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Effects of dietary restriction on mortality and age-related phenotypes in the short-lived fish *Nothobranchius furzeri*

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Summary

The short-lived annual fish *Nothobranchius furzeri* shows extremely short captive life span and accelerated expression of age markers, making it an interesting model system to investigate the effects of experimental manipulations on longevity and age-related pathologies. Here, we tested the effects of dietary restriction (DR) on mortality and age-related markers in *N. furzeri*. DR was induced by every other day feeding and the treatment was performed both in an inbred laboratory line and a longer-lived wild-derived line. In the inbred laboratory line, DR reduced age-related risk and prolonged maximum life span. In the wild-derived line, DR induced early mortality, did not reduce general age-related risk and caused a small but significant extension of maximum life span. Analysis of age-dependent mortality revealed that DR reduced demographic rate of aging, but increased baseline mortality in the wild-derived strain. In both inbred- and wild-derived lines, DR prevented the expression of the age markers lipofuscin in the liver and Fluoro-Jade B (neurodegeneration) in the brain. DR also improved performance in a learning test based on conditioning (active avoidance in a shuttle box). Finally, DR induced a paradoxical up-regulation of glial fibrillary acidic protein in the brain.

Key words: intermittent fasting; animal model; dietary restriction; age markers; neurodegeneration; annual fish.

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Introduction

Reduction of food availability, named dietary restriction (DR), was reported to increase life span in a variety of different organisms (Ingle *et al*., 1937; Austad, 1989; Jiang *et al*., 2000; Kealy *et al*., 2002) and in laboratory strains of all the major animal models: *Caenorhabditis elegans*, *Drosophila*, mouse and rat (McCay *et al*., 1935, 1939; Weindruch & Walford, 1988; Hekimi *et al*., 1998; Mair *et al*., 2003). In laboratory rodents, DR not only prolongs life span, but also reduces cancer incidence and retards age-related physiological decay of several organs (Weindruch & Walford, 1988). In particular, DR exerts a neuroprotective action and prevents some forms of age-related neuronal impairment (Hori *et al*., 1992; Pitsikas & Algeri, 1992; Eckles-Smith *et al*., 2000; Mattson, 2000; Fontan-Lozano *et al*., 2007; Adams *et al*., 2008).

DR effects on longevity are robust, but not universal. DR does not increase the life span of the medfly (*Ceratitis capitata*) (Carey *et al*., 2002) or of the common housefly (*Musca domestica*) (Cooper *et al*., 2004). In *Drosophila*, life extension is triggered by protein deprivation and not calorie restriction, and it is mainly the result of longevity–fertility trade-off mechanisms (Mair *et al*., 2005; Lee *et al*., 2008). Similar longevity–fertility trade-offs induced by protein restriction were recently described in the tephritid fruit fly, *Anastrepha ludens* (Carey *et al*., 2008). Even in laboratory mice, effects of DR are strain specific (Turturro *et al*., 1999; Forster *et al*., 2003). In addition, effects of DR are generally studied in laboratory strains of model organisms which are the result of tens or hundreds of generations of (involuntary) captive selection for high productivity in a benign environment. These conditions may lead to rapid evolution of laboratory strains characterized by fast growth and shorter life span (Promislow & Tatar, 1998; Sgró & Partrige, 2000; Miller *et al*., 2002; Swindell & Bouzat, 2006). Indeed, when effects of DR were investigated in mice directly stemming from wild progenitors, no extension of median longevity was observed (Harper *et al*., 2006).

Fish show indefinite growth, a phenotype not observed in any other model organisms used in aging research, and have the capability to restore growth after a period of food restriction or adverse environmental conditions (Comfort, 1963; Tsai *et al*., 2007). Studies of DR in fish were so far neglected. Early studies in laboratory guppies (*Poecilia reticulata*) reported that food deprivation early in life can increase longevity (Comfort, 1963). Later studies, which analysed four different lines of wild-derived guppies, however, could not detect a significant effect of food level on longevity (Reznick *et al*., 2004).

Here, we investigated the effects of DR on longevity and age-related phenotypes in the short-lived annual fish *Nothobranchius furzeri*. Annual fish of the genus *Nothobranchius*

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Table 1 Life-history characteristics of the strains used in the study

Population	Year of isolation	Habitat type	Age at sexual maturity	Mean/maximum life span	Size at 5 weeks (± 95% CI)	Size at 11 weeks (± 95% CI)	Size at 21 weeks (± 95% CI)
GRZ	1969	400 m s.l.	4 weeks	Mean: 9 weeks	♂: 0.68 ± 0.18 g (n = 14)	♂: 2.07 ± 0.36 g (n = 18)	♂: –
		Semi-arid		Maximum: 13 weeks	♀: 0.47 ± 0.08 g (n = 12)	♀: 1.04 ± 0.24 g (n = 24)	♀: –
MZM-04/10	2004	120 m s.l.	4 weeks	Mean: 20 weeks	♂: 0.94 ± 0.15 g (n = 8)	♂: 1.8 ± 0.29 g (n = 26)	♂: 3.42 ± 0.45 g (n = 21)
		Semi-arid		Maximum: 26 weeks	♀: 0.59 ± 0.06 g (n = 8)	♀: 0.74 ± 0.11 g (n = 29)	♀: 1.57 ± 0.2 g (n = 25)
MZM-04/03	2004	50 m s.l.	4 weeks	Mean: 24 weeks	♂: 1 ± 0.39 g (n = 13)	♂: 2.38 ± 0.7 g (n = 10)	♂: 2.44 ± 0.9 g (n = 10)
		Semi-humid		Maximum: 32 weeks	♀: 0.72 ± 0.3 g (n = 15)	♀: 1.02 ± 0.04 g (n = 8)	♀: 1.34 ± 0.19 g (n = 18)

Relevant characteristics of *N. furzeri* strains. Habitat type reports the elevation on the sea level (s.l.) and the climatic conditions. Semi-arid indicates an evaporo-precipitation ratio (E/P) of 4.5 to 3.5, semi-humid (E/P) of 3.5 to 2.5. Age at sexual maturity is defined as the age when females start to lay eggs and males start courtship behaviour. Mean and maximum life spans indicated for each strain are referring to experiments performed in Pisa and reported in Terzibasi *et al.* (2008). Body sizes recorded for 5-, 11- and 21-week-old animals are reported as weights (in grams) ± 95% confidence intervals (CI).

are a group of teleosts found in ephemeral bodies of water which form during the monsoon season in eastern and southern Africa. All surviving adults die when their habitat dries out and their maximum natural life span is limited to few or several months, making them among the shortest-lived vertebrates (Genade *et al*., 2005). The laboratory strain of *N. furzeri* was isolated in 1969 from the Gona Re Zhou (GRZ) National Park in Zimbabwe, a semi-arid area with scarce and erratic precipitation (Jubb, 1971). This strain is therefore highly inbred (as confirmed by measuring homozygosity on polymorphic loci; Reichwald *et al*., 2009) and adapted to breed in a benign environment.

