for antioxidant action in this test system as well. The catechol (quercetin) and the pyrogallol (myricetin) functions at the B-ring, however, seemed to be of minor significance for this action.

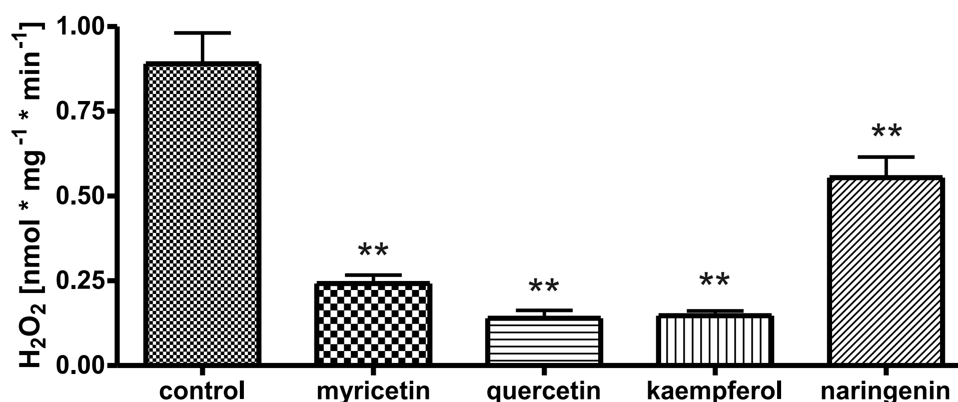


Fig. 20 Impact of flavonoids on H_2O_2-generation in isolated mitochondria
Generation of H_2O_2 from mouse skeletal muscles was assessed by Amplex Red assay with 100 µM of indicated flavonoids or solvent control. Each bar represents mean ± SEM of 4 individual mice analyzed in duplicates. Statistical significance of differences between control and intervention groups was determined by one-way ANOVA with Dunett's post-hoc test (**P<0.01).

As flavonoids are known to affect mitochondrial respiration, inhibition of the reverse electron-flow might be mechanistically responsible for their antioxidant action [177]. Therefore the impact of the different flavonoids on H_2O_2-generation was assessed with rotenone-blocked electron transfer. The strongly diminished H_2O_2-generation induced by rotenone was even further reduced by the flavonols while no effect was seen for naringenin. This finding is similar to the results obtained under rotenone-free conditions (Fig. 21). Thus, a blockade of the reverse electron flow seems unlikely to be the responsible factor for the antioxidant action of the flavonols in isolated mouse skeletal muscle mitochondria.

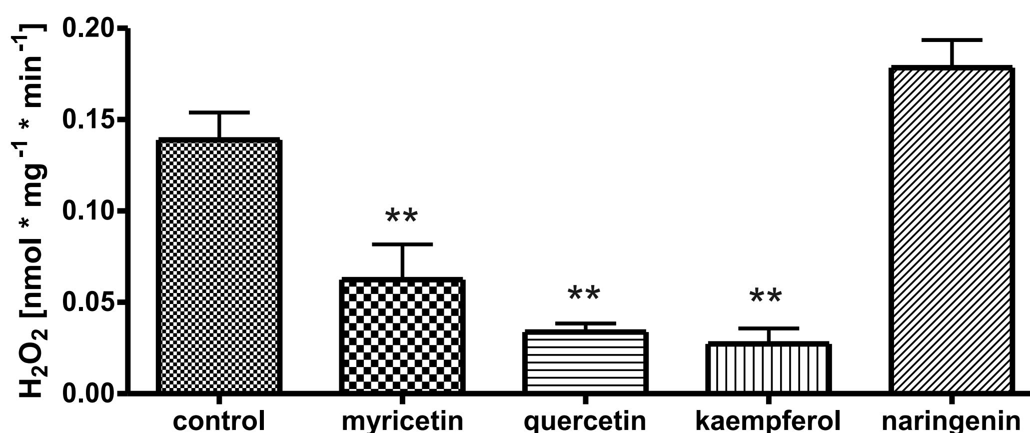


Fig. 21 Impact of flavonoids plus rotenone on mitochondrial H_2O_2-generation
Generation of H_2O_2 from mouse skeletal muscles was assessed by Amplex Red assay with 100 µM of indicated flavonoids or solvent control and rotenone. Each bar represents mean ± SEM of 4 individual mice analyzed in duplicates. Statistical significance of differences between control and intervention groups was determined by one-way ANOVA with Dunett's post-hoc test (**P<0.01).

4.3.2 ROS levels in *C. elegans*

Flavonols reduce mtROS

To analyze the effects of the flavonoids on mtROS levels in *C. elegans* the dye CM-H_2XRos, which selectively stains respiring mitochondria dependent on their superoxide-generation rate, was employed [160]. In wild-type *C. elegans*, incubated with 100 µM of the different flavonoids for 48 hours, quercetin and myricetin reduced mtROS strongest followed by kaempferol whereas naringenin did not show any effect (Fig. 22 and Fig. 23 for the 48 hour point of time). Here as well the 3-OH group and the double bond at the C-ring seem to define the antioxidant action of flavonoids which is intensified by an additional catechol function at the B-ring. The additional hydroxyl group of myricetin obviously can not further increase the antioxidant action.

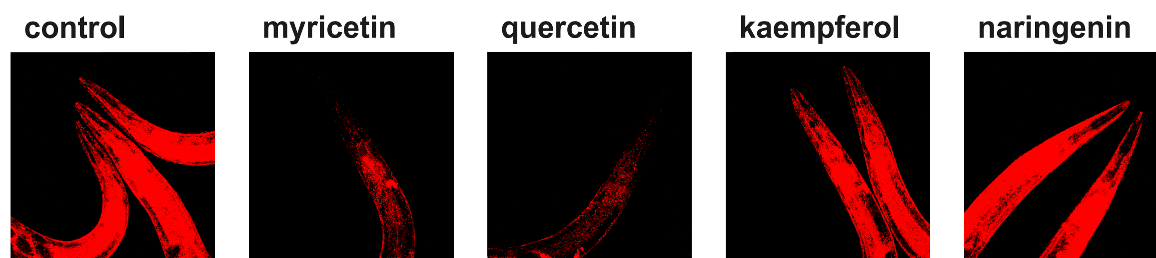


Fig. 22 Impact of flavonoids on mtROS in wild-type *C. elegans*
Representative microscopic fluorescence images at 40-fold magnification of CM-H_2XRos in L4 wild-type *C. elegans* pharyngeal area. The anterior end of the nematodes is located to the top. Worms were incubated with 100 µM of indicated flavonoids or solvent control for 48 h. Pictures represent typical results from 3 independent experiments with at least 10 worms analyzed. The anterior end of the nematodes is located to the top.

To assess the time-dependency of the antioxidant action, *C. elegans* were incubated with 100 µM of the flavonoids for 2, 6, 24 and 48 hours, respectively. After 2 hours of exposure to myricetin and quercetin, mtROS levels were already reduced by ~25% and ~15% and declined further by ~60% and ~70%, after 48 hours respectively. Treatment with kaempferol resulted in a slight increase of fluorescence (~15%) detectable after two hours. All longer incubation reduced mtROS by ~20%. Incubation with naringenin did not show any significant effect on the CM-H_2XRos fluorescence intensity in wild-type *C. elegans* (Fig. 23). Thus the flavonols already exerted their main antioxidant activity after treatment for 2 - 6 hours which was further enhanced with prolonged incubation times.

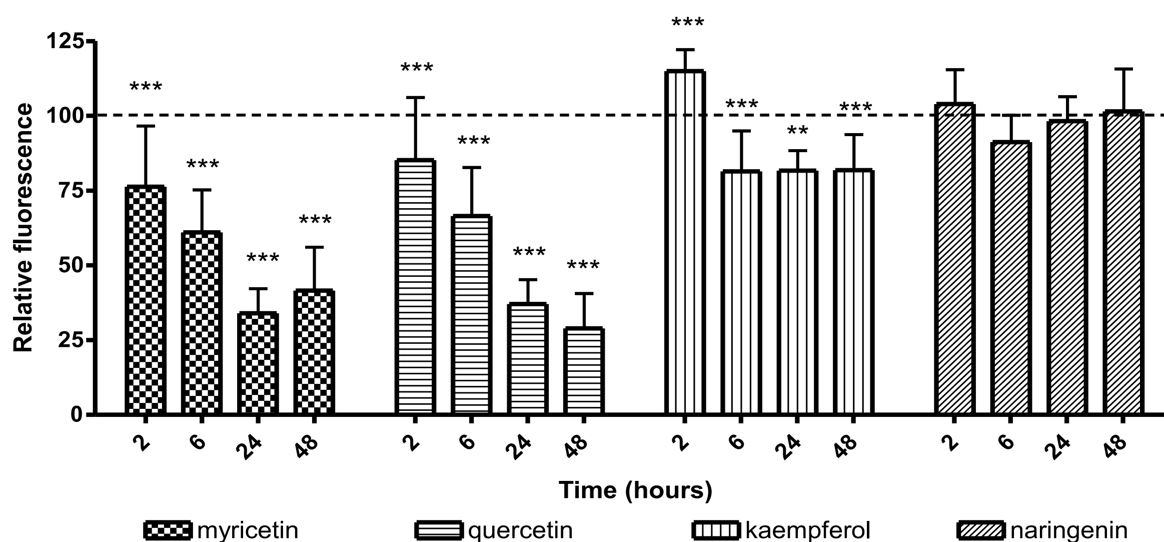


Fig. 23 Time-dependent impact of flavonoid exposure on mtROS in wild-type *C. elegans*
Relative fluorescence of CM-H_2XRos in wild-type *C. elegans* incubated with 100 µM of indicated flavonoids or solvent control for 2, 6, 24 and 48 h. Each bar represents mean ± SD of 2 - 3 individual experiments per point of time with at least 10 worms analyzed. Statistical significance of differences between according control (dotted line) and intervention groups was determined by two-way ANOVA with Bonferroni post-hoc test (**P<0.01, ***P<0.001).

Mitochondrial ROS-reduction is independent of initial ROS status

To elucidate whether this antioxidant action could also be observed in strains with initially altered ROS-production, the impact of the flavonoids on mtROS in the *mev-1(kn1)* and *isp-1(qm150);ctb-1(qm189)* strains was determined. As expected *mev-1(kn1)* worms showed ~50% increased levels of mtROS when compared to wild-type nematodes. Treatment with 100 µM of the four flavonoids for 48 hours resulted in similar influences on mtROS as observed in wild-type N2. Briefly, quercetin and myricetin treatment reduced mtROS by ~60% and ~45%, respectively, a decrease even below the concentration observed in wild-type *C. elegans* controls. Kaempferol reduced mtROS levels by approximately 23%, whereas naringenin again failed to show any effect (Fig. 24 A). In contrast to *mev-1(kn1)* the concentration of mtROS was reduced by ~31% in *isp-1(qm150);ctb-1(qm189)* when compared to wild-type *C. elegans*. Additional treatment with 100 µM myricetin or quercetin reduced mtROS by 28% and 47%, respectively, whereas neither kaempferol nor naringenin had any effect (Fig. 24 B). As a broadly similar structure-activity relationship was obtained in wild-type, *mev-1(kn1)* and *isp-1(qm150);ctb-1(qm189) C. elegans*, the reduction of mtROS seems independent of the initial mitochondrial ROS load.

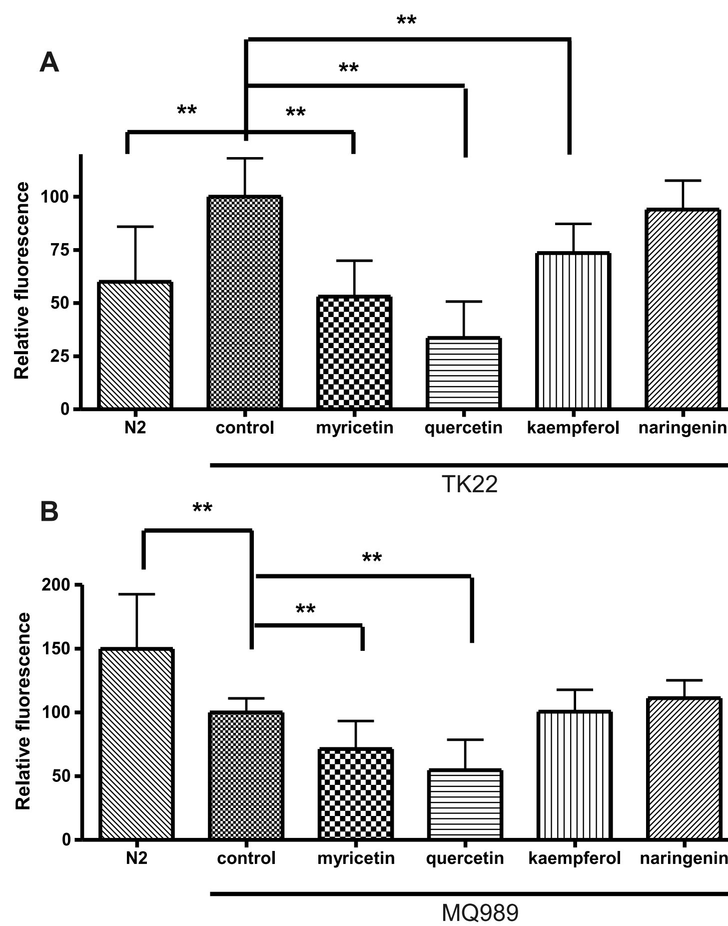


Fig. 24 Impact of flavonoids on mtROS in *mev-1(kn1)* and *isp-1(qm150);ctb-1(qm189) C. elegans*

Relative fluorescence of CM-H_2XRos in *mev-1(kn1)* (TK22) (A) and *isp-1(qm150);ctb-1(qm189)* (MQ989) (B) *C. elegans* incubated with 100 µM of indicated flavonoids or solvent control for 48 h. Each strain was analyzed together with wild-type N2 treated like control group. Each bar represents means ± SD of 2 - 3 individual experiments with at least 10 worms analyzed. Statistical significance of differences versus control group was determined by one-way ANOVA with Dunett's post-hoc test (**P<0.01).

4.4. Impact of flavonoids on protein-carbonyl content

To assess whether the antioxidant action of the flavonoids is reflected in reduced oxidative damage to macromolecules, the protein-carbonyl content was analyzed in *C. elegans*. To validate this method, the age-dependent PCC was determined in wild-type *C. elegans* and strains with described elevated levels of carbonylated proteins, namely *mev-1(kn1)* and *daf-16(mu86)* mutants [117]. All three strains showed higher PCC at day 11 of life compared to their third larval stage. Additionally, increased amounts of protein-carbonyls were observed in *mev-1(kn1)* and *daf-16(mu86)* nematodes compared to wild-type *C. elegans* (Fig. 25). As *mev-1(kn1)* mutants were shown to have increased levels of mtROS (see 4.3.2) and *daf-16* mutants are known to have reduced expression levels of antioxidant defense enzymes [117], these data suggest ROS-stress to induce protein-carbonylation.

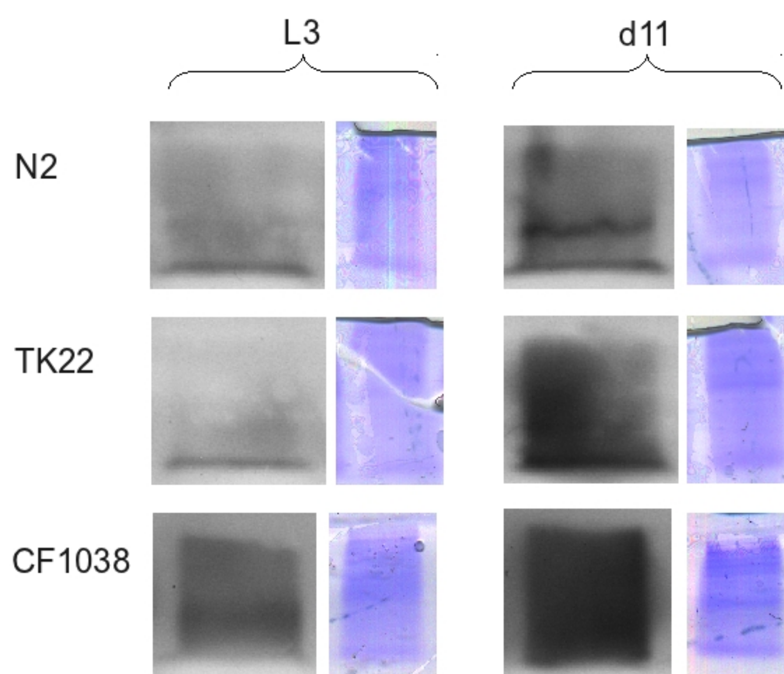


Fig. 25 Carbonylated proteins in wild-type, *mev-1(kn1)* and *daf-16(mu86) C. elegans* at different ages
Carbonylated proteins in L3 larva and 11-day old (d11) wild-type (N2), *mev-1(kn1)* (TK22) and *daf-16(mu86)* (CF1038) *C. elegans*, determined by OxyBlot Protein Oxidation Detection Kit. Left panels shows radiographic picture of blotted protein-carbonyls and according right panels show parallel-run coomassie-stained loading-control gel. Each lane represents 10 µg of a biological sample.

Since the flavonols reduced mtROS load, their impact on protein-carbonyl levels was analyzed in 11-day-old wild-type *C. elegans*. Quercetin and myricetin treatment reduced protein-carbonyl levels by ~60% and 50%, respectively. Exposure to kaempferol or naringenin did not show any significant effect (Fig. 26). As quercetin and myricetin also reduced mtROS strongest, their antioxidant action seems to be reflected also in reduced PCC.

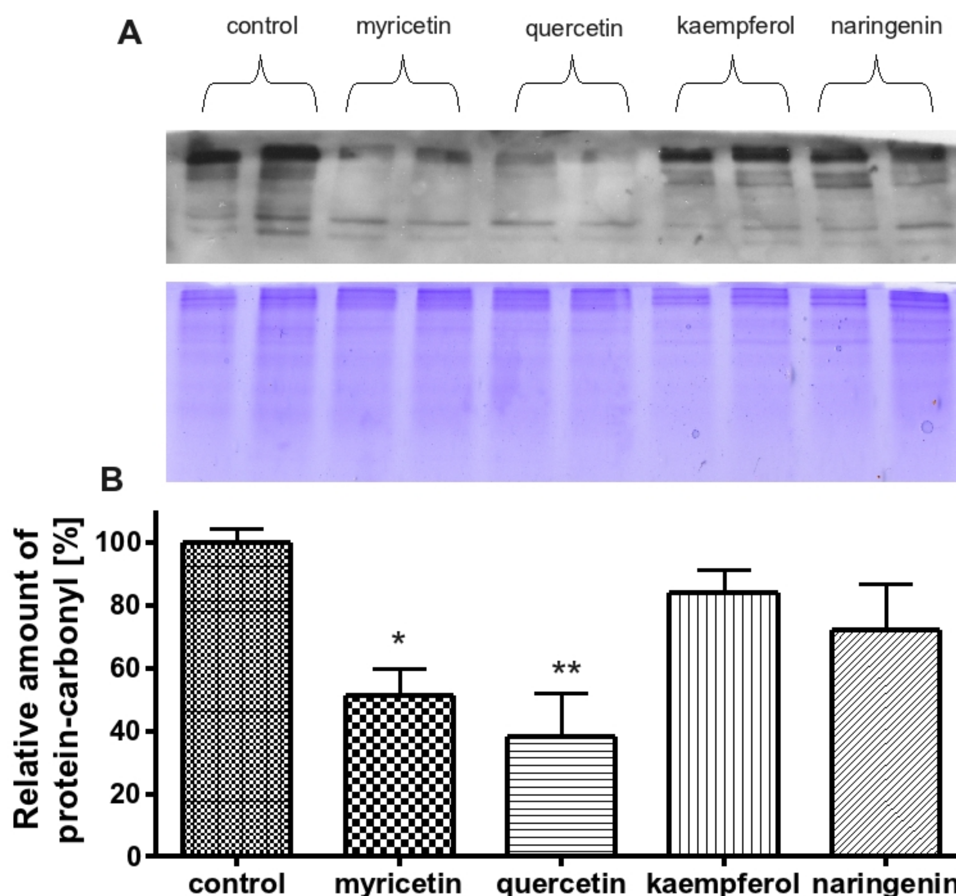


Fig. 26 Carbonylated proteins of flavonoid-treated wild-type *C. elegans*
(A) Carbonylated proteins of 11-day old wild-type *C. elegans* treated with 100 µM of indicated flavonoids or solvent control, determined by OxyBlot Protein Oxidation Detection Kit. Upper panel shows a radiographic image of blotted protein-carbonyls and lower panel shows a parallel-run coomassie-stained loading-control gel. Each lane represents 10 µg of a biological sample. (B) Semi-quantitative analysis of radiographic image by ImageJ gel analysis software after correction with loading-control. Each bar represents mean ± SD of two biological samples. Statistical significance of differences between control and intervention groups was determined by one-way ANOVA with Dunett's post-hoc test (*P<0.05, **P<0.01).

4.5. Flavonoids effects on exogenous stress

4.5.1 Paraquat-induced ROS in isolated mouse mitochondria

The herbicide paraquat was applied to test whether the flavonoids may also exert antioxidant action on exogenously induced ROS. As paraquat is thought to generate ROS in mitochondria by acceptance of electrons via complex I, the impact of the flavonoids on paraquat-treated mouse muscle mitochondria was tested [156]. The addition of 5 µM paraquat doubled the H_2O_2-generation rate from ~0.9 to ~1.9 nmol*mg^{-1}*min^{-1}. This effect was abolished when succinate was depleted, confirming mitochondrial respiratory chain as the major site of paraquat-induced ROS-generation. All flavonoids reduced the H_2O_2-generation induced by paraquat. The strongest reduction was seen for quercetin and kaempferol (0.29 and 0.37 nmol*mg^{-1}*min^{-1}) followed by myricetin (0.51 nmol*mg^{-1}*min^{-1}) and naringenin (0.94 nmol*mg^{-1}*min^{-1}) (Fig. 27). Thus, in paraquat-treated mouse mitochondria showing enhanced ROS-production a similar structure-activity relationship of the flavonoids with respect to ROS-scavenging was observed as in paraquat untreated mitochondria.

