Blueberry polyphenols increase lifespan and thermotolerance in *Caenorhabditis elegans*

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Summary

The beneficial effects of polyphenol compounds in fruits and vegetables are mainly extrapolated from in vitro studies or short-term dietary supplementation studies. Due to cost and duration, relatively little is known about whether dietary polyphenols are beneficial in whole animals, particularly with respect to aging. To address this question, we examined the effects of blueberry polyphenols on lifespan and aging of the nematode, *Caenorhabditis elegans*, a useful organism for such a study. We report that a complex mixture of blueberry polyphenols increased lifespan and slowed aging-related declines in *C. elegans*. We also found that these benefits did not just reflect antioxidant activity in these compounds. For instance, blueberry treatment increased survival during acute heat stress, but was not protective against acute oxidative stress. The blueberry extract consists of three major fractions that all contain antioxidant activity. However, only one fraction, enriched in proanthocyanidin compounds, increased *C. elegans* lifespan and thermotolerance. To further determine how polyphenols prolonged *C. elegans* lifespan, we analyzed the genetic requirements for these effects. Prolonged lifespan from this treatment required the presence of a CaMKII pathway that mediates osmotic stress resistance, though not other pathways that affect stress resistance and longevity. In conclusion, polyphenolic compounds in blueberries had robust and reproducible benefits during aging that were separable from antioxidant effects.

Key words: Aging; blueberry; *Caenorhabditis elegans*; lifespan; proanthocyanidin; thermal stress.

Introduction

Plants synthesize an array of chemical compounds that are not involved in their primary metabolism. These `secondary compounds' instead serve a variety of ecological functions, ultimately to enhance the plant's survival during stress (Winkel-Shirley, 2002). In addition, these compounds may be responsible for the beneficial effects of fruits and vegetables on an array of health-related measures (Liu, 2003). Previous research suggested that the combination of antioxidant/anti-inflammatory polyphenol compounds found in fruits and vegetables may show efficacy in reversing aging (Joseph et al., 2005). In particular, 8-week dietary supplementation with spinach, strawberry or blueberry (BB) extracts was effective in reversing age-related deficits in neuronal function and behavior in aged (19 month) F344 rats (Joseph et al., 1999). However, only the BB-supplemented group exhibited improved performance on tests of motor function. Specifically, the BB-supplemented group displayed improved performance on two motor tests that rely on balance and coordination, rod walking and the accelerating rotarod, while none of the other supplemented groups differed from controls on these tasks. A subsequent study using a BB-supplemented diet replicated these findings (Youdim et al., 2000). Results from both studies indicated that the significant effects of BB on behavior were due to a multiplicity of actions, in addition to those involving antioxidant and anti-inflammatory activity.

To further investigate the effects of blueberries on parameters related to aging, we designed a study to determine whether BB polyphenols can delay aging and prolong lifespan in a whole organism. For these studies, we required an organism with relatively short lifespan that could be assayed reproducibly and robustly, and for which the genetic and environmental factors affecting lifespan were well defined. The experimental organism that could best accommodate these requirements was the nematode, *Caenorhabditis elegans*, which has become a popular model for studying aging and longevity, due to its short 2- to 3-week lifespan, rapid generation time and experimental flexibility (Guarente & Kenyon, 2000).

Studies have shown that specific genetic and environmental factors influence aging and lifespan of *C. elegans* (Gems & Riddle, 2000; Garigan et al., 2002; Herndon et al., 2002). In addition, aspects of aging are similar between nematodes and mammals, including humans. For instance, sarcopenia, the loss of muscle mass, is a major feature of aging in humans and *C. elegans* and contributes to aging-related behavioral declines in both (Herndon et al., 2002; Glenn et al., 2004). Second, calorie restriction, the only known intervention that successfully extends lifespan in mammals, can prolong *C. elegans* lifespan (Lakowski & Hekimi, 1998). Finally, oxidative stress appears to...
be a major factor limiting lifespan in both *C. elegans* and humans (Larsen, 1993; Finkel & Holbrook, 2000). These findings show that studies of aging in *C. elegans* provide useful stepping stones for identifying genes and compounds that can prolong lifespan in humans.

Here, we report that treatment with total BB polyphenols, or a proanthocyanidin (PAC)-enriched fraction from BB, could prolong adult lifespan and delay aging in *C. elegans*. These treatments increased thermotolerance, but did not improve resistance to oxidative stress. BB treatment was correlated with reduced basal levels of *hsp* mRNA, indicating these compounds had direct or indirect effects on gene expression. Our genetic analysis indicated that BB polyphenols may act through a CaMKII signaling pathway to affect *C. elegans* lifespan. These findings show that natural compounds from blueberries can provide antiaging benefits in vivo in an intact organism.

