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# Experimental Gerontology

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## Glycerol extends lifespan of *Brachionus manjavacas* (Rotifera) and protects against stressors



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### ARTICLE INFO

#### Article history:

Received 8 March 2014

Received in revised form 3 May 2014

Accepted 5 May 2014

Available online 14 May 2014

Section Editor: T.E. Johnson

#### Keywords:

Lifespan

Glycerol

Longevity

2-Deoxyglucose

Swimming

### ABSTRACT

Diet has profound effects on animal longevity and manipulation of nutrient sensing pathways is one of the primary interventions capable of lifespan extension. This often is done through caloric restriction (CR) and a variety of CR mimics have been identified that produce life extending effects without adhering to the rigorous CR dietary regimen. Glycerol is a dietary supplement capable mimicking CR by shifting metabolism away from glycolysis and towards oxidative phosphorylation. Glycerol supplementation has a number of beneficial effects, including lifespan extension, improved stress resistance, and enhanced locomotory and mitochondria activity in older age classes. Using rotifers as a model, we show that supplements of 150–300 mM glycerol produced 40–50% extension of mean lifespan. This effect was produced by raising glycerol concentration only three times higher than its baseline concentration in rotifer tissues. Glycerol supplementation decreased rotifer reliance on glycolysis and reduced the pro-aging effects of glucose. Glycerol also acted as a chemical chaperone, mitigating damage by protein aggregation. Glycerol treatment improved rotifer swimming performance in older age classes and maintained more mitochondrial activity. Glycerol treatment provided increased resistance to starvation, heat, oxidation, and osmotic stress, but not UV stress. When glycerol was co-administered with the hexokinase inhibitor 2-deoxyglucose, the lifespan extending effect of glycerol was enhanced. Co-administration of glycerol with inhibitors like 2-deoxyglucose can lower their efficacious doses, thereby reducing their toxic side effects.

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### 1. Introduction

Aging is an evolutionarily conserved, regulated process that is controlled by a complex signaling network (Gems and Partridge, 2013). This network consists of multiple parallel pathways, so that interactions are likely (Amrit and May, 2010). A key discovery from work on laboratory model organisms has been that lifespans can be extended and health and function improved, by simple interventions like diet and drugs that influence metabolism. Nutrient sensing is one of the key systems that organisms use to match their metabolism to environmental conditions (Libert and Pletcher, 2007). Slowing metabolism when food is scarce and accelerating it when food is abundant is a common adaptation shared by many animals (de Magalhaes et al., 2012).

Diet has profound effects on longevity, and caloric restriction (CR) without malnutrition extends lifespan in most animals thus far tested (Testa et al., 2014). CR of 20–40% extends lifespan in many organisms (Bordone and Guarente, 2005; Fontana et al., 2010; Mair and Dillin, 2008; Sinclair 2005), including rotifers (Gribble and Mark Welch, 2013; Snell, 2014). The comparative approach using a variety of

model organisms has been productive in advancing understanding of the mechanisms of aging (Austad, 2009; Deweerdt, 2012). Besides restricting caloric intake, a less studied approach to extending lifespan is shifting metabolism from greater reliance on glycolysis towards more reliance on oxidative phosphorylation (Johnson et al., 2013a, 2013b; Wei et al., 2009). Metabolic shifts probably mimic CR by modulating the same nutrient sensing pathways that produce CR lifespan extension, but the mechanisms are not understood (Wei et al., 2009). Campisi (2013) has argued that metabolic shifts may link processes like aging and cancer, and a well known element of age-related tumor progression is the metabolic shift to glycolysis (Warburg effect).

There are a variety of approaches to reducing utilization of pro-aging carbon sources like glucose. One is by inhibiting glycolysis, and some inhibitors, like the hexokinase inhibitor 2-deoxyglucose, can have significant lifespan extending affects (Ingram and Roth, 2011). An alternative approach is to provide alternate substrates that suppress glycolysis and favor oxidative phosphorylation. One such substrate is glycerol. Glycerol supplementation has a number of beneficial effects, including lifespan extension, improved stress resistance, and enhanced mitochondria activity (Wei et al., 2009).

In this work, we explore the benefits of glycerol supplementation on aging using rotifers as a model animal. Rotifers have long been employed in aging studies (Snell, 2014), and they are particularly well suited for

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studies of dietary supplementation because of their short lifespan and ease of culture. We demonstrate that modest supplements of 150–300 mM glycerol can produce 40–50% extension of mean lifespan. This occurs because glycerol shifts rotifer metabolism away from glycolysis and towards greater reliance on oxidative phosphorylation. Glycerol also acts as a chemical chaperone, mitigating damage by protein aggregation. Besides extending lifespan, glycerol treatment improves swimming performance in older age classes and maintains more mitochondrial activity. Glycerol treatment provides increased protection from a variety of stressors. The inhibitor 2-DG enhances the glycerol effect, suggesting that it could be co-administered to obtain greater lifespan extension with fewer toxic side effects.

## 2. Methods

### 2.1. Rotifer cultures

All experiments were conducted using the rotifer species *Brachionus manjavacas* originally collected from the Azov Sea region of Russia. This strain has been cultured in the laboratory since 1983 with periodic resting egg production, collection, and storage. The *Brachionus plicatilis* originated from Spain (Smith and Snell, 2012) and *Brachionus calyciflorus* from Florida (Snell et al., 1991). For each experiment, resting eggs were hatched in 25 mL of 15 ppt ASW (artificial sea water, Instant Ocean) under constant fluorescent illumination (2000 lux) at 25 °C. The resting eggs hatched after 18 to 20 h into a physiologically uniform cohort of neonates. All animals were fed *Tetraselmis suecica* cultured in F medium (Guillard, 1983). Algae were grown in a 560 mL chemostat with ¼ daily replacement under constant illumination (2000 lux) at 25 °C. To simplify life table experiments, rotifers were also treated with 20 µM 5-fluoro-2-deoxyuridine (FDU) to prevent the hatching of amictic eggs (Snell et al., 2012).

### 2.2. Experimental design and treatments

All chemical treatments were first tested in a 3-day reproductive range-finding test (Snell et al., 2012) to determine the highest dose that does not decrease reproduction. Based on these tests, the following concentrations were used in the life table experiments: 20 µM 2-deoxyglucose (2-DG), 20 µM bromopyruvate, 222 mM glyceraldehyde, 10 µM lonidamine, and 10 µM metformin. Glycerol exposures ranged from 13.7 mM (0.1%) to 274 mM (2%).

Full cohort life tables were performed with 120 female rotifers per treatment. Animals were kept in 24-well plates with 5 females per well in a 1 mL medium. The medium contained  $6 \times 10^5$  cells/mL *T. suecica* diluted in 15 ppt ASW, 20 µM FDU, and appropriate test compound. Plates were monitored daily for mortality until all animals were dead, and all animals were transferred to a fresh medium on day 8. Plates were maintained at 22 °C in low light for the duration of the experiment. Data is reported as mean, median and maximum lifespan (age of 95% mortality).

A few life table experiments with reproduction also were conducted without FDU using 24 female rotifers per treatment, each isolated singly in wells of a 24-well plate. Each well had 1 mL of medium containing  $2 \times 10^5$  cells/mL *T. suecica* and the appropriate test compound. Offspring were counted and removed daily, and the original parthenogenetic mothers were transferred to a fresh medium on day 6. These plates were also maintained at 22 °C in low light.

