

Cocoa confers life span extension in *Drosophila melanogaster*

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Abstract

Cocoa is thought to be an excellent source of antioxidants. Here, we investigated the effects of cocoa supplementation on *Drosophila melanogaster* life span under different oxidative stress conditions. Our results illustrate that a moderate supplementation of cocoa under normoxia increases the average life span, whereas, at higher concentrations, average life span is normal. Under hyperoxia or in a Cu/Zn-superoxide dismutase-deficient background, cocoa exhibited a strong antioxidant activity, significantly increasing the average life span. Nevertheless, cocoa supplementation in a Mn-superoxide dismutase-deficient background enhanced an earlier mortality accompanied by a loss of climbing ability, indicating that cocoa may act as a pro-oxidant in mitochondria under conditions of extreme oxidative stress. Finally, we illustrate that cocoa also acts as a metal chelator in the presence of excess heavy metals, enhancing larval survival to the adult stage on copper or iron-supplemented medium. Taken together, our results document the antioxidative, pro-oxidative, and metal-chelating effects of cocoa on *Drosophila melanogaster* life span.

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Keywords:

Drosophila melanogaster; Cocoa; Aging; Oxidative stress; Heavy metals

Abbreviations:

ROS, reactive oxygen species; SOD, superoxide dismutase; UAS, upstream activating sequence.

1. Introduction

Reactive oxygen species (ROS) are inevitable byproducts of aerobic respiration and are known to cause modifications to DNA bases, enhance lipid peroxidation, and result in loss of protein enzymatic activity [1–3]. Under the barrage of ROS, oxidative damage builds up over time and eventually causes a gradual loss of normal physiologic function, a process known as senescence [4]. Because of the severe consequences associated with oxidative damage, aerobic organisms have developed a diversity of antioxidant defense mechanisms. Nevertheless, even under normal physiologic conditions, ROS production transcends endogenous defenses over time and inflicts damage [5].

Diet-derived antioxidants play a major role in maintaining ROS homeostasis, with the combination of dietary antioxidants and endogenous defense enzymes resulting in a highly

effective defense network against oxidative stress [6]. Cocoa and its products such as dark chocolate are known to be excellent sources of polyphenol and flavonoid antioxidants that may significantly contribute to the total antioxidant capacity of diet [7–9]. Other *in vivo* studies revealed that cocoa consumption increases the antioxidant capacity of serum and prevents lipid peroxidation and low-density lipoprotein oxidation [10–12]. Nevertheless, despite cocoa's antioxidative role in defense against ROS being well established, its contributions to longevity and aging have not been thoroughly investigated. The objectives of this study were to investigate the oxidative properties of cocoa and their effects on life span in *Drosophila melanogaster*. We hypothesize that cocoa supplementation has the potential to extend *D melanogaster* life span, particularly in the presence of stress-related oxidative damage. The relatively short life span of this model organism, the ability to control genetic background and experimental conditions, and, finally, the availability of different antioxidant-deficient transgenic lines should facilitate a further understanding of antioxidant contributions of dietary cocoa in defense against

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ROS and aging. Considering that the fruit fly has been used as a model for studies on human nutrition, the results of this study should provide useful insights into the potential roles of dietary cocoa in human longevity.

2. Methods and materials

2.1. Fly food

Drosophila melanogaster culture medium was prepared as described previously [13]. Cocoa-supplemented media were prepared by dissolving 5 or 10 g of cocoa powder (Master Choice brand, Toronto, Ontario, Canada; containing 200 g protein, 200 g fat, 200 g fiber, and 16.75 kJ of energy per 1 g cocoa) in 100 mL of normal culture medium.