## Results

**Blueberry polyphenols extend *C. elegans* lifespan**

Adult wild-type animals grown under our standard laboratory conditions at 25 °C have a mean lifespan of 12.7 days and average maximum lifespan of 19.7 days. On media containing either crude BB extract (*Vaccinium angustifolium*) or a C18 column fraction containing their bulk polyphenols, mean lifespan of wild-type animals was lengthened by 28% (Fig. 1A, Table 1). Maximum lifespan was also increased by an average of 14% in all trials (*P* = 0.007; 0 µg mL⁻¹, *n* = 17 trials; 200 µg mL⁻¹, *n* = 16 trials). Similar results were obtained using a crude extract of a different species of blueberry (*Vaccinium ashei*) (not shown).

Lifespan in *C. elegans* is affected by temperature (Gems et al., 1998). Animals grown at 25 °C have shorter adult lifespan than animals grown at 15 °C, although both temperatures are considered within the normal range for *C. elegans*. The effects of BB polyphenols on *C. elegans* lifespan were also temperature dependent. BB treatment prolonged lifespan in animals grown at 25 °C or 20 °C, but no significant benefits were observed upon lifespan at 15 °C (Table 1).

By several measures, BB treatment slowed aging in *C. elegans*, rather than simply improving survival at old age. One measure of aging in *C. elegans* is the speed of pharynx contraction, or pumping (Bolanowski et al., 1981; Huang et al., 2004). Young adult animals pump 250–300 times per minute and pumping declines gradually with increasing age. BB treatment was associated with higher pumping rates at adult days 8 and 10 (Fig. 1B).

BB treatment also delayed the accumulation of aging-related cellular damage. One marker for cellular damage during aging is the intracellular level of lipofuscin, autofluorescent material that accumulates in aging cells. (Brunk & Terman, 2002). Lipofuscin levels increase with aging in many organisms, including *C. elegans* (Hosokawa et al., 1994). We examined lipofuscin levels in the intestines of control and BB-treated adult day 16 animals. Consistent with the expectation that BB polyphenols could slow aging, intestinal lipofuscin levels were reduced 20% in BB-treated animals. Consistent with the expectation that BB polyphenols could slow aging, intestinal lipofuscin levels were reduced 20% in BB-treated animals.

### Fig. 1 Blueberry polyphenols extend lifespan and slow aging in *Caenorhabditis elegans*. (A) Treatment with blueberry polyphenols (67 µg mL⁻¹ (orange) or 200 µg mL⁻¹ (blue)) extended mean lifespan in fem-1(hc17) animals grown at 25 °C (untreated control, green). (B) Blueberry polyphenols slowed the decline in pharynx pumping during aging. Open circles, untreated; filled circles, treated with 200 µg mL⁻¹ blueberry polyphenols. Average pumping rate (pumps per minute) in 14 animals scored for two trials; error bars indicate SEM among individual animals scored; t-test, untreated vs. treated, day 8, *P* = 0.023, day 10, *P* = 0.004.

| Genotype     | Treatment | Control          | Treated          | Change |
|--------------|-----------|------------------|------------------|--------|
| fem-1(hc17)  | BB crude  | 12.1, 0.63 (73)  | 16.6, 0.46 (46)  | 1.37*  |
| fem-1(hc17)  | “         | 12.7, 0.23 (362)| 16.2, 0.23 (350)| 1.28** |
| fem-1(hc17)  | “         | 16.1, 0.26 (237)| 18.7, 0.26 (210)| 1.16** |
| fem-1(hc17)  | “         | 22.9, 0.74 (44) | 22.2, 0.92 (38) | 0.97   |
| fem-1(hc17)  | “         | 13.1, 0.66 (60) | 17.5, 0.56 (66) | 1.34** |
| fem-1(hc17)  | “         | 13.1, 0.66 (60) | 18.1, 0.46 (70) | 1.38** |
| mev-1(kn1)   | BB        | 9.1, 0.28 (101)| 8.6, 0.28 (98)  | 0.95   |
| sir-2(ok34)  | BB        | 12.2, 0.54 (76) | 15.9, 0.58 (68)| 1.30** |
| osr-1(m1)    | BB        | 16.3, 0.31 (151)| 15.7, 0.37 (78)| 0.96   |
| unc-43(n1186)| BB        | 13.1, 0.31 (108)| 12.8, 0.48 (61)| 0.98   |
| sek-1(ga1)   | BB        | 11.7, 0.73 (33) | 10.9, 0.64 (26)| 0.93   |
| daf-16(mdF50)| BB        | 10.2, 0.38 (53) | 12.3, 0.34 (42)| 1.21** |
| “          | “         | 11.5, 0.38 (53) | 12.7, 0.38 (53)| 1.10*  |
| “          | “         | 11.5, 0.38 (53) | 14.7, 0.38 (53)| 1.28** |
| skn-1(2u67) | BB        | 9.9, 0.52 (36)  | 13.1, 0.54 (43)| 1.32** |