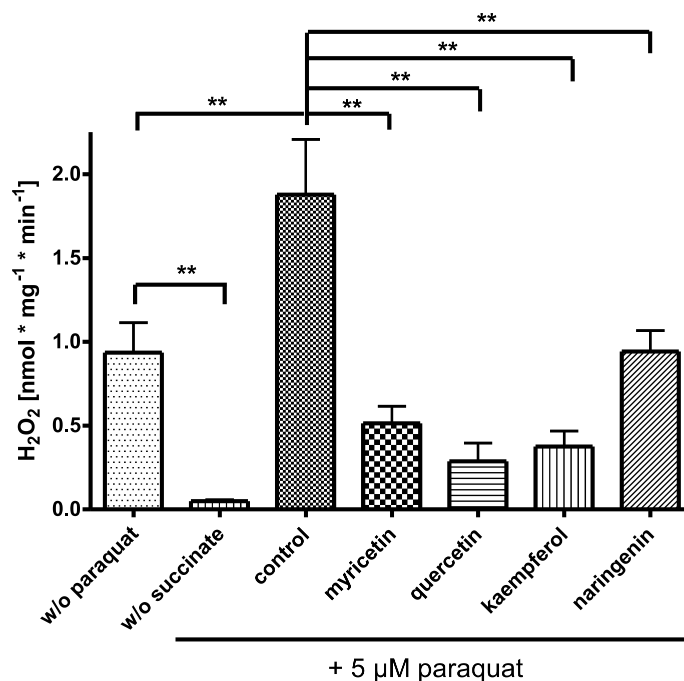


Fig. 27 Paraquat-induced H_2O_2-generation in isolated mouse mitochondria
Generation of H_2O_2 from mouse skeletal muscles was assessed by Amplex Red assay with or without 5 µM paraquat and 100 µM of indicated flavonoids or solvent control, respectively. Each bar representing means ± SEM of 4 individual mice analyzed in duplicates. Statistical significance of differences between control and intervention groups was determined by one-way ANOVA with Dunett's post-hoc test (**P<0.01).

4.5.2 Effects on paraquat-induced stress in *C. elegans*

To assess whether this antioxidant effect against paraquat-induced ROS-production can also be observed in *C. elegans*, the impact on paraquat-resistance was analyzed next in the nematodes. Wild-type, *mev-1(kn1)* and *isp-1(qm150);ctb-1(qm189) C. elegans* were exposed on NGM plates to 100 µM of different flavonoids plus 1.6 mM paraquat. In wild-type nematodes the mean survival of ~7.4 days was reduced by co-exposure with myricetin to ~83% while the other flavonoids did not have any effect (Fig. 28; Table 14 appendix). This was a surprising finding since myricetin greatly reduced paraquat-induced ROS-generation in isolated mitochondria and showed the strongest lifespan-extending effects under standard conditions.

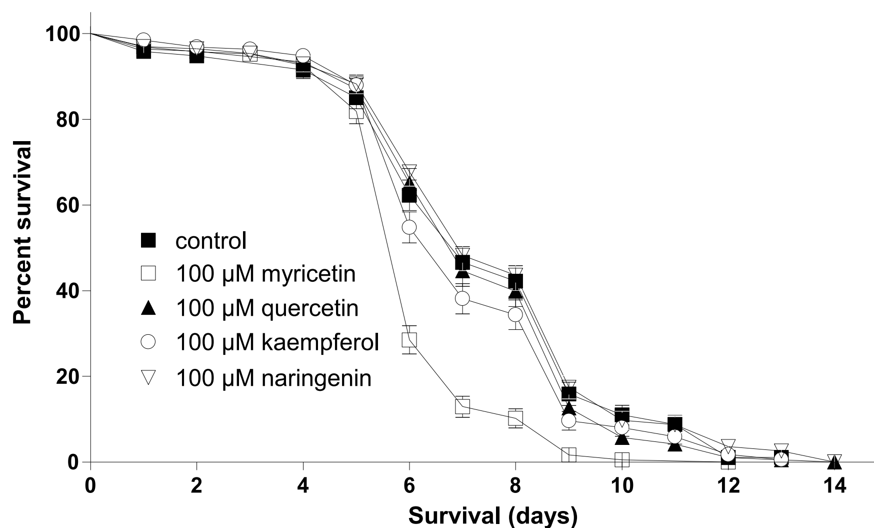


Fig. 28 Effects of flavonoids on paraquat-resistance in wild-type *C. elegans*
Effects of 100 µM of the selected flavonoids on resistance in wild-type N2 *C. elegans* against 1.6 mM paraquat. Data was compared vs. control by Log rank test and plotted as Kaplan-Meier survival curves with SEM based on two individual experiments with at least 90 individuals per group. Details see Table 14 appendix.

In the *mev-1(kn1)* strain, which was isolated based on its sensitivity against paraquat [102], an expected reduced mean survival of ~2.7 days when compared to wild-type N2 was observed. Like in wild-type nematodes, myricetin and additionally quercetin reduced resistance against paraquat to approximately 85% while the other flavonoids did not show any effect (Fig. 29; Table 15 appendix).

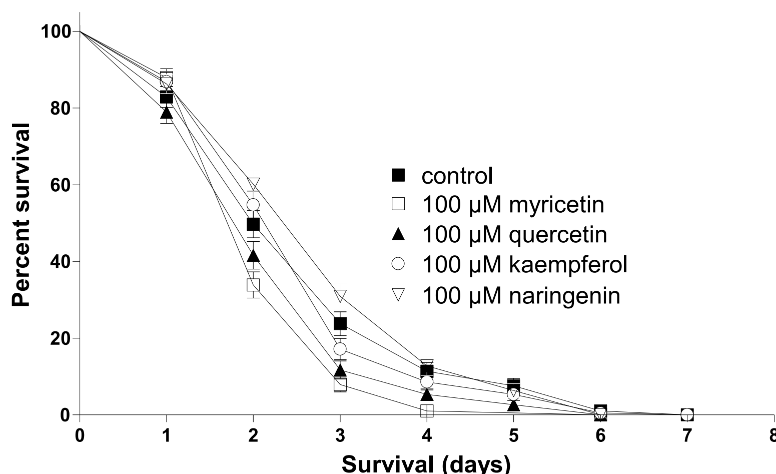


Fig. 29 Effects of flavonoids on paraquat-resistance in *mev-1(kn1) C. elegans*
Effects of 100 µM of the selected flavonoids on resistance in *mev-1(kn1) C. elegans* against 1.6 mM paraquat. Data was compared vs. control by Log rank test and plotted as Kaplan-Meier survival curves with SEM based on two individual experiments with at least 90 individuals per group. Details see Table 15 appendix.

In *isp-1(qm150);ctb-1(qm189) C. elegans* mean survival in the presence of paraquat was ~7 days which was solely decreased by kaempferol (to ~92%) while for the other flavonoids no significant effect was observed (Fig. 30; Table 16 appendix). Thus in all three strains, none of the flavonoids showed any protective effect on paraquat resistance despite decreasing the H_2O_2-generation in isolated mitochondria. More strikingly, the flavonols that exerted strongest antioxidant action even reduced paraquat survival.

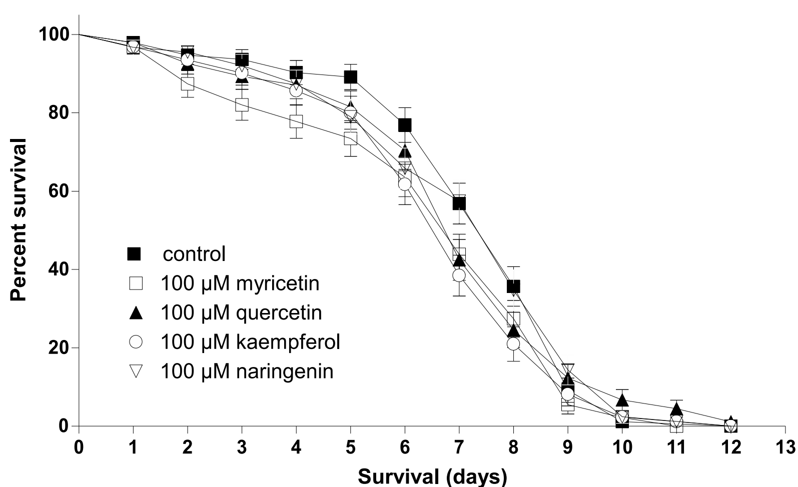


Fig. 30 Effects of flavonoids on paraquat-resistance in *isp-1(qm150);ctb-1(qm189) C. elegans*
Effects of 100 µM of the selected flavonoids on resistance in *isp-1(qm150);ctb-1(qm189) C. elegans* against 1.6 mM paraquat. Data was compared vs. control by Log rank test and plotted as Kaplan-Meier survival curves with SEM based on at least 90 individuals per group. Details see Table 16 appendix.

To elucidate whether the co-exposure scenario of paraquat with the flavonoids might have led to inadvertent interactions and thus to the unexpected findings, nematodes were next treated in a consecutive manner. Wild-type *C. elegans* were first exposed to 100 µM of the different flavonoids for 24 and 48 hours, respectively, the incubation times showing strongest reduction in mtROS (see 4.3.2). Subsequently the animals were transferred to M9 buffer containing OP50 feeding bacteria and 100 mM paraquat for additional 24 hours. Pre-incubation with quercetin and kaempferol for 24 hours elevated the survival rate from ~24% in control worms to ~52% and ~55%, respectively. Also naringenin increased the survival to ~39% while myricetin did not show any significant effect. Extension of flavonoid-exposure to 48 hours further increased the survival rate only in case of naringenin while treatment with the other compounds did not show any additional effect. Again, myricetin had no protective effect on paraquat resistance (Fig. 31).

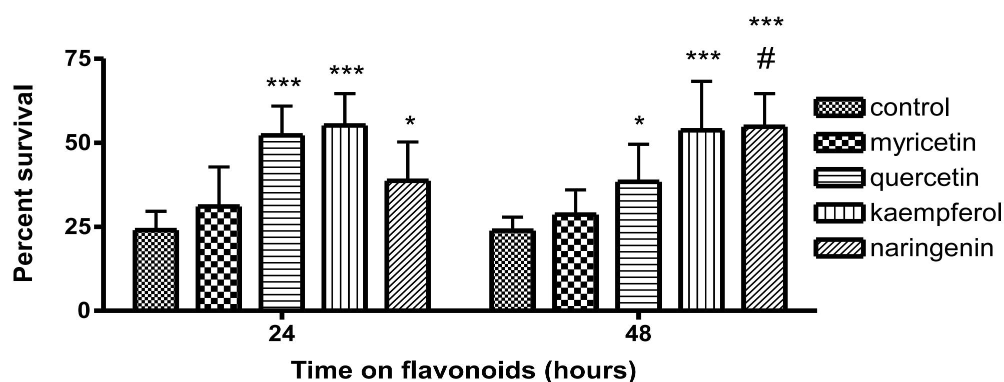


Fig. 31 Survival of *C. elegans* under acute paraquat-stress
Survival of wild-type *C. elegans* after 24 and 48 hours on 100 µM of indicated flavonoids followed by incubation in 100 mM paraquat for 24 hours. Each bar represents mean ± SD of 2 individual experiments with at least 90 worms analyzed. Statistical significance of differences between control and intervention groups (*P<0.05, **P<0.01, ***P<0.001) and between the incubation times ($^{\#}$P<0.05) were determined by two-way ANOVA with Bonferroni post-hoc test, respectively.

Taken together, these data demonstrate that the flavonoids can reduce paraquat-induced ROS-generation in isolated mitochondria according to their antioxidant activity. Co-exposure of the flavonoids with paraquat induces no or even adverse effects on the survival rate of *C. elegans*. This lack of effect during simultaneous exposure remains to be clarified but most likely arises from interaction effects of the flavonoids with paraquat. However, when treatment was done consecutively, the protective effects of the flavonoids became

obvious. Yet, it cannot solely be explained with the antioxidant action, since myricetin did not increase stress-resistance despite having shown strong antioxidant action.

4.5.3 Impact on heat-resistance

Thermal stress is often employed as a surrogate to elucidate the effects of plant-derived compounds on *C. elegans* lifespan [178] [179]. Additionally, heat-stress is thought to lead to increased formation of ROS [180]. Therefore the effect of flavonoids on thermal stress at 34 °C was analyzed in wild-type *C. elegans*. All flavonols increased thermal resistance when compared to control conditions while naringenin only slightly reduced heat-stress-resistance (Fig. 32). The structural properties at the C-ring seem to determine the effects on thermal-stress-resistance.

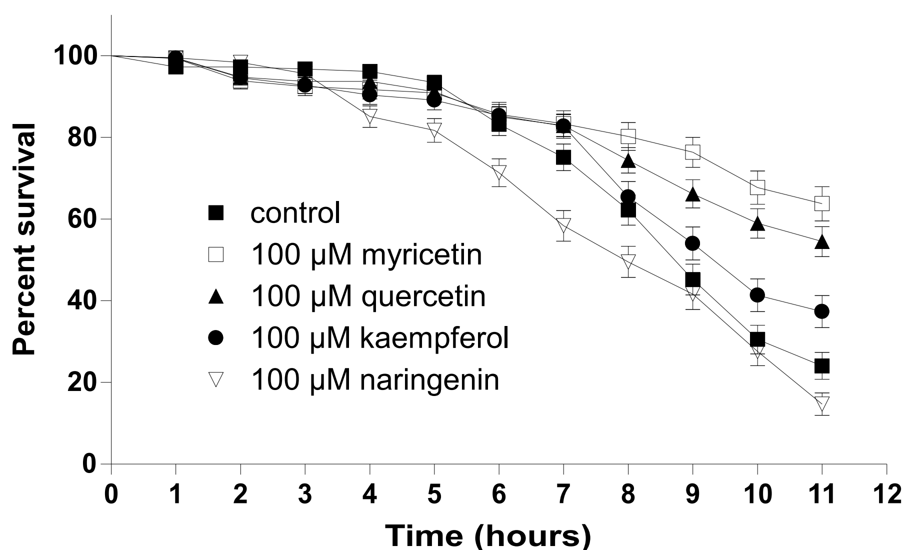


Fig. 32 Effects of flavonoids on heat-resistance in wild-type *C. elegans*
Effects of 100 µM of the selected flavonoids on resistance in wild-type N2 *C. elegans* against 34 °C heat-stress. Data was compared vs. control by Log rank test and plotted as Kaplan-Meier survival curves with SE based on two individual experiments with at least individuals per group. Details see Table 17 appendix.

4.6. Impact of flavonoids on metabolic parameters

4.6.1 Effects on the pharynx pumping rate

Caloric restriction is a well-known treatment to induce lifespan extension in *C. elegans* [88] [89]. To test whether the flavonoids might induce food-aversion and thus reduce food intake, the pumping rate of the pharynx was assessed in animals exposed to the compounds. In *eat-4(ky5)* mutants pharyngeal contractions were delayed, as described previously [88]. Amongst the flavonoid tested only myricetin reduced pharyngeal pumping rate slightly by ~4% (Fig. 33). Whether this decrease alone is sufficient to explain the lifespan extension seems questionable, as the other flavonols did not show a comparable effect but also increased lifespan.

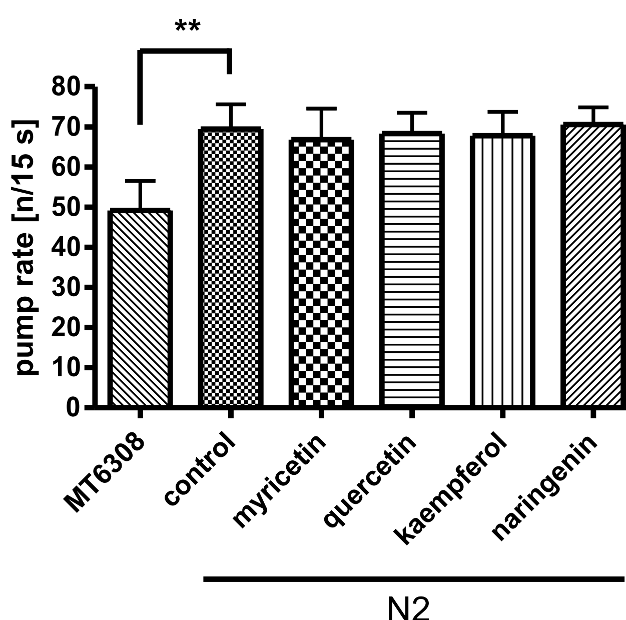


Fig. 33 Pharynx pumping rate after flavonoid-exposure
Pharynx pumping rate in *eat-4(ky5)* (MT6308) and wild-type (N2) *C. elegans* after exposure to 100 µM of indicated flavonoids for 48 hours. Each bar represents the mean ± SD of 3 individual experiments with 20 – 30 worms analyzed. Statistical significance of differences versus N2 control group was determined by one-way ANOVA with Dunett's post-hoc test (**P<0.01).

In *mev-1(kn1)* mutants a reduced overall pumping rate was observed when compared to wild-type N2. As pharyngeal pumping is also a good marker for general fitness as its rate declines with age [181], the senescent phenotype of *mev-1(kn1)* mutants is confirmed by

this assay. All flavonoids, however, partially rescued the reduced pumping rate, implying an increased fitness due to flavonoid treatment that seems independent of their structural properties (Fig. 34).

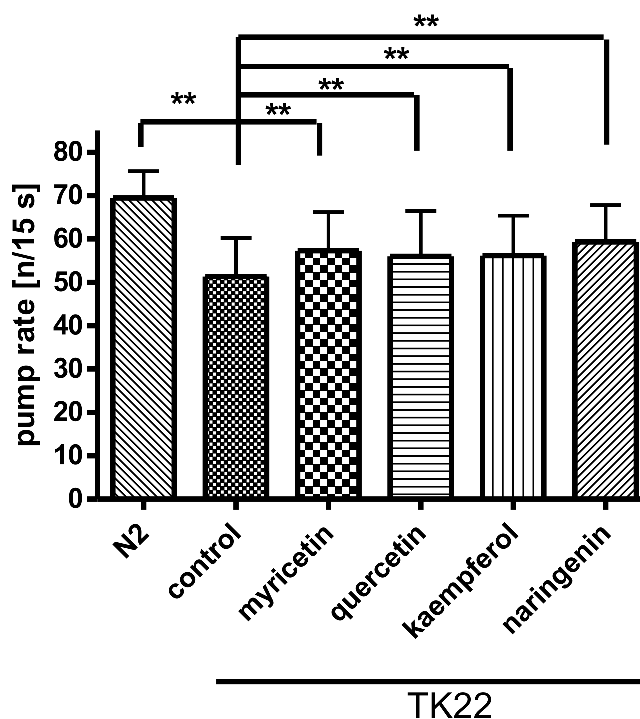


Fig. 34 Pharynx pumping rate after flavonoid-exposure in *mev-1(kn1) C. elegans*
Pharynx pumping rate in *mev-1(kn1)* (TK22) and wild-type (N2) *C. elegans* after exposure to 100 µM of indicated flavonoids for 48 hours. Each bar represents the mean ± SD of 3 individual experiments with 20 - 30 worms analyzed. Statistical significance of differences versus *mev-1(kn1)* control group was determined by one-way ANOVA with Dunett's post-hoc test (*P<0.05, **P<0.01).

4.6.2 Effects on respiration rate

Several *C. elegans* strains with alteration in lifespan are also described to have altered oxygen consumption rates [109] [182] [183]. To assess whether respiratory effects might account for the flavonol-induced lifespan extension, the impact on oxygen consumption was determined by means of electro-chemical detection. A strong correlation between the number of worms (determined as protein) and the oxygen consumption rate was observed (Fig. 35).

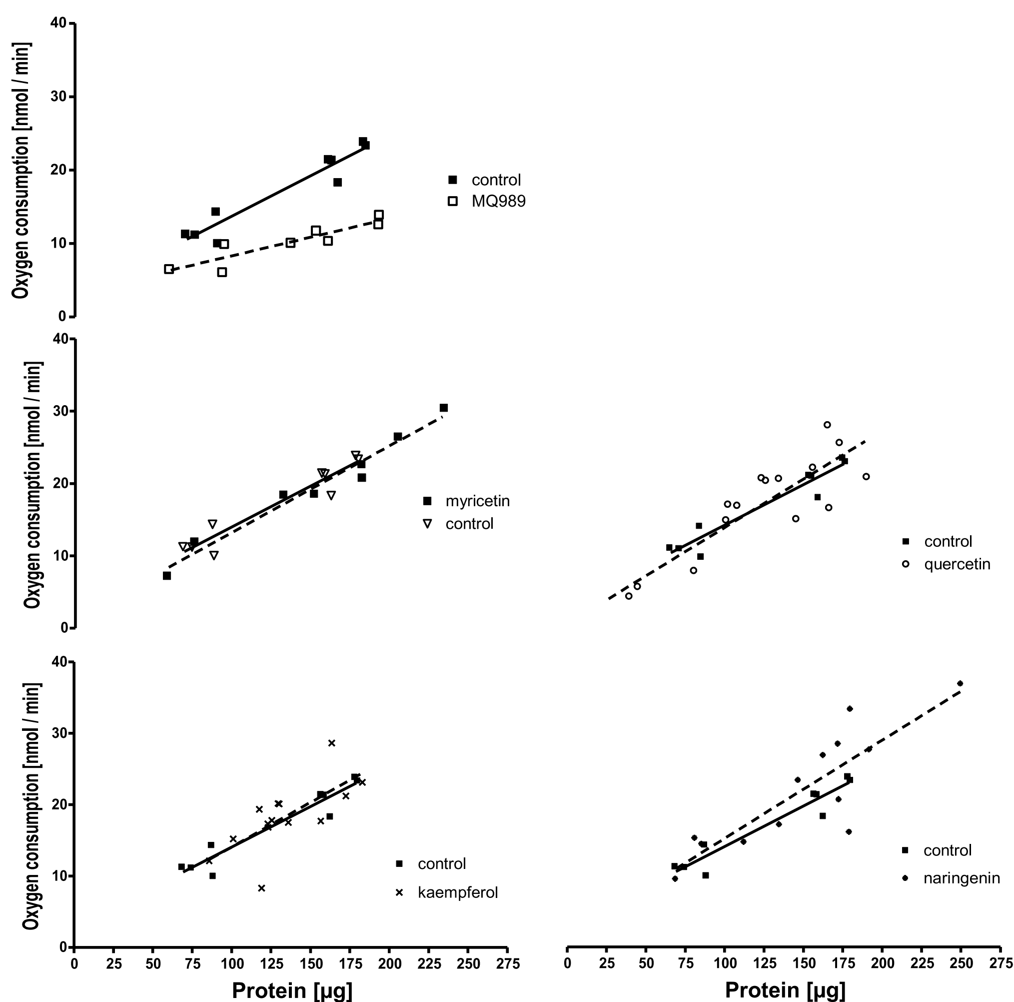


Fig. 35 Oxygen consumption in *C. elegans* depending on sample size
Oxygen consumption in *isp-1(qm150);ctb-1(qm189)* (MQ989) and wild-type *C. elegans* after treatment with 100 µM of indicated flavonoids or solvent depending on sample size. Line of best fit of 8 – 16 experiments per group is given with each point representing a single experiment.

