Late-Onset Temperature Reduction Can Retard the Aging Process in Aged Fish Via a Combined Action of an Anti-Oxidant System and the Insulin/Insulin-Like Growth Factor 1 Signaling Pathway

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Abstract

Two different mechanisms are considered to be related to aging. Cumulative molecular damage caused by reactive oxygen species (ROS), the by-products of oxidative phosphorylation, is one of these mechanisms (ROS concept). Deregulated nutrient sensing by the insulin/insulin-like growth factor 1 (IGF-1) signaling (IIS) pathway is the second mechanism (IIS concept). Temperature reduction (TR) is known to modulate aging and prolong life span in a variety of organisms, but the mechanisms remain poorly defined. Here we first demonstrate that late-onset TR from 26°C to 22°C extends mean life span and maximum life span by approximately 5.2 and 3 weeks, respectively, in the annual fish *Nothobranchius guentheri*. We then show that TR is able to decrease the accumulation of the histological aging markers senescence-associated β -galactosidase (SA- β -Gal) in the epithelium and lipofuscin (LF) in the liver and to reduce protein oxidation and lipid peroxidation levels in the muscle. We also show that TR can enhance the activities of catalase, glutathione peroxidase, and superoxide dismutase, and stimulate the synthesis of SirT1 and FOXO3A/FOXO1A, both of which are the downstream regulators of the IIS pathway. Taken together, our findings suggest that late-onset TR, a simple non-intrusion intervention, can retard the aging process in aged fish, resulting in their life span extension, via a synergistic action of an anti-oxidant system and the IIS pathway. This also suggests that combined assessment of the ROS and IIS concepts will contribute to providing a more comprehensive view of the anti-aging process.

Introduction

AGING IS CHARACTERIZED BY A time-dependent progressive loss of physiological integrity, leading to impaired function and increased vulnerability to death. Two of the most studied and widely accepted conjectures on possible aging mechanisms are the oxidative stress hypothesis and the insulin/insulin-like growth factor 1 (IGF-1) signaling (IIS) pathway. Oxidative stress, an imbalance in the production and detoxification of reactive oxygen species (ROS), the by-products of oxidative phosphorylation, causes damage to lipids, proteins, DNA, and mitochondria, thereby impairing cellular integrity and functionality.^{1,2} Thus, it is hypothesized that aging results from ROS accumulation and its associated cellular damage, and thus ingestion of ROS-scavenger compounds, such as resveratrol, vitamin E, and ethosuximide, may prolong an organism's life span.^{3,4}

The IIS pathway participates in nutrient sensing and informing cells of the presence of glucose. Current available evidence supports the notion that anabolic signaling accelerates aging and reduced nutrient signaling or attenuation of IIS downstream effectors, such as target of rapamycin (TOR) and FOXO (a forkhead transcription factor), extends longevity.^{5,6} Consistent with the relevance of anabolic sensing as a hallmark of aging, dietary restriction (DR), a reduction in caloric intake, increases life span or health span in all eukaryote species examined, including non-human primates.^{7,8} Paradoxically, decreased IIS is a common characteristic of normal aging, as well as in mouse models of premature aging,⁹ and extremely low levels of IIS are incompatible with life.¹⁰ Moreover, there are cases of progeroid mice with very low levels of IGF-1, in which supplementation of IGF-1 can ameliorate premature aging.¹¹ These apparently contradictory observations can be accommodated under a unifying model

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by which IIS down-regulation reflects a defensive response aimed at minimizing cell growth and metabolism in the context of systemic damage.¹² Accordingly, organisms with decreased IIS can survive longer because they have lower rates of cell growth and metabolism and, hence, lower rates of cellular damage, whereas physiologically or pathologically aged organisms decrease IIS in an attempt to extend their life span.

The phenotype of an organism, which includes characteristics such as life span, is determined in part by the nucleotide sequence of its genes and in part by the regulation of these genes, which is often influenced by the genome as well as the environment. Therefore, non-intrusion interventions, such as CR and temperature reduction (TR), are also identified as possible approaches for prolonging an organism's life span.¹³ For example, temperature variations have been shown to modulate aging and life-history traits in poikilotherms such as worms,¹⁴ flies,^{15,16} and fish.^{17,18} However, the underlying mechanisms remain largely unclear.

Small annual fishes, especially the genus *Nothobranchius*, have many anatomical and histological characteristics similar to those of mammalian species and a relatively short life span, are commercially available, and are easily reared in captivity. Thus, they have become an emerging model organism for aging studies in recent years.^{19–21} In the present study, we sought to explore if late-onset TR, a sustained reduction in ambient temperature, has any influence on aging kinetics and aging biomarkers in the annual fish *Nothobranchius guentheri*, and if so, to investigate its underlying mechanisms.

