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Rhodiola: A Promising Anti-Aging Chinese Herb

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ABSTRACT

Using the fruit fly, *Drosophila melanogaster*, we investigated the effects of *Rhodiola* on lifespan. *Rhodiola* is a plant root used in traditional Chinese medicine that may increase an organism's resistance to stress. It has been proposed that *Rhodiola* can extend longevity and improve health span by alleviating oxidative stress. *Rhodiola* supplied every other day at 30 mg/mL significantly increased the lifespan of *Drosophila melanogaster*. When comparing the distribution of deaths between *Rhodiola*-supplemented and control flies, *Rhodiola*-fed flies exhibited decelerated aging. Although the observed extension in lifespan was associated with statistically insignificant reductions in fecundity, correcting for a possible dietary restriction effect still did not eliminate the difference between supplemented and control flies, nor does the effect of *Rhodiola* depend on dietary manipulation, strongly suggesting that *Rhodiola* is not a mere dietary restriction mimetic. Although this study does not reveal the causal mechanism behind the effect of *Rhodiola*, it does suggest that the supplement is worthy of continued investigation, unlike the other Chinese herbals, Lu Duo Wei (LDW), Bu Zhong Yi Qi Tang (BZYQT), San Zhi Pian (SZP, Three Imperial Mushrooms), Hong Jing Tian (*Rhodiola*) that were evaluated in this study.

INTRODUCTION

IN DEVELOPED COUNTRIES, chronological age is the most significant risk factor for noncontagious disease.¹ Thus, it is understandable that many suppose that understanding the aging process might play a major role in the development of therapies to slow or alleviate age-related diseases such as cancer, cardiovascular disease, and cognitive disorders. Some further imagine that such "anti-aging" measures include dieting, weight control, exercise, and stress management. Although these are all desirable interventions, patient compliance with

these measures is a considerable obstacle to overcome for health professionals.

With the advent of successful genetic interventions in aging among model organisms over the past 3 decades, pathways regulating aging and potential molecular targets for interventions have been identified.² Although the genetic methods applied to these model systems are not appropriately applied to humans, the pathways that they reveal are considered helpful clues for pharmacological intervention. The genetic mapping of aging in animal models has thus opened the door for "anti-aging" drug discovery.

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In recent years, increases in longevity have been achieved by supplementing pharmaceuticals and botanicals with antioxidative properties to various laboratory organisms. For example, superoxide dismutase/catalase mimetics have been shown to extend the mean lifespan of wild-type worms by 44%.³ Furthermore, there have been recent reports that supplements such as lipoic acid, resveratrol, melatonin, and Lu-Duo-Wei (traditional Chinese medicine herb) can extend *Drosophila melanogaster* lifespan, supposedly by reducing oxidative damage to macromolecules.⁴⁻⁶ These few cases of extended longevity resulting from drug exposure suggest both that aging can be slowed by targeted interventions and that the pharmacology of lifespan extension is a practical area of biomedical research.

Pharmacological approaches to postpone or slow aging have used several experimental systems. Nematodes, fruit flies, and mice have all provided useful data, and are candidate model systems for anti-aging pharmacology. The fruit fly, *Drosophila melanogaster*, is a particularly effective a model system for aging research. Fruit flies and humans share key metabolic pathways, such as superoxide metabolism, DNA repair, and insulin-like signaling. They also have similar pathophysiological outcomes of these pathways. Furthermore, *Drosophila* has been used as a model system for aging for more than 90 years,⁷ with notable successes in experiments specifically focused on postponing its aging.⁸ For these reasons, we have chosen to use fruit flies as a model system for screening of candidate anti-aging compounds.

Anti-aging pharmacological intervention is likely to involve secondary effects, given the multifold pathways that affect fruit fly aging.⁹⁻¹¹ Although various compounds that have been tested in *Drosophila* have increased the lifespan of these model systems, this work has typically lacked evaluation of secondary mechanisms that could have resulted in ostensible anti-aging effects artifactually.¹² Particular pharmaceuticals or botanicals that affect aging may do so through multiple pathways, not just the pathway that is of a priori interest. Most importantly, it is not appropriate to simply assume that a compound that affects adult survival *only* has such effects. Such compounds

might also have adverse secondary effects that would limit their clinical use in the treatment of aging. To date, anti-aging studies have not systematically evaluated the secondary adverse effects of potential anti-aging compounds, particularly negative effects on reproduction.^{4,5}

The *Rhodiola* root has long been used in the traditional medical systems in Europe and Asia to increase an organism's resistance to physical stress¹³; currently, it is widely thought to have antioxidative properties.¹⁴ Studies in cell cultures have demonstrated that *Rhodiola rosea* extract has a protective effect against free-radical oxidative damage in human red blood cells.¹³ This study was designed to examine the effect of *Rhodiola* and other Chinese Herb mixtures on the lifespan of adult *Drosophila*. In addition, we performed experiments to detect life extension confounds and artifacts.

METHODS

Drosophila population used

All *Drosophila melanogaster* stocks used in these experiments were ultimately derived from a sample (called "IV") of the Amherst, Massachusetts, Ives population that was collected in 1975 and cultured at moderate to large population sizes ever since.^{15,16} This population has been reared at controlled densities (50–80 eggs per vial) for more than 700 generations with discrete generations cultured every 2 weeks.

Maintenance of flies

All flies used in these assays were raised as larvae in 5 mL of standard banana-molasses food at densities of between 50 and 80 eggs per 8-dram vial. Populations were maintained at about 25°C with constant illumination. During the assays, adults were kept in standard 5-mL food vials containing banana-molasses food and 1 mg of yeast paste to promote egg laying.

All Chinese herbal compounds were supplied to adults only. The Chinese Herbs were mixed into the yeast paste; the adults preferentially consume this paste. Adults were transferred to fresh vials and survivors were

counted every 2 days. All assays were conducted on flies that had undergone two generations of controlled density rearing. When flies from different treatments were compared, all preliminary rearing was carried out in parallel.

Chinese herbal trials

The powder of each Chinese Herb mixture was mixed into the yeast paste. The calculated dose used in our data analyses reflects the final concentration of the herb in the yeast paste that flies consumed during the experiment. Compounds were obtained from Sun Ten, Inc., in powder format. For each trial, various doses of each compound were compared to a control. We screened the Chinese Herbal mixtures at the following doses: Lu Duo Wei (LDW) at 0, 2, 10, 50, 60, 100, 200 mg/mL, Bu Zhong Yi Qi Tang (BZYQT) at 0, 60, 100, 200 mg/mL, San Zhi Pian (Three Imperial Mushrooms) at 0, 15, 30, and 60 mg/mL, and Hong Jing Tian (*Rhodiola*) at 0, 15, 30, 60, 100, and 200 mg/mL.

Mortality assays

We evaluated the impact of each compound on mortality. For each assay, three doses of each compound were compared to a control group. For each dose, 320 males and 320 females were exposed to the compound. There were 4 males and 4 females in each vial, with a total of 80 vials per dose per sex, and the flies were transferred every other day during the aging phase, which lasts 4 weeks in IV stocks.¹⁷ We attempted to determine a dose-response relationship during the mortality assays. This required repeating testing of the same compound over a range of doses. Compounds demonstrating a beneficial effect on mortality were subjected to fecundity assays, as described below.