### 2.3. Quantification of glycerol in tissue

The concentration of glycerol present in rotifer tissue was quantified using a glycerol colorimetric assay (Caymen Chemical, #10010755). Animals were first cultured for three days in the glycerol treatment,  $6 \times 10^5$  cells/mL *T. suecica*, and 20 µM FDU. Treatments were 0, 13.7, 34, 67, 103, 137, 171, 206, 240, and 274 mM glycerol. After three days, the animals were washed to remove algae, and 100 animals were collected

in a 0.6 mL tube, with 3 replicates per treatment. Tubes were placed on ice, causing animals to stop swimming, fall to the bottom, and the liquid was pipetted off the top. Animals were re-suspended in 20 µL DI H<sub>2</sub>O and manually homogenized using a plastic pestle for about a minute. 10 µL of the crude rotifer homogenate was added to wells of a 96 well plate, and the colorimetric assay was then performed following kit protocol. Absorbance was measured at 542 nm, and values were compared to a standard curve to calculate the glycerol concentration (mg/L) in the tissue samples.

### 2.4. Rotenone dose–response experiment

We tested the effects of glycerol on sensitivity to rotenone, an oxidative phosphorylation inhibitor. Female rotifers were cultured for four days in  $6 \times 10^5$  cells/mL *T. suecica*, 20 µM FDU, and 274 mM glycerol. Control animals were not exposed to glycerol. Animals were then transferred to 24-well plates at 25 °C with  $6 \times 10^5$  cells/mL *T. suecica*, 20 µM FDU, and rotenone. Each well contained 1 mL medium and 10 rotifers, with 40 animals per treatment. Rotenone treatments ranged from 0 to 0.5 µM in 0.1 µM increments. Survival was recorded 72 h after rotenone exposure, and these data were used to construct a dose–response curve. Rotenone dose–response curves were compared between control animals and rotifers treated with 274 mM glycerol to determine whether glycerol caused a shift in rotenone sensitivity.

### 2.5. Estimation of rotifer swimming speed

To quantify swimming speed, approximately 40 female rotifers were cultured for 10 days in 24-well plates with 2 to 4 animals per well in  $6 \times 10^5$  cells/mL *T. suecica*, 20 µM FDU, and the appropriate chemical treatment. Plates were maintained at 22 °C in low light. Treatments were 100 mM glycerol, 200 mM glycerol, 100 mM glycerol + 20 µM 2-deoxyglucose, and an untreated control. After 10 days, the swimming speed of 15 randomly chosen females from each treatment was recorded at 15× magnification using a Pixelink camera attached to a stereomicroscope. For each video, one animal was placed on a slide in 15 µL of 15 ppt ASW and swimming recorded for 30 s. Videos were converted to image sequences using VirtualDub ([www.virtualdub.org](http://www.virtualdub.org)), and ImageJ was used to remove background noise from the sequences. The ImageJ plugin MTrack2 (<http://valelab.ucsf.edu/~nico/IJplugins/MTrack2.html>) was used to track animal movement and calculate speed in pixels per frame. This measurement was converted to millimeters per second using the video capture speed of the camera (50 frames per second) and the conversion 102.3 pixels per mm.

### 2.6. Estimation of mitochondrial activity

MitoTracker® Red (Invitrogen) was used to estimate mitochondrial activity of rotifers. Female rotifers were cultured in 24-well plates in  $6 \times 10^5$  cells/mL *T. suecica*, 20 µM FDU, and the appropriate chemical treatment for four days. Plates were maintained at 22 °C in low light, and treatments were 274 mM glycerol, and an untreated control. Before staining, animals were rinsed in 15 ppt ASW for 1 h to remove algae from their stomachs and reduce auto-fluorescence. Animals were then transferred to 0.5 mL fresh 15 ppt ASW and 5 µM MitoTracker and incubated at 22 °C in the dark for 30 min. After staining, the animals were rinsed with 15 ppt ASW, anesthetized with 1 mL club soda, fixed with 20 µL of 20% formalin, and rinsed again. To ensure that there was no non-specific binding of MitoTracker, 15 animals from each treatment were anesthetized, fixed with formalin, and incubated at room temperature for 4 h before staining and imaging. This allowed us to compare mitochondrial staining in live and dead rotifers. Images were taken at 200× magnification and 5 ms exposure with an Alexa 486 nm filter using a Zeiss Imager.Z1 microscope. ImageJ was used to measure the average pixel intensity of each rotifer and subtract the background. For

each treatment, 15 animals were imaged and the mean pixel intensity was calculated.

### 2.7. Stressor challenge experiments

To test the effects of glycerol and glycolysis inhibitors on different types of stress resistance, five different stressor challenge experiments were performed using starvation, osmotic shock, heat shock, UV exposure, and oxidative stress. First, female rotifers were cultured for four days in 24-well plates with 1 mL medium and 5 animals per well. The medium consisted of  $6 \times 10^5$  *T. suecica*, 20  $\mu$ M FDU, and the appropriate chemical treatment. Plates were maintained at 22 °C in low light. Treatments were 100 mM glycerol, 20  $\mu$ M 2-deoxyglucose, 20  $\mu$ M bromopyruvate, 100 mM glycerol + 20  $\mu$ M 2-deoxyglucose, and an untreated control. After four days, the animals were removed from the algae containing medium, and challenged with one of the five stressors.

The osmotic shocked animals were transferred from 15 to 60 ppt ASW for 1 h. Heat shocked animals were exposed to 40 °C for 1 h. UV stressed animals were transferred to a Petri dish with 5 mL ASW and exposed to 20 min of UV-B radiation, 25 cm from an 8 W source (UVP, model UVM-28 EI) with an intensity peak at 302 nm. After the stressors were applied, these animals were transferred to 24-well plates at 25 °C with 1 mL 15 ppt ASW, 20  $\mu$ M FDU, and no algae, to follow post-stress survival for 72 h. In the starvation treatment, animals were deprived of all food for 72 h. In the oxidative stress treatment, rotifers were exposed to 0.1  $\mu$ M juglone for 72 h. These stressor treatments were all chosen to produce considerable mortality in the control after 72 h, but not 100% mortality. All plates contained 10 female rotifers per well with a total of 120 animals per treatment. After 72 h, the plates were scored for survival.

### 2.8. Statistics

Test concentrations in the reproductive range finding tests were compared by ANOVA with Dunnett's test to compare mean fecundity in treatments to control using the statistical package JMP 8 (SAS Institute). Survival curves were compared using the JMP 8 reliability and survival analysis, with Wilcoxon's test calculated to compare survival curves of control and treatments. Rotenone LC50s were calculated from linear regressions of dose–response curves estimated with JMP 8. Swimming speed and MitoTracker staining intensities were compared by ANOVA with Dunnett's test to compare treatments to control. Survival in the stressor challenge treatments was compared to control using Fisher's Exact Test with Bonferroni correction for multiple comparisons.