When related to the sample size *isp-1(qm150);ctb-1(qm189) C. elegans* consumed less oxygen with rates of 0.081 ± 0.018 nmol*μg^{-1}*min^{-1}*mL^{-1} than wild-type *C. elegans* (0.138 ± 0.019 nmol*μg^{-1}*min^{-1}*mL^{-1}), as described previously [109]. Exposure to the test compounds did not alter the oxygen consumption in wild-type *C. elegans* (Fig. 36). This indicates that the mechanisms which are responsible for the lifespan extension and mtROS reduction observed in flavonol-exposed nematodes are independent from effects on the respiratory chain activity suggesting other mechanisms to be involved.

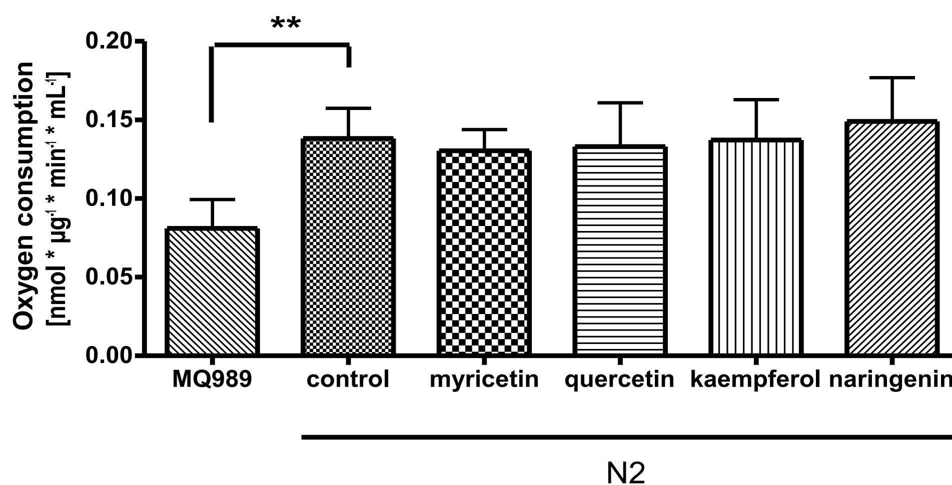


Fig. 36 Relative oxygen consumption rate in *C. elegans* after treatment with flavonoids
Oxygen consumption in *isp-1(qm150);ctb-1(qm189)* (MQ989) and wild-type (N2) *C. elegans* after treatment with 100 µM of indicated flavonoids or solvent relative to protein content. Each bar represents the mean ± SD of 8 – 16 samples. Statistical significance of differences versus N2 control was determined by one-way ANOVA with Dunett's post-hoc test (**P<0.01).

4.7. Impact of flavonoids on the IIS-cascade

4.7.1 DAF-16 sub-cellular localization

Several cell-signaling cascades are discussed as targets by which flavonoids could mediate their effects [176]. Among these the IlS cascade is known to be a central regulator of lifespan and stress-resistance in *C. elegans,* controlling the phosphorylation status of the transcription factor DAF-16 [56]. To assess whether the beneficial, i.e. lifespan-extending, antioxidant and heat-resistance effects of the flavonoids might be mediated via DAF-16, the *gfp* reporter strain TJ356 was employed to visualize DAF-16 localization. All tested flavonoids increased the nuclear localization of DAF-16 to a similar extent (Fig. 37). This indicates a reduction of the DAF-16 phosphorylation status after flavonoid treatment independent from their structural features.

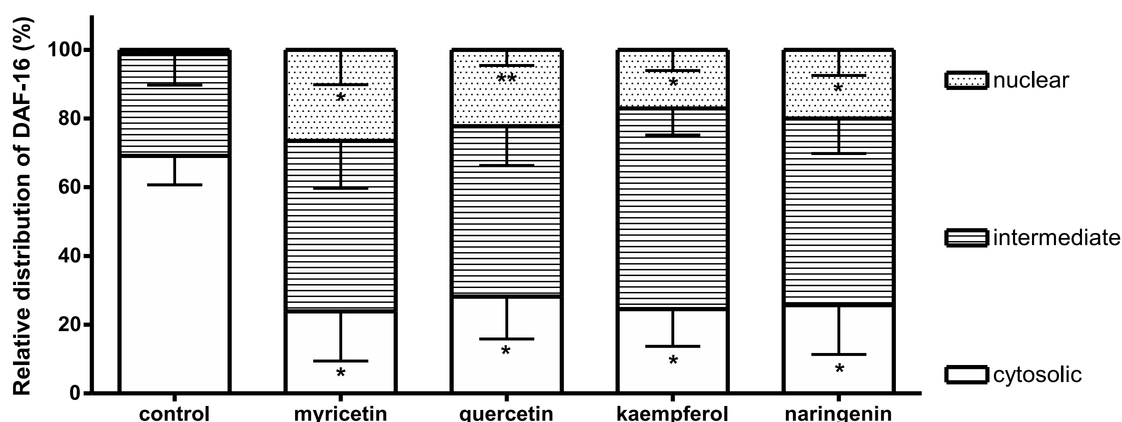


Fig. 37 Effects of flavonoids on DAF-16 transcription factor localization
Relative distribution of GFP in *daf-16::gfp* reporter *C. elegans* after exposure of worms to 100 µM of the flavonoids or solvent for 48 h. Each bar represents the mean ± SEM of 4 individual experiments with at least 100 worms analyzed. GFP localization was classified into the categories “nuclear”, “intermediate” and “cytosolic”. Statistical significance of differences between control and treated groups was determined by students t-test (*P<0.05, **P<0.01).

4.7.2 Involvement of DAF-16 linked-genes

To test whether the increased nuclear localization of DAF-16 might contribute to the beneficial effects observed after flavonoid exposure of worms, the promoter activities of the DAF-16/IlS linked genes *sod-3, hsp-16.2* and *hsp-70* (C12C8.1) were assessed [64] [170] [184]. Therefore strains expressing either a *sod-3::gfp*, a *hsp-16.2::gfp* or a *hsp-70::gfp* reporter were employed. Additionally the *daf-16* mutant strain CF1038 was used to elucidate the role of DAF-16 on the antioxidant and lifespan effects of the flavonoids.

Feeding the ROS-generator juglone, used as a positive control, induced promoter activity of the gene *sod-3* encoding a mitochondrial SOD, as reported previously [185]. The promoter activity was also increased upon exposure of nematodes to the different flavonoids (Fig. 38). Thus, the increased *sod-3* promoter activity was independent of the attached moieties and thereby confirmed the results obtained in the *daf-16::gfp* reporter strain.

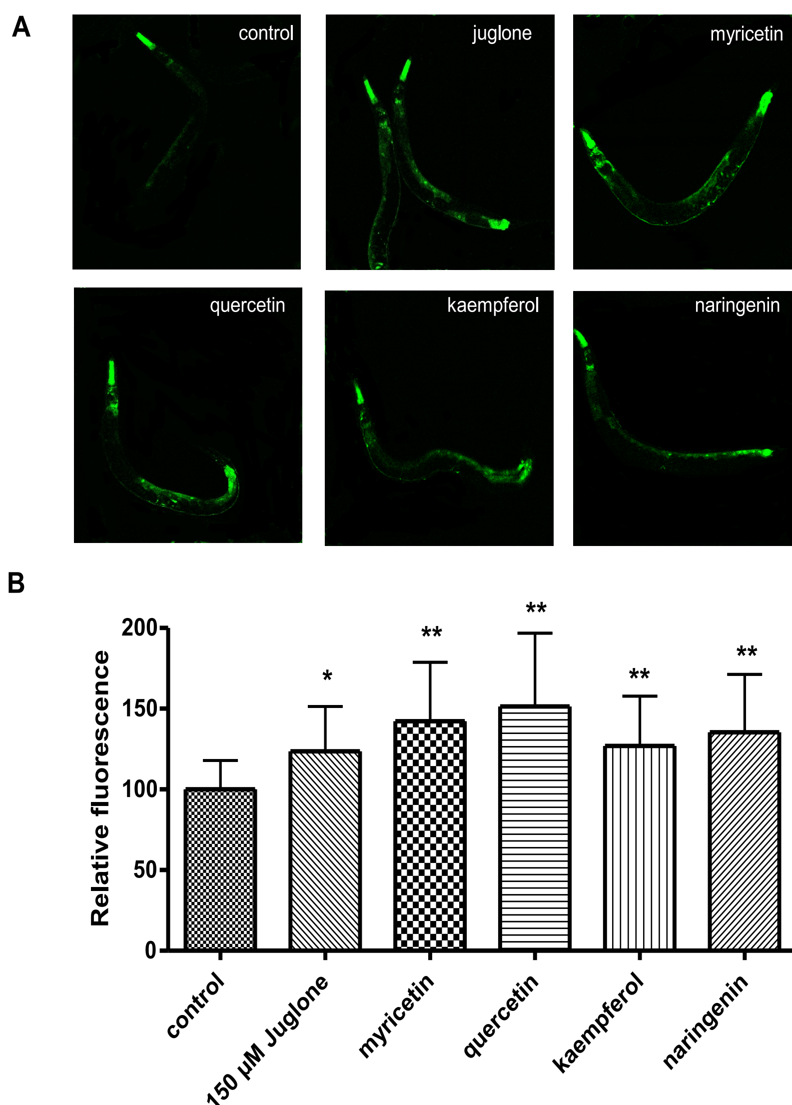


Fig. 38 Effects of flavonoids on *sod-3* promoter activity
(A) Representative microscopic fluorescence images of *sod-3::gfp* reporter *C. elegans* exposed to solvent (control), 150 µM juglone or 100 µM of indicated flavonoids. Pictures at 20-fold magnification with the anterior end of the nematodes located to the upper left corner. (B) Relative fluorescence intensity of GFP in the *sod-3::gfp* reporter strain exposed to solvent (control), 150 µM juglone or 100 µM of the indicated flavonoids. Each bar represents the mean ± SD of 2 - 3 individual experiments with at least 10 worms analyzed. Statistical significance of differences between control and treatment groups was determined by one-way ANOVA with Dunett's post-hoc test (*P<0.05, **P<0.01).

In *hsp-16.2::gfp C. elegans* neither treatment with flavonoids nor solvent alone induced any measurable GFP::promoter signal (data not shown). This indicates that despite DAF-16 translocation, flavonoid exposure does not affect *hsp-16.2* promoter activity. As treatment with juglone was demonstrated to induce *hsp-16.2* promoter activity [172], suggesting a protective effect of this chaperone under ROS-stress, the impact of the flavonoids after treatment with this redox-cycling agent was analyzed. As expected,

juglone-induced *hsp-16.2::gfp* fluorescence. Flavonol-treatment, however, did not result in any effect on juglone-induced *hsp-16.2* promoter activity (Fig. 39). This might suggest that this chaperone is not involved in the increased ROS-resistance observed after flavonoid exposure of the animals.

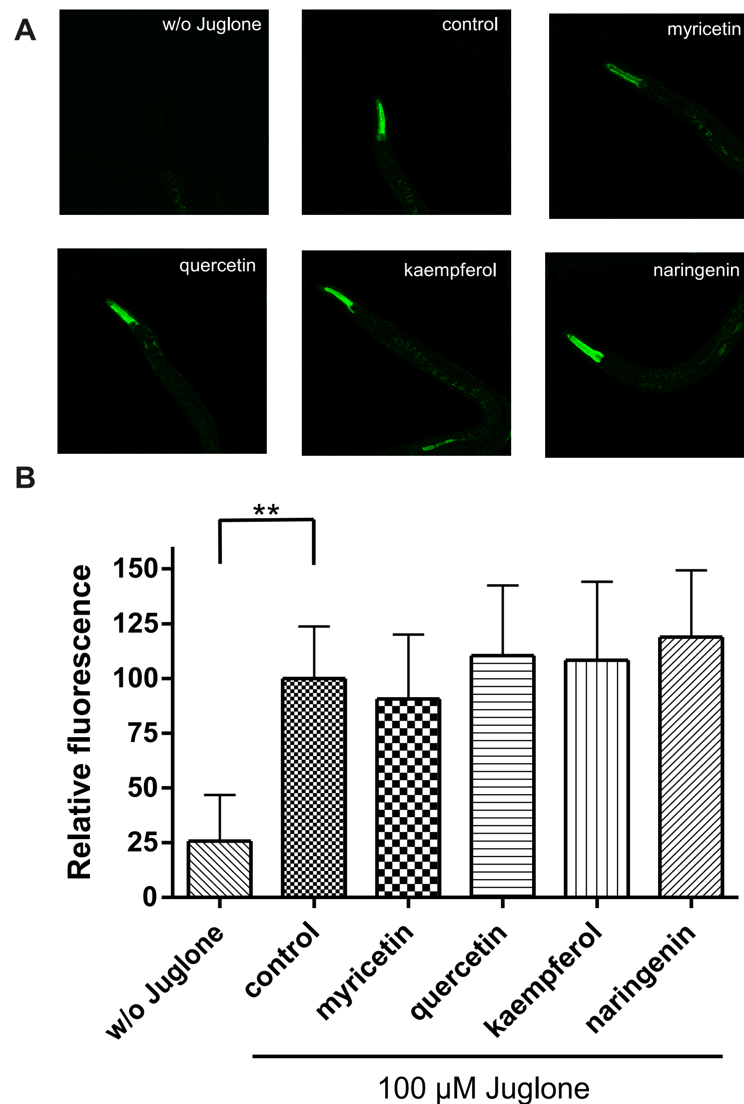


Fig. 39 Effects of flavonoids on juglone-induced *hsp-16.2* promoter activity
(A) Representative microscopic fluorescence images of *hsp-16.2::gfp C. elegans* exposed to solvent (w/o juglone), 100 µM juglone (control) or 100 µM of indicated flavonoids and 100 µM juglone. Pictures at 20-fold magnification with the anterior end of the nematodes located to the upper left corner. (B) Relative fluorescence intensity of GFP in the *hsp-16.2::gfp* strain exposed to solvent (w/o juglone), 100 µM juglone (control) or 100 µM of the indicated flavonoids and 100 µM of juglone. Each bar represents the mean ± SD of 3 individual experiments with at least 10 worms analyzed. Statistical significance of differences versus control group was determined by one-way ANOVA with Dunett's post-hoc test (**P<0.01).

Besides oxidative stress, HSP are far better known to be induced by the presence of proteins misfolded upon heat-stress. To test whether the heat-shock proteins HSP-16.2 and HSP-70 (C12C8.1) might contribute to the observed heat-resistance, the impact of the flavonoids on the heat-induced promoter activity in *hsp-16.2::gfp* and *hsp-70::gfp C. elegans* was analyzed. In *hsp-16.2::gfp* mutants fluorescence increased 50 min after heat-shock reaching a plateau after 200 min. Similar to the findings after juglone-induced stress, none of the flavonoids showed any significant effect on heat-induced *hsp-16.2*-promoter activity (Fig. 40) suggesting that *hsp-16.2* is not involved in any of the processes that contribute to the increased heat-resistance observed after flavonol exposure of the nematodes.

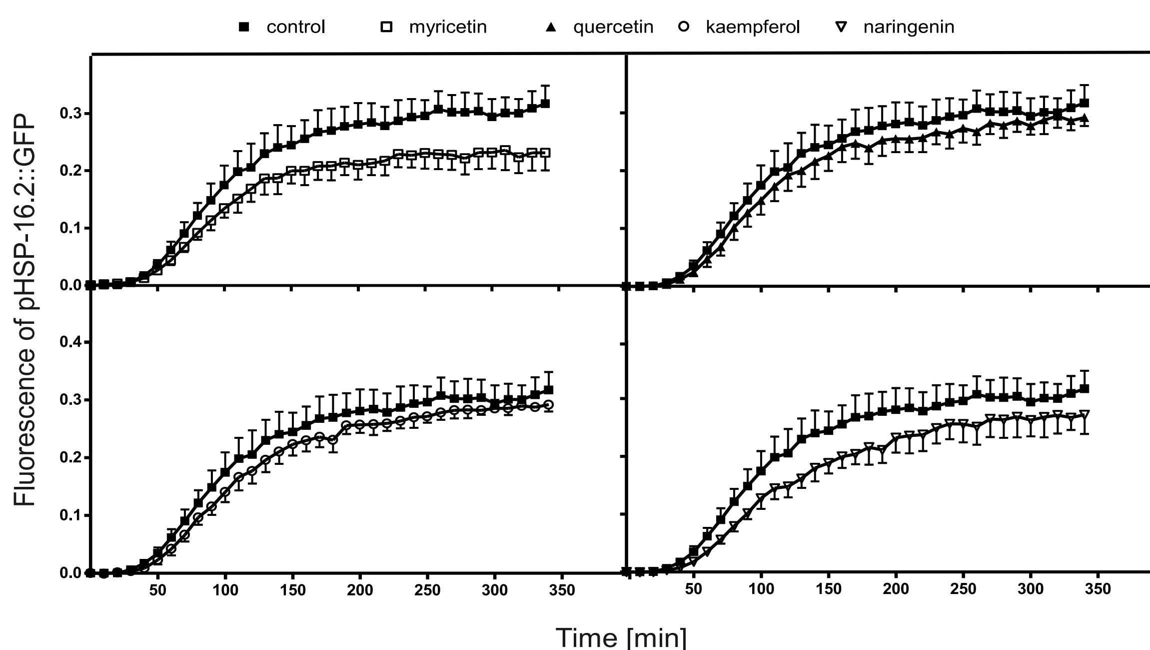
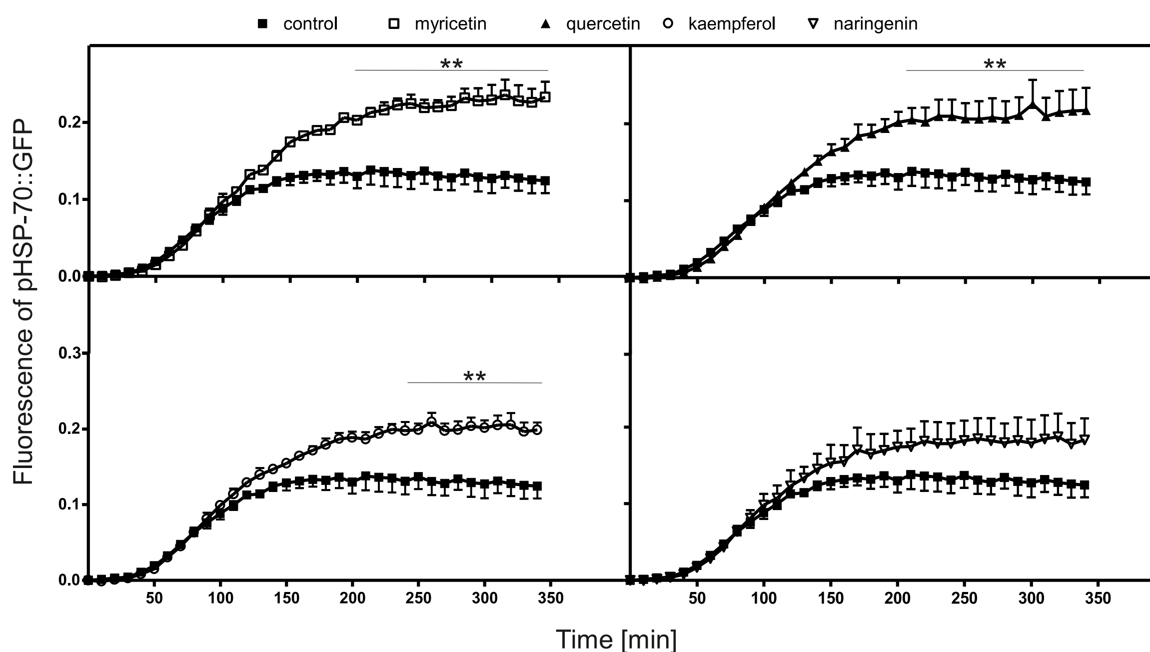


Fig. 40 Effects of flavonoids on heat-induced *hsp-16.2* promoter activity
hsp-16.2::gfp C. elegans exposed to solvent (control) or 100 µM of indicated flavonoids were heat-shocked at 34°C for 2 x 30 min. Fluorescence of pHSP-16.2::GFP was subsequently analyzed for 340 min. Each data point represents the mean ±SEM of three individual experiments with 4 x 50 worms analyzed. Statistical significance of differences between control and intervention groups was determined by two-way ANOVA with Bonferroni post-hoc test.

In *hsp-70::gfp C. elegans* fluorescence started to increase 50 minutes after heat-shock and a plateau was reached after 200 min. All flavonols further increased the heat-induced fluorescence when compared to control. Briefly, myricetin showed the strongest effects with increased promoter activity after 190 min post heat shock followed by quercetin

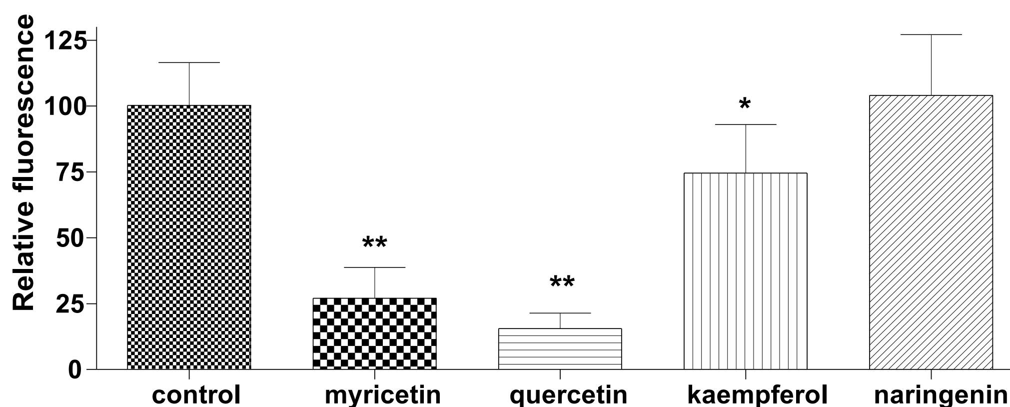
(200 min) and kaempferol (230 min). Solely naringenin failed to show an significant effect (Fig. 41). This indicates that the increased promoter activity is dependent on the additional structural features of the flavonols.



hsp-70::gfp C. elegans exposed to solvent (control) or 100 µM of indicated flavonoids were heat-shocked at 34°C for 2 x 30 min. Fluorescence of pHSP-70::GFP was subsequently analyzed for 340 min. Each data point represents the mean ±SEM of 3 - 4 individual experiments with 4 x 50 worms analyzed. Statistical significance of differences between control and intervention groups was determined by two-way ANOVA with Bonferroni post-hoc test (**P<0.01).

4.7.3 Contribution of DAF-16 on the beneficial effects

To elucidate whether DAF-16 might contribute to the flavonoid antioxidant effects at all, the mtROS load was also analyzed in *daf-16(mu86) C. elegans.* Determination of CM-H_2XRos fluorescence revealed similar effects as observed in N2, *mev-1(kn1)* and *isp-1(qm150);ctb-1(qm189) C. elegans (*Fig. 42). This suggests that the antioxidant effects of the flavonols could be independent from DAF-16 and thus possibly also independent from other DAF-16 regulated antioxidant enzymes.