*P ≤ 0.05, **P ≤ 0.001, log-rank. Aging assays performed at 25 °C, unless noted, except mev-1(kn1) which was carried out at 25 °C and 20 °C, with or without FUDR, with similar results. Treatments: crude at 1.5 mg mL⁻¹; BB, BB polyphenols at 200 µg mL⁻¹; amp, ampicillin at 100 µg mL⁻¹.
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in BB-treated animals, compared with controls (Fig. 2A,B). 4-Hydroxynonenol (4-HNE) is a lipid peroxidation product that accumulates with aging in many animals (Chiarpotto et al., 1995). In *C. elegans*, a correlation between 4-HNE levels and aging has not yet been demonstrated, although levels of 4-HNE-modified protein have been correlated with lifespan (Ayyadevara et al., 2005). BB treatment also reduced levels of 4-HNE in 14-day adults (Fig. 2C,D).

**BB treatment delayed aging-related increase in heat-shock protein mRNA levels**

We next examined the effects of BB treatment on a transcriptional marker of aging. Several independent analyses of gene expression during aging in *C. elegans* have revealed that heat-shock protein mRNA levels increase with age (Lund et al., 2002; Golden & Melov, 2004). Using real-time PCR, we confirmed that expression of *hsp*-12.6, -16.1, -16.49 and -70 increased markedly in control animals between days 0 and 4 of adulthood under our laboratory conditions (Fig. 3). BB treatment blocked this increase and mRNA levels for these *hsp*s remained at basal levels through to adult day 4. From this experiment, we conclude that BB polyphenols delayed changes in *hsp* expression that are normally associated with aging in *C. elegans*, consistent with the observation that BB treatment delayed morphological features of aging.

**Proanthocyanadin components of blueberries enhance longevity**

Blueberries contain a mixture of different polyphenol compounds that can be separated into three primary fractions enriched in either anthocyanins (ATC), proanthocyanidins (PAC) or hydroxycinnamic esters, mainly chlorogenic acid (CA). Major components of each of these fractions have been shown to confer significant antioxidant activity and ATC can protect cells against oxidative stress in vitro (Youdim et al., 2000; Zheng & Wang, 2003). To determine which fraction(s) delayed aging, we assayed their effects on *C. elegans* lifespan. Neither the ATC-enriched fraction nor purified CA had any significant effect on longevity (Fig. 4A) (control, 12.0 ± 0.34 days; ATC, 11.7 ± 0.36 days, $P = 0.96$ vs. control; CA, 11.7 ± 0.39 days, $P = 0.99$). However, treatment with the PAC-enriched fraction increased lifespan to a similar extent as the starting BB polyphenol mixture or the remixed fractions (Fig. 4A,B) (PAC, 14.4 ± 0.36 days, $P < 0.0001$ vs. control; start, 14.8 ± 0.36 days, $P < 0.0001$; remix, 14.0 ± 0.48 days, $P < 0.0001$; complete statistics for lifespan trials with PAC-enriched fraction are presented in Table 2). Thus, components
were linked, as thermotolerance was increased by treatment 
with BB polyphenols was correlated with a 2.5-fold increase in 
significantly by BB treatment. In wild-type animals, treatment 
resistance of BB-treated animals. Thermotolerance was increased 
& Riddle, 2003). To test this possibility, we examined stress 
conditions of heat or oxidative stress (Lithgow 
Fig. 4 A PAC-enriched fraction of BB contained components sufficient to 
extend Caenorhabditis elegans lifespan. The total blueberry polyphenols were 
fractionated by C18 and Sephadex LH20 to produce fractions enriched in 
anthocyanins (ATC), proanthocyanidins (PAC) or chlorogenic acid (CA). 
Purchased, purified CA was used for CA experiments. Each fraction was 
asayed for effects on C. elegans longevity at a concentration of 67 µg mL⁻¹, 
similar to that in the complete extract. (A) Only the PAC-enriched fraction 
(orange) prolonged lifespan. No longevity benefits were observed for the 
ATC-enriched fraction (purple) or purified CA (blue); 0 µg mL⁻¹, n = 97 
animals, 2 trials; ATC, n = 99, 2; CA, n = 91, 2; PAC, n = 88, 2. Complete 
statistics for PAC trials are presented in Table 2. (B) The reconstituted extract, 
produced by mixing equal mass ratios of fractions in (A) also conferred the 
same lifespan extension as the starting mix of polyphenols (remix, n = 40 
animals, 1 trial; start, n = 98, 2).