## 3. Results

Glycerol concentrations of 685 mM or higher had a negative effect on rotifer reproduction (Fig. 1). After 48 h exposure, significantly lower reproduction was observed when there was 685 mM glycerol

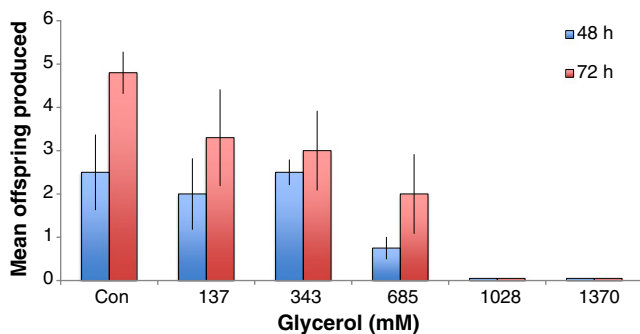


Fig. 1. Reproductive ranging finding test for glycerol toxicity to *B. manjavacas*. Mean offspring produced are the number of females produced by a single parthenogenetic mother in 48 or 72 h at 25 °C.

(5%) in the medium, but not 343 mM. At 1028 mM glycerol, no reproduction was observed after 48 or 72 h. This dose–response relationship set the upper boundaries for exploring the positive effects of glycerol supplementation on rotifer lifespan while avoiding toxic effects.

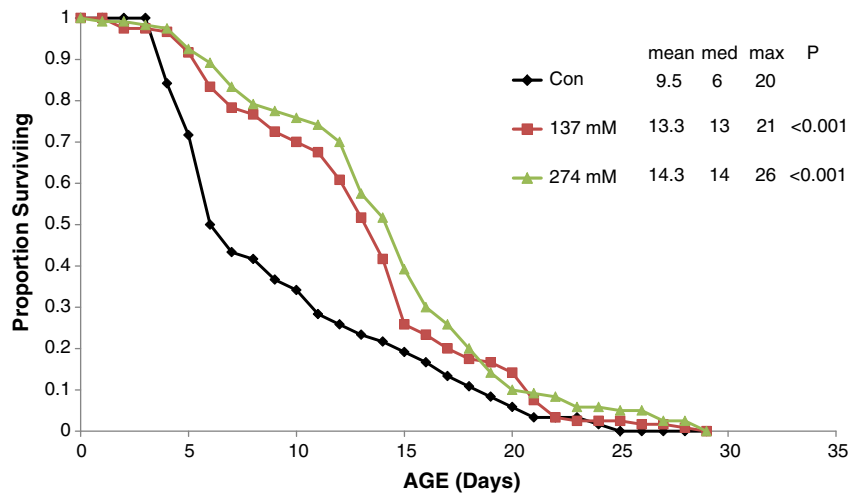
Glycerol supplementation of 137 or 274 mM in the medium significantly extended rotifer lifespan (Fig. 2). Mean lifespan at 137 mM glycerol was 40% longer than the control and 51% longer in the 274 mM treatment. Median lifespan (50% mortality) was 117% and 133% longer in 137 and 274 mM glycerol, respectively. In contrast, the age when only 5% of the cohort remained alive (0.05 lx), was only 5% longer in the 137 mM glycerol treatment and 30% at 274 mM glycerol. Comparison of the pattern of age-specific mortality in controls versus 137 mM glycerol illustrates that glycerol treatment shifts peak mortality from day 7 in the control to day 15 (Fig. 3). This shifts the peak of age-specific mortality from the reproductive phase of the life cycle in the control to the post-reproductive phase in the glycerol treatment.

Since rotifers are dosed by adding glycerol to their medium, it is important to know how much glycerol actually is absorbed into rotifer tissues. The relationship between glycerol exposure and concentration in rotifer tissues is linear from 0 to 274 mM glycerol supplementation (Fig. 4). In the absence of glycerol supplementation, rotifers have about 45 mg/L glycerol in their tissues from normal metabolic activity. Significant rotifer life extension was observed at 137 mM glycerol supplementation (Fig. 1), which produces a 3.3-fold increase in glycerol concentration in rotifer tissues over baseline (Fig. 4). This demonstrates that the efficacy dose for life extension (150 mM) is only about 20% of the reproductive toxicity dose (685 mM). Moreover, these observations provide guidelines for investigating the life extension effects of glycerol in other animal species. Our expectation is that life extension effects may be observed when glycerol is provided as a dietary supplement so that baseline tissue concentration is raised about 3-fold.

Glycerol supplementation up to 274 mM in the medium had no negative effect on rotifer reproduction (Fig. 5). Female lifetime fecundity ( $R_0$ ) ranged from 13.2 offspring in controls to 18.8 in the 274 mM glycerol treatment. Fecundity in the 274 mM treatment was actually 42% greater than control, which is significant by *t*-test ( $df = 46$ ,  $P = 0.02$ ).

Sensitivity to rotenone varies among three brachionid species, with *B. calyciflorus* exhibiting the greatest sensitivity ( $LC_{50} = 0.074 \mu$ M) and *B. manjavacas* the most resistant ( $LC_{50} = 0.35 \mu$ M, Fig. 6). When the dose–response curves of control and 274 mM glycerol supplemented rotifer cultures are compared in *B. manjavacas*, the glycerol treatment is more sensitive to rotenone, especially at lower concentrations (Fig. 7). This pattern of inhibition demonstrates that glycerol supplemented rotifer cultures are less dependent on glycolysis and more dependent on aerobic respiration. In other words, glycerol supplementation shifts rotifer metabolism away from glycolysis and toward oxidative phosphorylation. Direct inhibition of glycolysis with 20  $\mu$ M of the hexokinase inhibitor 2-deoxyglucose does not change with glycerol supplementation (data not shown). This suggests that glycerol supplementation does not directly affect glycolysis as much as aerobic respiration.

Brachionid rotifers swim continuously throughout their lives, so rotifer swimming speed is a measure of their vitality. Newborn *B. manjavacas* females swim at about 0.8 mm/s (Fig. 8). Swimming speed increases with maturity, peaking at 2.5 mm/s at age 4 days, which corresponds to the age of maximum reproduction. As females age, swimming speed declines, so that by age 12 days it falls to about 1 mm/s. As senescence proceeds, swimming slows further so that rotifers eventually cannot maintain themselves in the water column, they sink to the bottom and die. When rotifers are supplemented with 100 mM glycerol, their swimming speed at age 10 days is 37% greater than controls ( $P = 0.02$ , Fig. 8). Supplementation with 200 mM glycerol preserved swimming vitality by 60% greater than controls ( $P < 0.01$ ). The slightly greater preservation of swimming speed in older age classes was achieved with a treatment that combined 100 mM glycerol with 20  $\mu$ M 2-deoxyglucose, which yielded 61% faster swimming at age 10 days ( $P < 0.01$ ).



**Fig. 2.** Survival curves for *B. manjavacas* continuously exposed to 0, 137 or 274 mM glycerol. Proportion surviving represents the fraction of an initial cohort of 120 rotifers surviving to the indicated age. Mean, median and maximum lifespan (5% surviving) are reported in days. *P* refers to the Wilcoxon test probability that the treatment survival curves are similar to control.

The activity of rotifer mitochondria was quantified using MitoTracker (data not shown). Females were treated for two days, exposed to MitoTracker, and then imaged at 200 $\times$  magnification for epifluorescence. Those treated with 274 mM glycerol had 77% higher MitoTracker binding than controls ( $P = 0.02$ ). The positive effect of glycerol vanished if rotifers were dead for 4 h prior to incubating in MitoTracker.

