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RESEARCH ARTICLE

# Quercetin mediated lifespan extension in *Caenorhabditis elegans* is modulated by *age-1, daf-2, sek-1* and *unc-43*

**Kerstin Pietsch · Nadine Saul · Ralph Menzel · Stephen R. Stürzenbaum · Christian E. W. Steinberg**

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**Abstract** The nematode *Caenorhabditis elegans* responds to flavonoid-rich diets with improved health and longevity. The precise mechanism(s) responsible for this remains to be identified, but is believed to be linked to the highly antioxidative properties of flavonoids. This study provides a dissection of lifespan modulation by the flavonoid quercetin. In detail, quercetin was shown not to act as a simple antimicrobial agent or exclusively via radical scavenging capacities. Likewise, lifespan extension had no effect on reproduction and body length. Furthermore, neither a caloric restriction mimetic nor a sirtuin (*sir-2.1*) dependence was identified as a likely mode of action. However, four genes were pinpointed to be required for the quercetin derived lifespan extension, namely *age-1, daf-2, unc-43* and *sek-1*. The latter two have, to date, not been linked to quercetin-mediated lifespan extension.

**Keywords** *C. elegans* · Quercetin · Lifespan · *age-1* · *daf-2* · *unc-43* · *sek-1*

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K. Pietsch (✉) · N. Saul · R. Menzel · C. E. W. Steinberg
Laboratory of Freshwater and Stress Ecology, Department of Biology, Humboldt-Universität zu Berlin, Späthstr. 80/81, 12437 Berlin, Germany
e-mail: kpietsch@gmx.de

S. R. Stürzenbaum
Pharmaceutical Science Division, School of Biomedical & Health Sciences, King's College London, 150 Stamford Street, London SE19NH, UK

## Introduction

Aging research, with particular focus on unraveling underlying mechanisms of longevity, is rapidly gaining momentum. The dream of longevity has a long tradition and a multitude of reviews have addressed the process of aging in detail (Kenyon 2005; Kirkwood 2005, 2008a, b; Antebi 2007; Bishop and Guarente 2007; Patridge and Gems 2007; Greer and Brunet 2008; Guarente 2008; Murphy and Patridge 2008; Piper et al. 2008; Zimniak 2008).

Ever since long-lived *Caenorhabditis elegans* mutants were isolated (Friedman and Johnson 1988; Kenyon et al. 1993), a plethora of research has attempted to identify further lifespan-modulating genes. However, arguably equally challenging is the identification of molecular pathways and interacting metabolic processes that affect aging. In this context, studies have assessed the beneficial effects of natural organic geopolymers (Steinberg et al. 2007) and pharmacological active compounds of herbal origin (Wu et al. 2002; Brown et al. 2006; Wilson et al. 2006; Kampkötter et al. 2007a, b, 2008; Saul et al.

2008; Wiegant et al. 2008). A matter of particular interest is the identification of molecules capable of significantly decelerating deleterious impacts of aging in vivo. Promising candidates are the antioxidant and anti-inflammatory polyphenols (Joseph et al. 1999, 2005; Wilson et al. 2006). Indeed, the bioavailability and bioefficacy of certain polyphenols have been demonstrated in humans (Manach et al. 2004, 2005; Nielsen et al. 2003; Rechner et al. 2002). Flavonoids, a subgroup of polyphenols, are thought to possess neuroprotective, cardioprotective and chemopreventive properties, via a yet to be identified mode of action. Whilst previous studies have focused on the antioxidative properties of the flavonoids (Delgado et al. 2001; Lee and Lee 2006), there is also evidence that flavonoids and their metabolites exert age-modulating actions in cells via protein and lipid kinase signaling pathways (Williams et al. 2004).

Quercetin is a flavonoid that is omnipresent in herbal food, including apples, red grapes, onions, broccoli, blueberries, and cherries. Recently, Kampkötter et al. (2007b, 2008) and Saul et al. (2008) identified that quercetin increases stress-resistance and extend lifespan in *C. elegans*. Using cell-free assays and transgenic nematodes, Kampkötter et al. (2008) verified that quercetin accumulates in *C. elegans* and exerts radical scavenging activities, the likely causative contributor of the observed increased fitness of the nematodes. Kampkötter et al. (2007b) argue that this process is regulated by the activation of the FoxO transcription factor DAF-16, however, an in-depth analysis revealed that quercetin-mediated longevity remains unchanged in a *daf-16(mgDf50)* loss-of-function mutant (Saul et al. 2008). This latter finding questions the notion that the reduction of internal oxidative stress is the exclusive role of quercetin. This paper re-addresses this issue by investigating whether quercetin may possess general microbicidal properties (as identified for *Trypanosoma*, Mamani-Matsuda et al. 2004) or caloric restriction (CR) mimetic features. As reproduction and body size remained unaffected this paper challenges the disposable soma theory (Kirkwood 1988) as an explication for extended lifespan, likewise, transgenerational, adaptive and additive effects could be negated. However, the core of this work evolves around 13 different mutant strains to study the impact of genetic background on quercetin induced changes in aging and stress resistance.

## Materials and methods

### Strains and growth conditions

All strains were maintained (unless otherwise stated) at 20°C in appropriate incubators on nematode growth medium (NGM) seeded with *Escherichia coli* feeding strain OP50 according to Brenner (1974). All NGM plates were 35 mm in diameter and inoculated with 100 µl OP50 ($OD_{595}$ 3.4–3.6). Strains used in this study were: N2, Bristol (wild type); GR1307, *daf-16(mgDf50)*; VC199, *sir-2.1(ok434)*; AM1, *osr-1(rm1)*; AU1, *sek-1(ag1)*; MT2605, *unc-43(n498n1186)*; TK22, *mev-1(kn1)*; DR1572, *daf-2(e1368)*; VC8, *jnk-1(gk7)*; TJ1052, *age-1(hx546)*; DR20, *daf-12(m20)*; EU1, *skn-1(zu67)*; VC204, *akt-2(ok393)*; AE501, *nhr-8(ok186)*. All *C. elegans* strains as well as the OP50 strain were obtained from the Caenorhabditis Genetics Center (CGC), University of Minnesota, USA.

