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Mitochondrial DNA copy number and function decrease with age in the short-lived fish *Nothobranchius furzeri*

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Summary

Among vertebrates that can be kept in captivity, the annual fish *Nothobranchius furzeri* possesses the shortest known lifespan. It also shows typical signs of aging and is therefore an ideal model to assess the role of different physiological and environmental parameters on aging and lifespan determination. Here, we used *Nothobranchius furzeri* to study whether aging is associated with mitochondrial DNA (mtDNA) alterations and changes in mitochondrial function. We sequenced the complete mitochondrial genome of *N. furzeri* and found an extended control region. Large-scale mtDNA deletions have been frequently described to accumulate in other organisms with age, but there was no evidence for the presence of detectable age-related mtDNA deletions in *N. furzeri*. However, mtDNA copy number significantly decreased with age in skeletal muscle, brain, liver, skin and dorsal fin. Consistent with this finding, expression of *Pgc-1α* that encodes a transcriptional coactivator of mitochondrial biogenesis and expression of *Tfam* and *mtSsbp* both encoding mtDNA binding factors was downregulated with age. The investigation of possible changes in mitochondrial function revealed that the content of respiratory chain complexes III and IV was reduced in skeletal muscle with age. In addition, ADP-stimulated and succinate-dependent respiration was decreased in mitochondria of old fish. These findings suggest that despite the short lifespan, aging in *N. furzeri* is associated with a decline in mtDNA copy number, the downregulation of mtDNA-associated genes and an impairment of mitochondrial function.

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Introduction

Mitochondria are the primary energy suppliers of most eukaryotic cells harbouring the oxidative phosphorylation system (OXPHOS) that generates the energy currency ATP. Mitochondria have been suggested for decades to be implicated in aging (Harman, 1972), and it has been observed in a variety of organisms and tissues that mitochondrial function declines with age (Navarro & Boveris, 2004; Marcinek *et al.*, 2005; Hebert *et al.*, 2010). In humans, age-related mitochondrial dysfunction has been most often described in skeletal muscle tissue (Boffoli *et al.*, 1994; Short *et al.*, 2005).

Mitochondria are unique organelles in that they contain their own DNA. A typical vertebrate mitochondrial genome has a size of ~16 kb and harbours a set of 37 genes encoding two ribosomal RNAs, 22 transfer RNAs and 13 proteins that are essential for mitochondrial respiration and ATP production. It also contains two main noncoding regions, the control region or displacement loop and the light-strand replication origin, which are involved in replication and transcription of the mitochondrial DNA (mtDNA) (Clayton, 1992). The gene number and order are usually highly conserved among vertebrates, although a number of mtDNA rearrangements have been described in birds, reptiles, amphibians and fishes (Boore, 1999).

The occurrence of damage to mtDNA and clonal expansion of somatic mutations with aging has been reported in several organisms (Corral-Debrinski *et al.*, 1992; Melov *et al.*, 1995; Yui *et al.*, 2003). For example, there are several hundred types of mtDNA deletions described in humans (Samuels *et al.*, 2004), and a 4977-bp deletion (referred to as the 'common deletion') has been frequently observed to accumulate with age in heart, skeletal muscle and various regions of the brain (Meissner *et al.*, 2008). Interestingly, a mouse model that harbours a proofreading-deficient copy of mtDNA polymerase (the so-called mutator mouse) accumulates a high number of mitochondrial point mutations and deletions (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005). This leads to a reduced lifespan and a premature onset of aging-related phenotypes, although it has to be noted that the described mutation frequency in those mice is much higher than during normal aging (Vermulst *et al.*, 2007; Ameur *et al.*, 2011).

The turquoise killifish *Nothobranchius furzeri* has an exceptionally short lifespan of 3–8 months. This is the shortest reported maximum lifespan of a vertebrate that can be bred in captivity (Valdesalici & Cellerino, 2003). *N. furzeri* is therefore emerging as an alternative model organism for age research (Terzibasi *et al.*, 2007). The natural habitat of *N. furzeri is* seasonal ponds in south-east Africa, which are filled with water during the rainy season, and *N. furzeri* survives the dry season in form of protected eggs. Time and amount of rainfall can greatly vary from year to year, which makes the occurrence of this habitat erratic (Terzibasi *et al.*, 2008). One of the predictions from the classical aging theory suggests that low levels of extrinsic hazards should lead to the evolution of slow aging, whereas high extrinsic mortality should result in fast aging (Austad & Kirkwood, 2008). Consistent with this theory, previous studies have shown that the short lifespan of *N. furzeri* is associated with fast aging

such as an early onset of aging biomarkers (e.g., lipofuscin accumulation and β-galactosidase activity), a decline in learning/behavioural capabilities and age-related telomere shortening (Terzibasi *et al.*, 2007, 2008; Hartmann *et al.*, 2009). In this study, we addressed the questions of whether different aspects of mitochondria biology are affected during aging of *N. furzeri*. We therefore performed a comprehensive study analysing the content of mtDNA, mitochondria-related gene transcripts as well as the amount and function of mitochondrial proteins upon aging.