Supplementation of the culture medium with 20  $\mu$ M 2-deoxyglucose or bromopyruvate significantly extended mean rotifer lifespan by 22% (Fig. 9), with no effect on reproduction (data not shown). However, supplements of 10  $\mu$ M lonidamine or metformin had no significant effect on lifespan. Similarly, supplementing the medium with 150  $\mu$ M D-glucose, L-glucose, or pyruvate had no significant effect on rotifer lifespan, nor did 222 mM glyceraldehyde (data not shown).

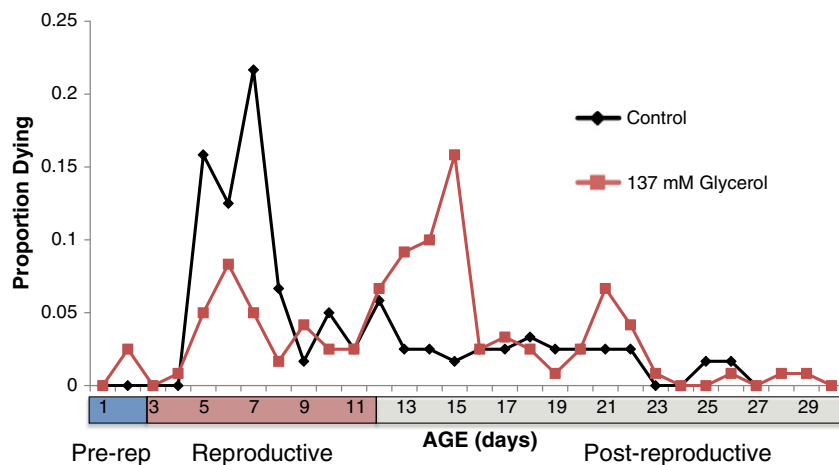
Glycerol supplementation of the culture medium was protective for a variety of stressors. Rotifers were challenged with one of five stressors, starvation, heat, oxidation, UV, or osmotic stress, and their survival followed for 72 h (Fig. 10). Supplementation of the rotifer medium with 100 mM glycerol enhanced resistance to starvation so that 57.5% of animals survived after 72 h, in comparison to 15.8% in controls. If rotifers were exposed to 42  $^{\circ}$ C for 1 h, survival after 72 h in the glycerol treatment was 42.5% compared to 3.3% in controls. Continuous exposure to 0.1  $\mu$ M juglone caused oxidative stress, leading to 56.7% survival in the glycerol treatment versus 43.3% survival in controls. Glycerol exposure did not

confer significant resistance to 20 min UV exposure, producing 15% survival versus 11.4% in the control. Glycerol exposure provided significant protection from osmotic stress (60 ppt for 1 h), with 25% survival in the glycerol treatment compared to 1.5% in controls. Treatment of rotifers with 20  $\mu$ M 2-deoxyglucose or bromopyruvate did not confer significant resistance to any of the five stressors. Likewise, adding 2-deoxyglucose to glycerol supplementation did not significantly enhance protection from the stressors.

A life table experiment was conducted to investigate interactions between glycerol supplementation and exposure to 2-deoxyglucose. Exposure to 100 mM glycerol did not significantly extend mean rotifer lifespan ( $P = 0.20$ ), but exposure to 20  $\mu$ M 2-deoxyglucose caused significant life extension (9%,  $P = 0.04$ ) (Fig. 11). The combined treatment of 100 mM glycerol and 20  $\mu$ M 2-deoxyglucose significantly extended rotifer lifespan compared to the control (12%,  $p < 0.001$ ) and compared to glycerol treatment alone (7%,  $P = 0.05$ ).

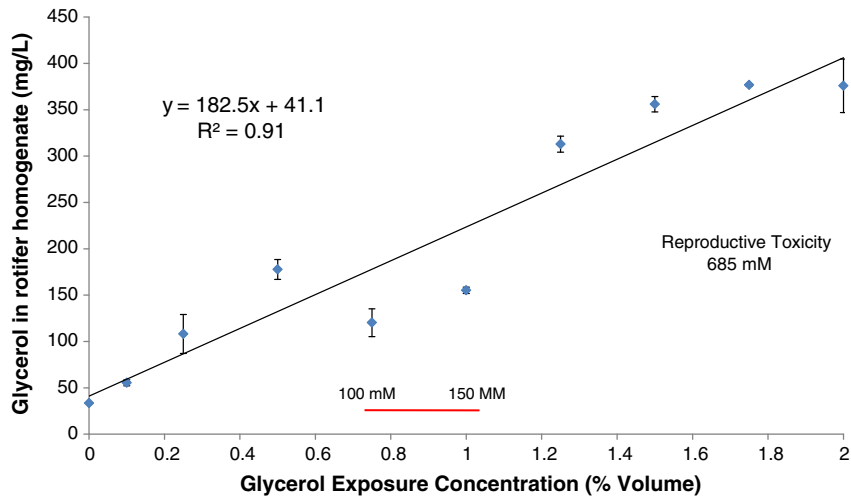
#### 4. Discussion

One of our principal findings is that treatment of rotifers with 150–300 mM glycerol extends lifespan 40–50%. This dose is only about three times the baseline tissue concentration of glycerol and produces a positive effect at about 20% of that causing reproductive toxicity. Insight into the mechanism by which glycerol treatment extends lifespan



**Fig. 3.** Age-specific mortality of *B. manjavacas* continuously exposed to 0 or 137 mM glycerol. Proportion dying represents the fraction of an initial cohort of 120 rotifers dying in the indicated age interval. Pre-rep is the number of days in the mean lifespan spent in the pre-reproductive stage, repro is the reproductive stage, and post-rep is the number of days spent post-reproductive.





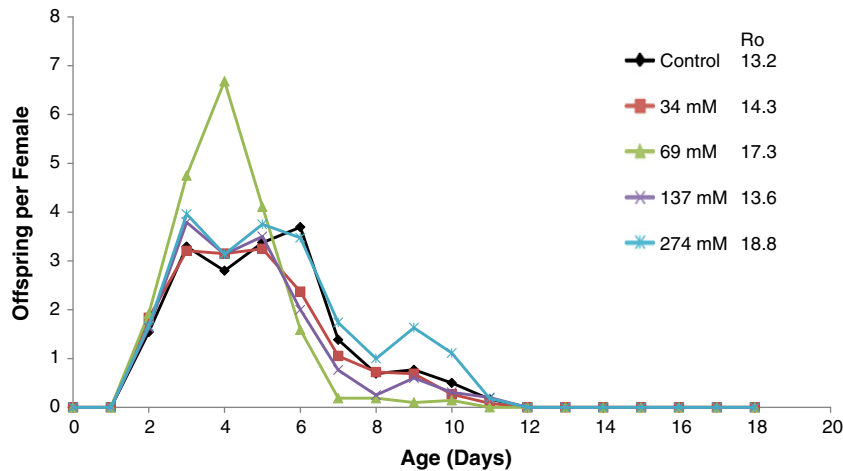
**Fig. 4.** Dose–response curve for glycerol exposure and glycerol concentration in rotifer tissue. Exposure concentrations are reported as percent volume glycerol in the medium. Glycerol in rotifer tissue homogenate is in mg/L. The 100–150 mM line indicates the range of glycerol concentrations where rotifer lifespan extension is observed.

is provided by the experiment with rotenone inhibition. Rotenone is a broad spectrum insecticide and piscicide that selectively inhibits electron transfer from electron transfer chain complex I to ubiquinone. It is useful for probing cellular bioenergetic pathways to estimate the relative contributions of glycolysis and aerobic respiration (Wu et al., 2007). Rotenone selectively inhibits oxidative phosphorylation and 2-DG inhibits glycolysis. Comparing dose–response curves for rotenone in the presence and absence of glycerol provides evidence that glycerol makes rotifers more sensitive to rotenone inhibition because they are more heavily utilizing aerobic respiration. In contrast, the lack of modification of the 2-DG dose–response curve by glycerol suggests that it has little effect on glycolysis.