### Quercetin

Quercetin (Quercetin dihydrate; Sigma–Aldrich, Germany) was dissolved in DMSO (Dimethylsulfoxid; Applichem, Germany) and added (final concentration 100 or 200 µM) to the NGM medium and the OP50 bacterial feeding suspension. A final DMSO concentration of 0.3% (v/v) was maintained in control and quercetin containing plates.

### Lifespan assay

Lifespan of wild type *C. elegans* was tested in several exposure scenarios. The 1st generation (P0) started at L4 larval stage on either control or treatment plates. Eggs of the 2nd generation (F1) developed in the pre-exposed parent and hatched in the corresponding condition and were exposed during their entire lifetime. Nematodes of the 3rd generation (F2) were either used to investigate the presence of additional or adaptive effects of quercetin, or transferred (as eggs) onto control plates to determine transgenerational responses. Concentrations implemented were 0, 100, and 200 µM quercetin for the wild type and 0 and 200 µM for the mutants, respectively. For each experimental condition at least two independent trials were performed, each comprising ten small agar plates with at least 100 nematodes. The first day of

adulthood was defined as day 1. During the reproductive period, adult nematodes were transferred daily to new treatment plates to avoid overcrowding. Following post-reproduction, transfer occurred every third day, until the impact of aging disallowed handling of the nematodes. Alive and dead animals were scored daily until all had died. Animals were considered dead when no response was observed following a gentle touch by a platinum wire. Nematodes which had escaped the plates or died after internal hatch were subtracted from the total number. All lifespan assays were carried out at 20°C, except where mentioned otherwise. Usually lifespan assays were performed with F1 nematodes, yet, only for ad libitum fed wild type from all three generations.

To verify if quercetin acts only via antibacterial effects or as a CR mimetic, lifespan assays were also conducted with heat-killed *E. coli* (30 min at 65°C, according to Gruber et al. 2007) or without a bacterial lawn. To avoid excessive loss, only 6 day old *C. elegans* (F1 generation) were transferred to either control or quercetin plates.

### Growth alterations

The growth assay utilized 20 nematodes of the F1 generation per concentration and trial. Nematodes were cultured at 20°C. Following 6 days of adulthood, nematodes were killed by heat exposure and the length of each individual nematode measured using a microscope equipped with a graduated eyepiece.

### Reproduction assay

Ten nematodes (L4 larvae of the F1 generation) per concentration were placed onto individual treatment plates. Nematodes were cultivated at 20°C and transferred daily to new plates until reproduction was complete, typically day 5 of adulthood. The offspring of each individual nematode was counted once grown to L2 or L3 stage.

### Thermotolerance test

F1 generation nematodes were cultured as described above from L4 to the sixth day of adulthood at 20°C either on control or quercetin treated plates. The thermal resistance test was performed with approximately 120 nematodes for each concentration in each trial. At the sixth day of adulthood, plates were switched to 35°C. After 8 h for wild type N2 and *unc-43(n498n1186)*, 9 h for the *age-1(hx546)*, 10 h for *daf-2(e1368)* and 6 h for *sek-1(ag1)* dead and alive nematodes were counted.

### Data interpretation and statistical analysis

For each condition mean, median, and maximum lifespan was assessed. Alterations in lifespan values of treated versus untreated nematodes were specified in percentage. Statistical significance was calculated by means of the log-rank test (Bioinformatics at the Walter and Eliza Hall Institute of Medical Research; http://bioinf.wehi.edu.au/software/russell/logrank/). Statistical analyses were only applied to mean lifespan data. Mean values were calculated for the reproduction and body length assays. Statistical significance was evaluated by one way ANOVA (Sigma Stat 3.5, SPSS Inc., USA) and deemed significant at $P < 0.05$. For the thermotolerance assay, mean survival rates and alterations in comparison to the control were calculated for each trial. Statistical significance was evaluated with the chi square test (Sigma Stat 3.5, SPSS Inc., USA). Variances were considered significant at $P < 0.001$.

## Results

### Lifespan extension of the wild type in different exposure scenarios

Quercetin enhanced lifespan in all generations tested (Fig. 1a–c), an effect that was statistically significant in the F1 and F2 generation. This may be a result of differing exposure times (the P0 generation was exposed only from L4 stage onwards) or an effect triggered during embryonic development, a question that clearly warrants further investigations. Nevertheless, lifespan extension was modulated in a quercetin dose responsive manner. In detail, the mean lifespan of the F1 generation was prolonged by 11 and 18% in the presence of 100 and 200 μM quercetin, respectively (Table 1). The median lifespan was expanded by up to 21% (Supplementary Table 1).

The experimental setup was purposefully designed to test for additive (mean lifespan of F2 > mean lifespan of F1) or adaptive effects (mean lifespan of

