



Published in final edited form as:

*Free Radic Biol Med.* 2011 June 1; 50(11): 1669–1678. doi:10.1016/j.freeradbiomed.2011.03.011.

## NECTARINE PROMOTES LONGEVITY IN *DROSOPHILA MELANOGASTER*

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### Abstract

Fruits containing high antioxidant capacities and other bioactivities are ideal for promoting longevity and healthspan. However, few fruits are known to improve the survival and healthspan in animals, let alone the underlying mechanisms. Here we investigate the effect of nectarine, a globally consumed fruit, on lifespan and healthspan in *Drosophila melanogaster*. Wild-type flies were fed the standard, dietary restriction (DR) or high fat diets supplemented with 0–4% nectarine extract. We measured lifespan, food intake, locomotor activity, fecundity, gene expression changes, and oxidative damage indicated by the level of 4-Hydroxynonenal-protein adduct in these flies. We also measured lifespan, locomotor activity and oxidative damage of *sod1* mutant flies on the standard diet supplemented with 0–4% nectarine. Supplementation of 4% nectarine extended lifespan, increased fecundity and decreased expression of some metabolic genes, including a key gluconeogenesis gene PEPCK, and oxidative stress response genes, including peroxiredoxins, in female wild-type flies fed the standard, DR or high fat diet. Nectarine reduced oxidative damage in wild-type females fed the high fat diet. Moreover, nectarine improved the survival and reduced oxidative damage in female *sod1* mutant flies. Together, these findings suggest that nectarine promotes longevity and healthspan partly through modulating glucose metabolism and reducing oxidative damage.

### Keywords

Aging; Nectarine; Peach; Lifespan; Oxidative stress; Reproductive aging; JNK signaling pathway; Nutraceutical; 4-Hydroxynonenal-protein adduct

### INTRODUCTION

Aging is a multi-faceted process associated with a gradual decline of physiological function and an increased incidence of various diseases, including cancer, neurodegenerative disease and diabetes [1, 2]. Increasing evidence has demonstrated that many fruits and their extracts contain high levels of phytochemicals, and fruit consumption promotes health and prevents

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Abbreviations of other genes are in the supplementary Table S3

or delays age-related diseases [3–6]. However, few preclinical studies in animals have emerged demonstrating that specific fruits when supplemented in the diet promote longevity and healthspan.

Numerous genetic and environmental factors have been implicated in modulating aging processes [1, 2]. Among many hypotheses of aging, the free radical hypothesis proposed more than 50 years ago states that cumulative oxidative damage to macromolecules in the cell is a causal factor of aging [7]. Since then, numerous lines of evidence have been discovered to support this hypothesis but also point out that the effect of oxidative stress on lifespan depends on the environmental context [8, 9]. Genetic studies have identified hundreds of genes that are involved in modulating lifespan in yeast, worms, flies and rodents. These genes can be categorized into a number of signaling pathways, including the insulin/insulin-like, target of rapamycin (TOR), Jun kinase (JNK) and sirtuin signaling pathways, which are functionally conserved across diverse species [1, 2, 10, 11]. Single nucleotide polymorphisms in some longevity-associated genes, such as insulin-like growth factor receptor, have been linked to human longevity since they are enriched in centenarians [12–15]. Some of the longevity-associated pathways, such as the JNK signaling pathway, mediate oxidative stress response in the cell. This is consistent with free radical hypothesis of aging, although increasing resistance to oxidative stress does not necessarily result in longer lifespan.

Food consumption, an environmental factor, plays a pivotal role in modulating lifespan of an organism. Dietary restriction (DR), a potent environmental intervention, can extend the lifespan and healthspan in various species, although DR does not always promote longevity [1, 16–18]. DR is also effective in reducing the incidence of cancer, diabetes and other diseases in mammals. Lifespan extension by DR may be mediated through several non-exclusive genetic pathways, such as sirtuin, SKN-1 and TOR pathways [1, 2, 10].

Another prolongevity intervention is to supplement the regular diet with health promoting nutraceutical compounds and extracts derived from plants and fruits. A prominent feature of nutraceutical extracts is that they have high levels of polyphenols, which possess high antioxidant activities and other health-promoting properties [4, 19]. Polyphenols have been shown to reduce the incidence of various types of cancer, type II diabetes, and other diseases [4, 19]. Several nutraceutical compounds and extracts have been shown to promote longevity in diverse species. Resveratrol, a polyphenol compound, can extend lifespan in yeast, worms, flies and fishes on their respective regular diets, and mice on a high fat diet [20–23], through activating several longevity associated genes, including sirtuins [24]. Blueberry extract has been shown to extend the lifespan in *C. elegans* partly through an osmotic stress resistance related  $Ca^{2+}$ /calmodulin-dependent protein kinase pathway [25]. Depending on the composition of diets, a mixture of cranberry and oregano extracts can extend lifespan without compromising fecundity in Mexican fruit flies [26]. Pulp extract of açai, a berry indigenous to the Amazon River region, can improve survival of flies on a high fat diet partly through activating the JNK pathway and reducing expression of genes in gluconeogenesis [27]. However, the number of prolongevity nutraceutical compounds and extracts identified so far is still small. In some cases, their effectiveness in promoting longevity has been challenged. Some other studies in yeast, worms and flies have shown that no or marginal lifespan extension was induced by resveratrol [28, 29]. Moreover, the mechanisms by which nutraceutical agents promote longevity remain elusive.

Nectarine (*Prunus persica* var. nectarine) is a subspecies of peach that is grown and consumed worldwide [30]. Nectarine contains a number of nutritionally important health promoting ingredients, including dietary fiber, meaningful amounts of vitamin C and  $\beta$ -carotenoids, as well as high contents of polyphenols [31–36]. A mouse study shows that

both cytosolic and mitochondrial enzymatic activities in repairing 8-Oxoguanine (8-oxoG) DNA lesions are significantly improved in aged mice fed the diet supplemented with a nectarine extract compared to the age-matched control animals on the non-supplemented diet [37]. Impairment of DNA repair capacities is linked to aging, cancer and neurological diseases [38], and the mouse study supports the health benefits of nectarine consumption for its enhancement of DNA damage repair. However, there is no experimental evidence supporting any longevity promoting property of nectarine at the organismic level.

Here we describe a series of studies designed to examine the effects of nectarine supplementation in diets on lifespan in *Drosophila melanogaster*. *D. melanogaster* is an excellent model system to investigate the longevity promoting properties of compounds and nutraceutical extracts since it has a short lifespan, can be cultured on simple diets, has a rich genetic resource with a fully sequenced genome, and more importantly over half of the fly genes have mammalian homologs [39, 40]. In this study, we demonstrate that nectarine can improve the survival of flies and that the extent of lifespan extension depends on gender, dietary conditions and stress levels in flies.

