

Resveratrol Prolongs Lifespan and Retards the Onset of Age-Related Markers in a Short-Lived Vertebrate

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Summary

Resveratrol, a natural phytoalexin found in grapes and red wine [1], increases longevity in the short-lived invertebrates *Caenorhabditis elegans* and *Drosophila* [2–5] and exerts a variety of biological effects in vertebrates, including protection from ischemia and neurotoxicity [6–10]. Its effects on vertebrate lifespan were not yet known. The relatively long lifespan of mice, which live at least 2.5 years [11], is a hurdle for life-long pharmacological trials. Here, the authors used the short-lived seasonal fish *Nothobranchius furzeri* with a maximum recorded lifespan of 13 weeks in captivity [12, 13]. Short lifespan in this species is not the result of spontaneous or targeted genetic mutations [14], but a natural trait correlated with the necessity to breed in an ephemeral habitat and tied with accelerated development and expression of ageing biomarkers at a cellular level [12, 13]. Resveratrol was added to the food starting in early adulthood and caused a dose-dependent increase of median and maximum lifespan. In addition, resveratrol delays the age-dependent decay of locomotor activity and cognitive performances and reduces the expression of neurofibrillary degeneration in the brain. These results demonstrate that food supplementation with resveratrol prolongs lifespan and retards the expression of age-dependent traits in a short-lived vertebrate.

Results

Effects of Resveratrol on Longevity

We hatched and raised fishes ($n = 157$) under standard conditions (for details refer to [13]) until 4 weeks, when

they reach sexual maturity [12]. Throughout the remaining part of their life, 110 fishes were fed food supplemented with resveratrol at three different concentrations: 24 $\mu\text{g/g}$ food (0.1 μM ; $n = 30$), 120 $\mu\text{g/g}$ food (0.5 μM ; $n = 60$), and 600 $\mu\text{g/g}$ food (2.5 μM ; $n = 20$), while 47 control fishes continued to receive standard food. Fishes were not fed ad libitum, but received a defined dose of food twice a day (see **Experimental Procedures**). Therefore, it can be excluded that resveratrol effects are confounded by food aversion behaviors that result in dietary restriction. Survival of control-fed fishes is almost identical to the survival of a larger number of untreated individuals that represent our reference data ($n = 132$, **Figure 1A**). Supplementation of resveratrol resulted in a statistically significant increase of longevity that was dose dependent: the lowest dose (24 $\mu\text{g/g}$ food) was ineffective in increasing lifespan compared to both control-fed and untreated fishes (**Figure 1A**). In two independent trials, 120 $\mu\text{g/g}$ food caused an increase of median and maximum lifespan of 33% and 27%, respectively ($p < 0.001$, log rank test; **Figure 1A**), and 600 $\mu\text{g/g}$ food induced 56% and 59% increase in median and maximum lifespan, respectively ($p < 0.001$, log rank test; **Figure 1A**). 600 $\mu\text{g/g}$ food was significantly more effective than 120 $\mu\text{g/g}$ food in prolonging lifespan ($p = 0.01$, log rank test) (maximum lifespan in demographic studies is defined as the 10th percentile in survival). Life extension was observed both in males and females (**Figure 1B**) and did not differ between the two sexes. Longevity was not linked to loss of fertility: resveratrol-treated females continued to lay eggs and males were still fertile (i.e., were able to fertilize eggs) at age 12 weeks when all controls had died. Eggs laid by resveratrol-treated fishes developed into normal adults (not shown).

Demographic data can be analyzed by an exponential fit of age-dependent mortality rates that separate two components: an age-independent mortality (mortality unrelated to aging) and an exponential increase of mortality rates (which is thought to reflect time-dependent accumulation of irreversible biochemical damage [15]). Resveratrol changed the slope of the mortality curve, slowing down the age-dependent increase in death rate, and interestingly increases the intercept, i.e., raises the mortality rates of treated fishes over those of control-fed fishes during the first weeks after administration (**Figure 1C**). This datum suggests an early hormetic role of resveratrol as a possible mechanism of action for the induction of slowed aging and prolonged lifespan.

