Comparative Biochemistry and Physiology, Part C 300 (2026) 110389

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Research article

Non-lethal heat shock induces cross-tolerance to different stressors in two strains of *Brachionus koreanus* (Rotifera: Monogononta): Mechanisms of increased tolerance to hydrogen peroxide

Luana Granada [a,b,*], Inês F.C. Morão [a], Marco F.L. Lemos [a], Peter Bossier [b], Sara C. Novais [a,*]

[a] *MARE – Marine and Environmental Sciences Centre & ARNET – Aquatic Research Network, ESTM, Polytechnic of Leiria, 2520-641, Peniche, Portugal*
[b] *Laboratory of Aquaculture & Artemia Reference Center, Department of Animal Sciences and Aquatic Ecology, Faculty of Bioscience Engineering, Ghent University, Ghent, 9000, Belgium*

ARTICLE INFO

Edited by Martin Grosell

Keywords:
Epigenetic effects
Gene expression
Histone modifications
qPCR
Swimming capacity
Western blot

ABSTRACT

The rotifer *Brachionus* sp. is of great importance for aquaculture, as the reproduction cycle under rearing conditions of many economically important species larvae depends on the use of rotifers as first live feed. Establishing a protocol that results in an improved tolerance of rotifers to environmental stressors will allow for a more stable rotifer production. The exposure to non-lethal heat shocks (NLHS) already proved to enhance the tolerance, not only to heat stress, but also to other stressors in several aquatic species, by activating the heat shock response and epigenetic mechanisms. This study aimed to determine the potential of a single NLHS to induce tolerance to different abiotic stressors in two strains of *B. koreanus* (MRS10 and IBA3) and to evaluate possible molecular mechanisms involved in the achievement of increased tolerance to hydrogen peroxide induced by NLHS. Cross-tolerance was achieved for both strains, namely to high salinity, cadmium chloride, and hydrogen peroxide. Scale-up tests resulted in increased tolerance to hydrogen peroxide only for MRS10. During the exposure to this substance, heat-shocked MRS10 rotifers showed an up-regulation of genes related to oxidative stress response and histone modifications, increased production of HSP70, and higher levels of total acetylation of histone H3. A single NLHS proved to induce epigenetic effects when rotifers were exposed to other stressor later in life. However, further studies should elucidate if the NLHS conditions used in this study can yield a persistent outcome, allowing the establishment of tolerant rotifer strain lines and, consequently, a more stable production.

1. Introduction

The monogonont rotifer *Brachionus* spp. has been grown commercially and enabled the stable production of several marine species since the 1960's (Hagiwara and Yoshinaga, 2017), being an important food source for fish, bivalves, and crustacean larvae reared in hatcheries (Dhont et al., 2013). Therefore, enhancing the stability of rotifer cultures is of great importance for aquaculture sustainability.

Parameters such as salinity and water temperature are of great importance for aquaculture and if, on one hand, their correct regulation enhances the survival, growth, and fecundity of rotifers, on the other hand, unexpected variations can result in culture crashes (Kim et al., 2020; Wang et al., 2019). Moreover, rotifers are often exposed to antibiotics and other therapeutants, such as hydrogen peroxide, which is used in the control and treatment of external infections of cultivated organisms and eggs (Schmidt et al., 2006) and in the establishment of axenic rotifer cultures (Suga et al., 2011).

Rotifers, and especially *B. plicatilis*, have been used as a model for ecotoxicological studies and as indicator of water quality, by assessing lethal effects of toxicants through ISO 19820:2016, that recommends copper sulfate as reference substance.

It has been demonstrated that is possible to modulate the response of organisms to stressful conditions by exposing them to environmental variations (Hua et al., 2013), promoting the development of advantageous traits (Granada et al., 2018). In rotifers, exposure to hypoxia resulted in extended longevity and lifetime reproduction in *B. manjavacas* (Snell et al., 2019), and acute heat shock resulted in tolerance to lethal temperatures in *B. koreanus* (Granada et al., 2025).

---

* Corresponding authors at: Edifício CETEMARES, Avenida do Porto de Pesca, 2520-630, Peniche, Portugal.
E-mail addresses: luana.almeida@ipleiria.pt (L. Granada), sara.novais@ipleiria.pt (S.C. Novais).

https://doi.org/10.1016/j.cbpc.2025.110389
Received 31 May 2025; Received in revised form 28 October 2025; Accepted 11 November 2025

Heat shock exposure also showed to have a positive effect on the response of *Macrobrachium rosenbergii* to hypersalinity and heat (Rahman et al., 2004), as well as of *Artemia* to heat (Norouzitallab et al., 2014) and toxic compounds (Pestana et al., 2016), and of *Penaeus vannamei* to heat, ammonia, heavy metals (Sung et al., 2018) and pathogen infections (Junprung et al., 2017).

The heat shock response is a highly conserved defence mechanism, that allow organisms to cope with stressful conditions, such as exposure to heat, osmotic stress and chemicals (Xiang et al., 2017), and is characterized by the activation of gene expression and synthesis of several heat shock proteins (HSPs) (Liang et al., 2020). The HSP70 is considered the most highly conserved among the heat shock protein families (Mukhopadhyay et al., 2003), being involved in folding, transport and repair of other proteins (Liang et al., 2020). Heat stress also demonstrated to enhance the production of reactive oxygen species (ROS) and, therefore, to cause oxidative stress (Slimen et al., 2014). In addition, exposure to abiotic stressors can also have epigenetic effects that, by controlling gene activity, allow the organisms to cope with changes in the environment and adapt to new conditions (Lee et al., 2020b). DNA methylation and histone modifications are the most studied epigenetic mechanisms. As *B. plicatilis* lacks important genes involved in the process of DNA methylation, it is suggested that the main epigenetic mechanisms in this organism are histone modifications (Lee et al., 2020a, 2020b). There are several histone modifications (e.g., methylation, acetylation, ubiquitination, and phosphorylation) and variants, being histone acetylation and methylation the best characterized (Granada et al., 2018). Histone methylation is controlled by histone lysine methyltransferases (HMT) and demethylases (HDM), and act by activating or repressing gene activity. Histone acetylases (HAT) and deacetylases (HDCA) control histone acetylation, and result in gene activity or silencing, by opening or repressing the chromatin structure, respectively (Granada et al., 2018; Lee et al., 2020b; Norouzitallab et al., 2014). The induction of tolerance mediated by epigenetic mechanisms is particularly advantageous, as altered epigenetic states and resultant phenotypes can be inherited by the following generations (Norouzitallab et al., 2019), perpetuating tolerant strain lines.

Considering this, the main objective of this study was to assess if the exposure of rotifer neonates to a non-lethal heat shock could result in increased tolerance to other stressors (salinity, hydrogen peroxide, copper sulfate, and cadmium chloride) later in life in two strains of *B. koreanus*, and to better understand the molecular mechanisms underlying this phenotypic outcome. Upon induction of tolerance to hydrogen peroxide by non-lethal heat shock (NLHS), the present study addressed general stress responses, by evaluating the expression of oxidative stress and cellular damage related genes and the production of HSP70; and the role of epigenetic mechanisms, through the expression of genes related to histone modifications and DNA methylation, and quantification of multiple modifications of histone H3.

2. Material and methods

2.1. Rotifer cultures

Two strains of clone cultures (MRS10 and IBA3) of the monogonont rotifer *Brachionus koreanus* were used in this study (Granada et al., 2022). Organisms were maintained at 25 ppt (autoclaved artificial seawater, ASW, Instant Ocean Sea Salt with deionized water), room temperature of 25 ± 1 °C, and placed in front of cool white tube lights under constant light intensity of 34 μmol m^{-2} s^{-1}. Cultures were fed daily with *Tetraselmis* sp. at a final concentration of 10^5 cells mL^{-1} (Granada et al., 2022).

2.2. Confirmation of non-lethal heat shock conditions

Non-lethal heat shock conditions determined by Granada et al. (2025) to induce thermotolerance were used. In short, groups of 0–3 h neonates were abruptly exposed to a range of temperatures (42 °C to 46 °C) for 30 min, to calculate the temperature causing 50 % mortality (LT_{50}). Then, neonates were exposed to temperatures that did not cause any visible effects (40 °C to 42 °C) to determine the temperature of non-lethal heat shock (NLHS), and different hours of recovery at control conditions were tested. The NLHS conditions were obtained by exposing all the organisms to the LT_{50} calculated previously, and comparing survival obtained for Reference (rotifers continuously maintained at control conditions) and NLHS treatments. Determinations of LT_{50}, NLHS temperature, and recovery period were done for both strains.