Materials and Methods

Fish, culture, and survival observation

The fish *N. guentheri* used in this study were bred in our own laboratory. All of the fish were reared (at a density of five fishes per 10-liter tank) under an ambient photoperiod at $26 \pm 1^\circ\text{C}$, and fed with live blood worms twice a day. Because the fish have median survival of approximately 12 months,²² 9-month-old male *N. guentheri* were used as model of aged fish in the following experiments.

A total of 100 9-month-old male fish were divided into two groups (50 each); one group remained at $26 \pm 1^\circ\text{C}$, and the other group was moved to $22 \pm 1^\circ\text{C}$. They were both reared under an ambient photoperiod and fed with live blood worms twice a day. The health of each fish was surveilled every day, and the survival of the fish was recorded until the death of all the fish. In parallel, 135 of the 9-month-old male fish were divided into three groups. One group of 45 was immediately anesthetized by cooling on ice and killed for the following experiments. The remaining two groups (45 each) were maintained at $26 \pm 1^\circ\text{C}$ and $22 \pm 1^\circ\text{C}$, respectively, for 1 month and then killed (at 10 months) as above for the following experiments.

Assays for histological markers

Previous experiments showed that the caudal fin and liver of *N. guentheri* were suitable tissues for detection of senescence-associated β -galactosidase (SA- β -Gal), a putative marker of cellular senescence, and lipofuscin (LF), the aging pigment,^{20,23,24} respectively. Therefore, the caudal fins and

livers were dissected from male fish, fixed in 10% formalin at room temperature for 12 hr, and washed in 50% ethanol for 3 min. After dehydration, these tissues were embedded in paraffin and sectioned at 6 mm.

For SA- β -Gal, de-paraffinized sections of the caudal fins were washed three times with phosphate-buffered saline (PBS), stained at 37°C (without CO_2) overnight by immersing in SA- β -Gal stain solution,²⁵ and observed under bright-field microscopy. For LF detection, unstained sections of the livers were viewed by fluorescence light microscopy.²⁶ Auto-fluorescence of LF was evaluated with blue (450–490 nm) excitation light and 520-nm emission filters, respectively.²⁷ Examination was performed under a fluorescence microscope (Olympus BX51). The areas of SA- β -Gal staining and LF fluorescence were determined using ImageJ software (<http://rsb.info.nih.gov/ij/>).

Assay for protein oxidation

The protein oxidation assay followed the method of Sohal et al.²⁸ A total of 1 gram of muscle, including red and white ones, from freshly killed fish was homogenized in 5 mL of 50 mM PBS (pH 7.5) containing protease inhibitors (leupeptin at 0.5 mg/mL, aprotinin at 0.5 mg/mL, pepstatin at 0.7 mg/mL, and phenylmethylsulfonyl fluoride [PMSF] at 40 mg/mL) by using a Polytron and sonicator. The homogenate was centrifuged at $5000 \times g$ for 10 min at 4°C . The protein concentration of the supernatant was determined with a BCA Protein Assay Kit (CWBIO, China). Aliquots of 300 μL of the resulting supernatant with 2–2.5 mg of protein were treated with 300 μL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2 M HCl or with 2 M HCl in the controls. The mixtures were incubated at room temperature for 1 hr (with agitation every 10 min), precipitated with 10% trichloroacetic acid (final concentration), and centrifuged at $12,000 \times g$ at 4°C for 15 min. The pellets were washed three times with 1 mL of ethanol/ethyl acetate (vol/vol 1:1) and re-dissolved in 1 mL of 6 M guanidine in 10 mM PBS/trifluoroacetic acid (pH 2.3). Any trace insoluble material was removed by centrifugation at $12,000 \times g$ for 15 min. The difference in absorbance between the DNPH- and HCl-treated materials was determined at 366 nm and expressed as nanomoles of carbonyl groups per milligram of protein, using the excitation coefficient of $22 \text{ mM}^{-1} \text{ cm}^{-1}$ for aliphatic hydrazones.

Assay for lipid oxidation

To estimate lipid peroxidation levels, a lipid peroxidation MDA Assay Kit (Beyotime, China) was used to quantify the generation of malondialdehyde (MDA). In brief, 1 gram of the fish body (FB) from pectoral fin to tail fin containing only bones and red and white muscles, and excluding head and internal organs was homogenized in 10 mL of 50 mM PBS (pH 7.5) using a Polytron and sonicator. The homogenate was centrifuged at $5000 \times g$ for 10 min at 4°C . The supernatant (100 μL) was mixed with 200 μL of thiobarbituric acid (TBA) working solution in a test tube, and the mixture was incubated in a boiling water bath for 15 min. After cooling in tap water, the mixture was centrifuged at $1000 \times g$ for 10 min, and its absorbance was measured at 532 nm. The concentration of MDA was expressed as micromoles per gram of protein.²⁹ The protein concentration of

the supernatant was determined with a BCA Protein Assay Kit (CWBIO, China).