Effects of blueberry polyphenols on intrinsic stress resistance

One possible explanation for the beneficial effects of BB polyphenols 
on aging in C. elegans is that these compounds were able to 
increase cellular stress resistance. In several studies, increased 
longevity was closely associated with improved survival under 
conditions of heat or oxidative stress (Lithgow et al., 1995; Muñoz 
& Riddle, 2003). To test this possibility, we examined stress 
resistance of BB-treated animals. Thermotolerance was increased 
significantly by BB treatment. In wild-type animals, treatment 
with BB polyphenols was correlated with a 2.5-fold increase in 
16-h survival at 35 °C (Fig. 5A). Lifespan and thermotolerance 
were linked, as thermotolerance was increased by treatment 
with the PAC-enriched fraction, but not the ATC-enriched fraction 
or CA (Fig. 5D).

Interestingly, BB treatment did not improve survival under mild 
to severe oxidative stress. Resistance to oxidative stress was 
examined by exposing animals to hydrogen peroxide or paraquat, 
an intracellular free-radical-generating compound. BB treatment 
was not associated with any increase in survival in the 
presence of hydrogen peroxide or paraquat (Fig. 5B,C). To fur-
ther examine whether BB polyphenols could protect against 
acute oxidative stress, we examined mev-1(kn1) animals, which have 
a mutation in the cytochrome b large subunit of mito-
chondrial complex II (Ishii et al., 1998). This mutation results in 
overproduction of superoxide and increased oxidative stress, 
along with accelerated aging and reduced lifespan (Hosokawa 
et al., 1994; Senoo-Matsuda et al., 2001). Consistent with the 
finding that BB did not protect against extrinsic oxidative stress, 
BB treatment also did not significantly affect lifespan of mev-1(kn1) 
animals (Table 1).

Effects of BB treatment on expression of stress-
inducible genes

We next considered the possibility that BB treatment acted as 
a mild stressor that induced expression of protective enzymes 
and this increased gene expression delayed aging (Lithgow et al., 
1995). To investigate this possibility, we examined the effects 
of BB treatment on the expression of several stress-inducible 
genes. Although BB treatment improved thermotolerance, 
the mRNA levels for a number of stress-inducible genes were not 
altered by BB treatment in any consistent way (Supplementary 
Table S1). In this analysis, we were careful to select stress-inducible 
genes regulated by several different pathways. In addition, the 
earlier experiment showed that mRNA levels of inducible hsp- 
s were not increased in BB-treated animals (Fig. 3), nor was BB 
treatment associated with consistently greater hsp mRNA induc-
tion following heat-shock compared with untreated controls 
(Supplementary Table S2). Together, these gene expression 
analyses show that BB treatment did not cause a general induc-
tion of stress-response genes under normal growth conditions, 
but appeared to increase overall health, which may have promoted

Table 2 Effect of PAC-enriched fraction of BB polyphenols on fem-1(hc17) 
adult lifespan at 25 °C, results for individual and combined trials

| Trial | Treatment | Adult lifespan, 25 °C (mean, SE) | (log-rank) P vs. control |
|-------|-----------|----------------------------------|-------------------------|
| 1     | Control   | 12.2 days, 0.47 (40)             |                        |
| PAC 67 µg mL⁻¹ | 13.4 days, 0.66 (29)             | 0.063                   |
| PAC 200 µg mL⁻¹ | 13.2 days, 0.53 (33)             | 0.171                   |
| 2     | Control   | 11.8 days, 0.47 (57)             |                        |
| PAC 67 µg mL⁻¹ | 14.8 days, 0.42 (59)             | < 0.0001                |
| PAC 200 µg mL⁻¹ | 14.0 days, 0.43 (59)             | 0.0026                  |
| 1 + 2 | Control   | 12.0 days, 0.34 (97)             |                        |
| PAC 67 µg mL⁻¹ | 14.4 days, 0.36 (88)             | < 0.0001                |
| PAC 200 µg mL⁻¹ | 13.7 days, 0.34 (92)             | 0.0008                  |
greater survival under thermal stress. However, we cannot rule out the possibility that BB polyphenol treatment may increase expression levels of stress-response genes during old age.