## MATERIALS AND METHODS

### Materials and media

The cornmeal food for flies was made from cornmeal, sugar, yeast, and agar according to the published protocol [41]. The standard sugar-yeast extract (SY) diet contained 10% sugar, 10% yeast extract and 1.5% agar. The high fat diet was prepared by incorporating 2% palmitic acid (w/v) and 1% Tween-80 (v/v) into the standard SY diet as previously described [27]. The calorie-restricted diet contained 2.5% sugar, 2.5% yeast extract and 1.5% agar. The freeze-dried nectarine extract was kindly provided by Paul Neipp at USDA (Parlier, CA, USA) and stored at  $-80^{\circ}\text{C}$  until it was added to the standard, high fat, and calorie-restricted diets at the final concentrations of 2, 4 and 8% (w/v).

Wild type *D. melanogaster* strain *Canton S* was obtained from the Bloomington Drosophila Stock Center (Bloomington, IN, USA). Mutant flies with the reduction-of-function of *sod1* were generated using the Gal4-UAS system combined with the RNA interference (RNAi) technique [42]. Specifically, double-stranded RNA of *sod1* was induced from a UAS-*sod1*-inverted-repeat (UAS-*sod1IR*) line by a ubiquitously expressed Gal4, daughterless-Gal4 (da-Gal4). The UAS-*sod1IR* stock (strain F103) was originally generated by J. Phillips (University of Guelph, Canada) [42]. Both UAS-*sod1IR* and da-Gal4 stocks were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN). All fly stocks were maintained on the cornmeal medium at  $18^{\circ}\text{C}$  or room temperature before being used for various assays.

### Lifespan, food intake, locomotor activity and fecundity assays

To obtain adult flies for lifespan, food intake, locomotor activity and fecundity assays, parental flies were allowed to mate and lay eggs on the cornmeal medium. After approximately two weeks, adult progeny flies of mixed sex within 24 h of eclosion were collected into new bottles with the standard SY medium. After mating for 24 h, males and females were sorted under light  $\text{CO}_2$  anesthesia and placed separately into vials with approximately 5 mL of the standard SY medium. Each vial contained approximately 20 male or female flies. After another 24 h, flies were transferred to their respective treatment diets for lifespan, food intake, locomotor activity and fecundity assays.

For the lifespan assay, flies were transferred to fresh food once every 2–3 days, and the number of dead flies was counted at each transfer. Lifespan data were recorded using

Microsoft Excel (Microsoft, Redmond, WA). Around 100–120 flies in 5–6 vials were included for each lifespan experiment. Each lifespan assay was repeated at least twice.

The capillary feeder method (CAFE) was employed to measure food intake as previously described [43]. Female flies were first treated with the standard, calorie-restricted and high fat diets supplemented with 0 or 4% nectarine for 11 days. Subsequently, eight flies were selected from each treatment and individually housed in the capillary feeding chamber. Each capillary was filled with the same food that each fly was fed before the feeding assay, except that the food in the capillaries contained no agar so that it would remain a liquid. Two capillaries with the food were set up in two separate feeding chambers without flies to account for evaporation of liquid food. Food intake was measured within 24h. Final values of food intake were calculated by subtracting the evaporation and averaging food intake of 6–8 live flies. Food intake was excluded for flies that died before the end of the experiment.

For the locomotor activity assay, spontaneous activity of 14-day old females was measured after consumption of the standard, DR or high fat diets supplemented with or without 4% nectarine using the Drosophila Activity Monitor System (DAMS) from TriKinetics Inc. (Waltham, MA, USA) according to the manufacturer's suggested protocol. The DAMS measures the number of times that a fly breaks the infrared beams by walking back and forth in a horizontal glass vial. Specifically, a glass vial with approximately 10 flies and the food supplemented with or without nectarine was inserted into the DAMS. The number of times that flies broke the infrared beams was recorded once every 20 seconds during a 24-hour recording window. At the end of recording, total number of surviving flies was recorded in each vial. Because the flies were undisturbed during the recording period, we operationally defined this activity as spontaneous locomotor activity. The 24h spontaneous activity level was calculated by dividing the total number of beam breaks in 24h by the total number of surviving flies in the vial. Recording spontaneous activities of females on each treatment was replicated 5–6 times with 5–6 vials, each with 10 females.

For the fecundity assay, after female flies were mated with males for 24 h, they were separated from males, placed on their respective diets and then transferred to vials with fresh food once every 2–3 days. The number of dead flies was counted at each transfer. The vials with old food and eggs on them were kept for egg counting. The number of eggs laid was counted and recorded in Microsoft Excel® (Microsoft, Redmond, WA) until all the flies were dead in a vial. Each egg laying assay was repeated 5–6 times in separate vials. The lifetime fecundity was calculated by dividing the lifetime egg production in each vial by the number of females (approximately 10 flies per vial) at the beginning of the experiment.

### Quantitative polymerase chain reaction (qPCR)

Three-day old female flies were fed the standard SY, DR or high fat diets supplemented with 0%, 2% or 4% nectarine until 14 days old. Fly heads and bodies were frozen with liquid nitrogen and separated using a sieve. Total RNA was prepared from heads using the Trizol reagent from Invitrogen Inc. according to the manufacturer's suggested protocol (Carlsbad, CA). Quality and quantity of total RNA were assessed using the Nanodrop 1000 from Thermo Scientific (Wilmington, DE). cDNA was synthesized from the total RNA by using Superscript reverse transcriptase from Invitrogen Inc. (Carlsbad, CA). qPCR was performed using the StepOnePlus real-time PCR system from Applied Biosystems (Foster City, CA) according to the manufacturer's suggested protocol. The primer sequences for genes tested in this study were designed using the Primer3 program at <http://primer3.sourceforge.net/> and listed in the supplementary table (Table S2). Each qPCR measure was repeated 3–4 times with 3–4 biologically independent samples. Rp49 transcript was used as the reference for quantifying the relative transcript level of each selected gene.

## Protein preparation

Mitochondrial and cytosolic proteins were prepared using the mitochondria isolation kit from MitoSciences (Cat# MS850). Briefly, approximately forty 14-day-old *Canton S* or *sod1RNAi* female flies fed the standard or high fat diet supplemented with or without 4% nectarine extract were homogenized in ice cold isolation buffer containing protein inhibitor cocktail (Roche Cat# 04693159001) using a glass dounce homogenizer. Each homogenate was centrifuged at 1000g at 4°C for 10 min. The supernatant was then transferred to a new tube, and centrifuged again at 12,000g at 4°C for 15 min to separate cytosolic and mitochondrial proteins. The supernatant were collected as the cytosolic protein sample, while the pellet was washed and re-suspended in 30µl isolation buffer as the mitochondria protein sample. Protein concentration was determined with the BCA protein Assay kit (ThermoFisher, Cat# 23225) according to the manufacturer's suggested protocol.