Assay for catalase activity

Catalase (CAT) activity was estimated as described by Hsu et al.³⁰ A total of 1 gram of FB was homogenized in 10 mL of 50 mM PBS (pH 7) by using a Polytron and sonicator. The homogenate was centrifuged at $5000 \times g$ at 4°C for 10 min. The assay reaction consisted of 50 mM potassium phosphate buffer (pH 7), 100 μ L of 30% hydrogen peroxide (H_2O_2), and the resulting supernatant in a total volume of 1 mL. The reaction was carried out at 25°C. A blank control was prepared with 900 μ L of 50 mM potassium phosphate buffer and 100 μ L of 30% H_2O_2 . The rate of absorbance change ($\Delta A/\text{min}$) at 240 nm was recorded, which indicated the decomposition of H_2O_2 . CAT activities were calculated by using the molar extinction coefficient of H_2O_2 at 240 nm, 43.59 l/mol cm. Units of CAT were expressed as the amount of enzyme that decomposes 1 μ mol of H_2O_2 per min at 25°C. The specific activity was expressed in terms of micromoles per minute per milligram of protein.

Assay for glutathione peroxidase activity

Glutathione peroxidase (GPX) activity assay was measured by the method of Hsu et al.³⁰ Briefly, 1 gram of FB was homogenized in 10 mL of 50 mM Tris-HCl buffer (pH 7.4) containing protease inhibitors (0.5 μ g/mL leupeptin, 0.5 μ g/mL aprotinin, 0.7 μ g/mL pepstatin, 40 μ g/mL PMSF) by using a Polytron and sonicator. The homogenate was centrifuged at $5000 \times g$ at 4°C for 10 min. An aliquot of 10 μ L supernatant was mixed with 10 μ L of GPX working solution (5 mM nicotinamide adenine dinucleotide phosphate [NADPH], 42 mM reduced glutathione, and 20 U glutathione reductase), and then with 176 μ L GPX assay buffer (50 mM Tris-HCl containing 2 mM EDTA, pH 7.6). All of the solutions were pre-incubated at 25°C before mixing. The reaction was initiated by adding 4 μ L of 15 mM

tert-butyl-hydroperoxide (t-Bu-OOH; Beyotime, China) in Milli-Q-grade water. The absorbance (optical density [OD]) was recorded at 340 nm every 30 sec. One unit of GPX activity was defined as the amount of enzyme that hydrolyzes 1.0 μ mol of NADPH into $NADP^+$ per minute under the conditions described.³¹

Assay for total superoxide dismutase

The assay of superoxide dismutase (SOD) was based on the reduction of nitroblue tetrazolium (NBT) to water-insoluble blue formazan.³² Briefly, 1 gram of FB was homogenized in 10 mL of 50 mM PBS (pH 7.4) by using a Polytron and sonicator. The homogenate was centrifuged at $5000 \times g$ at 4°C for 10 min, and the supernatant was pooled. Total SOD activity was assayed according to the instructions of the SOD Assay Kit (Beyotime, China). The rate of NBT reduction was monitored at 560 nm at 25°C. One unit of SOD was defined as the amount of protein that resulted in 50% inhibition of the rate of NBT reduction.

Cloning of gene fragments and quantitative real-time PCR

Red and white muscles dissected out of freshly killed *N. guentheri* were ground in RNAiso Plus (TaKaRa) and kept at -70°C until use. Total RNAs were isolated from the frozen samples according to the manufacturer's instructions. After digestion with recombinant DNase I (RNase free; TaKaRa) to eliminate the genomic contamination, cDNAs were synthesized with a reverse transcription kit (TaKaRa) with oligo(dT) primer. The reaction was carried out at 42°C for 1 hr and inactivated at 75°C for 15 min. The cDNAs were stored at -20°C until use.