**Blueberry polyphenols do not appear to prolong lifespan through antimicrobial effects**

One contributor to late-age mortality in *C. elegans* is the detrimental effect of the bacterial food source (Gems & Riddle, 2000; Garigan *et al*., 2002). Accordingly, *C. elegans* lifespan could be increased approximately 30% when bacterial growth was arrested by ampicillin (Table 1). We observed that lifespan of BB-treated wild-type animals was not further extended by the presence of ampicillin to arrest bacterial growth (Table 1), indicating that BB may have extended lifespan by relieving microbial stress. To investigate this possibility, we first tested whether the BB polyphenols that prolong lifespan inhibited bacterial growth. We monitored growth of the bacterial lawn under conditions identical to *C. elegans* aging assays, 11 days on NGM agar at 25 °C, in the presence or absence of BB. During this period, untreated bacterial lawns completed approximately one population doubling, while bacterial growth was arrested after ampicillin treatment. In contrast, BB polyphenols had no effect on bacterial population growth at the doses tested in lifespan assays (Fig. 6A). Furthermore, *C. elegans* nematodes grown on ampicillin-treated bacteria displayed no increase in thermotolerance, nor did the combination of ampicillin and BB together enhance thermotolerance (Fig. 6B). Together these findings show that ampicillin and BB polyphenols had distinct effects during aging, supporting the conclusion that BB polyphenols do not extend *C. elegans* longevity through simple antimicrobial effects.
Genetic requirements for increased survival from BB treatment

Pathways for induction of stress-response genes that affect lifespan have been identified in C. elegans. Because BB treatment might improve survival by acting through these genes, we examined whether mutations in the four major stress response and longevity pathways impaired the ability of BB to prolong lifespan. The premise of these experiments was that BB treatment would not extend lifespan in mutant animals missing a gene required for BB’s beneficial effects.

Treatment with the polyphenol, resveratrol, or related compounds, can increase C. elegans lifespan through sir-2.1, which encodes a histone deacetylase-like protein that integrates metabolic status with lifespan (Tissenbaum & Guarente, 2001; Wood et al., 2004). BB treatment extended lifespan of sir-2.1(ok434) animals which lacked sir-2.1 gene activity, showing that BB polyphenols and resveratrol did not act through the same mechanism to increase C. elegans lifespan (Table 1).

Survival in hyperosmotic environments requires the activity of a novel protein, OSR-1, which couples to SEK-1/MAPKK through UNC-43/CaMKII (Solomon et al., 2004). SEK-1/MAPKK is also required for resistance to pathogenic bacteria and oxidative stress (Kim et al., 2002; Kondo et al., 2005). BB treatment did not prolong lifespan of osr-1(tm1) animals, suggesting that BB polyphenols might act through osr-1 (Fig. 7A, Table 1). Similarly, BB treatment did not affect lifespan of sek-1(ag1) or unc-43(t1186) animals (Fig. 7B, C, Table 1). These results implicate the OSR-1/UNC-43/SEK-1 pathway as a target for BB polyphenols in C. elegans.

In C. elegans, two transcription factors, DAF-16 and SKN-1, promote expression of antioxidant or detoxification enzymes. The DAF-16/FOXO transcription factor promotes expression of genes that confer extended longevity and enhanced stress resistance (Lee et al., 2003; Murphy et al., 2003). SKN-1, which is related to vertebrate Nrf proteins, promotes expression of detoxification enzymes in response to oxidative stress (An & Blackwell, 2003). BB treatment prolonged lifespan of both daf-16(mdg50) and skn-1(zeus67) animals, showing that BB may act independently of these genes (Table 1, Supplementary Fig. S1A). BB treatment also significantly improved thermotolerance in daf-16(mdg50) animals (Supplementary Fig. S1B). Interestingly, BB and ampicillin had additive effects on daf-16(mdg50) lifespan, further supporting the conclusion that BB polyphenols do not act through simple antimicrobial effects (Fig. 6C).

