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Non-lethal heat shock increases tolerance to metal exposure in brine shrimp

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ABSTRACT

Pollution and temperature increase are two of the most important stressors that aquatic organisms are facing. Exposure to elevated temperatures and metal contamination both induce heat shock proteins (HSPs), which may thus be involved in the induced cross-tolerance in various organisms. This study aimed to test the hypothesis that exposure to a non-lethal heat shock (NLHS) causes an increased tolerance to subsequent metal exposure. Using gnotobiotic cultures of the brine shrimp *Artemia franciscana*, the tolerance to Cd and Zn acute exposures was tested after a prior NLHS treatment (30 min exposure to 37 °C). The effects of NLHS and metal exposure were also assessed by measuring 70 kDa-HSPs production, along with the analysis of epigenetic markers such as DNA methylation and histone H3 and histone H4 acetylation. Our results showed that heat-shocked *Artemia* had increased acute tolerance to Cd and Zn. However, different patterns of HSPs were observed between the two metal compounds and no epigenetic alterations were observed in response to heat shock or metal exposure. These results suggest that HSP production is a phenotypically plastic trait with a potential role in temperature-induced tolerance to metal exposure.

1. Introduction

In the last decades environmental scientists have been developing and applying methods to evaluate the ecological effects of complex mixtures of different chemical stressors (Altenburger et al., 2015; Neumann et al., 1994). This has been achieved not only with respect to multiple contaminants but also with the concomitant exposure of contaminants and natural biotic and abiotic stressors such as temperature, UV light, salinity, predation and parasitism (Altenburger et al., 2015; De Coninck et al., 2013; Pestana et al., 2010; Sokolova and Lannig, 2008; Tetreau et al., 2014).

However, the vast majority of studies have focused on simultaneous exposures and few studies have investigated how stress induced by an environmental factor can alter the response to chemical stress (Altenburger et al., 2015; De Coninck et al., 2013; Holmstrup et al., 2010; Neumann et al., 1994; Pestana et al., 2010; Sokolova and Lannig, 2008; Tetreau et al., 2014). Recent research has shown that chemical tolerance cannot be explained solely on the basis of constitutive tolerance (Chaumot et al., 2009; Jansen et al., 2011; Silvestre et al., 2012). Organisms produce a range of phenotypes allowing them to cope and exploit a broader range of environmental conditions. Phenotypic plasticity in the physiological mechanisms of defense against environmental stressors can also allow for increased tolerance (Pigliucci, 2005; Silvestre et al., 2012). Thus, organismal tolerance can result from effects of multiple factors on physiological processes. This is because exposure to one stressor can alter the tolerance of organisms to other stressors (i.e. induced tolerance and cross-tolerance) (Hua et al., 2014). It is possible that many combinations of stressors can elicit such responses but little investigation has been conducted despite the potentially major conservation implications for populations that are sequentially exposed to a multitude of environmental stressors.

This is increasingly urgent in the face of future climate change scenarios and given the possibility that organisms will unpredictably experience exposure to multiple stressors (IPCC, 2007). Temperature increase and exposure to contaminants such as pesticides and metals are stressors that aquatic organisms will

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increasingly face in a near future. In fact, temperature variability and the frequency of extreme events such as heat waves are expected to increase along with an increase in global average temperatures (Rahmstorf and Coumou, 2011). Phenotypic plasticity can thus have a major influence on the capacity of organisms to rapidly respond and acclimate to future environmental conditions (Hoffmann and Sgrò, 2012).

It is thus of fundamental importance to increase our knowledge on the physiological basis of induced cross-tolerance but this phenomenon has rarely been considered in the context of how climate change drivers, such as temperature, affect tolerance to chemical stressors. This induced cross-tolerance has already been observed for a number of different stressors after acclimation or short exposure to elevated temperatures (Baruah et al., 2012; Chen and Stillman, 2012; Hoffmann et al., 2003; Hofmann and Todgham, 2010; Kellett et al., 2005; Norouzitallab et al., 2015a; Sung et al., 2007).

Short-term exposures to elevated temperatures set off complex reactions in aquatic organisms leading to the up regulation of heat shock proteins (HSPs) (Feder and Hofmann, 1999; Hoffmann et al., 2003). Responses to transient non-lethal heat shock (NLHS) can alter thermotolerance and improve survival to subsequent heat stress (Aleng et al., 2015; Arias et al., 2012). This is because most HSPs play a crucial role in maintaining protein homeostasis and allow organisms to cope with stress (Feder and Hofmann, 1999). HSPs function as molecular chaperones and are involved in protein folding/unfolding, translocation, and degradation of misfolded or aggregated proteins (Feder and Hofmann, 1999).

HSPs have important functions in the protection against stressors that can cause cellular damage. Investigations have confirmed that HSPs expression is induced by a range of natural and anthropogenic stressors besides temperature such as insecticides, heavy metals, desiccation, disease, parasites and inbreeding (Sørensen et al., 2003). Moreover, it has also been documented that NLHS induce HSPs in a variety of aquatic organisms and increase their subsequent tolerance to heat (Aleng et al., 2015), pathogen infections (Norouzitallab et al., 2015a; Sung et al., 2007), osmotic stress (DuBeau et al., 1998) and toxic compounds (Sung et al., 2014). All these studies show that NLHS increase HSPs production and can protect aquatic organisms against subsequent environmental stress.

