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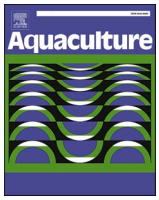

Molecular mechanisms of induced thermotolerance in two strains of *Brachionus koreanus* (Rotifera: Monogononta)

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ABSTRACT

Temperature plays an important role in the occurrence and performance of organisms in aquatic ecosystems and is also one of the main environmental factors affecting species' survival and growth in aquaculture. As an important species for aquaculture sustainability, the rotifer *Brachionus* sp. benefits from inducible phenotypic traits that allow the organisms to cope with environmental stress. The exposure to high temperature has shown to increase production of heat shock proteins (HSPs) and histone modifications in several organisms, resulting in induced thermotolerance. This study aimed to evaluate the potential of non-lethal heat shock (NLHS) to induce thermotolerance in two strains of *B. koreanus* and pinpoint some of the molecular mechanisms involved in the process. Exposure of organisms to 42 °C for 30 min, with a subsequent recovery at 25 °C for 8 h demonstrated to increase thermotolerance in up to three-fold when organisms were, in a posterior phase, subjected to a lethal temperature. This study also showed that one of the strains is tendentially more thermotolerant than the other. Indeed, NLHS exposure resulted in increased mRNA expression of different *Hsp* genes and production of HSP70 in general, but different patterns of expression were observed between strains. However, a single NLHS showed to have no effects in epigenetic mechanisms, suggesting that the induced capacity to tolerate heat stress was transient and some more cycles of NLHS may be needed to promote a persistent effect.

1. Introduction

In aquatic ecosystems, temperature has a great impact in the occurrence, performance, and evolution of organisms. Thermal history to which a population has been exposed, especially in zooplankton, plays a major role in the life-history strategies of the organisms (Liang et al., 2020; Xiang et al., 2017). Rotifers comprise several species complexes. Formerly considered generalists and widely distributed, it is now known that this group encompass cryptic species with more limited distribution, being temperature one of the main ecological constraints (Paraskevopoulou et al., 2020). Several studies have shown that temperature strongly affects lifespan, survival and fecundity of rotifers and, subsequently, population growth rate (Johnston and Snell, 2016; Paraskevopoulou et al., 2020).

In aquaculture, regulation of water temperature is of main importance for survival and growth of cultured species, and undesired variations in this environmental factor can cause serious biological and economic losses (Wang et al., 2019). Among the monogonont rotifers, several *Brachionus* spp. are grown commercially to be used as live feed for several marine species larvae (Lawrence et al., 2012). The study of life-history characteristics of different rotifer lineages and strains under different environmental settings allows to determine the best culture parameters and avoid production crashes (Dooms et al., 2007). As crashes and disease outbreaks are a significant limitation to the development of aquaculture sector (Baruah et al., 2015), and being rotifers crucial to a production of aquatic seedling independent of wild resources (Hagiwara and Yoshinaga, 2017), it is extremely valuable to study conditions that allow inducing more advantageous phenotypic traits (Hagiwara et al., 2007).

According to Hua et al. (2013), induced tolerance can be more likely achieved by exposing organisms to environmental variations, since the maintenance of inducible traits enable a continuous adaptive response to environmental stress. Contrariwise, a continuous exposure to altered conditions can result in constitutive tolerance and loss of inducible traits

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(Hua et al., 2013). It has been proven that exposure to acute non-lethal heat shock (NLHS) improves survival to not only subsequent heat stress (Norouzitallab et al., 2014), but also ammonia (Sung et al., 2014), pathogen infections (Junprung et al., 2017), toxic compounds (Pestana et al., 2016), and osmotic stress (Rahman et al., 2004) in different species.

Induced tolerance is achieved mainly through the heat shock response triggered by NLHS, which generally includes an increased mRNA expression of heat shock proteins (HSPs), a well conserved mechanism found in organisms from bacteria to mammals (Liang et al., 2020). HSPs are considered molecular chaperones that, by interacting with stress-induced denatured proteins, repair and minimize macromolecular damage. HSPs comprise several families of proteins with different molecular weights (kDa): small HSP (sHSP), HSP10, HSP40, HSP60, HSP70, and HSP90 (Feder and Hofmann, 1999). Besides heat stress, production of these proteins is also activated by exposure to other stressors, such as hypoxia and food deprivation (Cara et al., 2005), chemical compounds (Baruah et al., 2015; Kim et al., 2016), or UV-B radiation (Kim et al., 2011). Consequently, HSPs are considered stress proteins and have been extensively used as bioindicators of environmental stress in many organisms, including rotifers (Ravaux et al., 2016; Smith et al., 2012).

Phenotypic alterations can also be induced by epigenetic mechanisms in response to environmental cues. Post-translational modification of histone proteins (e.g., methylation and acetylation) is a major epigenetic mechanism that is able to control gene activity without changing DNA nucleotide sequence, by regulating the association between histones and DNA (Granada et al., 2018). This mechanism has shown to increase phenotypic variability in aquatic organisms (Lee et al., 2020b). For instance, Norouzitallab et al. (2014) showed that exposure to NLHS can alter histones H3 and H4 acetylation patterns in *Artemia* sp., with positive phenotypic outcome, such as increased thermotolerance, with effects lasting several generations. Histone modifications have shown to play a key role in multigenerational plasticity to ocean acidification in rotifers (Lee et al., 2020a), and there is evidence that this is the main epigenetic mechanism in these organisms (Lee et al., 2020b).

Considering all the above, the main objective of the present study was to determine if increased thermotolerance could be achieved through the exposure to heat stress in rotifer *B. koreanus*, and identify some molecular mechanisms involved in this phenotypic alteration. Besides its importance to aquaculture, the fact that *Brachionus* sp. reproduction occurs mainly via parthenogenesis, originating female clones (Gómez et al., 1997), makes this species a good model to study the influence of early environment on several mechanisms such as epigenetic modifications and gene expression, and subsequently in the tolerance of these organisms to non-optimal conditions. Therefore, in this study the expression of genes encoding several HSP and histone modifications, the production of HSP70, and the determination of histone H3 total acetylation were assessed. Moreover, given the fact that different clones or strains of the same species may have different tolerances (Rico-Martínez et al., 2016), two strains of *B. koreanus* were used and the outcomes of NLHS exposure and underlying mechanisms compared.

2. Material and methods

2.1. Rotifer cultures

Living samples of two rotifer strains (MRS10 and IBA3) of *Brachionus koreanus* were obtained from the Laboratory of Aquaculture and Artemia Reference Center (Ghent University, Belgium). Species identification was previously confirmed by amplifying a portion of the 16S rRNA gene (Granada et al., 2022). One clone culture from each strain (MRS10.5 and IBA3.4), isolated in 2017 from individual females carrying at least one amictic egg, were used in this study. Clone cultures were maintained in stocks, in falcon tubes with 30 mL of autoclaved 25 PSU artificial seawater (ASW, Instant Ocean Sea Salt with deionized water) and placed in front of cool white tube lights with a constant light intensity of 2500 Lux and at a room temperature of 25 ± 1 °C. Every two weeks, stock cultures were restarted, and clone cultures cleaned. Cultures were fed daily with *Tetraselmis* sp. at a final concentration of 10^5 cells mL^{-1}. This microalgae was semi-continuously cultured in autoclaved 32 PSU natural seawater with F/2 medium, with a 16:8 light:dark photoperiod of 3000 Lux LED lights. Before feeding the rotifers, the microalgae were concentrated by centrifugation and stored at 4 °C (Granada et al., 2022; Xiang et al., 2016). Invertebrate animals used for this study do not require specific approval from the ethical committee.

2.2. Determination of non-lethal heat shock conditions

2.2.1. Lethal temperature

To determine the temperature causing 50% lethality (LT_{50}) to each rotifer strain, 0–3 h neonates were exposed to six different temperature treatments for 30 min, ranging from 43.5 °C to 46 °C. Organisms were exposed inside 0.2 mL tubes with 100 μL ASW 25 PSU (30 organisms per tube), in the dark, using a T100 PCR Thermal Cycler (Bio Rad). Control organisms (Reference treatment) were maintained at 25 °C. After the heat treatment, all organisms were transferred to control conditions (25 °C, ASW 25 PSU) in 48-well microplates with 1 mL ASW, placed in the dark, and mortality was determined 6 h later (Sung et al., 2018). Each temperature treatment consisted of six replicates with 10 neonates per replicate. Rotifers were considered dead if not showing any movement during 10 s of observation (ISO 19820:2016). At the end of the experiment, the highest temperature that did not cause any effects (in survival and behavior) was considered as a starting point for the subsequent determination of the best non-lethal heat shock (NLHS) temperature, and the LT_{50} was calculated as further described (Sung et al., 2018).