2.3. NLHS-induced cross-tolerance

2.3.1. Chemicals

Hydrogen peroxide (30 %; H_2O_2; Merck KGaA, Darmstadt, Germany), copper sulfate ($CuSO_4.5H_2O$; 249.68 g mol^{-1}; Alfa Aesar, Ward Hill, MA, USA; 99 % purity), and cadmium chloride ($CdCl_2$; 183.32 g mol^{-1}; Merck, Darmstadt, Germany; purity ≥99.99 %). Test solutions with different concentrations were prepared by diluting in ASW highly concentrated stock solutions prepared in ASW (H_2O_2 and $CdCl_2$) or Milli-Q water ($CuSO_4$), which were the same for both strains (Granada et al., 2023). The range of concentrations chosen were based on the lethal and sub-lethal toxicity results obtained by Granada et al. (2023).

2.3.2. Experimental design

To test the impact of NLHS during the initial life stages on the ability of rotifers to better cope with stressors beyond temperature, groups of 0–3 h old neonates were exposed to the NLHS temperature established previously (41 °C for MRS10 and 42 °C IBA3), for 30 min, in a T100 Thermal Cycler (Bio Rad) (NLHS treatment; Granada et al., 2025). Organisms maintained at 25 °C were used as controls (Reference treatment). After the heat shock, all organisms were transferred to 48-well microplates with 1 mL ASW and held in the dark, at control conditions (25 °C, ASW 25 ppt) for 8 h. This was the recovery period that showed better results for thermotolerance (Granada et al., 2025). Later, organisms from both Reference and NLHS treatments were exposed during 24 h to several stressors: high salinity (60, 65, 70, 75, 80, and 85 ppt), hydrogen peroxide (0, 1.36, 1.62, 1.92, 2.29, and 2.72 mg L^{-1}), copper sulfate (0, 0.50, 0.78, 1.22, 1.92, and 3 mg L^{-1}), and cadmium chloride (0, 60.0, 106.7, 189.7, 337.4, and 600 mg L^{-1}). For this, organisms were transferred to new 48-well microplates, with 500 μL of the respective test medium per well. Six treatments of a given stressor were tested, each treatment consisting of six replicates of five rotifers. Exposures were conducted in a climatic room at 25 ± 1 °C, with no light. At the end of the experiment, the survival curves for each stressor, within reference and NLHS organisms, were determined and compared, along with the calculation of concentrations causing 50 % lethality (LC_{50}). Rotifers not showing any movement for 10 s of observation were considered dead (Granada et al., 2023; ISO, 2016). The cross-tolerance induction was considered successful if the survival of the organisms from NLHS treatment was statistically higher when compared to Reference treatment ($P < 0.05$).

2.4. Analysis of molecular responses to an oxidative stressor after NLHS exposure

2.4.1. Experimental design

To study possible mechanisms responsible for the increased tolerance to hydrogen peroxide after exposure to NLHS (gene expression, HSP70 production, and histone H3 modifications), scale-up tests were performed with both strains. For this, rotifer cultures were up-scaled in several 5 L cultures, and maintained with light aeration, and at control conditions (25 °C, ASW 25 ppt, constant light) for at least three weeks. For the experiments, cultures were filtered with a sieve with 100 μm of diameter to obtain rotifers with eggs that were incubated at control conditions (no aeration) for 5 h. Neonates (0–5 h old) were collected and concentrated with a sieve with 30 μm of diameter, and equally divided by six 1 L Schott flasks, all with a density of 300 neonates per mL (Fig. S1). Three flasks were placed in a temperature-controlled water bath (Precision SWB 15, Thermo Fisher Scientific, USA) accurate to ±0.1 °C and an abrupt 30 min NLHS was given at 41 °C for MRS10 and 42 °C for IBA3 (NLHS treatment; Granada et al., 2025). Three flasks were maintained at 25 °C, in the dark, for 30 min (Reference treatment). Then, after making up the volume to 1 L in all six flasks, all replicates were placed at 25 °C, in the dark, during 8 h for recovery (Granada et al., 2025). After this period, a concentrated solution of hydrogen peroxide was added to all replicates, from both NLHS and Reference treatments, in order to expose the organisms to a concentration equivalent to the LC_{20} of the Reference treatment (determined before). Exposure to the hydrogen peroxide lasted for 18 h, in the same conditions of the recovery phase (25 °C, 25 ppt, no aeration, in the dark) (Fig. S1).

Sampling for the mechanistic analysis was done at 2 h, 6 h, and 18 h of exposure (Fig. S1). At each sampling point, in each replicate, three samples with a minimum of 4000 rotifers were collected using a sieve with 20 μm of diameter. All samples were immediately frozen in liquid nitrogen and stored at −80 °C. Production of HSP70 was assessed for all sampling points, gene expression was evaluated at 6 h, and total acetylation of histone H3 was determined for 6 h and 18 h of exposure to hydrogen peroxide.

To validate the positive effect of NLHS when exposed to other stressor later in life on a larger scale, 90 min after the beginning of the exposure, some neonates from each replicate of both Reference and NLHS treatments were transferred to a 48-wells microplate, six replicates of 5 neonates per treatment, with 500 μL of medium per well. The microplate was incubated in the same conditions as in the 1 L Schott flasks. After a total of 24 h of exposure to hydrogen peroxide, differences in swimming capacity between treatments were determined (Fig. S1). Swimming capacity was assessed as described in Granada et al. (2023). The scale-up test was considered valid if the swimming capacity of the organisms from NLHS treatment was statistically higher when compared to Reference treatment ($P < 0.05$).

2.4.2. RNA, proteins, and histone extraction

Total RNA, proteins, and histones were extracted as described in Granada et al. (2025). Total RNA and proteins were extracted from the same replicates using TRIzol® reagent (Invitrogen, Belgium), and total histones were extracted using the Histone Extraction Kit (ab113476; Abcam, UK), according to the manufacturer's instructions.

The quantity and quality of the extracted RNA was determined by performing spectrometric analyses at 230, 260, and 280 nm using the Nanodrop 2000 (Thermo Scientific, USA), and its contamination with gDNA was determined using the Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, USA). RNA integrity of all samples was validated in a 1 % agarose gel electrophoresis (as in Granada et al., 2025).

Total protein and histones were quantified by Comassie Plus™ (Bradford) Assay Kit (Thermo Scientific, USA) at 595 nm, and its integrity was verified by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Granada et al., 2025).

2.4.3. Gene expression

Total RNA was converted into cDNA as described by Granada et al. (2025). To evaluate the effect of the NLHS on the rotifers' capacity to cope with oxidative stress derived from H_2O_2 exposure, the primer sequences were designed for 9 target genes related with antioxidant responses, cellular damage, and epigenetic proteins involved in histone and DNA modifications, plus 1 housekeeping gene, using Oligo Explorer software (version 1.4, Gene Link), according to gene sequences obtained from National Center for Biotechnology Information (NCBI) database. Primer sequences, respective properties and concentrations used are presented in Table S1. Specificity and efficiency of all primer sets were assessed and carried out as described by Granada et al. (2025). Amplification reactions were performed in triplicates for all samples on a 96-well plate (Biorad, Multiplate® PCR Plates) using iTaq™ Universal SYBR® Green Supermix (Biorad) on a CFX Connect™ Real-Time PCR System (BioRad). For the amplification reactions, Master Mixes were prepared including 4 μL of nuclease-free water, 2 μL of both forward and reverse primers, and 10 μL of iTaq™ Supermix. For each well, 18 μL of Master Mix were added to 2 μL of the cDNA template, making a total of 20 μL in each well. Reaction conditions consisted of one initial cycle of 30 s at 95 °C (activation step), and 40 cycles of a combined denaturation (5 s at 95 °C) and annealing (30 s at 60 °C) step. Melting curves were generated by an additional cycle at 65 °C for 5 s, followed by increasing steps of 0.5 °C, and a final cycle for 5 s at 95 °C. Technical controls were performed to verify the presence of primer dimers (non-template controls, NTC) and the possible influence of gDNA on the qPCR amplification (cDNA synthesis without reverse transcriptase, −RT controls).

Expression values of the target genes were normalized by the expression of 18S ribosomal RNA (*18S rRNA*), the housekeeping gene (HK). The stability of the housekeeping gene was validated by assessing the maximum amplification variation (ΔC_T) between samples, including the different strains and treatments ($\Delta C_T = 1.69$ cycles). The relative gene expression of each target gene, after 6 h of exposure to hydrogen peroxide, was calculated using the equation: Gene expression ratio = $(E_{GOI})^{\Delta Ct\ GOI} / (E_{HK})^{\Delta Ct\ HK}$, where E is the efficiency of the primer for each gene of interest (GOI) and housekeeping genes (HK), and ΔC_T is the difference between the minimum C_T of Reference treatment and the C_T of each sample of the Reference and NLHS treatment, for each strain.