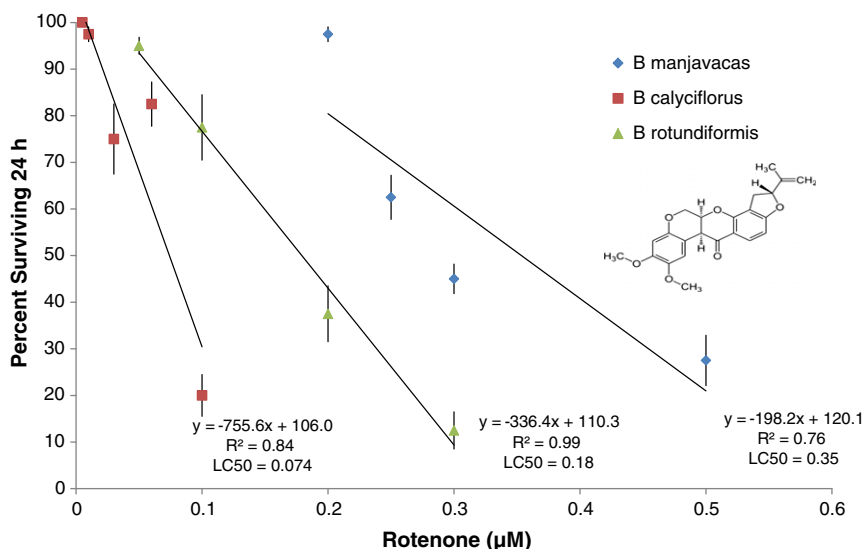
In addition to shifting metabolism away from glycolysis, glycerol acts as a chemical chaperone, protecting cells from the accumulation of protein aggregates (Deocaris et al., 2008). The mechanism of glycerol action is not completely understood, but it is thought that glycerol stabilizes native proteins by altering the solvent properties of water, protein polarity and diffusion, and reducing side chain reactions that lead to misfolding and aggregation (Diamant et al., 2001). Treatment of human cells with 200–400 mM glycerol induced re-folding of heat-denatured luciferase and helped maintain cell proliferation in the presence of oxidative stress (Deocaris et al., 2008). Glycerol treatment also induced expression of mtHSP70, which accounted for some of its

beneficial effect. Deocaris et al. also tested the effect of glycerol on *Caenorhabditis elegans* and found that it reduced the accumulation of age-related lipofuscins and improved thermo-tolerance, but it failed to extend lifespan. The doses of glycerol in the Deocaris et al. study (200–400 mM) are similar to those we found efficacious for extending rotifer lifespan (100–300 mM glycerol). There are some indications that it may be difficult to achieve these tissue concentrations in mice and humans by oral dosing because of the rapid metabolism of glycerol (Bai et al., 1998). Perhaps achieving such high plasma concentrations is not necessary for human lifespan extension. As we argue below, maybe the lifespan extending effects of glycerol can be achieved by simply maintaining plasma glycerol concentration about three times above baseline values. According to Nelson et al. (2011), this is only about 327 μM in humans and is quite feasible with oral dosing. Clearly there are species differences in glycerol uptake and metabolism, so more work needs to be done before optimal glycerol dosing is determined.

Some rotifer species are capable of shifting metabolism from aerobic to anaerobic pathways because they are adapted to live in low oxygen environments. For example, various rotifer species have been found in low oxygen environments in meromictic lakes (Miracle and Vicente, 1983). Moreover, *B. plicatilis* can maintain high population densities even when oxygen is <1 mg/l (Esparcia et al., 1989). This tolerance of hypoxia is likely due to the ability of this rotifer to shift to anaerobic



**Fig. 5.** Age-specific reproduction with glycerol treatment. Offspring per female refers to the average number of offspring produced daily by a cohort of 48 females.  $R_0$  is the mean total offspring produced by a female over her lifetime.



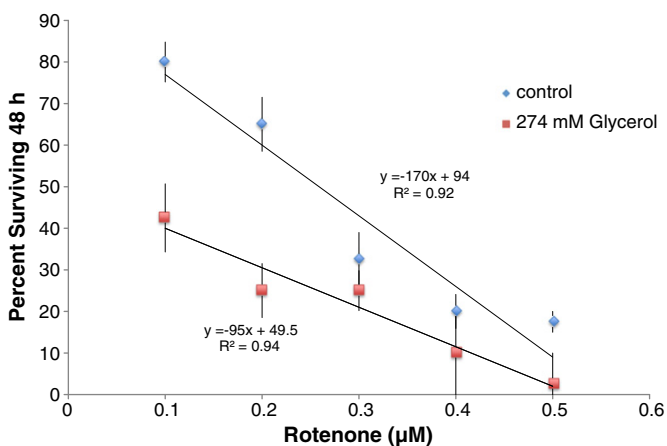
**Fig. 6.** Dose–response curves to rotenone of *B. manjavacas*, *B. rotundiformis*, and *B. calyciflorus*. Each data point represents the mean percent survival of 4 replicate populations of 10 rotifers after 24 h exposure to rotenone. Vertical lines represent standard errors for each rotenone concentration. Trend lines are least-squares fit linear regressions. The concentrations where there is 50% mortality (LC50) are calculated from the respective regression equations.

metabolism by activating lactate and glucose–succinate pathways in hypoxic conditions (Esparcia et al., 1992). Calorically restricted *B. plicatilis* were significantly more tolerant of extreme hypoxia (7.5–11 h at <0.1% O<sub>2</sub>) than *ad libitum* fed rotifers (Ozaki et al., 2010). The CR rotifers up-regulated the glycolytic enzymes glyceraldehyde-3 phosphate dehydrogenase and enolase which enhanced their capacity for anaerobic metabolism and contributed to their greater tolerance of hypoxia. Oxidative stress was induced in *Brachionus* sp. by exposure to 0.1 mM H<sub>2</sub>O<sub>2</sub> for 24 h. This treatment caused more than a 3-fold up-regulation of hsp-20 mRNA production, as well as significantly increased glutathione peroxidase, reductase, and transferase enzyme activity (Rhee et al., 2011). We presented data in this paper suggesting that rotifer lifespan is extended by reducing glycolysis and enhancing aerobic respiration. We accomplished this by supplementation with glycerol, which may have increased the availability of pyruvate for direct incorporation into the oxidative phosphorylation pathway. Glycolysis was further reduced when rotifers were exposed to 2-DG to inhibit hexokinase activity. Because of their complimentary modes of action,

