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Short Report

The prolongevity effect of resveratrol depends on dietary composition and calorie intake in a tephritid fruit fly

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ABSTRACT

Several studies have shown that resveratrol can extend lifespan in yeast, worm, fruit fly and short-lived fish, as well as mice under a high-fat diet, probably acting through molecular pathways similar to dietary restriction. However, the putative prolongevity effect of resveratrol has not been observed in other studies. To evaluate the robustness of the prolongevity effects of resveratrol, we designed a nutritional study to address the question, Under what nutritional conditions does resveratrol affect lifespan and reproduction? We fed 2592 individual tephritid fruit fly of the species, *Anastrepha ludens*, 24 diets of different sugar:yeast ratios supplemented with or without 100 μM resveratrol. Sex-specific survival and daily egg laying in females were recorded. Resveratrol was found to have no or little effect on lifespan of males in all the treatments, as well as on lifespan and reproduction of females. Only under one diet combination, resveratrol appears to increase mean lifespan of females but not at a statistically significant level after multiple comparison adjustment. These findings suggest that the prolongevity effect of resveratrol is at most limited to a narrow range of dietary composition and calorie content in this fruit fly. Coupled with a recent study indicating that resveratrol does not extend lifespan of mice fed the standard diet, our findings further question the ability of resveratrol to increase lifespan in organisms under normal conditions.

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1. Introduction

Dietary restriction (DR) has been shown to extend lifespan in a variety of species from yeast, worm, fly, fish, rodents and dogs, and recent studies also support the potential health benefits of DR in nonhuman primates and humans (Guarente, 2005; Masoro, 2005; Partridge et al., 2005; Sinclair, 2005; Ingram et al., 2006a,b, 2007; Tatar, 2007). Health benefits of DR include reducing cancer incidence and improving behavioral function (Ingram et al., 2006a,b; Ingram et al., 2007). However, it would be a major challenge for humans to adopt DR as a lifestyle.

An alternative is to develop DR mimetics, which induce similar beneficial effects of DR without requiring markedly restriction the diet (Lithgow et al., 2005; Ingram et al., 2006a,b). Resveratrol, a polyphenolic compound found naturally in many plants and fruits, with an especially high concentration in red grapes and Japanese knotwood, has been suggested to be such a mimetic (Baur and Sinclair, 2006). Resveratrol has been shown to extend lifespan of yeast *Saccharomyces cerevisiae*, worm *Caenorhabditis elegans* and fly *Drosophila melanogaster* under standard full diets but not restricted diets in several studies, suggesting resveratrol extends lifespan through pathways mediated by DR (Howitz et al., 2003; Wood et al., 2004). Resveratrol can also extend lifespan and improve health of a short-lived fish *Nothobranchius furzeri* and mice on a high-fat diet beginning in middle age (Baur et al., 2006; Valenzano et al., 2006). The beneficial effect of resveratrol appears to be mediated by genes involved in DR pathways, including sirtuins, AMP kinase and PGC-1α, although the precise mechanisms remain unidentified (Baur et al., 2006; Lagouge et al., 2006). Several studies have demonstrated that resveratrol could not extend lifespan of yeast, worms or flies carrying mutations in their sirtuin genes, Sir2, Sir-2.1 and dSir2, respectively, which suggest a critical role for this genetic pathway (Howitz et al., 2003; Wood et al., 2004). However,

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the prolongevity effect of resveratrol has been challenged by several recent studies that failed to observe lifespan extension associated with resveratrol treatments in several models, including *S. cerevisiae*, *C. elegans* and *D. melanogaster* (Kaeberlein et al., 2005; Bass et al., 2007). Bass et al. even found that resveratrol extended lifespan of *sir-2.1* mutant worms (Bass et al., 2007). It was proposed that the discrepancy is due to the weak and/or species-specific effect of resveratrol. However, many factors, including environmental and dietary variables, remain to be investigated.

To evaluate the robustness of resveratrol on lifespan and healthspan, we conducted a parametric study using the tephritid fruit fly, *Anastrepha ludens*, commonly known as the Mexican fruit fly (mexfly). Here we addressed two questions: Under what nutritional conditions does resveratrol extend lifespan and how does resveratrol affect reproduction given the potentially inverse relationship between DR and reproduction? The reproduction was monitored to assess the effect of resveratrol on healthspan. The tephritid fruit fly was selected for this study since extensive demographic data have been published for this species (Carey et al., 2005). Based on the limited sequences available in the Genbank, most of the mexfly proteins share >70% sequence similarity with the standard lab fly, *D. melanogaster*. More importantly, the mexflies are relatively bigger in size compared to *D. melanogaster*, which has permitted development of a method that provides accurate evaluation of food and calorie uptake of individual flies (Carey et al., 2008). This advantage permits studies that can parallel mammalian DR studies by providing relatively precise amounts of food, and allows precise control of drug dosage for any intervention.