## Western blot analysis for 4-hydroxynonenal-protein adduct quantification

20 µg/lane of protein sample was separated by electrophoresis with 7% NuPAGE® Novex Tris-Acetate SDS Mini gel (Invitrogen, Cat# EA03585BOX) under a reducing condition according to manufacturer's suggested protocol. After electrophoresis, protein were transferred to 0.2-µM PVDF membrane for 7.5 min with iBot Dry blotting system (Invitrogen, Cat# IB1001). Membrane-immobilized proteins were blocked with 5% non-fat dried milk in 1x Tris-buffered saline (pH 7.5) containing 0.1% Tween 20 (TBST) for 1 h at room temperature, and then incubated overnight at 4°C with anti-N<sup>α</sup>-acetylysine-4-hydroxynonenal (4-HNE) fluorophore rabbit polyclonal primary antibody (Calbiochem, Cat# 393206) at a 1:4000 dilution in the blocking solution to detect HNE-protein adducts [44]. The blots were washed three times (10 min for each) with TBST and incubated in horseradish peroxidase-labeled goat anti-rabbit IgG diluted 1:3000 with the blocking solution for 90 min at room temperature. The immunoreactive proteins were detected with Amersham ECL Plus Western Blotting detection reagents (GE Healthcare, Cat# RPN2132) and quantified using the ImageQuantTL software in Typhoon Trio+ imager system (GE healthcare). The blots were then stripped and incubated with anti β-actin antibody (Abcam, Cat# ab8224) and anti-Complex I subunit NDUFS3 antibody (Mitosciences, Cat# MS112) both at a 1:2000 dilution to detect β-actin and NDUFS3 as the loading controls for cytosolic and mitochondrial proteins respectively. For each protein sample, at least three biological replicates were used for immunostaining. For 4-HNE-protein adduct measurement, the protein region between molecular weight (MW) 460 and 268 KDa was quantified. Relative 4-HNE level was calculated by first normalizing the 4-HNE intensity value to that of β-actin and the ratios were adjusted again so that the ratio for the non-supplemented control group was "1" for each comparison between supplemented and non-supplemented groups.

## Statistical analysis

All data were analyzed using Statview version 5.0 software (SAS, Cary, NC). For the lifespan data, Mantel-Cox log rank tests were performed by comparing flies on the nectarine supplemented diets to the non-supplemented control diet. Maximum lifespan analysis was conducted on the longest-lived 10% of flies in each treatment. The two independent sets of lifespan data were analyzed either separately or by using data pooled from the two replicated experiments. For the spontaneous activity, food intake, fecundity, qPCR and 4-HNE data, unpaired t-tests were performed by comparing flies on the nectarine supplemented diets to the non-supplemented controls. The Bonferroni correction was conducted to adjust multiple comparisons. Statistical significance was set at  $p \leq 0.05$ . All mean and maximum lifespan, food intake, locomotion, fecundity, qPCR and 4-HNE values are presented as means ± standard error (s.e.). In addition, for expression of a gene to be considered significantly altered by supplementation of nectarine, the percentage of changes at the transcript level had to meet a stringent and operationally set level  $\geq 20\%$ .

## RESULTS

### The effect of nectarine extract on lifespan of flies fed a standard diet

Nectarine is rich in polyphenols and other antioxidants [32–34]. We hypothesized that nectarine may counteract oxidative damage and therefore extend the lifespan in flies. To test this hypothesis, we supplemented the standard SY diet for wild type *Canton S* flies with 2 or 4% nectarine extract. No significant lifespan increase in male flies under the tested conditions was observed (Fig. 1A, Table 1 for results from combining two replicates and online supplementary Tables S1 and S2 for replicated experiments). Based on the pooled data, Supplementation of 4% nectarine slightly decreased mean and maximum lifespan of males fed the standard diet but by <8% and 2.5% respectively ( $p < 0.01$ , Table 1). Supplementation of 4% but not 2% nectarine significantly increased the mean lifespan of female flies by approximately 14–22% ( $p < 0.01$ ) but not consistently the maximum lifespan (Fig. 1B, Table 1, and online supplementary Tables S1 and S2). In one trial, supplementation of 2% nectarine decreased lifespan of females (Table S2). To determine whether food intake played a role in lifespan extension in female flies by nectarine, food intake within 24h was measured. Supplementation of 4% nectarine did not significantly change the amount of food intake in females (Fig. 1C). These findings suggest that nectarine can promote longevity without affecting food intake, and this lifespan extension depends on gender and dosage of nectarine.

### The effect of nectarine on lifespan of flies fed the DR diet

DR has been shown to extend lifespan in a wide variety of species [1]. To determine if nectarine and DR acts on the same or different pathways, we measured the lifespan of *Canton S* flies fed the DR diet supplemented with or without 2 or 4% nectarine. Supplementation of nectarine at both concentrations did not consistently affect the mean and maximum lifespan of males when compared to the non-supplemented controls (Fig. 2A, Table 1 and supplementary Tables S1 and S2). However, supplementation of nectarine at 2 or 4% significantly extended the mean lifespan of females by 7–11% and the maximum lifespan by 10–14% ( $p < 0.001$ , Fig. 2B, Table 1 and supplementary Tables S1 and S2). No significant difference in food intake within 24h was found between females fed the DR diets supplemented with or without 4% nectarine (Fig. 2C). These results suggest that the prolongevity effect of nectarine is mediated at least partly through DR-independent pathways.

### The effect of nectarine on lifespan of flies fed on a high fat diet

Consumption of high fat diets is often associated with an increase of oxidative damage and a decrease of lifespan. To evaluate whether nectarine could promote the survival of flies fed a high fat diet, we compared the lifespan of flies fed a high fat diet supplemented with or without nectarine. Supplementation of 2 or 4% nectarine significantly increased the mean and maximum lifespan of males but only marginally by up to 5% in most cases when compared to the non-supplemented controls ( $p < 0.05$ , Fig. 3A, Table 1 and supplementary Tables S1 and S2). However, supplementation of nectarine at 8% did not extend the mean and maximum lifespan of males (Table 1). On the other hand, supplementation of nectarine at 2, 4 or 8% significantly extended both mean and maximum lifespan of females fed the high fat diet by 10–20% ( $p < 0.01$ , Fig. 3B, Table 1 and supplementary Tables S1 and S2). Food intake was measured for females fed the high fat diet supplemented with 0, 2 or 4% nectarine. Although there is a trend of slight decrease in food intake by nectarine, no statistically significant differences were found among the three groups of females ( $p > 0.1$ , Fig. 3C). These findings suggest that nectarine can limit the adverse effects of the high fat die and promote the survival of flies on the high fat diet.