Results

The mitochondrial genome of *Nothobranchius furzeri* harbours an extended control region

We sequenced the mitochondrial genome of the *N. furzeri* laboratory strain GRZ (acc. no.: NC_011814) and determined a size of 19 527 bp (Table 1). This is considerably longer than most other teleost fish mitogenomes (Boore, 1999). While gene order and structure of the *N. furzeri* mitogenome are typical for a vertebrate, the noncoding control region is remarkably extended. The control region includes the $tRNA^{Pro}$ gene (position 17 681–17 749), which in other species is found adjacent to the control region, an additional copy of the $tRNA^{Glu}$ gene (pos. 18 933–19 000) and two almost identical sequence blocks of 964 and 961 bp (99% identity) that are separated by 1075 bp (Fig. 1A). Other species of the genus *Nothobranchius* (*N. rachovii* and *N. palmquisti*) also contain an extended control region, while West African killifishes (*Aphyosemion australe* and *Epiplatys chaperi*), the nonannual genera most closely related to *Nothobranchius* and the more distantly related American killifishes (*Kryptolebias* (formerly *Rivulus*) *marmoratus* and *Jordanella floridae*) as well as the Japanese medaka (*Oryzias latipes*), lack the extended control region (Fig. 1B).

We also performed Southern blotting analysis using digested DNA of *N. furzeri* and medaka (see Fig. 1A for restriction sites) and a species-specific *Cytb* probe. The size of the resulting fragments of 2.6 kb for medaka and 6.7 kb for *N. furzeri* confirmed the PCR amplification and sequencing data (Fig. 1C). Comparison of *N. furzeri's* mitogenome (without control region) to other fish species revealed sequence identities of 68.3–69.8% (Table 1) with zebrafish being most distantly related (68.3%). As expected, the level of identity between the mtDNA of human and *N. furzeri* is even lower (60.7%) than among fish.

It has recently been postulated that the frequency with which cysteine is encoded by mtDNA is a specific and phylogenetically ubiquitous molecular indicator of longevity: long-lived species synthesize respiratory chain complexes depleted of cysteines (Moosmann & Behl, 2008). We calculated the cysteine content of the mitochondrial proteome of *N. furzeri,* and as expected for a short-lived species, we found a higher cysteine content (0.79%) than in other model fish species (0.66–0.76%, Table 1).

No evidence for mtDNA deletions in *N. furzeri*

Mammalian species accumulate mtDNA deletions in various tissues with aging. To search for the presence of mtDNA deletions in *N. furzeri*, we amplified different parts of the mtDNA using long-range PCR (see Fig. 1A for primer positions). As template, we used DNA from muscle, skin and brain of six old fish (31 weeks old). Using different primer combinations, we found no shorter products in any of the tissue samples (PCR results with muscle DNA are shown in Fig. 1D). Another approach to search for large-scale mtDNA deletions is to use real-time PCR with different mitochondrial primers. When a primer pair binds in a mtDNA region that is more frequently deleted, this would be detectable by real-time PCR. We tested several primers and chose three mitochondrial primer pairs that bind within the D-loop, the *16S rRNA* gene and the *ND4* gene (see Fig. 1A for primer positions). For normalization, we chose two primer pairs that amplify nuclear DNA, namely a part of the *Cdkn2a/b* gene locus and of the *Insr* gene locus. As template, we used muscle DNA samples of aged fish. We observed some variation between individual fish; however, there was no significant difference in mtDNA content when using different mitochondrial and nuclear primers (Fig. 1E). Thus, with two different approaches, we could not detect any evidence for the occurrence of large-scale, age-associated mtDNA deletions in *N. furzeri*.

Mitochondrial DNA copy number decreases with age

Next, we determined and compared the relative mtDNA content in various tissues of young (5 weeks old) and old (31 weeks old) fish. For that, we used the above-described real-time PCR with primers for the mitochondrial gene *16S rRNA* and for the nuclear *Cdkn2a/b* locus. From nine examined tissue types, five tissues showed a significant decrease in relative mtDNA content (Fig. 2A). Liver tissue of young fish had a mean of 1026 mtDNA copies per cell, which represents the highest value of all examined tissues. Liver of old fish had a mean of 575 mtDNA copies per cell indicating an age-related reduction of about 44% (*t*-test, $P < 0.01$). Mean mtDNA copies also significantly decreased with age by 42% in skeletal muscle (from 891 to 520), by 51% in brain (from 631 to 307), by 32% in skin (from 429 to 291) and by 22% in fin tissue (from 256 to 199; *t*-test, $P < 0.05$). No significant age-related change in mtDNA content was observed in kidney, gill, eye and spleen (Fig. 2A).

Keeping fish in single tanks allowed us to monitor mtDNA content of the same individual during aging. For this, we measured mtDNA content

Table 1 Mitochondrial genome characteristics and maximum lifespan of *Nothobranchius furzeri*, four model fish species and human

Species	Size of mitochondrial genome (bp)	Size of mitochondrial control region (bp)*	Identity of mitochondrial DNA between *N. furzeri* and other species (%)†	Cysteine frequency in mitochondrially encoded proteins (%)‡	Lifespan (years)‡
Nothobranchius furzeri (killifish)	19 527[a]	3938	–	0.79	0.5
Oryzias latipes (medaka)	16 714[b]	1073	69.8	0.76	5
Danio rerio (zebrafish)	16 596[c]	950	68.3	0.76	5
Gasterosteus aculeatus (stickleback)	16 538[d]	885	69.6	0.66	8
Tetraodon nigroviridis (pufferfish)	16 462[e]	818	69.5	0.66	10
Human	16 569[f]	1122	60.7	0.58	100

Accession number – a: NC_011814, b: NC_004387, c: NC_002333, d: NC_003174 and AB054361, e: NC_007176, f: NC_012920.

*Control region of *N. furzeri* contains two tRNA genes.

†Without control region.