2. Materials and methods

2.1. Measurement of lifespan and reproduction

Virgin males and females were collected and individually housed in 4 × 4 × 10 cm plexiglass cages at environmental conditions, 24 ± 2 °C, 65 ± 9% relative humidity and 12:12 h light–dark cycle. Cages (24) were organized into one unit. Fifteen microliters of one of the following 24 diets and a 15-μl droplet of water were provided daily to each fly on glass slides. The 24 diets were designed by combination of four different sugar:yeast ratios, 1:0 (sugar only), 24:1, 9:1 and 3:1, each at three dilution levels, 1× (the full diet), 0.5× and 0.25× of the full diet, with or without 100 μM resveratrol. Sugar and yeast extract were dissolved in water and the concentration of total sugar and yeast was 20% by weight for all the full diets. Diets (0.5× and 0.25×) were prepared by diluting the full diet by 50% and 75%, respectively. Resveratrol was first dissolved in 100% ethanol and then added to each sugar:yeast mixture to the final concentration of 100 μM. Resveratrol (>98%) was kindly provided by Dr. Rafael de Cabo and was originally from Orchid Pharmaceuticals (Aurangabad, India) (Baur et al., 2006). A total of 54 males or females were used in each treatment, and were randomly assigned into the 24-unit cages while placing females and males in alternate cages to avoid mixing eggs from two females. Each cage had a mesh for females to lay eggs. Sex-specific survival and egg laying of females were recorded daily. After the lifespan experiments, the purity and stability of resveratrol stored at −80 °C were assessed by measuring its Raman spectrum using a Renishaw inVia confocal Raman microscope controlled by the WiRE 3.0 software with a 785 nm near-IR laser (Renishaw Inc., U.K.), which was based on a published method (Billes et al., 2007).

For measuring lifespan of the mexflies in populations, approximately 2000 adult males and females were housed together and fed the solid 9:1 sugar:yeast diet with or without resveratrol in each population cage based on a previously described protocol (Zou et al., 2006). The final concentrations of resveratrol tested were 100 and 200 μM. Dead flies were sorted out and counted by sex every day for measuring lifespan. For each treatment, two population cages were used, and the expectation of life and lifespan curve were derived from approximately 2000 males or females in total.

2.2. Data analysis

Event life graphs and response surfaces were generated using DeltaGraph 5 software (Red Rock Software Inc. Salt Lake City, Utah, USA). ANOVA analysis and logistic analysis were conducted to assess the statistical significance of life span and reproduction data. A p-value of $p < 0.05$ after Bonferroni adjustment for multiple comparisons is considered statistically significant.

3. Results

3.1. Lifespan and reproduction of individually housed flies

In this study, 2592 virgin tephritid fruit flies were individually housed and fed one of the 24 diets of four sugar:yeast (SY) ratios, 1:0 (sugar only), 24:1, 9:1 and 3:1, each at three dilution levels, 1× (the full diet), 0.5× and 0.25× of the full diet, with or without 100 μM resveratrol (see detailed experiment procedures in Supplementary materials). The selection of resveratrol concentration was based on previous reports demonstrating that 100 μM of the compound was effective for *S. cerevisiae*, *C. elegans* and *D. melanogaster* (Howitz et al., 2003; Wood et al., 2004). Fifteen microliters of a diet and a 15-μl droplet of water were provided daily to each fly according to a previously published DR/calorie restriction (CR) protocol (Carey et al., 2008). Normally the food was completely consumed by a fly in a day, which allowed achievement of CR when using the diluted food. We recorded the daily sex-specific survival and egg laying, a parameter representing reproduction since virgin females can lay eggs even individually housed. Event history charts were generated for females to show relationship of cohort survival and individual-level reproduction in all the treatments, and lifespan and reproduction were calculated (Fig. 1 and Table 1, Carey et al., 1998).

3.2. The effects of diet on lifespan and reproduction

Under the diets without resveratrol, both mean and maximum lifespan of the fruit flies initially increased with increasing yeast ratios and then decreased at the high yeast ratio diet (Table 1 and Supplementary Fig. S1). The maximum mean lifespan was observed with the sugar:yeast 9:1 diets for both males and females. In general CR increased lifespan in both males and females with the treatments of the sugar only and SY 24:1 diets, but not significantly for 9:1 and 3:1 diets. Reproduction steadily increased with increasing yeast ratios (Table 1 and Supplementary Fig. S1), but CR had little effect on reproduction (Tables 1 and S7). This indicates that factors affecting longevity and reproductive fitness varied according to different dietary compositions, which is consistent with a previous DR/CR study in this tephritid fruit fly (Carey et al., 2008). Similar effects of diets on lifespan and reproduction have also been observed in *D. melanogaster* and crickets *Teleogryllus commodus*, although the response patterns, such as the maximum mean lifespan, are not the same (Lee et al., 2008; Maklakov et al., 2008; Skorupa et al., 2008).