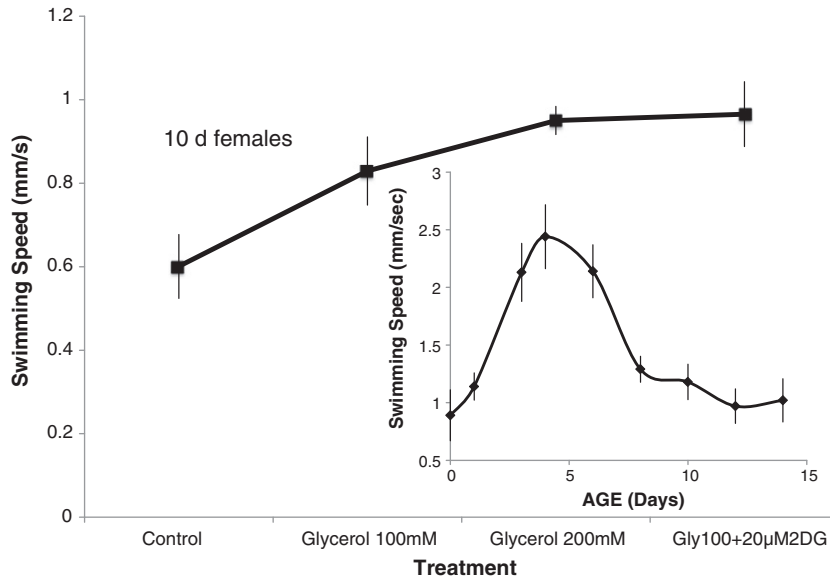
co-administration of both glycerol and 2-DG produced greater life extension than either alone.

The effect on lifespan of shifting of metabolism from anaerobic to aerobic pathways has been explored in other model organisms. Exposure of *C. elegans* to 2% (112 mM) D-glucose in the agar medium shortened lifespan by about 20% by inhibiting activity of the DAF-16/FOXO and HSF-1 transcription factors (Lee et al., 2009). The life-shortening effect of glucose is likely mediated through changes in glycerol metabolism through the aquaporin gene *aqp-1*, which encodes a glycerol channel. Nematodes exposed to 1% glycerol in the medium, decreased lifespan 23–36%, similar to the glucose effect. Glycerol feeding suppressed *aqp-1* expression, which is a regulator of insulin/IGF-1 signaling. Yeast responded oppositely to nematodes when given glucose or glycerol as a sole carbon source (Brisson et al., 2001). Yeast grown on glucose triggered catabolite repression, stimulated glycolysis, and inhibited respiration. In contrast, growth on glycerol relieved catabolite repression and stimulated respiration by conversion of glycerol to pyruvate. If nematodes had responded similarly to glycerol as yeast, Lee et al. (2009) thought that glycerol should lengthen, not shorten, *C. elegans* lifespan. These authors asserted that glucose and glycerol are metabolically interchangeable in *C. elegans*, which is clearly not true for yeast. Indeed, we observed life extension in rotifers by glycerol dietary supplementation, which is precisely the response that was observed yeast. Pathways of carbohydrate metabolism clearly differ between nematodes and rotifers, and provide a cautionary tale about assuming that findings in *C. elegans* are representative of all animals.

Glycerol accumulation is associated with extended lifespan in yeast as evidenced by *sch9* mutants accumulating glycerol (Wei et al., 2008). In yeast, Tor1 is a key regulator of a nutrient sensing pathway that switches from reliance primarily on aerobic respiration to glycolysis and glycerol biosynthesis. Increased glycerol production is a key metabolic change that produces lifespan extension in yeast. Down-regulation of the Tor1 pathway mimics caloric restriction by lowering the glucose content of the medium. Effects of glycerol were investigated by examining mutants that accumulated glycerol rather than ethanol. In these experiments, glycerol was produced endogenously rather than supplied externally as a supplement in the medium. Long-lived mutants had higher expression of several genes involved with glycerol biosynthesis. Deletion of glycerol synthesis genes reversed life extension and resistance to heat, osmotic and oxidative stress. However, supplementation of the growth medium with 0.1 or 1% glycerol did not result in life



**Fig. 7.** Rotenone dose–response curves of *B. manjavacas* in the absence (control) and presence of 274 mM glycerol. Each data point represents the mean percent survival of 4 replicate populations of 10 rotifers after 48 h exposure to rotenone. Vertical lines represent standard errors for each rotenone concentration. Trend lines are least-squares fit linear regressions.



**Fig. 8.** Estimating differences in swimming speed of 10 d old rotifers exposed to various glycerol treatments. Each data point is the average swimming speed (mm/s) of 15 rotifers in each treatment. Vertical lines indicate standard error.

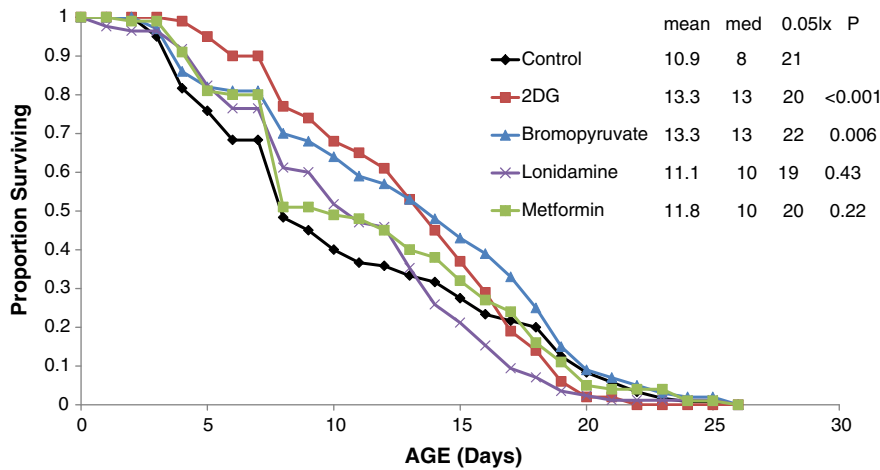
extension for wild type yeast. Glycerol, unlike glucose or ethanol, did not adversely affect the life extension effect induced by caloric restriction. This suggests that carbon source substitution may be an alternative to caloric restriction as a strategy for delaying aging. Glycerol seems to slow aging in rotifers as it does in yeast, and may have similar effects in other animals.

Brachionid rotifers are planktonic herbivores that swim continuously throughout their lifespan. Swimming speed is age-dependent and is therefore a general measure of animal vitality (Snell et al., 2012). Besides extending lifespan, glycerol treatment also preserved rotifer vitality in older age-classes, maintaining swimming speed 60% higher than in controls. The mechanism by which glycerol extends vitality and promotes healthy aging is not known and is likely to be a profitable area of research.