The data were analyzed by examining the number of surviving flies at the end of week 4. Significant differences between the control and each drug treatment were assessed by the Pearson chi-square test.

Survival probability

Rhodiola resulted in a beneficial effect on mortality; therefore, a total survivorship assay

was conducted to reaffirm the result. This assay also allowed for the determination of mean lifespan change as a result of the herb. Survivorship was determined with every-other-day transfers until every fly was dead. The most efficacious dose from the mortality assay, 30 mg/mL of *Rhodiola*, was compared to a control. For each group (control vs. drug treatment), 2400 males and 2400 females were used in sets of 8 (4 males: 4 females) per vial. The flies from one group were housed in 15 separate racks.

Gompertz mortality. In this formulation we will let the index i indicate one of the 15 racks, j indicate sex (1 = female, 2 = male), k indicates drug treatment (1 = control, 2 = drug treated), and t indicate age. Then the predicted mortality between ages t and $t + 1$ is y_{ijkt} . The basic nonlinear model is given by,

$$y_{ijkt} = f(\Psi_{ijk}, t) + \varepsilon_{ijkt}, \quad (1)$$

where Ψ_{ijk} is the vector of parameters, t is the age, and ε_{ijkt} is the within population variation. The function f is the Gompertz model,

$$f(\Psi_{ijk}, t) = 1 - \exp\left\{\frac{A_{ijk}}{\alpha_{ijk}} [\exp(\alpha_{ijk}t) - \exp(\alpha_{ijk}(t + 1))]\right\}. \quad (2)$$

The parameter A is sometimes called the age-independent parameter of the Gompertz and is a reflection of background mortality that does not change with age. On the other hand, α is called the age-dependent parameter and measures the rate at which mortality increases with age, e.g., senescence. We assume that the parameters of the Gompertz equation may be affected by the fixed effects, sex and drug treatment, and the random rack environment. These assumptions translate into a system of equations,

$$\begin{aligned} A_{ijk} &= \beta_1 + \gamma_1\delta_j + \phi_1\delta_k + b_{1i} \\ \alpha_{ijk} &= \beta_2 + \gamma_2\delta_j + \phi_2\delta_k + b_{2i}, \end{aligned} \quad (3)$$

where $\delta_i = 0$ if $i = 1$, or 1 otherwise. To test for significant effects of sex on A and α , we determined whether γ_1 or γ_2 is significantly different from 0, respectively. Likewise, a test for the

effects of drugs on A and α corresponds to a test for whether ϕ_1 or ϕ_2 is significantly different from 0. Model (3) could be expanded to include interactions between sex and drugs. Although we tested for such interactions; since they were never significant, we do not include them in the basic model described here.

The variance of mortality is expected to change with the mean value of mortality. The general formulation for the variance of ε_{ijkt} is,

$$\text{Var}(\varepsilon_{ijkt}) \cong \sigma^2 g^2(\hat{u}_{ijkt}, t), \quad (4)$$

where $\hat{u}_{ijkt} = E(y_{ijkt} | \mathbf{b}_i)$. In this analysis we used $g(\cdot) = |y_{ijkt}|^\delta$. The \mathbf{b}_i were distributed as,

$$\mathbf{b}_i \sim N\left(\mathbf{0}, \begin{bmatrix} \sigma_1 & 0 \\ 0 & \sigma_2 \end{bmatrix}\right). \quad (5)$$

The parameters in Equations (3–5) were estimated from a nonlinear mixed effects model as implemented by the *nlme* package of R (r-project.org; version 2.4.0; see also Pinheiro and Bates, 2000, chapter 7).¹⁸

Two-stage Gompertz mortality. Large cohorts of fruit flies exhibit departures from the Gompertz mortality dynamics in the form of a leveling off of mortality rates at advanced ages.^{19,20} We have developed a model, called the two-stage Gompertz, which shows Gompertz dynamics at young ages and then at an advanced age, called the break day, mortality rates plateau at a constant value.¹⁷ The discontinuity in the two-stage Gompertz model caused by the break day makes it especially difficult to obtain estimates of the parameters for this model.²¹ As a practical matter, the same methods used to infer the drug effects on the Gompertz model cannot be used with the two-stage Gompertz.

To overcome this difficulty, we have used bootstrap samples as a means of determining whether there were significant differences in the two-stage Gompertz parameters due to drug treatment. For the two-stage Gompertz model, the probability of surviving to age t is

$$p(t) = \begin{cases} \exp(A(1 - \exp(\alpha t))/\alpha) & \text{if } t \leq bd \\ \exp(A(1 - \exp(\alpha bd))/\alpha) + A_2(bd - t) & \text{if } t > bd \end{cases} \quad (6)$$

where bd is the break day, A and α have similar interpretations as in the Gompertz, and A_2 is the plateau mortality rate. The mortality rate between ages t and $t + 1$ is

$$1 - \frac{p(t+1)}{p(t)}. \quad (7)$$

Least-squares estimates of the parameters $\theta = (A, \alpha, bd, A_2)$ are obtained by minimizing the sum of squared differences between the observed and predicted mortality (Eq. 7). In the following discussion, we will also refer to the components of θ as $\theta_1, \theta_2, \theta_3, \theta_4$. Because the least-squares surface can sometimes be very flat, we used four very different initial conditions to begin our numerical search for the least-squares estimates. The initial condition that leads to estimates with the smallest sum of squares is used for the final estimate of θ .

One set of observations consists of a vector of ages of death, $T = (t_1, t_2, \dots, t_m)$, the number of deaths at each of those ages, $D = (d_{t_1}, d_{t_2}, \dots, d_{t_m})$, and a total sample size of $N = \sum_i d_{t_i}$. To create bootstrap samples, we need to define the probability of dying on any one of the m -days in T as $P_i = d_{t_i}/N$. One bootstrap sample then consists of N individuals whose ages of death are equal to one of the days in T . Thus, the chance of choosing day t_i as the day of death in the bootstrap sample is P_i .

This sampling process was used to create B bootstrap samples, $D^{*1}, D^{*2}, \dots, D^{*B}$. From each of these samples, a least-squares, θ^{*i} , estimate was made by the methods described above. The bootstrap parameter estimates are given by

$$\hat{\theta}^* = \left(B^{-1} \sum_i \theta_1^{*i}, B^{-1} \sum_i \theta_2^{*i}, B^{-1} \sum_i \theta_3^{*i}, B^{-1} \sum_i \theta_4^{*i} \right). \quad (8)$$

We generated B bootstrap samples for the control samples, θ_c^* and the drug-treated sample, θ_d^* . From these we computed the difference between the drug treatment parameter values and the control treatment parameter values, $\hat{\theta}_d^* - \hat{\theta}_c^*$. To test for significant differences between the drug and control, we created a random permutation, without replacement, of the $2B$ control and drug bootstrap vectors. We then computed B differences between the first B val-

ues of the permuted vector and the last B values. This process was repeated 1000 times to create an empirical distribution to judge the statistical significance of the differences $\hat{\theta}_d^* - \hat{\theta}_c^*$.