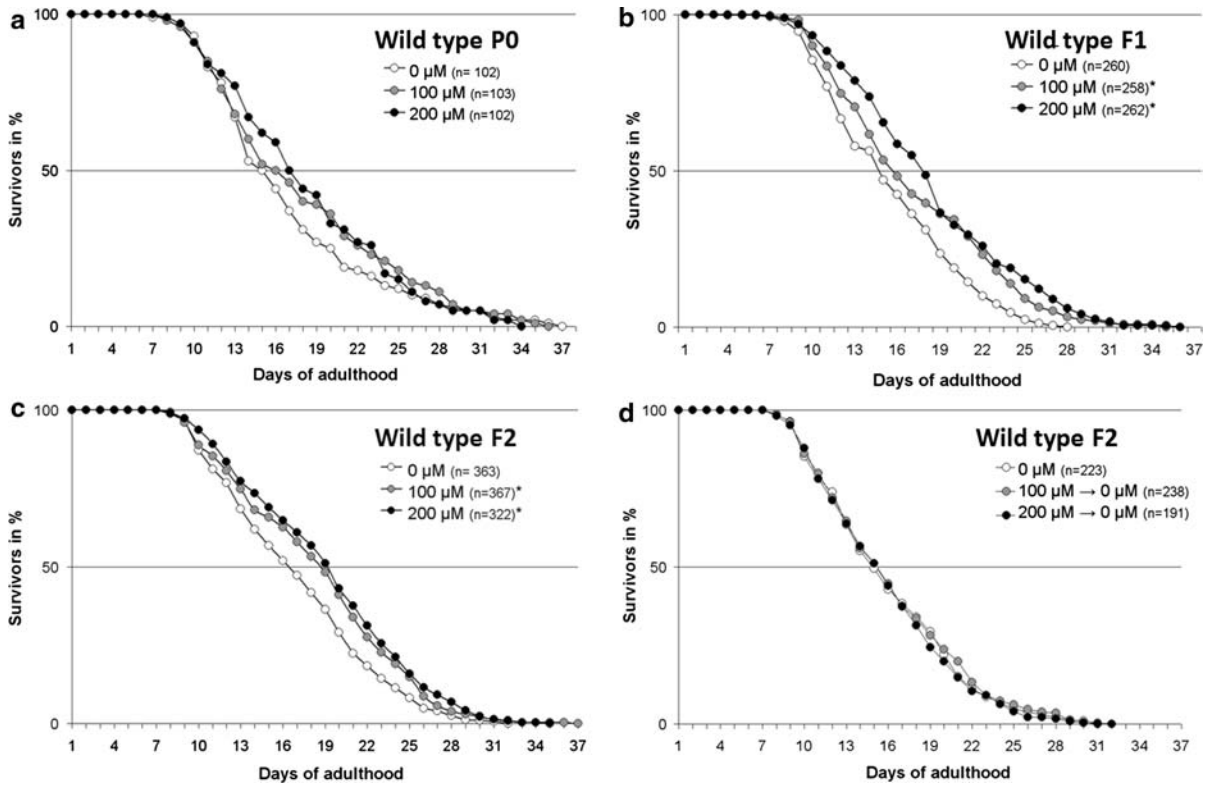


**Fig. 1** Quercetin significantly extends the lifespan in *C. elegans*, shown are survival curves of nematodes of the first (P0) (**a**), second (F1) (**b**) and third (F2) (**c**) generation exposed to 0, 100, and 200 µM quercetin, respectively. Quercetin treatment did not show transgenerational effects in the F2 generation, when nematodes were replaced to non-treatment plates as eggs (**d**). *Graphs* combine 2–3 independent trials, for further details see Table 1, * $P < 0.001$ (log-rank test)

**Table 1** Mean lifespan of untreated control and quercetin treated wild type *C. elegans*

| **Quercetin (µM)** | **Generation** | Mean lifespan (d) ± SEM (*n*) | Change | Trials | Change in single trials |
|---|---|---|---|---|---|
| 0 | | 17.06 ± 0.72 (102) | | | |
| 100 | P0 | 18.42 ± 0.16 (103) | 1.08 | 2 | 1.13, 1.03 |
| 200 | | 18.73 ± 0.10 (102) | 1.10 | | 1.15, 1.05 |
| 0 | | 15.72 ± 0.70 (260) | | | |
| 100 | F1 | 17.47 ± 0.03 (258) | 1.11* | 2 | 1.06, 1.17* |
| 200 | | 18.54 ± 0.15 (262) | 1.18* | | 1.10*, 1.25* |
| 0 | | 17.11 ± 1.22 (363) | | | |
| 100 | F2 | 16.73 ± 1.11 (367) | 1.08* | 3 | 1.05, 1.08, 1.13 |
| 200 | | 19.05 ± 1.54 (322) | 1.11* | | 1.07, 1.14*, 1.11 |
| 0 | | 16.15 ± 1.05 (223) | | | |
| 100→0 | F2 | 16.40 ± 0.92 (238) | 1.01 | 2 | 1.01, 1.03 |
| 200→0 | | 16.09 ± 0.74 (191) | 1.00 | | 0.97, 1.02 |
| 0 | F1[a] | 26.57 ± 1.35 (250) | | 2 | |
| 200 | | 28.76 ± 1.33 (218) | 1.09* | | 1.09, 1.08 |
| 0 | F1[b] | 28.91 ± 7.13 (271) | | 3 | |
| 200 | | 32.24 ± 9.31 (236) | 1.10* | | 1.16*, 1.05, 1.09* |

* Statistical significance was calculated by log-rank testing, changes in mean lifespan are considered significant at $P < 0.001$

[a] Fed with heat-killed *E. coli* bacteria from the sixth day of adulthood

[b] No food from the sixth day of adulthood (food deprivation)

F2 < mean lifespan of F1). Although the first experimental trials suggested that lifespan was extended in the F1 generation (compared to F2), further seven experimental trials could not confirm this perceived trend (compare Table 2). However, life extension of the F1 and F2 generation were comparable (Fig. 1c, Table 1; median and maximum lifespan data are shown in Supplementary Table 1). Overall this suggests that quercetin displays no additive or adaptive effects in lifespan regulation. Similarly, no beneficial transgenerational impact were observed, i.e., when the ancestor (P0 and F1) but not the offspring generation (F2) is treated with quercetin (Fig. 1d, Table 1).

## Quercetin does not cause lifespan extension due to antimicrobial properties

Proliferating bacteria can cause harmful effects on *C. elegans* by producing deleterious metabolites (Garigan et al. 2002), a finding that is supported by Gems and Riddle (2000). However, the lifespan extension observed here was not linked to a bacterial effect, as nematodes fed with heat-killed *E. coli* cells displayed analogous quercetin-mediated longevity (Fig. 2a), with the median and mean lifespan of wild type significantly extended by 8–9% (Table 1, Supplementary Table 2). Interestingly, compared to wild-type nematodes fed healthy *E. coli* ad libitum, the availability of only heat-killed *E. coli* resulted in an enhanced lifespan of 60%. This may resemble food deprivation, an effect known to substantially extend lifespan, a notion that was tested in the following experiment.

## Quercetin does not appear to prolong lifespan by mimicking caloric restriction

Nematodes of the F1 generation were deprived of food following day six of adulthood (after reproduction had ceased and to ensure that nematodes remain healthy and fit). The lifespan of starved adult wild-type nematodes was extended by a statistically significant 10% (Fig. 2b, Table 1). Indeed, compared to ad libitum fed wild type, starved nematodes lifespan was prolonged by about 75%. As quercetin mediated lifespan extension was observed in the absence of food, suggests that quercetin does not act as a CR mimic.