We recorded a median life span for the GRZ strain of 9 and 11.5 weeks in two different laboratories, and this short life span is coupled to fast growth and accelerated expression of age-related markers (Valdesalici & Cellerino, 2003; Genade *et al*., 2005; Terzibasi *et al*., 2008). Life span of *N. furzeri* GRZ can be prolonged by reducing water temperature (Valenzano *et al*., 2006a) and by addition of the natural compound resveratrol to the food (Valenzano *et al*., 2006b). More recently, we established wild-derived lines of *N. furzeri* originating from individuals collected 150 km and 300 km downstream of the original habitat of the GRZ strain. These populations show a median life span of 20–24 weeks and a retarded expression of age-related markers when compared to the GRZ strain (Terzibasi *et al*., 2008). Intrigued by the different responses induced by DR in inbred and wild-derived mice, we set to test the effects of DR in the inbred laboratory strain and in a wild-derived line of *N. furzeri*.

We measured longevity, age-dependent cognitive decay, accumulation of lipofuscin (an auto-fluorescent pigment which accumulates in age-dependent manner in many species) in the liver and neurodegeneration in the brain. In addition, we analysed glial acidic fibrillary protein (GFAP) expression in the brain as a marker of reactive gliosis.

Results

Life-history of inbred and wild-derived strains of *N. furzeri*

In this paper, three strains of *N. furzeri* were studied. The laboratory strain GRZ was isolated in 1969 from a semi-arid, high-land (400 m elevation) habitat and is highly homozygous, as determined by typing of polymorphic markers (Reichwald *et al*. submitted). The MZM-04/10P strain was collected in 2004 in a semi-harid habitat located 150 km downstream of the GRZ collection point (120 m elevation). The MZM-04/03 strains was collected in an habitat which was close to semi-humid located at sea level on a different fluvial system about 300 km from the GRZ collection point. Relevant life-history traits of the tree strains are reported in Table 1. The MZM-04/10P and MZM-04/03 strains have similar life span and are both much longer-lived than the GRZ strain (Terzibasi *et al*., 2008). All three strains reach sexual maturity at 4 weeks of age and the large differences in life span are not reflected into large differences

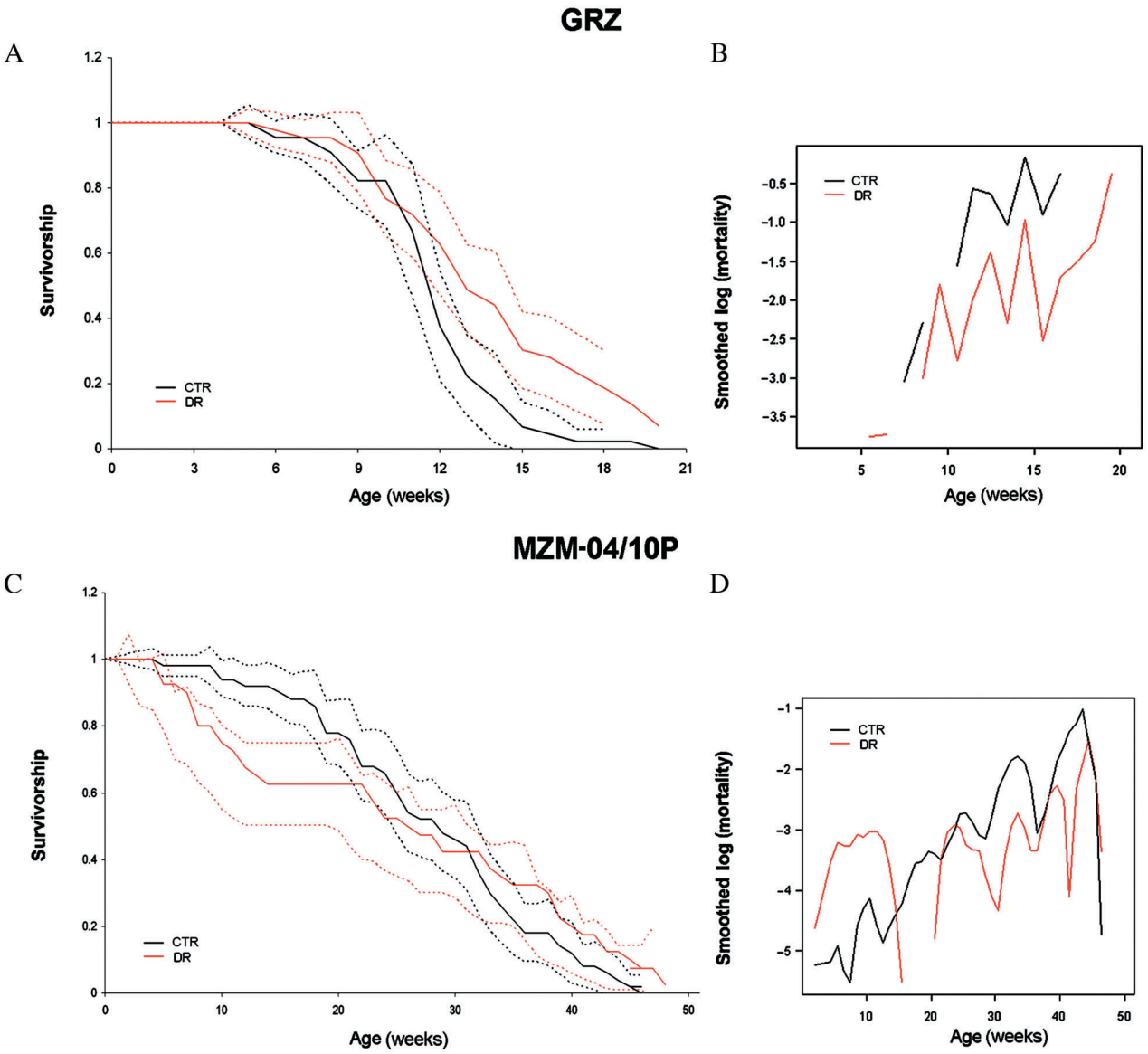

Fig. 1 Survivorship curves. (A) Survivorship of Gona Re Zhou (GRZ) inbred strain. Red solid line represents dietary restricted (DR) and black solid line controls. Dotted lines correspond to 95% confidence intervals as obtained by Cox analysis of proportional hazards. Results here reported are the average of three different experiments. Results of single experiments are shown in Fig. S1 (Supporting Information). (B) Mortality plot of GRZ inbred strain. Red solid line represents DR and black solid line controls. (C) Survivorship of MZM-04/10P wild-derived strain. Red solid line represents DR and black solid line controls. Dotted lines correspond to 95% confidence intervals as obtained by Cox analysis of proportional hazards. Results here reported are the average of two different experiments. Results of single experiments are shown in Fig. S1. (D) Mortality plot of MZM-04/10P wild-derived strain. Red solid line represents DR and black solid line controls.

for maturation. More details of the aging phenotypes for these strains can be found in Terzibasi *et al*. (2008).