Fecundity assays

Some compounds may increase lifespan simply by substantially depressing fecundity; depressed fecundity will increase longevity by and of itself in *Drosophila*.^{12,22} Therefore, a fecundity assay is an important check for artifactual lifespan enhancement. We evaluated age-specific fecundities using the same dosing protocols as above, except that the flies were handled with one female and one male in each assay vial. The number of eggs laid each day by each individual female was recorded for a period of 10 days. The standard fecundity assay was used to measure fecundity rates. At less than 12 hours from pupa emergence, flies are collected as newly

TABLE 1. FRACTION THAT DIED DURING AGING PHASE WITH LDW, BZYQT, AND SZP

Chinese Herb	Dose (mg/ml)	Mortality rate \pm SD	p-value	
LDW	Male			
	0 (Control)	0.69 \pm 0.03	—	
	0.016	0.65 \pm 0.03	0.26	
	0.08	0.63 \pm 0.03	0.12	
	0.4	0.68 \pm 0.03	0.76	
	Female			
	0 (Control)	0.64 \pm 0.03	—	
	0.016	0.61 \pm 0.03	0.58	
	0.08	0.56 \pm 0.03	0.05	
	0.4	0.70 \pm 0.03	0.01	
	BZYQT	Male		
		0 (Control)	0.67 \pm 0.03	—
0.0625		0.70 \pm 0.04	0.51	
0.1		0.59 \pm 0.04	0.10	
0.2		0.65 \pm 0.04	0.73	
Female				
0 (Control)		0.44 \pm 0.03	—	
0.0625		0.45 \pm 0.04	0.78	
SZP	Male			
	0 (Control)	0.62 \pm 0.03	—	
	0.125	0.87 \pm 0.02	0.00	
	0.25	0.82 \pm 0.02	0.00	
	0.5	0.99 \pm 0.01	0.00	
	Female			
	0 (Control)	0.60 \pm 0.03	—	
	0.125	0.63 \pm 0.03	0.39	
	0.25	0.64 \pm 0.03	0.26	
	0.5	0.76 \pm 0.03	0.00	

TABLE 2. FRACTION THAT DIED DURING AGING PHASE WITH RHODIOLA ROSEA

Replicate	Dose (mg/ml)	Mortality rate \pm SD	p-value
1	Male		
	0 (Control)	0.61 \pm 0.03	—
	15	0.56 \pm 0.03	0.23
	30	0.38 \pm 0.03	0.00
	60	0.51 \pm 0.03	0.01
	Female		
	0 (Control)	0.62 \pm 0.03	—
	15	0.48 \pm 0.03	0.00
	30	0.44 \pm 0.03	0.00
	60	0.61 \pm 0.03	0.82
2	Male		
	0 (Control)	0.71 \pm 0.03	—
	60	0.48 \pm 0.03	0.00
	100	0.43 \pm 0.03	0.00
	200	0.42 \pm 0.03	0.00
	Female		
	0 (Control)	0.69 \pm 0.03	—
	60	0.50 \pm 0.03	0.00
	100	0.51 \pm 0.03	0.00
	200	0.52 \pm 0.03	0.00
3	Male		
	0 (Control)	0.63 \pm 0.03	—
	30	0.48 \pm 0.03	0.00
	Female		
0 (Control)	0.84 \pm 0.02	—	
30	0.65 \pm 0.03	0.00	
4	Male		
	0 (Control)	0.28 \pm 0.03	—
	30	0.24 \pm 0.03	0.35
	Female		
0 (Control)	0.43 \pm 0.03	—	
30	0.27 \pm 0.03	0.00	

emergent adults. Adult flies are transferred to 8-dram charcoal-food vials containing 75 μ l yeast solution at a density of one female and one male fly per vial and allowed to lay eggs for 24 hr. The experimental groups were supplemented with a mixture of yeast solution and *Rhodiola*, whereas the controls were supplemented with yeast solution only. The flies to be assayed were transferred daily at regular intervals for 10 days to fresh charcoal-food vials with *Rhodiola* supplement. For 10 days, the number of eggs laid was counted daily and recorded using 16 \times light stereoscopes. The number of flies assayed for fecundity rates was 160 females per experiment. Forty females per dose (low, medium, and high) were evaluated including 40 females for the control group. Thus, we examined 160 females at a time, assaying fecundity rates over the first 10 days of life, with daily transfers. We analyzed

the total number of eggs laid over 10 days for each female. The impact of drugs on fecundity was assessed by a one-way analysis of variance (ANOVA). We also compared the 10-day fecundity of control females to the pooled data from all three doses using a standard *t*-test on the mean values.

Diet assays

Two assays were performed to evaluate the impact of interaction of diet with *Rhodiola* on the mortality rate. The same methodologies (i.e., number of flies, density in each vial, frequency of transfers, etc.) used in the mortality assay were employed for the diet assays. In one assay, control assay, flies were exposed to the following concentrations of yeast: 0.5 mg/vial, 1.0 mg/vial, and 1.5 mg/vial. The yeast solutions contained 50 mL water, 2 mL 1% acetic acid, and the respective amount of yeast. In the other, *Rhodiola*-supplemented, assay, flies were exposed to the above concentrations of yeast plus 30 mg/mL of *Rhodiola* in each vial.

Metabolic rate assays

As a further test for artefactual effects, compounds that had a beneficial effect on mortality, but did not significantly depress fecundity, were assayed for their effect on metabolic rate. This assay was used to ascertain whether there had been an artefactual decrease in mortality due to

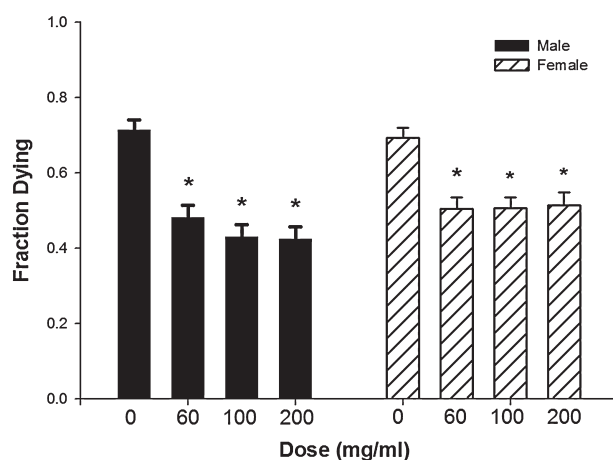


FIG. 1. Fraction dying with *Rhodiola* at 60, 100, and 200 mg/mL. Decrease in fraction dying with 60, 100, and 200 mg/mL in male flies and a decrease in fraction dying with 60, 100, and 200 mg/mL in female flies ($p < 0.05$). * $p < 0.05$.