‡All values are taken from Moosmann & Behl (2008) except for *N. furzeri.*

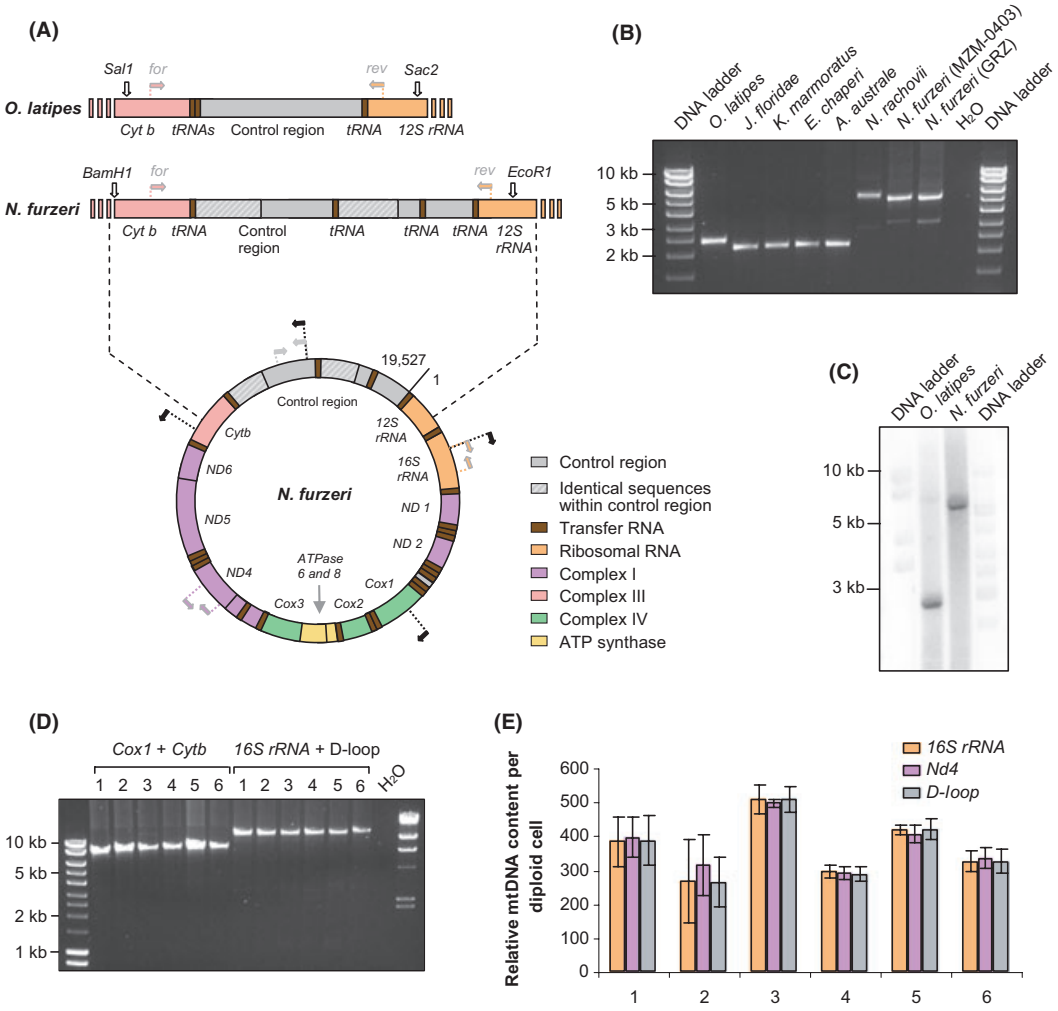

Fig. 1 Organization of the mitochondrial genome of *Nothobranchius furzeri*. (A) The control region is considerable extended as shown in comparison with the medaka fish (*Oryzias latipes*) and contains an additional *tRNA* gene and two almost identical sequence blocks of 964 and 961 bp (shaded areas). The positions of the restriction sites and degenerated primers used in *B* and *C* are shown in the upper part, and the real-time and long-range PCR primers used in *D* and *E* are shown in the lower part. (B) Amplification of the control region showed an extended control region in two *Nothobranchius* species, but no extension in medaka or other killifish species. The faint second band in the *N. furzeri* lane was sequenced and emerged as a PCR artefact. Medaka: *Oryzias latipes*, American killifishes: *Jordanella floridae*, *Kryptolebias* (formerly *Rivulus*) *marmoratus*, African killifishes: *Epiplatys chaperi*, *Aphyosemion australe*, *Nothobranchius rachovii* and *Nothobranchius furzeri*. (C) Southern blot analysis using species-specific probes and restriction enzymes that cut next to the control region revealed a fragment of 2624 bp in *O. latipes* and of 6685 bp in *N. furzeri* confirming the extended control region. (D) Using DNA of six aged fish for long-range PCR amplification revealed full-length products, but no shorter fragments that might have resulted from mitochondrial DNA (mtDNA) deletions. (E) Relative mtDNA copy number of muscle (n = 6, 31 weeks old) was determined using real-time PCR with primers from different mtDNA regions and with the nuclear primers of the *Cdkn2a/b* gene for normalization.

in the dorsal fin at the age of 5 weeks, 25 weeks and, when possible, at the age of 35 weeks. In the group of fish that died between the age of 25–35 weeks (n = 9), mtDNA copy number significantly decreased from a mean of 229 at the age of 5 weeks to a mean of 117 at the age of 25 weeks (Fig. 2B). Fish that lived longer than 35 weeks (n = 11) had a mean mtDNA copy number of 231 at the age of 5 weeks, 201 at the age of 25 weeks and 136 at the age of 35 weeks. Interestingly, in this group, the mtDNA copy number did not significantly decrease between 5 and 25 weeks but was significantly reduced at 35 weeks (Fig. 2C).