Relative fluorescence of CM-H_2XRos in *daf-16(mu86) C. elegans* incubated with 100 µM of indicated flavonoids or solvent control for 48 h. Each bar represents the mean ± SD of 3 individual experiments with at least 10 worms analyzed. Statistical significance of differences versus control group was determined by one-way ANOVA with Dunett's post-hoc test (**P<0.01).

To assess whether the lifespan-extending effects of the flavonoids are also independent from DAF-16, the lifespan in *daf-16(mu86) C. elegans* was determined. In these animals myricetin and quercetin increased mean adult lifespan by ~6% and ~4%, respectively, while kaempferol and naringenin did not show any significant effect (Fig. 43, Table 13 appendix). The lifespan-extending effects of the flavonols seem therefore also not to require DAF-16 despite the fact that upon flavonoid-exposure DAF-16 translocation into the nucleus is increased and selected target genes show elevated activity, suggesting other underlying mechanisms to be involved.

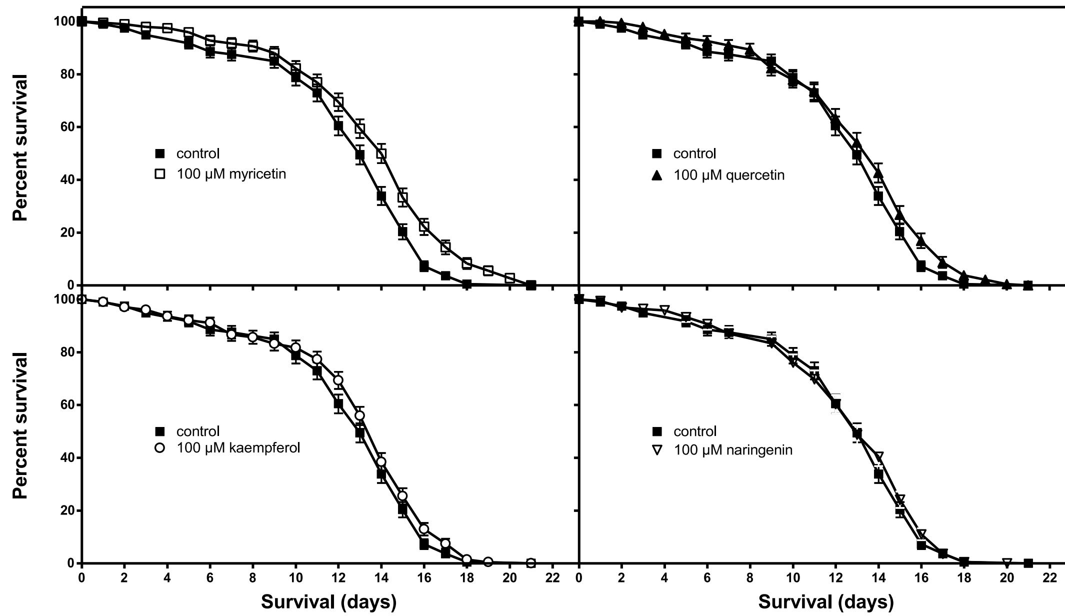


Fig. 43 Effects of flavonoids on adult lifespan in *daf-16(mu86) C. elegans*
Effects of 100 µM of the selected flavonoids on adult lifespan in *daf-16(mu86) C. elegans*. Data is plotted as Kaplan-Meier survival curves with SE based on two individual experiments with at least 90 individuals per group. Details see Table 13, appendix.

5. Discussion

The flavonoids are bioavailable to C. elegans

Bioactivity of any substance depends on its bioavailability. This includes absorption by the organism, distribution to the target tissues and cellular uptake. In *C. elegans* the bioavailability of the flavonoids was demonstrated by a method that allows their visualization *in vivo* by using 2-aminoethyl diphenyl borate and by a HPLC method to quantify the parent compounds and metabolites. Despite the lack of *in vivo* staining in case of naringenin, HPLC/DAD analysis confirmed its bioavailability. As 2-aminoethyl diphenyl borate forms chelates with oxo-groups of polyphenols [154], the 3-OH group at the C-ring seems important for the chelate-formation and the labeling efficiency *in vivo* as the fluorescence of kaempferol was enhanced but not that of naringenin. In the present study, the majority of fluorescence was always observed in the gut epithelial cells as the site of absorption but obviously also of accumulation. This is in accordance to data from mammals, demonstrating highest concentrations of flavonoids in gut and liver after oral administration [186] [187]. HPLC/DAD analysis revealed total flavonoid concentrations to range between ~19 and 37 $nmol*mg^{-1}$ protein. As a rough estimate these concentrations are in a comparable magnitude to concentrations described in *in vitro* models. In dermal fibroblast, Caco-2 and cardiomyoblast cell lines quercetin concentrations between 0.23 $nmol*mg^{-1}$ protein and 4 $nmol*mg^{-1}$ protein were recovered after exposure of cells to 10 µM to 50 µM of this flavonol [188] [189] [190]. Highest concentrations in nematodes were found for the flavanone naringenin. This confirms data reported by Spencer *et al.* for different cell types demonstrating up to 10-fold higher uptake of naringenin than for quercetin or kaempferol [191]. This seems not surprising, considering the structural differences between flavonols and flavanones, determining their polarity. Accumulation of kaempferol in worms was ~15% lower than that of the other flavonols, a finding that was also observed in cell culture systems [191].

HPLC/DAD analysis indicates that a large fraction of the compounds taken up into *C. elegans* undergoes extensive bio-transformation to conjugates that can be cleaved again by glucuronidase and arylsulfatase. This is in accordance to data from human studies demonstrating quercetin-3-glucuronide, 3'-methyl-quercetin-3-glucuronide and quercetin-3'-sulfate as major plasma metabolites after consumption of a quercetin-rich diet [192].

Accordingly, after consumption of a naringenin-rich beverage 7- and 4'-glucuronides were detected in the urine [193] demonstrating extensive metabolism of flavonoids in humans. Although the precise nature of the metabolites in *C. elegans* is not yet known there are indications that A-ring conjugates might account for the majority of the detected metabolites. Conjugated flavonoids are generally thought to be less potent antioxidants than their aglycones [133]. Thereby the site of conjugation at the flavonoid backbone is of major importance. Quercetin-3-glucuronide and -4'-glucuronide, for example, were shown to have lower *in vitro* antioxidant capacity than quercetin-7-glucuronide [194]. As the flavonols exerted antioxidant effects in *C. elegans,* the OH-groups at the B-ring and C-ring are most likely unconjugated. Furthermore, as the 3-OH group was demonstrated to be necessary for proper enhancement of auto-fluorescence by 2-aminoethyl diphenyl borate, conjugation at this site seems also unlikely. Consequently, the A-ring OH-groups seem to be the most probable sites for conjugation. This is supported by data of *C. elegans* which were exposed to genistein and mainly A-ring conjugates were detected (personal communication by Prof. S. Kulling, Karlsruhe). These data also demonstrate glucosides to be the major conjugates of genistein in *C. elegans*. This is a surprising finding since glucuronidation and sulfatation are thought to be the most prominent conjugation reactions of flavonoids in mammals [195] [196] [191]. However, recently, Laing *et al.* demonstrated glucosides to be main conjugates of the anti-helmintic drug albendazole in *C. elegans*, suggesting glucosidation to play a prominent role in phase II metabolism in *C. elegans* [197]. Whether this also accounts for conjugation of flavonoids has to be further analyzed.

Flavonoids display antioxidant activity in isolated mouse mitochondria and C. elegans

The flavonols revealed effects on lifespan and are also known to possess a high *in vitro* antioxidant capacity [132]. To be causal for the longevity effects, their *in vitro* antioxidant capacity has to be translated into antioxidant action in more complex systems. Therefore ROS-generation was assessed in isolated mitochondria, the major source of ROS, and in *C. elegans*.

The ETC complexes I and III are discussed to be the major sites of mitochondrial ROS-generation [18]. For complex I this was confirmed in isolated mouse skeletal muscle mitochondria with the complex II substrate succinate also indicating reverse electron flow from complex II to I as the most likely source of ROS. This finding has also been described in succinate-fed rat brain mitochondria [198] and in *Drosophila melanogaster*

mitochondria when supplied with complex I and III substrates [199]. Despite the fact that the H_2O_2-generation was of comparable magnitude to values described in literature [198], it might not reflect the real physiological rates. In *ex vivo* systems 0.15% to 2% of the electron flow is thought to generate ROS, a rate that is thought to be less *in vivo* due to lower oxygen partial pressure and continuous ATP synthesis [200] [201]. However, as mice lacking mitochondrial SOD rapidly die after birth [24], even the lower ROS levels *in vivo*, if not properly detoxified, seem to be lethal. Despite the differences to the physiological situation, isolated mitochondria are by far more complex than any *in vitro* detection system for antioxidant activities and are closer the *in vivo* setting.

Exposure of mitochondria to the flavonoids revealed their ROS-scavenging activity to depend mainly on the 3-OH group and the double bond at the C-ring since the flavonols reduced H_2O_2-generation much stronger than the flavanone naringenin. This is in accordance with data from isolated rat mitochondria where a similar assay was used [202]. As neither quercetin nor myricetin further reduced the H_2O_2-generation, the catechol and pyrogallol functions appear to be irrelevant for scavenging mtROS in this *ex vivo* system. This is in contrast to data obtained in *in vitro* assays in which myricetin and quercetin were shown to possess an *in vitro* antioxidant capacity higher than that of kaempferol and naringenin [132] [133] [147]. It has to be taken into account that comparative methods such as TEAC or FRAP rely on other mechanisms as the assay used in the mitochondria model. These *in vitro* methods are single electron transfer reactions where electrons are transferred from the antioxidant to the probe [203]. In the Amplex Red assay used here, a horseradish peroxidase catalyzes the conversion of Amplex Red to the fluorescent dye resorufin. Besides the different nature of the quenched radical (e.g. $ABTS^{\circ}$ in the TEAC assay and H_2O_2 in the Amplex Red assay) the tested flavonoids might also interact with the horseradish peroxidase [203]. However, all data collected confirmed the structural properties attached to the C-ring to play a prominent role in antioxidant action *ex vivo* but also emphasizes the limitations for extrapolating *in vitro* data to more complex biological systems.

To analyze the antioxidant activity of the four test compounds *in vivo* in *C. elegans*, the dye CM-H_2XRos was employed. The flavonols reduced mtROS up to 70%, an effect that was stronger than those described for other plant compounds. EGCG and *Ginkgo biloba* extract for example reduced ROS levels in *C. elegans* by just 15-30% as determined by the

widely-used dye 2.7-dichlorofluororescein diacetate [204] [172] [179]. These differences might be explained by the high specificity of CM-H_2XRos to stain mitochondrial ROS while the frequently used dyes MitoSOX^{TM} and 2.7-dichlorofluororescein diacetate also stain cytosolic ROS [205]. In accordance with that quercetin was demonstrated to accumulate preferably in mitochondria of lymphocytes [206], which may suggest a higher antioxidant action in these organelles. Among the flavonoids, the flavonols but not naringenin reduced mtROS levels significantly, demonstrating the structural properties at the C-ring as important prerequisites for antioxidant activity also in *C. elegans*. When taking the higher levels of naringenin in worms into account, this compound seems to have a negligible antioxidant activity in the nematodes. A possible explanation for the crucial role of the 3-OH group might be that these flavonoids are planar while compounds lacking this structural feature show a torsion of the B-ring with respect to the base of the molecule. A planar structure generally seems to promote delocalization of electrons and thus increases antioxidant activity [207]. Furthermore the 3-OH group of flavonols was shown to have a low oxidation potential which is oxidized irreversibly thus preventing redox-cycling [208]. Although HPLC based quantification revealed kaempferol levels to be lowest among the flavonols in worms it seems to have a slightly higher antioxidant activity. All flavonols carrying the catechol function reduced mtROS levels stronger implying an additional antioxidant activity for this structural group, as suggested *in vitro* [135]. Accordingly the hydrogen-donating reaction rates are higher for flavonoids carrying this structural characteristic [209]. The pyrogallol function of myricetin, however, did not have an additional effect. These antioxidant effects were independent of the initial ROS status of the nematodes as in the mutant strains with altered mitochondrial ROS-generation similar effects were observed. The reduction of mtROS became apparent already after 2 hours of incubation. As Kampkötter and colleagues could not detect quercetin in *C. elegans* by means of HPLC analysis after a 3 hours exposure [153], rather low cellular concentrations seem to be already effective to reduce mtROS. Taken together the data show ROS-scavenging activities in isolated mouse mitochondria and *C. elegans* that depend on the compounds structure.

Protein-carbonyls were determined in *C. elegans* to assess whether the flavonoids antioxidant action is reflected in the levels of oxidatively damaged proteins. Since the flavonols with the strongest effect on mtROS levels (myricetin and quercetin) also reduced PCC, a direct link to their antioxidant potential seems plausible. In accordance with that,

C. elegans strains carrying gene knock-outs such as *mev-1, sod-1, sod-2* or *daf-16* which are described to possess (and shown here) to have elevated ROS levels and/or a reduced ROS-defense capacity all display also increased PCC [117] [182] [96]. Conversely, mutants of genes such as *daf-2, isp-1* or *age-1* are long-lived, resistant against ROS-stress and possess reduced protein-carbonyl levels [210] [182] [211]. Furthermore, in yeast and in erythrocytes, quercetin was shown to reduce ROS-induced PCC [212] [213]. This suggests the antioxidant action of the flavonoids to be translated into protective effects on protein modifications as well. It has to be acknowledged that no statement can be made on beneficial effects on other macromolecules such as lipids or DNA. Furthermore, besides ROS PCC is also regulated by degradation mechanisms enhancing their removal such as by proteasome-mediated pathways that were not analyzed here. However, the data strongly indicate that the structural prerequisites determining the antioxidant activity of flavonoids *in vitro* is reflected *in vivo* and is translated into markers of oxidative damage in *C. elegans*.

To elucidate whether the flavonoids might exert their effects also on ROS levels induced by an exogenous stressor, the herbicide paraquat was employed in mouse mitochondria and *C. elegans*. Paraquat is thought to exert its toxic effects by stimulation of superoxide anion radical production at complex I in the mitochondrial ETC [156]. This was confirmed in the isolated mitochondria used here, since ROS were induced only when succinate was provided and thus a reverse electron flow from complex II to complex I got established. The paraquat-induced H_2O_2-generation was reduced by co-exposure with the flavonoids in a similar way as under paraquat-free conditions with a structure-activity pattern – confirming the structural features necessary for antioxidant action also under ROS-induced conditions in this system. In line with the isolation criteria of *mev-1(kn1) C. elegans,* an increased sensitivity to paraquat was observed in these mutants [102]. The resistance of *isp-1(qm150);ctb-1(qm189)* mutants, however, was not different from wild-type nematodes. Feng *et al.* also investigated the effect of paraquat in these mutant animals and observed an increased resistance. As they only determined the influence during larval development but not on adult animals, protective mechanisms of these mutations may only be present during development [109]. The flavonoids showed protective effects in the nematodes when consecutively exposed to paraquat and then the effects seemed to be uncoupled from their antioxidant action since myricetin did not exert any effect. In accordance with that, several secondary plant metabolites such as resveratrol, various plant

adaptogens and quercetin were also shown to increase the resistance against paraquat [214] [152], but this was not necessarily correlated to their *in vitro* antioxidant capacity [211] [173]. More strikingly, myricetin even displayed adverse effects when administered with paraquat simultaneously. In analogy to the findings here, the flavonoid hesperidin was demonstrated to protect yeast only when administered prior but not after paraquat treatment [215]. As antioxidant compounds can also act as pro-oxidants, the adverse effect of myricetin might be explained by such mechanisms. Myricetin, quercetin but not kaempferol were shown to generate ROS by auto-oxidation *in vitro,* with highest rates described for myricetin demonstrating its pyrogallol function at the B-ring to have a major impact [216]. This process is greatly enhanced by addition of transition metals such as iron [216]. Oxidative effects of paraquat were exacerbated in rats when the antioxidant ascorbic acid was administered after but not prior paraquat-exposure, hence when the tissue was already injured and transition metals were released [217]. It therefore seems plausible that a co-exposure of *C. elegans* to paraquat and flavonoids can cause enhanced auto-oxidation especially of myricetin leading to a reduced stress-resistance. Whether such mechanisms might account for the observed effects was beyond the scope of the present work and were only found in the nematodes but not in isolated mouse mitochondria.

Antioxidant mechanisms alone are not sufficient to explain lifespan extension

With a maximum of 18%, even the strongest increase in mean adult lifespan after myricetin exposure is rather low, when compared to *C. elegans* genetic models such as the mutants *daf-2* or *daf-2;pept-1* that show an extension of lifespan of more than 100% [51] [218]. The observed effects are comparable in magnitude as described for other compounds, including the best-characterized substance resveratrol, administered to wild-type *C. elegans* (Table 9).

Table 9. Lifespan extension of selected plant compounds administered to *C. elegans*

Compound (administered dose)	Mean lifespan extension	Reference
Epigallocatechin gallate (220 µM)	10%	[204]
Ginkgo biloba extract EGb761 (100µg*mL^{-1})	12%	[178]
Catechin (100 µM)	17%	[174]
Quercetin (200 µM)	10%	[219]
Resveratrol (100 µM)	14%	[220]
Blueberry extract (200 µg*mL^{-1})	16%	[221]
Cocoa polyphenols (4 mg*mL^{-1})	20%	[222]
Tannic acid (100 µM)	18%	[223]

Besides mean lifespan also maximum lifespan was increased by the flavonols. This implies the aging process may be delayed rather than providing an improvement of living conditions and fitness of the nematodes. Whether secondary plant compounds might exert similar effects also in vertebrates remains largely unknown. Lifespan-extending effects, for resveratrol for example, were described also in *S. cervisiae, D. melanogaster*, *Nothobranchius furzeri* (a seasonal fish) and in mice fed a high-fat diet [224] [220] [225] [226]. On the contrary, Jones and Hughes demonstrated lifespan-reducing effect of quercetin-fed mice [227]. Possibly the high dose used in this study, estimated with around 285 mg*kg^{-1}*d^{-1} which is 20 times higher than concentrations commonly used in human studies, resulted in these adverse effects. Other plant phenols such as tannic acid and ellagic acid were also demonstrated to unfold adverse effects when administered in high concentrations to *C. elegans* [223], demonstrating the narrow concentration range for protective versus fatal effects. In the *C. elegans* study here, however, all flavonols but not naringenin showed effects (although modest) that in addition could be related to the structural properties of the C-ring and the increasing number of OH-groups attached to the B-ring as major determinants. As these structural features also characterize the flavonoids antioxidant action, a causal relationship, as suggested by the "free radical theory of aging", seems plausible. Lifespan effects have also been demonstrated in different *C. elegans* strains carrying mutations in genes such as *age-1, isp-1* or *nuo-6* in combination with reduced ROS levels [228] [109] [229]. The same holds true for foreign substances, administered to the nematodes such as superoxide dismutase mimetics, *Ginkgo biloba* extract, fisetin and lipoic acid [230] [178] [231] [232]. Life-prolonging effects of synthetic antioxidants have already been described also in mice more than 40 years ago [233] [234].

However, from these studies it is not clear whether such compounds might exert their effects also by other mechanisms than ROS-scavenging. In the present study, the flavonols also increased the resistance of *C. elegans* against heat, a stressor which deleterious effects are also thought to be mediated by increased ROS-production [180]. Similarly, plant adaptogens, quercetin, rutin and *Ginkgo biloba* extract were demonstrated to increase thermal resistance in worms accompanied with antioxidant action [214] [179] [178]. However, whether heat-resistance is a suitable surrogate for oxidative stress seems questionable, since *mev-1* mutants do not show reduced sensitivity against this stressor [117].

Recent findings have challenged again the concept of a causal link between increased ROS-production and accelerated aging [3] [10]. For example no or even an lifespan-increasing effect has been demonstrated in *C. elegans* mutants of the mitochondrial superoxide dismutases *sod-2* and *sod-3,* despite the fact that the animals are sensitive against ROS-stress [235] [114] [11]. Conversely, over-expression of individual *sods* and catalases failed to increase the lifespan in *C. elegans* or mice [114] [236]. Moreover *C. elegans* mutants of genes including *mev-3*, *mev-6* and *mev-7* are known to posses increased resistance against ROS in the absence of a lifespan extension [237] [238]. On the other hand *C. elegans* and mouse mutants of *clk-1*, a gene coding a hydroxylase involved in ubiquinone synthesis, are long-lived despite not being ROS-resistant [64]. In accordance with these inverse relations between ROS defense and lifespan, moderate levels of the ROS-generating substance juglone were shown to induce longevity in *C. elegans*, indicating ROS to induce a hormetic dose-response [171] [185]. Furthermore, Ristow and co-workers suggested a controversially discussed theory termed “mitohormesis”. This theory states that a transient increase in mtROS (e.g. by glucose deprivation) induces enzymatic defense mechanisms as a response that gets abolished by exogenous antioxidant compounds [239] [240]. Despite the fact that a transient increase in mtROS was observed after kaempferol exposure here, this seems not to account for the effects of the other flavonoids. Additionally, all flavonoids elevated promoter activity of the mitochondrial *sod-3* in a comparable manner. Thus it is questionable whether mitohormetic mechanisms account for the observed lifespan-effects in the present study. However, as a linear correlation between ROS levels and aging seems unlikely, it is also questionable whether the reduction of physiological ROS levels by antioxidant xenobiotics is the cause for their longevity-inducing effects. Accordingly, Pun and colleagues screened several plant

compounds with known *in vitro* antioxidant activities, demonstrating no correlation to their lifespan effects in *C. elegans* [211]. Moreover, Xue *et al.* did also not find a relation between the antioxidant potential and lifespan effects of different quercetin conjugates in *C. elegans* [241]. Similarly here, quercetin reduced the mtROS-load and PCC levels to the same extend as myricetin but its longevity-effect was lower than that of myricetin. This was also confirmed in *mev-1(kn1) C. elegans* where neither quercetin nor kaempferol increased lifespan despite reducing the elevated mtROS levels to those found in wild-type nematodes. The lack of a lifespan alteration by quercetin in this strain, however, could also be due to the experimental design as 200 µM of quercetin in the 2nd generation of exposed *mev-1(kn1)* nematodes was demonstrated to prolong their lifespan [219]. Nevertheless, lifespan extension by quercetin was lower than that of myricetin suggesting longevity not to be caused solely by antioxidant mechanisms. Additionally Gruber *et al.* in a generalized manner proposed the following criteria that a compound should meet for its antioxidant action to cause lifespan-effects. It should: a) reduce oxidative damage, with PCC as the to date best marker in *C. elegans*, b) be protective under oxidative stress (e.g. PQ-stress or in *mev-1(kn1)*), c) not induce other mechanisms such as CR, reduced metabolism or interfere with ETC and d) be independent from other antioxidant mechanisms such as *sod-3, hsp-16.2* [242]. As not all of these criteria were met by the flavonoids it is doubtful whether their antioxidant action alone is sufficient to explain the longevity effects, calling for other mechanisms to be involved.