2.2. Fly stocks and maintenance

Unless specified otherwise, *rosy*⁺⁵ wild-type flies were used in all experiments. For RNA-interference experiments, *UAS-SOD1-IR* [14] and *UAS-SOD2-IR* [15] transgenic lines were crossed to *da*^{G32}*Gal4* driver stock (FlyBase: P{GAL4-*da.G32*}); the F1 progeny carrying both the GAL4 driver and the UAS-transgene experienced the corresponding silencing of *SOD1* or *SOD2* genes. For longevity studies, a total of 200 newly eclosed males (initially 20 per vial) were collected for each life span experiment. Flies were maintained at 25°C on a 12:12 light/dark cycle with survivors transferred to fresh food vials every 2 days. The control and the experimental flies were fed on normal culture medium and cocoa-supplemented media, respectively.

2.3. Gustatory assay

For the adult feeding assay, we used a similar protocol to what has been described previously [13,16]. Newly eclosed females were collected (15 per vial) and aged on a fresh culture medium for 3 to 4 days. Afterward, flies were starved for 20 hours on water-soaked Whatman paper and subsequently transferred to fresh vials containing cocoa-supplemented medium mixed with 0.2% sulforhodamine B sodium salt (Acid-Red) for 2 hours. (For control flies, culture medium was only supplied with 0.2% Acid-Red.) The degree of abdomen redness for each fly was blind scored using a subjective grading scale ranging from grade 0 (colorless abdomen) to grade 5 (fully red abdomen). The degree of abdomen redness was used as an indicator of the amount of food taken by the insect.

2.4. Hyperoxia aging assay

For the hyperoxia assay, we used a similar protocol to that described previously [13]. Newly eclosed males were aged on cocoa-supplemented medium for 10 days and, afterwards, exposed to a steady flow of 100% oxygen bubbled through water in a sealed chamber. A total of 200 flies were tested for each survival curve, with survivors transferred to fresh cocoa-supplemented food vials every 2 days. Control flies were fed on normal food throughout the entire experiment.

2.5. Superoxide dismutase activity assay

Superoxide dismutase (SOD) activity was measured using the in-gel nitroblue tetrazolium assay as described previously [15]. The intensity of the bands was quantified using Scion Image software (Scion Corp, Frederick, Md) and was normalized as ratios to that of *da*^{G32}*Gal4* control band.

2.6. Climbing assay

For the adult climbing assay, we used a similar protocol to that described previously [17]. A maximum of 20 flies were placed at the bottom of a 4-in glass vial with an identical glass vial placed on the top. After 30 seconds, glass vials were separated from each other and the number of flies in each vial was counted. The climbing index was expressed as percentage of the number of flies that climbed to the top vial relative to the total number of flies tested ($n > 50$).

2.7. Statistical analysis

Data are expressed as mean values, with error bars representing SEM. Statistical analyses were performed using SAS software (SAS 9.1.3, SAS Institute, Inc, Cary, NC). The significance of the difference between means was assessed using the 1-way analysis of variance statistical test. Survival curves were analyzed using the Kaplan-Meier log-rank statistical test.

3. Results and discussion

3.1. Gustatory assay

Feeding behavior and nutritional constituents of a culture medium are 2 important factors in *D melanogaster* life span determination [18]. To ensure that any changes in

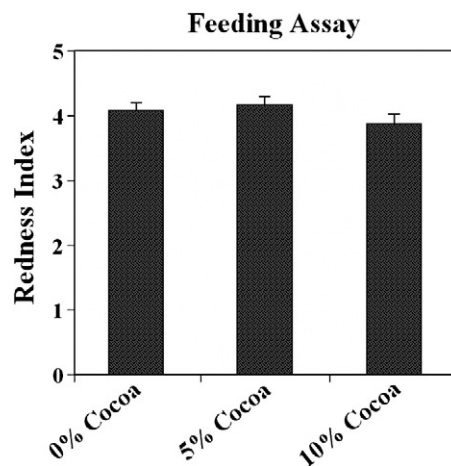


Fig. 1. Cocoa supplementation had no significant effect (1-way analysis of variance: $F_{2,176} = 1.18$, $P = .31$) on adult feeding behavior. Flies were fed on different media supplemented with 0.2% sulforhodamine B sodium salt and the degree of abdomen redness was estimated using a subjective grading scale. Bar graphs with errors represent means \pm SEM of at least 50 flies.

life span are attributed solely to the nutritional constituents of the food, we tested adults' gustatory behavior on cocoa-supplemented medium. As Fig. 1 illustrates, adults' feeding is not affected by cocoa supplementation within the range used, indicating that any alterations in the life span are attributed to the nutritional constituents of the food rather than an altered feeding behavior.