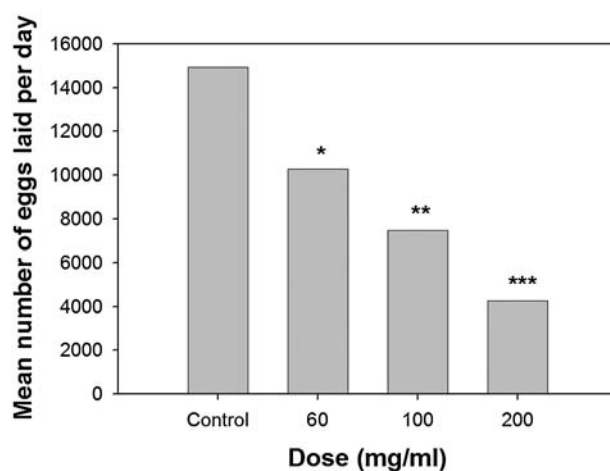


FIG. 2. The impact of *Rhodiola* at 60, 100, and 200 mg/mL doses on fecundity. A dose response decrease in fecundity is observed. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

hypometabolism. In addition, metabolic rate is a useful surrogate for a number of physiological and behavioral functions that could potentially be impaired by medications, such as locomotor activity. CO₂ production in drugged flies was compared to that of a control group handled in parallel and assayed simultaneously. We used flow-through respirometry to measure the rate of CO₂ release from groups of flies following the methods of Williams et al.²³ Room air was passed through a small chamber containing soda lime, two silica gel chambers, and a Drierite/Ascarite/Drierite column (Drierite; W.A. Hammond Drierite Co.; Ascarite (Arthur H. Thomas Co.)). The air was directed by a series of computer-controlled valves (Sable Systems, Las Vegas, NV) that allowed six individual flies in separate chambers to be measured in turn. The flies were measured while in vials in the presence of food and the appropriate pharmacological agent. Measurements of CO₂ were made using a Licor LI-6260 gas analyzer. CO₂ levels were averaged and recorded every second using data acquisition software (Sable Systems). Each vial was measured for 20 minutes. During periods when they were not being measured, the flies were kept in a stream of dry, CO₂-free air by flushing with a separate air stream. The last 5 minutes of each 20-minute recording of CO₂ release was averaged to provide an estimate of relative metabolic rate. The effects of drug treatments on metabolic rates were analyzed with a one-way ANOVA using drug level treated as a fixed effect.

Mating success assay

Male virility was used as an assay for a variety of possible secondary drug effects on fly behavior, including reduced activity and other forms of central nervous system depression. In order to administer the herbs to the males, they were maintained in vials containing the herb/yeast solution for 10 days prior to the virility assay, using the same protocol as that for the mortality assays. The control (no-herb) males were maintained in parallel, with all conditions of rearing and maintenance identical except for the absence of the herb in the yeast paste given to the control males.

For each assay of virility, two male flies—one that was exposed to the Chinese Herb and one that was not exposed to the Herb—were placed in a mating vial with a virgin female fly, who had not been exposed to the Chinese Herb. Among the mating vials, half had marked drugged males; the other half had marked control males. The marking procedure used a felt-tip marker to color the tip of one wing.

Each virility assay used 120 vials, with 40 vials for each dose. Virility was scored according to the number of vials in which the drugged male mated with the test female. The scoring of a successful mating required mounting for at least 30 seconds.

In this experiment, each male was scored according to his drug status (control or drugged),

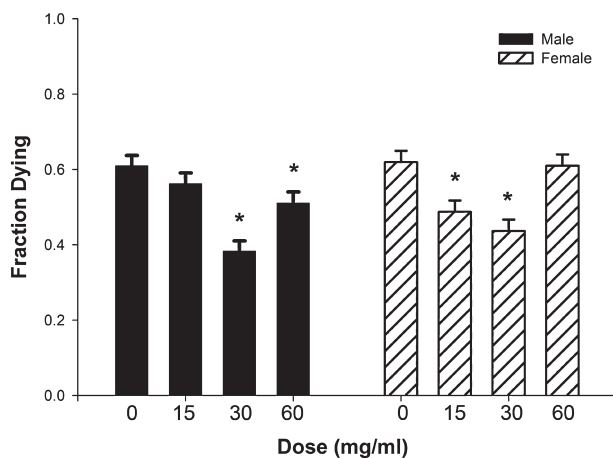


FIG. 3. Fraction dying with *Rhodiola* at 15, 30, and 60 mg/mL. Decrease in fraction dying with 30 mg/mL and 60 mg/mL in male flies and a decrease in fraction dying with 15 mg/mL and 30 mg/mL in female flies ($p < 0.05$).

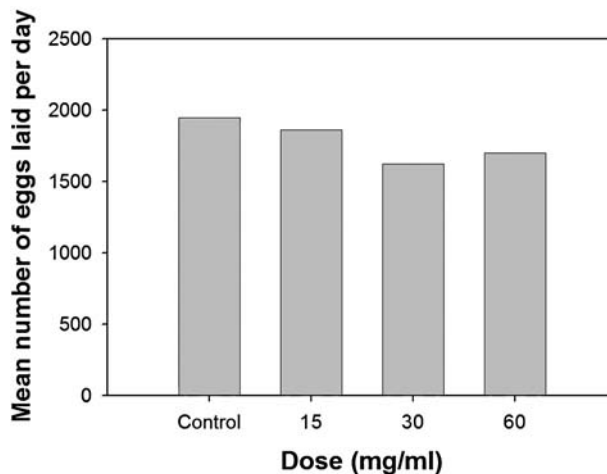


FIG. 4. The impact of *Rhodiola* at 15, 30, 60 mg/mL doses on fecundity. There is no impact on fecundity at any of these doses.

marked status (marked or not marked), and mating status (mated or not mated). The data were inserted into a contingency table that was analyzed using a log likelihood model. The basic model included only the main effects (drug status, marked status, and mating status). The fit of this model was compared to a model with the main effects and interactions between herb status and mating status as well as marked status and mating status. For each herb, if the model with interactions provided a better fit, we then determined whether this improvement was due to the herb effects, the marking effects, or both.

RESULTS*Negative results*

LDW, BZYQT, and San Zhi Pian (SZP) did not result in statistically significant increases in the number of flies surviving to the end of the aging phase (Table 1). As a result of this absence of a net beneficial effect on survival during aging over the range of doses used, we did not proceed with further testing over a finer range of doses, nor did we proceed with tests of the side effects of these Chinese Herbs on fecundity, metabolic rate, or mating success.

Positive results

Mortality and confounds. *Rhodiola* resulted in a statistically significant decrease in mortality.

We were able to replicate these mortality results four times in four different assays (Table 2). Consequently, we proceeded with further testing of fecundity, metabolic rate, mating success, and total longevity. At the higher dose range (0, 60, 100, and 200 mg/mL) a significant mortality decrease was observed (Fig. 1) along with a dose-dependent decrease in fecundity (Fig. 2). At lower doses (0, 15, 30, and 60 mg/mL), at which a significant mortality benefit was observed (Fig. 3), there was no statistically significant difference in fecundity relative to the control (Fig. 4).

We proceeded on to a metabolic rate assay. As shown in Tables 3 and 4, *Rhodiola* did not have a significant adverse effect on metabolic rate at any dose on either sex. In fact, metabolic rate showed a statistically nonsignificant increase. Accordingly, we continued to an assay of male mating success. As shown in Table 5, there was no significant decrease in mating success from this assay.

Longevity. The mean longevity for the entire experiment was used to determine whether there were significant differences between the flies given drugs and the controls (Table 6). *Rhodiola* increased survival by 3.5 days in males and 3.2 days in females. All differences in longevity were statistically significant because of the very large number of flies used in these experiments.