Involvement of metabolic mechanisms

Caloric restriction is well-known to induce longevity in several species including *C. elegans* [89]. Therefore pharyngeal pumping rate was assessed to elucidate whether the flavonoids might act as CR mimetics i.e. via food aversion. Myricetin was found to slightly reduce the pharyngeal pumping rate but the other flavonols did not exert a similar effect, implying longevity to be independent from reduced food intake. Although other plant compounds were similarly shown to increase *C. elegans* lifespan independently of effects on food intake [211] [243] [174], an impact on other CR-mediated pathways can not be excluded. Several pharmacological compounds such as 2-deoxyglucose, fisetin and resveratrol are described to exert CR-like activity and thus were termed “CR mimetics” [240] [220]. Resveratrol, as the best characterized CR mimetic, was demonstrated to exert its beneficial effects via activation of sirtuins that are proposed to mediate CR-induced

longevity in yeast, *C. elegans* and *D. melanogaster [224]* [220] [152]. Recent data suggest that the activation of sirtuins, either by CR, sirtuin over-expression or activators such as resveratrol, increases longevity by stimulating autophagy [244], a process that can reduce cellular protein aggregates under stress conditions [245]. *In vitro* data, however, suggests that quercetin and myricetin might also be able to influence sirtuin activity providing an indication that CR mechanisms might be involved in the longevity effects of these compounds [246]. As it correlates with age, pharyngeal pumping rate is also regarded as a good measure of age-dependent fitness [181]. Several longevity-inducing plant compounds such as blueberry polyphenols, rosmaric acid and caffeic acid were shown to increase pharyngeal pumping rate in *C. elegans* [221] [173]. Under the conditions used here such beneficial effects were only observed in *mev-1(kn1) C. elegans*, indicating a positive effect only under stressful conditions. As all flavonoids increased pharyngeal pumping in *mev-1(kn1) C. elegans* similarly, this effect seems to be independent from their structural properties and thus their antioxidant and lifespan effects.

To test whether a slow-down of the metabolic rate as suggested in the rate-of-living theory [4], might be responsible for the observed lifespan effects of the flavonoids, the oxygen consumption rate was determined in *C. elegans* after flavonoid exposure. In long-lived *C. elegans* mutants such as *isp-1(qm150);ctb-1(qm189)* a diminished oxygen consumption rate was associated with reduced mtROS levels [109], a finding that was confirmed here. In accordance with the mitochondrial free radical theory of aging the reduced oxygen consumption in this strain might reflect a diminished electron-flow in the ETC that results in lower mtROS levels and a subsequently increased lifespan. Despite a mtROS reduction induced by the flavonols this seems unlikely to be the sole reason for the longevity effects. The flavonols could reduce the metabolic rate with a reduction of the respiration rate. According to the disposable soma theory such a reduced metabolism could as trade-off provide energy resources in favor of body maintenance [2], with diminished mtROS as a secondary outcome. Dorta *et al.*, for example, demonstrated that quercetin can inhibit respiration in isolated rat liver mitochondria [177] while the F_0F_1 ATP synthase was shown to be a potential target within the ETC of bovine heart mitochondria [247]. However, as none of the flavonoids altered oxygen consumption in the study here, an impact on metabolism seems unlikely as a cause of longevity and the antioxidant effects. Additionally this also demonstrates that reduced respiration and thus a reduced electron

flow is unlikely to be responsible for the reduction in mtROS suggesting other mechanisms to be involved.

Flavonoids affect molecular targets

As neither the antioxidant action alone nor metabolic effects sufficiently explain the flavonol-induced longevity, other mechanisms were elucidated. There is growing evidence that flavonoids exert their effects by interaction with cell-signaling cascades [176]. In this regard the IlS cascade seemed a promising target as it was shown to be a key control system for aging not just in *C. elegans* [56] but also in *D. melanogaster* and mice [71] [72]. Also in humans there is evidence that the genetics of the IGF receptor or a reduced IlS might be involved in longevity of certain centenarian populations [248] [249]. In *C. elegans,* several polyphenols such as quercetin, fisetin and EGCG were shown to increase the nuclear localization of DAF-16, the endpoint of the IlS cascade, suggesting an involvement in their beneficial effects [153] [179] [250]. In accordance with that, all flavonoids tested here also increased nuclear translocation of DAF-16 as a more generalized effect independent of their structural features. This finding strongly suggests a reduced phosphorylation state of DAF-16 possibly induced by reduced IlS activity. Flavonoid interaction with the IlS cascade might originate at different levels. Myricetin, quercetin and, to some lesser extend, kaempferol were shown to inhibit the activity of upstream PI 3-kinase in epidermoid cancer cells [140]. Also physiological doses of naringenin were shown to inhibit this enzyme in adipocytes [141]. Moreover, quercetin is the lead compound on which specific PI 3-kinase inhibitors such as LY294002 were designed on [251]. The underlying mechanism seems to be steric interaction with the ATP-binding pocket of this kinase as revealed by X-ray crystallographic analysis [252]. In recombinant PI 3-kinase the C_2-C_3 double-bound and the number of hydroxyl groups at the B-ring were shown to correlate with the flavonoids inhibitory effects [253]. However, this can not explain the findings in the nematodes as DAF-16 translocation was similar for all flavonoids. And therefore other points of interaction with the IlS cascade have to be postulated. Myricetin, for example, was demonstrated to bind to the downstream target AKT-1 inhibiting its phosphorylation *in vitro* [254]. Also PTEN, an inhibitor of PI 3-kinase, might be identified as a target of the flavonoids, as quercetin was demonstrated to increase its expression in breast cancer cells leading to a reduced phosphorylation of AKT [255].

Whether the DAF-16 translocation found is in any respect linked to the observed beneficial effects seems questionable since all four compounds affected DAF-16 similarly but displayed different effects on lifespan and stress-resistance. Not just the location and phosphorylation status of DAF-16 regulates its activity but also interaction with other transcription factors, thus increasing possible sites of flavonoids interaction [78]. Increased nuclear localization must therefore not lead to increased longevity as seen in mutations in the presumed phosphatase *smk-1*, known to increase nuclear translocation of DAF-16 without increasing longevity [256]. The existence of several DAF-16 regulators is further supported by the fact that almost 80% of all genes in *C. elegans* possess a DAF-16 binding element region [257] but only a small number of them are regarded as “true” DAF-16 targets. For example, using chromatin immunoprecipitation Oh *et al.* identified just some more than 100 direct target genes of DAF-16 [68]. To get further insight into the outcome of this DAF-16 translocation in our *C. elegans* models, promoter activities of the down-stream targets *sod-3* and *hsp-16.2* were assessed [64] [170]. In accordance with the DAF-16 nuclear abundance all flavonoids increased *sod-3* promoter activity independent of their structural features. In contrast to that, Kampkötter *et al.* found quercetin to reduce the promoter activity of *sod-3* despite an increased nuclear accumulation of DAF-16 [153]. Possibly the liquid medium used for quercetin exposure in that study might explain the observed differences. However, whether the elevated promoter activity contributes to the beneficial effects in particular in the mtROS reduction seems questionable since all tested flavonoids had similar impact on this reporter expression but different effects on lifespan and mtROS levels. Furthermore, *sod-3* contribution to mtROS reduction might be relatively low, as its mRNA level, its contribution to overall SOD activity and Mn-SOD protein amount were demonstrated to be rather low when compared to the other *C. elegans* SOD isoforms [114]. Additionally in *daf-16(mu86)* mutants, mtROS were affected by the flavonoids similarly to wild-type animals, emphasizing the antioxidant effects to be independent from DAF-16.

As part of the heat-shock response *hsp-16.2* expression is thought to be initiated by the presence of misfolded proteins, which themselves are induced by various stresses [168]. This should make its promoter activity a good marker for such deleterious effects [150]. As the flavonoid treatment failed to alter *hsp-16.2* reporter activity under standard conditions, a general toxic effect or induction of cellular stress-response by the flavonoids seems unlikely. In accordance with the time-dependent reduction of the mtROS levels this also

casts doubt on hormesis effects to be involved in the beneficial effects of the test compounds. Mechanistically, the absence of *hsp-16.2* promoter activity despite increased nuclear DAF-16 localization implicates further factors to be necessary for *hsp-16.2* expression. A promising candidate in this regard might be the heat-shock factor 1 HSF-1, which was shown to be required for *hsp-16.2* promoter activity [258] and, as DAF-16, for lifespan extension of IlS mutants [65]. The heat-shock response in the nematodes was initiated by oxidative and by heat stress but here flavonoid treatment as well failed to show an effect on promoter activity ruling out that the beneficial effects of the flavonoids on these stress-responses involve this chaperone.

The promoter-activity of *hsp-70* (C12C8.1) as putative contributor of increased heat-resistance was also assessed here. Despite the fact that this chaperone was demonstrated to be partially necessary for longevity of the IlS mutant *age-1* [151], RNA interference against *hsf-1* but not against *daf-16* reduced its heat-induced mRNA levels [259]. In the study here, heat-induced promoter activity of *hsp-70* was further increased by prior exposure to the flavonols. This might suggest an increased protection from heat-induced protein-aggregation and elevated heat-tolerance. Mechanistically this contrasts findings in HeLa and breast cancer cell lines in which quercetin was demonstrated to reduce heat-induced *hsp-70* and *hsf-1* expression [260] [261]. Species differences in the function of HSF-1 might explain this discrepancy since *hsf-1 C. elegans* mutants show a reduced lifespan [151] whereas embryonic cells from *hsf-1* mouse mutants display no altered survival under non-heat-shock conditions [262]. Taken together these data show that the flavonoids do have an impact on promoter activity of protective enzymes although this may not necessarily translate into effects on their activity.

To assess whether a functional DAF-16 is involved at all in the beneficial effects, the *daf-16(mu86)* mutant strain was employed. In this strain the preservation of the lifespan-extending and antioxidant effects was observed as well and this strongly suggests other mechanisms to be responsible in the lifespan extension and antioxidant effects than DAF-16. This is in accordance with findings by Saul *et al.,* demonstrating a prolonged lifespan induced by quercetin in a *daf-16* knock-out strain [263]. Besides the IlS cascade other molecular targets are described to mediate flavonoids beneficial effects in *C. elegans*. Pietsch and colleagues, for example, demonstrated in addition to the IlS-linked genes *daf-2* and *age-1*, *sek-1* (a MAP-kinase-kinase acting downstream of Toll and the interleukin-1

receptor) and *unc-43* (a type II calcium/calmodulin-dependent protein kinase) to modulate quercetin-induced lifespan extension [219]. The mRNA level of *skn-1,* a transcription factor regulating the expression of several protective enzymes [86], was found elevated upon EGCG exposure [264], proposing it also as a putative target for the flavonoids. The lifespan extension observed here after flavonol treatment therefore calls for further studies that link the beneficial effects to other signaling processes in nematodes.

6. Summary and conclusions

Flavonoids represent a major subgroup of secondary plant compounds, present in various foodstuffs, with a broad spectrum of attributed biochemical properties claimed to affect health and aging. With more than 9000 described structures they possess a magnificent diversity which consequently will determine their functions. To elucidate for a small subset of compounds the structure-activity relationship *in vivo,* a comparative approach was used by exposing the nematode *Caenorhabditis elegans* to four structurally related flavonoids and measured their impact on lifespan and stress-resistance. The flavonols kaempferol, quercetin and myricetin, differing from each other in an increasing number of OH-groups attached to the B-ring, and the flavanone naringenin (lacking hydroxylation and a double bond at the heterocyclic C-ring) were chosen. These structural features are well-known to be necessary for a high antioxidant capacity *in vitro*. By means of HPLC/DAD analysis their apparent bioavailability, a prerequisite for any biological action, was confirmed and their extensive metabolization was demonstrated. Additionally, a novel *in vivo* approach that allows visualization of flavonoids by enhancing their auto-fluorescence was established, confirming the gut as major site of flavonoid accumulation in the nematodes.

Reactive oxygen species (ROS) are thought to contribute, at least partially, to aging processes and various diseases. Flavonoids possess a high antioxidant capacity, a characteristic often associated with their proposed beneficial effects on health. This prominent feature, however, is usually determined by *in vitro* methods, permitting conclusions on their structure-activity relationship, but give no information on the impact in more complex systems or whole organisms. Therefore the antioxidant activity of the test compounds was determined in isolated mouse skeletal muscle mitochondria and in *C. elegans*. Hydroxylation and a double bond at the heterocyclic C-ring in the compounds were demonstrated to have ROS-scavenging activity in isolated mitochondria. An additional catechol function at the B-ring (3', 4'-OH) even increased this effect in *C. elegans* and in turn also reduces the levels of oxidatively damaged proteins. Hence, the structural prerequisites that determine the *in vitro* antioxidant capacity of the compounds were largely confirmed in the *ex vivo* model of the isolated mitochondria and *in vivo* in *C. elegans*. All flavonols but not the flavanone naringenin increased lifespan and resistance against heat-stress in *C. elegans*, demonstrating that antioxidant activity can also exert

beneficial effects on aging and stress resistance. Only myricetin was able to prolong lifespan in the mutant *mev-1(kn1)*, possessing an intrinsic high level of ROS-production, despite the fact that all flavonols reduced the high mitochondrial ROS levels. Thus, the flavonols antioxidant action alone is not sufficient to explain their beneficial effects. Any effects on food intake and thus a caloric restriction phenotype as well as on respiratory activity could be excluded as causal mechanisms asking for other molecular targets.

The insulin/IGF-like signaling (IlS) cascade was demonstrated to be a key control system for aging and stress-resistance in *C. elegans, Drosophila melanogaster* and mice. The prime endpoint of this cascade is the FOXO transcription factor DAF-16. In absence of phosphorylation, DAF-16 can translocate into the nucleus eliciting expression of a large set of defense enzymes such as superoxide dismutases or heat-shock proteins and thereby extend the nematodes lifespan. All flavonoids were able to cause a DAF-16 translocation into the nucleus and to increase promoter-activity of one of its target *sod-3* but not of *hsp-16.2*, demonstrating alteration of gene expression control beyond DAF-16. By employing a *daf-16* mutant strain the lifespan-extending and antioxidant effects of the flavonols were found preserved and thus shown to be independent of DAF-16. This consequently calls for further studies elucidating other potential mechanisms by which the flavonoids can exert their effects.

In summary the present work demonstrates that flavonoids in a structure specific manner can increase *C. elegans* lifespan. As major requirements for their effectiveness their bioavailability was demonstrated and first insights were gained into the metabolism of the compounds. A variety of mechanisms and pathways by which they could exert their effects was tested. This is the first time that such studies are done *in vivo* to reveal structure-activity relationship of the selected model compounds. Finally, in search of dietary constituents that may modify the aging-trajectory and prevent age-dependent impairments, we have found compounds that can modestly – but as good as the most discussed caloric-restriction mimetic resveratrol – increase mean and maximal lifespan in *C. elegans*.

7. Zusammenfassung und Schlussfolgerungen

Flavonoide sind eine der größten Untergruppen von sekundären Pflanzenstoffen und damit in zahlreichen Nahrungsmitteln vorzufinden. Mit mehr als 9000 beschriebenen Substanzen besitzen diese Polyphenole eine enorme strukturelle Vielfalt die folglich auch ihre ebenso vielfältigen biologischen Wirkungen bestimmt. Zur Klärung einer Struktur-Wirkungs-Beziehung wurden hier vier strukturell verwandte Flavonoide in ihren Effekten auf das Altern und die Stressresistenz im Fadenwurm *Caenorhabditis elegans in vivo* untersucht. Dazu wurden die Flavonole Kaempferol, Quercetin und Myricetin, die sich voneinander in einer steigenden Anzahl von OH-Gruppen am B-Ring unterscheiden eingesetzt. Zusätzlich wurde das Flavanon Naringenin, welchem die bestimmende „Flavonolgruppe“ (Hydroxylierung und Doppelbindung am heterocyclischen C-Ring) fehlt, gewählt. Die spezifischen strukturellen Eigenschaften der „Flavonolgruppe“ werden auch oft mit ihrer hohen antioxidativen Kapazität in Verbindung gebracht. Mit Hilfe von HPLC/DAD-Analytik wurde die Bioverfügbarkeit als notwendige Voraussetzung für biologische Wirkung demonstriert und eine umfangreiche Metabolisierung in *C. elegans* nachgewiesen. Zusätzlich wurde eine für *C. elegans* neuartige *in vivo* Methode etabliert die eine Visualisierung der Anreicherung der Flavonoide im Wurm ermöglicht. In *C. elegans* scheinen vor allem die Darmepithelzellen die Substanzen zu akkumulieren.

Eine erhöhte Bildung von reaktiven Sauerstoff Spezies (ROS) werden mit einer Vielzahl von Erkrankungen und mit Alterungsprozessen in Verbindung gebracht. Die hohe antioxidative Kapazität der Flavonoide wird daher als protektives Prinzip diesen Substanzen zugeschriebenen. Diese wird jedoch oft nur mittels *in vitro* Methoden bestimmt, was zwar die Analyse von Struktur-Wirkungsbeziehungen-Beziehung erlaubt, jedoch nur bedingte Aussagen über deren Effekte im biologischen System bzw. in Organismen zulässt. Daher wurde hier die antioxidative Wirkung der Flavonoide in isolierten Mitochondrien des Skelettmuskels der Maus sowie in *C. elegans* und damit *in vivo* bestimmt. Dabei zeigte sich, dass die „Flavonol-bestimmende Gruppe“ hauptsächlich verantwortlich ist für eine Absenkung der ROS-Spiegel in den isolierten Mitochondrien. In *C. elegans* wurde dieser Effekt zusätzlich durch die Catecholfunktion am B-Ring (3', 4'-OH) verstärkt. Hier waren die Schutzeffekte auch an einer reduzierten Menge an Protein-Carbonyl als Surrogatmarker für oxidativ geschädigtes Protein zu sehen. Alle Flavonoide außer Naringenin erhöhten die mittlere und maximale Lebensdauer sowie die

Hitzeresistenz in *C. elegans*. Dies weißt darauf hin, dass die strukturellen Voraussetzungen, welche die antioxidative Aktivität tragen, auch für diese Eigenschaften verantwortlich zeichnen. Es scheint aber letztlich kein kausaler Zusammenhang zwischen den beiden Effekten zu bestehen, da lediglich Myricetin die Lebensdauer in der *mev-1(kn1)* Mutante mit intrinsisch hohem ROS-Spiegel verlängerte, aber alle Flavonole die mitochondrialen ROS Mengen reduzierten. Somit ist die antioxidative Wirkung der Flavonole allein nicht ausreichend um die protektiven Wirkungen zu erklären. Effekte der Substanzen auf die Nahrungsaufnahme sowie damit möglicherweise verbundene Kalorienreduktion sowie Wirkungen auf die mitochondriale Respirationsrate konnten als kausale Ursachen ausgeschlossen werden.

Die Insulin/IGF-like-signaling (ILS)-Kaskade ist ein nachgewiesener zentraler Regulator der Alterung und Stress-Resistenz in *C. elegans, Drosophila melanogaster* und Säugern mit dem FOXO-Transkriptionsfaktor DAF-16 als zentralem nukleären Target. Im nicht-phosphorylierten Zustand kann DAF-16 in den Zellkern translozieren und die Expression einer Vielzahl von protektiven Enzymen wie Superoxiddismutasen oder Hitzeschock-Proteinen induzieren. Alle Flavonoide erhöhten die DAF-16-Translokation in den Zellkern und führen zu erhöhter Promotoraktivität eines DAF-16 Zielgenes, der *sod-3*, jedoch nicht von *hsp-16.2*. Dies ließ vermuten, dass die Flavonoidwirkungen – zumindest partiell – durch Einfluss auf die IlS-Kaskade und eine erhöhte Translokation von DAF-16 in den Kern vermittelt werden. In einer DAF-16 Mutante von *C. elegans* zeigte sich jedoch, dass die Lebensdauer-Verlängerung und die antioxidative Wirkung der Flavonoide konserviert waren und ihre protektiven Effekte damit DAF-16-unabhängig sind. Weitere Studien sind daher nötig um die zugrunde liegenden Mechanismen der Wirkung dieser Flavonoide im Organismus besser zu verstehen.

Zusammenfassend lässt sich feststellen, dass die ausgewählten Flavonoide in einer strukturspezifischen Weise die Alterung und die Stress-Resistenz im Modellorganismus *C. elegans* reduzieren können und eine moderate Lebenszeitverlängerung ermöglichen. Die Effekte der nutritiven Verbindungen auf die Lebensspanne sind im Gegensatz zu genetischen Modellen in *C. elegans* nur moderat aber ähnlich groß, wie die von Resveratrol, das als Muttersubstanz von „caloric restriction mimetics“ gilt.