Effects of DR on life span and demographic parameters

In the inbred laboratory strain GRZ, DR starting from week 4 induced significant reduction of age-related risk with increase of median life span from 11.5 to 13 week (Log-rank test: $\chi^2 = 7.98$, d.f. = 1, $p = 0.005$) and 10% survivorship increased from 15 to 20 weeks ($\chi^2 = 4.21$, d.f. = 1, $p = 0.04$). Figure 1A reports the survivorships of the two groups with confidence intervals, as obtained by Cox analysis of proportional hazards. In MZM-04/10P, DR starting from week 4 induced a wave of early mortality between week 5 and week 15 which eliminated 37% of the experimental animals (Fig. 1C). This effect was observed in two tanks independently (Fig. S1, Supporting Information). In this strain, we also recorded age-dependent increase in size for control and DR animals. DR fish of both sexes remained smaller than controls throughout their life span (Fig. S2, Supporting Information). General age-related risk was not decreased by DR in MZM-04/10P (Log-rank test: $\chi^2 = 0.798$, d.f. = 1, $p = 0.41$). Median life span in the DR group was reduced from 29 to 27 weeks ($\chi^2 = 2.41$, NS) and 10% survivorship was extended from 41 to 46 weeks ($\chi^2 = 5.41$, d.f. = 1, $p = 0.02$). Figure 1C reports the survivorships of the two

Table 2 Maximum likelihood estimates of demographic parameters

Group	Baseline mortality, *a*	*a*, 95% CI	Rate parameter, *b*	*b*, 95% CI
GRZ DR	0.00457	(0.00173–0.0121)	0.252	(0.195–0.327)
GRZ Control	0.00375	(0.00148–0.00947)	0.338	(0.272–0.419)
MZM-04/10P DR	0.0174	(0.00713–0.0303)	0.0434	(0.0221–0.0852)
MZM-04/10P Control	0.00336	(0.00140–0.00811)	0.107	(0.0804–0.143)

Survivorships curves of the four groups were modelled on the Gompertz equation ($\ln\mu_x = a + bx$, where x is time and μ_x is mortality) using WinModest. The square brackets indicate 95% confidence intervals (CI).

groups with confidence intervals, as obtained by Cox analysis of proportional hazards. Mortality during the early weeks of treatment in the DR groups is arguably not related to aging. In order to measure the impact of DR on aging-related mortality, we analysed the longevity of animals that survived past week 15. On this subset of survivors (n = 25), DR caused significant reduction of age-related risk (Log-rank, χ^2 = 7.88, d.f. = 1, p = 0.005).

We used WinModest software to obtain maximum likelihood estimates (MLEs) of the demographic parameters for DR and control groups (Table 2). In GRZ, DR induced a 25% decrease in the demographic rate of aging (the *b* parameter of the model), which failed to reach statistical significance (likelihood ratio test: likelihood of a Gompertz model with *b* free to vary = –235.10; likelihood of a Gompertz model with *b* identical in both groups = –236.59, d.f. = 1, p = 0.08). In MZM-04/10P, DR caused a significant 60% decrease in the demographic rate of aging (likelihood ratio test: likelihood of a Gompertz model with *b* free to vary = –320.44; likelihood of a Gompertz model with *b* identical in both groups = –324.97, d.f. = 1, p = 0.003) and a significant 410% *increase* in baseline mortality (the *a* parameter of the model) (likelihood ratio test: likelihood of a Gompertz model with *a* free to vary = –320.44; likelihood of a Gompertz model with *a* identical in both groups = –323.75, d.f. = 1, p = 0.01).

Quantification of age-dependent fecundity was beyond the scope of this paper. We can report the qualitative observation that DR animals of both strains did not show apparent impairments in reproductive activity and produced eggs which developed into vital embryos. We can conclude that this protocol of food restriction does not abrogate reproductive activity, although we cannot exclude a quantitative reduction of reproductive output.

DR prevents behavioural age-dependent decay

Age-dependent impairment of learning and memory is a hallmark of aging in complex animals and is observed in model organisms including *Drosophila* (Horiuchi & Saitoe, 2005) and zebrafish (Yu *et al*., 2006). A form of age-dependent learning decline was described in *N. furzeri* using a conditioning protocol (active avoidance) in a modified version of the well-established shuttle box test (Valenzano *et al*., 2006b). This age-related behavioural phenotype is observed in the inbred GRZ strain and in the wild-derived MZM-04/10P strain (Terzibasi *et al*., 2008). We quantified learning performances in a subset of the animals (n = 10, 5 males and 5 females) used for analysis of age-dependent mortality. DR and control animals from GRZ and MZM-04/10P were analysed at age 9 weeks. Instantaneous frequencies of avoids are reported in Fig. 2(A,C). The temporal series were fitted with a Ln curve [GRZ (DR: $y = 0.118\text{Ln}(x) + 0.036$, $R^2 = 0.39$, $p < 0.0001$; CTR: $y = 0.071\text{Ln}(x) - 0.074$, $R^2 = 0.29$, $p < 0.0001$); MZM-04/10P (DR: $y = 0.130\text{Ln}(x) - 0.137$, $R^2 = 0.55$, $p < 0.0001$; CTR: $y = 0.047\text{Ln}(x) - 0.059$, $R^2 = 0.19$, $p < 0.005$)]. The slopes of the two curves were compared following Zar (1984) and significant differences were found for MZM-04/10P (F = 14.38; $p < 0.001$). For GRZ, the slopes only showed a non-significant trend (F = 3.06; p = 0.08). The two elevations were compared according to Zar (1984) and significant differences were found (F = 108.9; $p < 0.001$). These results indicate that DR accelerates acquisition of the task in both strains. A second statistical test was performed using peak frequency of avoids as variable. DR improved this parameter in both groups (Student's *t*-test, $p < 0.01$ for both strains; Fig. 2B,D).

We also tested effects of DR on exploratory activity in the GRZ inbred strain using open-field exploration (Valenzano *et al*., 2006b). Exploratory activity showed a significant decrease through time in DR ($y = -0.129 + 2.43$, $R^2 = 0.35$, $p < 0.0001$), but not in CTR ($y = -0.013 + 1.59$, $R^2 = 0.004$, $p > 0.5$). The slopes of the two equations were compared following Zar (1984) and showed significant differences (F = 6.54; $p < 0.02$) (Fig. S3, Supporting Information). This datum demonstrates that improved performance in the shuttle box test was not due to increased exploratory activity.

DR reduces age-dependent neurodegeneration

N. furzeri shows age-dependent expression of the neurodegeneration marker Fluoro-Jade B (Valenzano *et al*., 2006b). Expression of Fluoro-Jade B is accelerated in the short-lived inbred strain GRZ as compared with the longer-lived wild-derived lines (Terzibasi *et al*., 2008). We used Fluoro-Jade B to investigate the effects of DR on age-dependent neurodegeneration in GRZ and in the MZM-04/03 wild-derived line. The MZM-04/03 line was chosen because life span is similar to that of the MZM-04/10P strain we used for the survivorship assay (Table 1) and expression of histological age markers was investigated in detail previously (Terzibasi *et al*., 2008). In MZM-04/03, levels of Fluoro-Jade B staining at 21 weeks of age are comparable to those in GRZ at

