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# **Influence of temperature on age-related lipid peroxidation in a short-lived vertebrate (*Nothobranchius furzeri*)**

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## **Abstract**

In ectotherms, temperature modulates oxidative stress, a key driver of aging. However, the effects of temperature on oxidative stress have not been investigated at several life stages of an ectotherm. In order to improve understanding of aging processes, we conducted a cross-sectional study in short-lived ectotherm vertebrates, the killifish *Nothobranchius furzeri*. Malondialdehyde contents were assessed at three stages of life, in the liver and muscles of fish acclimatized to optimal or sub-optimal temperatures during all their life cycle. In accordance with the “free-radical theory”, our results highlighted an increase in lipid peroxidation in senescent organisms. In the liver, this lipid peroxidation increase was more intense in senescent fish acclimatized to sub-optimal temperature than in fish acclimatized to their optimal temperature.

## Introduction

In the mid-50's, the "free radical theory" was proposed; this theory states that radical oxygen species (ROS) generated in cells result in damage which leads to an age-related decline in biological functions (Harman 1956). Although subsequent research has emphasised that ageing is a multifactorial process, oxidative stress is still suggested to be a key factor in senescence (Preedy 2014). While genetic determinism partly explains age-related oxidative stress, studies have also shown the effects of environmental insults (reviewed in Finkel and Holbrook 2000). For example, the influence of caloric restriction has been intensively researched (e.g., Sohal and Weindruch 1996; Mattison et al. 2012). Conversely, although temperature modulates oxidative stress in ectotherms (reviewed in Lushchak et al. 2011), few studies have considered the influence of this factor on age-related oxidative stress (Malek et al. 2004; Valenzano et al. 2006; Hsu et al. 2009; Hemmer Brepson et al. 2014; Wang et al. 2014). Moreover, these studies have investigated the influence of temperature on oxidative stress using a single measurement point in adult or senescent ectotherms, and thus, no approach has considered the effect of temperature on lipid peroxidation using two temperatures, and at several phases of an ectotherm's life. Consequently, the preliminary study reported here is important for aging research as it investigates the influence of temperature on oxidative stress at different life phases of a vertebrate ectotherm. The species chosen for investigation was the short-lived killifish *Nothobranchius furzeri*, given its suitability as a model species for aging studies (Lucas-Sanchez et al. 2014; Cellarino et al. 2016). Age-related oxidative stress was estimated through lipid peroxidation in the liver and muscles, at two temperatures: 26°C, considered as the optimal temperature for the fish since it induces a higher growth rate (Genade et al. 2005); and 22°C, considered as a sub-optimal temperature.

## Material and methods

During experiment, the life cycle of *N. furzeri* (strain MZM 0410, with a maximum lifespan of 32 weeks, as described in Terzibasi et al. 2008) was entirely controlled. From hatching to 10 days post-hatching (dph), juveniles were fed twice a day with *nauplii* of *Artemia salina*, and water (24°C) was renewed daily. At 10 dph, individuals were gently transferred to rearing tanks and acclimatised gradually (by mixing water) at  $22.1 \pm 0.8^\circ\text{C}$  or  $25.6 \pm 0.5^\circ\text{C}$  (mean