2.4.4. Quantification of HSP70 production

The production of HSP70 was quantified by Western Blot, as described by Granada et al. (2025). HeLa (heat-shocked) cells (6 μg; Enzo Life Sciences, Farmingdale, NY, USA) were loaded in one of the wells of each gel as technical control to later calculate the relative production of HSP70 in the samples. The relative signal intensity was quantified by densitometry, comparing the signal intensity of each sample with the intensity of HeLa cells, using the BioRad Image Lab 6.1 software.

2.4.5. Determination of histone H3 modifications

Histone samples were analyzed using the Histone H3 Total Acetylation Detection Fast Kit (ab131561; Abcam, UK) and the Histone H3 Modification Multiplex Assay Kit (ab185910; Abcam, UK), according to manufacturer's instructions. Histone H3 total acetylation was measured, in triplicates, in 1 μg histone samples, at 6 h and 18 h of exposure to hydrogen peroxide, for both Reference and NLHS treatments, for MRS10 and IBA3 strains. The different histone H3 modifications were measured using a single kit with only one measurement per modification, in 100 ng histone samples of the MRS10 strain (the one showing a positive effect of NLHS in the capacity of organisms to respond to the exposure to hydrogen peroxide), at 6 h of exposure to hydrogen peroxide, for both Reference and NLHS treatments.

2.5. Statistical analysis

To assess the impact of NLHS in the ability of rotifers to better cope with other stressors, the response curves of Reference and NLHS treatments were compared. Firstly, 24 h-LC_{50} values (50 % reduction in survival compared to control treatments), and respective 95 % confidence intervals, were calculated based on log concentrations by fitting four-parameter logistic dose-response curves (Y=Bottom+(Top-Bottom)/$(1 + 10^{(\mathrm{LogEC50}-X)*\mathrm{HillSlope}})$), where "Y" is the response (% mortality for LC_{50} estimation), "Bottom" is the basal response (0 %), "Top" is the maximal response (100 %), "X" is the logarithm of concentration, and "HillSlope" is the slope of the logistic curve. Secondly, the global fitting (extra sums of squares F-test) was used to compare the response curves obtained for each stressor between treatments. Survival analysis and global fitting were done using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California, USA). This was done for both strains.

To validate the scale-up experiments, independent samples *t*-tests were performed to compare the swimming capacity between Reference treatment and NLHS treatment. Two-Way ANOVA analyses were performed to determine differences in gene expression using strain (MRS10 and IBA3) and treatment (Reference and NLHS) as fixed factors, and for each strain to determine differences in the HSP70 production or total acetylation of histone H3 using exposure period (three or two sampling points, respectively) and treatment (Reference and NLHS) as fixed factors. Homogeneity of variances and data normality were checked

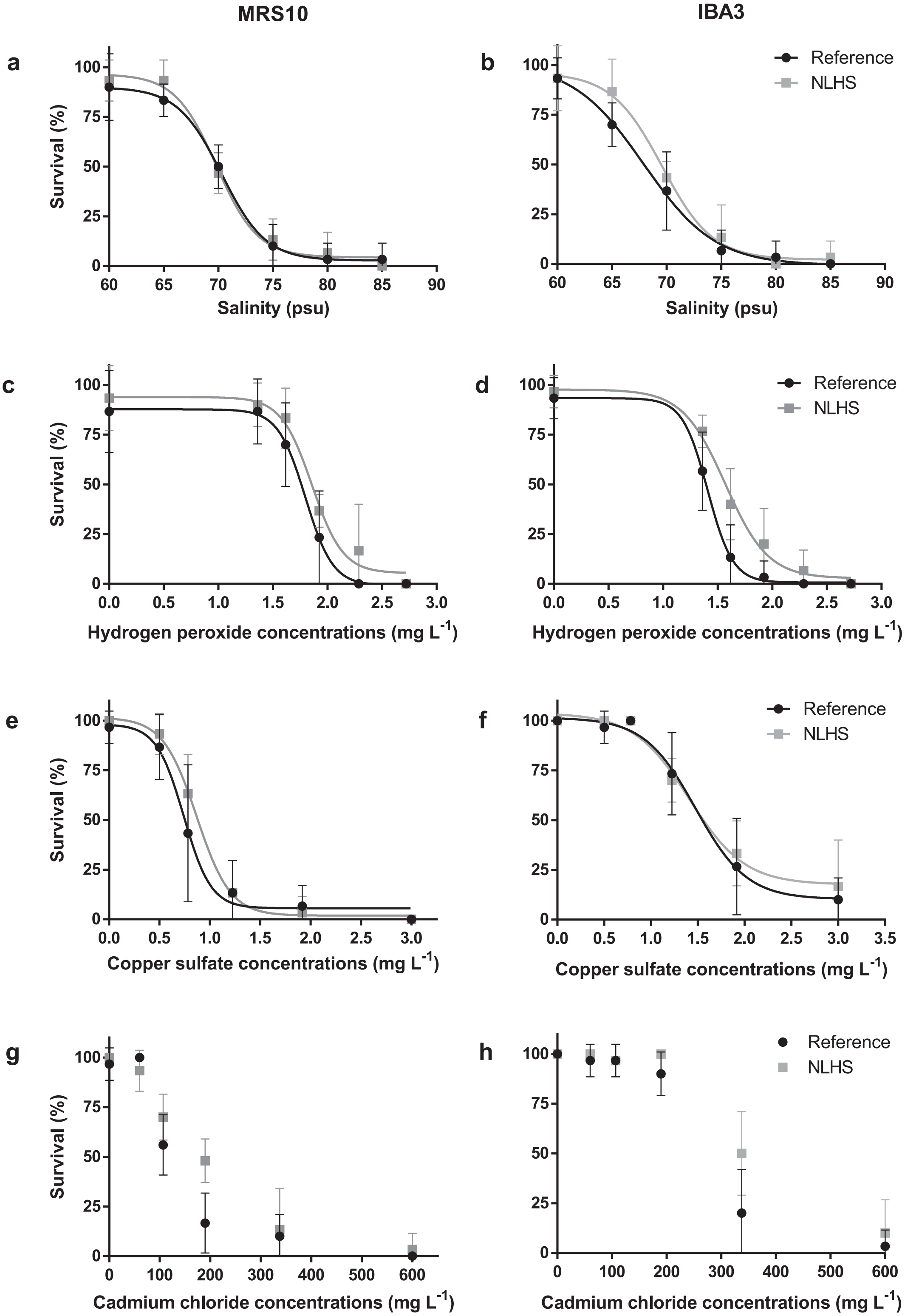

Fig. 1. Dose-response curves representing the survival effects (%) of reference (maintained at control conditions) and non-lethal heat shocked (NLHS) MRS10 (a, c, e, g) and IBA3 (b, d, f, h) rotifers (*Brachionus koreanus*), when challenged with exposure to: a–b) high salinity; c–d) hydrogen peroxide; e–f) copper sulfate; g–h) cadmium chloride.

through Levene's test and Shapiro-Wilk test, respectively. LSD test was used for post hoc comparisons. These calculations were performed using IBM SPSS Statistics version 28 for Windows. For all these tests, the significance level was set at $P \leq 0.05$.

Results are presented as mean ± SD. All graphics were made using IBM SPSS Statistics version 28 for Windows or GraphPad Prism version 6.00 for Windows.

3. Results

3.1. Effects of NLHS on rotifers' tolerance to several stressors

All lethality tests fulfilled the validity criteria as described by the standard guideline (ISO 19820:2016). The 24 h-LC_{50} values, along with the global fitting comparisons between treatments are given in Table S2.

Concerning the exposure to high salinity conditions, responses between Reference and NLHS treatments did not show significant differences in the case of MRS10 ($F_{2,68} = 0{,}5027$, $P = 0.6071$). However, in the case of IBA3, organisms that were exposed to a previous NLHS treatment showed increased tolerance to the high salinity conditions ($F_{2,68} = 3412$, $P = 0.0387$) (Fig. 1a, b; Table S2).

Likewise, when MRS10 were exposed to hydrogen peroxide, no differences were observed between Reference and NLHS treatments ($F_{2,68} = 3101$, $P = 0.0514$), while for IBA3 the organisms previously exposed to a NLHS treatment showed increased tolerance to this stressor ($F_{2,68} = 16{,}36$, $P < 0.0001$) (Fig. 1c, d; Table S2).