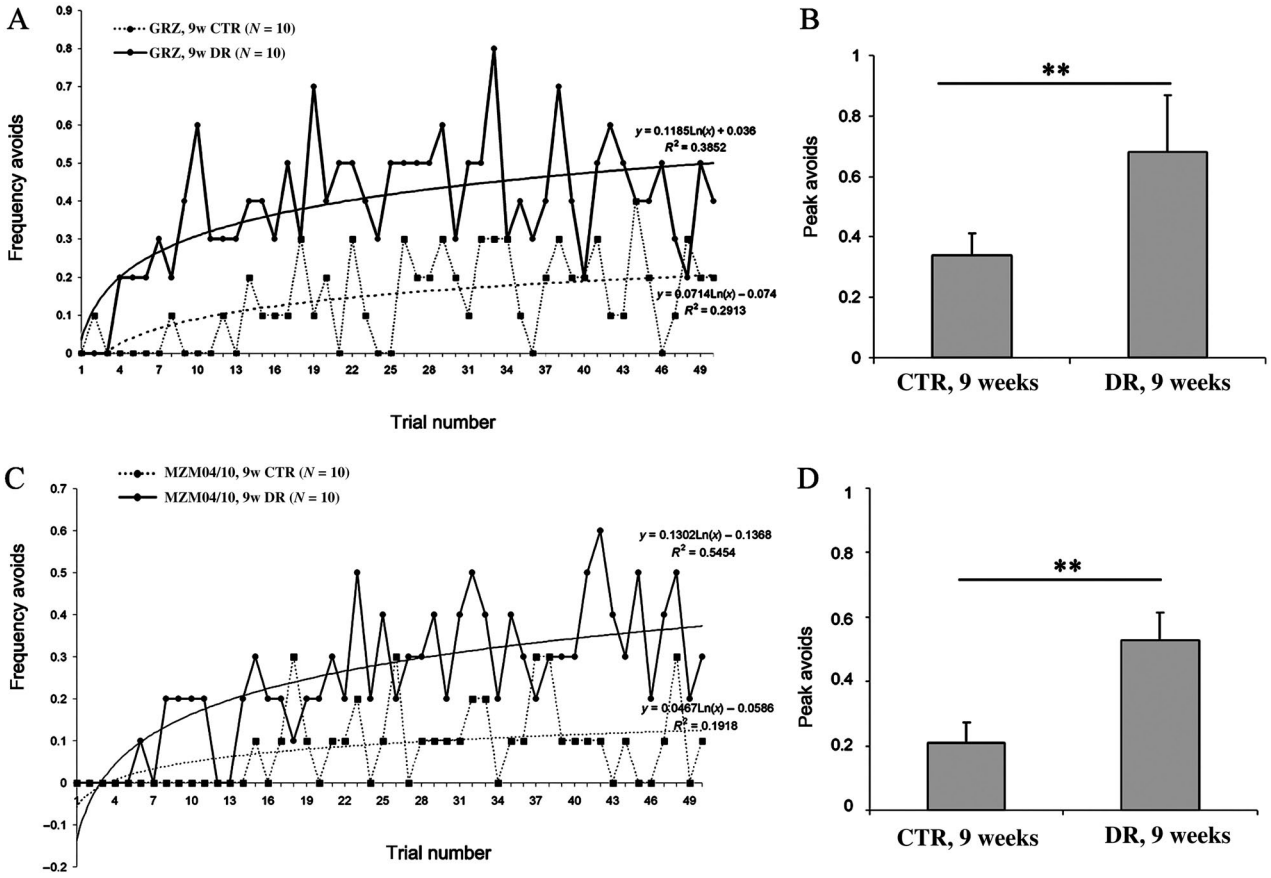

Fig. 2 Active avoidance test. (A) Gona Re Zhou (GRZ) inbred strain, learning curves of controls (dotted) and dietary restricted (DR; solid line) groups; the instantaneous frequency of avoids (average of ten animals per group) is reported as function of the trial number. (B) Peak frequency of avoids for the GRZ inbred strain. (C) MZM-04/10P wild-derived strain, learning curves of controls (dotted) and DR (solid line) group; the instantaneous frequency of avoids (average of 10 animals per group) is reported as function of the trial number. (D) Peak frequency of avoids for the MZM-04/10P wild-derived strain. Error bars are standard errors of means. $^{**}p < 0.01$, Student's *t*-test.

11 weeks (Terzibasi *et al.*, 2008), we therefore used 21 weeks as temporal endpoint of analysis for this longer-living line. Intensity of fluorescent labelling was quantified by confocal microscopy in both strains (GRZ, 5 controls, 5 DR; MZM-04/03, 10 controls, 5 DR). DR reduced age-dependent neurodegeneration: Telencephalon (percent area over threshold, Student's *t*-test: GRZ, NS; MZM-04/03 $p < 0.001$), Optic Tectum (percent area over threshold, Student's *t*-test: GRZ, $p < 0.01$; MZM-04/03 $p < 0.01$), Hindbrain (percent area over threshold, Student's *t*-test: GRZ, $p < 0.05$; MZM-04/03 $p < 0.01$) (Fig. 3).

DR induces up-regulation of glial fibrillary acidic protein

In mammals, neuronal degeneration during aging is coupled to gliosis and up-regulation of GFAP, an effect which is mitigated by DR (Bronson *et al.*, 1993; Nichols *et al.*, 1995; Morgan *et al.*, 1997; Sharma & Kaur, 2007). We analysed GFAP immunoreactivity using a monoclonal antibody raised against human GFAP. The sections analysed were derived by the same animals used for Fluoro-Jade B staining. In GRZ, DR from week 4 to week 11 induced up-regulation of GFAP immunoreactivity (5 controls, 5 DR). Only low levels of labelling were detected in control animals, whereas DR-animals presented clearly-visible glial processes positive for GFAP immunoreactivity (Fig. 4). Up-regulation of fluorescent labelling was quantified by confocal microscopy and was significant in all three brain regions analysed (percent area over threshold, Student's *t*-test: Optic Tectum, $p < 0.05$; Telencephalon, $p < 0.05$; Hindbrain, $p < 0.01$). Up-regulation of GFAP immunoreactivity in GRZ under DR regimen was confirmed by use of a second monoclonal antibody raised against porcine GFAP (Fig. S4, Supporting Information). In MZM-04/03 (5 controls, 5 DR), DR from week 4 to week 21 induced up-regulation of GFAP, although less extensively than in GRZ. This effects was significant in the Telencephalon (percent area over threshold, Student's *t*-test $p < 0.05$), close to significance in the Hindbrain ($p = 0.09$) and non-significant in the Optic Tectum.

DR reduces lipofuscin accumulation in the liver

Lipofuscin is an auto-fluorescent pigment that accumulates over time in a large variety of organisms (Brunk *et al.*, 1992). We previously reported age-dependent expression of lipofuscin in the liver of *N. furzeri* (Genade *et al.*, 2005; Valenzano *et al.*,