2.2.2. Non-lethal heat shock temperature

To determine the temperature of NLHS capable of inducing the best thermotolerance results in each strain, 0–3 h neonates were exposed to three heat shock (HS) temperatures for 30 min, ranging from 40 °C to 42 °C, inside 0.2 mL tubes with 100 μL ASW 25 PSU (30 organisms per tube), in the dark, using a T100 PCR Thermal Cycler (Bio Rad). Neonates held at 25 °C were used as controls (Reference treatment). After the heat treatment, all organisms were transferred to control conditions (25 °C, ASW 25 PSU) in 48-well microplates with 1 mL ASW, in the dark. Each temperature treatment consisted of six replicates with 10 neonates per replicate. After a 6 h recovery period, all neonates were then transferred to 0.2 mL tubes and exposed for 30 min to the previously calculated LT_{50} for each strain, using a T100 PCR Thermal Cycler (Bio Rad). Neonates were transferred again to 48-well microplates with 1 mL ASW and, after 6 h in control conditions, mortality was determined. The temperature at which the survival was higher was selected as NLHS temperature.

2.2.3. Recovery period for non-lethal heat shock

To determine the effect of different recovery periods, 0–3 h neonates were exposed to the previously determined NLHS temperature for 30 min, inside 0.2 mL tubes with 100 μL ASW 25 PSU (30 organisms per tube), in the dark, using a T100 PCR Thermal Cycler (Bio Rad). Neonates held at 25 °C were used as controls (Reference treatment). After the heat treatment, all organisms were transferred to control conditions (25 °C, ASW 25 PSU) in 48-well microplates with 1 mL ASW, in the dark, for different recovery periods (2 h, 4 h, 6 h, and 8 h). Each temperature treatment consisted of six replicates with 10 neonates per replicate. All neonates were then transferred to 0.2 mL tubes and exposed for 30 min to the previously calculated LT_{50} for each strain, using a T100 PCR Thermal Cycler (Bio Rad). Neonates were transferred again to 48-well microplates with 1 mL ASW and, after 6 h in control conditions, mortality was determined.

2.3. Induced thermotolerance assay and molecular mechanisms of response

2.3.1. Experimental design

Scale-up tests were performed with both strains to study the mechanisms underlying the increased tolerance to high temperature induced by NLHS, namely gene expression, HSP70 production, and histone H3 total acetylation. For this, clone cultures were up-scaled and rotifers were cultured for at least three weeks in several 5 L cultures, with light aeration, and at control conditions (25 °C, ASW 25 PSU, and constant light). For the experiments, rotifers with eggs were obtained by sieving the cultures with a sieve with 100 μm of diameter, and after 5 h of incubation at control conditions (no aeration), 0–5 h old neonates were collected. Total number of neonates was calculated to confirm the minimum number of organisms to proceed with the experiment. Neonates were concentrated with a sieve with 30 μm of diameter, and equally divided by six 1 L Schott flasks, three replicates for Reference treatment, and three replicates for NLHS treatment (Fig. 1). At this point, the density in each replicate was 300 neonates per mL, the same density used to perform the NLHS in the Thermal Cycler in the tests described above. Abrupt 30 min NLHS was given at the temperature determined previously for each strain (41 °C for MRS10 and 42 °C for IBA3) in a temperature-controlled water bath (Precision SWB 15, Thermo Fisher Scientific, USA) accurate to ±0.1 °C. Control organisms were maintained at 25 °C, in dark, during 30 min (Reference treatment). After the 30 min, the volume of every replicate was made up to 1 L, and the flasks were placed at 25 °C, in the dark, during 8 h for recovery (Fig. 1).

Sampling for the molecular mechanisms' analyses was done at 1 h, 4 h, and 8 h of recovery (Fig. 1), to capture the time period where the thermotolerance induction occurred as well as the eventual time-lapse between the first mechanistic responses at the gene level, the responses at the protein level and finally the phenotypic alterations. Depending on the total number of neonates obtained to perform the assay, three samples with a minimum of 4000 rotifers were collected in each replicate, at every sampling point, using a sieve with 20 μm of diameter. Collected samples were immediately frozen in liquid nitrogen and stored at −80 °C.

To validate the NLHS effect on survival of organisms in the scale-up test, after 8 h of recovery, 30 neonates from each replicate of both Reference and NLHS treatments were transferred to 0.2 mL tubes with 100 μL ASW 25 PSU and exposed in the Thermal Cycler to the LT_{50} (calculated previously) for 30 min. Then, all organisms were transferred to control conditions (25 °C, ASW 25 PSU) in 48-well microplates with 1 mL ASW, in the dark. Each treatment consisted of six replicates with 10 neonates per replicate. After 6 h, mortality was determined (Fig. 1). The scale-up test was considered valid if the survival of the organisms from NLHS treatment was higher, with statistical significance ($p < 0.05$).

2.3.2. RNA, proteins, and histone extraction

Total RNA and proteins were extracted from the same replicates using TRIzol® reagent (Invitrogen, Belgium), according to the manufacturer's instructions. Organisms were homogenized using disposable pestles in 0.5 mL of TRIzol® reagent. After RNA isolation, total RNA was purified with DNase treatment, using the DNaseI and RiboLock RNase Inhibitor from Thermo Fisher Scientific (USA). Spectrometric analyses using the Nanodrop 2000 (Thermo Scientific, USA) were performed at 230, 260, and 280 nm in order to measure the quantity and quality of the extracted total RNA. Contamination with gDNA was determined by using the Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, USA). Before proceeding with the qPCR assays, RNA integrity of all samples was validated in a 1% agarose gel electrophoresis. Samples were stored at −80 °C until use. Regarding the proteins' isolation, following the TRIzol® protocol after the RNA isolation, DNA was precipitated and discarded. Protein was then precipitated and washed, being then resuspended in Urea buffer (30 mM Tris-HCl, 7 M urea, 2 M thiourea, 4% CHAPS, pH 8.5) with protease inhibitor cocktail (Merck KGaA, Germany). Total protein was quantified by Coomassie Plus™ (Bradford) Assay Kit (Thermo Scientific, USA) at 595 nm, and its integrity was verified by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Total histones were extracted from different replicates using the Histone Extraction Kit (ab113476; Abcam, UK), according to the manufacturer's instructions. Organisms were homogenized using disposable pestles in the pre-lysis buffer. Total histone was quantified with Bradford method (595 nm) and its integrity verified by SDS-PAGE.

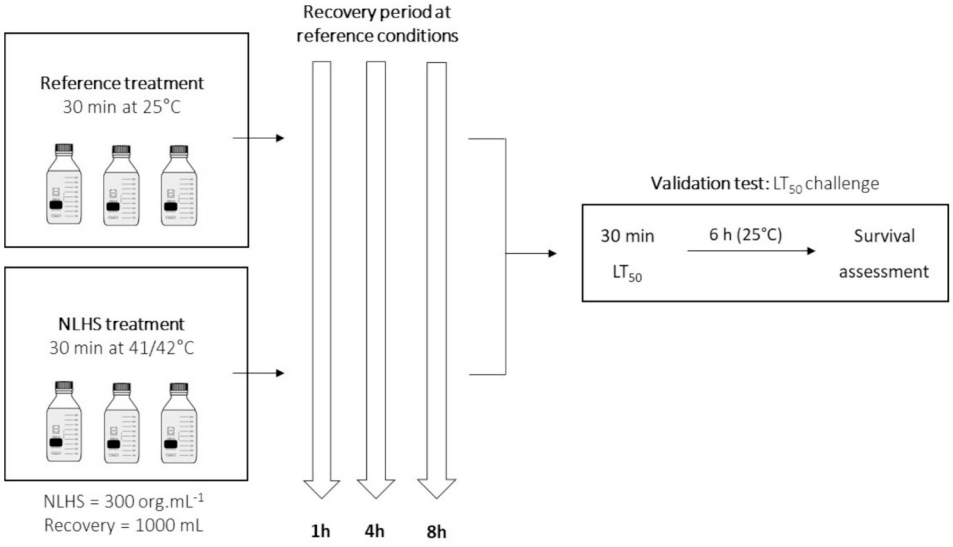

Fig. 1. Experimental design for the induced-thermotolerance assay and the determination of the molecular mechanisms involved after the non-lethal heat shock. Organisms were divided into Reference (non-heat shocked) and NLHS (heat shocked) treatments. During the recovery period at control conditions, organisms were collected for the analyses of differential expression of several genes (heat shock proteins and epigenetic modifications), production of HSP70, and total acetylation of histone H3. After 8 h of recovery, a set of organisms were exposed to a lethal temperature to validate the NLHS thermotolerance induction. This experiment was performed for two different strains of *Brachionus koreanus* (IBA3 and MRS10).