When exposed to copper sulfate, neither MRS10 ($F_{2,68} = 2999$, $P = 0.0565$) nor IBA3 ($F_{2,68} = 0{,}7830$, $P = 0.4611$) showed significant differences between the response of Reference and NLHS treatments to this stressor (Fig. 1e, f; Table S2).

Regarding cadmium chloride, both MRS10 ($F_{2,68} = 8342$, $P = 0.0006$) and IBA3 ($F_{2,68} = 12{,}50$, $P < 0.0001$) showed increased tolerance to this stressor after being exposed to the NLHS (Fig. 1g, h; Table S2).

3.2. Molecular mechanisms responsible for cross-tolerance

3.2.1. Validation of the scale-up experiment

During the scale-up experiments, MRS10 and IBA3 neonates were subjected to a NLHS (at 41 °C and 42 °C, respectively) and were subsequently exposed to the Reference LC_{20} of hydrogen peroxide (1.71 mg L^{-1} and 2.23 mg L^{-1}, respectively). To validate the cross-tolerance effects of the NLHS against hydrogen peroxide during the scale-up experiments, differences in behaviour between Reference and NLHS treatments were recorded after the 24 h of exposure (Table 1). For MRS10, it was possible to observe that rotifers exposed to the NLHS had a better swimming capacity after the exposure to hydrogen peroxide ($P = 0.021$). For IBA3, NLHS did not significantly influenced the capacity of rotifers to cope with hydrogen peroxide in terms of swimming behaviour (Table 1). Discrimination of rotifers as swimming, adhesive, and inactive are presented in Table S3.

3.2.2. Mechanisms of NLHS-induced tolerance to H_2O_2

3.2.2.1. General stress responses. Relative expression of genes related to oxidative stress (catalase, superoxide dismutase, and glutathione peroxidase) and cellular damage (autophagy-related protein 2) in organisms under the Reference or NLHS treatments are shown in Fig. 2. Data showed that, for MRS10, after 6 h of exposure to H_2O_2, *MnSOD* was significantly up-regulated in rotifers previously exposed to NLHS ($P = 0.006$) in comparison with Reference treatment. Although not significant, the same trend for an up-regulation in NLHS treatment was seen in MRS10 for the other genes (*CAT*, *GPx*, and *ATG2*), while for IBA3 there were no significant differences between treatments and even an opposite trend for a down-regulation in *GPx* expression under the NLHS treatment was observed.

Table 1
Average swimming velocity (μm s^{-1}) (±SD) of reference (maintained at control conditions) and non-lethal heat shocked (NLHS) MRS10 and IBA3 rotifers (*Brachionus koreanus*) after exposure for 24 h to the Reference's LC_{20} of hydrogen peroxide for validation of scale-up cross-tolerance tests.

Strains	Reference	NLHS
MRS10	43.56 ± 15.23	96.64 ± 13.71*
IBA3	13.14 ± 10.58	44.72 ± 65.86

* Significance between Reference and NLHS treatments ($P < 0.05$).

Western blot results showed a higher production of HSP70 in MRS10 under NLHS treatment in comparison to Reference treatment after exposure to hydrogen peroxide during all the time points assessed ($P < 0.05$) (Figs. 3a, S2). For IBA3, there were only statistically significant differences between treatments after 2 h of exposure to hydrogen peroxide ($P = 0.017$), with production of HSP70 in NLHS treatment showing a tendency to decrease along the exposure period (Figs. 3b, S2). However, no statistically significant differences were observed between time points within treatments, in both strains.

3.2.2.2. Epigenetic markers. The effect of H_2O_2 exposure in rotifers after being subjected, or not, to a NLHS was also studied at the level of gene expression related to epigenetic markers (Fig. 4), including genes encoding histone lysine methyltransferase (*KMT3E*), demethylase (*KDM1*), acetyltransferase (*KAT6*), and deacetylase (*SIRT6*), and a DNA methyltransferase (*DNMT2*). For MRS10, genes *KMT3E*, *KDM1*, and *KAT6* were significantly up-regulated ($P < 0.05$) in heat shocked rotifers, after 6 h of exposure to H_2O_2. There were no differences in gene expression between treatments for IBA3 also for this set of genes (Fig. 4).

Results of total acetylation of histone H3 for Reference and NLHS treatments at 6 h and 18 h of exposure to H_2O_2 can be seen in Fig. 5. For MRS10, data showed an increase of H3 acetylation levels in heat shocked rotifers ($P < 0.001$) at 6 h of exposure, with a decrease to Reference levels at 18 h (Fig. 5a). NLHS did not alter significantly the acetylation levels of histone H3 in IBA3 when exposed to H_2O_2 (Fig. 5b).

Based on the evidence presented earlier from the gene expression and histone H3 acetylation results, showing that NLHS did not result in epigenetic modifications in IBA3, a comprehensive analysis of various histone H3 modifications were exclusively assessed for MRS10, for 6 h of exposure to H_2O_2 (Fig. 6). A total of 21 histone H3 modifications were measured using a multiplex assay kit, including 15 lysine methylations, 4 lysine acetylations, and 2 serine phosphorylations. In general, the majority of lysine methylation and acetylation levels appeared to be higher in NLHS treatment, while higher levels of serine phosphorylation were observed in Reference treatment, although no statistical analysis was possible.

4. Discussion

As the exposure to heat stress showed positive effects in the survival of rotifers when subjected to lethal temperatures later in life (Granada et al., 2025), understanding how this can also improve the capacity of organisms to cope with other stressors relevant for aquaculture would be very advantageous for this industry. Using two strains of *B. koreanus*, this study provided evidence that the exposure to a single NLHS results in increased acute tolerance to several abiotic stressors, and that epigenetic mechanisms are involved in the achievement of cross-tolerance to hydrogen peroxide, a therapeutant widely used in aquaculture to prevent external fungal and bacterial infections in farmed fish and respective eggs (Schmidt et al., 2006).

4.1. NLHS-induced cross-tolerance

Induced tolerance to high salinity and hydrogen peroxide was

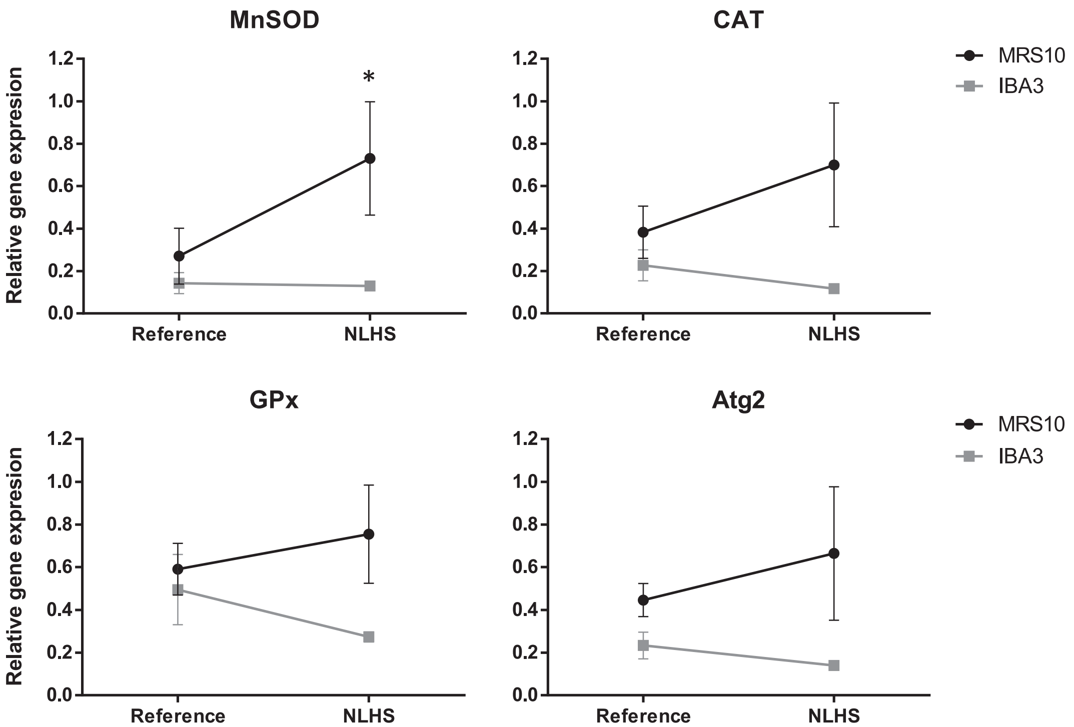

Fig. 2. Relative gene expression of oxidative stress (superoxide dismutase – *MnSOD*, catalase – *CAT*, glutathione peroxidase – *GPx*) and cellular damage (autophagy-related protein 2 – *Atg2*) related genes in Reference (maintained at control conditions) and non-lethal heat shocked (NLHS) MRS10 and IBA3 rotifers (*Brachionus koreanus*), after 6 h of exposure to the Reference's LC_{20} of hydrogen peroxide. * indicates statistical significance between treatments, using a housekeeping gene as internal standard ($P < 0.05$).