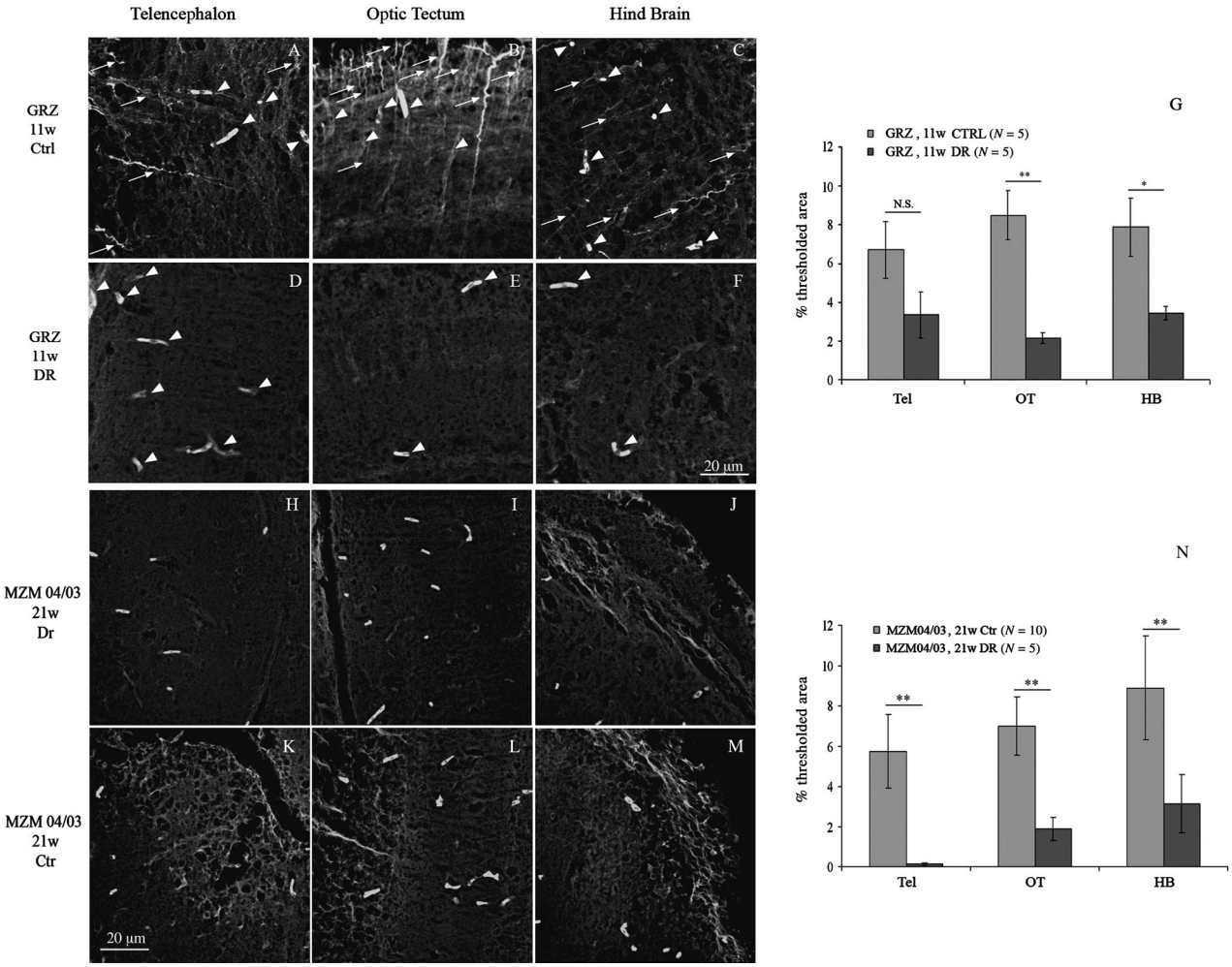

Fig. 3 Fluoro-Jade B staining in the brain. Confocal images taken at an excitation wavelength of 488 nm. Images are projections of seven confocal planes at a distance of 1 µm. White arrows denote labelled neuronal processes and arrowheads point to autofluorescent erythrocytes, which were excluded from the analysis. Gona Re Zhou (GRZ) inbred line, control case: (A) Telencephalon, (B) Optic tectum, (C) Hindbrain; GRZ inbred line, dietary restricted (DR) case: (D) Telencephalon, (E) Optic tectum, (F); MZM-04/03 wild-derived line, control case: (H) Telencephalon, (I) Optic tectum, (J) Hindbrain; MZM-04/03 wild-derived line DR case: (K) Telencephalon, (L) Optic tectum, (M) Hindbrain. (G,N) Quantification of fluorescence intensity. Light grey bars correspond to controls and dark grey bars to DR. The metric used for statistics is percentage of area over threshold. Error bars are standard errors of means. NS, not significant. $*p < 0.05$, $**p < 0.01$, Student's *t*-test.

2006a). Lipofuscin accumulation is accelerated in the GRZ strain as compared to the MZM-04/03 line (Terzibasi *et al.*, 2008). We analysed the effects of DR on lipofuscin expression in both GRZ and MZM-04/03. The short-lived GRZ strain was analysed at 11 weeks of age after 7 weeks of DR (5 controls, 5 DR) and the longer-lived strain at 21 weeks after 17 weeks of DR (10 controls, 5 DR). Lipofuscin was quantified by confocal microscopy and age-dependent accumulation was significantly reduced by DR in both strains (percent area over threshold, Student's *t*-test $p < 0.01$; Fig. 5).

Discussion

Effects of DR on age-related mortality

We investigated effects of DR in *N. furzeri* and compared the responses of a highly inbred captive strain and a line recently derived from wild individuals.

In the inbred GRZ strain, DR induced a significant decrease of age-related risk as measured by log-rank statistics and an increase of maximum life span. This result is in line with the accepted notion that DR consistently induces life extension in laboratory strains of model organisms.

In the wild-derived line MZM-04/10P, DR induced an early wave of mortality in the first 10 weeks of treatment and a significant increase in baseline mortality. However, DR also significantly decreased demographic rate of aging. As a result of the opposing effects of increased baseline mortality and decreased demographic aging rate, the general age-dependent risk is not reduced, but maximum longevity is extended. It is intriguing that these same effects (enhanced mortality at early ages, reduced demographic rate of aging and extension of maximal longevity) were previously described for wild-derived mice under DR (Harper *et al.*, 2006). Three possible mechanisms can be proposed to explain these opposing actions of DR:

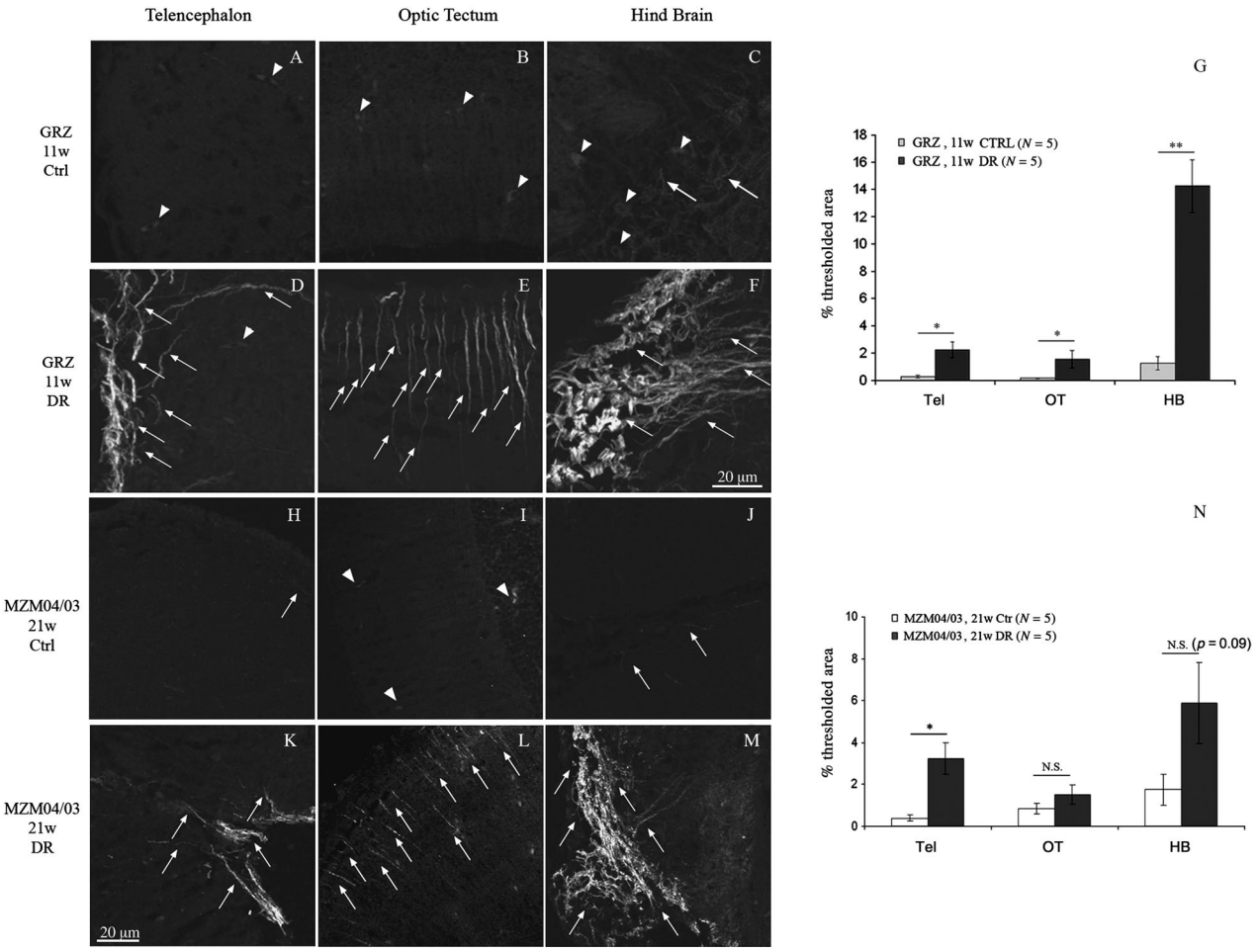

Fig. 4 Glial fibrillary acidic protein (GFAP) immunostaining in the brain. Confocal images taken at an excitation wavelength of 488 nm. Images are projections of seven confocal planes at a distance of 1 µm. White arrows denote labelled glial processes and arrowheads point to autofluorescent erythrocytes, which were excluded from the analysis. Gona Re Zhou (GRZ) inbred line, control case: (A) Telencephalon, (B) Optic tectum, (C) Hindbrain; GRZ inbred line, DR case: (D) Telencephalon, (E) Optic tectum, (F); MZM-4/03 wild-derived line, control case: (H) Telencephalon, (I) Optic tectum, (J) Hindbrain; MZM-4/03 wild-derived line DR case: (K) Telencephalon, (L) Optic tectum, (M) Hindbrain. (G,N) Quantification of fluorescence intensity. Light grey bars correspond to controls and dark grey bars to DR. The metric used for statistics is percentage of area over threshold. Error bars are standard errors of means. ns = not significant. $*p < 0.05$, $**p < 0.01$, Student's *t*-test.

1. Genetic variation in natural populations results in individual variation in the response to DR: the same regime of DR is detrimental to some individuals and beneficial to others and this variation is heritable (see also Discussion in Harper *et al*., 2006). It was described indeed that DR effects in inbred laboratory mice depend on the genetic background (Turturro *et al*., 1999; Forster *et al*., 2003). The heritability of DR response can be estimated experimentally by comparing the effects of DR on the F_1 progeny of several different pairs of wild fish. We plan a new collection trip to obtain wild *N. furzeri* in order to perform this experiment.
2. DR might have acted as a selective force which eliminated early in life for individuals with weaker phenotypes and selected for individuals more resistant to stress and ultimately to age-induced damage.
3. DR might act as a stressor. Some individuals will die as result of the stress, but survivors will develop resistance to age-induced damage due to up-regulation of stress-defence mechanisms.

The beneficial effects of a low-dose stressor (or toxin) are well-described and named hormesis (Henschler, 2006). It was proposed by several authors that the effects of DR (and other life-extending treatments) are due to hormesis (Neafsey *et al*., 1988; Boxenbaum, 1991; Masoro, 1998; Hayflick, 2001; Rattan, 2001; Lamming *et al*., 2004; Mattson, 2007). This is supported by the observation that DR increases corticosterone levels (Han *et al*., 2001; Ottinger *et al*., 2005; Harper *et al*., 2006) and induces heat shock proteins (Heydari *et al*., 1993, 1996; Aly *et al*., 1994; Ehrenfried *et al*., 1996; Selsby *et al*., 2005).

Effects of DR on age-related markers

Aging in *N. furzeri* is associated with expression of several age-related markers, both at the level of behaviour and histology (Terzibasi *et al*., 2007). Here, we quantified learning using an active-avoidance protocol and we further investigated age-dependent changes in the liver and brain by histology.