Effects of Resveratrol on Functional Markers

Age-related reduction of locomotor efficiency is a marker for neuromuscular decay [16]. We quantified spontaneous locomotor activity by scoring of videotapes starting from week 5. Statistical analysis revealed reduction in spontaneous swimming at 9 weeks in control animals (**Figure 2A**, Kruskal-Wallis rank-based ANOVA, $p < 0.001$); this reduction is retarded in resveratrol-treated fishes (120 $\mu\text{g/g}$ food, differences between treated and control group at 9

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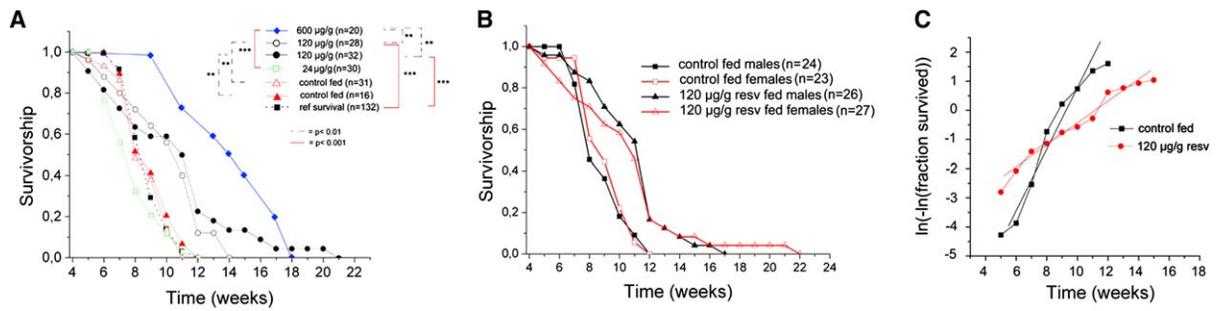


Figure 1. Age-Dependent Survival of Treated and Untreated *Nothobranchius furzeri*

The fraction of animals survived is plotted on the ordinate, and the age (in weeks) is plotted on the abscissa. The origin of the curves is at age 4 weeks, when the fishes are sexually mature and the treatment starts.

(A) Survival curves of five separate experiments and of a reference set for a total of 289 animals. Two trials of control-fed fishes (solid and empty triangles, total number = 47); reference survival of untreated fishes (black dotted, n = 132, a total of 5 replicates); 24 µg/g resveratrol-treated fishes (green, n = 30); two trials of 120 µg resveratrol-treated fishes (solid and empty black circles, total number = 60; 4 fishes were sacrificed for histological analysis and censored at age 9 weeks); 600 µg resveratrol-treated fishes (blue, n = 20).

(B) Comparison between the age-dependent survival of males and females in controls and 120 µg/g resveratrol-treated fishes. Control males (black line, n = 22) and females (dotted black line, n = 25); 120 µg/g resveratrol-treated males (blue line, n = 30) and females (dash and dot line, n = 27). Three animals could not be sexed upon death.

(C) Death trajectories in controls (black, $y = 1.0452x - 4.4726$; $R^2 = 0.9402$) and 120 µg/g resveratrol-treated fishes (red, $y = 0.3761x - 2.3177$; $R^2 = 0.9739$). The linear interpolations are performed for both groups from week 6 to the 10% survivorship age (week 11 for controls, week 14 for treated fishes).

weeks significant by two-way ANOVA, Tukey post hoc test: $p < 0.001$). Open-field exploration, a standard behavioral test in the rodent that quantifies the exploration of a new environment [17], was quantified by automated video tracking. Age-dependent decrease in time spent moving and average velocity was detected at 9 weeks in control fishes when compared to 5-week-old fishes (Figures 2B and 2C, Kruskal-Wallis rank-based ANOVA, $p < 0.01$). Locomotor function was preserved in 9-week-old treated fishes (two-way ANOVA, Tukey's post hoc test: $p < 0.001$). The swimming performance increased, instead of decreasing, in resveratrol-treated fishes until the 10th week and decayed in 13-week-old fishes (Kruskal-Wallis rank-based ANOVA, $p < 0.001$).