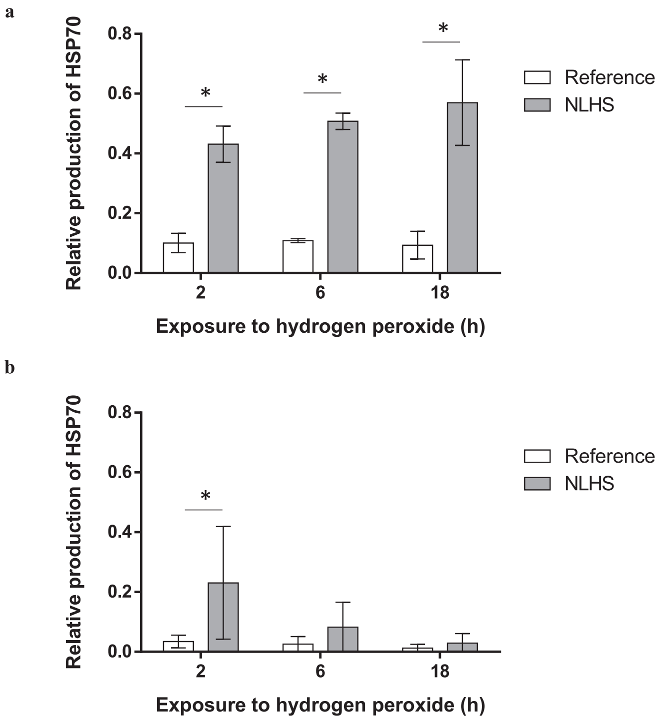

Fig. 3. Relative quantification of HSP70 production levels in a) MRS10 and b) IBA3 rotifers (*Brachionus koreanus*), maintained at control conditions (Reference) and exposed to a non-lethal heat shock (NLHS), after 2, 6 and 18 h of exposure to the Reference's LC_{20} of hydrogen peroxide. HeLa (heat shocked) cells were used as positive control and for calculating the relative amount of HSP70 in each sample. * indicates statistical significance between Reference and NLHS treatments ($P < 0.05$).

successfully achieved for IBA3, and to cadmium chloride for both strains (Fig. 1, Table S2). The fact that NLHS allowed to induce cross-tolerance to more stressors for IBA3 may be related to the molecular alterations that occurred during the recovery period in consequence of acute heat shock exposure. In the study by Granada et al. (2025), it was possible to observe that, for IBA3, NLHS resulted in higher levels of HSP70 production and fold change expression of *Hsp40* in the NLHS treatment, comparing to MRS10. It has been already shown for other species that induction of HSP70 upon NLHS resulted in increased tolerance to other stressors, such as ammonia and heavy metals (Pestana et al., 2016; Sung et al., 2018), and that one of the main roles of HSP40 in *Brachionus* sp. may be the regulation of HSP70 activity (Paraskevopoulou et al., 2020; Smith et al., 2012). Moreover, IBA3 showed an up-regulation in *Sirt6* expression immediately after the NLHS, which was not observed for MRS10 (Granada et al., 2025). Sirtuin 6 is a histone lysine deacetylase involved in repair of DNA damage and resistance to oxidative stress (Beauharnois et al., 2013). This may be an indication that histone epigenetic alterations play a major role in cross-tolerance in *B. koreanus*.

4.2. *Mechanisms involved in increased tolerance to hydrogen peroxide after NLHS*

After successfully achieving increased tolerance to three of the four studied stressors, scale-up experiments were performed with hydrogen peroxide to understand the molecular mechanisms involved in induced cross-tolerance mediated by NLHS. In the previous experiments, NLHS showed to induce tolerance to hydrogen peroxide in only one of the strains (Fig. 1c, d); this way, by comparing the results on molecular mechanisms between strains, it would be possible to understand if the differences observed in the mechanisms studied between Reference and NLHS treatments could be a direct effect of NLHS. As the purpose of the scale-up experiments was to observe responses throughout the exposure to hydrogen peroxide, organisms were exposed to the previously calculated LC_{20} for this compound for the Reference treatment, for each

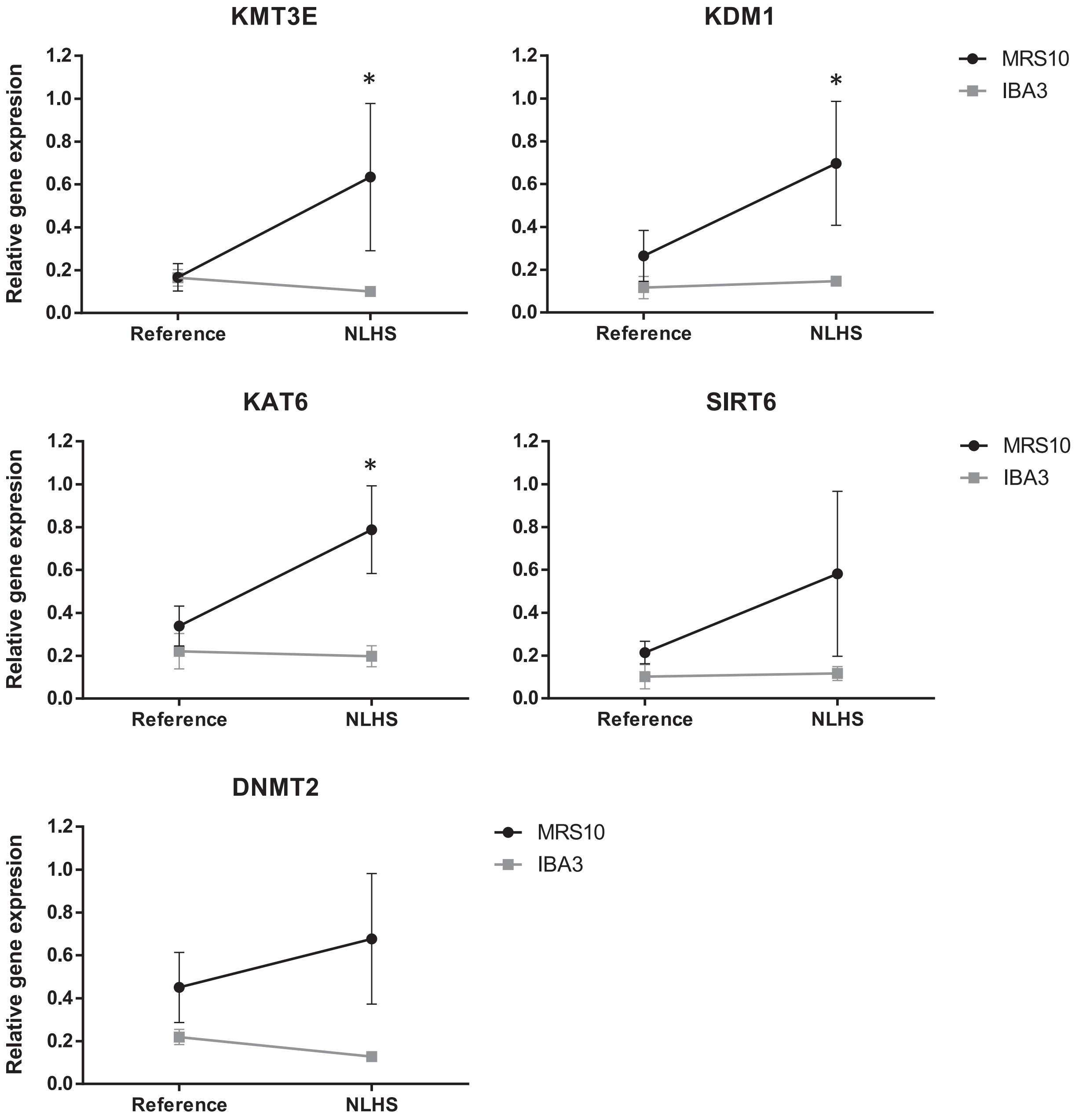

Fig. 4. Relative gene expression of histone H3 modifications (histone methyltransferase – *KMT3E*, histone demethylase – *KDM1*, histone acetyltransferase – *KAT6*, histone demethylase – *SIRT6*) and DNA methylation (DNA methyltransferase – *DNTM2*) related genes in Reference (maintained at control conditions) and non-lethal heat shocked (NLHS) MRS10 and IBA3 rotifers (*Brachionus koreanus*), after 6 h of exposure to the Reference's LC_{20} of hydrogen peroxide. * indicates statistical significance between treatments, using a housekeeping gene as internal standards ($P < 0.05$).

strain, and experiments were validated through a behavioural parameter.