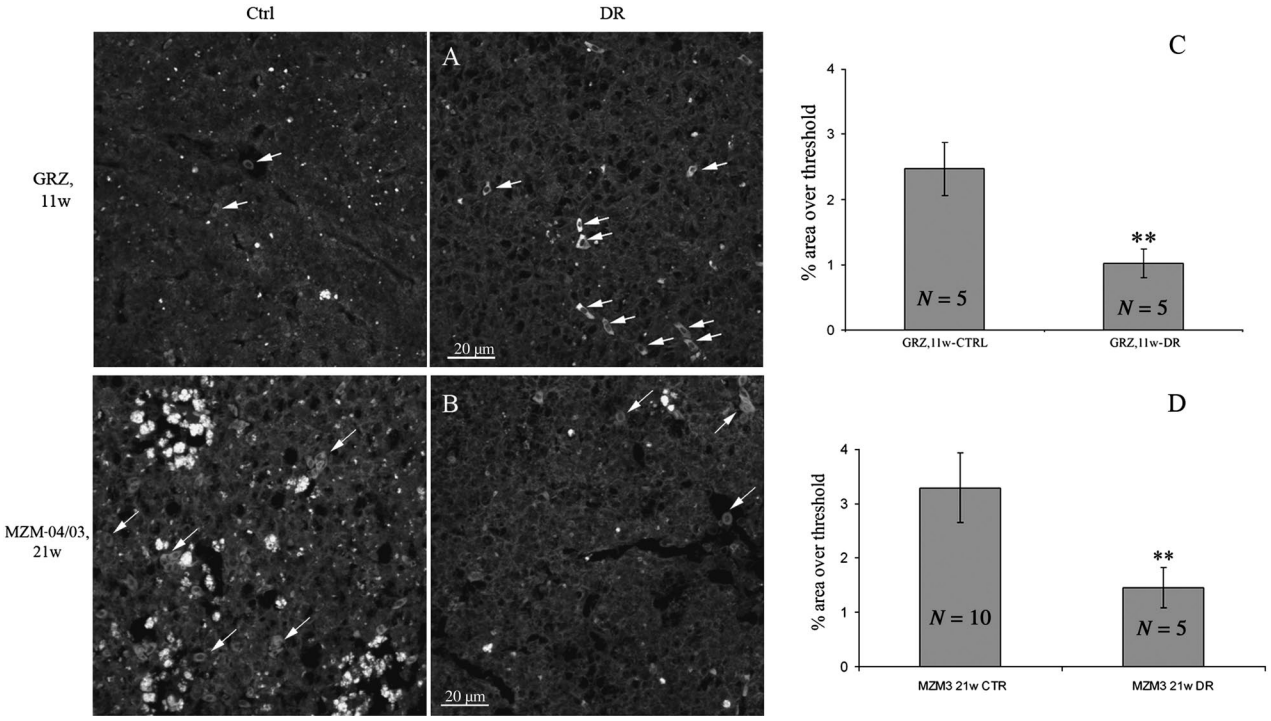

Fig. 5 Lipofuscin auto-fluorescence in the liver. Confocal images taken at an excitation wavelength of 488 nm. Images are projections of seven confocal planes at a distance of 1 μm. White arrows denote autofluorescent erythrocytes, which were excluded from the analysis. (A) Gona Re Zhou (GRZ) inbred line, the left image depicts a representative control case and right image a dietary restricted (DR) case. (B) MZM 04/03 wild-derived line, the left image depicts a representative control case and right image a DR case. (C,D) Quantification of fluorescence intensity. Light grey bars correspond to controls and dark grey bars to DR. The metric used for statistics is percentage of area over threshold. Error bars are standard errors of means. NS, not significant. $*p < 0.05$, $**p < 0.01$, Student's *t*-test.

Despite differences between the inbred laboratory strain and a wild-derived strain in the impact of DR on age-related risk, these markers were improved by DR in both strains. We can conclude that DR retards accumulation of age-dependent damage in brain and liver. It is interesting to notice a parallel with wild-derived mice, where DR reduces tumour incidence even if median life span is not prolonged (Harper *et al*., 2006). Therefore, DR seems to exert similar effects in wild-derived *N. furzeri* and wild-derived mice, both at the level of demography and age-related pathology.

Paradoxical effects of DR on glial cells

DR induced up-regulation of GFAP in glial cells. GFAP is a glia-specific intermediate filament protein which is up-regulated in the mammalian brain in response to neuronal injury and is commonly used as a marker of reactive gliosis (Norton *et al*., 1992). The reaction of glial cells to neuronal injury is much less studied in fish. However, it was reported that optic nerve injury induces GFAP in the goldfish optic nerve (Levine, 1991, 1993). Age-dependent up-regulation of GFAP is well described in the rodent and human brain (Nichols *et al*., 1993; Szenborn, 1993; David *et al*., 1997; Cruz-Sanchez *et al*., 1998) and it is mitigated by DR (Bronson *et al*., 1993; Nichols *et al*., 1995; Morgan *et al*., 1997; Sharma & Kaur, 2007). It is therefore surprising that DR *increased* GFAP immunoreactivity in *N. furzeri*, despite mitigation of age-dependent neurodegeneration. This datum introduces the interesting notion that DR induces a biological response in glial cells. The role of GFAP in the reaction of teleost brain to injury is still not well understood, the functional significance of this response remains unclear.

Conclusions

This is the first systematic study of DR effects on age-related phenotypes in a teleost species. In both inbred- and wild-derived lines of *N. furzeri*, DR prevented the expression of the age markers lipofuscin in the liver and Fluoro-Jade B (neurodegeneration) in the brain. DR also improved performance in a learning test based on conditioning (active avoidance in a shuttle box). Therefore, several beneficial effects of DR observed in rodent models are replicated in this short-lived fish. *N. furzeri* can represent an alternative model system to investigate the mechanisms of DR.

Experimental procedures

Survival assays

Eggs were maintained on wet peat moss at room temperature in sealed Petri dishes. When embryos had developed, eggs were hatched by flushing the peat with tap water at 16–18 °C. Embryos were scooped with a cut plastic pipette and transferred to a clean vessel. Fry were fed with newly hatched *Artemia nauplii* for the first 2 weeks and then weaned with finely chopped *Chironomus*

larvae. Starting at the fourth week of life, fish were moved to 40-l tanks at a maximum density of 20 fish per tank equipped with air-driven sponge filters. The aquarium room's temperature was set at a constant 26 °C. Twice a week the bottom of the tanks was siphoned and 50% of the water was exchanged with tempered tap water.

To record survivorship, the number of surviving fish was counted once a week starting at the fourth week of life. To compute differences among treatments, we used commercially available GraphPad program.

Demographic analysis was performed using WinModest software (http://www.hcoa.org/scott/softw-winmodest.asp), which estimates demographic parameters using maximum likelihood analysis instead of linear regression of log-transformed mortality. Only mortality occurring after age 5 weeks was used for estimating the parameters.

The protocols of fish maintenance were approved by the local authority in the State of Thuringia (Veterinär- und Lebensmittelüberwachungsamt).

Dietary restriction

DR in mice is generally induced by reducing food availability to 60% of the *ad libitum* feeding rate. Providing *ad libitum* feeding in fish is technically complicated, because food rapidly deteriorates in water. For this reason, it is not feasible to perform 60% DR as it is routine in mice. We opted for a protocol of every other day feeding which in mice reproduces many effects of DR (Goodrick *et al*., 1982, 1990). Over all the experimental time, control fish followed a standard diet regime, that means they were fed normally twice a day with *Chironomus* larvae, while DR fish were fed twice a day every other day. Food delivery to each tank was adjusted to a quantity that the fish consumed in a 30-min period leaving no uneaten food. This procedure was identical between control and DR fish. Therefore, food delivery was dependent on the fish appetite, not directly on the number of fish in the tank. It should also be noted that *N. furzeri* shows apparent age-dependent reduction of food intake and we diminished the dose of food progressively, so that no uneaten food was remaining on the tank bottom at any time.

Histology and histochemistry

Fish were euthanized with MS-222 and cooled on crushed ice for 5 min before dissection. Target tissues were dissected and fixed by immersion in 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.4). Then they were included in paraffin and cut at 10 μm on a microtome.

For lipofuscin detection, unstained sections were deparaffinized and mounted using a water-based mounting medium. Lipofuscin is autofluorescent and emits when excited at 488 nm without the need for any staining. This auto fluorescence can be quantified directly using a confocal microscope.

For Fluoro-Jade B histochemistry, sections were deparaffinized and staining was performed following the manufacturer's instruction (Chemicon, now part of Millipore, Schwalbach, Germany). Fluoro-Jade B staining product fluoresces when excited at 488 nm and staining can be quantified with a confocal microscope like lipofuscin.

Immunohistochemistry

Before starting immunolabelling, slides were subject to antigen unmasking using 'target-retrieval Solution' (Dako, Hamburg, Germany). After deparaffinization, slides were placed in a vessel filled with the retrieval solution and heated in a microwave oven until it boiled for 5 min. This unmasking cycle was repeated twice. Omission of this step resulted in lack of labelling.

Two different monoclonal anti-GFAP antibodies were used: anti-GFAP anti-human mouse monoclonal, clone 6F2 (from Dako, diluted 1 : 100), and anti-GFAP anti-pig mouse monoclonal, clone GA5 (from Sigma-Aldrich, diluted 1 : 100). Both antibodies were raised against natural full-length GFAP purified from brain. Sections were incubated with normal serum blocking solution (BSA 5%, Triton 0.3% dissolved in PBS) for 2 h to block non-specific binding of immunoglobulin. Sections were then incubated with primary antibody at appropriate dilution in primary antibody dilution buffer (BSA 1%, Triton 0.1% in PBS) overnight at 4 °C. The following day, sections were rinsed with PBS (3×5 min) and incubated at room temperature for 1 to 2 h with secondary antibody Alexa Fluor 488 (Invitrogen, diluted 1 : 400) in the same dilution buffer used for the primary antibodies. Finally, section were rinsed again with PBS (3×5 min) and mounted with a water-based fluorescence-specific mounting medium.