**Table 2** Effect of 200 μM quercetin treatment on wild type and mutant *C. elegans* mean lifespan compared to untreated control

| Genotype | Mean lifespan ± SEM (n) | | Change | Trials | Change in single trials |
|---|---|---|---|---|---|
| | Control [d] | Treated [d] | | | |
| N2 | 16.43 ± 0.77 (806) | 18.05 ± 0.84 (865) | 1.10* | 7 | 1.07*, 1.25*, 1.04, 1.07, 1.12*, 1.03, 1.06 |
| N2[a] | 19.73 ± 0,32 (146) | 21.48 ± 0,54 (122) | 1.09* | 2 | 1.11*, 1.07 |
| *age-1(hx546)* | 20.19 ± 0.81 (643) | 20.50 ± 0.53 (599) | 1.02 | 5 | 0.93, 1.01, 1.06, 1.09, 0.98 |
| *akt-2(ok393).* | 18.42±0.95 (284) | 20.21± 0.82 (241) | 1.10* | 2 | 1.08, 1.11* |
| *daf-2(e1368)* | 25.08 ± 0.92 (378) | 25.57 ± 0.84 (353) | 1.01 | 4 | 1.05, 1.00, 1.06, 0.97 |
| *daf-12(m20)* | 11.53 ± 0.36 (207) | 13.35 ± 0.86 (214) | 1.16* | 2 | 1.11, 1.20* |
| *daf-16(mgDf50)* | 11.07 ± 0.74 (475) | 12.72 ± 0.85 (512) | 1.15* | 3 | 1.15*, 1.15*, 1.14* |
| *jnk-1(gk7)* | 13.82 ± 0.87 (305) | 15.26 ± 1.1 (307) | 1.11* | 2 | 1.09*, 1.11* |
| *mev-1(kn1)* | 8.38 ± 0.46 (205) | 9.25 ± 0.15 (179) | 1.10* | 2 | 1.16*, 1.05 |
| *nhr-8(ok186)* | 14.73 ± 0.19 (324) | 16.49 ± 0.22 (344) | 1.12* | 2 | 1.08, 1.16* |
| *osr-1(rm1)* | 15.90 ± 1.76 (156) | 17.74 ± 1.84 (223) | 1.12* | 2 | 1.13, 1.11 |
| *sek-1(ag1)*[a] | 16.82 ± 0.11 (133) | 17.58 ± 0.81 (101) | 1.05 | 2 | 1.05, 0.97 |
| *sir-2.1(ok434)* | 14.72 ± 0.57 (262) | 15.92 ± 0.64 (261) | 1.08* | 3 | 1.07, 1.05, 1.13* |
| *skn-1(zu67)* | 12.01 ± 0.64 (262) | 14.19 ± 1.11 (283) | 1.18* | 2 | 1.14*, 1.21* |
| *unc-43 (n498n1186)* | 11.50 ± 1.60 (283) | 11.27 ± 1.35 (293) | 0.98 | 2 | 1.01, 0.96 |

Genetic backgrounds preventing significant quercetin effects on *C. elegans*' lifespan are highlighted in gray

* Statistical significance was calculated by log-rank testing, changes in mean lifespan are considered significant at $P < 0.001$

[a] Incubated at 15°C

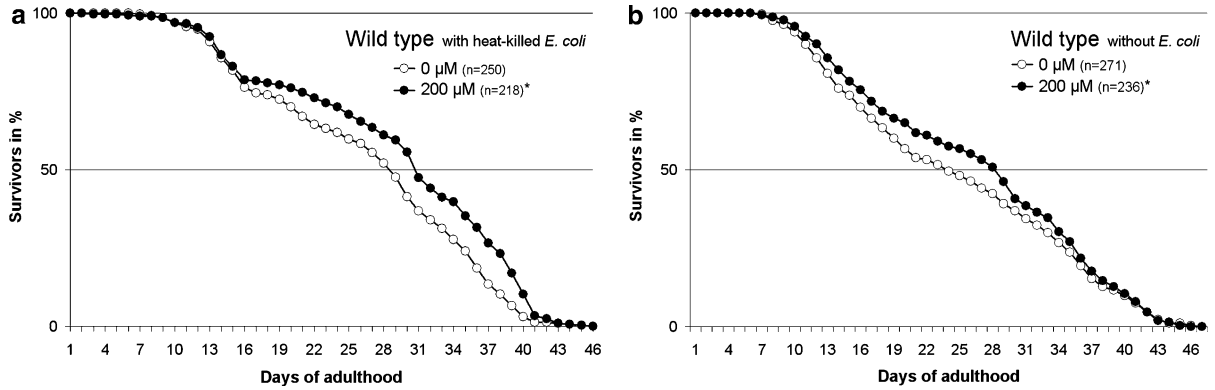


**Fig. 2** Quercetin does not appear to extend *C. elegans* lifespan through antimicrobial effects or due to being a CR mimetic, because it continued to prolong lifespan of wild type when grown on heat-killed OP50 (**a**) or in conditions of food deprivation (**b**). The 6 days old F1 adult wild type nematodes were placed on either control or treatment plates in the presence or absence of dead OP50, respectively. *Graphs* combine 2–3 independent trials, for further details see Table 1, * $P < 0.001$ (log-rank test)

## Influence of quercetin on reproductive capacity and body length

Quercetin-mediated lifespan extension did not alter other fitness parameters. In detail, exposure to quercetin did not affect daily reproductive output. Likewise, total brood size did not change (control: 275, 100 µM quercetin: 278 and 200 µM quercetin: 271) (Fig. 3a). The body length of the nematodes was also not affected by quercetin treatment, averaging 1.31, 1.32 and 1.29 µm in controls, 100 and 200 µM quercetin treated nematodes, respectively (Fig. 3b).

## Studies of the genetic background on quercetin-mediated longevity

Several stress response pathways have been shown to affect *C. elegans* lifespan (Table 2; for background information refer to Tables 3 and 4). To investigate possible interactions between these pathways and quercetin treatment, experiments were performed with the F1 generation of mutant strains exposed to 200 µM quercetin.