Discussion

Dietary consumption of compounds in blueberries and other fruits and vegetables can attenuate age-related declines in several physiological and functional indices (Joseph et al., 2005). Using the short-lived nematode, C. elegans, we have established a genetic system to examine the effects of BB polyphenols upon longevity and aging. This work shows that treatment with BB polyphenols, or a PAC-enriched fraction alone, produced moderate extensions of both the mean and average maximum adult
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Figure 7: Increased lifespan from blueberry polyphenol treatment is blocked by mutations in the osmotic stress resistance pathway. Lifespan in osr-1(rm1) (A), sek-1(ag1) (B), and unc-43(n1186) (C) was not lengthened by treatment with 200 µg mL⁻¹ blueberry polyphenols. Lifespan survival statistics are contained in Table 1.

lifespan as well as improved thermotolerance. While this effect was modest as compared with mutations in longevity genes, it was significant, reliable and robust (Kenyon et al., 1993). Finally, we showed that the beneficial effects of BB treatment appear to require the activity of the osr-1 pathway that also governs osmotic stress resistance.

By using C. elegans for these experiments, a genetic analysis of the beneficial effects of BB was possible. We showed that BB could not protect animals against the elevated oxidative stress imposed by the mev-1(kn1) mutation, consistent with our finding that BB also did not protect wild-type animals against oxidative stress from paraquat or hydrogen peroxide treatments. In addition, we found that three stress-response pathways were dispensable for the beneficial effects of BB polyphenols upon lifespan. These pathways were represented by: (i) sir-2.1, which promotes longevity during calorie restriction, (ii) daf-16, which promotes longevity and stress resistance, and (iii) skn-1, which promotes oxidative stress resistance. Specifically, BB treatment was able to extend lifespan of animals lacking any of these three genes. In contrast, BB treatment did not prolong lifespan of animals with defects in the osr-1/unc-43/sek-1 pathway that promotes resistance to osmotic stress. One interpretation of this latter finding is that the effects of BB treatment are mediated, at least in part, through these genes. Alternatively, mutations in the osr-1 pathway could place animals under some stress that is not affected by BB treatment. To date, osr-1 pathway mutants have not been thought to suffer from different causes of death than wild-type animals, suggesting that the latter explanation is less likely. Therefore, we propose that BB exerts beneficial effects through interactions with the osr-1 pathway.

Fractionation of the bulk polyphenols showed that BB’s benefits on longevity and thermotolerance cofractionated with the PAC-enriched fraction, and not with ATC or CA, two other major constituents of blueberries that have antioxidant activity in vitro. These findings may either show that (i) the antioxidant effects of these compounds in vitro are not relevant to their in vivo effects in this model, or (ii) differential bioavailability of these compounds is a major determinant of their effects in whole animals. Due to the structural diversity in these fractions, we did not attempt to directly measure the levels of polyphenols inside the animals. However, we did observe the accumulation of BB pigments (primarily ATC) inside the intestines of treated animals, leading us to conclude that components of BB extracts could enter the body of these animals. Our analysis showed that BB did not protect animals against oxidative stress from paraquat, hydrogen peroxide or genetic mutation [mev-1(kn1)], which increases superoxide production in vivo (Senoo-Matsuda et al., 2001). These findings are consistent with the hypothesis that in vitro antioxidant activities of these compounds are not necessarily strong predictors of their benefits in vivo in whole animals.

As a part of this study, we examined possible mechanisms for the beneficial effects of BB treatment. As mentioned, BB treatment was not correlated with increased oxidative stress resistance, nor did BB appear to act solely by relieving microbial stress. One reasonable hypothesis is that BB’s antioxidant activity was only sufficient to cope with low-level oxidative stress, such as induced by thermal stress or during aging. Alternatively, BB PACs might have other activities that protected cells specifically during thermal stress.

Given this evidence, we propose the following model for BB’s effects on C. elegans lifespan. BB appears to protect animals against some aging-associated stress that overlaps with the stress imposed by high temperature. One possibility is that BB polyphenols protect cells from low levels of free radicals. Alternatively, BB polyphenols might alter the activity of signaling pathways required for response to thermal stress. Given the beneficial effect of BB on lifespan and thermotolerance, it was
somewhat unexpected that the osr-1/unc-43/sek-1 pathway for osmotic stress resistance was required for these effects of BB polyphenols, especially since osr-1 has not been found to affect thermotolerance (Solomon et al., 2004). One possibility is that BB and osr-1 differentially affect outputs that can increase resistance to several types of stress. Further investigation of the cellular effects of BB treatment may reveal the nature of this genetic interaction.