Expression of *Pgc-1α*, *Tfam* and *mtSsbp* declines with aging

To analyse the expression of genes associated with aging and mitochondrial biogenesis, we compared gene expression in young and old muscle and skin tissue using real-time PCR. Expression of the aging-associated genes insulin receptor (*Insr*), sirtuin 1 (*Sirt1*) and forkhead box O 3a (*Foxo3a*) was not significantly altered in muscle or skin between the age of 5 and 21 weeks (Fig. 3A + C). However, the PPAR-γ coactivator 1α (*Pgc-1α*) gene that is involved in the regulation of mitochondrial biogenesis was significantly downregulated in 21- vs. 5-week-old muscle and skin tissue (–2.0-fold for muscle and –2.6-fold for skin). To validate the results and to test known target genes of *Pgc-1α*, we compared 31- vs. 5-week-old tissue and found a significant decrease in *Pgc-1α* expression in 31-week-old muscle and skin tissue (–2.0-fold for muscle and –1.8-fold for skin; Fig. 3B + D). In addition, the expression of transcription factor A, mitochondrial (*Tfam*), a target of Pgc-1α, was significantly downregulated in 31-week-old muscle tissue (–1.4-fold), and mitochondrial single-stranded DNA-binding protein (*mtSsbp*) was significantly downregulated

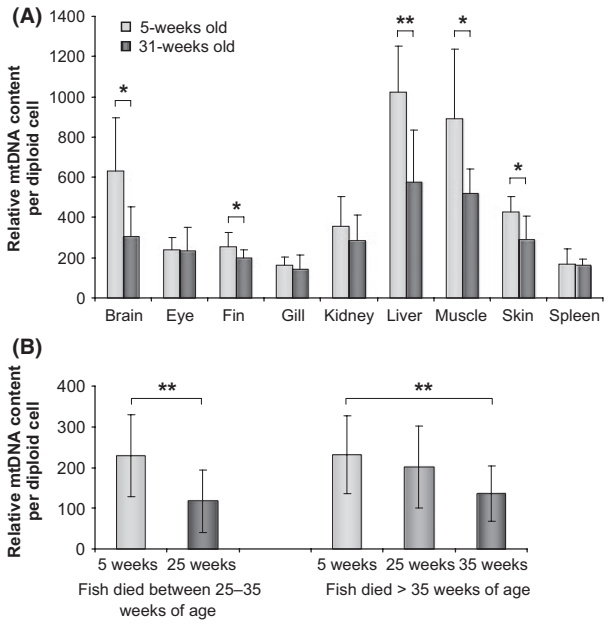

Fig. 2 Mitochondrial DNA (mtDNA) content declines with ageing in *Nothobranchius furzeri*. The relative mtDNA copy number was determined using quantitative real-time PCR with primers for the *16S rRNA* gene (mitochondrial) and the *Cdkn2a/b* gene locus (nuclear). (A) Mean mtDNA copy number significantly decreased with age in brain, liver, muscle, skin and fin tissue (six young fish and six old fish were used with the exception of muscle and skin where 10–12 young fish and six old fish were used; unpaired *t*-test, $*P < 0.05$, $**P < 0.01$; error bars indicate standard deviation). (B) MtDNA copy number also significantly decreased with age in the dorsal fin of individual fish. In the group of fish that died at the age of 25–35 weeks ($n = 9$), the reduction in mtDNA copy number between the age of 5 and 25 weeks was significant (paired *t*-test, $P = 0.004$). In the long-lived group ($n = 11$, lifespan longer than 35 weeks), the reduction was significant between the age of 5 and 35 weeks (paired *t*-test, $P = 0.006$), but not between 5 and 25 weeks (paired *t*-test, $P = 0.431$).

in 31-week-old skin samples (−1.6-fold). Both Tfam and mtSsbp bind to mtDNA and are considered to be involved in mtDNA replication (Anderson & Prolla, 2009).

Old muscle tissue displays decreased content of respiratory chain complexes

We employed blue native gel electrophoresis (BNE) in combination with in-gel activity and haem stains to quantify mitochondrial complexes of the OXPHOS in skeletal muscle of six young and six old fish using digitonin for solubilization (Fig. 4). We identified the complexes I, V, III and IV as well as the small supercomplex S that usually consists of complexes I and III (Schagger & Pfeiffer, 2000). Supercomplex S, complex I and complex III were quantified densitometrically directly in Coomassie-stained 1-D BNE gels (Fig. 4A + D). The relative protein contents of complex IV was assessed using a specific haem stain (Fig. 4C + F). The amount of supercomplex S and complex I was also quantified using an in-gel NADH:NTB reductase activity assay (Fig. 4B + E). In the lane of complex V, we detected extra proteins that could not be identified when analysing isolated mitochondria suggesting that we had solubilized an additional unknown complex with the same native mass that comigrated with the mitochondrial ATP synthase (complex V) in BNE (data not shown). To ensure selective quantification of ATP synthase from muscle homogenates, we used individual subunits α and β for densitometric analysis in 2-D gels (Fig. 4A lower panel and Fig. 4D). While the significance level was only reached for complexes III and IV, the amount of all OXPHOS complexes was decreased in old muscles.

Mitochondrial bioenergetics is impaired in old muscle

To further investigate whether respiratory chain activity changes with age, we measured oxygen consumption in isolated mitochondria from white muscle of 5- and 32-week-old animals. Muscle tissue of several young and old animals was pooled to obtain 500-mg tissue. One young and one old sample were always prepared and measured side by side. State 4 respiration was measured with malate and glutamate as substrates and in the absence of ADP. There was no significant change in state 4 respiration as a function of age (Fig. 5A), which is consistent with findings in other organisms (Ferguson *et al.*, 2005). State 3 respiration was monitored in the presence of excess substrate and ADP and is considered to be the active state of mitochondrial respiration. State 3 respiration was reduced by approximately 65% in old skeletal muscle of *N. furzeri*. As a consequence, the respiratory control ratio (state 3/state 4 oxygen consumption rate) that reflects mitochondrial coupling was also significantly higher in young muscle (Fig. 5B). Furthermore, complex II-dependent respiration with succinate as substrate was reduced by approximately 65% in old muscle (Fig. 5A) indicating that also the activity or content of the downstream respiratory chain complexes was reduced in mitochondria of old *N. furzeri*. This is in good agreement with the results obtained by blue native electrophoresis that revealed a reduced content of complexes III and IV. Overall, these results indicate that oxidative phosphorylation is compromised in skeletal muscle of old fish.

