Ecotoxicology and Environmental Safety 140 (2017) 18–23

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

HSP70 expression in *Biomphalaria glabrata* snails exposed to cadmium

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ARTICLE INFO

Keywords:
Protein induction
Biomarker
Bioindicator
Mollusk
Acquired resistance

ABSTRACT

In this study, the effects of the heavy metal cadmium on the stress protein HSP70 are investigated in freshwater mollusks *Biomphalaria glabrata*. Adult snails were exposed for 96 h to $CdCl_2$ at concentrations ranging from 0.09 to 0.7 mg L^{-1} ($LC_{50/96\ h}$ = 0.34 (0.30–0.37). Time and concentration-dependent increases in the expression of HSP70 were observed at sub-lethal levels in the immunoblotting assay. Further, an increased survival to a lethal heat shock was observed in animals pre-exposed to a nonlethal concentration of cadmium, evidencing the induction of acquired tolerance. The present study demonstrated the inducibility of *B. glabrata* HSP70 by cadmium, a relevant environmental contaminant, at non-lethal levels, providing evidences that the assessment of HSP70 in *B. glabrata* can be regarded as a suitable biomarker for ecotoxicological studies.

1. Introduction

The heat shock response, firstly described in drosophila upon exposure to elevated temperature (Ritossa, 1962), involves the induction of a set of proteins known as stress proteins or heat shock proteins (HSPs) in response to environmental insults (Lindquist, 1986). Heat shock proteins can be broadly placed into five major families according to their molecular weight, amino acid sequence homologies and functions and are frequently referred to as HSP100, HSP90, HSP70, HSP60 and the small HSP stress-proteins. The HSP70 family proteins differ from other classes of heat shock proteins being one of most highly conserved, with more than 50% of homology between man, drosophila, mouse, yeast and bacteria and the first to be induced under stress conditions. It is one of the most widely studied and well defined classes of HSPs (Lindquist, 1986; Gupta et al., 2010).

At cellular level, HSPs are involved in the maintenance of protein homeostasis and their role in protecting cells from stress is strongly suggested by the character of an emergency response, being extremely rapid and very strong (Parsell and Lindquist, 1993). Although the role of HSPs at the organismal level is not well established, some studies suggest the association of aberrant heat shock responses with diseases including cancer, neurodegenerative disorders, ischemia or hypoxia, virus infection, inflammation and wound healing (Kim et al., 2007).

Exposure to a wide range of physical and chemical stressors proved to elicit the cellular stress response with increases in HSP70 levels (Sanders, 1993; Bierkens, 2000; Gupta et al., 2010). Despite the non-specific nature of the stress response, heat shock proteins have been postulated as possible biomarkers in ecotoxicology by their role in the recovery of damaged proteins, characterizing an initial response that can be used in protocols to evaluate damage to organisms subjected to environmental aggressors (Sanders, 1993; Bierkens, 2000). In addition, HSP70 reflects a direct link between exposure and cellular damage, and its highly conserved nature among different species supports its use as an early marker of environmental stress (Mukhopadhyay et al., 2003).

Studies with organisms from the most diverse phyla proposed the use of HSPs, especially the HSP70, as biomarkers in ecotoxicology (Nadeau et al., 2001; Ireland et al., 2004; Bednarek et al., 2016). In aquatic species, HSP70 has been applied as indicator of alterations and biological damage, either in natural environment or in laboratory (Sanders, 1993; Sanders et al., 1995; Williams et al., 1996; Tedengren et al., 2000; Singer et al., 2005; Radlowska and Pempkowiak, 2002; Piano et al., 2004; Pruski and Dixon, 2007; Ivanina et al., 2009; Cantinha et al., 2013; Wali and Balkhi, 2016).

Freshwater snails *B. glabrata* have been reared in our laboratory for more than 30 years and data from our former studies showed that this is a suitable species for studies on biological effects of chemical contaminants (Nakano et al., 2003; Estevam et al., 2006; Tallarico et al., 2014). In addition to toxicity analysis, endpoints for detection of effects

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http://dx.doi.org/10.1016/j.ecoenv.2017.02.026
Received 3 November 2016; Received in revised form 13 February 2017; Accepted 16 February 2017
Available online 27 February 2017
0147-6513/

on the reproductive potential were established. Results from the dominant lethal assay with reference compounds showed that *B. glabrata* can absorb chemical contaminants from water and metabolically activate indirect mutagens (Nakano et al., 2003).