### The effect of nectarine on spontaneous activities of flies

Locomotor activity generally declines with age [45]. To test whether the lifespan extension by nectarine was associated with any changes in locomotor activity, we measured spontaneous locomotor activity of 14 day-old females fed the standard, DR or high fat diets supplemented with or without 4% nectarine. No obvious changes of the spontaneous locomotor activity were found between flies fed the diets supplemented with or without nectarine (Fig. 4A). These findings suggest that nectarine is not detrimental to the mobility of flies.

### The effect of nectarine on fecundity

Lifespan extension in animals in some cases is associated with a decrease in reproduction [46, 47]. To determine if the extension of lifespan by nectarine was associated with a decrease in reproduction, we determined the fecundity of female flies on the standard, DR or high fat diets supplemented with 0, 2 or 4% nectarine by measuring the egg production during the life of female flies. The lifetime egg production by flies fed the diet supplemented with 2 or 4% nectarine was significantly increased when compared to the non-supplemented controls under all three dietary conditions (Fig. 4B). These findings indicate that supplementation of nectarine not only increases the lifespan but also maintains or even increases reproduction of female flies, suggesting that nectarine can delay reproductive aging.

### The effect of nectarine on expression changes of genes associated with metabolism, oxidative stress and longevity

To investigate the molecular mechanisms by which nectarine promotes lifespan and healthspan, we measured expression changes of genes associated with metabolism, oxidative stress and longevity, including those in insulin-like, JNK, sirtuin and TOR signaling pathways, in heads of female flies fed the standard, DR or high fat diets supplemented with 0, 2 or 4% nectarine. The relative transcript levels of the genes tested in this study are listed in the supplementary Table S3 and expression patterns of some genes with significant changes induced by nectarine are depicted in Fig. 5.

For metabolism, a significant decrease of the transcript level of *phosphoenolpyruvate carboxykinase (PEPCK)*, a key enzyme in gluconeogenesis, was found in flies fed the standard, DR or high fat diet supplemented with nectarine when compared to the respective non-supplemented controls. *Iron regulatory protein 1B (Irp-1B)*, which is involved in iron metabolism, was down-regulated by nectarine in flies under all three dietary conditions.

For the oxidative stress response pathway, *lethal (2) essential for life (l(2)efl)* and *heat shock protein 68 (Hsp68)* were down-regulated, while *metallothionein A (MtnA)* was up-regulated by supplementation of 4% nectarine in flies fed the DR or high fat diets but not the standard diet. These genes are downstream targets of the JNK pathway. However, the expression of other genes in the JNK pathway, including *basket (bsk, the Drosophila JNK gene)*, *puckered (puc)* and *glutathione S transferase D1 (GstD1)*, was not changed by supplementation of nectarine. Among other stress response genes examined, mitochondrial *superoxide dismutase 2 (Sod2)* was down-regulated by nectarine in flies fed the high fat diet, and *catalase (cat)* was down-regulated by nectarine in flies fed the standard diet, while *female-specific independent of transformer (fit)*, which is induced under various stress conditions, was down-regulated by nectarine in flies fed the DR and high fat diets.

Peroxiredoxins (Prx) are a family of proteins with peroxidase activity and play an important role in oxidative stress response [48, 49]. Transcript levels of four Prx genes, *Prx2540*, *Prx5*, *Prx6005* and *Prx5037* were down-regulated by supplementation of 4% nectarine in flies fed

the high fat diet. In addition, *Prx6005* was down-regulated by nectarine in flies fed the standard but not the DR diet, whereas *Prx2540* was down-regulated by nectarine in flies fed the standard or DR diet.

For the TOR signaling pathway, the transcript level of *4E-BP* but not *TOR* or *S6K* was significantly reduced by the supplementation of 4% nectarine in flies fed the standard or high fat diet but not the DR diet. No significant alterations in gene expression were observed for genes involved in the insulin-like signaling (IIS) pathway, including three *Drosophila* insulin-like peptides (*dllp2*, *dllp3* and *dllp5*), Insulin-like receptor substrate (*chico*) and forkhead transcription factor (*Foxo*), and in mitochondrial biogenesis, including *nitric oxide synthase* (*Nos*), *mitochondrial assembly regulatory factor* (*Marf*) and *mitochondrial transcription factor A* (*TFAM*) (Supplementary Table S3). Together, these findings indicate that supplementation of nectarine significantly influences the expression of genes involved in glucose metabolism, oxidative stress response and detoxification.

### The effect of nectarine on lifespan of *sod1* mutant flies

To directly test whether the prolongevity effect of nectarine is related to the anti-oxidative stress, we examined whether supplementation of nectarine could promote the survival of *sod1* mutant flies. *Sod1* is a major cytosolic enzyme that scavenges highly reactive superoxides within the cell [50]. Loss- or reduction-of-function *sod1* mutants have higher amounts of oxidative damage to various macromolecules and shorter lifespan when compared to wild type flies [51]. We used the RNAi method to generate a reduction-of-function *sod1* mutant (*sod1RNAi*) and fed these flies the standard diet supplemented with 0, 2 or 4% nectarine. Similar to the findings in wild type *Canton S* flies, supplementation of up to 4% nectarine did not consistently extend the mean and maximum lifespan of *sod1RNAi* males (Fig. 6A, Table 1 and supplementary Tables S1 and S2). However, supplementation of nectarine at 2 or 4% significantly increased the mean and maximum lifespan of *sod1RNAi* females by 14–28% ( $p < 0.0001$ , Fig. 6B, Table 1 and supplementary Tables S1 and S2). Here the lifespan extension appears to be positively correlated with the concentration of nectarine. These results suggest that nectarine can alleviate the high level of oxidative damage and promote the survival in *sod1RNAi* female flies and also suggest that nectarine acts either downstream or independent of *Sod1* to promote longevity.