Swimming capacity was the behavioural parameter assessed to validate the effect of NLHS in the capacity of rotifers to cope with hydrogen peroxide. Contrarily to what was expected considering the previous results (Fig. 1c, d; Table S2), cross-tolerance was successfully induced for MRS10 and not IBA3, with MRS10 presenting a statistically significant higher swimming capacity after the exposure to hydrogen peroxide in NLHS treatment compared to Reference treatment (Table 1). Contrary to the rotifer cultures used prior to the experiments aimed at determining the NLHS conditions and their capacity to induce cross-tolerance, the conditions of the cultures used for the scale-up experiments were slightly different, including greater volumes and densities, and consequent aeration. Even though the final density of rotifers during the NLHS and recovery period was the same in both experimental setups, it is possible that the culturing conditions prior to the experiments could have interfered with the effect of the NLHS in rotifers' tolerance.

As expected, the values of swimming velocity observed after the exposure of the organisms to hydrogen peroxide, and especially under the reference treatment, were considerably lower than the values observed under control conditions for MRS10 and IBA3 strains (43.56 versus 138.00 $\mu m\ s^{-1}$ and 13.14 versus 150.67 $\mu m\ s^{-1}$, respectively) (Granada et al., 2023).

The analysis of molecular mechanisms showed that the exposure of MRS10 rotifer neonates to a single NLHS, after 6 h of exposure to hydrogen peroxide, enhanced the expression of *MnSOD* (Fig. 2), *KMT3E*, *KDM1*, and *KAT6* (Fig. 4) genes, and the levels of total acetylation of histone H3 (Fig. 5), along with the synthesis of HSP70 throughout the entire exposure period (Fig. 3a). These molecular alterations in the case of MRS10 strain were also followed by an increased swimming capacity

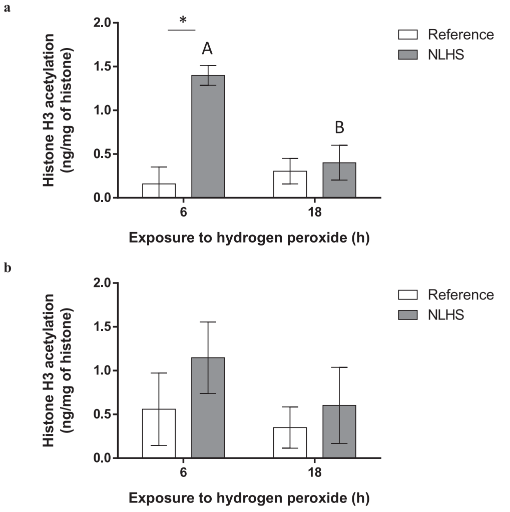

Fig. 5. Histone H3 total acetylation in a) MRS10 and b) IBA3 rotifers (*Brachionus koreanus*), maintained at control conditions (Reference) and exposed to a non-lethal heat shock (NLHS), after 6 and 18 h of exposure to the Reference's LC_{20} of hydrogen peroxide. * indicates statistical significance between Reference and NLHS treatments within each time point, and different capital letters indicate significance between time points for NLHS treatment ($P < 0.05$).

of up to two-fold, when compared to the Reference treatment. On the contrary, in the case of IBA3, results of the swimming capacity at the end of the experiment showed that cross-tolerance was not achieved for this strain, and this was corroborated by the lack of significant changes in the addressed molecular mechanisms between treatments for this strain.

Hydrogen peroxide generates intracellular non-radical reactive oxygen species (ROS) (Yang et al., 2013). Aerobic organisms have detoxification processes in which superoxide dismutase (SOD) converts superoxide anion into oxygen and hydrogen peroxide, which catalase (CAT) breaks down into water and oxygen; glutathione peroxidase (GPx) is also an alternative enzymatic route for the degradation of hydrogen peroxide into harmless molecules (Lemos, 2021). In this study, MRS10 showed an up-regulation of *MnSOD* in heat shocked organisms after exposure to hydrogen peroxide in comparison with the rotifers that did not go through a previous NLHS (Reference) (Fig. 2). Although a pattern of up-regulation was also observed for the other oxidative stress related genes, no differences were found for *CAT* and *GPx* between NLHS and Reference treatments (Fig. 2). In *Brachionus* sp., previous studies have shown that the exposure to oxidative stress produced by different substances (juglone, paraquat, hydrogen peroxide) can induce the expression of MnSOD and catalase (Kailasam et al., 2011; Kaneko et al., 2011; Yang et al., 2013). Zhang et al. (2016) observed that the exposure to polybrominated diphenyl ethers resulted in up-regulation of SOD in *Brachionus* sp. but had no effects on expression levels of catalase. On the other hand, Han et al. (2020) showed that rapid temperature changes resulted in increased levels of ROS in *B. plicatilis*, with no changes in levels of SOD but leading to a decrease in levels of catalase and an increase in levels of glutathione S-transferase (GST). The authors suggested that, although GST is involved in conjugation of xenobiotics, it may also play an important part in the mechanism involved in protection against oxidative stress caused by rapid temperature changes in *B. plicatilis* (Han et al., 2020). Therefore, the exposure to NLHS may have not only triggered a heat shock response (Granada et al., 2025), but also initiated the oxidative stress and consequent antioxidant response, that may have been advantageous to cope with the ROS further generated by the hydrogen peroxide exposure. Besides the antioxidant system, autophagy is also an important process in the response of organisms to toxicants. Autophagy-related gene 2 (*Atg2*) is involved in the formation of pre-autophagosomal structures and has shown to be up-regulated in response to cadmium-induced oxidative stress in *B. koreanus* (Kang et al., 2018). In the present study, however, NLHS showed to have no increased effect in *Atg2* expression levels in response to hydrogen peroxide exposure (Fig. 2).

Concerning heat shock response, previous results have already demonstrated that the exposure to NLHS induces the up-regulation of several *hsp* genes and increases the production of HSP70 in both rotifer strains (Granada et al., 2025). In the present study, MRS10 heat shocked rotifers showed a higher production of HSP70 than organisms from the Reference treatment at all time points assessed (Fig. 3a). However, there was no evidence for an increase in HSP70 levels along the exposure to hydrogen peroxide in NLHS treatment. This may suggest that the increase in HSP70 production was mainly due to the NLHS, as differences between NLHS and Reference treatments were achieved in MRS10 at the end of recovery period (seen in Granada et al., 2025, Fig. 5a), but the subsequent exposure to hydrogen peroxide maintained this altered state and may have contributed to the higher tolerance. This is corroborated by the fact that, although a higher production of HSP70 was also seen in IBA3 heat shocked rotifers at 2 h of exposure to hydrogen peroxide (Fig. 3b), these levels were not sustained during the rest of the exposure period. This suggests that when the exposure to a second stressor maintains or enhances the alterations resultant from the NLHS, the establishment of a positive phenotypic outcome is possible. This also suggests that HSP70 has an important role in the successful induction of

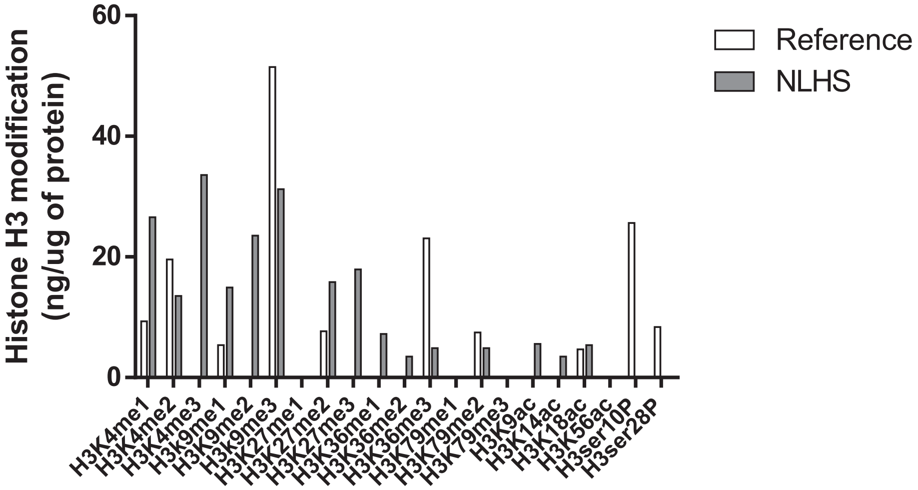

Fig. 6. Histone H3 modifications in MRS10 rotifers (*Brachionus koreanus*), maintained at control conditions (Reference) and exposed to a non-lethal heat shock (NLHS), after 6 h of exposure to the Reference's LC_{20} of hydrogen peroxide.

cross-tolerance in MRS10.