± standard error of the mean). In rearing tanks (0.50 x 0.30 x 0.25 m), fish were fed twice a day *ad libitum* with *Chironomus larvae*, and were exposed to an artificial light period of 12 hours per day. Seven fish were euthanized (using 0.008% eugenol) after seven weeks (juvenile), 18 weeks (adult) and 30 weeks (senescent) post-hatching, for each temperature of acclimatization. Fish weights were  $0.69 \pm 0.07$  g and  $0.85 \pm 0.16$  g at 7 weeks,  $1.44 \pm 0.07$  g and  $2.22 \pm 0.45$  g at 18 weeks and  $1.71 \pm 0.30$  g and  $2.48 \pm 0.48$  g at 30 weeks (respectively for 22.1°C and 25.6°C acclimatization temperatures). Due to high variability, no significant difference in average weights were observed between the acclimatization temperature groups. The liver and axial muscle were removed from each individual and stored at -80°C. Next, these tissues were homogenised in ice-cold phosphate buffer containing Halt protease inhibitor cocktail (Thermo Scientific). The homogenates were centrifuged at 12500 g at 4°C for 15 min, and the post-mitochondrial fractions (PMF) were used for assays. Total protein concentrations were determined using the method of Bradford (1976) with a BioRad assay kit (500-0002) containing bovine serum albumin as a standard and Brilliant Blue G 250 as a reactant. The reaction was measured spectrophotometrically at 570 nm. Lipid peroxidation was then evaluated via malondialdehyde (MDA) concentration using a commercially available MDA assay kit (Oxis International). The method was based on the reaction of a chromogenic reagent (n-methyl-2-phenylindole) with MDA measured at 586 nm (Gérard-Monnier et al. 1998). The results are presented in µmol of MDA/g of protein, and significant differences between the two temperatures and between the three ages were investigated using non parametric Mann-Whitney and Kruskal-Wallis tests, respectively. A Bonferroni correction was applied, and thus, differences were considered statistically significant if  $P < 0.01$ .

## Results and Discussion

Regarding the effect of age on lipid peroxidation, our results showed higher hepatic MDA levels (**Figure 1A**) in juveniles than in adult fish, for both temperatures of acclimatization. This finding highlights potential oxidative stress during the early phase of the life of the fish. This agrees with the hypothesis of developmental-phase related oxidative stress (proposed by Monaghan et al. 2009). This hypothesis suggests that there is a link between high metabolic activities for growth and ROS generation. Given the lifecycle of our model species, such a hypothesis is even more plausible. Indeed, Terzibasi et al. (2007) described the lifecycle of *N.*

*furzeri* as “tied with explosive growth and accelerated with sexual maturation”. Since the liver is highly irrigated and involved in nutrient synthesis and storage, the metabolic demand on this organ during development may have been intense, and consequently, may have induced particularly high oxidative stress. When considering both organs (**Figure 1A and 1B**), our results indicate an increase in MDA in senescent organisms (30 weeks), when compared to the adult phase (18 weeks). Such a pattern, obtained in both tissues, is in line with the “free-radical theory” of aging (Harman 1956), and more particularly, with recent studies conducted by Hsu et al. (2008) and Liu et al. (2012), both of which observed an increase in oxidative stress linked with a decrease in the response of antioxidant systems in aged *Nothobranchius rachovii* and *Nothobranchius guentheri* (respectively). In the current study, measurement of antioxidant enzymes in muscles and liver would have been of interest to provide a better understanding of the cellular mechanisms leading to an increase in MDA in senescent *Nothobranchius furzeri*.

Regarding the effects of temperature, the results did not fit with our expectations. Indeed, it is generally admitted that (i) a higher acclimatization temperature in ectotherm vertebrates induces higher metabolic rates (up to a limit of temperature above the optimal temperature, Portner and Farrell 2008), and (ii) metabolic activity is responsible for the production of free-radicals (Turrens 1997). Based on these two statements, we expected that lipid peroxidation would have been positively modulated by temperature (i.e., temperature-dependent oxidative stress mediated through variation of metabolic rate). However, in our study, this phenomenon was not observed in muscles; no significant difference in lipid peroxidation was observed between 26°C and 22°C acclimatized fish. Similar results have been reported by Malek et al. (2004) and Hemmer Brepson et al. (2014), who suggested that antioxidant defence systems (such as superoxide dismutase) neutralize the increases in ROS due to temperature.