On the basis of the conserved sequences of the *sirt1*, *foxo3a*, *hsp70*, and *hsf-1* genes, four pairs of specific primers, S1 and AS1, F1 and AF1, H1 and AH1, h1 and Ah1 (Table 1), were designed using the Primer Premier program (version 5.0) and used for PCR. The amplification products

TABLE 1. SEQUENCES OF THE PRIMERS USED IN THIS STUDY

Primers	Sequences (5'(3')	Sequences information
S1 (sense)	CAACCCCAAATAGGCTCTTACAGT	<i>Sirt</i> fragment cloning primers
AS1 (antisense)	CGAACAAGGCTCCAAAGGTG	
F1 (sense)	TCCAATGCCAGCACAGTCAGC	<i>foxo3a</i> fragment cloning primers
AF1 (antisense)	CAGAGATGAGCCTGTTTTGTGGG	
H1 (sense)	AGAGCATCAACCCAGACGAGG	<i>hsp70</i> fragment cloning primers
AH1 (antisense)	CGTTGGTGATGGTGATCTTGTC	
h1 (sense)	GTAACACCAGAGAAAGACGACACAG	<i>hsf-1</i> fragment cloning primers
Ah1 (antisense)	CTCGCTCTCCTGAAGGATGG	
S2 (sense)	TCGTCTTTTTTGGAGAGAACCTACC	<i>sirt1</i> RT-PCR primers
AS2 (antisense)	AGGAATGGAATTTGGTATGAGGGC	
F2 (sense)	TGGATGGAGCGGTTGTGGAG	<i>foxo3a</i> RT-PCR primers
AF2 (antisense)	CGGATGATCTGCTGGATGACTTG	
H2 (sense)	ACGACCATCCCAACCAAACAAAC	<i>hsp70</i> RT-PCR primers
AH2 (antisense)	GAGCGGGTGGAATACCAGTCAG	
h2 (sense)	ACAGGATCATGGGAGTCAAACGG	<i>hsf-1</i> RT-PCR primers
Ah2 (antisense)	TGGTCCAAGGTGAAAGGTCGG	
β -actin (sense)	CACCTTCTACAATGAGCTCCGT	β -actin RT-PCR primers
β -actin (antisense)	GCAGGAGTGTGAAGGTCTCAA	

were cloned into a pGEM-T Easy vector (TIANGEN) following the manufacturer's instructions and transformed into Trans5 α bacteria (TRANSGEN). The positive clones were selected and sequenced using an ABI PRISM 3730 DNA sequencer. The sequences were compared with the related sequences available in GenBank using BLASTx. The sequences we obtained were deposited in GenBank and their accession numbers are: *foxo3a*, KM229540; *hsf1*, KM229541; *hsp70*, KM229542; and *sirt1*, KM229543.

To detect the expression profiles of *sirt1*, *foxo3a*, *hsp70*, and *hsf-1* in the muscles from fish maintained at different temperatures, quantitative real-time PCR (qRT-PCR) was performed using the first-strand cDNAs as template, which was reverse-transcribed from the total RNAs extracted from the muscles. Four pairs of primers specific for *sirt1*, *foxo3a*, *hsp70*, and *hsf-1*, S2 and AS2, F2 and AF2, H2 and AH2, and h2 and Ah2 (Table 1), were designed using the Primer Premier program (version 5.0). The reaction mixtures (final volume 20 μ L) consisted of 10 μ L of SYBR[®] Premix Ex Taq[™] (Tli RNaseH Plus), 0.4 μ L ROX Reference Dye II, 0.5 μ L of template, and 200 nM each of sense and antisense primers. The β -actin gene was chosen as the reference for internal standardization. All qRT-PCR experiments were conducted in triplicate. The amplification was performed on an ABI 7500 Real-Time PCR System (Applied Biosystems) at 95°C for 15 sec, followed by 40 cycles of 95°C for 5 sec, 60°C for 15 sec, and 72°C for 35 sec. The expression levels of *sirt1*, *foxo3a*, *hsp70*, and *hsf-1* relative to that of the β -actin gene were calculated by the comparative threshold cycle (CT) method ($2^{-\Delta\Delta CT}$).³³

Western blotting

A total of 1 gram of muscle from freshly killed fish was homogenized as above in 5 mL of 50 mM PBS (pH 7.5) containing protease inhibitors, and centrifuged at 5000 $\times$ g for 10 min at 4°C. The supernatant was collected and subjected to electrophoresis on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The proteins on the gel were transferred to polyvinylidene difluoride (PVDF) membranes, which were blocked with 4% bovine serum albumin (BSA) in 10 mM PBS (pH 7.4) at room temperature for 2 hr. They were incubated with the following respective antibodies: SirT1 (1:1000, arp32386_t100; Aviva, San Diego, CA), FOXO1A (1:500, arp38094_t100; Aviva, San Diego, CA), HSP70 (1:1000, SC-24; Santa Cruz, Santa Cruz, CA), HSF-1 (1:500, SC-13516; Santa Cruz, Santa Cruz, CA), and actin (1:10,000, mab1501; Chemicon, Temecula, CA) at 4°C overnight. These antibodies were used previously by Hsu and Chiu in *N. rachovii*,¹⁷ a kin species of *N. guentheri*. After washing in 10 mM PBS (pH 7.4) containing 0.1% Tween-20, the membranes were then probed with a secondary horse-radish peroxidase (HRP)-labeled antibody (1:3000) at room temperature for 3 hr. The bands were visualized using 3,3'-diaminobenzidine (DAB).