If we use the observed variances, we can estimate the minimum required sample sizes to detect the observed differences. The largest mortality difference was 3.5 days and a sample of only 51 flies would have been sufficient to detect longevity differences of this magnitude. From the estimated confidence intervals (Table

TABLE 3. METABOLIC RATE ASSAY DATA FOR FEMALES WITH *RHODIOLA* AT 0, 15, 30, 60 MG/ML. *RHODIOLA* DID NOT CHANGE THE METABOLIC RATE IN FEMALE FLIES

	Control	15 mg/ml	30 mg/ml	60 mg/ml
Mean ($\mu\text{L CO}_2/\text{hour}$)	9.292	10.081	9.771	9.660
Standard Deviation	2.616	3.170	2.977	3.097
<i>p</i> -Value		0.939	0.985	0.993

TABLE 4. METABOLIC RATE ASSAY DATA FOR FEMALES WITH *RHODIOLA* AT 0, 15, 30, 60 MG/ML. *RHODIOLA* DID NOT CHANGE THE METABOLIC RATE IN FEMALE FLIES

	Control	15 mg/ml	30 mg/ml	60 mg/ml
Mean ($\mu\text{L CO}_2/\text{hour}$)	13.239	12.817	12.891	14.431
Standard Deviation	1.315	3.807	1.121	4.931
<i>p</i> -Value		0.991	0.995	0.842

6) it is apparent that differences in longevity as small as 0.53 days could have been detected. As discussed previously, the differences in mean longevity may be caused by a variety of changes in the age-specific mortality patterns. Making inferences about these changes is a statistically more challenging undertaking.

Distribution of deaths. If we plot the quantiles from the drugged flies versus the quantiles from the control flies (Fig. 5), we see that for both males and females the points are elevated above the $x = y$ line even at early ages. This suggests that drugged flies are dying more slowly even from the start of the experiment. This difference is also apparent in the survivorship curves (Fig. 6). This would be consistent with a smaller age-independent Gompertz parameter for flies treated with *Rhodiola*. In males, this departure from the $x = y$ line gets even more pronounced at later ages, suggesting a possible decline in the age-dependent parameter of the Gompertz for drugged males. We investigate these possibilities in more detail next.

Sampling units. A single sex, drug-level treatment consisted of 15 separate racks of

TABLE 5. MATING SUCCESS ASSAY WITH *RHODIOLA* AT 0, 15, 30, 60 MG/ML. *RHODIOLA* DID NOT IMPACT NERVOUS SYSTEM FUNCTION IN MALE FLIES

Drug dose	Frequency of mating successes	Significance of drug mating interaction
Control	0.44	
15 mg/ml	0.56	$p = 0.67$
Control	0.64	
30 mg/ml	0.36	$p = 0.68$
Control	0.44	
60 mg/ml	0.56	$p = 1$

TABLE 6. THE MEAN LONGEVITY AND 95% CONFIDENCE INTERVALS ON THE DIFFERENCES BETWEEN THE FLIES RAISED ON RHODIOLA (30 MG/ML) AND THE UNDRUGGED CONTROLS

	Mean	Variance	Difference (drug-control)	\pm 95% c.i. on difference
Males				
Control	25.0	72.93	3.50	± 0.53
Drug	28.5	90.53		
Females				
Control	26.9	128.2	3.21	± 0.68
Drug	30.1	143.2		

adult flies, with approximately 160 flies per rack. To some extent, each rack can be considered its own microenvironment that may differ slightly from one rack to the next. There are two very different ways these data might be analyzed. The observations from all 15 racks can be pooled to form one set of observations, which can then be used to estimate age-specific mortality. Alternatively, age-specific mortality can be estimated for each rack separately and then the results from all 15 racks can be pooled.

The parameters of the Gompertz model were estimated by regression analysis and thus the number of distinct ages and the variability of the underlying mortality estimates determine the variance of the parameter estimates. Pooling all 15 racks will roughly increase the total sample size (N) by a factor of 15. We expect that in the pooled sample the variance of the estimated mortalities should decrease because the variance is approximately binomial and thus proportional to the N^{-1} . However, the number of different ages will not increase linearly with increasing N , although it should be somewhat higher in the pooled sample than for any individual rack.

The control flies have actually been tested on two separate occasions or blocks: in the *Rhodiola* experiment and in a separate experiment not reported here testing the effects of Pioglitazone. Thus, we can analyze these results with two different models. In the first model, assume that there is random variation in the values of A and α due to each rack and that this level of variation was nested within the variation from each block. The second model pools the results from all 15 racks to create two samples, one per block. The estimates of A and α

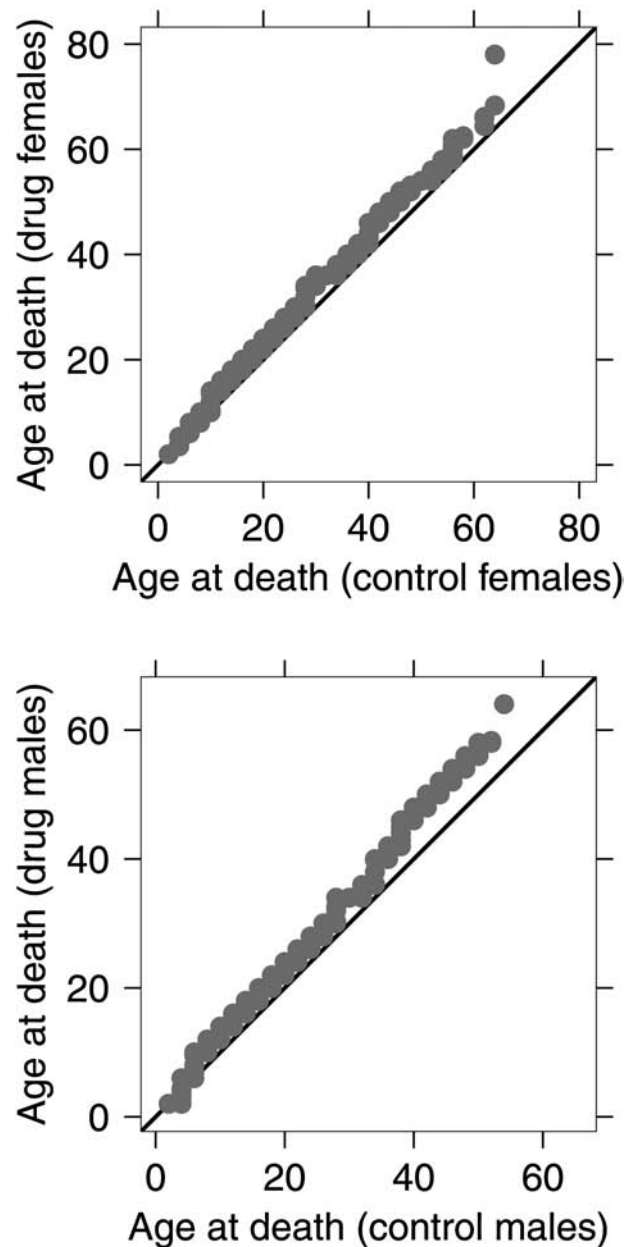


FIG. 5. The quantiles of drugged flies plotted against the control flies quantiles.

are each slightly different with each procedure (Fig. 7) although the differences are not statistically significant. However, the pooling procedure results in substantially higher standard errors for both parameters (Fig. 7).