2.3.3. Gene expression (qPCR)

Using iScript™ cDNA Synthesis Kit (Biorad), 200 ng of total RNA was converted into cDNA through a reverse transcription reaction in a T100 PCR Thermal Cycler (Bio Rad), in a total volume of 20 μL for each sample and according to the manufacturer's instructions. Amplification reactions were then 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).

To evaluate the effect of the non-lethal heat shock (NLHS treatment) in comparison to Reference Treatment, 9 target genes coding for heat shock proteins and epigenetic proteins involved in histone modifications, plus 2 housekeeping genes, were chosen and the primer sequences were designed 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 1. Specificity and efficiency of all primer sets were assessed through melting and standard curves, respectively. Primer efficiencies (%) were calculated by application of the equation: E = [10^(−1/slope)-1] x 100.

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 two housekeeping genes (HK), 18S ribosomal RNA (*18S rRNA*) and elongation factor 1 alpha (*EF1α*). Gene expression was calculated according to Hellemans et al. (2007), using an adaptation to the Pfaffl (2001) methodology, using the equation: Relative Fold Change (RFC) = $(E_{GOI})^{\Delta Ct\ GOI}$ / GeoMean[$(E_{HK})^{\Delta Ct\ HK}$], 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 mean C_T of Reference treatment and the C_T of each sample of the NLHS treatment. The geometric mean (GeoMean) between the two HK genes was used. Gene expression results for NLHS Treatment are shown as RFC to Reference Treatment, and $\log_2$ normalized. The relative gene expression of all HSP genes (*Hsp40*, *Hsp60*, and *Hsp90*) was calculated for the samples taken from the Reference treatment at 8 h of recovery, using the equation: Gene expression ratio = $(E_{GOI})^{\Delta Ct\ GOI}$ / $(E_{HK})^{\Delta Ct\ HK}$, where ΔC_T is the difference between the minimum C_T of Reference treatment and the C_T of each sample of the Reference treatment.

2.3.4. Quantification of HSP70 production by Western Blot

Protein samples (1.5 μg) were combined with loading buffer, vortexed, heated at 95 °C for 5 min, cooled and centrifuged at 2200 *g* for 1 min. Samples were then loaded into 4–20% Mini-PROTEAN TGX Stain-Free precast gels (BioRad) and electrophoresed at 130 V for 15 min and subsequently at 150 V for 45 min. HeLa (heat-shocked) cells (6 μg; Enzo Life Sciences, Farmingdale, NY, USA) were loaded in one of the wells of each gel as positive control to later calculate the relative production of HSP70 in the samples. Gels were transferred to methanol-activated polyvinylidene fluoride (PVDF) membranes (Immun-Blot; BioRad, USA) for protein blotting and antibody probing. Proteins were transferred from the gels to the membranes through electrophoresis at 100 V for 1 h. Membranes were then incubated with blocking buffer [50 mL of 1× phosphate buffered saline containing 0.2% (*v*/v) Tween-20 and 5% (*w/v*) bovine serum albumin] overnight at 4 °C. Then the membranes were incubated with the primary antibody, mouse monoclonal [3A3] to Hsp70 (Abcam, UK), at the recommended dilution of 1:5000. Subsequently, Goat Anti-Mouse IgG H&L (Abcam) was used as secondary antibody, at the recommended dilution of 1:3000, conjugated with Precision Protein StrepTactin HRP conjugate (BioRad) at the dilution of 1 μL per 10 mL of secondary antibody solution. Both incubations were done for 1 h at room temperature, shaking. Membranes were then treated with enhanced chemiluminescent (ECL) reagent (Clarity Western ECL Substrate; BioRad) for 5 min and the signals were detected using a ChemiDoc MP Imaging System (BioRad). The relative signal intensity was quantified by densitometry, comparing the intensity of samples signal with the intensity of HeLa cells, using the BioRad Image Lab 6.1 software.

Table 1
Primer properties for the housekeeping and target genes of rotifers tested in this study: NCBI accession number, primer direction (FW: forward, RV: reverse) and sequence (5′-3′), primer efficiency (%), R squared of standard curve, and primer concentration.

Gene abbreviation	Functional description	Accession	Primer sequences (5′-3′)	Efficiency (%)	r squared	Primer concentration
EF1α	Elongation factor 1 alpha	NA (Denekamp et al., 2009)	FW: GGTGTGGGTGAATTCGAAG RV: CGAATGGAATTTGTGCTGG	106.7	0.975	3 μM
18S rRNA	18S ribosomal RNA	KU314660.1	FW: CCTGCGGCTTAATTTGACT RV: ACCCACCGAATCAAGAAAG	92.7	0.999	1 μM
Hsp40	Heat shock protein 40	GU574483	FW: CTGCCGATGCGGAAGAACACAC RV: GACTGGCACATTGAACTCGTCTAC	97.9	0.998	3 μM
Hsp60	Heat shock protein 60	GU574485	FW: GAGCTATTGCCAAGGAGGGATTTC RV: ATTCGTTTACAACAGTCTCAACTGCG	95.3	0.998	3 μM
Hsp90α1	Heat shock protein 90 alpha 1	GU574488	FW: TTCGGTGTCGGTTTCTACTCTGC RV: CAGCAGCTGACTCCCAGATATATTG	98.5	0.997	3 μM
KDM1	Lysine-specific histone demethylase 1 A	MK234827	FW: AAGCAGAGGCGAGTCATTT RV: CTTCTCTGGGTTGAGGGACT	100.2	0.993	3 μM
KAT6	Lysine acetyltransferase KAT6A	MK234806	FW: CGAGCCCTTTCTGTTCTACG RV: TGATACTGCGGCATTACCAT	99.0	0.998	3 μM
KMT3E	Lysine *N*-methyltransferase SMYD3	MK234818	FW: CTAAATAGGCTACCTCAGATGC RV: ATACTGGACACACTTAGATA	100.2	0.999	3 μM
DNMT2	DNA methyltransferase 2	KP898248.1	FW: TGTGCTCAAGCTGAAGCA RV: ATCATGTCCCATGTCGTCA	98.6	0.984	3 μM
Sirt6	Sirtuin 6	MF945617	FW: ACATGCAGTCCAACTATCGG RV: ATCATAAAGGACCCCTCTGC	105.5	0.972	5 μM
Sirt7	Sirtuin 7	MF945618	FW: TGTGATTGACGAAGAGGACA RV: TCCTGCACCAGTATAAACCA	95.6	0.994	3 μM

NA = Not available.

2.3.5. Determination of histone H3 total acetylation

Histone H3 total acetylation was measured in the samples from 4 h and 8 h of recovery, for both Reference and NLHS treatments. Histone samples (1 μg) were analyzed using the Histone H3 Total Acetylation Detection Fast Kit (ab131561; Abcam, UK), accordingly to manufacturer's instructions.

2.4. Statistical analysis

For the determination of lethal temperature, LT_{50} values were calculated based on log temperatures by fitting four-parameter logistic dose-response curves ($Y=Bottom+(Top-Bottom)/(1+10^{(LogEC50-X)*HillSlope)}$), where "Y" is the response (% mortality for LC_{50} estimation), "Bottom" is the average survival at the highest temperature tested (0%), "Top" is the average survival at the lowest temperature (100%), "X" is the logarithm of temperature, and "HillSlope" the slope of the logistic curve. This analysis was done using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California, USA).