8. Appendix

Table 10. Adult lifespan of wild-type N2 *C. elegans* on plates containing 100 µM of indicated flavonoids

Treatment	Adult lifespan [days ± SE]			*P* vs. Control
	Mean	Maximum	n (censored)	Log-rank
Control	12.78 ± 0.44	20.00 ± 0.37	181 (9)	
Myricetin	15.08 ± 0.25	24.33 ± 0.92	169 (16)	<0.0001
Quercetin	13.52 ± 0.52	23.67 ± 1.38	171 (16)	0.0294
Kaempferol	13.50 ± 0.71	21.33 ± 1.15	173 (11)	0.0370
Naringenin	12.61 ± 0.34	20.67 ± 0.84	187 (4)	0.7141

Table 11. Adult lifespan of *mev-1(kn1) C. elegans* on plates containing 100 µM of indicated flavonoids

Treatment	Adult lifespan [days ± SE]			*P* vs. Control
	Mean	Maximum	n (censored)	Log-rank
Control	5.25 ± 0.34	7.44 ± 0.29	256 (23)	
Myricetin	6.08 ± 0.18	9.67 ± 0.44	261 (14)	0.0096
Quercetin	5.44 ± 0.28	9.33 ± 0.50	269 (8)	0.8365
Kaempferol	5.38 ± 0.14	9.11 ± 0.39	267 (11)	0.8804
Naringenin	5.57 ± 0.21	9.22 ± 0.28	278 (5)	0.6067

Table 12. Adult lifespan of *isp-1(qm150);ctb-1(qm189) C. elegans* on plates containing 100 µM of indicated flavonoids

Treatment	Adult lifespan [days ± SE]				*P* vs. Control
	Mean	±SE	Maximum	n (censored)	Log-rank
Control	11.51	0.49	18.00 ± 1.00	93 (16)	
Myricetin	13.15	0.64	24.67 ± 1.33	93 (10)	0.0104
Quercetin	12.56	0.67	22.33 ± 0.33	91 (8)	0.0564
Kaempferol	13.22	0.06	21.00 ± 1.00	97 (6)	0.1626
Naringenin	11.64	0.56	23.33 ± 1.33	92 (6)	0.9313

Table 13. Adult lifespan of *daf-16(mu86) C. elegans* on plates containing 100 µM of indicated flavonoids

Treatment	Adult lifespan [days ± SE]			*P* vs. Control
	Mean	Maximum	n (censored)	Log-rank
Control	12.09 ± 0.15	17.08 ± 0.27	198 (6)	
Myricetin	12.79 ± 0.22	19.33 ± 0.48	200 (16)	<0.0001
Quercetin	12.57 ± 0.30	18.33 ± 0.42	193 (9)	0.0336
Kaempferol	12.74 ± 0.19	17.92 ± 0.27	205 (4)	0.0780
Naringenin	12.53 ± 0.28	17.17 ± 0.17	194 (3)	0.5128

Table 14. Resistance of wild-type *C. elegans* against 1.6 mM paraquat on plates containing 100 µM of indicated flavonoids

	Survival at 1.6 mM paraquat [days]			*P* vs. Control
	Mean	± SE	n (censored)	Log-rank
Control	7.38	0.2	182 (10)	
Myricetin	6.13	0.18	186 (4)	<0.0001
Quercetin	7.39	0.33	191 (7)	0.3800
Kaempferol	7.43	0.27	186 (6)	0.1500
Naringenin	7.75	0.36	192 (5)	0.4800

Table 15. Resistance of *mev-1(kn1) C. elegans* against 1.6 mM paraquat on plates containing 100 µM of indicated flavonoids

	Survival time at 1.6 mM Paraquat			*P* vs. Control
	Mean	± SE	n (censored)	Log-rank
Control	2,67	0,23	193 (4)	
Myricetin	2,31	0,16	190 (1)	0,0001
Quercetin	2,36	0,19	190 (2)	0,0052
Kaempferol	2,68	0,12	190 (2)	0,7861
Naringenin	2,92	0,28	189 (1)	0,2470

Table 16. Resistance of *isp-1(qm150);ctb-1(qm189) C. elegans* against 1.6 mM paraquat on plates containing 100 µM of indicated flavonoids

	Survival time at 1.6 mM Paraquat			*P* vs. Control
	Mean	± SE	n (censored)	Log-rank
Control	7.00	0.40	97 (8)	
Myricetin	6.47	0.24	99 (7)	0.0582
Quercetin	6.82	0.29	96 (7)	0.4349
Kaempferol	6.44	0.27	94 (7)	0.0332
Naringenin	6.83	0.32	92 (8)	0.9809

Table 17. Resistance of wild-type *C. elegans* against 34 °C heat-stress on plates containing 100 µM of indicated flavonoids

	Survival time at 34 °C		*P* vs. Control
	Median	n (censored)	Log-rank
Control	9	185 (54)	
Myricetin	not def	157 (109)	<0.0001
Quercetin	not def.	116 (130)	<0.0001
Kaempferol	10	169 (73)	0.0262
Naringenin	8	195 (50)	0.0161

List of figures

Fig. 1 The mitochondrial electron transport chain.....8
Fig. 2 Life cycle of wild-type C. elegans.....13
Fig. 3 Simplified scheme of the insulin-like signaling cascade.....16
Fig. 4 Structures of selected flavonoids.....21
Fig. 5. Emission wavelengths of flavonoids with or without NSRA.....32
Fig. 6 Chemical structure of paraquat.....35
Fig. 7 H2O2 standard curve.....36
Fig. 8 Fluorescence of CM-H2XRos in isp-1(qm150);ctb-1(qm189) (MQ989), wild-type (N2) and mev-1(kn1) (TK22) C. elegans.....37
Fig. 9 Carbonylation and derivatization of an arginyl residue.....39
Fig. 10 Oxygen consumption of different sample size and killed C. elegans.....41
Fig. 11 DAF-16::GFP distribution.....42
Fig. 12 Induction of HSP::GFP fluorescence after heat shock.....43
Fig. 13 Enhanced auto-fluorescence of flavonoids in wild-type C. elegans.....45
Fig. 14 Closeup view of NSRA-enhanced auto-fluorescence of flavonols in C. elegans....46
Fig. 15 Effects of flavonoids on adult lifespan in wild-type C. elegans.....48
Fig. 16 Effect of 10 µM myricetin on adult lifespan in wild-type C. elegans.....49
Fig. 17 Effects of flavonoids on adult lifespan in mev-1(kn1) C. elegans.....50
Fig. 18 Effects of flavonoids on adult lifespan in isp-1(qm150);ctb-1(qm189) C. elegans. 51
Fig. 19 H2O2-generation in isolated mouse mitochondria under different conditions.....53
Fig. 20 Impact of flavonoids on H2O2-generation in isolated mitochondria.....54
Fig. 21 Impact of flavonoids plus rotenone on mitochondrial H2O2-generation.....54
Fig. 22 Impact of flavonoids on mtROS in wild-type C. elegans.....55
Fig. 23 Time-dependent impact of flavonoid exposure on mtROS in wild-type C. elegans56
Fig. 24 Impact of flavonoids on mtROS in mev-1(kn1) and isp-1(qm150);ctb-1(qm189) C. elegans.....57
Fig. 25 Carbonylated proteins in wild-type, mev-1(kn1) and daf-16(mu86) C. elegans at different ages.....58
Fig. 26 Carbonylated proteins of flavonoid-treated wild-type C. elegans.....59
Fig. 27 Paraquat-induced H2O2-generation in isolated mouse mitochondria.....61
Fig. 28 Effects of flavonoids on paraquat-resistance in wild-type C. elegans.....62
Fig. 29 Effects of flavonoids on paraquat-resistance in mev-1(kn1) C. elegans.....63
Fig. 30 Effects of flavonoids on paraquat-resistance in isp-1(qm150);ctb-1(qm189) C. elegans.....63
Fig. 31 Survival of C. elegans under acute paraquat-stress.....64
Fig. 32 Effects of flavonoids on heat-resistance in wild-type C. elegans.....65
Fig. 33 Pharynx pumping rate after flavonoid-exposure.....66
Fig. 34 Pharynx pumping rate after flavonoid-exposure in mev-1(kn1) C. elegans.....67
Fig. 35 Oxygen consumption in C. elegans depending on sample size.....68
Fig. 36 Relative oxygen consumption rate in C. elegans after treatment with flavonoids...69
Fig. 37 Effects of flavonoids on DAF-16 transcription factor localization.....70
Fig. 38 Effects of flavonoids on sod-3 promoter activity.....72
Fig. 39 Effects of flavonoids on juglone-induced hsp-16.2 promoter activity.....73
Fig. 40 Effects of flavonoids on heat-induced hsp-16.2 promoter activity.....74
Fig. 41 Effects of flavonoids on heat-induced hsp-70 promoter activity.....75
Fig. 42 Impact of flavonoids on mtROS in daf-16(mu86) C. elegans.....76
Fig. 43 Effects of flavonoids on adult lifespan in daf-16(mu86) C. elegans.....77

List of tables

Table 1. Equipment and Instruments....25
Table 2. Chemical and Supplier information....25
Table 3. Buffers and media for mouse mitochondria isolation....27
Table 4. Buffers and media for C. elegans and OP50 culture....27
Table 5. Solutions for oxyblot analysis....28
Table 6. C. elegans strains used....29
Table 7. HPLC/DAD Analysis....33
Table 8. Concentration of conjugated and free flavonoids in C. elegans....47
Table 9.Lifespan extension of selected plant compounds administered to C. elegans....84
Table 10. Adult lifespan of wild-type N2 C. elegans on plates containing 100 µM of indicated flavonoids....96
Table 11. Adult lifespan of mev-1(kn1) C. elegans on plates containing 100 µM of indicated flavonoids....96
Table 12. Adult lifespan of isp-1(qm150);ctb-1(qm189) C. elegans on plates containing 100 µM of indicated flavonoids....97
Table 13. Adult lifespan of daf-16(mu86) C. elegans on plates containing 100 µM of indicated flavonoids....97
Table 14. Resistance of wild-type C. elegans against 1.6 mM paraquat on plates containing 100 µM of indicated flavonoids....97
Table 15. Resistance of mev-1(kn1) C. elegans against 1.6 mM paraquat on plates containing 100 µM of indicated flavonoids....98
Table 16. Resistance of isp-1(qm150);ctb-1(qm189) C. elegans against 1.6 mM paraquat on plates containing 100 µM of indicated flavonoids....98
Table 17. Resistance of wild-type C. elegans against 34 °C heat-stress on plates containing 100 µM of indicated flavonoids....98

List of abbreviations

ADP	Adenosine-5'-diphosphate
age	aging alteration
APS	ammoniumpersulfate
ATP	Adenosine-5'-triphosphate
BSA	bovine serum albumine
clk-1	clock – abnormal timing
CP	Chappell-Perry buffer
CR	caloric restriction
ctb	cytochrome b
daf	abnormal dauer formation
dH_2O	distilled water
DMSO	dimethyl sulfoxide
DNA	desoxyribunucleic acid
DNPH	2,4-dinitrophenol hydrazine
ds	double-stranded
eat	abnormal pharynx pump rate
EGCG	epigallocatechin gallate
EGTA	ethylene glycol tetraacetic acid
ETC	electron transport chain
g	standard gravity
g	gram
GFP	green fluorescent protein
HPLC/DAD	High-performance liquid chromatography with diode array detection
HRP	horseradish peroxidase
HSF	heat-shock factor
HSP	heat-shock protein
IGF	insulin-like growth factor
IlS	insulin/IGF-like signaling
isp	iron-sulfur protein
JNK	c-Jun N-terminal kinases
L	liter
L1 – L4	*C. elegans* larval stages (1 - 4)
M	mol/L (molar)
MAP	mitogen-activated protein
mev	altered methyl-viologen sensitivity
mg	milligram
min	minute
mL	milliliter
mm	millimeter
mM	millimolar
mRNA	messenger RNA
mt	mitochondrial
N2	wild-type *C. elegans* var Bristol
NADH	nicotinamide adenine dinucleotide
nmol	nanomol
NSRA	Naturstoff reagent A (2-aminoethyl diphenyl borate)

PBS-T	phosphate buffered saline with TWEEN
PCC	protein-carbonyl-content
PIP_2	3,4-di-phosphate
PIP_3	3,4,5-tri-phosphate
pmol	picomol
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
ROS	reactive oxygen species
rpm	revolutions per minute
s	second
SD	standard deviation
SDS	sodium Dodecyl sulfate
SEM	standard error of the means
skn-1	skinhead
smk-1	suppressor of MEK null
SOD	superoxide dismutase
TOR	Target of Rapamycin
TRIS	2-Amino-2-hydroxymethyl-propane-1,3-diol
U	enzyme unit
unc	uncoordinated
UQ	ubiquinone
UQH_2	ubiquinole
UV-Vis	ultraviolet visible spectroscopy
V	volts
w/o	without
µg	microgram
µL	microliter
µM	micromolar

References

1 "aging." Encyclopædia Britannica. Encyclopædia Britannica Online. (20110324) *Encyclopædia Britannica*.
2 Kirkwood TB (1977) Evolution of ageing. *Nature* **270**, 301-304.
3 Van Raamsdonk JM & Hekimi S (2010) Reactive Oxygen Species and Aging in Caenorhabditis elegans: Causal or Casual Relationship? *Antioxid. Redox Signal* **13**, 1911-1953.
4 Pearl R (1928) *The rate of living: being an account of some experimental studies on the biology of life duration* A.A. Knopf.
5 HARMAN D (1956) Aging: a theory based on free radical and radiation chemistry. *J Gerontol* **11**, 298–300.
6 Harman D (1972) The biologic clock: the mitochondria? *J Am Geriatr Soc* **20**, 145-147.
7 Kirkwood TBL (2005) Understanding the Odd Science of Aging. *Cell* **120**, 437-447.
8 Campisi J, Kim SH, Lim CS & Rubio M (2001) Cellular senescence, cancer and aging: the telomere connection. *Exp. Gerontol* **36**, 1619-1637.
9 von Zglinicki T (2002) Oxidative stress shortens telomeres. *Trends Biochem. Sci* **27**, 339-344.
10 Lapointe J & Hekimi S (2010) When a theory of aging ages badly. *Cell. Mol. Life Sci* **67**, 1-8.
11 Raamsdonk JMV & Hekimi S (2009) Deletion of the mitochondrial superoxide dismutase sod-2 extends lifespan in Caenorhabditis elegans. *PLoS Genet* **5**, e1000361.
12 McCay CM, Crowell MF & Maynard LA (1935) The effect of retarded growth upon the length of life span and upon the ultimate body size. *JOURNAL OF NUTRITION* **10**, 63-79.
13 Mair W & Dillin A (2008) Aging and survival: the genetics of life span extension by dietary restriction. *Annu. Rev. Biochem* **77**, 727-754.
14 Lin S-J, Kaeberlein M, Andalis AA, Sturtz LA, Defossez P-A, Culotta VC, Fink GR & Guarente L (2002) Calorie restriction extends Saccharomyces cerevisiae lifespan by increasing respiration. *Nature* **418**, 344-348.
15 Houthoofd K, Johnson TE & Vanfleteren JR (2005) Dietary restriction in the nematode Caenorhabditis elegans. *J Gerontol A Biol Sci Med Sci* **60**, 1125-31.
16 Miwa S, Beckman KB & Muller FL (eds.) (2008) *Oxidative Stress in Aging* Humana Press, Totowa, NJ.
17 Wilson DF, Erecińska M & Dutton PL (1974) Thermodynamic relationships in mitochondrial oxidative phosphorylation. *Annu. Rev. Biophys. Bioeng* **3**, 203-230.
18 Turrens JF (1997) Superoxide production by the mitochondrial respiratory chain. *Biosci. Rep* **17**, 3-8.
19 Senoo-Matsuda N, Yasuda K, Tsuda M, Ohkubo T, Yoshimura S, Nakazawa H, Hartman PS & Ishii N (2001) A Defect in the Cytochrome b Large Subunit in Complex II Causes Both Superoxide Anion Overproduction and Abnormal Energy Metabolism in Caenorhabditis elegans. *J. Biol. Chem.* **276**, 41553 -41558.
20 Gus'kova RA, Ivanov II, Kol'tover VK, Akhobadze VV & Rubin AB (1984) Permeability of bilayer lipid membranes for superoxide (O2-.) radicals. *Biochim. Biophys. Acta* **778**, 579-585.
21 Yoshida H & Kisugi R (2010) Mechanisms of LDL oxidation. *Clin. Chim. Acta* **411**, 1875-1882.
22 Ragu S, Faye G, Iraqui I, Masurel-Heneman A, Kolodner RD & Huang M-E (2007)

Oxygen metabolism and reactive oxygen species cause chromosomal rearrangements and cell death. *Proc. Natl. Acad. Sci. U.S.A* **104**, 9747-9752.

23 McCord JM & Fridovich I (1969) Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem* **244**, 6049-6055.

24 Li Y, Huang TT, Carlson EJ, Melov S, Ursell PC, Olson JL, Noble LJ, Yoshimura MP, Berger C, Chan PH, Wallace DC & Epstein CJ (1995) Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat. Genet* **11**, 376-381.

25 Reaume AG, Elliott JL, Hoffman EK, Kowall NW, Ferrante RJ, Siwek DF, Wilcox HM, Flood DG, Beal MF, Brown RH Jr, Scott RW & Snider WD (1996) Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. *Nat. Genet* **13**, 43-47.

26 Roos D & Winterbourn CC (2002) Immunology. Lethal weapons. *Science* **296**, 669-671.

27 Lowenstein CJ & Snyder SH (1992) Nitric oxide, a novel biologic messenger. *Cell* **70**, 705-707.

28 Rhee SG (2006) Cell signaling. H2O2, a necessary evil for cell signaling. *Science* **312**, 1882-1883.

29 Leslie NR, Bennett D, Lindsay YE, Stewart H, Gray A & Downes CP (2003) Redox regulation of PI 3-kinase signalling via inactivation of PTEN. *EMBO J.* **22**, 5501-5510.

30 Chandel NS, McClintock DS, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM & Schumacker PT (2000) Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O2 sensing. *J. Biol. Chem* **275**, 25130-25138.

31 Hamanaka RB & Chandel NS (2010) Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. *Trends Biochem. Sci* **35**, 505-513.

32 Riddle DL (1997) *C. elegans II* CSHL Press.

33 Brenner S (1973) The genetics of behaviour. *Br. Med. Bull* **29**, 269-271.

34 Brenner S (1974) The genetics of Caenorhabditis elegans. *Genetics* **77**, 71–94.

35 Brenner S (2003) Nobel lecture. Nature’s gift to science. *Biosci. Rep* **23**, 225-237.

36 Sulston JE & Horvitz HR (1977) Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. *Developmental Biology* **56**, 110-156.

37 Sulston JE, Schierenberg E, White JG & Thomson JN (1983) The embryonic cell lineage of the nematode Caenorhabditis elegans. *Dev. Biol* **100**, 64-119.

38 Klass M & Hirsh D (1976) Non-ageing developmental variant of Caenorhabditis elegans. *Nature* **260**, 523-525.

39 The C. elegans Sequencing Consortium (1998) Genome Sequence of the Nematode C. elegans: A Platform for Investigating Biology. *Science* **282**, 2012 -2018.

40 Fire A, Albertson D, Harrison SW & Moerman DG (1991) Production of antisense RNA leads to effective and specific inhibition of gene expression in C. elegans muscle. *Development* **113**, 503-514.

41 Tabara H, Grishok A & Mello CC (1998) RNAi in C. elegans: soaking in the genome sequence. *Science* **282**, 430-431.

42 Timmons L & Fire A (1998) Specific interference by ingested dsRNA. *Nature* **395**, 854.

43 Mello CC, Kramer JM, Stinchcomb D & Ambros V (1991) Efficient gene transfer in C.elegans: extrachromosomal maintenance and integration of transforming sequences. *EMBO J* **10**, 3959-3970.

44 SHIMOMURA O, JOHNSON FH & SAIGA Y (1962) Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. *J Cell Comp Physiol* **59**, 223-239.
45 Chalfie M, Tu Y, Euskirchen G, Ward WW & Prasher DC (1994) Green fluorescent protein as a marker for gene expression. *Science* **263**, 802-805.
46 Chalfie M (2009) GFP: lighting up life (Nobel Lecture). *Angew. Chem. Int. Ed. Engl* **48**, 5603-5611.
47 Klass MR (1977) Aging in the nematode Caenorhabditis elegans: major biological and environmental factors influencing life span. *Mech. Ageing Dev* **6**, 413-429.
48 Friedman DB & Johnson TE (1988) A mutation in the age-1 gene in Caenorhabditis elegans lengthens life and reduces hermaphrodite fertility. *Genetics* **118**, 75-86.
49 Johnson TE, Tedesco PM & Lithgow GJ (1993) Comparing mutants, selective breeding, and transgenics in the dissection of aging processes of Caenorhabditis elegans. *Genetica* **91**, 65-77.
50 Riddle DL, Swanson MM & Albert PS (1981) Interacting genes in nematode dauer larva formation. *Nature* **290**, 668-671.
51 Kenyon C, Chang J, Gensch E, Rudner A & Tabtiang R (1993) A C. elegans mutant that lives twice as long as wild type. *Nature* **366**, 461-4.
52 Vowels JJ & Thomas JH (1992) Genetic analysis of chemosensory control of dauer formation in Caenorhabditis elegans. *Genetics* **130**, 105-123.
53 Dorman JB, Albinder B, Shroyer T & Kenyon C (1995) The age-1 and daf-2 genes function in a common pathway to control the lifespan of Caenorhabditis elegans. *Genetics* **141**, 1399-1406.
54 Kimura KD, Tissenbaum HA, Liu Y & Ruvkun G (1997) daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. *Science* **277**, 942-946.
55 Morris JZ, Tissenbaum HA & Ruvkun G (1996) A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in Caenorhabditis elegans. *Nature* **382**, 536-539.
56 Lin K, Dorman JB, Rodan A & Kenyon C (1997) daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. *Science* **278**, 1319–1322.
57 Ogg S, Paradis S, Gottlieb S, Patterson GI, Lee L, Tissenbaum HA & Ruvkun G (1997) The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. *Nature* **389**, 994-9.
58 Wolkow CA, Muñoz MJ, Riddle DL & Ruvkun G (2002) Insulin receptor substrate and p55 orthologous adaptor proteins function in the Caenorhabditis elegans daf-2/insulin-like signaling pathway. *J. Biol. Chem* **277**, 49591-49597.
59 Ogg S & Ruvkun G (1998) The C. elegans PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway. *Mol. Cell* **2**, 887-893.
60 Paradis S, Ailion M, Toker A, Thomas JH & Ruvkun G (1999) A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in Caenorhabditis elegans. *Genes Dev* **13**, 1438-1452.
61 Hertweck M, Göbel C & Baumeister R (2004) C. elegans SGK-1 is the critical component in the Akt/PKB kinase complex to control stress response and life span. *Dev. Cell* **6**, 577-588.
62 Padmanabhan S, Mukhopadhyay A, Narasimhan SD, Tesz G, Czech MP & Tissenbaum HA (2009) A PP2A regulatory subunit regulates C. elegans insulin/IGF-1 signaling by modulating AKT-1 phosphorylation. *Cell* **136**, 939-951.