If we examine the fraction of the variance in A due to racks versus blocks, we find that 80% is due to racks. For α , the fraction of variance due to racks is virtually 100%. We interpret this to mean that the small sample sizes of the racks leads to uncertainty in the estimated mortality rates and that this uncertainty affects α to a greater extent than A . Presumably if the number of flies in racks was reduced even further, the standard error of α estimated from individual racks would be higher than the variance from a pooled estimate. Although we have no general rule yet for an optimal size

for racks, it appears that in our study it is still better to analyze the data by keeping results from individual racks separate. Consequently, we have chosen to analyze the results of the experiments in this study by analyzing each rack separately.

Gompertz mortality dynamics. Males have a significantly lower value of A , the age-independent mortality parameter, than do females (γ_1 Table 7), but a significantly higher value of α (γ_2 Table 7), the rate-of-aging parameter. *Rhodiola* significantly lowers A by 20% in females and 33% in males (ϕ_1 , Table 7, Fig. 8). The effects of *Rhodiola* on α are smaller, a 5% reduction in females and a 3% reduction in males (Fig. 8). The effects of *Rhodiola* on α are not statistically significant (ϕ_2 , Table 7).

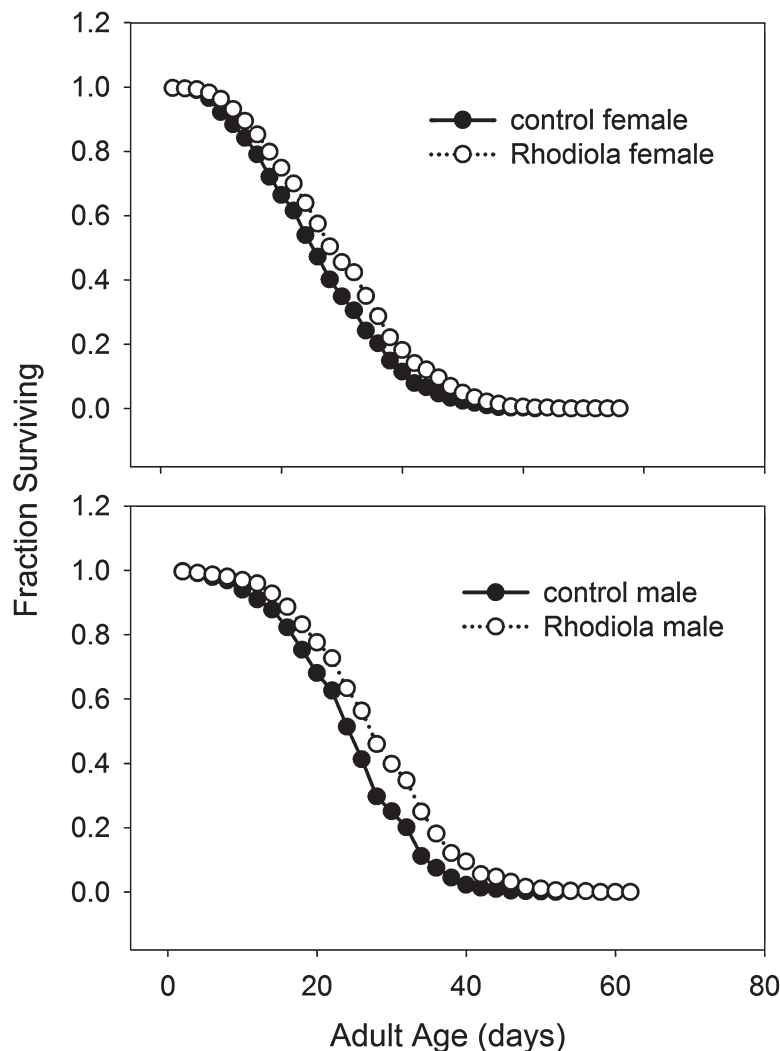


FIG. 6. The fraction of surviving males and females in cohorts exposed to food supplemented with *Rhodiola* and their controls.

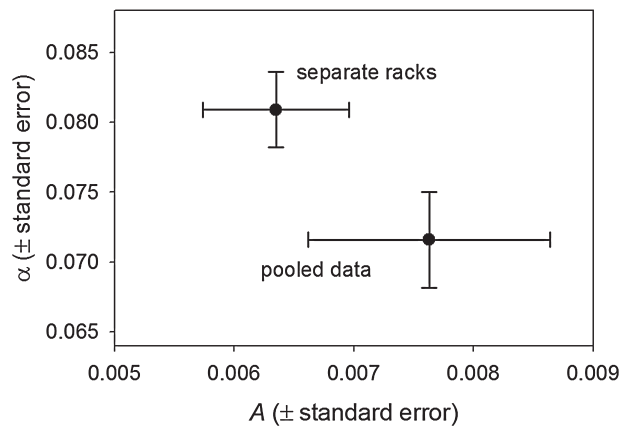


FIG. 7. The estimated parameters of the Gompertz equation for the control population and their standard errors. Two methods of estimation are shown. The first method estimated the parameters separately for each of 15 racks in the experiment while the second method pooled all 15 racks into one population.

Partial data set. For a population where mortality is well described, the Gompertz model may be possible to get good estimates of the model parameters with just the first few weeks of observations rather than waiting for every fly to die.²⁴ We repeated the analysis of the *Rhodiola* data set using only the first 28 days of data but otherwise using the same techniques as described previously. The numerical values of the parameters are different; however, the sign of the effects due to sex and drugs are the same for all the statistically significant parameters as with the full data set (Table 8). In addition, all of the significant parameters with the full data set are also significant with just 28 days of observations.

Although the parameter estimates obtained with 28 days of observation are somewhat different from those produced from the full data set, they give very similar predictions. For instance, the observed longevity of control females was 26.9 days, whereas the predicted mean from the Gompertz model utilizing all data is 26.6 days. The mean longevity predicted from the Gompertz model, utilizing only the first 28 days of data, is 24.2 days. We suggest that it may often be feasible to determine whether drugs have significant effects on longevity and mortality dynamics by using only partial datasets unless the drug effects are very small.

Two-stage Gompertz mortality dynamics. The estimation of the two-stage Gompertz parameters requires observations late in life to get reasonable estimates of the break day and plateau mortality rate. Consequently, we have not followed the previous protocol of obtaining individual estimates for each rack. Additionally, there are now four parameters to estimate rather than two and the estimation is done separately for each sex and drug treatment. We expect that our ability to infer differences between treatments when they exist will decline relative to the power available with the Gompertz model. The estimated parameters for the two-stage Gompertz show no significant differences between the control and *Rhodiola*-treated flies (Table 9). The control males have a higher α , lower A , and earlier break day than the *Rhodiola*-treated males (Table 9, Fig. 9). The higher value of α leads to a departure in the mortality kinetics late in life (Fig. 9), but again the difference is not statistically significant.

Control females show a 50% larger value of A than the *Rhodiola*-treated females, but this difference is not statistically significant. This difference does lead to slightly higher mortality rates at nearly all ages (Fig. 10).

One possible explanation for the increased survival of *Rhodiola*-treated flies is that they can sense the presence of the drug in the food and it reduces their intake of calories. The flies then experience increased survival due to caloric restriction, not due to a direct effect of the drug. In our nutrition experiment, we test this by comparing survival after 28 days at three different yeast levels, with and without *Rhodiola*.