For the determination of NLHS temperature, Levene's test was used to evaluate the homogeneity of the data. After this condition being met, data were subjected to analysis of variance (ANOVA) composed by one factor (temperature) to assess differences in survival between temperature treatments. The effect of recovery period on survival of Reference and NLHS rotifers was determined with a Two-Way ANOVA for each strain, using recovery period (four levels) and treatment (Reference and NLHS) as fixed factors. This test was also used to determine differences in the HSP70 production for both strains, and in the total acetylation of histone H3, for IBA3, using recovery period (three sampling points) and treatment (Reference and NLHS) as fixed factors. Homogeneity of variances and data normality were checked through Levene and Shapiro-Wilk tests, respectively. As data normality of total acetylation of histone H3 of MRS10 was not assumed, these results were analyzed with an ANOVA with Repeated Measures. LSD test was used for post hoc comparisons. These calculations were performed using IBM SPSS Statistics version 24 for Windows (SPSS Inc., Chicago, USA). The calculations for the differential gene expression analysis and respective statistical analyses were performed using Relative Gene Expression Software Tool version 2.0.13 (REST 2009, QIAGEN GmbH, Hilden, Germany). 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 24 for Windows or GraphPad Prism version 6.00 for Windows.

3. Results

3.1. Non-lethal heat shock conditions

Non-lethal heat shock conditions, namely LT_{50}, NLHS temperature, and recovery period, were determined based on the mortality (%) of rotifers after a 30 min heat shock and, at least, 2 h of recovery.

The same LT_{50} of 44.8 °C was determined for both MRS10 and IBA3 (Fig. 2). Between 43 °C and 44 °C, a low percentage of mortality was observed (2% at 43.5 °C and 0% at 44 °C). However, behavior alterations could be observed at these temperatures, namely lower swimming velocities (personal observations), and therefore 42 °C was determined as the maximum temperature for the following NLHS experiments.

Using 25 °C as Reference treatment, 40, 41, and 42 °C were tested as NLHS temperatures, being the exposure to 41 °C and 42 °C the conditions that resulted in a higher increase in rotifers' survival, for MRS10 and IBA3, respectively when later exposed to their LT_{50} (Table 2).

A heat shock at the previously calculated NLHS for 30 min was used to determine the recovery period allowing a higher difference between Reference and NLHS treatments. Data showed that better results were obtained after 6 h and 8 h of recovery, for MRS10 and IBA3, respectively (Table 3). Note that there was no statistically significant difference for survival observed in Reference treatment among recovery periods for both strains.

3.2. Molecular mechanisms of induced thermotolerance

3.2.1. Induced thermotolerance assay – validation of scale-up tests

After each scale-up experiment, some individuals were exposed to the LT_{50} for validation of the induced tolerance. For both strains, results showed that the NLHS treatment, even in a larger scale, allowed to increase the tolerance of rotifers to temperature (Table 4). In addition, the survival values obtained for the Reference treatment were significantly different for both strains ($t = -2.57$, $p = 0.028$), with higher survival observed for IBA3. The survival observed in NLHS treatment was similar for both strains, but the difference between Reference and NLHS treatments was higher for MRS10.

Table 2
Average survival (%) (±SD) of two strains of *Brachionus koreanus* neonates (MRS10 and IBA3) after exposure to control temperature (25 °C: Reference) and to different non-lethal heat shock (NLHS) temperatures for 30 min, followed by a challenge exposure to their LT_{50} (temperature causing 50% lethality under control conditions).

NLHS treatments (°C)	Survival (%)	
	MRS10	IBA3
25 (Reference)	42 ± 8 [a]	50 ± 9 [a]
40	75 ± 14 [b]	63 ± 12 [a,b]
41	**87 ± 15** [b]	73 ± 14 [b]
42	80 ± 9 [b]	**92 ± 10** [c]

Values in the same column showing the same superscript letter are not significantly different ($p > 0.05$).

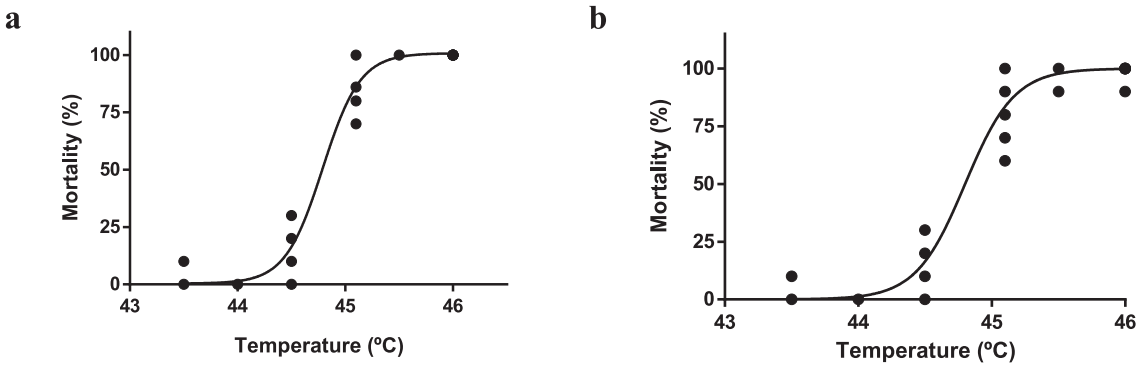

Fig. 2. Mortality (%) results determined for neonates of two strains of *Brachionus koreanus,* a) MRS10 and b) IBA3, after being exposed for 30 min to different heat shock treatments, followed by a 6 h period at control conditions. Each dot represents a replicate ($n = 6$).

Table 3
Average survival (%) (±SD) of two strains of *Brachionus koreanus* neonates (MRS10 and IBA3) after exposure to control temperature (Ref) and to the determined NLHS temperature (NLHS) for 30 min, with different recovery periods, and a subsequent challenge exposure to their LT_{50} (temperature causing 50% lethality under control conditions).

Recovery period (h)	MRS10		IBA3	
	Ref	NLHS	Ref	NLHS
2	38 ± 8^{a}	$63 \pm 10^{a,*}$	$50 \pm 15^{a,b}$	$72 \pm 12^{a,*}$
4	45 ± 10^{a}	$73 \pm 12^{a,*}$	58 ± 8^{a}	$83 \pm 8^{a,b,*}$
6	**35 ± 8^{a}**	**$88 \pm 8^{b,*}$**	45 ± 15^{b}	$80 \pm 6^{a,*}$
8	43 ± 12^{a}	$92 \pm 4^{b,*}$	**$50 \pm 6^{a,b}$**	**$95 \pm 8^{b,*}$**

Values in the same column showing the same superscript letter are not significantly different ($p > 0.05$); (*) indicates significance between HS and control treatments ($p < 0.05$).

Table 4
Average survival (%) (±SD) of two strains of *Brachionus* koreanus neonates (MRS10 and IBA3) in the scale-up induced thermotolerance assays by the non-lethal heat shock (NLHS) treatment, after exposure to their LT_{50} (temperature causing 50% lethality under control conditions).

Strains	Survival (%)	
	Reference	NLHS
MRS10	27 ± 10^{a}	$78 \pm 12^{a,*}$
IBA3	43 ± 12^{b}	$77 \pm 5^{a,*}$

Values in the same column showing the same superscript letter are not significantly different ($p > 0.05$); (*) indicates significance between NLHS and Reference treatments ($p < 0.05$).

3.2.2. Effect of NLHS in heat shock proteins production and gene expression

Results of relative fold change expression for genes encoding three heat shock proteins (*Hsp40*, *Hsp60*, and *Hsp90*) are shown in Fig. 3. Data showed that, for MRS10, mRNA expression of all genes increased significantly after the exposure to NLHS, and the number of transcripts appeared to increase along the recovery period, with higher fold changes at 4 h after the NLHS in relation to the reference treatment (Fig. 3a). For IBA3, exposure to NLHS significantly increased mRNA expression of *Hsp40* and *Hsp90*, but at 4 h the fold change was already decreasing for *Hsp40* (Fig. 3b).

Since there was evidence of a higher thermotolerance in IBA3 (Table 4), the relative gene expression of *Hsps* in organisms from Reference treatment, after 8 h of recovery (i.e., immediately before the exposure to LT_{50}), was evaluated and results are presented in Fig. 4. Comparing both strains, the gene expression of *Hsp40*, *Hsp60*, and *Hsp90* was significantly higher in MRS10 ($p \leq 0.001$), in organisms that were never exposed to a NLHS.