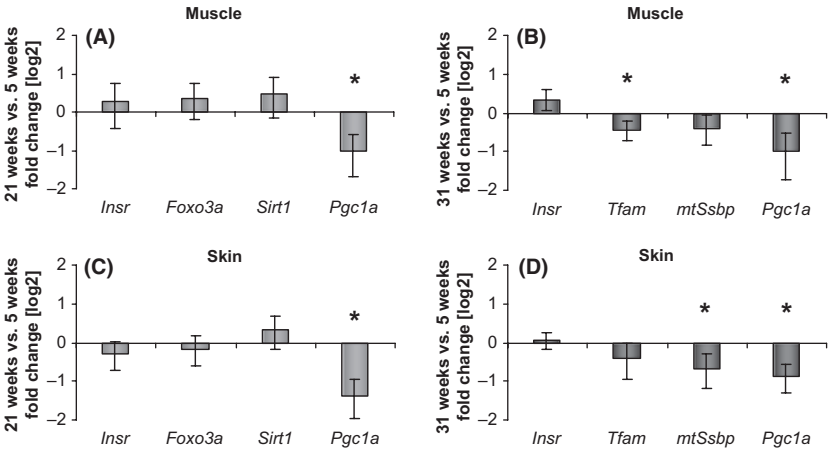

Fig. 3 Expression of genes associated with mitochondrial biogenesis is reduced with age in *Nothobranchius furzeri*. Expression of *Pgc-1α* was significantly downregulated in 21-week-old ($n = 9$) muscle and skin (A + C) and in 31-week-old ($n = 11$) muscle and skin (B + D) samples compared with 5-week-old samples ($n = 11$). The Pgc-1α target *Tfam* was also downregulated in old muscle (B), and *mtSsbp* was downregulated in old skin (D). Statistical significance was tested with the relative expression software tool (Pfaffl *et al.*, 2002; $*P < 0.05$; error bars indicate standard deviation).

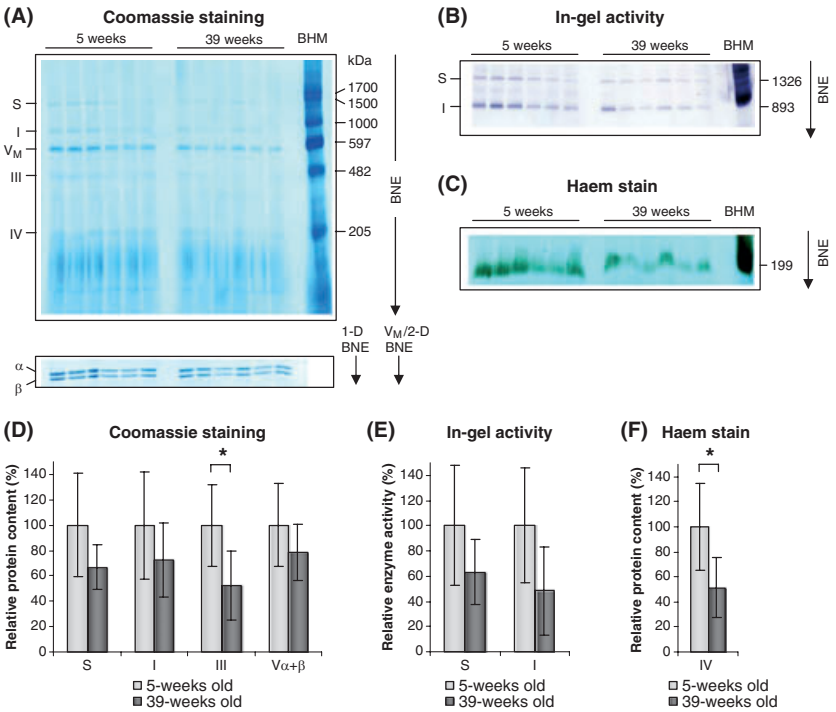

Fig. 4 Amount of oxidative phosphorylation system (OXPHOS) complexes is decreased with ageing in skeletal muscle. Mitochondrial OXPHOS complexes were solubilized with digitonin, separated by blue native electrophoresis (BNE) and analysed by densitometry. Complex intensities from six young and six old fish muscle specimens are expressed as per cent of young tissue obtained from a Coomassie-stained 1-D BNE gel for complexes S, I and III, and 2-D/V_MSDS gel for complex V (A + D), an in-gel NADH:NTB reductase activity assay for complexes S and I (B + E) and a complex IV-specific haem stain (C + F). Bovine heart mitochondria were used as molecular mass standard. Assignment of complexes: S, supercomplex composed of respiratory chain complexes I and III; I, complex I or NADH:ubiquinome oxidoreductase; V_M, monomeric complex V or ATP synthase; III, complex III or cytochrome *bc1* complex; IV, complex IV or cytochrome c oxidase. Asterisks mark significant differences (*t*-test, $*P < 0.05$); error bars indicate standard deviation.