TABLE 7. THE PARAMETER ESTIMATES FOR THE COMPLETE RHODIOLA DATASET WITH MODEL (3). EACH PARAMETER WAS TESTED TO DETERMINE IF IT DIFFERED FROM ZERO. THE STATISTICAL SIGNIFICANCE OF THIS T-TEST IS GIVEN IN THE COLUMN LABELED P-VALUE

Parameter	Value	Standard error	d.f.	p-value
β_1	0.0082	0.00043	1614	<0.0001
γ_1	-0.0034	0.00042	1614	<0.0001
ϕ_1	-0.0016	0.00036	1614	<0.0001
β_2	0.072	0.0022	1614	<0.0001
γ_2	0.038	0.0026	1614	<0.0001
ϕ_2	-0.0035	0.0027	1614	0.18

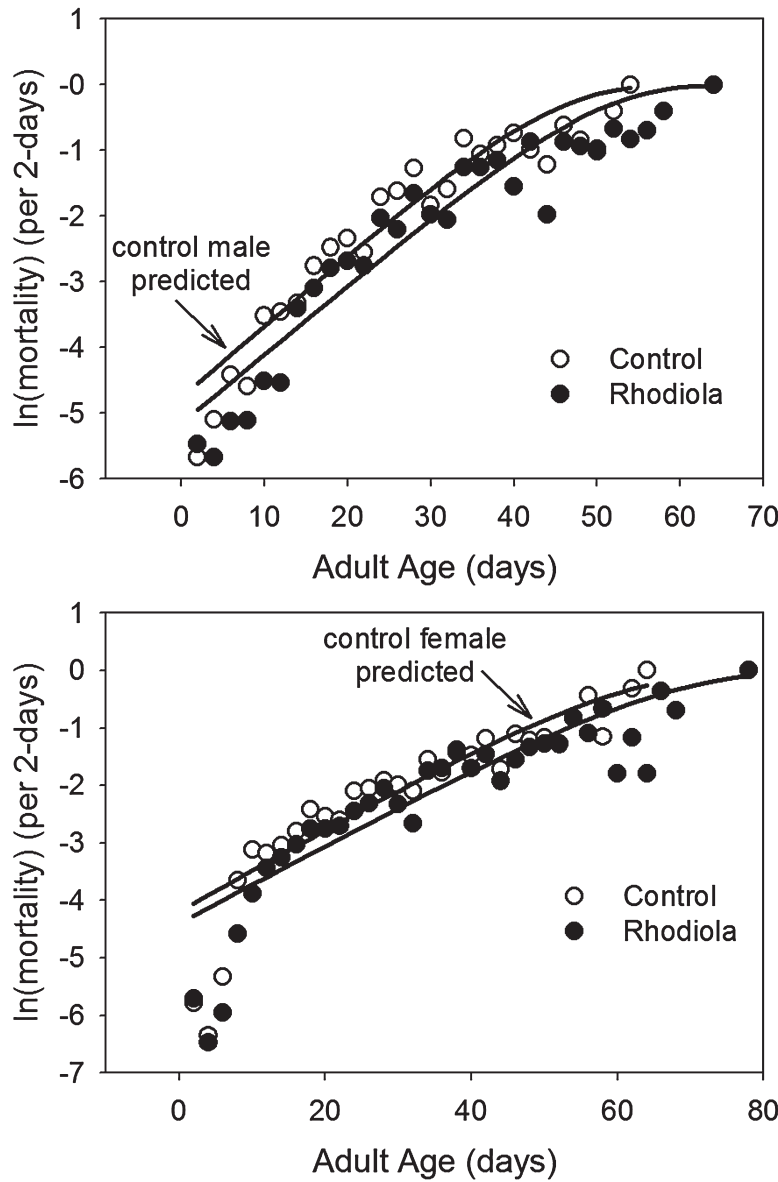


FIG. 8. The natural log of mortality versus age for control and *Rhodiola*-treated flies. The predicted values are not linear because the predictions are for 2-day intervals of mortality.

TABLE 8. THE PARAMETER ESTIMATES FOR THE PARTIAL (DAY 28) *RHODIOLA* DATASET WITH MODEL (3). EACH PARAMETER WAS TESTED TO DETERMINE IF IT DIFFERED FROM ZERO. THE STATISTICAL SIGNIFICANCE OF THIS *t*-TEST IS GIVEN IN THE COLUMN LABELED *p*-VALUE

Parameter	Value	Standard error	<i>d.f.</i>	<i>p</i> -value
β_1	0.0042	0.00031	805	<0.0001
γ_1	-0.0013	0.00028	805	<0.0001
ϕ_1	-0.0013	0.00031	805	<0.0001
β_2	0.12	0.0041	805	<0.0001
γ_2	0.032	0.0049	805	<0.0001
ϕ_2	0.0066	0.0051	805	0.20

We expect survival to decline as yeast levels increase. However, in the treatments with *Rhodiola*, food consumption should be reduced at all yeast levels if *Rhodiola* acts by affecting food consumption. Thus, in an analysis of survival, the effects of yeast should be radically different in *Rhodiola*-treated vials compared to vials without *Rhodiola*.

Using log linear models,²⁵ we compared two models. The first model (model 1) included the main factors of yeast (low, medium, high), drug (presence, absence), sex (male, female), and

TABLE 9. THE ESTIMATED PARAMETERS VALUES AND THE BOOTSTRAP TESTS FOR SIGNIFICANT DIFFERENCES BETWEEN CONTROL AND DRUG FLIES. THE DIFFERENCE IS COMPUTED AS THE DRUG FLY VALUE MINUS THE CONTROL FLY VALUE. THE *p*-VALUES ARE BASED ON 1000 BOOTSTRAP SAMPLES

Parameter	Control male	Drug male	Control female	Drug female
A	0.0092	0.0144	0.0153	0.0109
Difference		0.0051		-0.0044
<i>p</i> -value		0.21		0.19
α	0.0861	0.0618	0.0534	0.0570
Difference		-0.024		0.0036
<i>p</i> -value		0.22		0.32
A_2	0.358	0.429	0.129	0.187
Difference		0.0071		0.058
<i>p</i> -value		0.37		0.36
Break Day	35.0	52.5	55.6	55.0
Difference		17		-0.60
<i>p</i> -value		0.21		0.40

survival (to days 28 yes or no), and the two-way interactions between survival and sex, yeast, and drug. The second model (model 2) was the same as the first but also included the three-way interaction between yeast, drug, and survival. This three-way interaction is not significant (Table 10). We conclude that *Rhodiola* does not act by affecting food consumption, and thus the longevity-enhancing effects of *Rhodiola* are not produced by a caloric-restriction effect.