### The effect of nectarine on oxidative damage

To further examine the anti-oxidative stress property of nectarine, we determine the effect of nectarine on lipid oxidation as an indicator of oxidative damage by measuring the level of 4-HNE-protein adducts for wild-type *Canton S* or *sod1RNAi* female flies fed the standard SY or high fat diet supplemented with or without 4% nectarine for 11 days. 4-HNE-protein adducts accumulate with age and under oxidative stress, and are a commonly used biomarker of lipid oxidation [44, 52, 53]. We did not observe any significant change of the 4-HNE-protein adduct level in mitochondrial protein samples between any pair of diet-matched nectarine supplemented and control female flies (data not shown). Supplementation of 4% nectarine did not induce any statistically significant change of the 4-HNE-protein adduct level in cytosolic protein samples for female flies on the standard SY diet when compared to the non-supplemented control, either ( $p = 0.20$ , Fig. 7A). However, supplementation of 4% nectarine significantly reduced the 4-HNE-protein adduct level in the cytosolic protein samples by more than two-fold for flies on the high fat diet when compared to the diet-matched non-supplemented control ( $p < 0.01$ , Fig. 7B). In addition, supplementation of 4% nectarine significantly reduced the 4-HNE-protein adduct level by approximately two-fold for *sod1RNAi* female flies on the standard SY diet when compared to the non-supplemented control ( $p < 0.05$ , Fig. 7C). These findings further support the notion



that nectarine has anti-oxidative properties *in vivo* at least for flies on the high fat diet or oxidative stressed *sod1RNAi* flies.

## DISCUSSION

Free radicals play an important role in aging and age-associated diseases [8, 9]. Genetic mutations and environmental interventions that extend the lifespan of an organism are often associated with an increase in the resistance to oxidative stress in that organism. However, an alteration of oxidative stress response capacity alone does not necessarily lead to a change in lifespan, and the effect of oxidative stress response pathways on lifespan and aging depends on the environmental context and genetic background of an organism [9]. Nevertheless, reducing oxidative damage remains one of the promising interventions to delay the progression of aging and age-related diseases. Polyphenols are a family of phytochemicals possessing high antioxidant capacities and other health promoting bioactivities, such as activators of enzymes and ligand mimetics of receptors [4, 5, 19]. Many fruits contain significant amounts of polyphenols, and, therefore, are ideal candidates for longevity interventions. However, evidence directly demonstrating the longevity properties of fruits is scarce. In this study, we have demonstrated that an extract from nectarine can promote longevity in female flies under various dietary conditions, including the standard, DR and high fat diets. Supplementation of nectarine can also promote the survival of flies with high levels of oxidative damage resulting from a reduction-of-function mutation in *sod1*, a major superoxide scavenger in the cell [50]. In addition, nectarine can increase fecundity and duration of egg production (data not shown), which suggest that nectarine can delay reproductive aging. Furthermore, qPCR and Western blot analyses suggest that nectarine promote longevity at least partly through reducing oxidative damage. This study is the first demonstrating the longevity property of nectarine, a globally grown and consumed fruit.

Composition of dietary nutrients plays an important role in modulating lifespan [46, 54, 55]. DR has been shown to extend the lifespan in diverse species, although the genetic background of an organism also contributes to the DR lifespan extension effect [1, 17, 18]. Consumption of high fat diets induces higher levels of oxidative stress and result in shorter lifespan when compared to low fat diets [20, 56]. In the studies reported here, we have investigated how supplementation of nectarine affects the lifespan in flies fed three different diets, standard, DR and high fat. Supplementation of 4% nectarine is sufficient to extend lifespan of female flies under all the dietary conditions. Supplementation of 2% nectarine only consistently extends lifespan of females on the high fat diet. However, supplementation of nectarine up to 4% only marginally extends lifespan in males fed the high fat diet but not the standard or DR diet when compared to the respective non-supplemented controls. It is unclear why nectarine is much more effective in promoting longevity in females than males. Further study is needed to determine whether the differences in physiology, such as the energy requirement for reproduction, contribute to the gender-specific response [57]. Nevertheless, our findings suggest that the extent of lifespan extension by nectarine in flies depends on gender, dosage of nectarine and nutrient composition of the diet. This is consistent with previous observations using polyphenols and fruit extracts, e.g. the longevity effect of resveratrol in some organisms appears to depend on the dietary conditions [20, 22, 23, 58]. Resveratrol has been shown to promote the survival of mice on a high fat diet but not on the standard rodent diet [20]. Furthermore, resveratrol only extends the lifespan in female Mexflies on a restricted and high sugar diet [58]. In addition, a mixture of cranberry and oregano extracts can promote longevity in the Mexfly on high sugar diets but not high protein diets [26]. Açai pulp extract has been shown to improve the survival of female flies on a high fat but not a standard fly diet [27]. Collectively, these findings indicate that the dietary conditions are critical for the longevity effects of

nutraceutical agents and that the lifespan extension by different fruits may be mediated by different pathways.

Aging is associated with a decline of reproductive capacity [59]. We have found that supplementation of 2 or 4% nectarine under all dietary conditions significantly increases lifetime fecundity in females without an obvious change of food intake in these flies compared to the non-supplemented flies. Considering the calorie and protein contents of a typical nectarine [35], addition of 2% nectarine into the high fat diet with 10% sugar, 10% yeast extract and 2% palmitic acid results in <6% extra calorie and <2% extra protein when compared to the non-supplemented diet. However, the difference in lifetime egg production between flies on the high fat diets supplemented with or without nectarine is more than two fold. Therefore, it is unlikely that the difference in lifetime fecundity is mainly due to the differences in the calorie and protein contents in the food. It is possible that nectarine improves general health of animals, maintains health of reproductive organs, such as the ovary, or even directly regulates reproduction as hormone mimetics, and consequently extends the reproductive capacity and healthspan. Further molecular, cellular and genetic studies should reveal the mechanisms by which nectarine delays reproductive aging.

A number of genetic pathways have been shown to modulate lifespan in yeast, worms, flies, and rodents [1, 2], and many genes are altered at the transcript level with age [60, 61]. Some of these genes mediate the prolongevity effect of DR, and some are linked to human longevity. We have assessed whether nectarine promotes longevity through the DR pathways and have found that nectarine can further extend lifespan of flies on the DR diet. This suggests that the prolongevity effect of nectarine is at least partly mediated through DR-independent pathways. To determine the relationship between nectarine and longevity-associated pathways, we have surveyed the expression of a number of genes involved in various biological processes, including glucose metabolism, oxidative stress response and protein synthesis. Two prominent features emerge from this survey. One is that nectarine reduces expression of PEPCK, a key enzyme in gluconeogenesis, in flies fed all three diets [62]. We have previously observed a decrease of PEPCK expression by supplementation of açai pulp extract in flies fed the high fat diet. Inhibition of PEPCK activity by the drug fenofibrate is one of the therapeutic approaches to manage type II diabetes [63]. Although gluconeogenesis and the glucose level were not measured in our studies, our findings suggest that fruits rich in polyphenols have the potential to modulate glucose metabolism in a health-promoting manner.