3.3. The effects of resveratrol on lifespan and reproduction

We found that resveratrol had no or little significant impact on mean and maximum lifespan of males (Tables 1, S1 to S3). We ob-

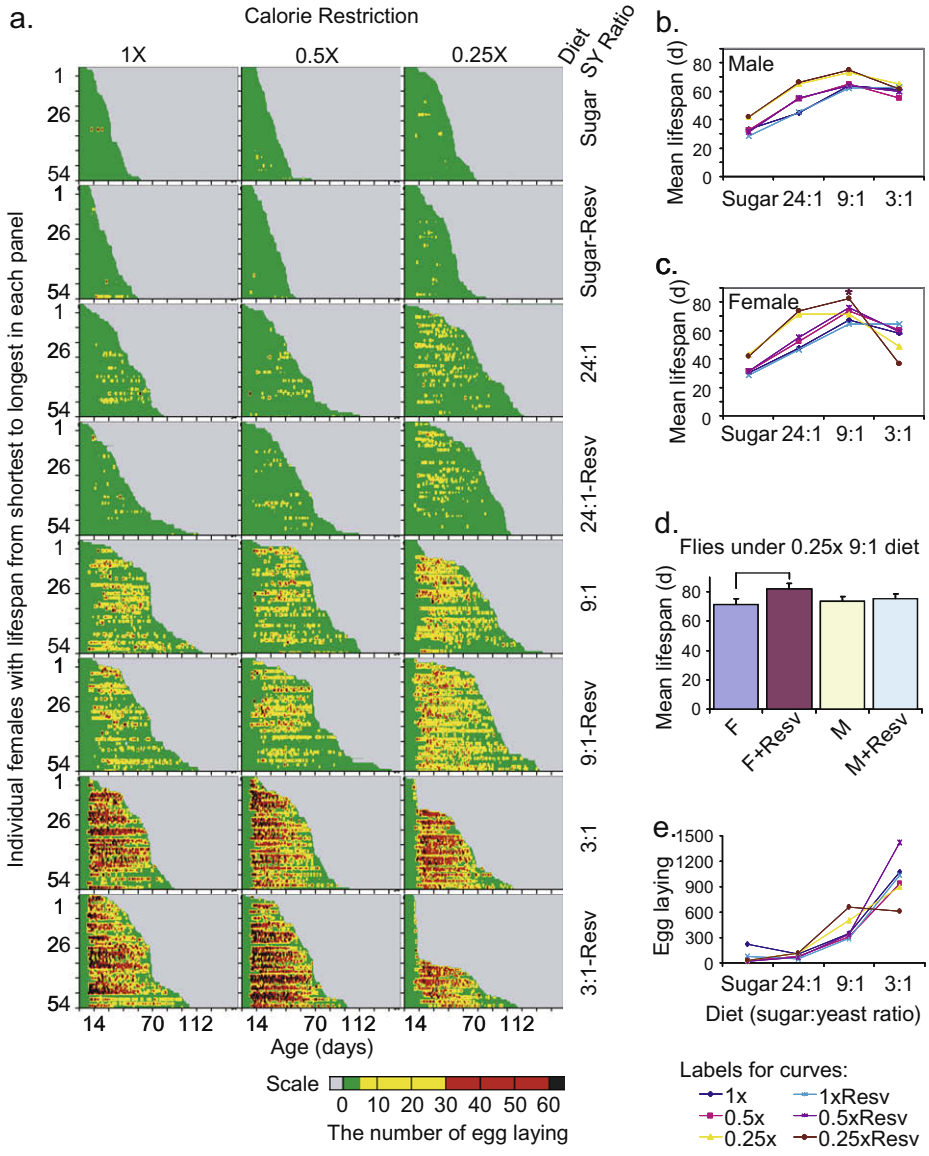

Fig. 1. (a) Event history charts for females showing individual lifespan and reproduction across treatments. Each horizontal colored line in a panel represents lifespan of a female ranked from shortest to longest lived on *Y* axis. The length of a colored line is in proportional to the lifespan of a female. The number of daily egg laying of a female is color coded by yellow and red as indicated in the scale. The numbers across the top represent the fraction of a full diet and the numbers at the right represent the sugar:yeast ratios without or with resveratrol (Resv). (b). Mean lifespan of males. (c). Mean lifespan of females. (d) Mean lifespan of flies under the 0.25× restriction SY 9:1 diet. F and M represents female and male, respectively. (e) Mean lifetime egg laying per fertile female. Each color coded curve in b, c and e represents a set of data from flies fed a full or restriction diet with or without resveratrol. *X* axis denotes the sugar:yeast ratio and *Y* axis denotes mean lifespan or mean lifetime egg laying per female. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