Western blot results showed significant differences in the production of HSP70 between Reference and NLHS treatments at 8 h of recovery, in MRS10 (Fig. 5a, Fig. S1). On the contrary, for IBA3 strain, Reference and NLHS treatments showed to be significantly different ($p < 0.05$) during all the time points assessed. Also, the results indicate an increase in HSP70 production at the end of the recovery period in the NLHS treatment for IBA3, while in the Reference treatment the HSP70 production decreased along time (Fig. 5b, Fig. S1).

3.2.3. Effect of NLHS in epigenetic markers

The effect of NLHS in the expression of genes related to epigenetic markers was studied (Fig. 6), including genes encoding histone lysine methyltransferase (*KMT3E*), demethylase (*KDM1*), acetyltransferase (*KAT6*), deacetylase (*SIRT6* and *SIRT7*), and a DNA methyltransferase (*DNMT2*). Even though there was a trend of increased average expression levels from 1 h to 4 h of recovery in MRS10, there was no differential expression of any of the targets after organisms were exposed to NLHS in relation to Reference treatment (Fig. 6a). On the contrary, for IBA3, mRNA expression of *SIRT6* increased significantly after 1 h of recovery, and *DNMT2* decreased significantly 4 h after the exposure to NLHS (Fig. 6b).

Results of total acetylation of histone H3 for Reference and NLHS treatments at 4 h and 8 h of recovery can be seen in Fig. 7. Despite the trend of higher acetylation levels in NLHS treatment for MRS10, especially at 8 h of recovery (Fig. 7a), NLHS did not significantly alter the acetylation levels of histone H3 in both MRS10 and IBA3.

4. Discussion

Rotifers, and *Brachionus* spp. in particular, have a ubiquitous distribution in the aquatic environment, and are known for their ability to tolerate environmental extremes including intense radiation (Boothby, 2019), hypoxia (Snell et al., 2019), desiccation (Jones et al., 2012), and large ranges of salinity (Lowe et al., 2005) and temperature (Denekamp et al., 2009). Nevertheless, the exposure to stressful environmental conditions during early life-stages can potentiate this natural tolerance later in life, allowing the organisms to cope better with environmental fluctuations (Norouzitallab et al., 2014). Hence, in the present study, the mechanisms involved in the induction of thermotolerance of two strains of *B. koreanus* were compared. The tolerance of MRS10 and IBA3 strains to acute heat stress was established and conditions of NLHS were optimized for maximum survival increase. Organisms used in this study were 0–4 h old neonates, since there is evidence that the exposure of early developmental stages to altered environmental conditions is important to the establishment and maintenance of epigenetic states of genes related to phenotypic variations (Granada et al., 2018; Johnston and Snell, 2016).

The studied neonates, from females maintained at 25 °C, survived temperatures up to 44 °C for at least 30 min (Fig. 2). Tolerance to a wide range of temperatures is well known in rotifers. As cosmopolitan aquatic organisms, rotifers can be found in shallow lakes and, therefore, many species can withstand daily thermal fluctuations (Dupuis and Hann, 2009). For example, *Rotaria rotatoria* is capable to reproduce at 12–40 °C, with no observed mortality (Xiang et al., 2017); and *B. plicatilis s.s.* reproduces at 8–30 °C (Yona, 2018). Moreover, MRS10 and IBA3 are capable to recover from a 24 h exposure to temperatures as low as 4 °C (data not shown). The response of both strains to the temperature gradient was very similar, as the temperature that caused lethality to 50% of the organisms (LT_{50}) was the same for MRS10 and IBA3 (44.8 °C), and the difference between no mortality and mortality above 80% was of 1 °C (44 °C vs 45 °C) (Fig. 2).

After determining the lethal temperatures for these strains, the establishment of conditions that enabled the improvement of thermotolerance was followed through acute heat stress exposure, in order to attain a state whereby organisms are capable to survive temperatures that were formerly considered lethal (Sung et al., 2018). When subjecting organisms to NLHS to improve their stress tolerance, it is important to determine the intensity of NLHS for these specific organisms to maximize the effect, since the ideal temperature can differ between species and even strains. In fact, NLHS at 40–42 °C resulted in equal survival for MRS10, whereas a better result was achieved for IBA3 when exposed to 42 °C, compared to lower temperatures (Table 2). In general, inducing thermotolerance in an organism is a two phase process, in which the exposure to an acute heat shock should be followed by several hours of non-stress conditions (Sung et al., 2011). Indeed, in this study it was possible to observe that the duration of recovery enlarged the effect of the NLHS, suggesting that the activation of molecular processes regulating the temperature-induced tolerance require some hours (Johnston and Snell, 2016; Xiang et al., 2017). It is, however, noteworthy that both strains showed a significant increase in survival when exposed to lethal heat stress after only two hours of recovery (Table 3).

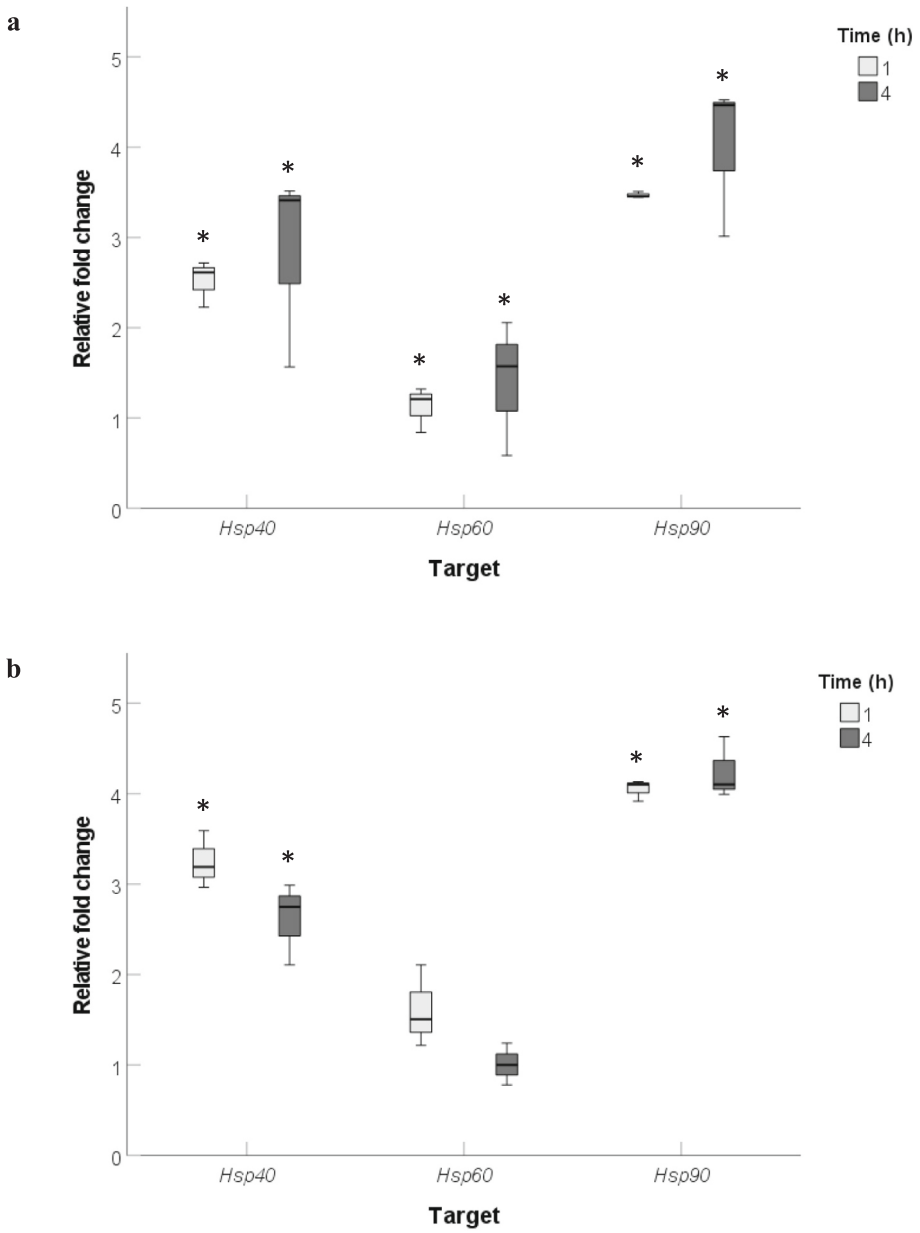

Fig. 3. Relative fold change (log_2) in expression of heat shock proteins related genes of a) MRS10 and b) IBA3 strains of *Brachionus koreanus*, after 1 and 4 h of recovery at control conditions from a non-lethal heat shock. (*) indicates differentially expressed genes compared to the Reference treatment ($p < 0.05$).

