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## Decreased mitochondrial superoxide levels and enhanced protection against paraquat in *Drosophila melanogaster* supplemented with *Rhodiola rosea*

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### Abstract

The root extract from *Rhodiola rosea* has been reported to have numerous health benefits in human and animal studies. Its molecular mechanism is currently unknown; however, it has been suggested to act as an antioxidant. This study found that a formulation of *R. rosea* extract, *SHR-5*, from the Swedish Herbal Institute (SHI) could extend both mean (24% in both sexes) and maximum (16% in males and 31% in females) life span in *Drosophila melanogaster* when compared to controls. It also found that it lowered mitochondrial superoxide levels and afforded elevated protection against the superoxide generator paraquat in both sexes. The extract *SHR-5* did not alter the activities of the major antioxidant enzymes, the superoxide dismutases or catalase, nor did it afford protection against H<sub>2</sub>O<sub>2</sub> or soluble iron. These results present a decrease in endogenous superoxide levels as a possible mode of action for the root extract of *R. rosea*.

**Keywords:** *Rhodiola rosea*, botanical extract, oxidative stress, life-span, ageing

### Introduction

The herbal extract from *Rhodiola rosea* (Golden Root) was previously identified in an unbiased screen for compounds which could decrease fly mortality. It was subsequently found to increase both mean and maximum life span of the fly and did so without any negative physiological effects on reproductive fitness, male virility or metabolic rate [1]. *R. rosea* has been used in traditional medicine in Eastern Europe and Asia where it has numerous physical and mental health benefits attributed to its consumption. It has been purported to improve stamina, memory and mood, as well as protect against high altitude sickness [2]. *R. rosea* has also been found to attenuate tumour progression in a rat model [3], lower blood glucose levels in diabetic mice [4] and improve

endurance in rats [5], demonstrating broad physiological benefits.

Despite documentation of a variety of positive effects, there is a paucity of knowledge regarding the molecular mechanism of *R. rosea*. However, significant antioxidant activities have been documented for various *Rhodiola* species extracts. Ohsugi et al. [6] reported that of 19 compounds present in *R. sacra*, eight had scavenging activities against superoxide (O<sub>2</sub><sup>•-</sup>) and seven against hydroxyl radicals. In addition *R. imbricata* has been shown to protect mice against gamma irradiation, demonstrating direct protective effects against free radicals *in vivo* [7]. These antioxidant properties of other *Rhodiola* species suggest a similar potential mechanism for *R. rosea*. Of the 28 different compounds identified in *R. rosea*, its activity is primarily attributed to

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p-tyrosol, salidroside and five salidroside-like glycosides (rhodiolin, rosiridin, rosarin, rosavin, rosin). Three of these (rosarin, rosavin, rosin) are found in the species *rosea* [2].

Because of the proposed ability of *R. rosea* extract to act as an antioxidant, along with the hypothesized role of reactive oxygen species (ROS) in ageing [8] and the increasing implication of mitochondrial dysfunction in age-related diseases [9], we undertook our mechanistic studies with a particular focus on mitochondria, mitochondrial ROS and oxidative stress. We also used a formulation of *R. rosea*, *SHR-5*, with higher concentrations of the putative active components (salidroside, rosin, rosarin and rosavin) than the one used previously by Jafari et al. [1]. We found that *SHR-5 R. rosea* supplementation extended life span and did so to a degree greater than the previously-used extract. *SHR-5* also decreased mitochondrial  $O_2^{\cdot-}$  levels and enhanced protection against the  $O_2^{\cdot-}$  generator paraquat in both sexes. These results suggest that *R. rosea* extract may work by lowering  $O_2^{\cdot-}$  levels *in vivo* and that the active ingredients of *R. rosea* extract may be salidroside and/or the rosavins.