Previously, we showed that a sublethal heat treatment could induce the stress response in freshwater snails *Biomphalaria glabrata*. Increases in the expression of HSP70 were observed in digestive glands of adult snails submitted to mild heating. Further, the same conditioning treatment increased the survival to a lethal heat shock, suggesting a protective effect of HSP70 (Cantinha et al., 2013).

The induction of temporary resistance by a nonlethal heat shock against a lethal heat shock was already described; furthermore, a strong correlation between the induction of HSPs and thermotolerance was demonstrated (Lindquist and Craig, 1988). Experimental evidence that stress proteins are involved in conferring tolerance to environmental extremes is abundant in both aquatic and nonaquatic species (Sanders, 1993). The stress response has been implicated in acquired tolerance, a phenomena in which a mild conditioning temperature confers tolerance, at the cellular and organismal levels, to subsequent temperatures that would otherwise be lethal (Lindquist and Craig, 1988). Thermotolerance induced by heat shock has been studied most extensively and has been demonstrated in organisms from diverse phyla, including bacteria, coelenterates, mollusks, echinoderms, fish, and mammals. Furthermore, other stressors that induce stress proteins, such as arsenite, cadmium, and ethanol, also induce thermotolerance (Sanders, 1993).

In the present study, the heavy metal cadmium, an environmental relevant chemical, was selected as stressor agent to evaluate the induction of HSP70 in *B. glabrata*. Although a relatively rare element, cadmium is extensively released into the environment mainly by anthropogenic sources, such as combustion of fossil fuels, iron and steel manufacturing, electroplating, fabrication of nickel–cadmium batteries, municipal solid waste burning, and as a by-product of phosphate fertilizers (Nriagu and Pacyna, 1988; Friberg et al., 1992). A persistent environmental pro-oxidant, cadmium produces a wide variety of detrimental effects and being non-degradable, it is accumulated in tissues of exposed organism at relatively low concentrations (Chandurvelana et al., 2013; Kafel et al., 2014).

The aim of the present study was to evaluate the induction of HSP70 by cadmium at sublethal concentrations. To correlate the stress response observed at cellular level to an organism level response, the induction of acquired tolerance by cadmium was assessed.

2. Material and methods

2.1. Animals

Pigmented adult, sexually mature, S. *mansoni* negative *B. glabrata* snails with shell diameter average of 14.4 (SE = 1.7) mm and aging between five to six months were used. The animals were collected from Barreiro (Belo Horizonte, MG) and reared in the Parasitology Laboratory of the Butantan Institute for several generations. Mollusks were maintained in plastic aquaria under controlled environmental conditions and daily fed with fresh lettuce (*Lactuca sativa*) *ad libitum*, weekly complemented with fish ration. Filtered tap water was used for rearing the organisms in laboratory conditions as well as for sample dilutions during the biological assays. (Tables S1 and S2).

2.2. Experimental design

The study was divided in three sets of experiments to: i) evaluate the induction of HSP70 by cadmium; ii) set the time-response pattern of the HSP70 induction; iii) evaluate the induction of acquired tolerance by cadmium.

i) Snails were exposed for 96 h to nominal concentrations of 0.09, 0.15, 0.22, 0.33, 0.5 and 0.7 mg L^{-1} of $CdCl_2$ (purchased from Sigma Aldrich, C3141) in groups of 10 animals. A control group with 10 snails was kept in filtered tap water. Animals were placed in groups of five in covered glass containers with a capacity of 180 mL of solution, renewed each 48 h at room temperature of 25 (± 2) °C and fed with fresh lettuce *ad libitum*. Snails were monitored under stereoscopy each 24 h and death was confirmed by the absence of heart beating. Exposure was done in four replicates. The LC_{50} was determined and surviving snails had the digestive glands dissected to the immunochemical analysis of HSP70 expression. Analysis was done in pools of 3–5 individuals depending on the number of survivors.

ii) To evaluate the time-response pattern of HSP70 after exposure to cadmium, four groups of ten individuals were exposed to 0.22 mg L^{-1} $CdCl_2$ for 96 h at room temperature. For comparison, five groups of 10 individuals were submitted to a heat shock of 33 °C in BOD climatic chamber for 120 h. Other five groups of 10 snails were kept as control immersed in dechlorinated filtered tap water at room temperature. Digestive glands were dissected to HSP70 analysis each 24 h until 96 h for the cadmium exposed groups and until 120 h for the 33 °C exposed groups.

iii) The acquired tolerance study was done with animals pre-exposed to a non-lethal concentration of cadmium and submitted to a lethal heat shock. A group of 35 animals exposed to 0.22 mg L^{-1} $CdCl_2$ for 96 h was submitted to 42 °C in a water-bath; a control group with 35 snails without cadmium pre-exposure was submitted to the lethal heat. Dead animals were scored each hour until all snails were dead.