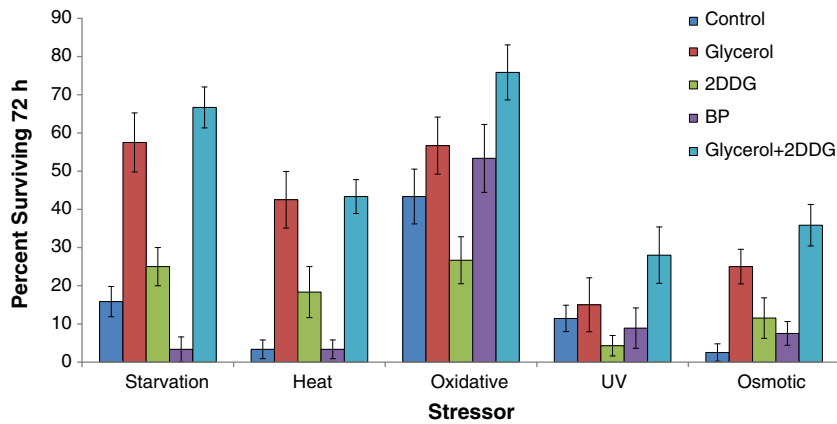
2-Deoxyglucose is a glucose analog that is a promising candidate as a CR mimic (Lane et al., 1998). 2-DG is easily taken up by cells and competitively inhibits glucose utilization because it blocks glycolysis at its first step, inhibiting hexokinase activity. This results in decreased energy production, mimicking the effect of reduced caloric intake. It also

shifts energy production towards heavier reliance on aerobic respiration. Our data showed that exposure of rotifers to 20 µM 2-DG consistently extended lifespan by about 20%. When *C. elegans* was treated with 5 mM 2-DG in agar medium, 17% mean lifespan extension was observed compared to control (Schulz et al., 2007). Impairing glycolysis with 2-DG increases ROS formation 3-fold and produces mitohormesis (Ristow and Zarse, 2010). This promoted lifespan extension and increased resistance to oxidative stress induced by exposure to 50 mM paraquat. In contrast, supplementation with 5 mM glucose decreased nematode mean lifespan about 6%.

2-Deoxyglucose restricts glycolysis in a wide variety of animals, including mammals (Ingram and Roth, 2011). Evidence of its efficacy is provided by rats, which experienced significant life extension when fed a diet supplemented 0.6% 2-DG by weight every other week (Ingram and Roth, 2011). Despite these early encouraging results, short-term toxicity studies and a long-term survival study found that doses of 0.2–0.4% 2-DG produced cardiotoxicity in both Fischer-344 and Brown–Norway rats (Minor et al., 2010). These results motivated our search for compounds that can be co-administered with 2-DG, lowering the dose required to



**Fig. 9.** Survival curves for *B. manjavacas* exposed to hexokinase inhibitors (2-DG, bromopyruvate, or lonidamine) or the drug metformin. Proportion surviving represents the fraction of an initial cohort of 120 rotifers surviving to the indicated age. Mean, median and maximum lifespans (5% surviving) are reported in days. *P* refers to the Wilcoxon test probability that the treatment survival curves are similar to control.



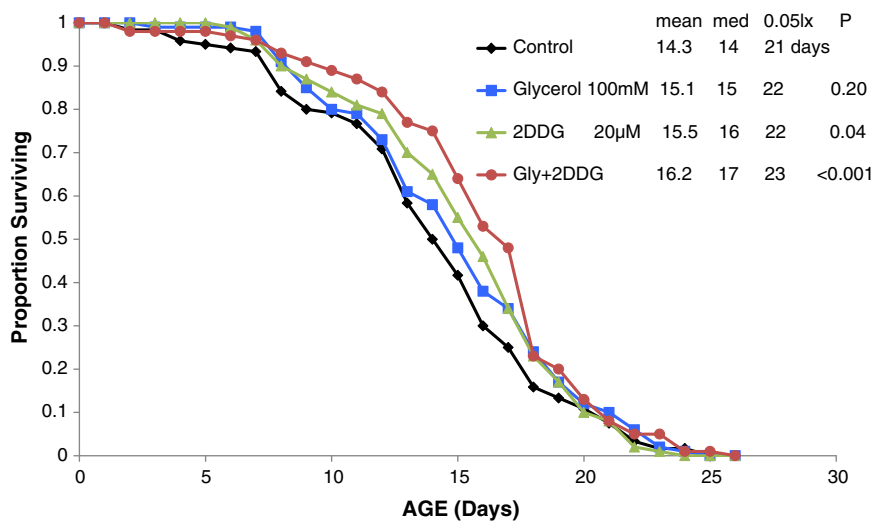
**Fig. 10.** Effect of stressor challenges on *B. manjavacas* survival. Test rotifers were exposed to 100 mM glycerol, 20  $\mu$ M 2-DG, 20  $\mu$ M bromopyruvate, or 100 mM glycerol + 20  $\mu$ M 2-DG for four days before the application of each stressor. The starvation test denied rotifers food for 72 h. The stress tests applied stressors for 20 min (UV), or 1 h (osmotic and heat), and the oxidative stress test exposed rotifers to 0.1  $\mu$ M juglone continuously for 72 h. Proportion surviving is the fraction of a cohort of 120 rotifers surviving 72 h after stressor exposure. Variance in each treatment is indicated by the standard error bars on top of each column.

achieve efficacy. Our data suggest that co-administering 140 mM glycerol and 20  $\mu$ M 2-DG produces greater lifespan extension in rotifers than either compound alone, without any toxic effects.

Caloric restriction remains one of the most effective interventions for lifespan extension (Fontana et al., 2010; Testa et al., 2014). One hypothesis about how caloric restriction works is that it suppresses glycolysis and enhances aerobic metabolism, a metabolic state that typically reduces proteotoxicity (Hippkiss, 2011a). Excessive glycolysis has been implicated as a major cause of proteotoxic stress through the generation of glyating metabolites like methylglyoxal and other low molecular weight carbonyl compounds (Hippkiss, 2011b). Others also have advocated the extension of longevity by enhancing cytoprotective functions like detoxification, innate immunity, oxidative stress response, and proteostasis (Shore and Ruvkun, 2013).

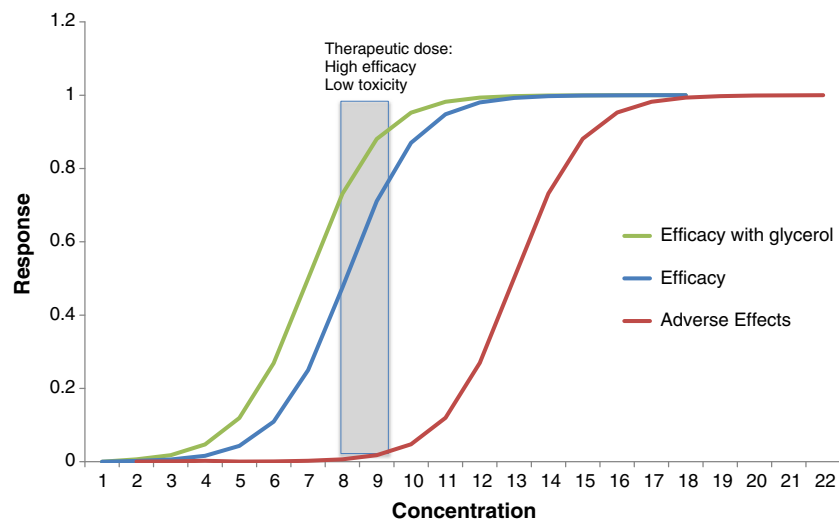
Rapamycin is a macrolide drug that specifically inhibits the activity of TOR kinase. It has been approved for human use as an immunosuppressant, cancer chemotherapy, and to prevent restenosis of arteries after cardiac angioplasty. Rapamycin also is broadly effective against aging and aging-related diseases (Johnson et al., 2013a; Menzies and Rubinsztein, 2010), and can extend lifespan in yeast, nematodes, *Drosophila* and mice (Bjedov et al., 2010; Blagosklonny, 2010; Harrison et al., 2009; Miller et al., 2011; Sharp, 2011; Wilkinson et al., 2012). TOR