We quantified operant learning in *Nothobranchius furzeri* by a modified shuttle-box originally developed

for goldfish and zebrafish [18, 19] (see Figure S1 in the Supplemental Data available with this article online), in order to evaluate an age-dependent cognitive decline. Young fishes (n = 10; 5 weeks old) learned the task effectively and after 50 consecutive trials attained 73% rate of successes (Figure 2D). Old fishes (n = 10; 9 weeks old) showed significant learning, but at the end of the training their success rate only reached 42% (Figure 2D). Age-dependent decay of cognitive performances was completely prevented in old fishes treated with resveratrol (120 µg/g food), which reached 74% rate of successes (n = 10; 9 weeks old, Kruskal-Wallis rank-based ANOVA, $p < 0.001$; Figure 2D; Figure S1).

Neurofibrillary degeneration was used as a histological marker of neuronal aging [20]. Young fishes (n = 4; 5 weeks old) did not show signs of neurofibrillary

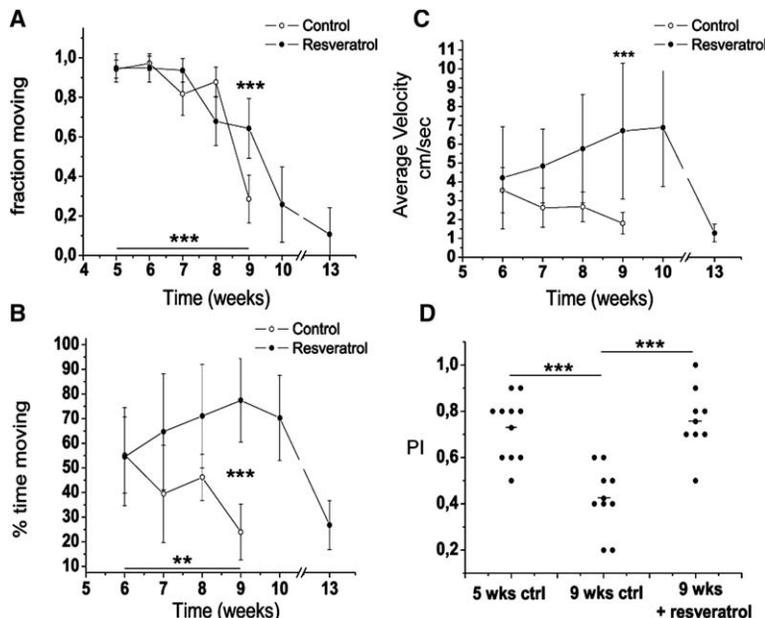


Figure 2. Functional Aging in Treated and Untreated *Nothobranchius furzeri*

(A–C) Locomotor activity.

(A) Fraction of fishes swimming in a 5 s time frame is reported on the ordinate.

(B) Average swimming velocity during open-field exploration.

(C) Time spent moving during open-field exploration. The age (in weeks) is reported on the abscissa. Open circles refer to controls and solid circles refer to 120 µg/g resveratrol-treated fishes. Error bars are standard errors. Pair-wise comparisons were performed by Kruskal-Wallis rank-based ANOVA, $**p < 0.01$, $***p < 0.001$.

(D) Cognitive test. Scatter of individual top scores in an active avoidance test (Figure S1). Solid circles refer to individual animals of the three groups and the barred circles to the averages of the three groups. Treated fishes are 120 µg/g food resveratrol-fed fishes. Pair-wise comparisons were performed with Kruskal-Wallis rank-based ANOVA, $***p < 0.001$.