2.3. Immunochemical assay of HSP70

Mollusks were pressed between two Petri dishes and, under stereoscopy, shell fragments were removed and the digestive gland was dissected. Tissues were macerated with RIPA buffer (25 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (75 U aprotinin, 2 mM PMSF, 5 mM sodium pyrophosphate, 10 mM NaF, and 5 mM sodium orthovanadate) for protein extraction. One hundred micrograms of protein were fractionated in a 10% SDS-PAGE. The prestained protein weight marker Precision Plus Protein™ Kaleidoscope Standards (161–0375) from BioRad was used. After transference to a nitrocellulose membrane, HSP70 was detected using an anti-HSP70 serum of *Trypanosoma brucei* produced in rabbit (1:1000) as primary antibody and an anti-rabbit IgG conjugated with peroxidase (1:2000) as secondary antibody. The anti-Tb HSP70 serum was kindly provided by Dr. James D. Bangs, University of Wisconsin-Madison and the secondary antibody was purchased at Sigma-Aldrich (A6154). The peroxidase was detected using a chemiluminescent kit from Thermo Scientific (ECL Western Blotting Substrate-32106). Epimastigota extract from *T. brucei* was employed as positive control to confirm the antibody specificity. Scanning densitometry, using ImageJ (Rasband, 2015), was used to determine the levels of HSP70 expression relative to the standard sample.

2.4. Data analysis

The Piecewise Exponential Estimator, PEXE (Kim and Proschan, 1991) was used to illustrate the survival performances. Since our data is continuous and the sample size is not large, we have chosen PEXE in the place of Kaplan-Meier standard estimator, KME. The latter shows a step graph that looks like a discrete survival function. PEXE can be viewed as a smoothing function of KME and is a continuous function as should be a proper estimator.

For the statistical inference about the mortality rates (failure rates for engineers) we consider exponential probability models in order to compare these rates. We adopt the Bayesian perspective that indicates

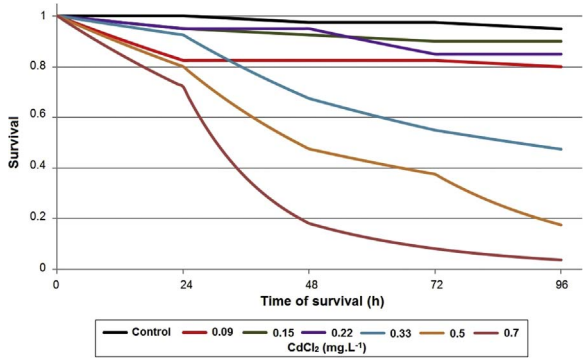

Fig. 1. Survival of *Biomphalaria glabrata* snails exposed for 96 h to $CdCl_2$.

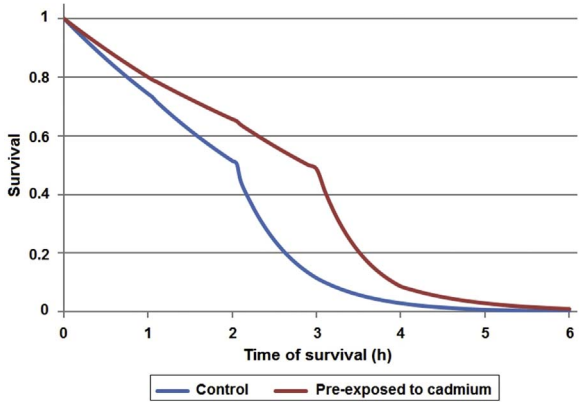

Fig. 2. Acquired tolerance in *Biomphalaria glabrata* snails challenged with heat shock after exposure to a sublethal concentration of $CdCl_2$.

the probability of one rate be higher than the others. As prior densities we use exponentials with rate one and as a consequence all the posteriors densities are the densities of Gamma Distributions. The statistics of the analysis is the Total Time on Test that is described in Barlow (1998). The evidence for the study of significance follows the method described in Pereira et al. (2008). This index is a Bayesian version of p-values.