## Materials and methods

### *Herbal extract characterization by HPLC*

*Rhodiola rosea* (*SHR-5*) extract was obtained from the Swedish Herbal Institute. An independent HPLC analysis of this extract was performed by Alkemists Pharmaceuticals (Costa Mesa, CA). Two hundred milligrams of test powder was dissolved in 8 mL methanol and sonicated at 50°C for 1 h. Volume was brought to a total of 10 mL and filtered through a 0.45  $\mu$ M PTFE membrane prior to HPLC. Twenty  $\mu$ L of samples were run on a Luna phenyl hexyl C18 column at 35°C at a flow rate of 1 mL/min in a liquid phase mixture of 0.01% TFA/H<sub>2</sub>O and acetonitrile. Compounds were detected at 254 nm and amounts were calculated from the retention times and concentrations of known standards (Chromadex, Irvine, CA).

### *Fly strain*

The flies used in this study were derived from the B line of Rose [10] and are equivalent, though maintained as a separate colony. The B flies were originally derived from the South Amherst, Massachusetts population established by Ives in 1975, from an initial collection of 200 males and 200 females [10,11]. These flies have been cultured at moderate-to-large population sizes and controlled densities (50–80 eggs per vial) for more than 700 generations, with discrete generations cultured every 2 weeks. Flies are maintained at ambient interior temperature (24–26°C) and humidity.

### *Feeding and life span assays*

Flies were fed *R. rosea* extract based on the methods described in Jafari et al. [1]. Concentrations of *R. rosea* extract listed were dissolved in yeast paste (1 g yeast/mL 1% acetic acid) and 75  $\mu$ L of this mixture was overlaid on standard *Drosophila* banana-molasses food. Flies were maintained at 25°C under constant illumination for all experiments. For life-span studies, 200 flies for each treatment group (100 per sex) were housed 10 to a vial (five males and five females). This density was maintained as long as feasible. Flies were given fresh food every 2 days and deaths were recorded at these times. Kaplan-Meier survival analyses were calculated based on the number of deaths recorded. For mitochondrial respiration,  $O_2^{\cdot-}$ , superoxide dismutase (SOD) and catalase assays, 800 flies for each treatment group (400 per sex) and experiment were fed for 2 weeks in a manner described above. This time frame was selected as it was sufficient to mediate a detectable difference in life-span, but permits the study of relatively young animals, which would minimize normal age-related changes.

### *Fecundity assay*

Flies were collected less than 12 h post-eclosion. One male and one female were placed in each 8 dram vial containing 5 mL of a sugar-charcoal media covered with 75  $\mu$ L yeast solution (1 g yeast/mL, 1% acetic acid). Forty pairs per treatment (control and 25 mg/mL) were permitted to lay eggs for 24 h and then transferred to new vials every 24 h for 10 days. Eggs were counted every 24 h.

### *Oxidative challenge*

Two hundred flies for each treatment group (100 per sex) were fed *R. rosea* at 125 or 25 mg/mL for 1 week after which they were subjected to oxidative challenge of 12.5 mM paraquat, 5% H<sub>2</sub>O<sub>2</sub> or 80 mM Fe-NTA in a 5% glucose solution on filter paper. Fe-NTA solution was mixed according to Awai et al. [12]. Flies (five males, five females per vial) were housed in 8 dram vials. Deaths were recorded every 4 h until the completion of the assay. Survival was measured by both the Kaplan-Meier survival analysis and Mann-Whitney analysis of average life spans.

### *Mitochondrial isolation*

Fifty flies per sample were homogenized in ice-cold isolation buffer (225 mM mannitol, 75 mM sucrose, 10 mM MOPS, 1 mM EGTA, 0.5% fatty acid free BSA, PH 7.2) using a glass-teflon dounce homogenizer and then filtered through two layers of cotton gauze. A mitochondrial enriched pellet was obtained by centrifugation for 10 min at 6000  $\times$  g and re-suspended in 150  $\mu$ L ice-cold isolation buffer.

Fifty  $\mu\text{L}$  of these suspensions were used for each respiration or superoxide measurement.