senses cellular nutrient and energy levels and regulates cell survival, growth, proliferation, motility, protein synthesis and transcription (Hay and Sonenberg, 2004; Stanfel et al., 2009). It is promising as a CR mimic, targeting downstream sites in nutrient regulation pathways (Ingram and Roth, 2011; Testa et al., 2014). Rapamycin exposure caused a metabolic shift from glucose utilization to fatty acid oxidation in L6 rat muscle cells without caloric restriction (Sipula et al., 2006). Skeletal muscle cells exposed to 15 nmol/L rapamycin for 24 h increased fatty acid oxidation by 60% by increasing activities of carnitine palmitoyltransferases I and II, the primary intracellular regulatory enzymes of the fatty acid oxidation pathway, while glycolysis was concomitantly reduced by 40%. This kind of metabolic switching between fatty acid and glucose metabolism for energy production in muscle is a normal physiological response to accommodate short-term changes glucose and fatty acid availability (Sipula et al., 2006). Increased fatty acid oxidation at the expense of glucose utilization also is characteristic of a fasting metabolic state. Intraperitoneal injections of 8 mg rapamycin/kg every other day beginning at weaning extended female and male mice lifespans 38 and 25%, respectively (Johnson et al., 2013b). Rapamycin inhibition of mTOR partly alleviated a mitochondrial disease in this mouse model, which was partially explained by rapamycin inducing a metabolic shift toward amino acid catabolism



**Fig. 11.** Interaction between glycerol and 2-DG. Rotifers were exposed to 100 mM glycerol, 20  $\mu$ M 2-DG, or 100 mM glycerol + 20  $\mu$ M 2-DG. Proportion surviving represents the fraction of an initial cohort of 120 rotifers surviving to the indicated age. Mean, median and maximum lifespans (5% surviving) are reported in days. *P* refers to the Wilcoxon test probability that the treatment survival curves are similar to control.





**Fig. 12.** Illustration of theoretical response curves for adverse effects (toxicity) and efficacy. X-axis is the concentration of a test compound and Y-axis is the response level. Low concentrations elicit low responses (toxicity or efficacy) and high concentrations elicit greater responses until saturation. Optimal therapeutic dose is the concentration range where there is high efficacy and low toxicity.

and away from glycolysis, reducing the buildup of glycolytic intermediates. Unfortunately, rapamycin has a number of adverse side effects (Johnson et al., 2013a; Testa et al., 2014), but some of these may be mitigated by the co-administration of other compounds.

Biological signaling is intrinsically complex, typically involving non-linear pathways, feedback loops, and compensatory mechanisms (de Magalhaes et al., 2012). Consequently, employing combinations of compounds to target multiple pathways is likely to be a more productive approach to improve efficacy and minimize toxicity (Hopkins, 2008). We have screened a variety of small molecule inhibitors for life extending ability in rotifers (Snell et al., 2014), many of these can be considered CR mimics since they inhibit similar pathways as CR (Testa et al., 2014). Inhibition of AKT, IGF-1R, AMPK, and PI3K signaling had no effect on rotifer lifespan, but inhibition of TOR and JNK gene expression both yielded significant life extension of 20–40%. In contrast, several experiments exposing rotifers to 20  $\mu$ M resveratrol failed to produce any significant life extension. Exposure to combinations of a few dietary antioxidants also produced modest rotifer life extension of 10–20% (Snell et al., 2012). In this paper, we report that treating rotifers with 10  $\mu$ M of the diabetes drug metformin had no lifespan extending effect.

The relationship between stress resistance and longevity has been well documented in yeast (Longo and Fabrizio, 2002), invertebrates (Johnson et al., 1996), birds (Harper et al., 2011) and mammals (Harper et al., 2007). What is less clear is why activation of these stress resistance pathways often has life extending effects. It has been suggested that the linkage between stress resistance and longevity indicates that accumulation of molecular damage may play a key role in aging (Longo and Fabrizio, 2002). Moreover, aging may be slowed by enhancing systems that repair or resist damage, like the xenobiotic metabolizing enzyme glutathione-S-transferase (Ayyadevara et al., 2005). However, recent work has suggested that accumulation of molecular damage may not be the whole story (Gems and Partridge, 2013). These authors believe that rather than being caused by the accumulation of damage, aging may result from over-activity of growth and reproduction processes (hyperfunction) that were beneficial earlier in life. Exposure of rotifers to glycerol significantly extended lifespan and provided some protection from starvation, high temperature, oxidative and osmotic stress, but not UV damage. Our data suggest that glycerol supplementation shifts metabolism away from glycolysis and towards oxidative phosphorylation. Independently suppressing glycolysis with the hexokinase inhibitor 2-DG did not provide protection from any of the five stressors. The mechanism of glycerol action in life extension and enhanced stress resistance is poorly understood. Glycerol exposure may reduce accumulation of molecular

damage or suppress some as yet undetermined hyperfunctionality, or both.

Whether a compound has a therapeutic value or not is largely determined by its dose. Much is known about how various doses of glycerol impact humans because of its use in sports drinks to maintain hydration (Frank et al., 1981; Nelson et al., 2011). Every compound has a characteristic dose–response curve for efficacy and adverse effects (see Fig. 12). The therapeutic dose is that narrow window of concentration that maximizes efficacy while minimizing adverse effects. Based on our observations in rotifers and human dose–response studies, we can predict a therapeutic dose of glycerol for testing its lifespan extension potential in humans. For example, normal serum concentrations of glycerol in adult humans are 0.05–0.1 mM/L (Lin, 1977). Based upon our findings in rotifers, we could assume that a dose with potential human lifespan extending effects would be approximately three times base line plasma glycerol concentration. Nelson et al. (2011) found that a dose of 0.032 g glycerol/kg lean body mass in human subjects produced a plasma concentration of 0.327 mM/L, which is 3.3 times baseline glycerol concentration. Higher doses resulted in plasma saturation and glycerol appearing in the urine. Glycerol has a density of 1.261 g/cm<sup>3</sup>, so the dose to raise the plasma glycerol of a 70 kg person to 3.3 times baseline is 1.78 mL glycerol. Since glycerol is cleared from plasma in about 2 h, this dose would have to be repeated three times daily to maintain a 3-fold elevated plasma glycerol concentration (Koenigsberg et al., 1995). This dose is only 8% of that recommended by van Rosendal et al. (2010) for trained athletes to achieve hyperhydration. These calculations illustrate that achieving a glycerol dose for testing the possibility of lifespan extending effects in humans may be feasible.

#### Conflict of interest

The authors have no conflicts of interests.

#### Acknowledgments

We are grateful for the support of the National Institute of Aging, grant R01 AG037960-02 for this work. Kristen Gribble and David Mark Welch made important comments that improved the paper. We also express our appreciation for the expert technical assistance provided by Leilani Barry, Shelby Rittweger, Brett Rabeneck, and Corey Rennolds.

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