In the liver of senescent fish, the results were contrary to our expectations; the lowest (and sub-optimal) acclimatization temperature, i.e., 22°C, led to the highest oxidative stress. A study conducted by Valenzano et al. (2006) showed a decrease in lipofuscin in the liver cells of senescent *N. furzeri* acclimatized to a lower temperature (22°C compared to 25°C). The authors suggested that oxidative stress varies positively with temperature, which is in direct contrast with our findings. Such a discrepancy could be due to the origin of this oxidative stress. Indeed, in our study, by measuring MDA we estimated the lipid peroxidation in polyunsaturated fatty acids (mainly contained in the cell membrane), while in the study of Valenzano et al. (2006), the

measurement of lipofuscin estimated oxidative stress in lysosomes of post-mitotic cells. Consequently, we suggest that a lower (and sub-optimal) acclimatization temperature in senescent fish could lead to an increase in oxidative stress in the cell membrane and, on the other hand, to a decrease in oxidative stress in lysosomes, and potentially in other intra-cellular components (Valenzano et al. 2006). Such a discrepancy could also be due to the husbandry and/or the fish strains used. Indeed, as previously described by Di Cicco et al. (2011), different strains (or rearing conditions) could cause differences of aging processes. For instance, the incidence of liver neoplasias was accelerated in a short-lived strain. While the fish strains and the husbandry conditions are not precisely described in Valenzano et al. (2006), these authors showed a median lifespan of 9 to 10 weeks for their fish, while our study showed a median lifespan of 34 weeks, suggesting that the husbandry and/or fish strains were different.

To explain the higher oxidative stress in the liver of senescent fish acclimatized to 22°C, we consider that decreasing efficiency of the antioxidant systems may be responsible for oxidative stress in senescent killifish (in accordance with Hsu et al. 2008). We postulate that this decrease was more important in fish acclimatized to the sub-optimal temperature than in fish acclimatized to their optimal temperature.

In conclusion, as our study was conducted at three phases of an ectotherm's life, it gives a novel insight into the influence of temperature on age-related lipid peroxidation in ectotherms. The increase in oxidative stress with aging in the two target tissues is consistent with the "free radical theory" of aging. The high level of lipid peroxidation in the liver of juveniles is in line with the hypothesis of a developmental-phase related oxidative stress. Finally, the results indicated higher lipid peroxidation in the liver of senescent fish acclimatized to the sub-optimal temperature, than in those acclimatized to the optimal temperature. This finding disagrees with the idea that oxidative stress varies positively with temperature, and consequently, is inconsistent with the idea that the variation in oxidative stress due to acclimatization temperature is mediated via the metabolic rate. However, these findings should be interpreted with caution since effects of oxidative stress were only investigated through lipid peroxidation in this study. Thus, further studies are required to provide a more comprehensive framework for understanding the influence of temperature on age-related oxidative stress. Indeed, cross-sectional and/or longitudinal approaches should be conducted in several ectotherms, using different indicators of oxidative stress (e.g., DNA damage, protein oxidation) in target organs.