#### *Mitochondrial respiration*

Mitochondrial respiration rates were measured using a Clark-type oxygen electrode and monitoring system (Hansatech Instruments, Norfolk, UK). An NADH-linked substrate (pyruvate in combination with malate, 5  $\mu\text{mol}$  each) was added to isolated mitochondria suspended in 1 mL of respiration buffer (225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris-HCl, 5 mM  $\text{KH}_2\text{PO}_4$ , pH 7.2) at 30°C. To this, 125 nmol ADP was added to generate state 3 respiration. State 3 respiration represents the maximal ability to generate ATP and is generally 5–6-fold higher than state 4 respiration which is determined after ADP is consumed. The ratio of state 3 to state 4 defines the respiratory control ratio (RCR) which is used to evaluate the functional quality of our mitochondrial preparations. Ratios of  $\sim 5$  or greater are considered to be functionally acceptable. Uncoupled (maximal rate of  $\text{O}_2$  consumption) respiration rates were measured after addition of 0.5 nmol of the uncoupler FCCP (Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone).

#### *Superoxide assay*

Rates of mitochondrial  $\text{O}_2^{\cdot-}$  production were measured with the fluorescent dye MitoSOX (Invitrogen, Carlsbad, CA) according to a protocol modified from Robinson et al. [13]. Briefly, mitochondria freshly isolated from 50 flies per sample were incubated in the presence of 10 mM succinate as an electron donor, 0.8  $\mu\text{M}$  MitoSOX and 2.5 mM ADP to induce state 3 respiration. Mitochondria were incubated for 5 min at 30°C, after which 15  $\mu\text{M}$  antimycin was added to block the electron transport chain and maximize  $\text{O}_2^{\cdot-}$  production. Superoxide production was then taken as the rate of MitoSOX oxidation measured at an excitation of 510 nm and emission of 579 nm and normalized to the amount of mitochondrial protein used.

#### *Enzyme activities and protein measurements*

Fifty flies per sample were homogenized in 2 mL of ice cold 50 mM potassium phosphate buffer, pH 7.0. Homogenates were centrifuged at 10 000  $\times g$  for 10 min at 4°C and the supernatants were used for enzymatic analyses. Superoxide dismutase activities were determined by an indirect method using xanthine/xanthine oxidase as a superoxide generator and nitroblue tetrazolium (NBT) as the target [14]. The SOD activities of the total fly homogenates were determined from a standard curve generated with purified Cu, Zn-SOD protein. One unit of SOD is defined as the amount of protein required to inhibit

NBT reduction (at 560 nm) by 50%. MnSOD levels are considered to be the level of SOD activity in the presence of 5 mM KCN which inhibits Cu, Zn SOD proteins. Catalase activity was measured by direct decomposition of  $\text{H}_2\text{O}_2$  (240 nm) in 50 mM potassium phosphate buffer, pH 7.0 [15]. Citrate synthase was measured by a method based on Williams et al. [16]. The reduction of dithionitrobenzoic acid is measured at 412 nm in the presence of 0.3 mM acetyl-coenzyme A and 0.5 mM oxaloacetic acid in 100 mM Tris-HCl, pH 8.0. SOD and catalase enzyme activities were reported as normalized to citrate synthase activity.

#### *Statistical analyses*

Data were presented as the mean  $\pm$  SEM. Statistical analyses were conducted using Prism software (GraphPad, La Jolla, CA). The tests used and sample sizes for each experiment are indicated in the figure captions and in the results section.

## **Results**

#### *Levels of rosavins and salidroside in Rhodiola rosea*

The active components of *R. rosea* are not known, but have been proposed to be the three rosavins (rosin, rosavin and rosarin) and salidroside [2]. The formulation used in a previous study on *Drosophila melanogaster* life-span [1] was composed of 60% *R. rosea* extract, which by HPLC analysis contained  $\sim 1\%$  salidroside, 0.2% rosavin and no detectable levels of rosin or rosarin. By comparison, the formulation used in this study, *SHR-5*, obtained from the Swedish Herbal Institute (SHI), contains 80% *R. rosea* extract and 20% maltodextrin, an inert filler. Via HPLC, we found *SHR-5* to have a salidroside content of 1.7% and a 4.5% content of total rosavins. Relative to the extract used previously, *SHR-5* had a 70% greater salidroside content and a greater than 20-fold increase in total rosavins per weight.