Quantification of fluorescence

Fluorescence intensity was quantified following a semiquantitative method: first, images were acquired using a Zeiss LSM confocal microscope at an excitation wavelength of 488 nm, with fixed imaging parameters (magnification, pinhole, photomultiplier, laser intensity, etc.). After acquisition, autofluorescent erithrocytes were manually erased using Adobe Photoshop. Erythrocytes could be unambiguously identified based on two characteristics: (i) their typical nucleated morphology and (ii) their location within blood vessels.

Curated images were then analysed using Metamorph® (Molecular Devices, Sunnyvale, CA, USA) to quantify percentage of area over threshold. During thresholding, all pixels below a defined greyscale value are transformed into 0 and all pixels above it are transformed into 1. The percentage of pixels with value 1 in the image is the metric used for the statistic. The threshold level is defined once by the experimenter so to exclude all background labelling and is then kept constant for all images to be compared. Measurements were performed by an operator blind to the experimental history of the analysed fish.

Exploratory activity assays

To measure exploratory activity, single fish were moved to a 20-l test-tank containing water at the same temperature as the home

tank and their swimming activity was recorded with a video camera placed above the centre of the tank. The water level was kept at 5 cm to minimize displacement on the *z*-axis, which would not be picked up by the camera while recording from above. Recordings were always made in the late morning (10:00–12:00 h). Fish were allowed to habituate for 5 min within the tank before the 5-min recording was started. Videoclips were analysed at a speed of 10 frames per second using an automated tracking system (Lolitrack, Loligo, Tjele, Denmark). This system tracks the geometric centre of the fish outline. The successive positions of the geometric centre of each fish in the successive frames were used to obtain their speed, which was then averaged over the total period of observation. Speed in cm s^{-1} was normalized by the length of each individual fish to transform it into length/second.

Active avoidance task

Active avoidance was measured using a modified version of the shuttle box (Valenzano *et al*., 2006b). A tank (38 cm × 23 cm × 18 cm) was divided in two by a hurdle with a rectangular hole (3 cm × 3 cm). The two compartments were wedged-shaped to funnel a fish through the hurdle. The tank was filled with water from the housing tank and the fish was left to acclimate for 15 min before starting the test. Then the conditioned stimulus (red light) was delivered in the compartment where the fish was present, followed by an adverse stimulus (swirling of a stick in the tank). To avoid operator bias in the test, the operator was blind as to the treatment of the fish. The fish always responded to the disturbance by moving to the other compartment. The aim of the test was to detect the acquisition of a strategy to escape from the adverse stimulus by crossing the hurdle upon presentation of the conditioned stimulus. The conditioned stimulus lasted for 30 s. If the fish did not move to the other compartment after 15 s, the adverse stimulus was delivered for 15 s. The fish moved to the other compartment, rested for 30 s, and then the cycle was repeated. If the fish crossed the hurdle within 15 s (i.e. before the onset of the adverse stimulus), the trial was scored as 'success', otherwise it was scored as 'failure'. A complete session consisted of 50 consecutive trials. Two indexes were scored to assess learning in each experimental group: the first measure was the *instantaneous frequency of avoids*, or average score (AS). This is the fraction of fish which produced an avoid response at each trials. Successful trials were scored as 1 and failures as 0. For each trial, AS was computed by averaging the response of all fish in the group. For example, for trial 1 in *n* fish, AS is:

$$AS_1 = \frac{1}{n}\sum_{i=1}^{n} p(i).$$

where $p(i)$ is 0 or 1. Instantaneous frequency of avoids is a measure of the evolution of learning as a function of the trial number in a group.

The second measure is the *peak frequency of avoids*. This variable is computed starting from the *performance index* (PI), which is the frequency of avoids averaged over a 10-bins sliding window. So each individual will described by a vector of dimension 40: $<PI> = (PI_{1–10}, PI_{2–11}, \ldots, PI_{41–50})$. PI is used to visualize the evolution of the performance as a function of the trial separately for each subject. The *peak frequency of avoids* is the maximum within <PI>. Compared to *instantaneous frequency of avoids*, the *peak frequency of avoids* provides an absolute measure of ability to succeed in a task, independent of the trial.

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Author contributions: E.T. designed the study, performed the experiments, wrote the paper and analysed the data, C.L. and P.D. analysed the locomotion, N.H and M.G. provided data concerning weight of the fish, A.C. designed the study, analysed the data, wrote the paper and supervised the study.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Survivorship of single trials. DR indicates the dietary restricted groups and CTR the control-fed groups. (A) Inbred line GRZ: each line represents a single experiment. DR1 (one tank, $n = 16$) and CTR1 (one tank, $n = 10$) were a 'cohort' hatched on the same day. DR2 (one tank, $n = 18$) and CTR2 (two tanks, $n = 35$) were a 'cohort' hatched on the same day. DR3 (one tank, $n = 15$) and CTR3 (two tanks, $n = 15$) were a 'cohort' hatched on the same day. (B) Wild-derived line MZM-04/10P. Each line represent a single experiment. DR1 (one tank, $n = 20$) and CTR1 (one tank, $n = 18$) were a 'cohort' hatched on the same day. DR2 (one tank, $n = 20$) and CTR2 (two tanks, $n = 32$) were a 'cohort' hatched on the same day.

Fig. S2 (A) Growth curves of MZM-04/10P, control and DR. Size (as volume, in cm^3) was estimated from digital pictures using the formula $V = 4/3\pi Rr^2$ were R and r are the major and minor axis respectively. Only animals of the DR1 and CTRL1 series (see Fig. S1) were analysed. The left graphs reports size of females and the right graph the size of males. Error bars are standard errors of means. (B) The histogram shows the mean values of weight (in grams) of GRZ males and females: 5-week-old controls ($n = 12$ females, $n = 14$ males; left columns) 11-week-old controls ($n = 24$ females, $n = 18$ males; centred columns) and 11-weeks-old DR ($n = 5$ females, $n = 8$ males; right columns). Error bars are standard errors of means. $***p < 0.001$, Student's t-test.

Fig. S3 Open-field test of exploratory activity. Fish were video recorded for 5 minutes. Average speed is reported as Lenghts/s. Each experimental group is composed of 10 fish. Small open circles represent single individuals and large filled circles the mean of the respective groups. Red indicates controls are blue dietary restricted. Exploratory activity showed a significant decrease through time in DR ($y = –0.129 + 2.43$, $R^2 = 0.35$, $p < 0.0001$), but not in CTR ($y = –0.013 + 1.59$, $R^2 = 0.004$, $p > 0.5$). The slopes of the two equations were compared following Zar (1984) andshowed significant differences ($F = 6.54$; $p < 0.02$).

Fig. S4 GFAP immunohistochemistry using anti-pig GFAP mouse monoclonal clone GA5 (Sigma). GRZ inbred strain control and DR subjects (11 weeks old). Note up-regulation of immunoreactivity in all three brain regions of the DR subject. Contrary to the clone 6F2 (Dako), this antibody also labels cell bodies of glial cells. The images are representative of five control and five DR cases.

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