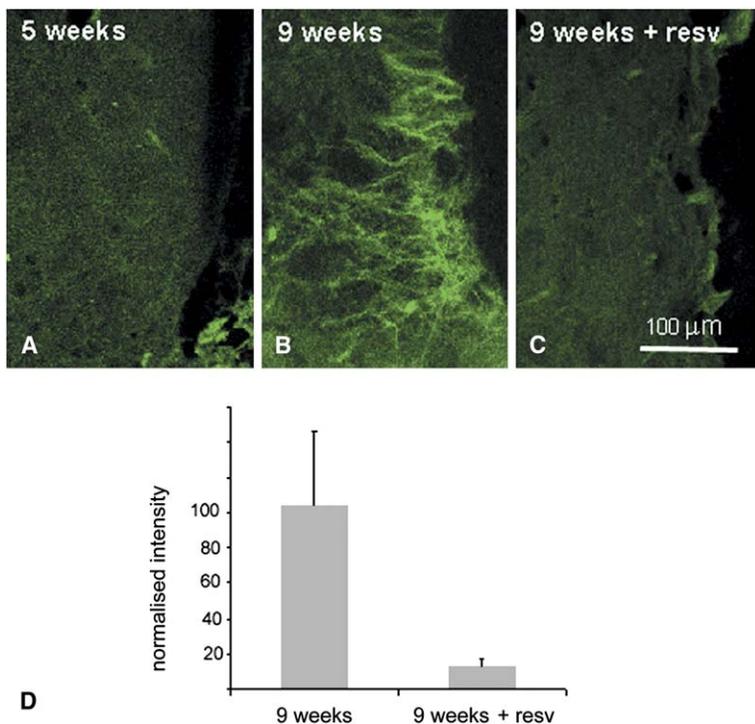


Figure 3. Neurofibrillary Degeneration in a Horizontal Section of Stratum Griseum Superficiale of the Optic Tectum

Lateral is right and medial is left. Fluoro-JadeB histochemistry. The specific reaction product is fluorescent green.

(A) 5-week-old fish. Note absence of specific signal.

(B) 9-week-old control fish. Note the bright labeling in neuronal processes orthogonal to the tectal surface.

(C) 9-week-old 120 μg/g resveratrol-treated fish.

(D) Quantification of labeling intensity. Gray level values are normalized with respect to the average gray value of controls, which is arbitrarily set to 100. Bars represent standard errors of means.

degeneration (Figure 3A), but old fishes ($n = 4$; 9 weeks old) showed neurofibrillary labeling of dendrites in the optic tectum (Figure 3B) as well as telencephalon and brainstem (not shown), as detected by Fluoro-JadeB staining. Labeling was absent in resveratrol-treated old fishes ($n = 4$, Figure 3C). This difference was quantified by image analysis (Figure 3D).

Discussion

In the present paper we tested the effects of resveratrol on the lifespan and on the onset of age-related markers in the short-lived fish *Nothobranchius furzeri*, a seasonal fish whose recorded median lifespan in captivity is 9 weeks [13]. This short captive lifespan is a natural trait observed also in one wild-derived population collected by the authors in 2004 (D.R.V., E.T., and A. Cellerino, unpublished data). Other longer-living natural populations of *Nothobranchius furzeri* exist, and they originate from a more humid habitat (unpublished data), in line with the relationship between length of the rain season and captive lifespan observed in *Nothobranchius* species [13]. The biological action of resveratrol, we report here, extend to vertebrates the longevity effect of resveratrol originally described in yeast, *Drosophila*, and *C. elegans* [3, 5]. The effects of resveratrol were not coupled to loss of fertility, in agreement with the effects found by others in *C. elegans* and *Drosophila* [5]. More importantly, this effect was observed with treatments starting in early adult life and effected functional parameters such as locomotor activity and cognitive deficit, which are observed during vertebrate and human aging [21–24]. A complex dietary supplement was shown to cause a small extension of longevity in mice [25], and supplementation of L-Acetyl-Carnitine was shown to retard rat locomotor and cognitive aging [22, 23].