#### *The effect of Rhodiola rosea on Drosophila life-span*

Dose-response was assessed through supplementation of *R. rosea* at 5, 25 and 125 mg/mL. *R. rosea* resulted in a significant increase in survival in both sexes at 25 mg/mL and 125 mg/mL (Figure 1 and Table I). At 5 mg/mL, however, only males exhibited an increase in survival ( $p < 0.01$ , Kaplan-Meier analysis and Table I). The maximum magnitude of increase in mean life-span was 24% for females at 25 mg/mL and 31% for males at 125 mg/mL. Because maximum life-span is defined by a single individual and is not statistically meaningful, we used the mean of the longest-lived 10% (90% mortality) as surrogate for maximum life-span. Using this criterion, the maximum life span was increased up to 16% in males

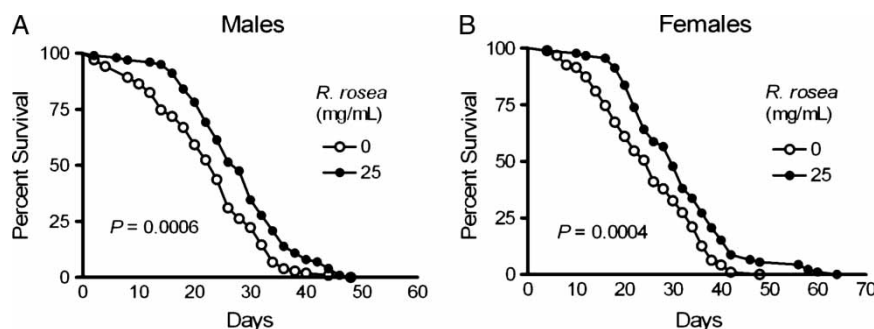


Figure 1. *Rhodiola rosea* supplementation and survival. Twenty-five mg/mL *R. rosea* increased survival in both (A) male and (B) female flies. *p*-values were calculated by Kaplan-Meier survival analysis,  $n=100$  for each treatment group and sex.

and up to 31% in females, both at 25 mg/mL. Because of the increased life-spans found in both sexes at doses of 25 and 125 mg/mL, these doses were used for further studies.

#### *The effect of Rhodiola rosea on fecundity*

As the disposable soma theory of ageing would predict a trade-off between longevity and reproductive fitness [17,18], we evaluated whether the *R. rosea* extract had such an impact on reproductive fitness via egg laying ability. We found a modest (15%), but statistically significant decrease in the number of eggs laid per female per day due to a diet supplemented with 25 mg/mL *R. rosea* extract (Figure 2).

#### *Protection against paraquat, but not H<sub>2</sub>O<sub>2</sub> or iron, by Rhodiola rosea*

Based on our initial hypothesis, we assessed *R. rosea* extract's ability to protect animals against oxidative insults. Fly survival was assayed after challenge to the superoxide generator paraquat, H<sub>2</sub>O<sub>2</sub> and iron-nitri- lotriacetic acid (Fe-NTA), which maintains iron in a soluble form that generates oxidative damage to lipids, proteins and nucleic acids [19]. *R. rosea* protected both sexes against paraquat (Figure 3), but not against H<sub>2</sub>O<sub>2</sub> or Fe-NTA ( $p > 0.05$  for both at both 25 and 125 mg/mL, data not shown).

#### *The effect of Rhodiola rosea on mitochondrial respiration and superoxide levels*

We next asked if *R. rosea* could modulate mitochondrial function as a potential explanation for its ability to increase stamina (i.e. elevated ATP production) or its ability to protect against paraquat (i.e. decreased ROS production). We found that *R. rosea* decreased superoxide production from isolated mitochondria in both males and females (Figure 4). However, it did not alter the major mitochondrial respiratory parameters (Table II), showing no appreciable effect on gross mitochondrial function. The RCR values reported here are nearly 5, which we consider to be functionally acceptable mitochondria, indicating that our respiration rates and superoxide production measurements are valid. These values were obtained in 2-week old flies, which already have a significant decrease in RCR values when compared to newly eclosed flies (data not shown).