For the statistical analysis of the effect of cadmium doses on the HSP70 expression we draw a graphic using a polynomial model with its 90% bands. The statistics is the standard minimum square estimator.

3. Results

3.1. $CdCl_2$ toxicity

$CdCl_2$ toxicity was determined by analyzing the survival of animals exposed for 96 h to increasing concentrations of $CdCl_2$ (Fig. 1) and $LC_{50/96\ h}$ value was determined as 0.34 (0.30 − 0.37) mg L^{-1}. The survival was inversely proportional to the concentrations of $CdCl_2$.

Table 1 presents the results of the statistical inference comparing the concentrations of $CdCl_2$ used for the exposure.

3.2. Acquired tolerance

The survival to a lethal heat shock was increased in the animals pre-exposed to a non-lethal concentration of cadmium (Fig. 2). Snails pre-exposed to 0.22 mg L^{-1} $CdCl_2$ survived one hour longer to the 42 °C temperature than the non-exposed ones. Using the exponential distribution, we calculate the evidence of the pre-exposed mortality rate ($fr_{pre} = 0.01$) be higher than the control rate ($fr_c = 0.02$): e-value = 0.1144.

3.3. Effects of $CdCl_2$ on HSP70

HSP70 expression was evaluated by western blot analysis in digestive glands of snails exposed to different $CdCl_2$ concentrations (Fig. 3A). A basal expression was detected in control animals and a greater amount of HSP70 in protein extracts after exposure to cadmium was observed. Induction of the protein was observed as increases in the HSP70 expression relative to the control group values. This increase was directly proportional to cadmium concentration up to 0.22 mg L^{-1}. The amount of HSP70 was remarkably low in the group exposed to 0.5 mg L^{-1} (Fig. 3B).

Table 1
Comparison among the concentrations of $CdCl_2$ tested in *Biomphalaria glabrata* snails.

Dose comparison	Significance for failure rates (e-values)
Fr 0.7 > Fr 0.5	0.000639538
Fr 0.5 > Fr 0.33	0.041728335
Fr 0.33 > Fr 0.22	0.002390802
Fr 0.22 > Fr 0.15	0.106764531
Fr 0.15 > Fr 0.09	0.179149403

3.4. Time-response analysis

Snails exposed to the sublethal conditions determined as the best inducers of HSP70 were assessed for the analysis of HSP70 expression at selected times. All exposed animals showed a time-dependent increase in the expression of HSP70 (Fig. 4A).

In snails exposed to 0.22 mg L^{-1} of $CdCl_2$, HSP70 expression increased up to 96 h. The animals submitted to the sub lethal temperature of 33 °C showed a rapid accumulation on HSP70 exhibiting a peak after 48 h that decreased thereafter up to 120 h (Fig 4B). Measurements were done related to the induction of HSP70 on the control group.

4. Discussion

We investigated the effects of the heavy metal cadmium on the stress protein HSP70 in *Biomphalaria glabrata*. The western blot analysis showed an increased expression of HSP70 in snails exposed to sublethal concentrations of cadmium chloride. Moreover, the HSP70 induction by cadmium could be correlated to an enhanced survival to a lethal heat shock.

A single immunoreactive band in the expected size range (~70 kDa) was recorded from digestive gland of both control and stressed snails; the intensity of this band was stronger in the exposed snails. These data point out the occurrence of a single HSP70 isoform in *B. glabrata* working as housekeeper in normal conditions and overexpressed under stress.

To date, the existence of different constitutive and inducible isoforms of HSP70 in *B. glabrata* is not well established. Laursen et al. (1997) firstly cloned and characterized an inducible HSP70 gene in the *B. glabrata* embryonic (Bge) cell line. Analysis of the predicted amino acid sequence revealed strongest identity (71–76%) and similarity (83–85%) with the HSP70s of various organisms including yeast, *Schistosoma mansoni*, Drosophila, and humans. Northern blot analysis indicated that the gene was either not constitutively expressed or expressed at very low levels at the baseline temperature. Nevertheless, in the protein analysis, a basal expression of HSP70 was observed in cells at control temperature. Zahoor et al. (2010) also observed a basal expression of HSP70 in haemocytes of *B. glabrata*, and Ittiprasert et al. (2009) have confirmed the constitutive expression of HSP70 by qualitative RT-PCR.