The second finding revealed by gene expression analysis is that genes involved in the oxidative stress response pathways tend to be down-regulated by nectarine. The extent of down-regulation depends on the dietary conditions. Nectarine decreases expression of two heat shock proteins, *l(2)efl* and *Hsp68*, but increases expression of *MtnA* in flies fed the high fat diet. These three genes are downstream targets of the JNK signaling pathway and are up-regulated in response to oxidative stress [64, 65]. Interestingly, açai pulp extract has been shown to increase expression of these three genes in flies fed the high fat diet. How nectarine differentially modulates expression of the JNK target genes is unclear. The JNK pathway has been implicated in modulating the lifespan in *D. melanogaster* [64, 65]. We postulate that nectarine indirectly modulates the JNK signaling pathway by influencing the redox status and reducing oxidative damage in the cell, while açai may directly activate the JNK pathway.

Our hypothesis is further supported by expression patterns of three Prxs, *Prx2540*, *Prx6005* and *Prx5037*, a family of antioxidant enzymes, involved in detoxifying peroxide in the cell [48, 66]. Nectarine decreases expression of all three Prxs in flies fed the high fat diet. Furthermore, nectarine decreases expression of *Prx2540* in flies fed the standard or DR diet,

and reduces the transcript level of *Prx6005* in flies fed the DR diet. Prxs tend to be up-regulated by increased oxidative stress. Consistent with the expression patterns of the JNK downstream genes, decreased expression of Prxs by supplementation of nectarine appears to be indirectly mediated by the effect of nectarine to reduce oxidative damage and/or improve the redox status. Furthermore, nectarine significantly reduces lipid oxidation as indicated by the 4-HNE-protein adduct level in cytosolic proteins of wild type female flies fed the high fat diet and *sod1RNAi* female flies fed the standard diet when compared to the respective non-supplemented controls. These results are also consistent with observations made in mice fed nectarine. Mice fed nectarine have a higher 8-oxoG DNA lesion repair capacity than the non-supplemented controls [37]. Several genes involved in detoxification, including *Sod1*, *glutathione peroxidase 1*, and *glutathione S-transferase alpha 3 (GSTA3)*, have been found to be down-regulated in 18–19 months old mice fed a nectarine-supplemented diet for 14–16 weeks when compared to non-supplemented controls [37]. Although nectarine does not necessarily reduce expression of the fly genes homologous to these mouse genes, these observations suggest that down-regulation of genes involved in detoxification is a conserved property of nectarine. Furthermore, both nectarine and açai significantly extend lifespan of *sod1RNAi* female flies with high levels of oxidative damage despite the difference in modulating expression of stress response genes. Taken together, these findings suggest that functional fruits such as nectarine and açai can promote the survival through reducing oxidative damage and/or activating stress response signaling pathways.

Consumption of fruits can provide numerous health benefits [5]. However, development of effective and relatively low cost longevity interventions with functional fruits is still in the early stage. A survey conducted in five European countries indicates that Europeans consume 2–4 nectarines and peaches per week on average [30]. Nectarine provides valuable macronutrients and micronutrients. An analysis on several nectarine cultivars in Australia has shown that the typical weight of a nectarine is approximately 90 grams and over 90% of it is edible with total energy content at approximately 1.6 KJ/g edible portion [35]. Among the edible part, more than 80% is water, approximately 7.5% is sugar, 2% is dietary fiber and 1% is protein [35]. More importantly, nectarine contains high contents of  $\beta$ -carotene and vitamin C, and meaningful amounts of polyphenols in both extractable forms by organic solvents and nonextractable but hydrolysable forms associated with dietary fiber and protein [31, 33, 34]. Our study indicates that supplementation of 4% nectarine to the food is sufficient to promote lifespan and healthspan in the fly. A recent mouse study indicates that supplementation of 8% nectarine to the food can induce a higher level of DNA repair activity [37]. These dosages of nectarine correspond to 5–8 nectarines per day for an average adult human [37]. Our study in *Drosophila* is the first to show lifespan extension by nectarine in an animal model, and thus provides a foundation for future research and development for nectarine as an effective intervention to promote lifespan and healthspan in mammals, including humans.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We would like to thank Paul Neipp for preparing the nectarine extract, Dr. Vilhelm Bohr for encouraging us to initiate this study, and Dr. Nan-ping Weng and Edwards Spangler for valuable comments and suggestions on the manuscript. This study was supported by the Intramural Research Program of the National Institute on Aging, NIH to S.Z.

## Abbreviations

<b>DR</b>	Dietary restriction
<b>sod1</b>	superoxide dismutase 1
<b>TOR</b>	target of rapamycin
<b>JNK</b>	Jun kinase
<b>8-oxoG</b>	8-Oxoguanine
<b>SY</b>	sugar-yeast extract
<b>RNAi</b>	RNA interference
<b>UAS-sod1IR</b>	UAS-sod1-inverted-repeat
<b>da-Gal4</b>	daughterless-Gal4
<b>CAFE</b>	capillary feeder method
<b>DAMS</b>	Drosophila Activity Monitor System
<b>qPCR</b>	Quantitative polymerase chain reaction
<b>4-HNE</b>	4-hydroxynonenal
<b>s.e</b>	standard error
<b>IIS</b>	insulin-like signaling
<b>PEPCK</b>	phosphoenolpyruvate carboxykinase
<b>Prx</b>	Peroxiredoxin

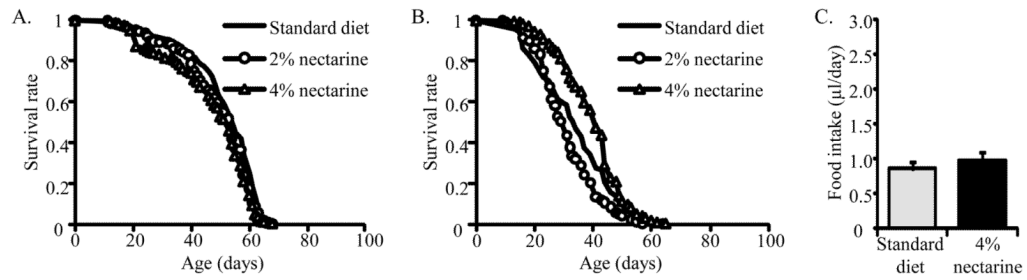
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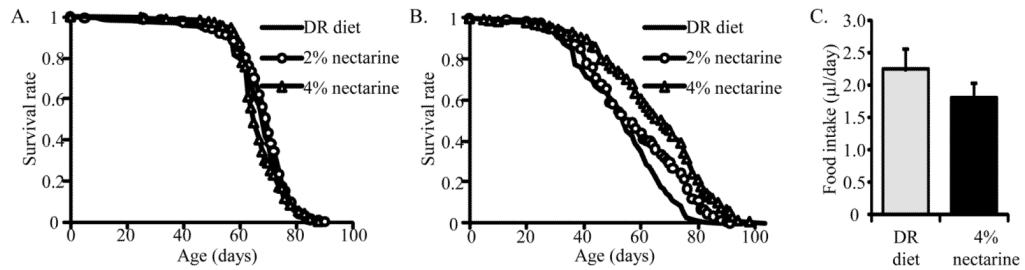
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**Fig. 1.**