Statistical analysis

All of the experiments were repeated at least three times. Statistical analysis was performed using SPSS 13.0 for Windows. The two-tailed *t*-test was used for the analysis of difference in the mean values between 9-month-old males reared at 26 $\pm$ 1°C followed by 1 month of culture at the same

temperature, as well as 10-month-old males reared at 26 $\pm$ 1°C and 10-month-old males reared at 22 $\pm$ 1°C. The data regarding survival curves were subjected to the log-rank test. A difference at $p < 0.05$ was considered significant. All the data were expressed as mean $\pm$ standard deviation (SD).

Results

TR extends life span

Our study also showed that 100 out of 104 4-month-old male *N. guentheri* cultured survived to 9 months old, indicating that no substantial mortality occurred before the experiments started. All 9-month-old male fish were divided into different groups at random, and cultured at different temperatures. Survivorship curves for 9-month-old (38 weeks) male *N. guentheri* maintained at 26°C and 22°C showed a temperature-dependence in life span (Fig. 1). The mean life span was 46.8 $\pm$ 1.5 weeks at 26°C and 52 $\pm$ 1.8 weeks at 22°C, respectively ($p < 0.005$). In accordance, the maximum life span was 53 $\pm$ 1.7 weeks at 26°C and 56 $\pm$ 1.2 weeks at 22°C, individually ($p < 0.005$). TR from 26°C to 22°C prolonged 5.2 weeks of the mean life span and 3 weeks of the maximum life span. It is apparent that TR is able to extend survivorship of male *N. guentheri*, confirming the previous observations by Hsu and Chiu and Valenzano et al.^{17,18}

TR is able to reduce accumulation of histological markers of aging

Cellular SA- β -Gal is stained blue with X-gal, and its activity is evidenced by the intensity of blue staining. The accumulation of SA- β -Gal in the tail epithelium of 3-, 6-, 9-, and 10-month-old males maintained at 26°C was detected (Fig. 2a, b, c, d), and the blue colors observed in the epithelia were 0.833 $\pm$ 0.208%, 1.6 $\pm$ 0.3%, 3.905 $\pm$ 1.035%, and 7.146 $\pm$ 1.143% (Fig. 2k), respectively. It was evident that SA- β -Gal accumulated significantly from 3- to 6-month-old males ($p < 0.05$), and extremely significantly from 6- to 9-month-old males ($p < 0.01$) as well as from 9- to 10-month-old males ($p < 0.01$). Here we selected 9- and 10-month-old males reared at 26°C as the subjects to compare with the 10-month-old males reared at 22°C. The blue color observed in the tail epithelium was clearly visible in 9-month-old males

FIG. 1 Survivorship curves. (Blue solid line) Survivorship curve of male *N. guentheri* kept at 26°C ($n = 50$); (red solid line) survivorship curve of male *N. guentheri* kept at 22°C ($n = 50$) ($p < 0.005$).

FIG. 2 Changes in histological markers. (a) Senescence-associated β -galactosidase (SA- β -Gal) accumulation in caudal fin of 3-month-old *N. guentheri* reared at 26°C. (b) SA- β -Gal accumulation in caudal fin of 6-month-old *N. guentheri* reared at 26°C. (c) SA- β -Gal accumulation in caudal fin of 9-month-old *N. guentheri* reared at 26°C. (d) SA- β -Gal accumulation in caudal fin of 10-month-old *N. guentheri* reared at 26°C. (e) SA- β -Gal accumulation in caudal fin of 10-month-old *N. guentheri* reared at 22°C. (f) Slight visible accumulation of LF in liver of 3-month-old *N. guentheri* reared at 26°C. (g) Visible accumulation of LF in liver of 6-month-old *N. guentheri* reared at 26°C. (h) Visible accumulation of LF in liver of 9-month-old *N. guentheri* reared at 26°C. (i) Dense accumulation of LF in liver of 10-month-old *N. guentheri* reared at 26°C. (j) Moderate accumulation of LF in liver of 10-month-old *N. guentheri* reared at 22°C. (k) Statistical analysis of the area of SA- β -Gal ($n=5$). (l) Statistical analysis of the area occupied by lipofuscin granules ($n=5$). Bar, 50 μ m. m, month.

reared at 26°C ($3.905 \pm 1.025\%$; Fig. 2c), whereas it was intensely increased in 10-month-old males reared at the same temperature ($7.146 \pm 1.143\%$; Fig. 2d). By contrast, the caudal epithelia of 10-month-old males reared at 22°C stained less blue ($5.3800 \pm 0.3939\%$; Fig. 2e) than those of 10-month-old males reared at 26°C (two-tailed t -test, $p < 0.05$; Fig. 2k). These data indicate that the SA- β -Gal accumulates with age in the caudal fin of *N. guentheri*, and TR is able to reduce the accumulation of SA- β -Gal in the tail epithelium.