### *Quercetin reduces internal oxidative stress and prolongs the lifespan of hypersensitive mutant mev-1*

The *kn1* allele contains a deletion within the *mev-1* gene, a subunit of complex II in the electron transport

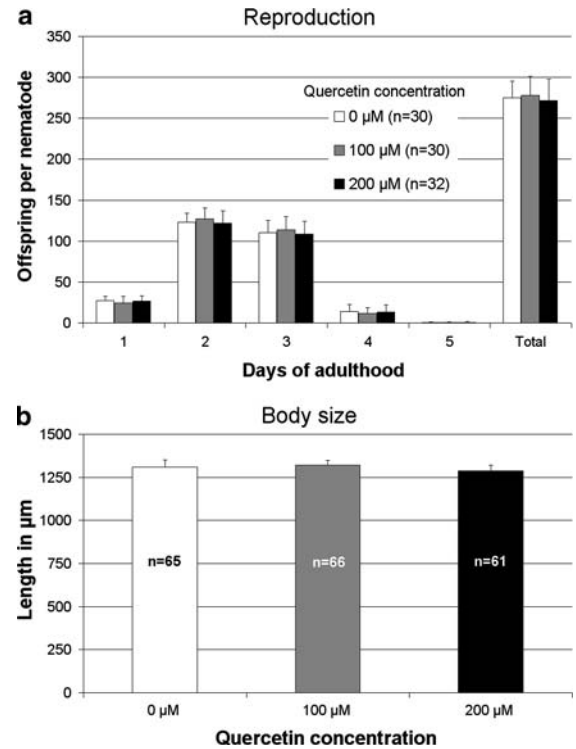


**Fig. 3** Treatment with either 100 or 200 µM quercetin does not alter the reproductive output or growth of the wild type F1 generation. (**a**) Shown are the mean daily offspring numbers per hermaphrodite over the broodperiod and the total broodsize. (**b**) Body size was measured of heat-killed 6-day-old adults using a graduated microscope eyepiece. Statistical analysis was performed by one way ANOVA, *error bars* represent the SEM

**Table 3** Genes not involved in quercetin-mediated lifespan extension and description of their common functions

| | |
|---|---|
| *daf-12* | Promotes gonad-dependent adult longevity (Gerisch et al. 2007) |
| *daf-16* | Encodes the central forkhead transcription factor of the insulin-mediated pathway responsible for general stress-response and dauer formation |
| *jnk-1* | Post-translational positive regulation of DAF-16 (Wolf et al. 2008) |
| *nhr-8* | Active in the xenobiotic defense system (Lindblom et al. 2001) |
| *osr-1* | Mediates survival in hyper-osmotic environments (Solomon et al. 2004) and was found responsible for lifespan extension mediated by blueberry polyphenols (Wilson et al. 2006) |
| *sir-2.1* | Controversially discussed, may (Tissenbaum and Guarente 2001; Wood et al. 2004) or may not (Kaeberlein et al. 2006; Lee and Lee 2006; Houthoofd and Vanfleteren 2006) promote longevity during caloric restriction |
| *skn-1* | Developmental transcription factor, responsible for the expression of various stress genes promoting oxidative stress resistance, shows striking similarities to DAF-16 mediated stress response, so far no regulatory link to IIS has been identified (An and Blackwell 2003; Inoue et al. 2005; Baumeister et al. 2006) |
| *akt-2* | Negative modulator of DAF-16 in IIS (Hertweck et al. 2004; Baumeister et al. 2006) |
| *mev-1* | Mutants showed a significant quercetin-mediated gain in lifespan. This mutant is characterized by an elevated accumulation of endogenous ROS (Ishii et al. 1998) and provides a special test system to prove an antioxidative capacity. It is conceivable that the *mev-1* mutant may respond with a lifespan extension due to antioxidative property of quercetin |

**Table 4** Genes putatively involved in quercetin's lifespan extending mode of action

| | |
|---|---|
| *age-1* & *daf-2* | AGE-1 and DAF-2 are both central players in IIS to inhibit DAF-16 activity. Mutations result in long-lived nematodes, armed with a robust stress-resistance machinery due to missing inhibition of DAF-16 translocation resulting in increased expression of its manifold target genes |
| *sek-1* | MAP2K, part of the MAP kinase pathway, acting downstream of TIR-1 (toll and interleucin receptor) and NSY-1 (MAP3K) and phosphorylates the *C. elegans* p38-family MAP kinase PMK-1, which results in elevated immune response to pathogen infection (Kim et al. 2002). NSY-1/SEK-1/PMK-1 cassette also functions via SKN-1 to control resistance against arsenic (An and Blackwell 2003) |
| *unc-43* | CaMKII ($Ca^{2+}$/calmodulin-dependent protein kinase II) mutations of the *unc-43* gene causes diverse behavioral defects by affecting locomotory activity, altering excitation of three muscle types, and changing the period of the motor output of a behavioral clock (Reiner et al. 1999). UNC-43, in association with voltage-activated calcium channels (UNC-2/UNC-36), presents a neuronal regulating signaling pathway upstream of TIR-1, NSY-1 and SEK-1, which is described to determine asymmetric *str-2* expression in the AWC olfactory neurons (Sagasti et al. 2001) |

chain (Ishii et al. 1998). It is hypersensitive to oxidative stress and displays a reduced lifespan, most likely due to *mev-1* mitochondria overproducing reactive oxygen species (ROS). Quercetin extended mean survival time by 10% in hypersensitive mutant *mev-1* (Fig. 4a, Table 2, for the corresponding medium and maximum survival times, please refer to Supplementary Table 2), indicating that quercetin is capable of improving survival during mild to severe oxidative stress.

*Long-lived and oxidative stress resistant daf-2 and age-1 mutants are not affected by quercetin*

It has been shown that the polyphenol resveratrol can increase lifespan via the sirtuin gene *sir-2.1*, which encodes a histone deacetylase-like protein that integrates the metabolic status and lifespan (Tissenbaum and Guarente 2001; Wood et al. 2004). Quercetin treatment extended lifespan of *sir-2.1(ok434)* (Table 2, Supplementary Table 2), suggesting that quercetin does not modulate this pathway.