#### *Antioxidant defenses in Rhodiola rosea fed flies*

It has also been suggested that *R. rosea* may act through hormesis [20], which is when a mildly toxic compound induces defense systems thereby conferring enhanced protection to further insults [21]. We asked whether *R. rosea* may be working through such a mechanism leading to the up-regulation of the major antioxidant defense systems in the fly, the SODs and catalase. We found no

Table I. Mean life span and 90% mortality values for *Rhodiola rosea* fed and control diet flies.

Sex	Dose (mg/mL)	<i>M</i>	<i>p</i>	90%	<i>p</i>
♂	0	22.5 ± 1.0		38.0 ± 1.5	
♂	5	26.0 ± 0.9	<0.05	41.6 ± 3.8	NS
♂	25	27.8 ± 0.9	<0.001	44.0 ± 0.8	<0.01
♂	125	29.2 ± 1.0	<0.001	43.6 ± 1.0	<0.05
♀	0	24.9 ± 1.0		40.6 ± 1.0	
♀	5	27.0 ± 1.0	NS	44.0 ± 0.5	NS
♀	25	31.0 ± 1.1	<0.01	53.1 ± 2.6	<0.001
♀	125	29.0 ± 1.0	NS	50.0 ± 1.6	<0.001

Values are means ± SEM. Units are days. Ninety per cent mortality is the mean life-span of the longest lived 10%. *p*-values were calculated by 1-way ANOVA and Dunn's multiple comparison test vs 0 mg/mL.

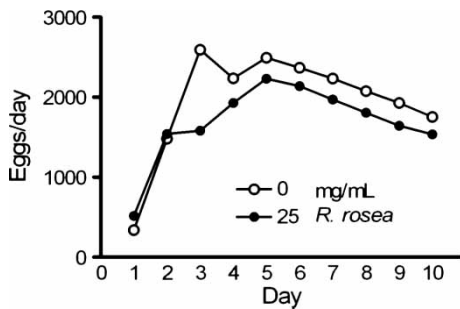


Figure 2. *Rhodiola rosea* supplementation and fecundity. Twenty-five mg/mL *R. rosea* negatively impacted fecundity.  $p < 0.05$ , repeated measures ANOVA,  $n = 40$ . The mean daily egg production per female was 49.4 for control and 42.2 for 25 mg/mL *R. rosea*.

measurable changes in the activities of these enzymes (Figure 5).

## Discussion

In addition to the numerous benefits attributed to *R. rosea* consumption in humans and in animal studies, we recently reported that it could also extend the life-span of the fruit fly *Drosophila melanogaster* [1]. Several studies have demonstrated the antioxidant properties of *R. rosea* extract [22–24] and one of its components, salidroside [25] which may explain its beneficial properties. Here, we have validated the positive effects of the botanical extract *R. rosea* on fly life-span and found that in both sexes it decreased mitochondrial  $O_2^{\cdot-}$  levels and protected against paraquat, a  $O_2^{\cdot-}$  generator.

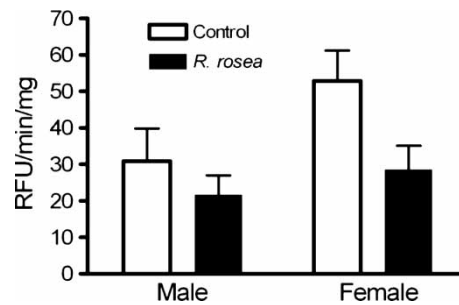


Figure 4. *Rhodiola rosea* and superoxide levels. *R. rosea* feeding decreased mitochondrial superoxide production from mitochondria isolated from both males and females.  $p < 0.05$  for diet,  $p = 0.07$  for sex, two-way ANOVA,  $n = 6$  for each group.