LF is visualized as bright green-colored auto-fluorescent dots in the cells. The accumulation of LF in the livers of 3-, 6-, 9-, and 10-month-old males maintained at 26°C was detected (Fig. 2f, g, h, i), and the auto-fluorescent dots in the livers were $0.16 \pm 0.034\%$, $0.245 \pm 0.022\%$, $0.3110 \pm 0.079\%$, and $0.5930 \pm 0.1293\%$ (Fig. 2l), respectively. It was evident that LF accumulated only slightly from 3- to 6-month-old males, and from 6- to 9-month-old males ($p > 0.05$), but significantly from 9- to 10-month-old males ($p < 0.05$), which is basically in line with previous observations by Mann et al.,³⁴ West et al.,³⁵ and Strauss.³⁶ Here we selected 9- and 10-month-old males reared at 26°C as the subjects to compare with the 10-month-old males reared at 2°C. Bright green-colored dots were clearly observed in the liver of 9-month-old fish kept at 26°C ($0.3110 \pm 0.079\%$; Fig. 2h),

whereas the number of green-colored dots was much denser in the liver of 10-month-old fish kept at the same temperature ($0.5930 \pm 0.1293\%$; Fig. 2i). By contrast, the liver of 10-month-old male fish kept at 22°C displayed less dense and brighter green-colored dots ($0.4234 \pm 0.08452\%$; Fig. 2j) than those of 10-month-old fish kept at 26°C (two-tailed t -test, $p < 0.05$; Fig. 2l). These data show that, like SA- β -Gal, LF increases with age in the liver of *N. guentheri*, and TR is able to reduce the accumulation of LF in the liver.

TR is able to reduce levels of protein oxidation and lipid peroxidation

The level of protein oxidation was assessed via determining the carbonyl contents of amino acids derived by using DNPH. The mean values of carbonyl-group content acquired by the muscles of 9- and 10-month-old males maintained at 26°C were 21.32 ± 2.79 and 25.87 ± 0.45 nM/mg protein (two-tailed t -test, $p < 0.05$; Fig. 3a), respectively. By contrast, the mean value of carbonyl-group content of 10-month-old males reared at 22°C was 23.19 ± 1.02 nM/mg protein, which is significantly lower than that of 10-month-old males maintained at 26°C (two-tailed t -test, $p < 0.05$; Fig. 3a). These results denote that the level of carbonyl-group

FIG. 3. Protein oxidation and lipid peroxidation levels in 9-month-old *N. guentheri* reared at 26°C, 10-month-old *N. guentheri* reared at 26°C and 10-month-old *N. guentheri* reared at 22°C, respectively. (a) Protein oxidation levels. (b) Lipid peroxidation levels. Data represent mean $\pm$ standard deviation (SD) ($n=5$). m, month.

content in the muscle increases with age and TR is able to reduce the protein oxidation level in the muscle of *N. guentheri*.

Lipid peroxidation was assessed via determining the levels of MDA, a metabolite of lipid peroxidation. The mean values of MDA obtained from the muscles of 9- and 10-month-old fish cultured at 26°C were 4.06 ± 0.16 and 6.07 ± 0.17 $\mu\text{M}/\text{mg}$ protein (two-tailed *t*-test, $p < 0.001$; Fig. 3b), separately. By contrast, the mean value of MDA obtained from the muscle of 10-month-old fish cultured at 22°C was 5.15 ± 0.52 $\mu\text{M}/\text{mg}$ protein, which is considerably lower than that of 10-month-old males maintained at 26°C (two-tailed *t*-test, $p < 0.05$; Fig. 3b). These results indicate that the level of MDA increases with age and TR is able to reduce the lipid peroxidation level in the muscle of *N. guentheri*.

TR is able to prevent decline of CAT, GPX, and SOD activities

Figure 4 shows the change in the activities of CAT, GPX, and SOD. The mean values of CAT activities obtained from 9- and 10-month-old fish reared at 26°C were 6.48 ± 0.45 and 4.85 ± 0.52 $\mu\text{M}/\text{min}$ mg protein (two-tailed *t*-test, $p < 0.05$; Fig. 4a), respectively. By contrast, the mean value of CAT activity obtained from 10-month-old fish reared at 22°C was 5.94 ± 0.42 $\mu\text{M}/\text{min}$ mg protein, significantly higher than that of 10-month-old fish maintained at 26°C (two-tailed *t*-test, $p < 0.05$; Fig. 4a). Similarly, the mean values of both GPX and SOD activities from 9- and 10-month-old fish cultured at 26°C were 9.16 ± 1.44 and 6.71 ± 0.48 nM/min mg protein, and 34.4 ± 4.41 and 24.67 ± 2.52 U/mg protein (two-tailed *t*-test, $p < 0.05$; Fig. 4b, c), respectively. By contrast, the mean values of GPX and SOD activities obtained from 10-month-old fish cultured at 22°C were 7.81 ± 0.39 nM/min mg protein and 31 ± 2 U/mg protein (two-tailed *t*-test, $p < 0.05$; Fig. 4b, c),