3.2. Cocoa supplementation in wild-type flies under normoxia

To test the effects of cocoa supplementation on life span, adult flies were raised on culture medium supplied with different concentrations of cocoa. As Fig. 2A illustrates, 5% cocoa supplementation significantly increased the average life span under normoxia, but at 10% concentration, cocoa had no visible effect on longevity compared to controls fed on normal food. In a human study, it was reported that moderate daily consumption of dark chocolate (4 kg of chocolate per month) is associated with an increase in the average life span, whereas higher supplements (>8 kg of chocolate per month) correlate with lower than average mean life span [19]. From these observations, we speculate that moderate consumption of cocoa under normoxia may be

beneficial to health, but at high concentrations, harmful effects of cocoa negate its beneficial effects and shorten life span. As we shall discuss later, it is a combination of cocoa's antioxidative, pro-oxidative, and metal-chelating activities that contributes to life span determination under various stress conditions.

3.3. Cocoa supplementation in wild-type flies under hyperoxia

To test the antioxidative effects of cocoa on life span, flies were fed on cocoa-supplemented medium under the acute oxidative stress of hyperoxia. As Fig. 2B illustrates, both 5% and 10% cocoa supplementations increased the average life span, indicating that antioxidant activities of cocoa have the potential to extend life span under the oxidative stress. The antioxidant nature of cocoa may be attributed to its polyphenol and flavonoid constituents [8,9], which have the capacity to transfer a hydrogen atom with a single electron to the oxygen radicals and prevent lipid peroxidation or oxidation of low-density lipoproteins [10,11,20].

3.4. Cocoa supplementation in SOD-deficient flies

Here we used a genetic approach to investigate the anti-pro-oxidant effects of cocoa on life span in *D melanogaster*. Using the UAS-GAL4 binary expression system [21] and the inverted-repeat transgenic constructs of SOD genes [14,15], we downregulated the expression of cytoplasmic Cu/ZnSOD (SOD1) and mitochondrial MnSOD (SOD2) in the adult flies (Fig. 3A and B). Our results illustrate that cocoa supplementation partially rescues the short life span of SOD1-deficient flies (Fig. 3C) but enhances an earlier mortality (Fig. 4) in SOD2-deficient flies.

The protective effects of dietary flavonoids, including their antioxidant capacity as well as metal-chelating properties, are attributed upon configuration and total number of their hydroxyl groups. Nevertheless, it is thought that multiple hydroxyl groups may also contribute to the production of extremely toxic hydroxyl radical through the Haber-Weiss reaction [20]. In light of cocoa's dual oxidative properties, we speculate that the antioxidant activity of flavonoids in the cytoplasm may contribute to the extension of life span in SOD1-deficient flies; on the other hand, the pro-oxidant activity of flavonoids toward mitochondria may be the main cause of an enhanced mortality along with the loss of climbing ability in SOD2-deficient flies. As an alternative explanation, the pro-oxidant activity of saturated fats in cocoa [7,8] may have caused the mitochondrial oxidation under oxidative stress.