4.1. Thermotolerance

Results from the validation of scale-up tests (Table 4) showed that, even though both strains achieved the same survival after the NLHS, MRS10 had a greater increment in survival in relation to the Reference treatment, comparing to IBA3, meaning the effect of NLHS was more pronounced in the organisms of the first strain. It was also possible to observe that, under the Reference treatment, IBA3 had higher survival to the same temperature exposure comparing to MRS10. In fact, the temperature of 44.8 °C (used as LT_{50} for both strains) caused approximately 75% mortality in MRS10, being closer to a LT_{75} than to a LT_{50} in this strain, while a mortality closer to 50% was observed in IBA3 (Table 4). This indicates that IBA3 presented higher natural thermotolerance than MRS10. This could be due to the fact that control IBA3 had more transcripts of some relevant stress genes than MRS10. Therefore, relative gene expression of *Hsp40*, *Hsp60*, and *Hsp90* was evaluated in organisms from Reference treatment immediately before the exposure to LT_{50}. Contrarily to what was expected, relative expression of all genes was significantly lower in IBA3 (Fig. 4). In addition, assuming this pattern would also apply to the expression of *Hsp70* (not evaluated in this work), a higher abundance of HSP70 in reference MRS10 would be expectable. However, there were no differences in HSP70 production levels between strains in the Reference treatment (8 h, Fig. 5). This could indicate that, in MRS10, processes of post-transcriptional regulation act so that expression of *Hsp* genes are not reflected at the protein level and, therefore, the temperature required for transcriptional induction of heat shock proteins can differ from the temperature needed for a quantifiable change in HSP production (Lewis et al., 2016).

As explained by Boothby (2019), high temperatures cause protein unfolding and formation of non-functional aggregates. Also, concerning the mechanisms involved, thermotolerance may be linked to desiccation tolerance in many organisms. Although HSPs are very important proteins in coping with thermal stress, other mechanisms not assessed in this study may be involved in the higher thermotolerance observed for IBA3. For instance, Late Embryogenesis Abundant (LEA) proteins have been associated with survival, not only to desiccation, but also to other

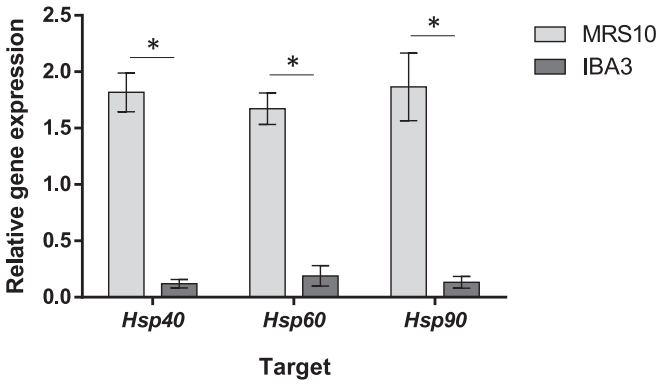

Fig. 4. Relative gene expression of heat shock proteins related genes at control conditions (Reference treatment) for two strains of *Brachionus koreanus* neonates, MRS10 and IBA3, using housekeeping genes as internal standards. (*) indicates statistical significance between strains ($p < 0.05$).

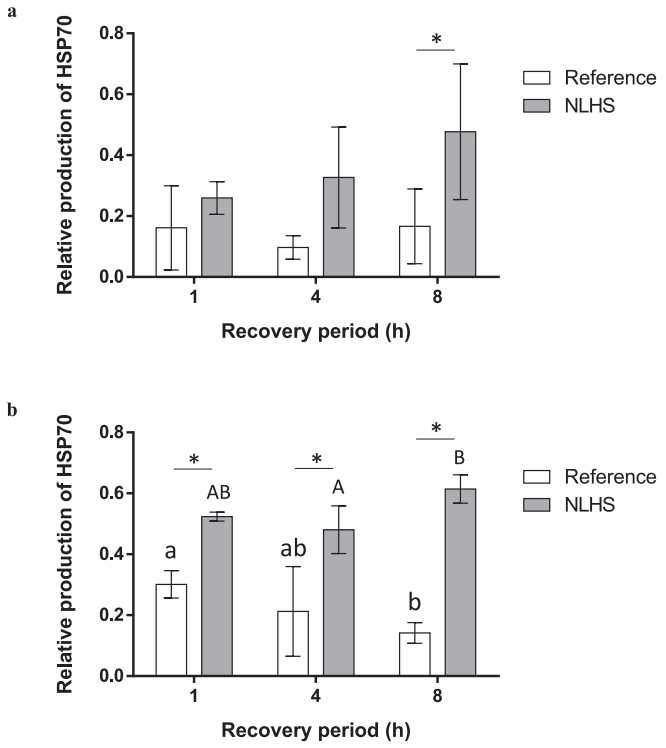

Fig. 5. Relative quantification of HSP70 production level in a) MRS10 and b) IBA3 strains of *Brachionus koreanus*, maintained either at control conditions (Reference) or exposed to a non-lethal heat shock (NLHS), after 1, 4 and 8 h of recovery at control conditions. HeLa (heat shocked) cells was used as technical control and for calculating the relative amount of HSP70 in each sample. (*) indicates statistical significance between Reference and NLHS treatments within each time point, different small letters indicate statistical significance between time points for Reference treatment, and different capital letters indicate statistical significance between time points for NLHS treatment ($p < 0.05$).

stressors such as temperature extremes (Jones et al., 2012) and acute salt stress (De Vos et al., 2021). Since LEA proteins have been proposed as molecular chaperones for their ability to prevent aggregation of cellular proteins (Jones et al., 2012), the up-regulation of LEA genes could explain the higher thermotolerance of IBA3. However, IBA3 strain comprises exclusively amictic females and, according to Denekamp et al. (2010), in rotifers, LEA transcripts and proteins degrade during hatching of resting eggs, not being expressed and synthesised in amictic females. Considering a different perspective, several studies have shown that extended lifespan is positively associated with antioxidant capacity and stress resistance in several species (Foley et al., 2019). The fact that IBA3 has a longer lifespan, comparing to MRS10 (Granada et al., 2022), could be an indication of its better performance and mechanisms of response to better deal with different stressors, including heat stress. The adaptation of organisms to stressful environments can depend on multiple complementary pathways, and we cannot exclude the possibility that IBA3 has constitutive thermotolerance and other proteins and mechanisms not addressed in our study that may be involved in the natural higher thermotolerance observed in IBA3.

4.2. Induced-thermotolerance

To understand the molecular mechanisms involved in induced-thermotolerance mediated by NLHS, the present study addressed the heat shock response, by evaluating the expression of heat shock protein 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 total acetylation of histone H3.

Concerning the heat shock response, the exposure of rotifer neonates to a single NLHS enhanced the expression of several *Hsp* genes in both strains (Fig. 3), and HSP70 synthesis, especially in the case of IBA3 (Fig. 5), resulting in increased survival to a lethal temperature up to three-fold in MRS10 and almost two-fold in IBA3, compared to the Reference treatments. Induction of *Hsp* genes in response to stress is a conserved mechanism, found from prokaryotes to higher eukaryotes (Paraskevopoulou et al., 2020). The production of heat shock proteins resultant from the exposure to stress conditions and respective functional significance in aquatic organisms was extensively reviewed by Sung et al. (2011) and Park et al. (2020), with emphasis in relevant species for aquaculture. Heat shock proteins are considered molecular chaperones and play an important role in maintaining cell's proper functioning, by mediating processes of protein folding, repair and degradation; interactions between proteins; and proteins translocation across membranes (Kim et al., 2016; Sung et al., 2014). More specifically, HSP40 targets proteins for degradation in the cytosol and regulates ATPase activity of HSP70, which modulates protein folding, transport and repair; HSP60 prevents protein denaturation under heat stress, and assists the folding and assembly of proteins imported from the cytoplasm into the mitochondrial matrix; and HSP90 participates in the protein folding and maintenance of structural integrity (Liang et al., 2020). Usually, heat shock response is fast, and alterations in expression and production of these proteins can be detected shortly after the heat stress event (Smith et al., 2012).