served marginally significant 3-way interaction among resveratrol, diet composition and diet restriction in females on mean ($p = 0.024$) and maximum ($p = 0.049$) lifespan but not reproduction ($p = 0.09$). Pairwise comparisons indicated that resveratrol slightly decreased the mean lifespan of females fed the 0.25× restricted 3:1 diet ($p = 0.011$), while a marginal increase in the mean lifespan by resveratrol was evident only for females fed the 0.25× restricted 9:1 diet from 71 to 82 days ($p = 0.014$) (Fig. 1d and Table S5). However, none of these differences reached a statistically significant level after simultaneous adjustment using the Bonferroni method (Table S5). These marginal lifespan changes were not associated with any significant changes in reproduction (Fig. 1e and Table S3). To confirm the purity and stability of resveratrol used in this study, we measured Raman spectrum of resveratrol using a Raman spectrometer, a sensitive method to assess the chemical structures, after the lifespan experiments were completed. We found that our resveratrol had a spectrum profile similar to a previously published one from resveratrol of 99% purity, suggesting that our resveratrol remained pure and structurally stable after a long-term storage (Fig. S2, Billes et al., 2007).

4. Discussion

This study investigated the effect of resveratrol on lifespan and reproduction of the tephritid fruit flies across a broad range of dietary treatments. The resveratrol effect was not found to be significant for any diet when adjusting for multiple comparisons. The diet combination coming closest to significance (but not reaching it) was a CR diet, 0.25× SY 9:1. Our findings present several important points. Firstly, resveratrol does not affect reproduction at the dose tested. Secondly, the prolongevity effect of resveratrol appears to be diet-dependent and at most modest for animals fed

Table 1
Mean and maximum lifespan, and reproduction.

Diet	Restriction w/o Resv			Restriction with Resv		
	1×	0.5×	0.25×	1×R	0.5 × R	0.25 × R
Mean lifespan (day)						
Females						
Sugar	30.3	31.4	43.1	28.6	31.4	41.9
24:1	47.6	52.0	71.2	46.5	55.2	73.9
9:1	67.5	73.9	71.3	64.2	75.8	82.4
3:1	57.9	60.6	49.0	64.4	59.2	36.6
Males						
Sugar	33.0	32.6	41.8	28.2	31.1	41.6
24:1	44.7	54.4	64.8	45.3	55.2	66.3
9:1	64.1	64.9	73.3	62.0	64.0	75.2
3:1	61.0	55.2	65.1	62.5	59.6	61.3
Maximum lifespan (day)[a]						
Females						
Sugar	45.0	46.6	62.0	49.6	45.0	59.0
24:1	68.6	80.0	91.4	86.8	85.8	96.6
9:1	92.2	99.6	98.8	97.2	117.0	121.0
3:1	98.2	70.6	96.6	96.8	82.4	87.4
Males						
Sugar	50.0	51.6	59.0	42.8	45.2	61.2
24:1	68.6	80.0	91.4	80.0	88.0	96.0
9:1	98.8	99.6	92.2	86.8	87.6	105.0
3:1	96.6	70.6	98.2	89.2	96.2	91.0
Reproduction[b]						
Females						
Sugar	220.0	27.0	27.6	77.7	21.8	31.5
24:1	107.6	79.8	112.8	48.5	68.5	111.5
9:1	348.6	311.6	501.2	285.8	335.0	657.4
3:1	1071.1	938.2	903.0	1034.4	1420.4	606.1

[a] Maximum lifespan: 90% percentile of the distribution of lifespan.
[b] Reproduction: mean number of eggs laid per fertile female.

standard diets. This is consistent with the finding by Bass et al. that resveratrol did not extend lifespan of *D. melanogaster* (Bass et al., 2007). It is also consistent with a recent study showing that resveratrol did not increase lifespan of mice fed a standard diet although the same study demonstrated a lifespan extension by resveratrol in mice fed a high-fat diet or under a CR diet (Baur et al., 2006; Pearson et al., 2008). Due to potential difference in physiology, the concentration of resveratrol tested in our study may not be optimal to be effective in *A. ludens*. However, this may not be the case since we have observed some effect of resveratrol on lifespan when interacting with diet composition and calorie content. In addition, we did not observe lifespan extension in the mexflies fed a higher dose of resveratrol (200 μM) in the 9:1 diet when they were housed together in population cages (Fig. S2b and c). The concentration tested here (100 μM) is an effective dose in the studies showing prolongevity effects of resveratrol in various model systems ranging from the single cell organism, *S. cerevisiae*, to worm, fly and fish. Nevertheless, our findings pose a challenge regarding the application of resveratrol as a DR mimetic with applicability to human aging. It is critical to understand the interaction of diet and a mimetic before any application in humans can be predicted.

Conflict of interest

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exger.2009.02.011.

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