An antioxidant mode of action is consistent with *R. rosea* extract's life-span extending effects in the fly, as free radicals have been proposed to be an underlying cause of the ageing process [8]. In this study we chose to use an *R. rosea* extract, *SHR-5* from the Swedish Herbal Institute, which was purported to be superior to the extract used previously in Jafari et al. [1]. We found that *SHR-5* had 70% more salidroside and 20-fold more rosavin content (the putative active components) than the extract used in Jafari et al. [1]. Consistent with our previous study, *R. rosea* was also able to increase life-span (Figure 1 and Table I) and it did so to a greater degree (24% vs 13%) when used at dose levels comparable to the previously used extract. While this is suggestive that salidroside and the rosavins are indeed the active components, with their higher levels resulting in the greater effect of *SHR-5*, more definitive experiments using individual compounds are currently being undertaken. The effects of

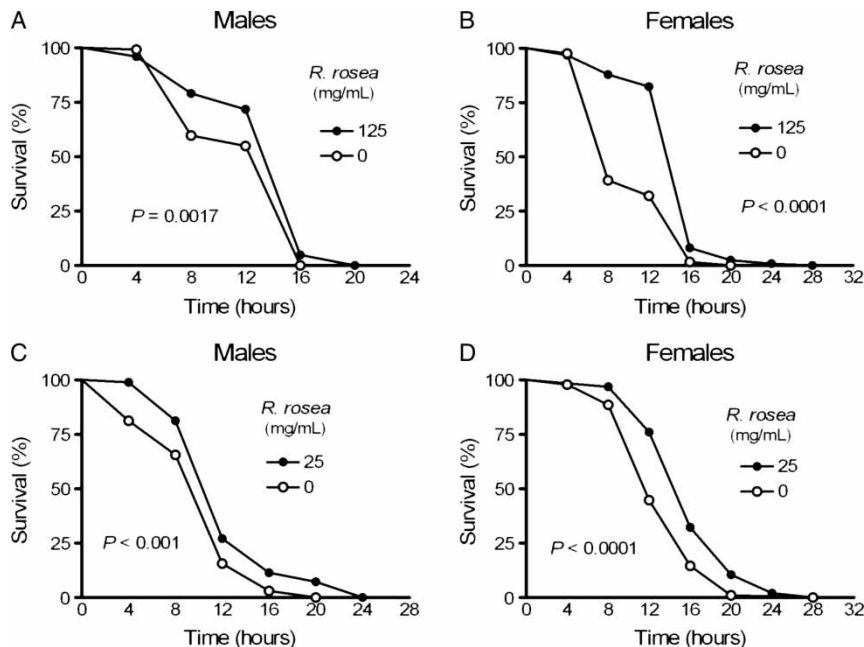


Figure 3. *Rhodiola rosea* supplementation and paraquat toxicity. Both males, (A) and (C), and females, (B) and (D), had enhanced survival when supplemented with *R. rosea* at the indicated doses.  $p$ -values were calculated by Kaplan-Meier survival analysis,  $n = 100$  for each treatment group and sex.

Table II. Respiratory parameters in mitochondria isolated from flies fed *Rhodiola rosea* at 25 mg/mL or control diet for 2 weeks.

Sex	Diet	State 3	State 4	RCR	Uncoupled
Male	Control	177 ± 16	37 ± 2	4.7 ± 0.4	192 ± 13
Male	<i>R. rosea</i>	174 ± 11	36 ± 3	4.9 ± 0.3	186 ± 21
Female	Control	204 ± 11	43 ± 3	4.8 ± 0.2	222 ± 18
Female	<i>R. rosea</i>	196 ± 13	40 ± 3	5.0 ± 0.3	202 ± 11
<i>P</i> -values	diet	0.68	0.52	0.65	0.43
2-way ANOVA	sex	0.07	0.10	0.84	0.16

Rates are mean ± SEM,  $n = 6$ , 50 flies per  $n$ .

Units are nmol O<sub>2</sub>/min/mg protein.

*SHR-5 R. rosea* extract on survival also appeared to be somewhat dose-dependent and possibly sex-specific. It increased survival in males at all three doses examined (Figure 1A and Table I), though the magnitude appeared to be smallest at 5 mg/mL (Table I). Female survival was only enhanced at the two highest doses, 25 and 125 mg/mL (Figure 1B and Table I).