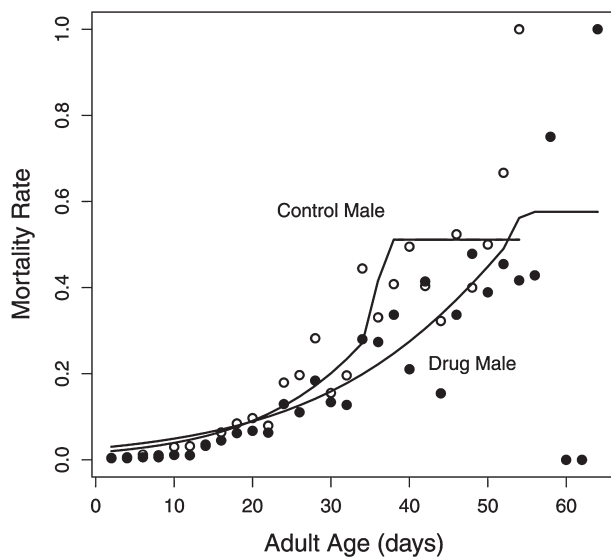


FIG. 9. The mortality rates versus age for males with predictions from the two-stage Gompertz model. The filled circles are drug-treated flies and the open circles are the control flies.

DISCUSSION

The present study demonstrates that *Rhodiola rosea* increases the lifespan of adult *Drosophila* without significantly affecting any secondary physiological mechanisms that may have resulted in an artefactual extension of lifespan. The observed anti-aging property of *Rhodiola* was not associated with statistically significant reductions in fecundity, metabolic rate, or male mating success.

We extensively replicated our *Rhodiola* trials, both by wholesale replication and by subsampling from a very large cohort study. There are many ways in which such data can be analyzed, and our analyses necessarily are limited to only some of the possibilities. However, we are confident in our conclusion that *Rhodiola* does significantly increase lifespan no matter how it is calculated or inferred. Whether the other substances tested here might be found efficacious by some other type of adult survival assays is an open question.

Because caloric restriction has been consistently found to increase longevity in mammals and invertebrates, there is the bare possibility that the presence of *Rhodiola* in the food may induce lower levels of food consumption. This could, in effect, mimic an effect similar to dietary restriction. It is well established that gross depressed fecundity is associated with dietary restriction sufficient to significantly increase *Drosophila* longevity.²⁶ In order to test possible caloric restriction effects in *Rhodiola*-induced

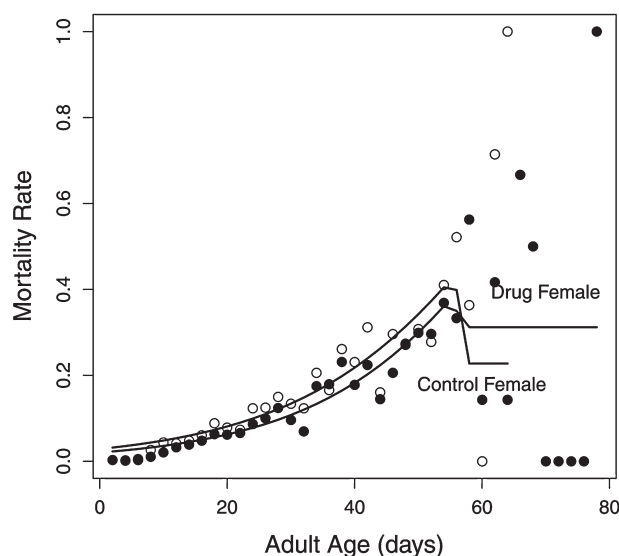


FIG. 10. The mortality rates versus age for females with predictions from the two-stage Gompertz model. The filled circles are drug-treated flies and the open circles are the control flies.

Drosophila, we measured the number of eggs produced over a 10-day period. As shown in Figure 4, supplementing *Drosophila* cohorts with various concentrations of the drug (0, 15, 30, and 60 mg/mL) produced no significant differences in treatment groups relative to the control, nor were there any notable differences between groups. In these measurements, there was no statistically significant decrement in reproductive output due to *Rhodiola* feeding. Because the dietary restriction enhancement of longevity found by Chippindale et al.²⁷ required a severalfold reduction in fecundity, we concluded that the extension of lifespan is not due to a dietary restriction effect mediated by reduced fecundity at doses of 15, 30, and 60

mg/mL *Rhodiola*. Because egg production is proportional to food level, the absence of a statistically significant decline in fecundity indicates that the *Drosophila* females were not lowering their levels of *Rhodiola*-supplemented food consumption.²⁸

However, let us numerically evaluate the possibility that the entire effect of *Rhodiola* is due to dietary restriction. We observed a statistically insignificant reduction in fecundity of 8.19 eggs per day in the 30 mg/mL treatment that has been our primary focus here. Taking the ratio between fecundity reduction and longevity increase found by Chippindale et al.,²⁹ which was 0.15 days of adult life per egg laid in a day, we can hypothetically reduce our estimate of the longevity benefit of 30 mg/mL of *Rhodiola* by 1.23 days. Given that the effect of this dose on average female longevity was 3.21 days with a confidence interval of ± 0.68 , this hypothetical, as opposed to substantiated, dietary effect is not enough to eliminate our estimate of the effect of the supplement on longevity.

As a further test of dietary artifact, we retested our flies at varying yeast levels with and without *Rhodiola*. Although varying yeast levels do indeed affect survival rates, they do not do so with a detectable interaction involving exposure to *Rhodiola*. Thus, it appears that the effect of *Rhodiola* on the longevity of adult flies was not mediated solely or primarily through dietary effects on reproduction.

It has been proposed that *Rhodiola* has versatile protective properties that counteract adverse physical, chemical, and biological stressors.³⁰ *Rhodiola* supplementation has been recommended for the treatment of depression,

TABLE 10. LIKELIHOOD RATIO TESTS FOR HIERARCHICAL, LOG-LINEAR MODELS OF ADULT SURVIVAL TO 28 DAYS AT THREE DIFFERENT YEAST LEVELS WITH AND WITHOUT *RHODIOLA*.

THE DEVIANCE IS THE LOG LIKELIHOOD RATIO BETWEEN THE SPECIFIC MODEL AND THE FULLY SATURATED MODEL. DELTA REPRESENTS THE DIFFERENCE IN THIS RATIO BETWEEN THE TWO MODELS COMPARED (MODEL 1 AND MODEL 2 OR MODEL 2 AND THE SATURATED MODEL). THE *P*-VALUE IS CALCULATED FROM A CHI SQUARE DISTRIBUTION WITH DELTA(Df) DEGREES OF FREEDOM. SEE THE TEXT FOR A DESCRIPTION OF THE TWO MODELS.

	Deviance	df	Delta (Dev)	Delta (df)	<i>P</i> > Delta (Dev)
Model 1	15.277	14			
Model 2	7.828	10	7.449	4	0.11
Saturated	0.000	0	7.827	10	0.65

headaches, hypertension, amenorrhea, asthenia, schizophrenia, male sexual dysfunction, as well as colds and flus.³¹ There is also clinical evidence that *Rhodiola* has anti-fatigue, anti-stress, anti-hypoxic, anti-cancer, anti-oxidant, and immune-enhancing effects.^{32,33} Additionally, cardioprotective effects have been inferred from reduced cardiac damage under stress.³⁴

In conclusion, we have presented evidence that pharmacological intervention using *Rhodiola rosea* can extend lifespan without statistically significant physiological tradeoffs that could generate an artefactual longevity benefit. The lifespan extension detected here might be due to antioxidative protection against a possible accumulation of free radicals that occurs with aging, but we have not shown that.

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