Several *C. elegans* transcription factors, including DAF-16, NHR-8 and SKN-1, promote the expression of antioxidant or detoxification enzymes. Quercetin treatment was able to prolong lifespan of *daf-16(mgDf50), nhr-8(ok186)* and *skn-1(zu67)* nematodes (Table 2, Supplementary Table 2), indicating that quercetin may act independently of these genes as well. A similar result was obtained for *jnk-1(gk7)*. Activation of JNK-1, which is localized in the neuronal system, drives the nuclear translocation of

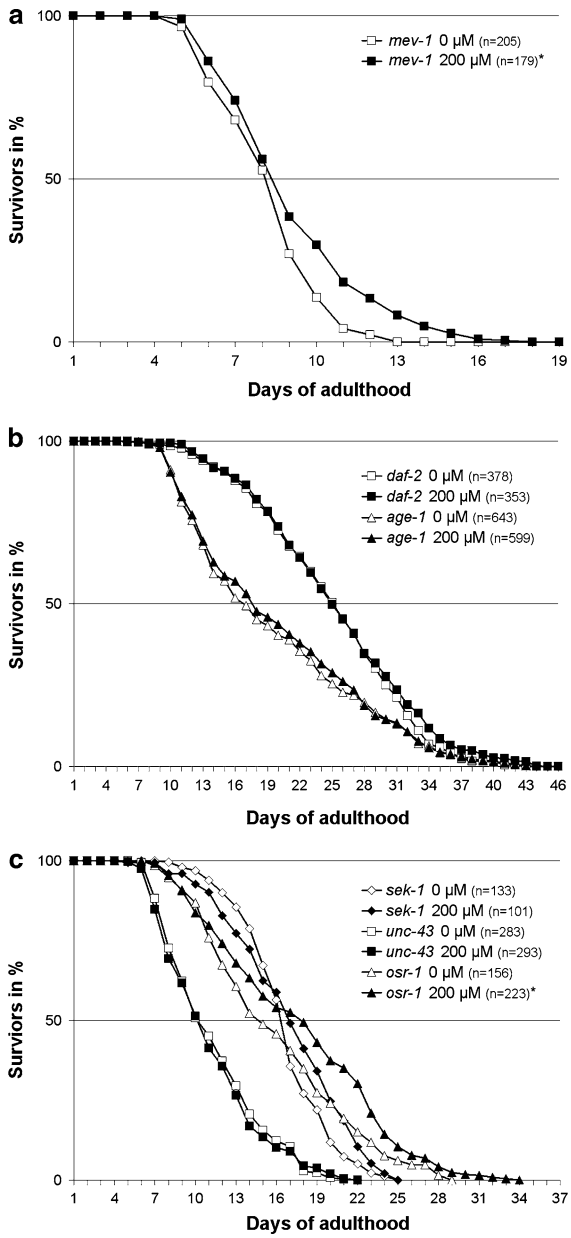


**Fig. 4** Impact of the genetic background on quercetin derived longevity. (**a**) Quercetin extends the life-span of *mev-1(kn1)*, which are hypersensitive against oxidative stress. (**b**) Lifespan of stress resistant and long-lived *age-1(hx546)* and *daf-2(e1368)* mutants could not be further extended by quercetin. (**c**) Similarly, *unc-43(n498n1186)* and *sek-1(ag1)* prevented quercetin induced longevity, whereas *osr-1(rm1)* was found to be significantly susceptible to quercetin. Shown are the survival curves of the F2 generation treated either with 0 or 200 µM quercetin. *Graphs* combine 2–5 independent trials, for further details see Table 2, * $P < 0.001$ (log-rank test)

DAF-16 within peripheral cells (Wolf et al. 2008). Furthermore, the lifespan of *daf-12(m20)* could also be enhanced by quercetin. DAF-12, a member of the steroid hormone receptor superfamily, influences dauer formation downstream of the TGF- and insulin signaling pathways, and affects gonad-dependent adult longevity together with DAF-16 (Gerisch et al. 2007).

In contrast to the aforementioned mutants, quercetin treatment did not prolong the lifespan of two long-lived and oxidative stress-resistant mutants, namely *daf-2(e1368)* and *age-1(hx546)* (Fig. 4b, Table 2). Both genes are key players of the insulin/IGF-I-like signaling (IIS) pathway, and mutations result in a dramatic increase in longevity. DAF-2 is the only insulin/IGF-1 like receptor in *C. elegans*, AGE-1 a catalytic subunit of a phosphoinositide 3-kinase (PI3K), is downstream of DAF-2. Both enzymes act at a similar point in the genetic epistasis pathway for dauer arrest and longevity and negatively regulate the activity of DAF-16. Given that DAF-16 was found to be non essential for lifespan regulation by quercetin, it is perhaps surprising to find other members of the IIS family to be involved in quercetin-mediated longevity.

### *A disrupted calmodulin kinase II (CaMKII) pathway seems to prevent quercetin induced longevity*

Survival in hyperosmotic environments requires the activity of several proteins of the CaMKII pathway, in which OSR-1 is coupled to SEK-1 (MAPKK) through UNC-43 (CaMKII) (Solomon et al. 2004). SEK-1 is also required for the resistance to pathogenic bacteria and oxidative stress by assisting the translocation of cytoplasmic DAF-16 into the nucleus (Kim et al. 2002; Kondo et al. 2005). All three genes were found to be fundamental to the blueberry extract-mediated longevity (Wilson et al. 2006). Similarly, quercetin treatment was not able to prolong the lifespan of *unc-43(n498n1186)* and *sek-1(ag1)* mutants (Fig. 4c, Table 2), strongly suggesting that this flavonoid may act via this pathway. Noteworthy however, was that *osr-1(rm1)* nematodes continued to display robust quercetin induced longevities (Fig. 4c, Table 2).

### Verification of genetic background results in thermotolerance assays

Because *sek-1(ag1)* mutants are very difficult to culture (a large proportion of nematodes die due to internal hatch) the results observed using the mutant strain test were re-assessed by thermal resistance testing (Fig. 5). The thermal resistance was significantly increased in wild type animals treated with 200 μM quercetin (according to Saul et al. 2008), a trend that could not be observed in *age-1(hx546), daf-2(e1368), sek-1(ag1)* and *unc-43(n498n1186)* mutants. These findings support the lifespan assays and strengthen the notion that quercetin mediated longevity is driven by *age-1, daf-2, sek-1* and *unc-43*.

## Discussion

Flavonoid polyphenols, including quercetin, have been shown to exert beneficial effects in a multitude of human diseases. Their biological actions have been mostly attributed to their antioxidant properties (Williams et al. 2004). On the other hand there is increasing evidence that they influence molecular signaling, both in mammals and invertebrates (Matter et al. 1992; Kong et al. 2000; Schroeter et al. 2001; Spencer et al. 2003). The basis for this assumption is that flavonoids and their metabolites are unlikely to act as major antioxidants in vivo, since the concentrations of other antioxidants (e.g., ascorbic acid, α-tocopherol) are much higher (Williams et al. 2004). Therefore, the precise mechanism by which quercetin exerts its beneficial effects remains unclear.