63 Paradis S & Ruvkun G (1998) Caenorhabditis elegans Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor. *Genes & Development* **12**, 2488 -2498.
64 Honda Y & Honda S (1999) The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans. *FASEB J* **13**, 1385–1393.
65 Hsu A-L, Murphy CT & Kenyon C (2003) Regulation of aging and age-related disease by DAF-16 and heat-shock factor. *Science* **300**, 1142–1145.
66 Barsyte D, Lovejoy DA & Lithgow GJ (2001) Longevity and heavy metal resistance in daf-2 and age-1 long-lived mutants of Caenorhabditis elegans. *FASEB J* **15**, 627-634.
67 Murphy CT, McCarroll SA, Bargmann CI, Fraser A, Kamath RS, Ahringer J, Li H & Kenyon C (2003) Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. *Nature* **424**, 277–283.
68 Oh SW, Mukhopadhyay A, Dixit BL, Raha T, Green MR & Tissenbaum HA (2006) Identification of direct DAF-16 targets controlling longevity, metabolism and diapause by chromatin immunoprecipitation. *Nat. Genet* **38**, 251-257.
69 Garsin DA, Villanueva JM, Begun J, Kim DH, Sifri CD, Calderwood SB, Ruvkun G & Ausubel FM (2003) Long-lived C. elegans daf-2 mutants are resistant to bacterial pathogens. *Science* **300**, 1921.
70 Murakami S & Johnson TE (1996) A genetic pathway conferring life extension and resistance to UV stress in Caenorhabditis elegans. *Genetics* **143**, 1207-1218.
71 Clancy DJ, Gems D, Harshman LG, Oldham S, Stocker H, Hafen E, Leevers SJ & Partridge L (2001) Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein. *Science* **292**, 104-106.
72 Blüher M, Kahn BB & Kahn CR (2003) Extended longevity in mice lacking the insulin receptor in adipose tissue. *Science* **299**, 572-574.
73 Dillin A, Crawford DK & Kenyon C (2002) Timing requirements for insulin/IGF-1 signaling in C. elegans. *Science* **298**, 830-4.
74 Baugh LR & Sternberg PW (2006) DAF-16/FOXO regulates transcription of cki-1/Cip/Kip and repression of lin-4 during C. elegans L1 arrest. *Curr. Biol* **16**, 780-785.
75 Wolkow CA, Kimura KD, Lee M-S & Ruvkun G (2000) Regulation of C. elegans Life-Span by Insulinlike Signaling in the Nervous System. *Science* **290**, 147 -150.
76 Libina N, Berman JR & Kenyon C (2003) Tissue-specific activities of C. elegans DAF-16 in the regulation of lifespan. *Cell* **115**, 489-502.
77 Lin K, Hsin H, Libina N & Kenyon C (2001) Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling. *Nat Genet* **28**, 139-145.
78 Landis JN & Murphy CT (2010) Integration of diverse inputs in the regulation of Caenorhabditis elegans DAF-16/FOXO. *Dev Dyn*.
79 Oh SW, Mukhopadhyay A, Svrzikapa N, Jiang F, Davis RJ & Tissenbaum HA (2005) JNK regulates lifespan in Caenorhabditis elegans by modulating nuclear translocation of forkhead transcription factor/DAF-16. *Proc Natl Acad Sci U S A* **102**, 4494-4499.
80 Baler R, Dahl G & Voellmy R (1993) Activation of human heat shock genes is accompanied by oligomerization, modification, and rapid translocation of heat shock transcription factor HSF1. *Mol. Cell. Biol* **13**, 2486-2496.
81 Tissenbaum HA & Guarente L (2001) Increased dosage of a sir-2 gene extends lifespan

in Caenorhabditis elegans. *Nature* **410**, 227-230.
82 Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, Tran H, Ross SE, Mostoslavsky R, Cohen HY, Hu LS, Cheng H-L, Jedrychowski MP, Gygi SP, Sinclair DA, Alt FW & Greenberg ME (2004) Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* **303**, 2011-2015.
83 Rogina B & Helfand SL (2004) Sir2 mediates longevity in the fly through a pathway related to calorie restriction. *Proc. Natl. Acad. Sci. U.S.A* **101**, 15998-16003.
84 Li J, Tewari M, Vidal M & Lee SS (2007) The 14-3-3 protein FTT-2 regulates DAF-16 in Caenorhabditis elegans. *Dev. Biol* **301**, 82-91.
85 van Hemert MJ, Steensma HY & van Heusden GP (2001) 14-3-3 proteins: key regulators of cell division, signalling and apoptosis. *Bioessays* **23**, 936-946.
86 An JH & Blackwell TK (2003) SKN-1 links C. elegans mesendodermal specification to a conserved oxidative stress response. *Genes Dev* **17**, 1882-93.
87 Tullet JMA, Hertweck M, An JH, Baker J, Hwang JY, Liu S, Oliveira RP, Baumeister R & Blackwell TK (2008) Direct inhibition of the longevity-promoting factor SKN-1 by insulin-like signaling in C. elegans. *Cell* **132**, 1025–1038.
88 Avery L (1993) The genetics of feeding in Caenorhabditis elegans. *Genetics* **133**, 897-917.
89 Lakowski B & Hekimi S (1998) The genetics of caloric restriction in Caenorhabditis elegans. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 13091 -13096.
90 Klass MR (1983) A method for the isolation of longevity mutants in the nematode Caenorhabditis elegans and initial results. *Mech. Ageing Dev* **22**, 279-286.
91 Houthoofd K, Braeckman BP, Johnson TE & Vanfleteren JR (2003) Life extension via dietary restriction is independent of the Ins/IGF-1 signalling pathway in Caenorhabditis elegans. *Exp. Gerontol* **38**, 947-954.
92 Kahn BB, Alquier T, Carling D & Hardie DG (2005) AMP-activated protein kinase: Ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metabolism* **1**, 15-25.
93 Greer EL, Dowlatshahi D, Banko MR, Villen J, Hoang K, Blanchard D, Gygi SP & Brunet A (2007) An AMPK-FOXO pathway mediates longevity induced by a novel method of dietary restriction in C. elegans. *Curr. Biol* **17**, 1646-1656.
94 Wullschleger S, Loewith R & Hall MN (2006) TOR signaling in growth and metabolism. *Cell* **124**, 471-484.
95 Jia K, Chen D & Riddle DL (2004) The TOR pathway interacts with the insulin signaling pathway to regulate C. elegans larval development, metabolism and life span. *Development* **131**, 3897-3906.
96 Henderson ST, Bonafe M & Johnson TE (2006) daf-16 protects the nematode Caenorhabditis elegans during food deprivation. *J Gerontol A Biol Sci Med Sci* **61**, 444-60.
97 Hansen M, Taubert S, Crawford D, Libina N, Lee S-J & Kenyon C (2007) Lifespan extension by conditions that inhibit translation in Caenorhabditis elegans. *Aging Cell* **6**, 95-110.
98 Fierro-González JC, González-Barrios M, Miranda-Vizuete A & Swoboda P (2011) The thioredoxin TRX-1 regulates adult lifespan extension induced by dietary restriction in Caenorhabditis elegans. *Biochemical and Biophysical Research Communications* **406**, 478-482.
99 Panowski SH, Wolff S, Aguilaniu H, Durieux J & Dillin A (2007) PHA-4/Foxa mediates diet-restriction-induced longevity of C. elegans. *Nature* **447**, 550-555.

100 Bishop NA & Guarente L (2007) Two neurons mediate diet-restriction-induced longevity in C. elegans. *Nature* **447**, 545-549.
101 Wang Y & Tissenbaum HA (2006) Overlapping and distinct functions for a Caenorhabditis elegans SIR2 and DAF-16/FOXO. *Mech Ageing Dev* **127**, 48-56.
102 Ishii N, Takahashi K, Tomita S, Keino T, Honda S, Yoshino K & Suzuki K (1990) A methyl viologen-sensitive mutant of the nematode Caenorhabditis elegans. *Mutat. Res* **237**, 165-171.
103 Ishii N, Fujii M, Hartman PS, Tsuda M, Yasuda K, Senoo-Matsuda N, Yanase S, Ayusawa D & Suzuki K (1998) A mutation in succinate dehydrogenase cytochrome b causes oxidative stress and ageing in nematodes. *Nature* **394**, 694–697.
104 Hartman PS, Ishii N, Kayser EB, Morgan PG & Sedensky MM (2001) Mitochondrial mutations differentially affect aging, mutability and anesthetic sensitivity in Caenorhabditis elegans. *Mech. Ageing Dev* **122**, 1187-1201.
105 Grad LI & Lemire BD (2004) Mitochondrial complex I mutations in Caenorhabditis elegans produce cytochrome c oxidase deficiency, oxidative stress and vitamin-responsive lactic acidosis. *Human Molecular Genetics* **13**, 303 -314.
106 Huang J & Lemire BD (2009) Mutations in the C. elegans Succinate Dehydrogenase Iron-Sulfur Subunit Promote Superoxide Generation and Premature Aging. *Journal of Molecular Biology* **387**, 559-569.
107 Wong A, Boutis P & Hekimi S (1995) Mutations in the clk-1 Gene of Caenorhabditis elegans Affect Developmental and Behavioral Timing. *Genetics* **139**, 1247–1259.
108 Braeckman BP, Houthoofd K, De Vreese A & Vanfleteren JR (1999) Apparent uncoupling of energy production and consumption in long-lived Clk mutants of Caenorhabditis elegans. *Curr. Biol* **9**, 493-496.
109 Feng J, Bussiere F & Hekimi S (2001) Mitochondrial electron transport is a key determinant of life span in Caenorhabditis elegans. *Dev Cell* **1**, 633–644.
110 Larsen PL (1993) Aging and resistance to oxidative damage in Caenorhabditis elegans. *Proceedings of the National Academy of Sciences* **90**, 8905 -8909.
111 Jensen LT & Culotta VC (2005) Activation of CuZn superoxide dismutases from Caenorhabditis elegans does not require the copper chaperone CCS. *J. Biol. Chem* **280**, 41373-41379.
112 Hunter T, Bannister WH & Hunter GJ (1997) Cloning, expression, and characterization of two manganese superoxide dismutases from Caenorhabditis elegans. *J. Biol. Chem* **272**, 28652-28659.
113 Fujii M, Ishii N, Joguchi A, Yasuda K & Ayusawa D (1998) A novel superoxide dismutase gene encoding membrane-bound and extracellular isoforms by alternative splicing in Caenorhabditis elegans. *DNA Res* **5**, 25-30.
114 Doonan R, McElwee JJ, Matthijssens F, Walker GA, Houthoofd K, Back P, Matscheski A, Vanfleteren JR & Gems D (2008) Against the oxidative damage theory of aging: superoxide dismutases protect against oxidative stress but have little or no effect on life span in Caenorhabditis elegans. *Genes & Development* **22**, 3236 -3241.
115 Lee SS, Kennedy S, Tolonen AC & Ruvkun G (2003) DAF-16 target genes that control C. elegans life-span and metabolism. *Science* **300**, 644–647.
116 Togo SH, Maebuchi M, Yokota S, Bun-Ya M, Kawahara A & Kamiryo T (2000) Immunological detection of alkaline-diaminobenzidine-negativeperoxisomes of the nematode Caenorhabditis elegans purification and unique pH optima of peroxisomal catalase. *Eur. J. Biochem* **267**, 1307-1312.
117 Yanase S, Yasuda K & Ishii N (2002) Adaptive responses to oxidative damage in three mutants of Caenorhabditis elegans (age-1, mev-1 and daf-16) that affect life span.

Mech Ageing Dev **123**, 1579–1587.
118 Petriv OI & Rachubinski RA (2004) Lack of peroxisomal catalase causes a progeric phenotype in Caenorhabditis elegans. *J. Biol. Chem* **279**, 19996-20001.
119 Joeng KS, Song EJ, Lee K-J & Lee J (2004) Long lifespan in worms with long telomeric DNA. *Nat. Genet* **36**, 607-611.
120 Kenyon C (2010) A pathway that links reproductive status to lifespan in Caenorhabditis elegans. *Ann. N. Y. Acad. Sci* **1204**, 156-162.
121 Treutter D (2006) Significance of flavonoids in plant resistance: a review. *Environ Chem Lett* **4**, 147-157.
122 Manach C, Scalbert A, Morand C, Rémésy C & Jiménez L (2004) Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr* **79**, 727-747.
123 Day AJ, Cañada FJ, Díaz JC, Kroon PA, Mclauchlan R, Faulds CB, Plumb GW, Morgan MR & Williamson G (2000) Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. *FEBS Lett* **468**, 166-170.
124 Gee JM, DuPont MS, Day AJ, Plumb GW, Williamson G & Johnson IT (2000) Intestinal transport of quercetin glycosides in rats involves both deglycosylation and interaction with the hexose transport pathway. *J. Nutr* **130**, 2765-2771.
125 Kottra G & Daniel H (2007) Flavonoid glycosides are not transported by the human Na+/glucose transporter when expressed in Xenopus laevis oocytes, but effectively inhibit electrogenic glucose uptake. *J. Pharmacol. Exp. Ther* **322**, 829-835.
126 Crozier A, Jaganath IB & Clifford MN (2009) Dietary phenolics: chemistry, bioavailability and effects on health. *Nat Prod Rep* **26**, 1001-1043.
127 Manach C, Williamson G, Morand C, Scalbert A & Rémésy C (2005) Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr* **81**, 230S-242S.
128 RusznyáK S & Szent-GyöRgyi A (1936) Vitamin P: Flavonols as Vitamins. *Nature* **138**, 27-27.
129 Bazzano LA, He J, Ogden LG, Loria C, Vupputuri S, Myers L & Whelton PK (2001) Legume Consumption and Risk of Coronary Heart Disease in US Men and Women: NHANES I Epidemiologic Follow-up Study. *Arch Intern Med* **161**, 2573–2578.
130 Hertog MG, Feskens EJ, Hollman PC, Katan MB & Kromhout D (1993) Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* **342**, 1007-1011.
131 Knekt P, Kumpulainen J, Järvinen R, Rissanen H, Heliövaara M, Reunanen A, Hakulinen T & Aromaa A (2002) Flavonoid intake and risk of chronic diseases. *Am. J. Clin. Nutr* **76**, 560-568.
132 Soobrattee MA, Neergheen VS, Luximon-Ramma A, Aruoma OI & Bahorun T (2005) Phenolics as potential antioxidant therapeutic agents: mechanism and actions. *Mutat Res* **579**, 200–213.
133 Tabart J, Kevers C, Pincemail J, Defraigne J-O & Dommes J (2009) Comparative antioxidant capacities of phenolic compounds measured by various tests. *Food Chemistry* **113**, 1226-1233.
134 Rice-Evans CA, Miller NJ, Bolwell PG, Bramley PM & Pridham JB (1995) The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radic. Res* **22**, 375-383.
135 Silva MM, Santos MR, Caroço G, Rocha R, Justino G & Mira L (2002) Structure-antioxidant activity relationships of flavonoids: a re-examination. *Free Radic. Res* **36**, 1219-1227.

136 Setchell KDR, Clerici C, Lephart ED, Cole SJ, Heenan C, Castellani D, Wolfe BE, Nechemias-Zimmer L, Brown NM, Lund TD, Handa RJ & Heubi JE (2005) S-equol, a potent ligand for estrogen receptor beta, is the exclusive enantiomeric form of the soy isoflavone metabolite produced by human intestinal bacterial flora. *Am. J. Clin. Nutr* **81**, 1072-1079.
137 Mortensen A, Kulling SE, Schwartz H, Rowland I, Ruefer CE, Rimbach G, Cassidy A, Magee P, Millar J, Hall WL, Kramer Birkved F, Sorensen IK & Sontag G (2009) Analytical and compositional aspects of isoflavones in food and their biological effects. *Mol Nutr Food Res* **53 Suppl 2**, S266-309.
138 Yang F, Oz HS, Barve S, de Villiers WJ, McClain CJ & Varilek GW (2001) The green tea polyphenol (-)-epigallocatechin-3-gallate blocks nuclear factor-kappa B activation by inhibiting I kappa B kinase activity in the intestinal epithelial cell line IEC-6. *Mol. Pharmacol* **60**, 528-533.
139 Aggarwal BB & Shishodia S (2006) Molecular targets of dietary agents for prevention and therapy of cancer. *Biochem. Pharmacol* **71**, 1397-1421.
140 Agullo G, Gamet-Payrastre L, Manenti S, Viala C, Rémésy C, Chap H & Payrastre B (1997) Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: A comparison with tyrosine kinase and protein kinase C inhibition. *Biochem Pharmacol* **53**, 1649-1657.
141 Harmon AW & Patel YM (2003) Naringenin inhibits phosphoinositide 3-kinase activity and glucose uptake in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* **305**, 229-234.
142 Schwarz D & Roots I (2003) In vitro assessment of inhibition by natural polyphenols of metabolic activation of procarcinogens by human CYP1A1. *Biochem. Biophys. Res. Commun* **303**, 902-907.
143 Fuhr U, Klittich K & Staib AH (1993) Inhibitory effect of grapefruit juice and its bitter principal, naringenin, on CYP1A2 dependent metabolism of caffeine in man. *Br J Clin Pharmacol* **35**, 431-436.
144 Zhang S, Qin C & Safe SH (2003) Flavonoids as aryl hydrocarbon receptor agonists/antagonists: effects of structure and cell context. *Environ Health Perspect* **111**, 1877-1882.
145 Pietro AD, Conseil G, Pérez-Victoria JM, Dayan G, Baubichon-Cortay H, Trompier D, Steinfels E, Jault JM, Wet H de, Maitrejean M, Comte G, Boumendjel A, Mariotte AM, Dumontet C, McIntosh DB, Goffeau A, Castanys S, Gamarro F & Barron D (2002) Modulation by flavonoids of cell multidrug resistance mediated by P-glycoprotein and related ABC transporters. *Cell Mol Life Sci* **59**, 307–322.
146 Chen C, Zhou J & Ji C (2010) Quercetin: A potential drug to reverse multidrug resistance. *Life Sciences* **87**, 333-338.
147 Re R, Pellegrini N, Proteggente A, Pannala A, Yang M & Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med* **26**, 1231-1237.
148 Honda S, Ishii N, Suzuki K & Matsuo M (1993) Oxygen-dependent perturbation of life span and aging rate in the nematode. *J Gerontol* **48**, B57–B61.
149 Henderson ST & Johnson TE (2001) daf-16 integrates developmental and environmental inputs to mediate aging in the nematode Caenorhabditis elegans. *Curr Biol* **11**, 1975-80.
150 Link CD, Cypser JR, Johnson CJ & Johnson TE (1999) Direct observation of stress response in Caenorhabditis elegans using a reporter transgene. *Cell Stress Chaperones* **4**, 235-242.

151 Morley JF & Morimoto RI (2004) Regulation of longevity in Caenorhabditis elegans by heat shock factor and molecular chaperones. *Mol Biol Cell* **15**, 657-64.
152 Gruber J, Tang SY & Halliwell B (2007) Evidence for a trade-off between survival and fitness caused by resveratrol treatment of Caenorhabditis elegans. *Ann N Y Acad Sci* **1100**, 530–542.
153 Kampkötter A, Timpel C, Zurawski RF, Ruhl S, Chovolou Y, Proksch P & Wätjen W (2008) Increase of stress resistance and lifespan of Caenorhabditis elegans by quercetin. *Comp Biochem Physiol B Biochem Mol Biol* **149**, 314–323.
154 Ernst IMA, Wagner AE, Lipinski S, Skrbek S, Ruefer CE, Desel C & Rimbach G (2009) Cellular uptake, stability, visualization by "Naturstoff reagent A", and multidrug resistance protein 1 gene-regulatory activity of cyanidin in human keratinocytes. *Pharmacol Res*.
155 Neu R (1957) Aromatic boron compounds IX. Chelates of diarylboronic acids with aliphatic hydroxy amines as reagents for detection of hydroxyphenylbenzo-g-pyrones. *Naturwissenschaften* **44**, 181-2.
156 Cochemé HM & Murphy MP (2008) Complex I is the major site of mitochondrial superoxide production by paraquat. *J Biol Chem* **283**, 1786–1798.
157 Brand MD & Nicholls DG (2011) Assessing mitochondrial dysfunction in cells. *Biochem. J.* **435**, 297-312.
158 Takahashi MA & Asada K (1983) Superoxide anion permeability of phospholipid membranes and chloroplast thylakoids. *Arch. Biochem. Biophys* **226**, 558-566.
159 Talbot DA & Brand MD (2005) Uncoupling protein 3 protects aconitase against inactivation in isolated skeletal muscle mitochondria. *Biochim. Biophys. Acta* **1709**, 150-156.
160 Hao L, Nishimura T, Wo H & Fernandez-Patron C (2006) Vascular Responses to {alpha}1-Adrenergic Receptors in Small Rat Mesenteric Arteries Depend on Mitochondrial Reactive Oxygen Species. *Arterioscler Thromb Vasc Biol* **26**, 819-825.
161 Kondo M, Senoo-Matsuda N, Yanase S, Ishii T, Hartman PS & Ishii N (2005) Effect of oxidative stress on translocation of DAF-16 in oxygen-sensitive mutants, mev-1 and gas-1 of Caenorhabditis elegans. *Mech. Ageing Dev* **126**, 637-641.
162 Levine RL (1983) Oxidative modification of glutamine synthetase. I. Inactivation is due to loss of one histidine residue. *J. Biol. Chem* **258**, 11823-11827.
163 Levine RL (2002) Carbonyl modified proteins in cellular regulation, aging, and disease,. *Free Radical Biology and Medicine* **32**, 790-796.
164 Nyström T (2005) Role of oxidative carbonylation in protein quality control and senescence. *EMBO J* **24**, 1311-1317.
165 Abramoff MD, Magelhaes PJ & Ram SJ (2004) Image processing with ImageJ. *Biophotonics international* **11**, 36–42.
166 Lee RY, Sawin ER, Chalfie M, Horvitz HR & Avery L (1999) EAT-4, a homolog of a mammalian sodium-dependent inorganic phosphate cotransporter, is necessary for glutamatergic neurotransmission in caenorhabditis elegans. *J. Neurosci* **19**, 159-167.
167 Furuyama T, Nakazawa T, Nakano I & Mori N (2000) Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochem. J* **349**, 629-634.
168 Richter K, Haslbeck M & Buchner J (2010) The heat shock response: life on the verge of death. *Mol. Cell* **40**, 253-266.
169 Mogk A, Schlieker C, Friedrich KL, Schönfeld H-J, Vierling E & Bukau B (2003)