The natural polyphenol resveratrol has received much attention for its possible pharmacological use. It was reported to have anticancer and antiinflammatory action in vitro [26, 27] and neuroprotective action in vitro [6, 7, 9, 10, 28] and in vivo [10]. The mechanisms of action of resveratrol are multiple [1]. It inhibits mitochondrial ATPase in mammals [29, 30] and was reported to activate the NAD-dependent histone deacetylase sirtuins in nematodes, flies, and rodents [5, 6]. The effects of resveratrol in nematodes and flies require the presence of a functional Sir2 gene [5]. Overexpression of the mammalian homolog of sir2, SIRT1, has reported to be neuroprotective in a variety of models, and this effect is mimicked by resveratrol [6, 7, 28]. Finally, dietary restriction, the best-studied life-extension treatment, causes overexpression of SIRT1 [31], and its effects on lifespan are not additive to those of resveratrol in *Drosophila* [5]. All these data raise the possibility that resveratrol mimics dietary restriction by decreasing the aging rate through activation of SIRT1. However, more recent results have shown that resveratrol increases Sir2 or SIRT1 activity only if the substrate is conjugated to a nonphysiological fluorescent moiety [32, 33]. So, at present, the mechanism underlying the life-extension property of resveratrol and its relationship with calorie restriction remain unclear. The effect of resveratrol is mimicked by overexpression of sirtuins [6, 7, 28], but does not seem to be mediated by direct action on sirtuins [32, 33].

Life extension by resveratrol is associated with a change in the slope of the mortality trajectory, i.e., the time-dependent increase in death risk is lowered in resveratrol-fed fishes compared to control-fed ones. A similar effect is observed in *Drosophila* when the temperature is reduced and was linked to a reduction in the age-dependent accumulation of biochemical irreversible damage [34]. This effect is different from the

life extension induced by dietary restriction, which, at least in *Drosophila*, is tied to time-restricted reduction in the acute risk of death without changes in the slope of the mortality rate [34]. If this interpretation can be extended to *N. furzeri*, it would further suggest caution when equating the effects of resveratrol with dietary restriction. We observed that resveratrol induced an early increase in death rate after administration, suggesting a possible hormetic role, i.e., its weakly toxic action would stimulate a stress response and eventually increase lifespan and retard ageing. This view is in agreement with the resveratrol-dependent activation of detoxification enzymes observed in *in vitro* studies [35].

Resveratrol has shown neuroprotective activity in a variety of paradigms both *in vivo* and *in vitro* [6, 7, 9, 10, 28]. Invertebrate studies have revealed the importance of the nervous system in regulating lifespan, as neuronal-specific gene manipulations and neuroprotective drugs can change the longevity of both worms and flies [2, 36–38]. Resveratrol-fed fishes showed remarkable preservation of operant learning and prevention of age-dependent neurodegeneration. The possibility cannot be excluded that life extension induced by resveratrol is secondary to a protective action on the nervous system.

The mechanisms by which resveratrol prolongs lifespan in model organism are not clear, but the observation that its supplementation with food extends vertebrate lifespan and delays motor and cognitive age-related decline could be of high relevance for the prevention of aging-related diseases in the human population.

Experimental Procedures

Fish Maintenance

Fishes used for this study are of the Gonarezhou strain of *Nothobranchius furzeri* kept at 25°C since the 4th week of life. We maintained a tank density of 20 fishes, which were fed twice a day with commercially available bloodworm larvae (*Chironomus sp.*). The water was changed once a week and was filtered by a sponge filter driven by air supply that provided a soft filtration. For details on raising *Nothobranchius sp.*, refer to [13].