Subjecting MRS10 to NLHS up-regulated the expression of *Hsp40*, *Hsp60* and *Hsp90*, increasing along the recovery period. In IBA3, NLHS resulted in increased mRNA expression of *Hsp40* and *Hsp90* after 1 h of recovery, with fold change decreasing for *Hsp40* at 4 h recovery. *Hsp60* did not show to be differentially expressed in response to NLHS in IBA3. A study with *B. manjavacas* (Smith et al., 2012) showed HSP40, HSP60, and HSP70 are needed for rotifer thermotolerance, suggesting a multiple chaperone pathway, as these HSPs were all individually necessary for the organisms to survive heat shock. Although Smith et al. (2012) results suggested HSP90 was not essential for rotifer thermotolerance, in our study, overall, *Hsp90* presented the highest fold changes in relation to Reference treatment, increasing along the recovery period for both strains, thus suggesting that it can play a relevant role in thermotolerance induction in *B. koreanus*. A study with *B. calyciflorus* s.s. and *B. fernandoi* (Paraskevopoulou et al., 2020) corroborates our results, as organisms showed up-regulation of *Hsp90* in response to high temperature exposure for both species (4 h at 32 °C and 26 °C, respectively). The same was observed for other aquatic organisms, such as copepods (Han et al., 2018) and common carp (Sung et al., 2014). Furthermore, although addressing exposure to UV-B radiation and not temperature, a

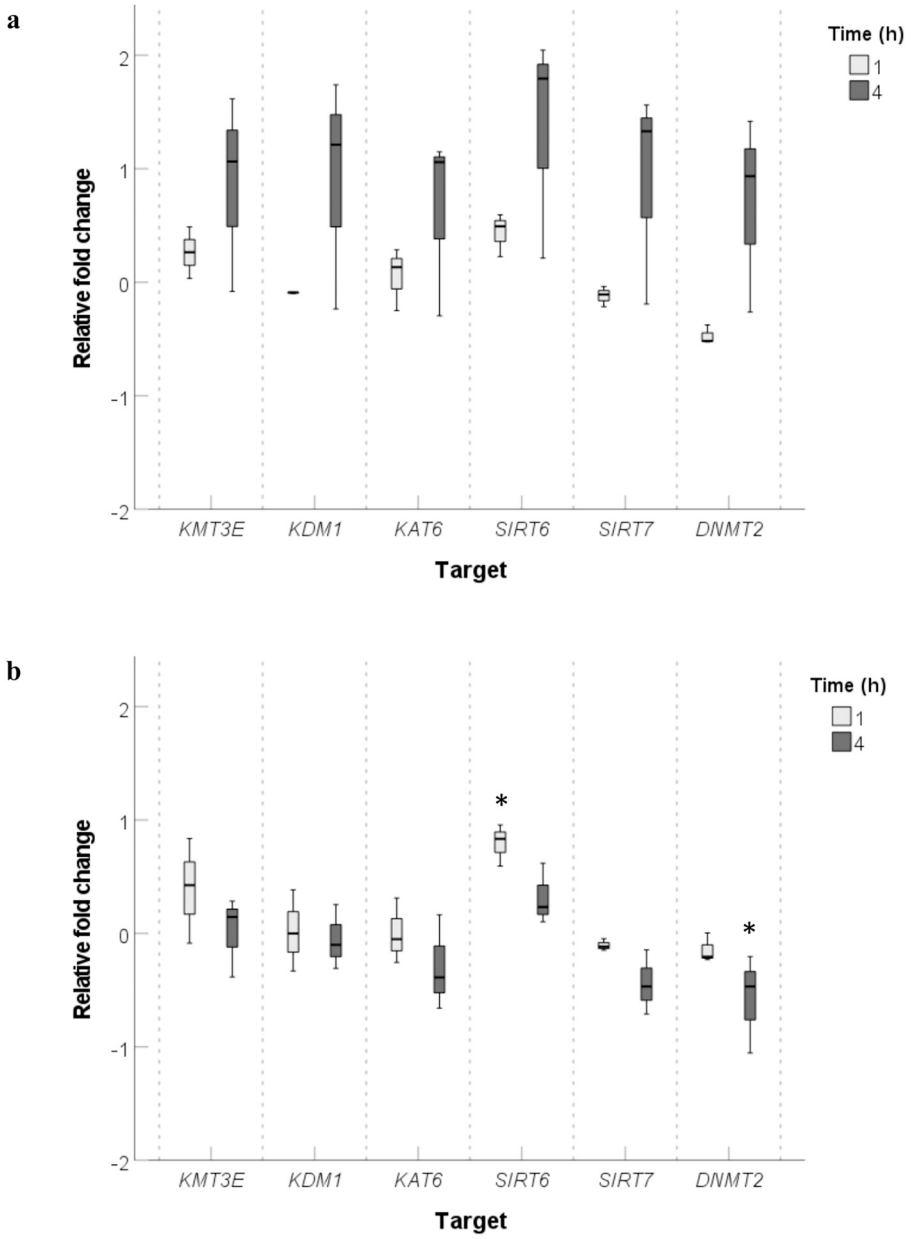

Fig. 6. Relative fold change (log_2) in expression of epigenetic modifications related genes of a) MRS10 and b) IBA3 strains of *Brachionus koreanus*, after 1 and 4 h of recovery at control conditions from a non-lethal heat shock. (*) indicates differentially expressed genes compared to the reference treatment ($p < 0.05$).

study with *Brachionus* sp. (Kim et al., 2011) revealed *Hsp90α1* may have an important role in this species.

Expression patterns of HSPs during the heat shock response also depend on the level of natural sensitivity of organisms to heat (Paraskevopoulou et al., 2020). These authors observed that, in the heat-tolerant *B. calyciflorus* s.s., most of the expression of *Hsp* genes (*Hsp40*, *Hsp60*, *Hsp70*) was induced by lower temperatures (20 °C), while in the heat-sensitive *B. fernandoi*, *Hsp70* and *Hsp90* expression increased under higher temperatures (26 °C) (Paraskevopoulou et al., 2020). Accordingly, in our study, in the scale-up experiment MRS10 showed to be more sensitive to temperature (control organisms had higher mortality compared to IBA3 when exposed to the same temperature). Also, as expected since the difference in survival between treatments was higher for MRS10, although both strains had differentially expressed *Hsp* genes when subjected to NLHS, MRS10 showed increasing fold changes in all *Hsp* genes along the recovery period, contrarily to IBA3. Taken together, the different expression patterns observed for *Hsp90* and the other HSPs in our study, and the evidence from the study with other Brachionids, indicate that proteins of the HSP90 family have an important contribution in heat response in rotifers (Paraskevopoulou et al., 2020). Also for other invertebrates, as *Penaeus vannamei*, the early increasing of mRNA expression of *Hsp70* and *Hsp90* after a heat shock suggest these genes might act as regulators of other genes (Junprung et al., 2017) and *Hsp40* is a responding factor to stress conditions, including temperature (Chen et al., 2018), being the same observed for *Pinctada fucata* (Wang et al., 2019).