The effect of NLHS during hydrogen peroxide exposure in the expression of genes related to histone modifications, total histone H3 acetylation and other histone H3 modifications was also addressed in this study. Several studies have shown the positive correlation between epigenetic modifications and increased capacity to cope with stress in aquatic organisms (reviewed in Granada et al., 2018). In this study, MRS10 rotifers previously subjected to NLHS, after 6 h of exposure to hydrogen peroxide, showed an up-regulation in genes *KMT3E* (histone methyltransferase), *KDM1* (histone demethylase), and *KAT6* (histone acetyltransferase) (Fig. 4), and an increase in total acetylation levels of histone H3 (Fig. 5). Results from multiple histone H3 modifications (Fig. 6) support these observations, as the majority of lysine methylation and acetylation levels appeared to be higher in NLHS treatment, although with no statistical support. Lee et al. (2020b) characterized histone modification genes and their role in transcriptional regulation in *B. koreanus* under low pH stress conditions. The authors observed a close relation between histone and DNA methylation systems and suggested that *KDM* genes may play an important role in the adaptation capacity of rotifers to several environmental stressors. Concordantly, in the present study, *KMT3E* and *KDM1* were the genes that presented higher fold changes in NLHS treatment (almost up to 4-fold change and 3-fold change, respectively).

In sum, NLHS significantly increased the tolerance to oxidative stress caused by hydrogen peroxide in MRS10. However, NLHS itself did not significantly affect the expression levels of genes related to histone modifications before exposure to hydrogen peroxide (seen in Granada et al., 2025). These results suggest that, despite not being a direct consequence of NLHS, the up-regulation of these enzymes under oxidative stress conditions relies on the previous exposure of rotifers to NLHS. This is concordant with the findings of Kailasam et al. (2011), whose results showed that *B. plicatilis* subjected to caloric restriction had higher tolerance to oxidative stress, associated to increased levels of MnSOD and catalase, but only after the exposure to the oxidative stressor. Additionally, present results on total acetylation of histone H3 showed that levels in heat shocked rotifers decreased to Reference levels by the end of the experiment (Fig. 5a). The exposure of rotifers to a single NLHS proved to be insufficient to cause epigenetic alterations (Granada et al., 2025) and, although a single NLHS showed to induce cross-tolerance mediated in part by epigenetic mechanisms, it remains uncertain whether this can yield a persistent outcome. To address this question, transgenerational studies are needed, as was done by Norouzitallab et al. (2014), that successfully induced tolerance of *Artemia* to several stressors and through three generations, concomitant to modifications in histones H3 and H4, by exposing the organisms to repeated heat shocks. Moreover, it remains uncertain whether the absence of gene expression changes in IBA3 was due to the failure in inducing increased tolerance during the scale-up experiment or due to strain-specific transcriptional regulation. To properly address this topic, it will be necessary to successfully induce increased tolerance in IBA3, in order to compare underlying molecular responses with the ones observed for MRS10.

5. Conclusion

Results demonstrate that the exposure of *B. koreanus* neonates to a non-lethal heat shock can generate cross-tolerance in both MRS10 and IBA3 strains, namely to high salinity, cadmium chloride, and hydrogen peroxide. The increased tolerance to hydrogen peroxide was associated with up-regulation of *MnSOD*, *KMT3E*, *KDM1*, and *KAT6* genes, and increased levels of HSP70 production and total acetylation levels of histone H3. However, it remains to be determined if the conditions of NLHS used can sustain a prolonged effect, and further studies should contribute to understand if a single exposure to heat stress results in improved tolerance to several stressors across various generations in rotifers. As in the previous studies, these results highlight the importance of characterizing strains from the same species, as the culture of strains with more easily induced positive phenotypic traits is more advantageous for aquaculture purposes.

CRediT authorship contribution statement

Luana Granada: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Inês F.C. Morão:** Investigation. **Marco F.L. Lemos:** Writing – review & editing, Resources, Funding acquisition. **Peter Bossier:** Writing – review & editing, Supervision, Resources, Methodology. **Sara C. Novais:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This study had the support of Fundação para a Ciência e a Tecnologia (FCT) through the Strategic Project granted to MARE – Marine and Environmental Sciences Centre (UIDB/04292/2020 and UIDP/04292/2020) and the project granted to the Associate Laboratory ARNET (LA/P/0069/2020). Luana Granada also wishes to acknowledge the financial support given by FCT (SFRH/BD/102036/2014). Sara Novais is funded by national funds (OE), through FCT – Fundação para a Ciência e a Tecnologia, I.P., in the scope of the framework contract foreseen in the numbers 4, 5 and 6 of the article 23, of the Decree-Law 57/2016, of 29 August, changed by Law 57/2017, of 19 July.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2025.110389.

References

Beauharnois, J.M., Bolívar, B.E., Welch, J.T., 2013. Sirtuin 6: a review of biological effects and potential therapeutic properties. Mol. BioSyst. 9, 1789–1806. https://doi.org/10.1039/c3mb00001j.

Dhont, J., Dierckens, K., Støttrup, J., Van Stappen, G., Wille, M., Sorgeloos, P., 2013. Rotifers, Artemia and copepods as live feeds for fish larvae in aquaculture. In: Allan, G., Burnell, G. (Eds.), Advances in Aquaculture Hatchery Technology. Woodhead Publishing Limited, pp. 157–202. https://doi.org/10.1533/9780857097460.1.157.

Granada, L., Lemos, M.F.L., Cabral, H.N., Bossier, P., Novais, S.C., 2018. Epigenetics in aquaculture – the last frontier. Rev. Aquac. 10, 994–1013. https://doi.org/10.1111/raq.12219.

Granada, L., Lemos, M.F.L., Bossier, P., Novais, S.C., 2022. Genetic identification and comparative study on life history parameters of two strains belonging to *Brachionus plicatilis* species complex (Rotifera: Monogononta). Aquac. Rep. 26, 101309. https://doi.org/10.1016/j.aqrep.2022.101309.

Granada, L., Lemos, M.F.L., Bossier, P., Novais, S.C., 2023. Swimming behaviour as an alternative endpoint to assess differences in abiotic stress sensitivities between strains of *Brachionus koreanus* (Rotifera: Monogononta). Environ. Sci. Pollut. Res. 30, 56137–56147. https://doi.org/10.1007/s11356-023-26190-3.

Granada, L., Morão, I.F.C., Lemos, M.F.L., Bossier, P., Novais, S.C., 2025. Molecular mechanisms of induced thermotolerance in two strains of *Brachionus koreanus* (Rotifera: Monogononta). Aquaculture 595, 741125. https://doi.org/10.1016/j.aquaculture.2024.741125.

Rotifers. In: Hagiwara, A., Yoshinaga, T. (Eds.), 2017. Fisheries Science Series. Springer Singapore, Singapore. https://doi.org/10.1007/978-981-10-5635-2.

Han, J., Lee, Jin Sol, Park, J.C., Hagiwara, A., Lee, K.W., Lee, Jae Seong, 2020. Effects of temperature changes on life parameters, oxidative stress, and antioxidant defense system in the monogonont marine rotifer *Brachionus plicatilis*. Mar. Pollut. Bull. 155, 111062. https://doi.org/10.1016/j.marpolbul.2020.111062.

Hua, J., Morehouse, N.I., Relyea, R., 2013. Pesticide tolerance in amphibians: induced tolerance in susceptible populations, constitutive tolerance in tolerant populations. Evol. Appl. 6, 1028–1040. https://doi.org/10.1111/eva.12083.

ISO, 2016. ISO 19820:2016(E) - Water Quality - Determination of the Acute Toxicity to the Marine Rotifer *Brachionus plicatilis*.