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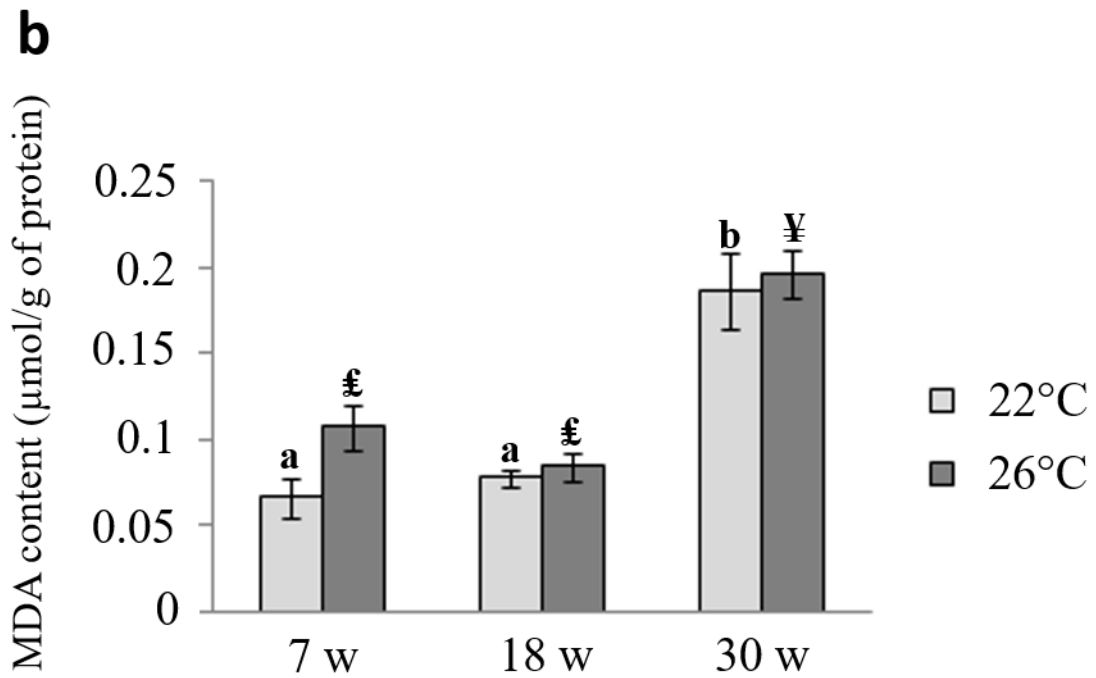
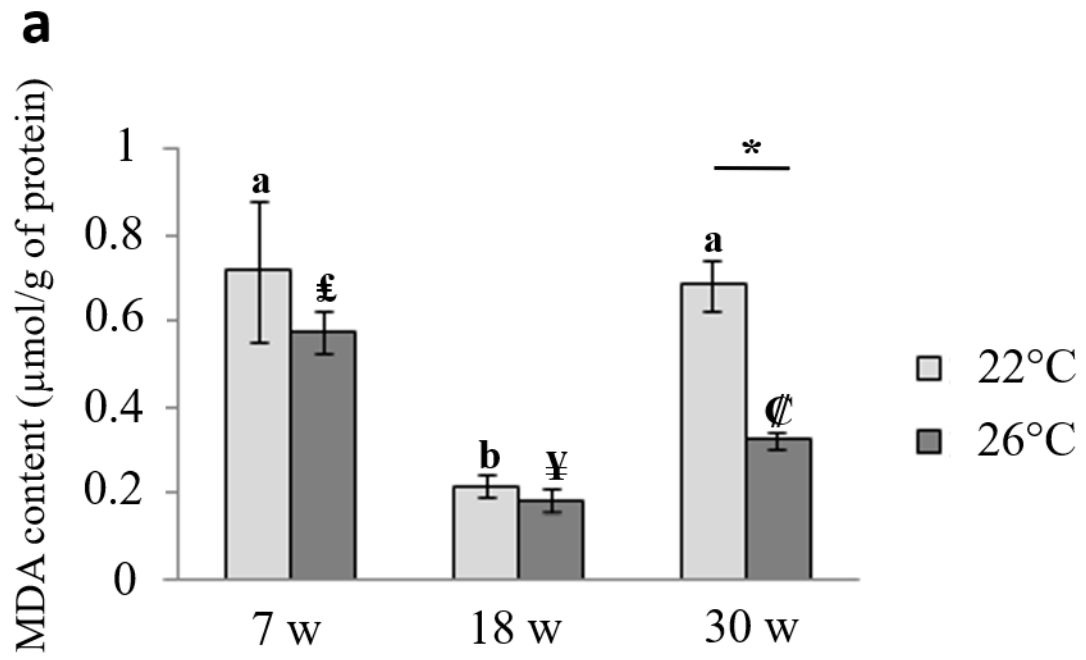
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## Figures captions

**Fig. 1:** MDA contents in the liver (**A**) and in the muscle (**B**) of *Nothobranchius furzeri* exposed to 22°C (grey) and 26°C (dark grey) during 7, 18 and 30 weeks (7 w, 18 w and 30 w). Values represent mean  $\pm$  standard error (n=7). For fish exposed to 22 °C, **different letters** above bars (**a**, **b** and **c**) indicate a **significant difference** ( $P < 0.01$ ) between the several age. For fish exposed to 26 °C, different symbols above bars (**£**, **¥** and **ℳ**) indicate a **significant difference** ( $P < 0.01$ ) between the several age. \*indicates a significant difference between same-age fish exposed to 22 and 26 °C.