Regardless of the specific mechanism involved, it is clear from these experiments that natural compounds available in blueberries can prolong lifespan of a whole organism, under certain conditions. This is a significant finding that lends support to previous experiments on cultured cells or short-term rodent studies showing beneficial effects in aging-related declines and stress resistance. Other studies have also shown that some other naturally available compounds can also prolong C. elegans lifespan under laboratory conditions (Harrington & Harley, 1988; Adachi & Ishii, 2000; Wu et al., 2002; Wood et al., 2004). With the exception of resveratrol, which acts through sir-2.1, the genetic requirements for these effects have not been examined (Wood et al., 2004). This work thoroughly examined in vivo the beneficial effects of compounds found in blueberries, and thus represents a significant advance in the study of the biological effects of natural compounds.

Experimental procedures

Strains and growth conditions

All strains were maintained at 15 °C on nematode growth medium (NGM) as described (Brenner, 1974). Strains used in this study were: N2, Bristol (wild-type); BA17, fem-1(hc17); GR1307, daf-16(mgDf50); TK22, mev-1(kn1); VC199, sir-2.1(ok434); EU1, skn-1(zu67)/nT1; AM1, osr-1(m1); AU1, sek-1(ag1); and MT2605, unc-43(f498n1186). Before analysis, the sir-2.1(ok434) strain was backcrossed twice against wild type.

Blueberry extracts and fractionation

Commercially prepared single strength wild blueberry juice (Vaccinium angustifolium) was applied to a preconditioned C18 column (Waters Canada Ltd, Mississauga, ON, Canada). The C18 column was washed with water to remove fructose, glucose and organic acids, which are abundant in blueberries, then with 100% methanol to obtain the total polyphenolic fraction. Methanol was removed under vacuum using a rotary evaporator (Buchi, Essen, Germany) at 30 °C. To obtain the proanthocyanidin fraction, the total polyphenol fraction was dissolved in 50% ethanol and applied to a column of Sephadex LH20 (Sigma-Aldrich, St. Louis, MO, USA), preconditioned with 50% ethanol. The column was washed with 60% ethanol until the eluant was colorless, and then with 70% aqueous acetone to elute the blueberry proanthocyanidins. To obtain the anthocyanin fraction, the total polyphenol fraction was dissolved in acidified water and washed four times with three volumes of ethyl acetate. Anthocyanins were partitioned into the water fraction, which was subsequently freeze dried. Pure chlorogenic acid (Sigma Chemical Company) was used instead of blueberry chlorogenic acid since the chlorogenic acid fraction obtained from blueberry fractionation is contaminated with minor flavonol, and flavonol glycoside components. These components are all contained in the ethyl acetate fraction obtained during anthocyanin isolation.

Phenotypic assays

For aging assays, synchronous populations were obtained by allowing 5–10 hermaphrodites to lay eggs for 4 h to overnight. For ease of analysis, we used the adult sterile strain, fem-1(hc17), as the wild-type strain to avoid progeny overgrowth in lifespan assays. Therefore, eggs were shifted to 25 °C, the nonpermissive temperature for fertility of fem-1(hc17). Lifespan scoring was initiated after hermaphrodites completed the final larval molt, on the first day of adulthood. For aging assays with BB extracts, treatments were added to NGM agar plates on the first day of the lifespan assay. For lifespan assays with fertile strains, hermaphrodites were transferred daily for the first 4 days of adulthood to avoid progeny overgrowth. In these cases, all treatment plates were prepared on day 0 of adulthood. Statistical analyses and survival plots of lifespan data were performed using JMP analysis software (SAS Institute Inc, Cary, NC, USA). Pharynx pumping rates were scored on adults at room temperature (24 °C) under a Nikon SMZ1500 stereomicroscope (Nikon, Melville, NY, USA).

Thermotolerance assays were performed with hermaphrodites on adult day 5, after the majority of egg-laying had ceased. Animals were transferred onto 3-cm NGM agar plates supplemented as indicated and then incubated at 35 °C for 16 h. Survival was scored as the number of animals responsive to gentle touch as a fraction of the original number of animals on the plate. Animals that had died from desiccation on the sides of the plate were censored. Paraquat-induced oxidative stress assays were performed with fem-1(hc17) hermaphrodites at 25 °C as for aging assays, except paraquat was added to NGM medium to 10 μM final concentration (ChemService, West Chester, PA, USA). For hydrogen peroxide, we scored 5-h survival of adult day 5 fem-1(hc17) animals in S-basal medium with indicated concentrations of hydrogen peroxide.