In summary, these findings illustrate that cocoa may act both as an antioxidant and a pro-oxidant upon various oxidative stress conditions and, consequently, extend or shorten life span. This is the first time, to the best of our knowledge, that cocoa's pro-oxidant activity toward the

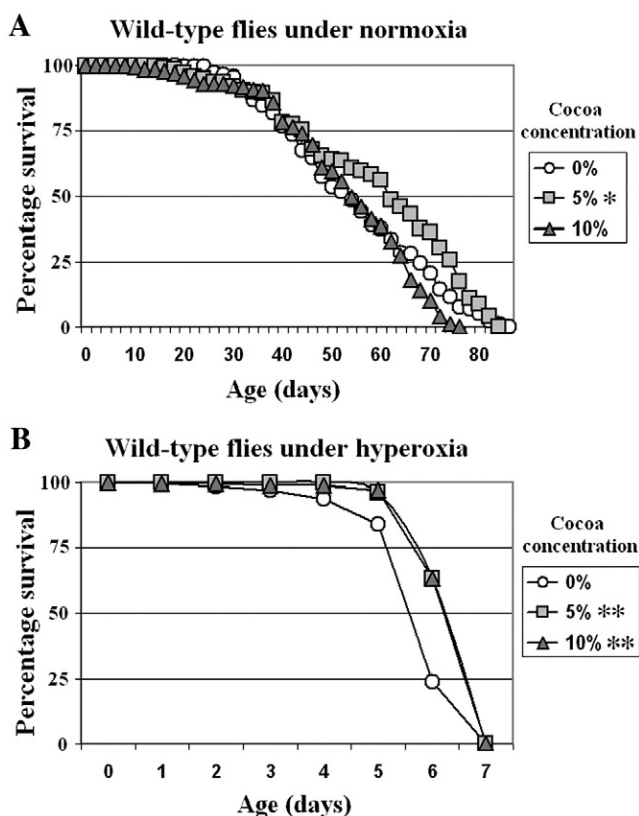


Fig. 2. Cocoa supplementation extended the average life span under normoxia (A) and under hyperoxia (B). The significance of the difference between survival curves ($n = 200$ flies per group) was analyzed using the Kaplan-Meier log-rank statistical test (* $P < .001$; ** $P < .00001$).