Resveratrol Treatment

Sample preparation for 120 mg/food: 1.2 mg/ μ l resveratrol stock was prepared in 5% ethanol and stored at 4°C in the dark. Frozen *Chironomus* larvae were thawed, left to drip dry, and aliquoted into portions of one feeding for 10 adult fish (1 g). 100 μ l of the 1.2 mg/ μ l stock was added to each *Chironomus* aliquot, which was left at 4°C for 1–2 hr to soak. 5% gelatine was added to the *Chironomus*/resveratrol aliquot, mixed, frozen, and stored at –20°C until use. For feeding, the frozen gelatine/*Chironomus* cube was thawed in water and fed to the fish. All uneaten food was removed. Fish received 2 feeding per day. Control-fed fishes received the same kind of food lacking resveratrol in the stock solution. The food consumption was approximately of 50 mg (food)/g (fish weight)/day.

Survival Assays

Fishes were counted once a week, and each day dead fishes were scored and removed from the tanks. The commercial available Graph Pad Prism was used for survival analysis. Death trajectories were computed as the $\ln(-\ln(\text{fraction survived}))$.

Locomotion Tests

Animals for the behavioral tests are a subset of the animals used for the survival curve. For analysis of spontaneous locomotion, fishes were filmed for 1 hr in their home tank at a frame rate of 1 frame per s. Ten couples of frames from the movie strain were extracted

in the following way: each frame couple consisted of two frames occurring 5 s apart. A fish was considered to be significantly displaced from one frame to the other if it moved for at least one half of its body length. The fraction of fishes actively swimming in each frame was scored manually and averaged. For open-field exploration, 5 males and 5 females were selected randomly from the survivors. Each fish was moved to a new tank and filmed. Clips were analyzed by standard procedures of Ethovision (Noldus, Waningen, The Netherlands).

Active Avoidance

Learning in fish can be studied with a protocol of operative conditioning with a modification of the shuttlebox [18]. The fish is placed in a tank with a divider and one red light in each compartment (see Figure S1). The fish is left to habituate to the new environment for about 1 hr before starting the test. When the fish is in one compartment, the light is switched on for 30 s, and after a delay of 15 s the fish receives a punishment if it remains within the same compartment. Each of these events is a trial. If the fish moves to the other compartment within 15 s from light onset, the fish avoids the punishment and the trial is scored as successful (Figure 2D). Frequency of success is averaged over ten consecutive trials and the top scores are used as Performance Index for statistical analysis. Details of the apparatus and quantifications are in Figure S1.

Histology

Fish were euthanized with MS-222 and placed on crushed ice for dissection. Tissues were immersion fixed, cryoprotected, included, and sliced by standard techniques. Horizontal sections from the optic tectum of 9-week-old controls ($n = 4$) and 9-week-old 120 μ g/g resveratrol-treated fishes ($n = 4$) were reacted for Fluoro-JadeB histochemistry in the same session and acquired at the confocal microscope at fixed laser intensity, photomultiplier parameters, and pinhole size during the same day. Fluorescence analysis was performed with Metamorph. For each subject, three rectangular frames of fixed size were acquired close to the pial surface and the images were thresholded at a fixed intensity value so that all pixels below threshold were excluded from the analysis. The average fluorescence intensity within the thresholded area was then calculated.

Supplemental Data

Supplemental Data include one figure and can be found with this article online at <http://www.current-biology.com/cgi/content/full/16/3/296/DC1/>.

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D.R.V. helped in designing the experiments; performed behavioral tests, statistical analysis, and survival assays; and cowrote the paper. E.T. performed the histology and survival assay. T.G. performed the survival assay and codeveloped the dosing protocol. A. Cattaneo and L.D. helped in designing the experiments. A. Cellerino designed the experiments and wrote the paper. This work was financed by Lay Line Genomics S.p.A., which holds the rights for commercial exploitation of the model.

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