Junprung, W., Supungul, P., Tassanakajon, A., 2017. HSP70 and HSP90 are involved in shrimp *Penaeus vannamei* tolerance to AHPND-causing strain of *Vibrio parahaemolyticus* after non-lethal heat shock. Fish Shellfish Immunol. 60, 237–246. https://doi.org/10.1016/J.FSI.2016.11.049.

Kailasam, M., Kaneko, G., Oo, A.K.S., Ozaki, Y., Thirunavukkarasu, A.R., Watabe, S., 2011. Effects of calorie restriction on the expression of manganese superoxide dismutase and catalase under oxidative stress conditions in the rotifer *Brachionus plicatilis*. Fish. Sci. 77, 403–409. https://doi.org/10.1007/s12562-011-0334-y.

Kaneko, G., Yoshinaga, T., Yanagawa, Y., Ozaki, Y., Tsukamoto, K., Watabe, S., 2011. Calorie restriction-induced maternal longevity is transmitted to their daughters in a rotifer. Funct. Ecol. 25, 209–216. https://doi.org/10.1111/j.1365-2435.2010.01773.x.

Kang, H.M., Lee, Jin Sol, Kim, M.S., Lee, Y.H., Jung, J.H., Hagiwara, A., Zhou, B., Lee, Jae Seong, Jeong, C.B., 2018. Genome-wide identification of 99 autophagy-related (Atg) genes in the monogonont rotifer *Brachionus* spp. and transcriptional modulation in response to cadmium. Aquat. Toxicol. 201, 73–82. https://doi.org/10.1016/j.aquatox.2018.05.021.

Kim, H.J., Ohtani, M., Kakumu, A., Sakakura, Y., Hagiwara, A., 2020. External factors that regulate movement in the marine rotifer *Brachionus plicatilis*. Fish. Sci. 86, 655–663. https://doi.org/10.1007/s12562-020-01438-w.

Lee, Y.H., Kang, H.M., Kim, M.S., Lee, Jin Sol, Wang, M., Hagiwara, A., Jeong, C.B., Lee, Jae Seong, 2020a. Multigenerational mitigating effects of ocean acidification on in vivo endpoints, antioxidant defense, DNA damage response, and epigenetic modification in an asexual monogonont rotifer. Environ. Sci. Technol. 54, 7858–7869. https://doi.org/10.1021/acs.est.0c01438.

Lee, Y.H., Kim, M.S., Jeong, H., Hagiwara, A., Lee, J.S., 2020b. Genome-wide identification and transcriptional modulation of histone variants and modification related genes in the low pH-exposed marine rotifer *Brachionus* koreanus. Comp. Biochem. Physiol. Part D Genomics Proteomics 36, 100748. https://doi.org/10.1016/j.cbd.2020.100748.

Lemos, M.F.L., 2021. Biomarker studies in stress biology: from the gene to population, from the organism to the application. Biology (Basel). 10, 1340. https://doi.org/10.3390/biology10121340.

Liang, Y., Gao, T., Shao, L., Min, Y., Yang, J., 2020. Effects of microcystin-LR and nitrite on the lifespan, reproduction, and heat shock responses of rotifer *Brachionus calyciflorus* at different temperatures. Aquat. Sci. 82, 74. https://doi.org/10.1007/s00027-020-00748-6.

Mukhopadhyay, I., Nazir, A., Saxena, D.K., Kar Chowdhuri, D., 2003. Heat shock response: hsp70 in environmental monitoring. J. Biochem. Mol. Toxicol. 17, 249–254. https://doi.org/10.1002/jbt.10086.

Norouzitallab, P., Baruah, K., Vandegehuchte, M., Van Stappen, G., Catania, F., Vanden Bussche, J., Vanhaecke, L., Sorgeloos, P., Bossier, P., 2014. Environmental heat stress induces epigenetic transgenerational inheritance of robustness in parthenogenetic *Artemia* model. FASEB J. 28, 3552–3563. https://doi.org/10.1096/fj.14-252049.

Norouzitallab, P., Baruah, K., Vanrompay, D., Bossier, P., 2019. Can epigenetics translate environmental cues into phenotypes? Sci. Total Environ. 647, 1281–1293. https://doi.org/10.1016/J.SCITOTENV.2018.08.063.

Paraskevopoulou, S., Dennis, A.B., Weithoff, G., Tiedemann, R., 2020. Temperature-dependent life history and transcriptomic responses in heat-tolerant versus heat-sensitive *Brachionus* rotifers. Sci. Rep. 10, 1–15. https://doi.org/10.1038/s41598-020-70173-0.

Pestana, J.L.T., Novais, S.C., Norouzitallab, P., Vandegehuchte, M.B., Bossier, P., De Schamphelaere, K.a.C., 2016. Non-lethal heat shock increases tolerance to metal exposure in brine shrimp. Environ. Res. 151, 663–670. https://doi.org/10.1016/j.envres.2016.08.037.

Rahman, M.M., Wille, M., Cavalli, R.O., Sorgeloos, P., Clegg, J.S., 2004. Induced thermotolerance and stress resistance in larvae of the freshwater prawn, *Macrobrachium rosenbergii* (de Man, 1879). Aquaculture 230, 569–579. https://doi.org/10.1016/J.AQUACULTURE.2003.10.010.

Schmidt, L.J., Gaikowski, M.P., Gingerich, W.H., 2006. Environmental Assessment for the Use of Hydrogen Peroxide in Aquaculture for Treating External Fungal and Bacterial Diseases of Cultured Fish and Fish Eggs, United States Geological Survey, Biological Resources Division Upper, Upper Midwest Environmental 54603, pp. 11545–11550. https://doi.org/10.1073/pnas.1411607111.

Slimen, I.B., Najar, T., Ghram, A., Dabbebi, H., Ben Mrad, M., Abdrabbah, M., 2014. Reactive oxygen species, heat stress and oxidative-induced mitochondrial damage. A review. Int. J. Hyperth. 30, 513–523. https://doi.org/10.3109/02656736.2014.971446.

Smith, H.a., Burns, A.R., Shearer, T.L., Snell, T.W., 2012. Three heat shock proteins are essential for rotifer thermotolerance. J. Exp. Mar. Biol. Ecol. 413, 1–6. https://doi.org/10.1016/j.jembe.2011.11.027.

Snell, T.W., Johnston, R.K., Jones, B.L., 2019. Hypoxia extends lifespan of *Brachionus manjavacas* (Rotifera). Limnetica 38, 159–166. https://doi.org/10.23818/limn.38.04.

Suga, K., Tanaka, Y., Sakakura, Y., Hagiwara, A., 2011. Axenic culture of *Brachionus plicatilis* using antibiotics. Hydrobiologia 662, 113–119. https://doi.org/10.1007/s10750-010-0488-0.

Sung, Y.Y., Rahman, N.A., Shazili, N.A.M., Chen, S., Lv, A., Sun, J., Shi, H., MacRae, T.H., 2018. Non-lethal heat shock induces Hsp70 synthesis and promotes tolerance against heat, ammonia and metals in post-larvae of the white leg shrimp *Penaeus vannamei* (Boone, 1931). Aquaculture 483, 21–26. https://doi.org/10.1016/J.AQUACULTURE.2017.09.034.

Wang, Q., Wei, W., Liu, Y., Zheng, Z., Du, X., Jiao, Y., 2019. HSP40 gene family in pearl oyster *Pinctada fucata martensii*: genome-wide identification and function analysis. Fish Shellfish Immunol. 93, 904–910. https://doi.org/10.1016/j.fsi.2019.08.029.

Xiang, X.L., Jiang, R.M., Chen, Y.Y., Ge, Y.L., Wen, X.L., Xi, Y.L., 2017. Regulation of heat shock protein 70 (Hsp70) levels in the bdelloid rotifer *Rotaria rotatoria* under temperature stress. Isr. J. Ecol. Evol. 63, 69–77. https://doi.org/10.1163/22244662-06301001.

Yang, Jianghua, Dong, S., Jiang, Q., Si, Q., Liu, X., Yang, Jiaxin, 2013. Characterization and expression of cytoplasmic copper/zinc superoxide dismutase (CuZn SOD) gene under temperature and hydrogen peroxide (H_2O_2) in rotifer Brachionus calyciflorus. Gene 518, 388–396. https://doi.org/10.1016/j.gene.2012.12.101.

Zhang, J., Wang, Y., Sun, K.M., Fang, K., Tang, X., 2016. A study of oxidative stress induced by two polybrominated diphenyl ethers in the rotifer *Brachionus plicatilis*. Mar. Pollut. Bull. 113, 408–413. https://doi.org/10.1016/J.MARPOLBUL.2016.10.032.