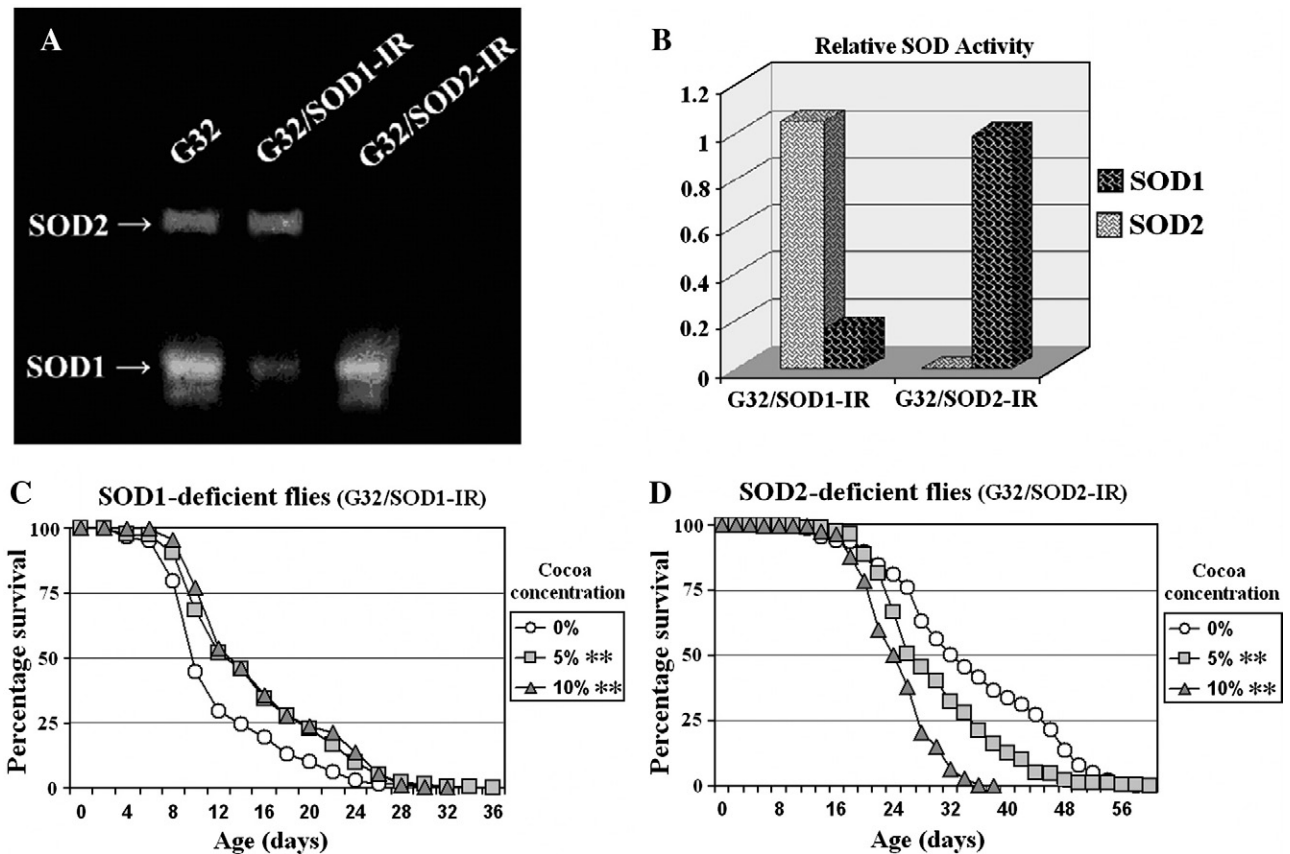


Fig. 3. Superoxide dismutase activity was measured using the in-gel nitroblue tetrazolium assay (A), and the intensity of SOD bands was normalized to that of *da^{G32}Gal4* (G32) control band (B). Cocoa supplementation under normoxia significantly increased the average life span of SOD1-deficient flies (C) but enhanced an earlier mortality in SOD2-deficient flies (D). The significance of the difference between survival curves ($n = 200$ flies per group) was analyzed using the Kaplan-Meier log-rank statistical test ($*P < .001$; $**P < .00001$). Genotypes were as follows: $w; +$; *da^{G32}Gal4/+* (G32), w ; *UAS-SOD1-IR/+*; *da^{G32}Gal4/+* (G32/SOD1-IR), and w ; *UAS-SOD2-IR/+*; *da^{G32}Gal4/+* (G32/SOD2-IR).