Since we are interested in identifying and characterizing compounds and extracts which extend longevity under relatively normal physiological conditions, we conducted our experiments with flies maintained as mixed sex populations. The relatively short life-span of the flies in this work, when compared to those typically seen by others, may be due to the marked negative effect of mating on life span [26] and the corresponding elevation of mortality rates [27]. The life-span of the flies used here (22.5 days for males and 24.9 days for females) compare favourably to the equivalent colony from which they were derived (20.6 days for males and 20.8 days for females), when maintained in a similar environment [10]. By comparison, Khazaeli et al. [28], in an independent study, showed that males of the B line exhibited a mean life span (61.0 days) similar to  $w^{1118}$  males (64.0 days) and greater than Canton S males (51.8 days), when maintained in the absence of females [28]. Males of the B line (0.30 mg) were also larger than either  $w^{1118}$  (0.26 mg) or Canton S (0.22 mg) males [28]. As a result, the relatively short life-spans of the flies used in this study are likely reflective of our decision to conduct experiments with animals maintained in the presence of the opposite sex and are not indicative of the use of a genetically compromised or parasitically infected strain.

While *R. rosea* may have a beneficial effect on life-span, it may do so by compromising other physiological parameters, in particular fecundity, which is negatively correlated to life-span [29,30]. Such an effect would also be predicted by the disposable soma theory of ageing [17,18]. We found that *SHR-5 R. rosea* did have a negative, though relatively modest, impact on fecundity at 25 mg/mL. This is in contrast to what was reported with the previously used extract which decreased fecundity at higher doses but had no

detectable impact on fecundity at lower doses that still increased lifespan [1]. This may be due to the relatively lower levels of rosavins and salidroside in the previously used extract, which also had a correspondingly lower effect on life-span as well. Even though *R. rosea* may negatively affect fecundity to some degree, the magnitude of life-span extension conferred by it may be too great to be explained solely by this mechanism. Chippindale et al. [30] have argued that the cost of producing one egg per day per fly is ~0.15 days of adult life. At 25 mg/mL of *R. rosea*, we found an average decrease of 7.2 eggs per day per fly compared to control-fed flies, which would correspond to only an additional 1.1 days of life. The increases in mean life span at this dose were ~6 days in females and 5 days in males. The mean increases at 90% mortality were over 12 days in females and 6 days in males. Thus, the increases in life-span observed due to *R. rosea* supplementation are much greater than what would be predicted from a decrease in fecundity alone.

In addition to increasing life-span, *R. rosea* extract protected both males and female flies against paraquat, which generates the superoxide anion (O<sub>2</sub><sup>•-</sup>). This is consistent with our hypothesis that *R. rosea* extract mediates its effects through an antioxidant mechanism. The lack of an up-regulation of the major antioxidant enzymes, SOD and catalase, is also consistent with our hypothesis, as it would not be expected for an antioxidant to elevate antioxidant defenses. This is in contrast to what is observed for green tea extract—which also extended fly life-span—where both SOD and catalase activities were elevated [31]. This suggests that *R. rosea* extract and green tea extract may work via different mechanisms. Recent *in vitro* assays have also supported the possibility that *R. rosea* extract may directly interact with O<sub>2</sub><sup>•-</sup> [6]. However, these studies must be interpreted with caution as pro-oxidant compounds, such as reduced metals, will also interact with O<sub>2</sub><sup>•-</sup> in these assays. Interestingly, another species of *Rhodiola*, *R. imbricata*, was found to protect mice against radiation toxicity [7], demonstrating the ability of *Rhodiola* extract to protect against free radicals *in vivo*. The antioxidant effect of *R. rosea* may also