The expression of newly synthesized HSP70 isoforms does not seem to be a common feature in mollusks submitted to stress. The induction of stress response is often detected as a significant enhancement in the

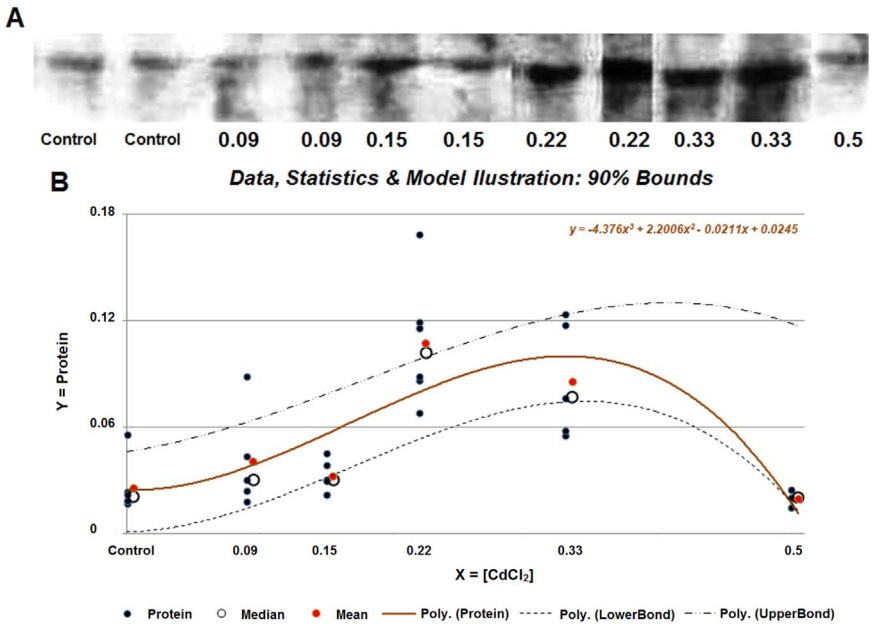

Fig. 3. Western blotting analysis of HSP70 in digestive glands of *B. glabrata* exposed to $CdCl_2$. Snails were exposed for 4 days to water (control) and different $CdCl_2$ concentrations (0.09–0.5 mg kg^{-1}). **A**.Western blotting. **B**. Levels of HSP70 induction on *Biomphalaria glabrata* snails exposed to $CdCl_2$.

expression of constitutive proteins of the 70-kDa family, as in *Mytilus galloprovincialis*, *Tapes philippinarum*, and *Scapharca inaequivalvis* (Piano et al., 2004). A mixture of constitutive and inducible HSP70 isoforms can also be observed, as in the mussels *M. edulis* (Tedengren et al., 2000; Radlowska and Pempkowiak, 2002; Pruski and Dixon, 2007) and *Dreissena polymorpha* (Singer et al., 2005).

To evaluate the induction of HSP70 by cadmium in the present study, a dose-response curve was established to set a range of non-lethal concentrations, since toxicity of $CdCl_2$ is well documented in *B. glabrata* (Allah et al., 2003; Ansaldo et al., 2006). Concentrations ranged from 0.09 to 0.7 mg L^{-1} to include non-lethal and 100% lethal levels. No increase in the mortality compared to the background levels was observed at concentrations up to 0.22 mg L^{-1}; with 0.7 mg L^{-1}, 100% of lethality was obtained. The concentration of 0.34 (0.30–0.37) mg L^{-1} was determined as the LC_{50} to adult snails exposed to $CdCl_2$ for 96 h.