In the present study, the effects of NLHS on *Artemia franciscana* tolerance to subsequent metal exposure were studied. *Artemia*, commonly used as live feed in aquaculture, is an extremophile animal that due to its availability, ease of maintenance and short life cycle has proven to be a useful model organism for studies on ecological and physiological aspects of stress responses (Baruah et al., 2012; Norouzitallab et al., 2014, 2015b; Nunes et al., 2006). Moreover, *Artemia* sp., are relatively tolerant to metal exposure being previously used as a model species to investigate effects of metal exposure including Cd and Zn on different physiological and biochemical stress responses (Brix et al., 2006; Kokkali et al., 2011; Seebaugh and Wallace, 2004). Using *Artemia* as a model provided us with the possibility of testing our hypothesis under axenic conditions, thus avoiding the possible interference of microbial communities present in the aquatic medium and facilitating the interpretation of the results (Baruah et al., 2013).

By examining the combined effects of heat shock and Cadmium and Zinc exposure on HSP70 production levels we aimed to verify the role of HSPs as mediators of metal tolerance in *Artemia*. Since previous studies have shown that NLHS can also trigger epigenetic alterations in *Artemia* that can be involved in the higher tolerance and can be transmitted to subsequent non-exposed generations (Norouzitallab et al., 2014), we also investigated the effects of both NLHS and metal exposure on acetylation of histones H3 and H4 and global DNA methylation.

2. Methods

2.1. Axenic hatching of Artemia franciscana

The Axenic system was set by hatching *Artemia* cysts axenically after decapsulation (Norouzitallab et al., 2015a). Briefly, *Artemia* cysts originated from the Great Salt Lake, Utah, USA (EG® Type, batch 21,452, INVE Aquaculture, Dendermonde, Belgium) were hydrated in sterile distilled water for 1 h, with aeration. After hydration, cysts were decapsulated by adding NaOH (32%) and NaOCl (50%) with a subsequent addition of $Na_2S_2O_3$ (10 g/L) to stop the reaction. The decapsulated cysts were then abundantly washed with sterile artificial seawater (35 g/L, Aquarium Systems, Sarrebourg, France), and were either suspended in different 50 ml Falcon™ tubes (for the acute tests) or 1 L glass bottles (for sampling) containing 30 ml and 800 ml sterile artificial seawater respectively. Decapsulated cysts were provided with filtered (0.2 μm) aeration and were incubated for 22 h at 28 °C with constant illumination of approximately 27 $\mu E/m^2/s$. Both decapsulation and hatching procedures were performed under a laminar flow hood and using autoclaved materials (121 °C for 20 min) for preserving axenic conditions. After the incubation, the axenicity of the hatched *Artemia* nauplii was verified by adding 10 μL of hatching water on Marine Agar medium (Difco, Detroit, USA) followed by the incubation at 28 °C for 5 days.

2.2. Experimental setup

2.2.1. Acute tests – effects of Cd and Zn with and without pre heat-shock treatment

The two metals independently tested in this study were added as cadmium chloride ($CdCl_2$) and zinc chloride ($ZnCl_2$). Stock solutions of each metal were prepared using sterile artificial seawater. After 48 h of exposure, samples for determination of dissolved Cd and Zn concentrations were collected in triplicate. All samples were acidified with 1% (v/v) 14 N HNO_3 and stored in the dark at 4 °C until analysis. Dissolved Cd and Zn concentrations were measured by atomic absorption spectroscopy (SpectrAA-100, Varian, Mulgrave, Australia).

After incubation for 22 h, decapsulated cysts were hatched and swimming nauplii at instar II stage (when the mouth is open) were obtained. The collected animals were homogeneously distributed into 2 groups. Each group was maintained in sterile 50 ml Falcon tubes containing 30 mL sterile artificial seawater. For hyperthermic treatment, animals in one group were exposed to a NLHS at 37 °C for 30 min in a water bath, followed by a recovery period of 6 h at 28 °C (Sung et al., 2007). During this period, constant illumination ($\approx 27\ \mu E/m^2/s$) was provided. Animals from the other group were maintained isothermally at 28 °C for the same 6h30min and considered as the reference group.

After the 6 h of recovery, animals from each group (NLHS and Reference sets) were collected and suspended in glass tubes containing 20 ml experimental metal solutions diluted in sterile artificial seawater. Animals were exposed to 8 concentrations of Cd (0-10-25-50-100-150-200-300 mg/L) and Zn (0-0.5-1-4-10-25-60-150 mg/L) for 48 h. Three replicates per treatment, with 10 organisms each, were used. The entire acute assay (contamination and exposure) was performed under axenic conditions. After 48 h of exposure, the survival of *Artemia* in each replicate was visually scored.

2.2.2. Sampling experiment for molecular analysis

To determine the combined effects of heat shock and metal exposure on HSP70 production and epigenetic markers, a similar gnotobiotic sequential exposure to heat stress (NLHS) and metal exposure was performed. After the 22 h hatching period, nauplii of