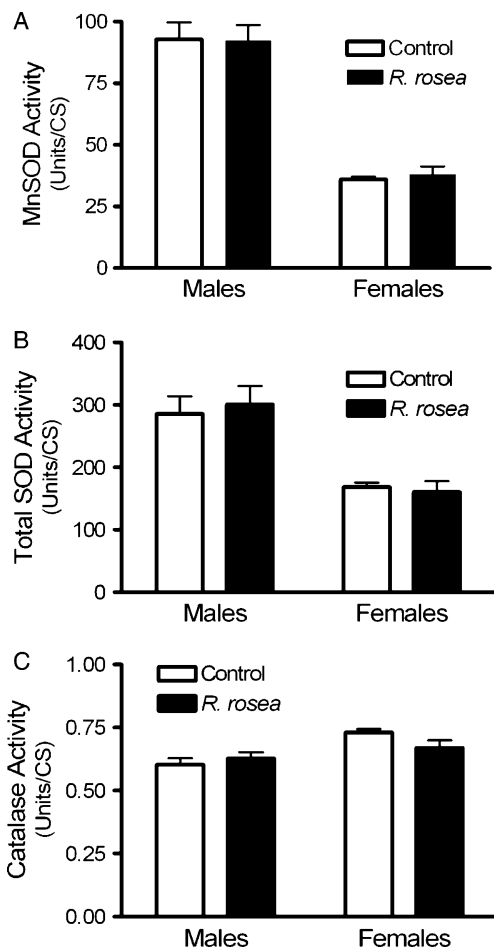


Figure 5. *Rhodiola rosea* supplementation and activities of the major antioxidant enzymes. (A) Mitochondrial superoxide dismutase ( $p=0.87$ ), (B) total SOD, which is predominantly the cytosolic Cu,ZnSOD ( $p=0.88$ ) and (C) catalase ( $p=0.50$ ) activities were unaffected by *R. rosea* feeding.  $p$ -values were calculated by 2-way ANOVA for diet,  $n=5$ . It is interesting to note that our results support a significant difference in SOD activity between males and females,  $p<0.0001$  for both mitochondrial SOD and total SOD, 2-way ANOVA.

be specific to  $O_2^{\bullet-}$  as evidenced by its inability to protect flies against  $H_2O_2$  or soluble iron.

Using the  $O_2^{\bullet-}$  sensitive dye MitoSOX, which localizes to the mitochondrial matrix, we found that *R. rosea* feeding could lower  $O_2^{\bullet-}$  levels generated within isolated mitochondria. This presents an alternative hypothesis that *R. rosea* may alter mitochondrial function in such a way as to decrease  $O_2^{\bullet-}$  production. This could occur by any mechanism which results in elevated electron flow, such as elevated ATP production (state 3) or elevated uncoupling (state 4), as oxygen consumption and ROS production are inversely related. However, the inability of *R. rosea* to alter any of the major respiratory parameters of mitochondria, including state 3 and state 4, argue against this. As mitochondrial SOD activities were also unaffected by *R. rosea* (Figure 5A), a decrease in  $O_2^{\bullet-}$  levels are likely occurring by direct interaction. If this were true, the

finding of lowered  $O_2^{\bullet-}$  levels in *isolated* mitochondria argue that *R. rosea* must be able to localize within the mitochondrion, in addition to the cytosol. However, we cannot definitely rule out changes in mitochondrial function at this time as the sensitivity of the fluorescent dye used is several orders of magnitude better than respiration measurements. As a result, we may miss subtle changes in oxygen consumption rates. Nevertheless, the findings that *R. rosea* can decrease mitochondrial  $O_2^{\bullet-}$  levels and protect against  $O_2^{\bullet-}$  insult, combined with findings that lowered  $O_2^{\bullet-}$  levels correlate with extended life span in *Drosophila* [32,33], argue that *R. rosea* may be acting, at least in part, through lowered levels of endogenous  $O_2$ .

In summary, our findings suggest removal of existing  $O_2^{\bullet-}$  and/or a decreased production of  $O_2^{\bullet-}$  as possible mechanisms for the mode of action of *R. rosea* extract. The finding that *R. rosea* can extend lifespan in a commonly used experimental organism, coupled with its safety profile in a large number of human studies, also presents it as an attractive agent with the potential to retard the ageing process and attenuate age-related disease in humans.

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**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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