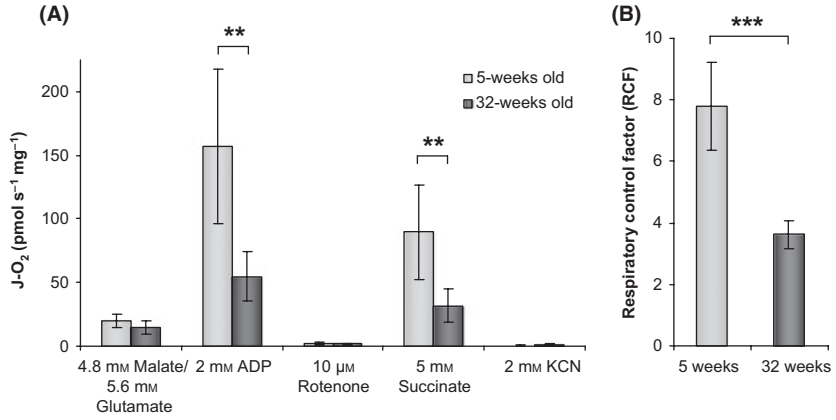

Fig. 5 Mitochondrial respiration and the respiratory control factor decline in old skeletal muscle. (A) Mitochondria were isolated from skeletal muscle tissue of young and old fish, and oxygen consumption was always determined from a young and an old sample in parallel ($n = 6$). State 4 respiration was measured using malate and glutamate as substrate, state 3 respiration was measured using ADP as substrate and after blocking complex I with rotenone, and complex II-dependent respiration was measured using succinate as substrate. (B) The respiratory control ratio was determined by the ratio of state 3 to state 4 respiration (*t*-test, $**P < 0.01$, $***P < 0.001$; error bars indicate standard deviation).

Discussion

Mitochondria are considered to play a major role during aging, but it is unclear whether this holds for vertebrates with an extremely short life-span like *N. furzeri*. The aim of the present study was to analyse whether age-related mtDNA alterations or changes in mitochondrial function occur in this fish species. As a prerequisite, we sequenced the complete mitochondrial genome of *N. furzeri* and detected an extended control region compared with other vertebrate mitogenomes (Boore, 1999). The extension was found in all examined tissues and animals of *N. furzeri* and in two additional *Nothobranchius* species. It remains to be determined whether the extended control region contributes to mtDNA stability and the fast aging phenotype of *Nothobranchius*.

A hallmark of aging is the occurrence of large-scale mtDNA deletions that have been described to accumulate with age in various organisms (Corral-Debrinski *et al.*, 1992; Melov *et al.*, 1995; Yui *et al.*, 2003; Meissner *et al.*, 2008). We searched for the occurrence of age-related mtDNA deletions in *N. furzeri* by long-range PCR covering the region around the *ND4* gene that is most commonly deleted in mammals (Samuels *et al.*, 2004), but we found no evidence for mtDNA deletions in muscle, skin and brain of old fish. In a second approach, we used real-time PCR with primers located in the *ND4* gene, in the D-loop that has been described to be deleted in rats with age (McInerny *et al.*, 2009) and in the 16S rRNA. However, there was no difference in the abundance of these mitochondrial regions indicating the absence of large-scale mtDNA deletions in *N. furzeri* with aging.

To our knowledge, this study is the first that analysed mtDNA content in different tissues of a fish species and demonstrated a significant decline of mtDNA content with age in several of those tissues. Most studies reporting on age-related changes in mtDNA abundance have investigated muscle, heart, liver or brain tissue of humans, rats or mice (Barazzoni *et al.*, 2000; Frahm *et al.*, 2005; Short *et al.*, 2005; McInerny *et al.*, 2009). There is considerable variation in those studies regarding the

influence of age on mtDNA copy number ranging from decrease to no change or increase. While some of the variations can be explained by species- and tissue-related differences, another reason for the discrepancies might be differences in the methods used for quantifying mtDNA abundance. In this study, a sensitive real-time PCR approach was used. Mean mtDNA copy number per diploid cell ranged from 140 in gill of old animals to 1026 in liver of young animals. This is somewhat lower than in mammals where mtDNA copy number is typically several thousand copies per cell (Trifunovic & Larsson, 2008), but it could be possible that warm-blooded mammals have a higher mtDNA abundance than fish.

High-energy demanding tissues such as liver, muscle and brain usually have a higher density of mitochondria. Given the 2–5 times higher mtDNA copy number in liver, muscle and brain compared with other tissues such as gill and spleen, there appears to be a strong link between mtDNA content and energy demand. In humans and mice, it has been reported that endurance training has a positive effect on mtDNA copy number in skeletal muscle (Lanza *et al.*, 2008; Safdar *et al.*, 2011). Furthermore, it is a common observation that old animals show a decline in behavioural activity. Therefore, we cannot distinguish whether the decrease in mtDNA content in muscle tissue is caused by a reduction in behavioural activity or by aging *per se*. However, the age-related decrease in mtDNA content in other tissues such as liver, brain and skin suggests that this is not the result of reduced physical activity. Interestingly, the age-related decline is most prominent in the tissues with higher mtDNA content. In future, it will be interesting to test whether the level or the rate of decrease in mtDNA content can be correlated with the lifespan of individual fish.

An interesting question is how the number of mtDNA molecules is controlled and regulated. A key player controlling mitochondrial biogenesis is the peroxisome proliferator–activated receptor γ coactivator α (Pgc-1α), which induces the mitochondrial transcription factor A (Tfam) (Wu *et al.*, 1999). It has been shown that the level of Pgc-1α is decreased during aging (Anderson & Prolla, 2009) and that the level of Pgc-1α and Tfam is directly proportional to the amount of mtDNA (Wu *et al.*, 1999; Ekstrand *et al.*, 2004). In line with these findings, we show here that the expression of *Pgc-1α* is decreased with age in skin and muscle and that also the expression of *Tfam* and *mtSsbp* is decreased in these tissues. This is consistent with the observed decline in mtDNA copy number with age suggesting Pgc-1α also to play an important role in mtDNA abundance in *N. furzeri*.