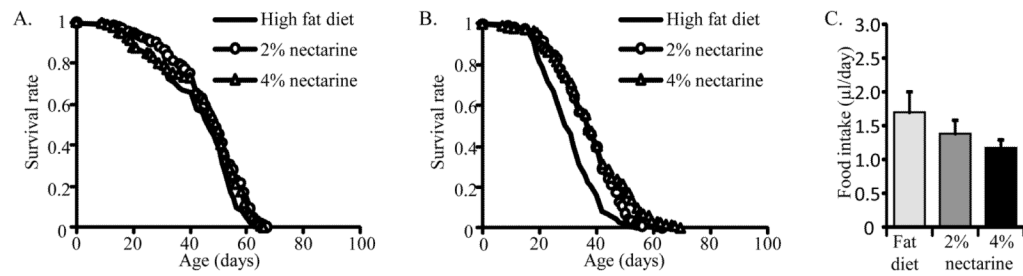
The effect of nectarine on lifespan and food intake of flies fed the standard diet. (A) The graph shows lifespan of *Canton S* males fed the standard diet (10% sugar and 10% yeast extract) supplemented with no nectarine, which is labeled as Standard diet, or with 2 or 4% nectarine extract as labeled. More than 90 males were used in each lifespan assay. (B) The graph shows lifespan of *Canton S* females fed the standard diet supplemented with 0, 2 or 4% nectarine. More than 90 females were used in each lifespan assay. (C) Food intake was measured in 6–8 individually housed female flies fed the standard liquid diet supplemented with or without 4% nectarine. Food intake in µl was calculated by averaging food intake of 6–8 flies within 24h after subtracting the evaporation of the food. Error bars indicate standard errors. The lifespan curves were derived from two independent experiments.





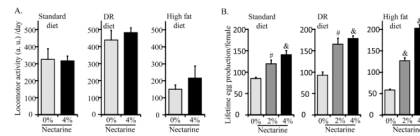
**Fig. 2.**

The effect of nectarine on lifespan and food intake of flies fed the DR diet. (A) The graph shows lifespan of *Canton S* males fed the DR diet (2.5% sugar and 2.5% yeast extract) supplemented with no nectarine, which is labeled as DR diet, or with 2 or 4% nectarine extract as labeled. More than 90 males were used in each lifespan assay. (B) The graph shows lifespan of *Canton S* females fed the DR diet supplemented with 0, 2 or 4% nectarine. More than 90 females were used in each lifespan assay. (C) Food intake was measured in 6–8 individually housed female flies fed the DR liquid diet supplemented with or without 4% nectarine. No agar was added in the food in the capillaries. Food intake in  $\mu\text{l}$  was calculated by averaging food intake of 6–8 flies within 24h after subtracting the evaporation of the food. Error bars indicate standard errors. The lifespan curves were derived from two independent experiments.



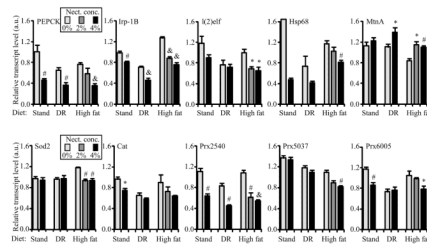
**Fig. 3.**

The effect of nectarine on lifespan and food intake of flies fed the high fat diet. (A) The graph shows lifespan of *Canton S* males fed the high fat diet (10% sugar, 10% yeast extract and 2% palmitic acid) supplemented with no nectarine, which is labeled as High fat diet, or with 2 or 4% nectarine as labeled. More than 90 males were used in each lifespan assay. (B) The graph shows lifespan of *Canton S* females fed the high fat diet supplemented with 0, 2 or 4% nectarine. More than 90 females were used in each lifespan assay. (C) Food intake was measured in 6–8 individually housed female flies fed the high fat liquid diet supplemented with or without 4% nectarine food. Food intake in  $\mu\text{l}$  was calculated by averaging food intake of 6–8 flies within 24h after subtracting the evaporation of the food. Error bars indicate standard errors. The lifespan curves were derived from two independent experiments.



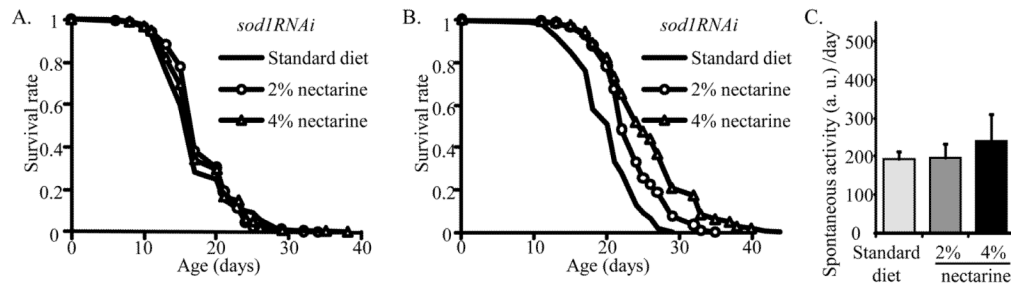
**Fig. 4.**

The effect of nectarine on locomotor activity and fecundity of female flies. (A). Locomotor activity as spontaneous activity was measured for 14-day of females fed the standard, DR or high fat diet supplemented with or without 4% nectarine. All females were pre-treatment for 11 days on the same diet on which the locomotion of flies was measured. Error bars indicate standard errors. a. u. refers to arbitrary unit. (B) Egg laid by females was counted once every 2–3 days during the lifetime of approximately 10 female flies in each vial. The lifetime egg production was calculated by dividing the total lifetime eggs production by the total number of females in each vial. Each egg production experiment was replicated 5–6 times. Error bars indicate standard errors. *p* values were calculated by comparing the nectarine fed flies to the non-supplemented controls. \**p*≤0.05; #*p*<0.01; &*p*<0.001.