To determine lipofuscin levels, adult hermaphrodites were anesthetized in 0.2% sodium azide and mounted on 2% agarose pads for visualization of intestinal fluorescence on a Nikon E800 microscope using an Endow GFP filter with a mercury UV source (Nikon). Images were captured using a constant exposure time with a Hamamatsu ORCA digital CCD camera (Hamamatsu, Bridgewater, NJ, USA) using OpenLab software (Improvision, Lexington, MA, USA). Lipofuscin levels were measured using ImageJ software (NIH Image) by determining average pixel intensity in each animal’s intestine.

To measure levels of 4-HNE, animals were collected, fixed with 4% formaldehyde and permeabilized by digestion with...
type IV collagenase (Sigma Chemical Company), as described (Loer & Kenyon, 1993). Fixed and permeabilized specimens were incubated with anti-4-HNE antisera (1 : 100 dilution, Genox Corp, Baltimore, MD, USA) for 2 h at 24 °C, washed and incubated overnight at 4 °C with Alexafluor-546-conjugated goat anti-mouse secondary antibody (1 : 100) (#A-11003, Invitrogen, Carlsbad, CA, USA). Stained animals were mounted on 2% agarose and fluorescence visualized as for lipofuscin with appropriate filter sets. 4-HNE immunofluorescence was measured in the pharynx terminal bulb and somatic gonad using ImageJ software.

Analysis of gene expression by reverse transcriptase PCR

For reverse transcriptase PCR (RT-PCR) analysis of gene expression, fem-1(hc17) animals were grown at an approximate density of 200 animals/plate in the presence or absence of 200 µg mL⁻¹ BB polyphenols. Animals were kept at 25 °C except for heat-stressed samples, which were transferred to 35 °C for 2 h before collection and processing. Animals were washed from plates with cold M9 buffer into Eppendorf tubes, and allowed to settle on ice. The worm pellet was resuspended in 300 µL. Absolutely RNA lysis buffer (Stratagene, La Jolla, CA, USA) and stored frozen at −80 °C. RNA was prepared as per kit instructions. Complementary DNA was prepared with the ProStar Ultra HF RT-PCR kit (Stratagene). Real-time PCR was performed in an MJ Research Opticon thermal cycler (BioRad, Hercules, CA, USA), using SybrGreen 2x master mix (Applied Biosystems, Foster City, CA, USA) and 0.5 µM primers and 0.5 µL cDNA in a 25-µL reaction volume using the following gene-specific primers (Hsu et al., 2003): actin (T04C12.6), GTGTGAAGGAGGTGCGCCCTTGGTGTAAGAC (F) and GGTAAGGATCTTCATGAGGTAATCAAAGC (R); hsp-12.6 (F38E11.2), ATGATGAGCGTTCCCGGTGACGACGAGGTTGCCGCTCTT-GAAGAATTCC (R); hsp-16.4 (T27E4.8), GTACTCTTACCATATT- TCCGTCCAGCTCATCAACGTTC (F) and CGTT TCGAAGAACTGTGTGCTGATCTATTCCGG (R); hsp-16.1 (T27E4.8), GTACTCTTACCATATT-TCCGTCCAGCTCATCAACGTTC (F) and CAACGGGCGCTTGCCTGGATTCTGTCCTAATGGAAATTTCC (R); hsp-16.2 (F38E11.2), ATGATGAGCGTTCCCGGTGACGACGAGGTTGCCGCTCTT-GAAGAATTCC (R); hsp-16.49 (Y46H3 A2), GCTCATGCTCCGTTCTCCATATTCTGATATTCAAATGC (F) and GCAACAAAAATTGATCGGAATAGAACGTGATGAG (R); and hsp-70 (F44E5.4), CGTTTGCAAAGAAGCTTGCTGTGATGCATCTCCTCG (G) and TTAATCA ACTTCCCTAACAAGAGTTGCTCTTGG (R).

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Supplementary material

The following tables and figure are available as supplementary material from: http://blackwell-synergy.com/

Table S1 Transcripts examined by RT-PCR in BB-treated animals

Table S2 hsp induction in BB-treated animals vs. untreated controls at adult days 4 and 8 is in BB-treated animals

Fig. S1 Beneficial effects of blueberry polyphenols did not require the activity of DAF-16/FOXO, which acts in the insulin-like signaling pathway in Caenorhabditis elegans. (A) Lifespan of daf-16(mgDf50) animals, completely lacking daf-16 activity, was extended by treatment with blueberry polyphenols at indicated doses. (B) Blueberry polyphenols increased thermotolerance in daf-16(mgDf50) animals. Thermotolerance was assayed as for Fig. 5(A).

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