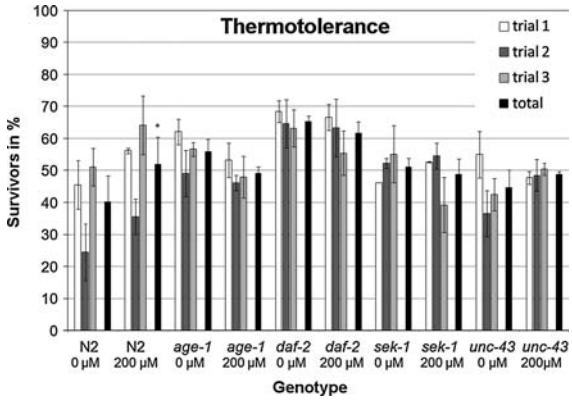


**Fig. 5** Treatment with 200 μM quercetin significantly improved thermotolerance in wild type N2 nematodes but not in *age-1(hx546), daf-2(e1368), sek-1(ag1)* and *unc-43(n498n1186)*. Shown are the 8 h-survival rates for N2 and *unc-43(n498n1186)*, 9 h-survival rates for *age-1(hx546)*, 10 h-survival rates for *daf-2(e1368)* and the 6 h-survival rate for *sek-1(ag1)* exposed to 35°C. The experiment was conducted with 6 days old adult nematodes treated with 0 or 200 μM quercetin, respectively. Shown are three independent trials and the resulting average values with 22–144 animals/experiment; *error bars* represent SEM; total number of animals tested (control/treated): N2 251/273, *age-1(hx546)* 303/337, *daf-2(e1368)* 305/309, *sek-1(ag1)* 98/111, *unc-43(n498n1186)* 299/341. * $P < 0.001$ (chi square test)

### Exposure scenarios

In the recent years three groups have published quercetin-modulated lifespan data with *C. elegans*. For instance, Wu et al. (2002) could not identify life-prolonging properties of quercetin. In contrast, Kampkötter et al. (2008) and Saul et al. (2008) showed a significant lifespan extension. This contradiction may stem from differences in methodological approaches, for instance the use of solid (Wu et al. 2002; Saul et al. 2008) versus liquid (Kampkötter et al. 2008) culture media or utilizing only a single generation of nematodes (Wu et al. 2002; Kampkötter et al. 2008). This study used agar plates and exposures spanning up to three generations. Indeed, our study confirmed that quercetin did not significantly expand the lifespan of the 1st generation (P0) of nematodes exposed on solid media. Only when nematodes were exposed throughout their entire life (i.e., including embryonic development) a significant lifespan extension could be observed. It is conceivable that the uptake and exposure dynamics are markedly different in liquid cultures thus explaining why effects may have been observed in the first generation. However, by using nematodes of the F1 and F2 generation, this study maintained some key advantages of cultures on solid medium, namely the ease of scoring phenotype alterations, reproductive capacity and growth rate, and the avoidance of hypoxia and stress.

### Underlying mechanisms

#### *Antimicrobial property or caloric restriction*

Quercetin does not exert longevity, at least primarily, through antimicrobial properties. Although trypanosome mortality increased following treatment with

100 μM quercetin (Mamani-Matsuda et al. 2004), this could not be confirmed for selected gram-positive and -negative strains exposed to 330 μM quercetin (Gatto et al. 2002). Feeding *C. elegans* with heat-killed *E. coli* dramatically prolonged lifespan; however, upon quercetin treatment this effect was further magnified. A similar effect was observed, when complete food deprivation was applied to post-reproductive nematodes. Therefore, unlike polyphenol resveratrol (Ingram et al. 2006; Chen and Guarente 2007), lifespan extension of quercetin cannot be attributed to antimicrobial effects or due to CR mimetic properties.

### *Impact on fitness parameters and antioxidant capacity*

The disposable soma theory, sensu Kirkwood (1977), states that a body must budget the available amount of energy for maintenance, growth and reproduction. Therefore, CR must have profound and complex impact upon reproductive health (Martin et al. 2008; Holliday 1989) and can also cause retardation of growth (McCay et al. 1935). Here, however, nematodes fed ad libitum in the presence or absence of quercetin showed no difference in body size or reproductive performance. This indicates that, at least for these fitness parameters, the disposable soma theory does not apply, thus supporting the notion that quercetin is not a CR mimetic.

It is well established that quercetin reduces ROS generation under thermal stress conditions (Kampkötter et al. 2007a). Moreover, quercetin was found to increase resistance to heat and oxidative stress (Kampkötter et al. 2007b; Saul et al. 2008), which is likely a direct consequence of its antioxidative properties. In addition, quercetin was able to repress the expression of the antioxidative enzymes *sod-3* and *gst-4* in *C. elegans* exposed to heat or oxidative stress (Kampkötter et al. 2007b, 2008).

### *Genetic parameters*

In total, 13 mutant strains were used, each selected for their involvement in well described stress and/or longevity pathways. Table 3 lists genes which were rejected as main mediators of quercetin induced longevity.

Four *C. elegans* mutants were identified where quercetin treatment did not prolong lifespan, nor increase thermotolerance, namely *age-1, daf-2, unc-43*, and *sek-1*. Table 4 provides an overview and short summary of known function(s). The results appear perhaps, in parts, unexpected and thus deserve a close inspection. For example, how is it possible that DAF-2 and AGE-1, both key players in IIS and inhibitors of DAF-16 activity, seem to be responsible for quercetin-dependent lifespan extension, while *daf-16* isn't? Similarly, why is *daf-16* not involved, even though Kampkötter et al. (2007b, 2008) showed a significant sub-cellular nucleus translocation of DAF-16 following the incubation with quercetin? It has been shown that the translocation of DAF-16, though essential, remains inactive if cofactors are missing (Henderson et al. 2006; Berdichevsky et al. 2006). This is at least in partial agreement with findings by Kampkötter et al. (2007b, 2008) who showed that both DAF-16 targets, *sod-3* and *gst-4*, were not induced, but repressed after quercetin treatment.

In addition to this, the findings of this study can be explained by a quercetin-dependent inhibition of DAF-2 and AGE-1. If DAF-2 and AGE-1 are inhibited, DAF-16 is translocated into the nucleus as described by Kampkötter (2007b, 2008). But results presented here (Fig. 4b, Table 2) show that the lifespan extension is independent of *daf*-16, Hence, lifespan extension and DAF-16 translocation coincide, but may not necessarily be causally connected.