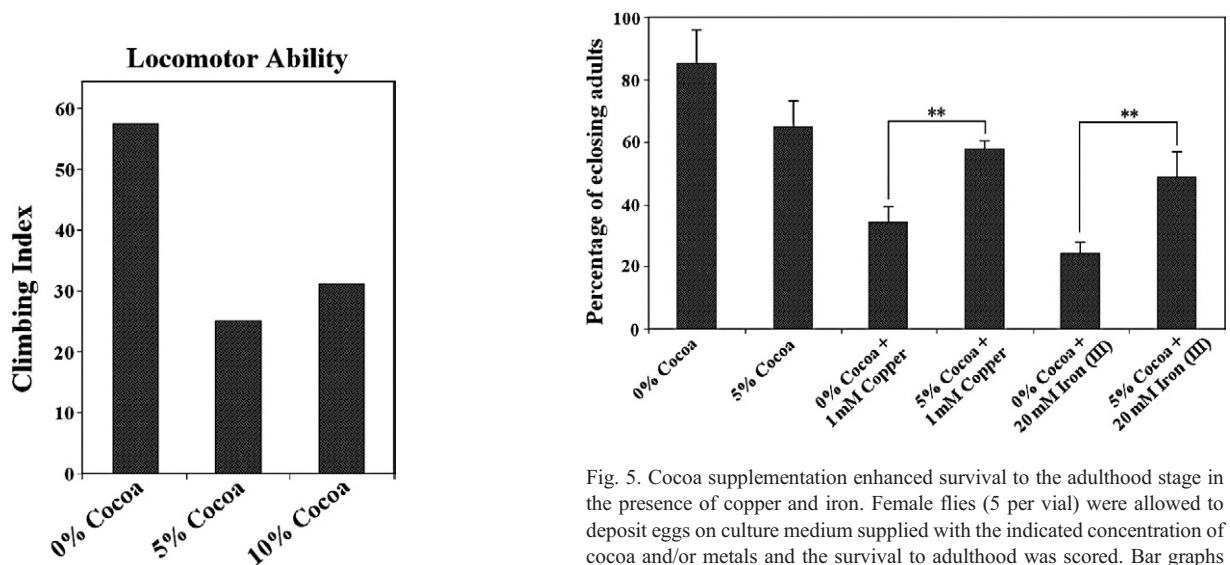


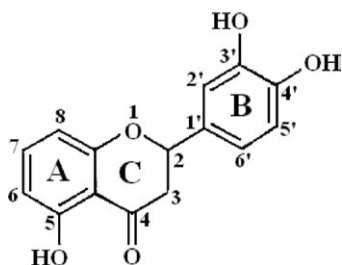
Fig. 4. Cocoa supplementation enhanced the loss of climbing ability in 30-day-old SOD2-deficient flies ($n > 50$ flies).

Fig. 5. Cocoa supplementation enhanced survival to the adulthood stage in the presence of copper and iron. Female flies (5 per vial) were allowed to deposit eggs on culture medium supplied with the indicated concentration of cocoa and/or metals and the survival to adulthood was scored. Bar graphs with errors represent means \pm SEM of at least 4 different vials. The significance of the difference between means was analyzed using the 1-way analysis of variance statistical test ($*P < .05$; $**P < .01$).

mitochondria is documented. Additional research is needed to further understand the mechanisms of this toxicity.

3.5. Cocoa supplementation in the presence of heavy metals

In addition to their antioxidant properties, flavonoids are known to exhibit a strong metal chelating activity, with the arrangement of 4-keto and 5-OH, or 3' and 4'-OH substituents resulting in the formation of chelating complexes between flavonoids and divalent cations (ie, copper and iron). The following generic structure of flavonoids represents the location of these substituents [22]:



To examine the metal-chelating properties of cocoa in *D melanogaster*, we tested larval survival to the adult stage on metal/cocoa-supplemented media. As Fig. 5 illustrates, cocoa supplementation significantly increased larval survival to adulthood on culture medium supplied with 1 mmol/L copper (II) sulfate or 20 mmol/L iron (III) sulfate hydrate, indicating that cocoa also acts as a strong metal chelator independent of its antioxidant activities. In a similar study, dietary tea was shown to act as an iron-chelating agent that prevents iron accumulation and extends life span in *Drosophila* [23]. Given this information, it is reasonable to argue that extension of life span on cocoa-supplemented medium may be partially attributed to the metal-chelating properties of cocoa, which decrease the accumulation of metal ions in flies and consequently prolong the life span. A limitation of this study, however, is the inability to distinguish between metal-chelating and anti/pro-oxidant contributions of cocoa to *Drosophila* life span.

Taken together, this study uncovers the effect of cocoa on *D melanogaster* longevity, which results from a combination of metal-chelating, antioxidative, and pro-oxidative activities. To our knowledge, this is the first time that the effects of cocoa on life span have been thoroughly studied in an animal model. Given that the fruit fly is an important model for studies on human nutrition and pharmacology, the results of this study suggest that moderate consumption of cocoa and its derivative products may have the potential to strengthen the mammalian antioxidant defense system and, consequently, extend their life span. However, considering the fact that cocoa may also exhibit pro-oxidant activities toward the mitochondria, the extent of life span extension may vary depending on genetic or environmental factors.

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