Non-lethal concentrations of $CdCl_2$ (0.09–0.22 mg L^{-1}) caused a dose-dependent increase in HSP70. To our knowledge, there is no data on the induction of HSP70 by cadmium in snails from *Biomphalaria* genus. Nevertheless, the sensitivity of *B. glabrata* was comparable to other aquatic organisms. Our results are in agreement with previous findings on the expression of HSP70 performed with standard immunoblotting techniques in other mollusk species at similar exposure levels. From the literature, oysters *Ostrea edulis* exposed to a range of 0.1–0.5 mg L^{-1} for 7 days of cadmium showed a dose-dependent enhancement of the HSP70 expression (Piano et al., 2004). Singer et al. (2005)

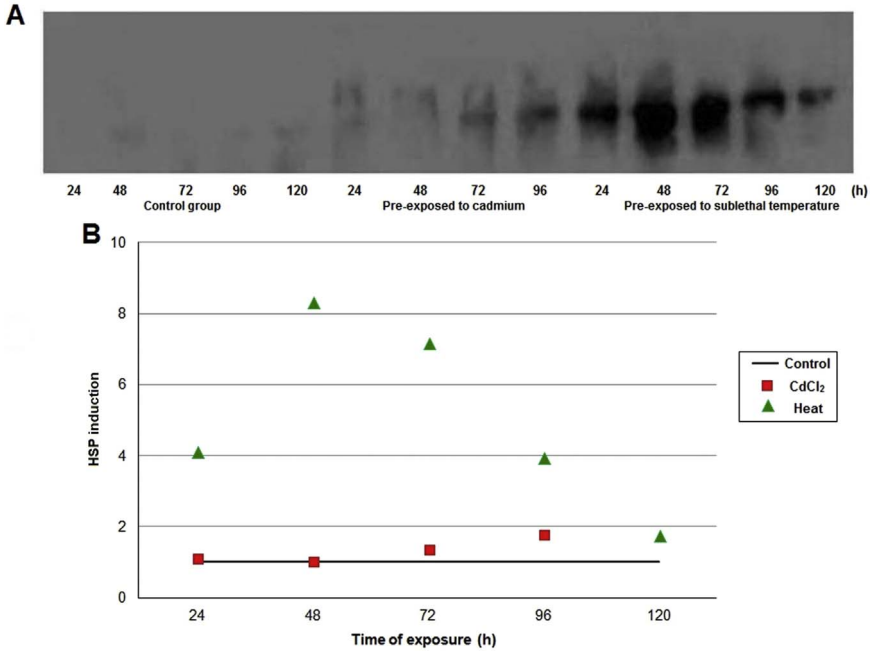

Fig. 4. Western blotting analysis of HSP70 time-dependent induction on *Biomphalaria glabrata* snails exposed to 0.22 mg L^{-1} of $CdCl_2$, and 33 °C. **A**. Western blotting. **B**. Levels of HSP70 induction on *Biomphalaria glabrata* snails exposed to $CdCl_2$ and heat, expressed as arbitrary units of HSP70 induction related to the control group.

exposed zebra mussels *Dreissena polymorpha* to cadmium at higher exposure levels – 0.5 mg L^{-1} for up to 10 weeks – detecting induction of HSP70. Pruski and Dixon (2007) found elevated HSP70 levels in edible mussels at exposure conditions similar to that used in our study – 0.2 mg L^{-1} of $CdCl_2$ for 4 days. Conversely, induction of HSP70 at lower levels of cadmium was observed in other mollusks. Exposure for 18–0.020 mg L^{-1} of cadmium caused an increase in HSP70 expression in Baltic Sea mussels *Mytilus edulis* (Tedengren et al., 2000). Ivanina et al. (2009) observed increased expression of HSP69 in oysters *Crassostrea virginica* with 50 μg/L of $CdCl_2$; however, the response was detected after a long term exposure of 45–50 days.

Increased levels of HSP70 in response to cadmium treatment have been described for several non-mollusk species, including *Physcia adscendens* (lichen), *Chironomus tentans* and *Spodoptera exigua* (insects), *Paracentrotus lividus* (sea urchin), *Pimephales promelas*, *Oncorhynchus mykiss*, and *Tanichthys albonubes* (fish) (Sanders et al., 1995; Williams et al., 1996; Geraci et al., 2004; Lee at al, 2006; Rustichelli et al., 2008; Jing et al., 2013; Kafel et al., 2014).

Other HSPs have also showed to be induced by cadmium as well as by heat shock. The transcription and expression of small heat shock proteins (sHSPs) HSP23, HSP24, HSP27 and HSP34, HSP40, HSP60 and HSP90 were increased in mollusks, insects and corals treated with these stressors (Park et al., 2015; Li et al., 2016; Seveso et al., 2016; Martin-Folgar and Martinez-Guitarte, 2017; Martínez-Paz et al., 2017).