Refolding of substrates bound to small Hsps relies on a disaggregation reaction mediated most efficiently by ClpB/DnaK. *J. Biol. Chem.* **278**, 31033-31042.
170 Walker GA & Lithgow GJ (2003) Lifespan extension in C. elegans by a molecular chaperone dependent upon insulin-like signals. *Aging Cell* **2**, 131–139.
171 Hartwig K, Heidler T, Moch J, Daniel H & Wenzel U (2009) Feeding a ROS-generator to Caenorhabditis elegans leads to increased expression of small heat shock protein HSP-16.2 and hormesis. *Genes Nutr* **4**, 59–67.
172 Strayer A (2003) Expression of the small heat-shock protein Hsp-16-2 in Caenorhabditis elegans is suppressed by Ginkgo biloba extract EGb 761. *The FASEB Journal*.
173 Pietsch K, Saul N, Chakrabarti S, Stürzenbaum SR, Menzel R & Steinberg CEW (2011) Hormetins, antioxidants and prooxidants: defining quercetin-, caffeic acid- and rosmarinic acid-mediated life extension in C. elegans. *Biogerontology*.
174 Saul N, Pietsch K, Menzel R, Stürzenbaum SR & Steinberg CEW (2009) Catechin induced longevity in C. elegans: from key regulator genes to disposable soma. *Mech Ageing Dev* **130**, 477–486.
175 YANASE S & ISHII N (2008) Hyperoxia Exposure Induced Hormesis Decreases Mitochondrial Superoxide Radical Levels via Ins/IGF-1 Signaling Pathway in a Long-lived age-1 Mutant of Caenorhabditis elegans. *J Radiat Res (Tokyo)* **49**, 211-218.
176 Williams RJ, Spencer JPE & Rice-Evans C (2004) Flavonoids: antioxidants or signalling molecules? *Free Radic Biol Med* **36**, 838–849.
177 Dorta DJ, Pigoso AA, Mingatto FE, Rodrigues T, Pestana CR, Uyemura SA, Santos AC & Curti C (2008) Antioxidant activity of flavonoids in isolated mitochondria. *Phytother. Res.* **22**, 1213-1218.
178 Kampkötter A, Pielarski T, Rohrig R, Timpel C, Chovolou Y, Wätjen W & Kahl R (2007) The Ginkgo biloba extract EGb761 reduces stress sensitivity, ROS accumulation and expression of catalase and glutathione S-transferase 4 in Caenorhabditis elegans. *Pharmacological Research* **55**, 139-147.
179 Kampkötter A, Nkwonkam CG, Zurawski RF, Timpel C, Chovolou Y, Wätjen W & Kahl R (2007) Investigations of protective effects of the flavonoids quercetin and rutin on stress resistance in the model organism Caenorhabditis elegans. *Toxicology* **234**, 113 - 123.
180 Bruskov VI, Malakhova LV, Masalimov ZK & Chernikov AV (2002) Heat-induced formation of reactive oxygen species and 8-oxoguanine, a biomarker of damage to DNA. *Nucleic Acids Research* **30**, 1354 -1363.
181 Huang C, Xiong C & Kornfeld K (2004) Measurements of age-related changes of physiological processes that predict lifespan of Caenorhabditis elegans. *Proc. Natl. Acad. Sci. U.S.A* **101**, 8084-8089.
182 Yang W, Li J & Hekimi S (2007) A Measurable increase in oxidative damage due to reduction in superoxide detoxification fails to shorten the life span of long-lived mitochondrial mutants of Caenorhabditis elegans. *Genetics* **177**, 2063-2074.
183 Zarse K, Schulz TJ, Birringer M & Ristow M (2007) Impaired respiration is positively correlated with decreased life span in Caenorhabditis elegans models of Friedreich Ataxia. *FASEB J* **21**, 1271-1275.
184 McElwee J, Bubb K & Thomas JH (2003) Transcriptional outputs of the Caenorhabditis elegans forkhead protein DAF-16. *Aging Cell* **2**, 111-121.
185 Heidler T, Hartwig K, Daniel H & Wenzel U (2010) Caenorhabditis elegans lifespan extension caused by treatment with an orally active ROS-generator is dependent on

DAF-16 and SIR-2.1. *Biogerontology* **11**, 183–195.
186 El Mohsen MA, Marks J, Kuhnle G, Rice-Evans C, Moore K, Gibson G, Debnam E & Srai SK (2004) The differential tissue distribution of the citrus flavanone naringenin following gastric instillation. *Free Radic. Res* **38**, 1329-1340.
187 Bieger J, Cermak R, Blank R, de Boer VCJ, Hollman PCH, Kamphues J & Wolffram S (2008) Tissue distribution of quercetin in pigs after long-term dietary supplementation. *J. Nutr* **138**, 1417-1420.
188 Spencer JPE, Kuhnle GGC, Williams RJ & Rice-Evans C (2003) Intracellular metabolism and bioactivity of quercetin and its in vivo metabolites. *Biochem. J* **372**, 173-181.
189 Yokomizo A & Moriwaki M (2006) Effects of uptake of flavonoids on oxidative stress induced by hydrogen peroxide in human intestinal Caco-2 cells. *Biosci. Biotechnol. Biochem* **70**, 1317-1324.
190 Angeloni C, Spencer JPE, Leoncini E, Biagi PL & Hrelia S (2007) Role of quercetin and its in vivo metabolites in protecting H9c2 cells against oxidative stress. *Biochimie* **89**, 73-82.
191 Spencer JPE, Abd El Mohsen MM & Rice-Evans C (2004) Cellular uptake and metabolism of flavonoids and their metabolites: implications for their bioactivity. *Arch Biochem Biophys* **423**, 148-161.
192 Day AJ, Mellon F, Barron D, Sarrazin G, Morgan MRA & Williamson G (2001) Human metabolism of dietary flavonoids: Identification of plasma metabolites of quercetin. *Free Radic Res* **35**, 941-952.
193 Mullen W, Archeveque M-A, Edwards CA, Matsumoto H & Crozier A (2008) Bioavailability and metabolism of orange juice flavanones in humans: impact of a full-fat yogurt. *J. Agric. Food Chem* **56**, 11157-11164.
194 Yamamoto N, Moon JH, Tsushida T, Nagao A & Terao J (1999) Inhibitory effect of quercetin metabolites and their related derivatives on copper ion-induced lipid peroxidation in human low-density lipoprotein. *Arch. Biochem. Biophys* **372**, 347-354.
195 Zhang W-D, Wang X-J, Zhou S-Y, Gu Y, Wang R, Zhang T-L & Gan H-Q (2010) Determination of free and glucuronidated kaempferol in rat plasma by LC-MS/MS: application to pharmacokinetic study. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci* **878**, 2137-2140.
196 Spencer JPE, Chowrimootoo G, Choudhury R, Debnam ES, Srai SK & Rice-Evans C (1999) The small intestine can both absorb and glucuronidate luminal flavonoids. *FEBS Letters* **458**, 224-230.
197 Laing ST, Ivens A, Laing R, Ravikumar S, Butler V, Woods DJ & Gilleard JS (2010) Characterization of the xenobiotic response of Caenorhabditis elegans to the anthelmintic drug albendazole and the identification of novel drug glucoside metabolites. *Biochem. J* **432**, 505-514.
198 Liu Y, Fiskum G & Schubert D (2002) Generation of reactive oxygen species by the mitochondrial electron transport chain. *J Neurochem* **80**, 780-787.
199 Sanz A, Stefanatos R & McIlroy G (2010) Production of reactive oxygen species by the mitochondrial electron transport chain in Drosophila melanogaster. *J. Bioenerg. Biomembr* **42**, 135-142.
200 St-Pierre J, Buckingham JA, Roebuck SJ & Brand MD (2002) Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J. Biol. Chem* **277**, 44784-44790.
201 Boveris A & Chance B (1973) The mitochondrial generation of hydrogen peroxide.

General properties and effect of hyperbaric oxygen. *Biochem. J* **134**, 707-716.
202 Lagoa R, Graziani I, Lopez-Sanchez C, Garcia-Martinez V & Gutierrez-Merino C (2011) Complex I and cytochrome c are molecular targets of flavonoids that inhibit hydrogen peroxide production by mitochondria. *Biochimica Et Biophysica Acta*.
203 Huang D, Ou B & Prior RL (2005) The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem* **53**, 1841-1856.
204 Abbas S & Wink M (2009) Epigallocatechin gallate from green tea (Camellia sinensis) increases lifespan and stress resistance in Caenorhabditis elegans. *Planta Med* **75**, 216-221.
205 Kuznetsov AV, Kehrer I, Kozlov AV, Haller M, Redl H, Hermann M, Grimm M & Troppmair J (2011) Mitochondrial ROS production under cellular stress: comparison of different detection methods. *Anal Bioanal Chem* **400**, 2383-2390.
206 Fiorani M, Guidarelli A, Blasa M, Azzolini C, Candiracci M, Piatti E & Cantoni O (2010) Mitochondria accumulate large amounts of quercetin: prevention of mitochondrial damage and release upon oxidation of the extramitochondrial fraction of the flavonoid. *The Journal of Nutritional Biochemistry* **21**, 397-404.
207 van Acker SA, de Groot MJ, van den Berg DJ, Tromp MN, Donné-Op den Kelder G, van der Vijgh WJ & Bast A (1996) A quantum chemical explanation of the antioxidant activity of flavonoids. *Chem. Res. Toxicol* **9**, 1305-1312.
208 Hendrickson HP, Sahafayen M, Bell MA, Kaufman AD, Hadwiger ME & Lunte CE (1994) Relationship of flavonoid oxidation potential and effect on rat hepatic microsomal metabolism of benzene and phenol. *J Pharm Biomed Anal* **12**, 335-341.
209 McPhail DB, Hartley RC, Gardner PT & Duthie GG (2003) Kinetic and stoichiometric assessment of the antioxidant activity of flavonoids by electron spin resonance spectroscopy. *J. Agric. Food Chem* **51**, 1684-1690.
210 Adachi H, Fujiwara Y & Ishii N (1998) Effects of oxygen on protein carbonyl and aging in Caenorhabditis elegans mutants with long (age-1) and short (mev-1) life spans. *J Gerontol A Biol Sci Med Sci* **53**, B240–B244.
211 Pun P, Gruber J, Tang S, Schaffer S, Ong R, Fong S, Ng L, Cheah I & Halliwell B (2009) Ageing in nematodes: do antioxidants extend lifespan in Caenorhabditis elegans? *Biogerontology*.
212 Belinha I, Amorim MA, Rodrigues P, de Freitas V, Moradas-Ferreira P, Mateus N & Costa V (2007) Quercetin increases oxidative stress resistance and longevity in Saccharomyces cerevisiae. *J. Agric. Food Chem* **55**, 2446-2451.
213 Pandey KB & Rizvi SI (2009) Protection of protein carbonyl formation by quercetin in erythrocytes subjected to oxidative stress. *Med Chem Res* **19**, 186-192.
214 Wiegant FAC, Surinova S, Ytsma E, Langelaar-Makkinje M, Wikman G & Post JA (2009) Plant adaptogens increase lifespan and stress resistance in C. elegans. *Biogerontology* **10**, 27-42.
215 Wilmsen PK, Spada DS & Salvador M (2005) Antioxidant activity of the flavonoid hesperidin in chemical and biological systems. *J. Agric. Food Chem* **53**, 4757-4761.
216 Canada AT, Giannella E, Nguyen TD & Mason RP (1990) The production of reactive oxygen species by dietary flavonols. *Free Radic. Biol. Med* **9**, 441-449.
217 Kang SA, Jang YJ & Park H (1998) In vivo dual effects of vitamin C on paraquat-induced lung damage: dependence on released metals from the damaged tissue. *Free Radic. Res* **28**, 93-107.
218 Meissner B, Boll M, Daniel H & Baumeister R (2004) Deletion of the intestinal peptide transporter affects insulin and TOR signaling in Caenorhabditis elegans. *J Biol Chem* **279**, 36739-45.

219 Pietsch K, Saul N, Menzel R, Stürzenbaum SR & Steinberg CEW (2009) Quercetin mediated lifespan extension in Caenorhabditis elegans is modulated by age-1, daf-2, sek-1 and unc-43. *Biogerontology* **10**, 565–578.
220 Wood JG, Rogina B, Lavu S, Howitz K, Helfand SL, Tatar M & Sinclair D (2004) Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature* **430**, 686-689.
221 Wilson MA, Shukitt-Hale B, Kalt W, Ingram DK, Joseph JA & Wolkow CA (2006) Blueberry polyphenols increase lifespan and thermotolerance in Caenorhabditis elegans. *Aging Cell* **5**, 59–68.
222 Martorell P, Forment JV, de Llanos R, Montón F, Llopis S, González N, Genovés S, Cienfuegos E, Monzó H & Ramón D (2011) Use of Saccharomyces cerevisiae and Caenorhabditis elegans as model organisms to study the effect of cocoa polyphenols in the resistance to oxidative stress. *J. Agric. Food Chem* **59**, 2077-2085.
223 Saul N, Pietsch K, Stürzenbaum SR, Menzel R & Steinberg CEW (2011) Diversity of Polyphenol Action in Caenorhabditis elegans: Between Toxicity and Longevity. *J Nat Prod*.
224 Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang L-L, Scherer B & Sinclair DA (2003) Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. *Nature* **425**, 191-196.
225 Valenzano DR, Terzibasi E, Genade T, Cattaneo A, Domenici L & Cellerino A (2006) Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate. *Curr. Biol.* **16**, 296-300.
226 Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K, Pistell PJ, Poosala S, Becker KG, Boss O, Gwinn D, Wang M, Ramaswamy S, Fishbein KW, Spencer RG, Lakatta EG, Le Couteur D, Shaw RJ, Navas P, Puigserver P, Ingram DK, de Cabo R & Sinclair DA (2006) Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* **444**, 337-342.
227 Jones E & Hughes RE (1982) Quercetin, flavonoids and the life-span of mice. *Exp. Gerontol.* **17**, 213-217.
228 Vanfleteren JR (1993) Oxidative stress and ageing in Caenorhabditis elegans. *Biochem J* **292**, 605-608.
229 Yang W & Hekimi S (2010) Two modes of mitochondrial dysfunction lead independently to lifespan extension in Caenorhabditis elegans. *Aging Cell* **9**, 433-447.
230 Melov S, Ravenscroft J, Malik S, Gill MS, Walker DW, Clayton PE, Wallace DC, Malfroy B, Doctrow SR & Lithgow GJ (2000) Extension of Life-Span with Superoxide Dismutase/Catalase Mimetics. *Science* **289**, 1567-1569.
231 Kampkötter A, Nkwonkam CG, Zurawski RF, Timpel C, Chovolou Y, Wätjen W & Kahl R (2007) Effects of the flavonoids kaempferol and fisetin on thermotolerance, oxidative stress and FoxO transcription factor DAF-16 in the model organism Caenorhabditis elegans. *Arch Toxicol* **81**, 849–858.
232 Brown MK, Evans JL & Luo Y (2006) Beneficial effects of natural antioxidants EGCG and alpha-lipoic acid on life span and age-dependent behavioral declines in Caenorhabditis elegans. *Pharmacol. Biochem. Behav* **85**, 620-628.
233 Comfort A, Youhotsky-Gore I & Pathmanathan K (1971) Effect of ethoxyquin on the longevity of C3H mice. *Nature* **229**, 254-255.

234 Harman D (1968) Free radical theory of aging: effect of free radical reaction inhibitors on the mortality rate of male LAF mice. *J Gerontol* **23**, 476-482.
235 Honda Y, Tanaka M & Honda S (2008) Modulation of longevity and diapause by redox regulation mechanisms under the insulin-like signaling control in Caenorhabditis elegans. *Exp. Gerontol* **43**, 520-529.
236 Pérez VI, Van Remmen H, Bokov A, Epstein CJ, Vijg J & Richardson A (2009) The overexpression of major antioxidant enzymes does not extend the lifespan of mice. *Aging Cell* **8**, 73-75.
237 Yamamoto K, Honda S & Ishii N (1996) Properties of an oxygen-sensitive mutant mev-3 of the nematode Caenorhabditis elegans. *Mutat. Res* **358**, 1-6.
238 Fujii M, Tanaka N, Miki K, Hossain MN, Endoh M & Ayusawa D (2005) Uncoupling of longevity and paraquat resistance in mutants of the nematode Caenorhabditis elegans. *Biosci. Biotechnol. Biochem* **69**, 2015-2018.
239 Ristow M & Schmeisser S (2011) Extending life span by increasing oxidative stress. *Free Radic Biol Med*.
240 Schulz TJ, Zarse K, Voigt A, Urban N, Birringer M & Ristow M (2007) Glucose restriction extends Caenorhabditis elegans life span by inducing mitochondrial respiration and increasing oxidative stress. *Cell Metab* **6**, 280–293.
241 Xue Y-L, Ahiko T, Miyakawa T, Amino H, Hu F, Furihata K, Kita K, Shirasawa T, Sawano Y & Tanokura M (2011) Isolation and Caenorhabditis elegans Lifespan Assay of Flavonoids from Onion. *J Agric Food Chem*.
242 Gruber J, Ng LF, Poovathingal SK & Halliwell B (2009) Deceptively simple but simply deceptive -Caenorhabditis elegans lifespan studies: Considerations for aging and antioxidant effects. *FEBS Lett*.
243 Saul N, Pietsch K, Menzel R, Stürzenbaum SR & Steinberg CEW (2010) The Longevity Effect of Tannic Acid in Caenorhabditis elegans: Disposable Soma Meets Hormesis. *J Gerontol A Biol Sci Med Sci*.
244 Morselli E, Maiuri MC, Markaki M, Megalou E, Pasparaki A, Palikaras K, Criollo A, Galluzzi L, Malik SA, Vitale I, Michaud M, Madeo F, Tavernarakis N & Kroemer G (2010) Caloric restriction and resveratrol promote longevity through the Sirtuin-1-dependent induction of autophagy. *Cell Death Dis* **1**, e10.
245 Madeo F, Tavernarakis N & Kroemer G (2010) Can autophagy promote longevity? *Nat. Cell Biol.* **12**, 842-846.
246 de Boer VCJ, de Goffau MC, Arts ICW, Hollman PCH & Keijer J (2006) SIRT1 stimulation by polyphenols is affected by their stability and metabolism. *Mech. Ageing Dev* **127**, 618-627.
247 Gledhill JR, Montgomery MG, Leslie AGW & Walker JE (2007) Mechanism of inhibition of bovine F1-ATPase by resveratrol and related polyphenols. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 13632-13637.
248 Suh Y, Atzmon G, Cho M-O, Hwang D, Liu B, Leahy DJ, Barzilai N & Cohen P (2008) Functionally significant insulin-like growth factor I receptor mutations in centenarians. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 3438-3442.
249 van Heemst D, Beekman M, Mooijaart SP, Heijmans BT, Brandt BW, Zwaan BJ, Slagboom PE & Westendorp RGJ (2005) Reduced insulin/IGF-1 signalling and human longevity. *Aging Cell* **4**, 79-85.
250 Bartholome A, Kampkötter A, Tanner S, Sies H & Klotz L-O (2010) Epigallocatechin gallate-induced modulation of FoxO signaling in mammalian cells and C. elegans: FoxO stimulation is masked via PI3K/Akt activation by hydrogen peroxide formed in cell culture. *Arch. Biochem. Biophys.* **501**, 58-64.

251 Vlahos CJ, Matter WF, Hui KY & Brown RF (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem* **269**, 5241-5248.
252 Walker EH, Pacold ME, Perisic O, Stephens L, Hawkins PT, Wymann MP & Williams RL (2000) Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine. *Mol. Cell* **6**, 909-919.
253 Kong D, Zhang Y, Yamori T, Duan H & Jin M (2011) Inhibitory activity of flavonoids against class I phosphatidylinositol 3-kinase isoforms. *Molecules* **16**, 5159-5167.
254 Kumamoto T, Fujii M & Hou D-X (2009) Akt is a direct target for myricetin to inhibit cell transformation. *Mol. Cell. Biochem* **332**, 33-41.
255 Waite KA, Sinden MR & Eng C (2005) Phytoestrogen exposure elevates PTEN levels. *Hum. Mol. Genet.* **14**, 1457-1463.
256 Wolff S, Ma H, Burch D, Maciel GA, Hunter T & Dillin A (2006) SMK-1, an essential regulator of DAF-16-mediated longevity. *Cell* **124**, 1039-53.
257 Kenyon C & Murphy CT (2006) Enrichment of regulatory motifs upstream of predicted DAF-16 targets. *Nat. Genet* **38**, 397-398; author reply 398.
258 Walker GA, Thompson FJ, Brawley A, Scanlon T & Devaney E (2003) Heat shock factor functions at the convergence of the stress response and developmental pathways in Caenorhabditis elegans. *FASEB J* **17**, 1960-1962.
259 Prahlad V, Cornelius T & Morimoto RI (2008) Regulation of the cellular heat shock response in Caenorhabditis elegans by thermosensory neurons. *Science* **320**, 811–814.
260 Hansen RK, Oesterreich S, Lemieux P, Sarge KD & Fuqua SA (1997) Quercetin inhibits heat shock protein induction but not heat shock factor DNA-binding in human breast carcinoma cells. *Biochem. Biophys. Res. Commun.* **239**, 851-856.
261 Nagai N, Nakai A & Nagata K (1995) Quercetin suppresses heat shock response by down regulation of HSF1. *Biochem. Biophys. Res. Commun.* **208**, 1099-1105.
262 McMillan DR, Xiao X, Shao L, Graves K & Benjamin IJ (1998) Targeted disruption of heat shock transcription factor 1 abolishes thermotolerance and protection against heat-inducible apoptosis. *J. Biol. Chem.* **273**, 7523-7528.
263 Saul N, Pietsch K, Menzel R & Steinberg CEW (2008) Quercetin-mediated longevity in Caenorhabditis elegans: Is DAF-16 involved? *Mech Ageing Dev* **129**, 611–613.
264 Zhang L, Jie G, Zhang J & Zhao B (2009) Significant longevity-extending effects of EGCG on Caenorhabditis elegans under stress. *Free Radic Biol Med* **46**, 414–421.

Danksagung

Solch eine Arbeit kann nie ohne die Hilfe und Unterstützung vieler Personen gelingen, daher gilt mein ganz besonderer Dank:

Frau Prof. Dr. Daniel, für das Überlassen dieses spannenden Themas sowie für das in mich gesetzte Vertrauen. Ihre motivierenden Worte und die fachlichen Diskussionen haben mich nicht nur für das Bearbeiten dieses Themas begeistert sondern auch wichtige Impulse für diese Arbeit geliefert.

Herrn Prof. Dr. Hauner für die Übernahme des Prüfungsvorsitzes sowie Herrn Prof. Dr. Klingenspor für die Erstellung des Gutachtens.

Britta, für Deine tatkräftige Betreuung und Unterstützung während der Zeit am Institut. Du hast mir nicht nur den Wurm näher, sondern mir auch wichtige Erkenntnisse beim „Puzzeln“ meiner Ergebnisse auf dem Institutsfußboden gebracht.

Frau Prof. Dr. Kulling und ihr Team, insbesondere Sebastian Sokoup, für die Analyse der Wurmproben. Kerstin Haas, für die Isolation und Analytik der Mausmitochondrien.

Jacki, für all die Aufmunterung während unserer gemeinsamen Zeit am Institut und insbesondere für das Abholen aus Lerchenfeld.

Kerstin, für alle die Ratschläge zu den wissenschaftlichen Fragestellungen und für die zu den alltäglichen Problemen.

Katrin, für all das Zählen der vielen lifespans und den ganzen Spaß den wir gemeinsam im Würfel hatten.

Allen Mitgliedern des Lehrstuhles für eine tolle Zeit am Institut. Vielen Dank auch Regina und Ines für Euer Engagement und Eure tolle Arbeit.

Markus und Carsten für das Sichern beim Klettern und dass durch Euch das kleine Freising ein Stück größer geworden ist.

Robert, für die vielen wissenschaftlichen Diskussionen, seit nunmehr über zehn Jahren, und für die Diskussionen über die unzähligen anderen wichtigen Themen.

Ein ganz besondere Dank gilt meinen Eltern Monika und Wolfgang, für Eure stete Unterstützung und den Glauben an mich.

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit

Structure-activity relationship of selected flavonoids on aging and stress-resistance in *Caenorhabditis elegans*

selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Die den benutzten Quellen wörtlich oder inhaltlich übernommenen Stellen sind als solche kenntlich gemacht. Die Arbeit hat in gleicher oder ähnlicher Form noch keiner anderen Prüfungsbehörde vorgelegen.

Singen, den

Gregor Grünz