individually, that are both markedly lower than those of 10-month-old fish maintained at 26°C. These data indicate that the activities of the anti-oxidant enzymes CAT, GPX, and SOD decrease with age, and TR is able to prevent the decline of the activities of the anti-oxidant enzymes.

TR is able to induce expression of *sirt1* and *foxo3a*

FOXO is a downstream target of the IIS pathway, the most conserved aging-mediating pathway in evolution, and its expression is modulated by the protein deacetylase SirT1.^{5,6,37,38} The FOXO family contains FOXO1, FOXO3, FOXO4, and FOXO6. HSP70, a major heat shock protein, can alleviate the cellular stresses imposed by hyperthermic stress,³⁹ and its expression is modulated by the HSF-1,⁴⁰ which itself is a crucial longevity transcription factor known to act downstream of IIS pathway.^{41,42} Partial cloning revealed the presence of *sirt1*, *foxo3a*, *hsp70*, and *hsf-1* in *N. guentheri*, which are highly identical to their counterparts in fish and mammalian species.

RT-PCR was used to examine the expression of *sirt1*, *foxo3a*, *hsp70*, and *hsf-1*. The dissociation curve of amplified products in all cases showed a single peak, indicating that the amplifications were specific (data not shown). As shown in Fig. 5, the expression of both *sirt1* and *foxo3a* in the muscle from 9-month-old fish kept at 26°C was remarkably higher than that in the muscle from 10-month-old fish reared at the same temperature (two-tailed *t*-test, $p < 0.01$; Fig. 5a, b). By contrast, the expression of *sirt1* and *foxo3a* in the muscle from 10-month-old fish kept at 26°C was significantly lower than that in the muscle from 10-month-old fish kept at 22°C (two-tailed *t*-test, $p < 0.01$; Fig. 5a, b). These results were also confirmed by western blotting, which showed that the levels of SirT1 and FOXO1A in the muscle from 10-month-old fish kept at 22°C was markedly higher than those from the same aged fish kept at 26°C (two-tailed *t*-test, $p < 0.05$; Fig. 5c–f). These data

demonstrate that both SirT1 and FOXO3A/FOXO1A decrease with age and TR is able to decelerate their decrease with aging in *N. guentheri*.

Figure 6 shows the expression of *hsp70* and *hsf-1* in *N. guentheri* cultured at different temperatures. The expression of *hsp70* and *hsf-1* in the muscle from 9-month-old fish kept at 26°C was only slightly higher than that in the muscle from 10-month-old fish reared at the same temperature (two-tailed *t*-test, $p > 0.05$). Similarly, the expression of *hsp70* and *hsf-1* in the muscle from 10-month-old fish kept at 22°C was not significantly different from that in the muscle from 10-month-old fish reared at 26°C (two-tailed *t*-test, $p > 0.05$). Western blotting also showed that the levels of HSP70 and HSF-1 in the muscle from 9-month-old fish reared at 26°C differed only slightly from those in the muscle from 10-month-old fish reared at the same temperature (two-tailed *t*-test, $p > 0.05$). These indicate that TR exerts little influence on the expression of HSP70 and HSF-1 in the muscle of *N. guentheri*.

Discussion

Late-onset TR prolongs fish life span

Temperature variations are known to extend the life span of different poikilotherms such as worms, flies,¹⁶ and fishes.^{13,18,44,45} In both flies and fishes, temperature affects life span by modulating the slope of age-dependent acceleration in death rate, which is thought to reflect the rate of age-related damage accumulation. In this study, we clearly demonstrate that late-onset lowering temperature from 26°C to 22°C increases both median and maximum life span in *N. guentheri*, suggesting that the life extension manifests slower natural aging of the fish, but not other non-age-dependent variables. It is therefore that late-onset TR is a simple intervention capable of retarding the rate of age-related damage accumulation in fish like *N. guentheri*.