The time course of HSP70 expression in *B. glabrata* exposed to cadmium was evaluated in the present study to identify the time window of the maximum stress response. The exposure to cadmium was performed concurrently with heat shock, used here as a reference stressor. Samples from the animals exposed to cadmium and those exposed to heat were blotted together, and a continuous exposure protocol was chosen to allow the accumulation of HSP70 at levels to be detected at the immunoblotting and the response was assessed at 24 h intervals. A time-dependent increase in HSP70 levels occurred in all exposed snails. After the nonlethal heat shock of 33 °C, HSP70 expression reached the highest level at 48 h and decreased thereafter without, however, resuming baseline levels. Likewise, stress proteins from oysters *Crassostrea gigas* and *C. virginica* peaked two days following a sublethal heat shock; control levels were reached after 14 days (Jackson et al., 2011). Also in *C. virginica*, Ivanina et al. (2009) observed the strongest induction of heat shock proteins after 48 h of recovery from heat shock. Following the exposure of *B. glabrata* to cadmium, the highest level of HSP70 was detected after 96 h. However, to determine the time for maximum induction of HSP70 and recovering of background levels, analysis of further time points is needed. Nonetheless, our data are in agreement with the study of Singer et al. (2005) with the zebra mussel *Dreissena polymorpha*. The effect of cadmium on HSP70 levels was recorded for 10 weeks and a weak induction of HSP70 was observed in the first 2–3 weeks; the maximum HSP70 concentrations were detected at day 39. In contrast, Radlowska and Pempkowiak (2002) found elevated HSP70 concentrations already after 2 days following exposure of *Mytilus edulis* to cadmium. Similarly, Piano et al. (2004) observed increased HSP70 levels in gill and digestive gland of *Ostrea edulis* after 7 days of exposure to cadmium.

To test for the hypothesis that the accumulation of HSP70 induced by cadmium could elicit a response at organism level, we assessed the occurrence of acquired tolerance. Previously, we have observed the occurrence of thermotolerance in *B. glabrata* and the possible involvement of HSP70 in the process (Cantinha et al., 2013). In the present study, we aimed to verify if a sublethal exposure to cadmium would induce a protective effect against a lethal heat shock.

Snails were pre-exposed to cadmium and then submitted to a lethal heat shock. The 0.22 mg L^{-1} concentration was selected with basis in the dose-response curve of cadmium as the nonlethal concentration that exhibited the greatest effect on the expression of HSP70. The analysis of survival showed that animals pre-exposed to cadmium had a significantly increased survival to the lethal heat shock.

The induction of HSPs as a critical factor in increasing the survival to a lethal stress is experimentally supported. In experiments measuring the rate of thermotolerance development, it closely parallels the rate of HSP accummulation. Moreover, the decay of thermotolerance when cells are returned to normal temperature, parallels the degradation of HSPs. Tolerance can also be induced by other types of conditioning treatments. These have in common the property of inducing HSPs. Exposure to ethanol, hypoxia, and heavy metal ions are commonly employed. Such treatments do not induce heat-shock proteins in all organisms, but when they do, they also induce thermotolerance (Lindquist and Craig, 1988).

Our results showing an increased survival to a lethal heat shock in animals pre-exposed to a sub-lethal concentration of cadmium corroborate this hypothesis and reinforce the relationships between contaminant exposure, organismal stress and stress protein accumulation.

5. Conclusion

The results obtained in the present study with a relevant environmental contaminant, the heavy metal cadmium, demonstrates the potential applicability of the HSP70 analysis in *Biomphalaria glabrata* as a biomarker for freshwater environmental monitoring. Snails could detect cadmium at sublethal levels through increases in HSP70 expression. This species has been used in our studies as a model in the assessment of freshwater contamination through the analysis of toxicity, genotoxicity and mutagenicity (Nakano et al., 2003; Estevam et al., 2006; Tallarico et al., 2014). By including the analysis of HSP70 induction, a general stress response, to the assessment protocol, a more effective monitoring strategy is expected to be achieved.

Funding

This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior (CAPES).

Acknowledgments

The authors would like to thank Dr. James D. Bangs, who gently provided the primary antiserum used in the experiments.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2017.02.026.

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