We further asked whether and how the fast aging of *N. furzeri* is associated with mitochondrial function. We found that the content of electron transport chain complexes III and IV is decreased in skeletal muscle with age. We identified and quantified the complexes in Coomassie-stained native gels and could show a reduced mitochondrial protein content upon aging. Other parameters of mitochondrial function include the measurement of the rate of oxygen consumption in isolated mitochondria (Hebert *et al.*, 2010). We found that complex I and complex II-dependent respiration in isolated mitochondria was decreased with age, suggesting age-related impairment of mitochondrial function. This is in line with other reports demonstrating an age-related decline in content or activity of respiratory chain complexes in mammalian skeletal muscle (Navarro & Boveris, 2007). Mitochondrial respiration and ATP synthesis have been also described to decrease with age in skeletal muscle of mammals (Drew *et al.*, 2003; Mansouri *et al.*, 2006).

One concept of the mitochondrial hypothesis of aging is the age-related decline in mitochondrial energetic competence to produce ATP. This hypothesis is supported by a number of reports as reviewed in (Navarro & Boveris, 2007). In the present study, we show a decline in the components of the respiratory chain and a reduction in state 3 respiration in old skeletal muscle of the very short-lived fish *N. furzeri*, which is consistent with the mitochondrial hypothesis of aging. A general observation is the accumulation of mtDNA mutations with aging, and many reports have linked mtDNA mutations and deletions with reduced mitochondrial respiration and ATP synthesis (Trifunovic & Larsson, 2008). Rather than mtDNA deletions, we have observed a significant age-related decrease in mtDNA abundance in muscle and other tissues of *N. furzeri*. One future aim is to establish transgenesis in *N. furzeri*. This will allow us to manipulate mitochondrial function, e.g., by overexpression of *Pgc-1α* and to study the consequences on lifespan.

Experimental procedures

Fish strains and maintenance

The *N. furzeri* laboratory strain GRZ was used to sequence the mitochondrial genome. The strain was originally collected in Zimbabwe in 1968, has an extremely short lifespan of 12–16 weeks and is highly inbred. Another *N. furzeri* strain (MZM-0403) has a maximum lifespan (10% survivors) of 29–31 weeks when kept in groups and a slightly longer lifespan when kept in single tanks. The MZM-0403 strain was collected during a field trip in Mozambique in 2004, is heterozygous in regard to numerous genetic markers (Reichwald *et al.*, 2009) and thus reflects a more wild-derived population. MZM-0403 was used to determine the influence of age on mtDNA copy number, gene expression and mitochondrial function. Fish were either kept in groups (12–14 fish per 40-litre tank) or individually (two separated fish per 5-L tank) at 26°C under a light regime of 12:12 hours light:dark. All fish were fed on red mosquito larvae (Chironomidae) *ad libitum* once a day. All animal experiments were performed according to the 'Principles of laboratory animal care' as well as to the current version of the German Law on the Protection of Animals.

Sequencing of the mitochondrial genome

Mitochondrial DNA was amplified in two overlapping PCR products using the Expand Long Template PCR System (Roche, Mannheim, Germany). Primers were designed on the basis of conserved regions of the *Cytb* and *ND2* genes from other fish species (see Table S1 in Supporting Information for primer sequences). The two PCR products were ~10 and ~11 kb in size and overlapped by ~1.5 kb. Shotgun libraries of PCR products were generated as described (Reichwald *et al.*, 2009), and about 70 clones were sequenced. Sequence gaps were closed by primer walking, genomic PCR and direct sequencing. Sequences were assembled, edited using the GAP4 module of the Staden Sequence Analysis Package (Staden, 1996) and deposited at GenBank (acc. no.: NC_011814). Pairwise global alignments were created by needle from the EMBOSS package (Rice *et al.*, 2000).

Measurement of mtDNA content

The relative mtDNA copy number was determined by real-time PCR with primers for the mitochondrial 16S rRNA gene and with primers for the nuclear *Cdkn2a/b* gene locus that was analysed in parallel. Total DNA was extracted from muscle and skin using the QIAamp Mini kit and from all other tissues using the QIAamp Micro kit (Qiagen, Hilden, Germany). PCR amplification was performed with the iCycler iQ5 detection system (Bio-Rad, Dreieich, Germany) in 25 μL volume containing 1 unit DreamTaq DNA polymerase (Fermentas, St. Leon-Rot, Germany), 200 nM of each primer (see Table S1 in Supporting Information for primer sequences), 10 nM fluorescein (Bio-Rad) for calibration, 0.75 μL Sybr Green I (5× stock solution) for detection and 20 ng DNA as template. The thermal cycling conditions included an initial denaturing step at 94°C for 1 min and then

40 cycles of 94°C for 20 s, 58°C for 30 s, 72°C for 20 s and 80°C for 20 s. After another denaturing step at 95°C for 30 s, the melting curve from 72 to 95°C (fluorescence reading every 0.5°C for 15 s) was recorded. All reactions were performed in triplicates, and negative controls (without template) were always included. Serial dilutions of at least standard DNA samples were also included in every run to determine PCR efficiency. All PCR products had an amplification efficiency of 95–105% and a size of around 150 bp. Cycle threshold (Ct) values of mitochondrial PCR products were normalized to Ct-values of a nuclear locus according to the following equation: Relative mtDNA copy number per diploid cell = $2 \times 2^{\Delta Ct}$, where ΔCt is $Ct_{Cdkn2a/b\ locus} - Ct_{mitochondrial\ locus}$.