How may the quercetin-dependent lifespan extension and increased stress resistance in *daf-16* mutants be explained as observed by Saul et al. (2008)? This finding can be interpreted by the antioxidative properties of quercetin, as already discussed with the *mev-1* mutants. Short-lived *daf-16* mutants are hypersensitive to both thermal and oxidative stress, because they lack the central IIS transcription factor and must carry the burden of elevated internal oxidative stress and down-regulated innate immune response (Chávez et al. 2007). The elevated antioxidative activity of quercetin attenuates the oxidative stress and thereby causes a physiological gain, resulting in a partial "rescue" to native lifespan levels.

On the other hand, *daf-2* as well as *age-1* long-lived mutants are armed with robust stress-resistance machineries. It is conceivable that the positive effect of quercetin could be masked, since oxidative stress

resistance and life extension may already be close to their maxima. And how do the *akt-2* results fit into this picture? Because AKT-2 is located downstream of DAF-2 and AGE-1 in IIS, one may expect *akt-2* not to respond to quercetin. However, our results reveal the opposite. Hertweck et al. (2004) and Tullet et al. (2008) offer a possible answer. They report that SGK-1 is a critical control factor in stress response and lifespan, which is mediated through IIS rather than AKT/PKB kinase complex. While SGK-1 is the mediator for stress response, AKT/PKB is more important for dauer formation. Hertweck et al. (2004) show that *akt-2* mutants are less stress-resistant than *sgk-1* mutants and in consequence the antioxidative property of quercetin may still expand the lifespan.

The story is made even more complex by *unc-43* and *sek-1* as they seem to be involved with the molecular mechanism of the quercetin response. Although both genes were shown to be essential in blueberry polyphenol-derived longevity of *C. elegans* (Wilson et al. 2006), their function cannot be explained by the antioxidative properties of the polyphenols alone. UNC-43 is a type II $Ca^{2+}$/Calmodulin-dependent kinase (CaMKII) and SEK-1 a MAP kinase (MAP2K) that plays a major role in innate immunity. Both act together in a pathway to determine neuronal cell fate (Millet and Ewbank 2004; Young and Dillin 2004). The quercetin action in *unc-43* and *sek-1* mutants is most likely mediated through altered molecular signaling via the p38 MAP kinase pathway and by sensory perception through olfactory neurons.

Figure 6 is a hypothetical model that depicts how quercetin is involved in lifespan regulation. The left side displays the MAP kinase and $Ca^{2+}$/Calmodulin pathways which leads to an elevated immune response and longevity through altering sensory perception. The middle section shows how the antioxidative properties of quercetin may reduce internal ROS accumulation and trigger an extension of lifespan. The mechanism on the right side of the model remains ambiguous. One possible explanation is that the stress resistance of *age-1(hx546)* and *daf-2(e1368)* mutants counteract the quercetin effect. The effects mediated through the *unc-43*/*sek-1* pathway may be masked in these mutated animals. If true, the

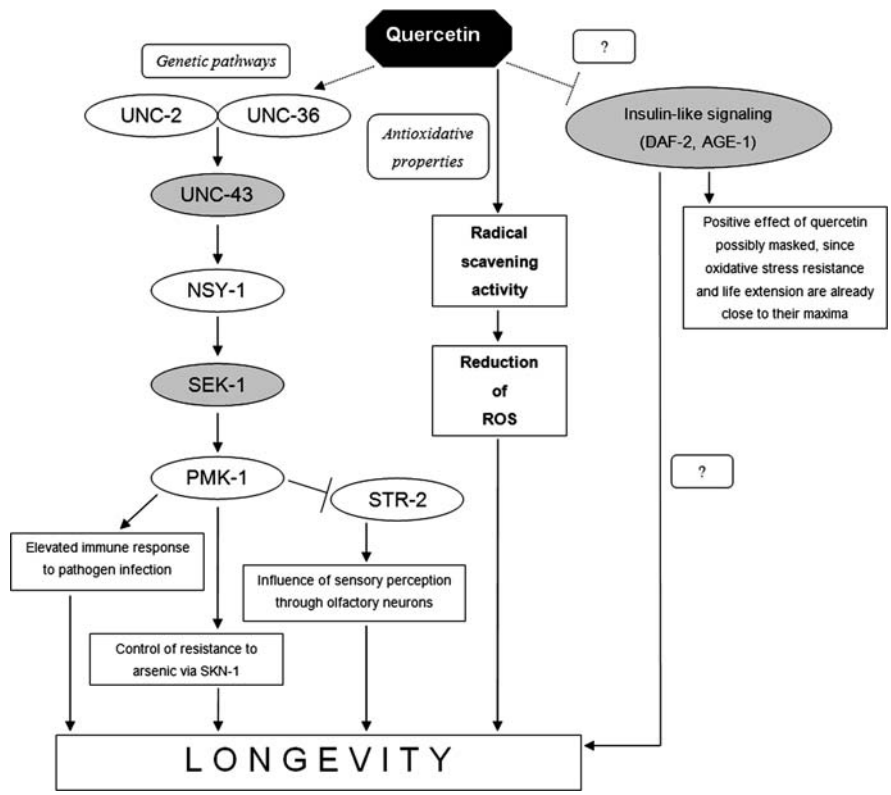


**Fig. 6** Proposed model of quercetin's mode of action. *Oval elements* highlighted in *gray* represent experimentally proven modulators, *oval elements* highlighted in *white* have yet to be verified. For more information see text

latter explanation raises the question if lifespan assays with different mutant strains can be compared and interpreted without considering specifics of the underlying internal physiology.

## Conclusion

Quercetin seems to display no transgenerational effects in lifespan extension. Although antimicrobial effects and caloric mimicking can be ruled out as underlying mechanism, this study has unraveled a diversity of modes of action upon quercetin exposure. First, it has strong antioxidative properties, which can explain the lifespan extension in stress-sensitive mutants, such as *daf-16* and *mev-1*, but also explain the reversal of the gain in the long-lived, stress-resistant *age-1* and *daf-2*. Second, the network of modulated signaling pathways is complex, as presented by the p38 MAP kinase and CaMKII pathways as well as various options of interaction. Although further detailed investigations are called for, our results provide first tantalizing insights on quercetin's multifaceted function in vivo.

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