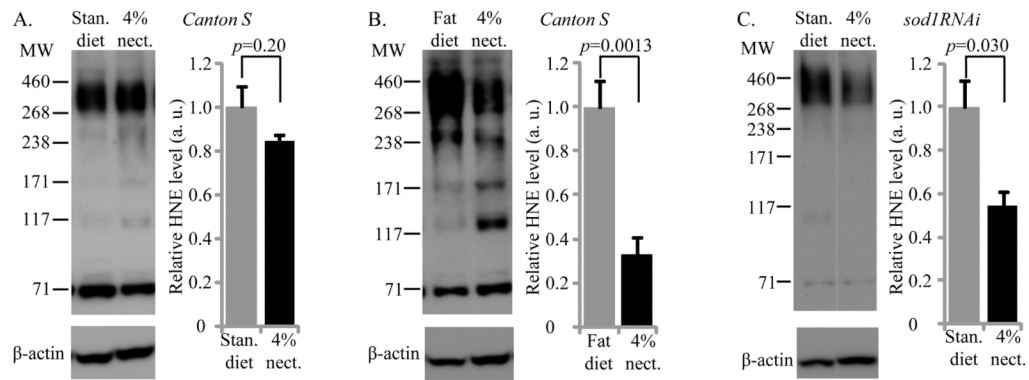
**Fig. 5.**

The effect of nectarine on relative transcript levels of metabolism, stress and longevity associated genes. RNA was prepared from heads of 14-day old females after treatment on the standard, DR or high fat diets supplemented with or without nectarine for 11 days. Rp49 was used as the reference gene to calculate relative transcript levels of the tested genes. Gene names are shown at the upper left corner of each expression panel. Error bars indicate standard errors. *p* values were calculated by comparing the nectarine fed flies to the non-supplemented controls. a. u. refers to arbitrary unit. \**p*≤0.05; #*p*<0.01; &*p*<0.001.



**Fig. 6.**

The effect of nectarine on the lifespan and locomotor activity of *sod1RNAi* flies fed the standard diet. (A) The graph shows lifespan of *sod1RNAi* males fed the standard diet (10% sugar and 10% yeast extract) supplemented with no nectarine, which is labeled as Standard diet, or with 2 or 4% nectarine as labeled. More than 90 males were used in each lifespan assay. (B) The graph shows lifespan of *sod1RNAi* females fed the standard diet supplemented with 0, 2 or 4% nectarine. More than 90 females were used in each lifespan assay. (C). Locomotor activity was measure for 14-day old *sod1RNAi* females fed the standard liquid diet supplemented with 0, 2 or 4% nectarine. All flies were pre-treated for 11 days on the same diet on which locomotion activity of flies was measured. Error bars indicate standard errors. a. u. refers to arbitrary unit. The lifespan curves were derived from two independent experiments.



**Fig. 7.**

The effect of nectarine on lipid oxidation. Lipid oxidation was determined by measuring the level of 4-HNE protein adducts. Representative Western blot images for cytosolic proteins are shown in this figure. Intensity in the area between molecular weight (MW) 460 and 268 KDa was measured as the 4-HNE protein adduct level of flies in each treatment.  $\beta$ -actin was used as protein loading control to normalize the 4-HNE protein adduct level. Relative HNE levels were calculated from 3–5 biologically independent samples for each treatment and are shown as bar graphs on the right side of the gel images. For each comparison, the average HNE level of the non-supplemented group was adjusted to the value “1”. (A) *Canton S* female flies were fed the standard SY diet supplemented with or without 4% nectarine (biological replicates, n=3). (B) *Canton S* female flies were fed the high fat diet with or without 4% nectarine (n=5). (C) *sod1RNAi* female flies were fed the standard SY diet supplemented with or without 4% nectarine (n=3).

Table 1

Lifespan of flies fed nectarine from combing two independent experiments.

Strain	Gender	Diet <sup>a</sup>	Total number of flies	Mean lifespan (days±se) <sup>b</sup>	P value for all flies <sup>c</sup>	# flies for Max. lifespan	Max. lifespan (days±se) <sup>b</sup>	P value for top 10% flies <sup>c</sup>
<i>Canton S</i>	Females	Standard diet	219	33.6±0.9		22	54.6±0.9	
		Standard+2% nect	215	31.1±0.7	<b>0.002</b>	22	48.1±1.5	<b>0.0091</b>
		Standard+4% nect	218	39.5±0.7	<b>0.0003</b>	22	56.1±0.9	0.3535
	Males	Standard	260	51.1±0.8		26	64.5±0.4	
		Standard+2% nect	222	49.3±0.9	0.057	22	64.1±0.4	0.3376
		Standard+4% nect	245	47.1±0.9	<b>0.0002</b>	24	62.9±0.4	<b>0.0042</b>
<i>Canton S</i>	Females	DR diet	213	53.1±1.0		22	76.2±0.9	
		DR+2% nect	216	57.3±1.2	<.0001	22	82.5±1.2	<b>0.0002</b>
		DR+4% nect	205	64.7±1.3	<.0001	22	91.3±0.9	<.0001
	Males	DR diet	225	66.4±0.8		22	79.0±1.0	
		DR+2% nect	211	68.3±0.7	<b>0.0532</b>	23	80.2±1.1	0.2116
		DR+4% nect	238	66.8±0.6	0.4731	24	78.5±1.5	0.3262
<i>Canton S</i>	Females	High fat	244	30.5±0.6		26	46.7±0.7	
		High fat+2% nect	232	36.6±0.7	<.0001	24	52.3±0.7	<.0001
		High fat+4% nect	234	38.1±0.8	<.0001	24	59.5±1.0	<.0001
	Males	High fat+8% nect	112	40.9±0.9	<.0001	12	54.6±0.5	<.0001
		High fat	238	42.5±0.9		25	59.4±0.5	
		High fat+2% nect	235	46.8±0.8	<.0001	24	63.1±0.4	<.0001
Females	High fat+4% nect	240	45.2±0.9	<b>0.0003</b>	25	62.4±0.4	<.0001	
	High fat+8% nect	109	46.9±1.0	0.1530	11	56.7±0.3	<.0001	
	<i>sod1/RNAi</i>	Females	Standard	234	20.1±0.3		24	26.8±0.3
Standard+2% nect			227	23.5±0.3	<.0001	23	31.7±0.4	<.0001
Standard+4% nect			226	25.9±0.4	<.0001	23	36.9±0.8	<.0001
Males		Standard	263	17.4±0.3		27	27.3±0.5	
		Standard+2% nect	267	18.3±0.3	0.0885	28	25.4±0.6	0.0265
		Standard+4% nect	265	17.9±0.3	0.3865	27	25.3±0.8	0.0316

## Note:

- <sup>a</sup> Standard refers to the standard diet containing 10% sugar and 10% yeast extract, and nect refers to nectarine and the percentage value indicates the final concentration nectarine in the food.
- <sup>b</sup> Lifespan values are expressed as  $\text{days} \pm \text{standard error (se)}$ .
- <sup>c</sup> p values were calculated by comparing flies on the nectarine supplemented diet to the corresponding non- supplemented controls. Significant p values after the Bonferroni adjustment are italicized in bold.