Late-onset TR retards the aging process in aged fish

SA-β-Gal and LF are routine histological markers of aging, and protein oxidation and lipid peroxidation are routine molecular markers of aging. TR is known to delay lipid oxidative damage in the fly *Drosophila melanogaster*¹⁶ and reduce the accumulation of LF in the fish *Nothobranchius furzeri*.^{17,18,30} Similarly, TR is known to reduce both protein oxidation and lipid peroxidation levels in *N. rachovii*.¹⁷ Here we show that TR is able to reduce the accumulation of SA-β-Gal in the epithelium and LF in the liver as well as the protein oxidation and lipid peroxidation levels in the muscle, indicating that late-onset TR delays the development of aging markers in aged fish. It is clear that late-onset TR can delay aging process in fish.

FIG. 4. Changes in activities of catalase (CAT), glutathione peroxidase (GPX), and superoxide dismutase (SOD) in 9-month-old *N. guentheri* reared at 26°C, 10-month-old *N. guentheri* reared at 26°C and 10-month-old *N. guentheri* reared at 22°C. (a) CAT activity. (b) GPX activity. (c) SOD activity. Data represent mean ± standard deviation (SD) ($n = 5$). m, month.

FIG. 5. Levels of SirT1 and FOXO in the muscles. (a) Expression profile of *sirt1*. (b) Expression profile of *foxo3a*. β -actin was chosen as the internal control for normalization. Relative expression data was calculated by the method of $2^{-\Delta\Delta CT}$. Data represent mean $\pm$ standard deviation (SD) ($n=5$). (c and d) Level of SirT1. (e and f) Level of FOXO1A. β -Actin served as loading control. Data represent mean $\pm$ standard deviation (SD) ($n=5$). m, month.

FIG. 6. Levels of HSP70 and HSF-1 in the muscles. (a) Expression of *hsp70*. (b) Expression of *hsf-1*. β -actin was chosen as the internal control for normalization. Relative expression data was calculated by the method of $2^{-\Delta\Delta CT}$. Data represent mean $\pm$ standard deviation (SD) ($n=5$). (c and d) Levels of HSP70. (e and f) Levels of HSF-1. β -Actin served as loading control. Data represent mean $\pm$ SD ($n=5$). m, month.

Late-onset TR affects both the anti-oxidant system and IIS pathway

Both oxidative stress and the IIS pathway are associated with life span. On one hand, the anti-oxidant enzymes CAT, GPX, and SOD all play a key role in scavenging ROS, which can induce protein oxidation, lipid peroxidation, and DNA damage. TR is known to induce the expression of SOD and CAT in *N. rachovii*.¹⁷ We show here that late-onset TR can enhance the expression of *cat*, *gpx*, and *sod* and induce increases in the activities of CAT, GPX, and SOD, thereby benefiting scavenging ROS, which is likely to lead to longevity. On the other hand, reduced IIS confers life span extension in many species, potentially including humans.^{6,40} FOXO is a downstream target of the IIS pathway, and its expression is under the control of the protein deacetylase SirT1. We show that both SirT1 and FOXO3A/FOXO1A reduce with age, which agrees with the general notion that the IIS pathway decreases with aging.⁹ However, our results demonstrate that late-onset TR slows down reduction of SirT1 and FOXO3A/FOXO1A in aged *N. guentheri*. Consistent with our observations, TR is also known to up-regulate the expression of SirT1 and FOXO in relatively aged *N. rachovii* (4-month-old fish vs. mean life span 6 months).¹⁷ It is highly likely that in these aged fishes, IIS signaling is extremely low so that it cannot support normal anabolic signaling. Therefore, enhancement of SirT1 and FOXO induced by TR may benefit maintenance of anabolic sensing, which eventually leads to life extension. However, we cannot rule out the possibility at the moment that maintenance of the IIS pathway directly contributes to the life extension. Taken together, our study suggests that late-onset TR induces an increased life span via combined action of the anti-oxidant system and IIS pathway in aged fish.

HSP70 is known to alleviate the cellular stresses imposed by hyperthermic stress.³⁹ The expression of HSP70 is modulated by the HSF-1,⁴⁰ which itself is a crucial longevity transcription factor known to act downstream of the IIS pathway.^{41,42} Interestingly, our results reveal that late-onset TR has little influence on HSP70 and HSF-1 levels in the muscle of *N. guentheri*. Similarly, the expression of *hsp70* shows no marked difference at varied temperatures in *N. rachovii*.¹⁷ These results suggest that late-onset TR exerts no significant effects on the change in life span in fish.

In summary, our study highlights that late-onset TR retards the aging process in aged fish, resulting in an increased median and maximum life span in *N. guentheri* via a combined action of the anti-oxidant system and IIS pathway. It also shows that TR is a simple non-intrusion intervention capable of slowing down the rate of age-related damage accumulation in fish.

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Author Disclosure Statement

No competing financial interests exist.

Shicui Zhang designed the research and wrote the manuscript; Xia Wang and Qingyun Chang performed the re-

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