Regarding the HSP60, other conditions besides heat shock are known to lead to an increase in its gene expression, as *Hsp60* genes also showed an increasing in *B. koreanus* when organisms were exposed to low temperature (20 °C; 25 °C control conditions) (Park et al., 2020), and in *B. plicatilis* exposed to environmental pollutants (Dahms et al., 2011). As a mitochondrial chaperonin, HSP60 is involved in stress protection in eukaryote mitochondria, by mediating the refolding of proteins imported into the mitochondrial matrix (Liang et al., 2020). There is

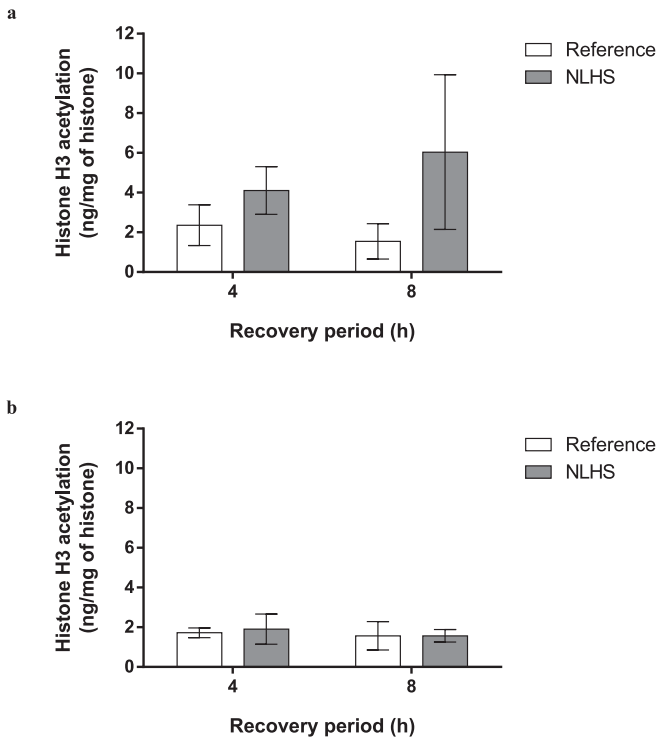

Fig. 7. Histone H3 total acetylation in a) MRS10 and b) IBA3 strains of *Brachionus koreanus*, either maintained at control conditions (Reference) or exposed to a non-lethal heat shock (NLHS), after 4 h and 8 h of recovery at control conditions.

evidence of high homology between mitochondrial HSP60 identified in *Brachionus* spp. and *Homo sapiens* (Park et al., 2020), where these proteins are constitutively expressed under normal conditions (Gammazza et al., 2015). In the present study, *Hsp60* was the gene with least differential expression in response to NLHS (Fig. 3). In IBA3, this gene was not differentially expressed in response to NLHS, even though its expression level under Reference conditions was lower compared to MRS10 (Fig. 4). As mentioned previously, HSP60 prevents protein denaturation under heat stress and is important for rotifer thermotolerance, so these results seem to corroborate the hypothesis that IBA3 may have other mechanisms not addressed in this study, contributing to its constitutive thermotolerance.

From the group of stress proteins, HSP70 is the most widely studied, as this family was considered the classical cellular stress response for a long time (Dahms et al., 2011). Besides its role in targeting damaged proteins for degradation and protein refolding, preventing aggregation, HSP70 already demonstrated to be positively correlated to increased immunity against several diseases (Baruah et al., 2015). Differences in HSP70 protein production between Reference and NLHS treatments were observed for IBA3, with levels increasing along the recovery period in heat shocked organisms, while for MRS10 differences between treatments were only observed at the end of the recovery period (Fig. 5). HSP70 has shown to be consistently inducible by NLHS, with positive physiological outcomes in species with aquaculture importance, such as increased tolerance to heat, ammonia, and heavy metals, as observed for *Artemia* sp. (Pestana et al., 2016) and *P. vannamei* (Sung et al., 2018). In the present study, IBA3 presented higher levels of HSP70 upon NLHS, but also higher fold change expression of *Hsp40* comparing to MRS10. In fact, it has been suggested that one major role of HSP40 is to regulate the HSP70 activity (Fan et al., 2003), including in *Brachionus* sp. (Paraskevopoulou et al., 2020; Smith et al., 2012).

The role of some epigenetic mechanisms in NLHS-induced thermotolerance was also addressed in this study. As mechanisms of regulation of gene expression, epigenetic modifications are crucial for phenotypic plasticity (Paraskevopoulou et al., 2020), since by activating or supressing transcription as response to environmental cues, they create phenotypic variability, allowing the organisms to adapt to changes in the environment (Lee et al., 2020b). Several studies have correlated epigenetic modifications with increased tolerance to several stressors in aquatic organisms, including temperature (reviewed in Granada et al., 2018). Two main epigenetic modifications are DNA methylation and histone modifications. DNA methylation is of critical importance in several biological functions, by regulating gene activity patterns, and DNA replication and repair, and is catalysed by a family of DNA methyltransferases (DNMTs) (Zuo et al., 2009). However, although *B. koreanus* has a gene encoding DNMT2, it lacks genes encoding the important DNMT1 and DNMT3. It is, therefore, suggested that histone modifications are the main epigenetic mechanisms in this organism (Lee et al., 2020a, 2020b). Histone methylation is usually involved in activation and suppression of transcription, while acetylation guide transcription by opening the chromatin structure (Lee et al., 2020b; Norouzitallab et al., 2014).

In this study, MRS10 showed no differential expression of any genes encoding histone modifications and DNA methylation (Fig. 6a), although a trend of increased fold change expression at 4 h of recovery was observed for all targets. The same was observed for total acetylation of histone H3 in MRS10 (Fig. 7a), with NLHS treatment showing a trend of higher acetylation levels at 8 h of recovery. It should also be noted that the acetylation levels were, in general, higher for MRS10 than IBA3, regardless the recovery period and treatment. However, contrarily to MRS10, IBA3 showed an increased mRNA expression of *Sirt6* and decrease in *DNMT2*, 1 h and 4 h after exposure to NLHS, respectively (Fig. 6b). Further analyses on histone modifications are necessary to fully understand if these epigenetic markers were involved in the increase of thermotolerance in IBA3.

Given the lack of DNA methylation process, a study by Lee et al. (2020b) highlighted the importance of histone modification genes in the epigenetic regulation in *B. koreanus* under stressful circumstances, using low pH conditions as reference. The authors concluded histone lysine methylation (KMT) could be the main epigenetic mechanism in this species, given the evidence of correlation between histone and DNA methylation systems. A study by Paraskevopoulou et al. (2020) also showed that the exposure to high temperature induced an increase in the expression of histone methyltransferase genes in *B. fernandoi*, a heat-sensitive species, but had no effect in the expression of these genes in the heat-tolerant *B. calyciflorus* s.s.. In addition, Lee et al. (2020b) suggested *B. koreanus* would be able to successfully adapt to different environmental conditions, since histone lysine acetylation (KAT) provide great capacity of gene transcription regulation to this species (Lee et al., 2020b). Histone acetylation also already proved to be crucial for thermal stress tolerance in *Artemia* sp. (Norouzitallab et al., 2019). Overall, a single NLHS seemed to be insufficient for effects in epigenetic related-genes expression and histone acetylation, suggesting that successive exposures to NLHS may be needed to induce epigenetic modifications, with possible transgenerational effects, as already observed for *Artemia* sp. (Norouzitallab et al., 2014; Pestana et al., 2016) and *Penaeus vannamei* (Junprung et al., 2017).

Further studies should be done to determine if NLHS provides a persistent survival effect in *B. koreanus*. In rotifers exposed to heat shock, the HSP70 synthesis declined after some hours, indicating capacity to resist heat stress is maintained for a short period of time (Xiang et al., 2017). This information and the results from the present work on gene expression suggest longer or consecutive exposures to heat stress are required to a persistent effect in the exposed organisms and, possibly, in subsequent generations.

5. Conclusion

Results demonstrate that a short heat stress at an initial stage of development in *B. koreanus* increased their capacity to survive higher

temperatures in subsequent exposures. In general, induced thermotolerance as a response to acute heat stress was concomitant with up-regulation of *Hsp40* and *Hsp90* genes and synthesis of HSP70. However, given the results concerning natural thermotolerance, this study highlights the possibility that (1) thermotolerance after NLHS is controlled by HSP production, while natural thermotolerance is not, and that (2) for the same species, different lineages and strains may evolve separately and develop different mechanisms of tolerance.

A single NLHS proved insufficient to cause epigenetic effects, indicating that the response to these heat stress conditions may be temporary, not leading to multigenerational increase in thermotolerance. Further studies should contribute to understand if this conditions of NLHS may also generate cross-tolerance, namely to abiotic and chemical stress, and if continuous exposure to heat stress endows several generations with higher tolerance to environmental stressors, as this would be extremely advantageous for aquaculture, as more resistant species will imply more resilient aquaculture practices.

Statements and declarations

The authors have no relevant financial or non-financial interests to disclose.

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. Original data of gene expression used in this study is available at Mendeley Data (https://doi.org/10.17632/j3m89pk5k8.1).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.741125.

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