Gene expression analysis

Comparison of gene expression between young and old tissue was measured using real-time PCR with the iCycler iQ5 detection system. RNA was isolated from muscle and skin samples using the RNeasy Mini kit (Qiagen). Synthesis of cDNA was performed in a 20 μL volume using 500 ng total RNA, 500 ng Oligo-dT primer and 200 U SuperScript II reverse transcriptase (Invitrogen, Darmstadt, Germany). PCR conditions were the same as described above (Measurement of mtDNA content), and the primer sequences are described in the Table S1 (Supporting Information). Ct-values of the analysed genes were normalized to the housekeeping gene *Tbp* (TATA box–binding protein), and the relative expression software tool was used (Pfaffl *et al.*, 2002).

Electrophoresis and quantitation of mitochondrial membrane complexes

1-D BNE (blue native electrophoresis) and 2-D SDS–PAGE were performed as described (Schagger, 2006; Wittig *et al.*, 2006). Conditions for solubilization were adapted to fish skeletal muscle specimens. Briefly, fish muscles were homogenized in sucrose buffer (250 mM sucrose, 20 mM sodium phosphate, 2 mM 6-aminohexanoic acid and 1 mM EDTA, pH 7.5) using a motor-driven, tightly fitting 2-mL glass/Teflon Potter-Elvehjem homogenizer. Aliquots corresponding to 20 mg of tissue (wet weight) were centrifuged for 10 min at 22 000 ***g*** to obtain sediments containing mitochondria, nuclei and larger cell fragments. Pellets from 20-mg skeletal muscle were solubilized in 40 μL solubilization buffer (50 mM NaCl, 50 mM imidazole/HCl, 2 mM 6-aminohexanoic acid and 1 mM EDTA, pH 7.0) and 15 μL 20% digitonin or 7.5 μL 20% dodecylmaltoside (DDM). Following 20-min centrifugation at 22 000 ***g***, the supernatant was supplemented with 7.5 or 4 μL of a 5% Coomassie blue G-250 suspension in 500 mM 6-aminohexanoic acid; 25 μL of each sample, corresponding to 10-mg tissue, was applied to the gel wells (0.15 × 0.5 cm) of a 4–13% acrylamide gradient gel with a 3.5% sample gel on top. Following BNE, native gels were used to quantify mitochondrial complexes by Coomassie stain or in-gel activity stains of 1-D BNE. For 2-D SDS–PAGE, native gel strips were placed on a glass plate and wetted with 1% SDS, and a Tricine–SDS gel was cast for the second dimension. 2-D SDS gels were stained with Coomassie or silver (Rais *et al.*, 2004). NADH:NTB reductase activity and staining for haem of complex IV were performed as described (Zerbetto *et al.*, 1997) with some modifications (Wittig *et al.* 2007). The Quantity One software (BioRad) was used for densitometric quantitation of in-gel activity, haem stain and Coomassie-stained protein complexes in 1-D or 2-D gels.

Preparation of *N. furzeri* mitochondria from muscle

Mitochondria were isolated from skeletal muscle of freshly killed fish. All steps were performed on ice using precooled buffers and equipment. Muscle tissue (0.5 g) of several young (5 weeks old) and old (32 weeks old) MZM-0403 fish was cut into small pieces and transferred into mitochondrial isolation buffer consisting of 250 mM sucrose, 140 mM KCl, 10 mM EDTA, 5 mM $MgCl_2$, 20 mM HEPES and 0.5% BSA (fatty acid free), pH 7.0. The tissue was treated with trypsin (~0.1 mg mL^{-1}) for 15 min and was homogenized twice with a Teflon homogenizer. After adding soybean trypsin inhibitor (~0.3 mg mL^{-1}), the extracts were filtered through a layer of cheesecloth and centrifuged at 600 ***g*** for 15 min at 4°C. The supernatant was centrifuged at 5700 ***g*** for 15 min at 4°C, and the mitochondrial pellet was resuspended in mitochondrial isolation buffer lacking BSA.

Analysis of mitochondrial respiration

Oxygen consumption of isolated mitochondria was determined at 25°C using an Oxygraph-2K high-resolution respiratory system (Oroboros, Innsbruck, Austria) equipped with two chambers and DatLab software. Mitochondria from young and old muscle tissue were isolated and measured in parallel, and each preparation and measurement was performed in six independent experiments. All measurements of mitochondrial respiration were normalized to protein content of mitochondria preparations (range of total protein amount of samples from young muscles: 1.6–2.3 mg and from old muscles: 1.6–2.8 mg). Measurements were performed in a buffer containing 200 mM sucrose, 10 mM potassium phosphate, 10 mM Tris-HCl, 10 mM $MgSO_4$ and 2 mM EDTA, pH 7.0. State 4 respiration was measured using malate (4.8 mM) and glutamate (5.6 mM) as substrates, and state 3 respiration was measured after the addition of ADP (2 mM). Complex I was inhibited by adding rotenone (10 μM). Subsequently, complex II-dependent respiration was measured after adding succinate (5 mM). Finally, KCN (2 mM) was added to inhibit complex IV activity.

Data analysis

Statistical analysis of gene expression data was performed with the relative expression software tool (Pfaffl *et al.*, 2002). This software tool uses a mathematical model that compares unknown and control samples. Significance is tested by a randomization test (pairwise fixed reallocation randomization test). The paired Student's *t*-test was used to compare mtDNA copy number of the same individual at young and old age. The unpaired Student's *t*-test was used to calculate *P*-values for all other data sets.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1 Primer list.

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