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# Assessment of thermal stress adaptation by monitoring Hsp70 and MnSOD in the freshwater gastropod, *Bellamya bengalensis* (Lamark 1882)

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**Abstract** Expression of the stress biomarkers 70-kDa heat shock proteins (Hsp70) and manganese superoxide dismutase (MnSOD) was measured as the molecular basis of adaptive response against increased experimental temperatures (32–40 °C for a span of 24–72 h) on the fresh water molluscan species, *Bellamya bengalensis* (Lamark 1882). The experimental snail specimens were collected during summer and winter seasons from two contrasting wetlands: an ecorestored (free from human interference) site (SI) and other experiencing anthropogenic stresses (SII). The mortality rate of the *B. bengalensis* and the immunoblotting of MnSOD and Hsp70 of their digestive glands were performed at regular intervals during the period of heat stress. The SI provided a lower stress environment based on physicochemical parameters such as pH, dissolved oxygen (DO), biological oxygen demand (BOD), chemical oxygen demand (COD), and alkalinity for the survival of test species, although both sites experienced mortality due to thermal stresses. The parity in protein expressions displayed a uniform mode of adaptive impact to temperature elevations in both field and laboratory exposure. The Hsp70 expression was minimal at lower thermal stress, but increased with a rise in temperature. It is very likely that higher Hsp70 levels are not directly related to survival or adaptation. In contrast, MnSOD levels appeared to be an indicator of adaptive responses vis-a-vis survival of the animals. So, the expression levels of a universal free radical scavenger like MnSOD are recognized as a potential biomarker in a bioindicator species like *Bellamya*.

**Keywords** Heat shock protein · SOD · Stress · Biomarker · Wetland · *Bellamya bengalensis*

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## Introduction

Global climate change and the environmental pollution can lead to an increase in the temperature of water bodies. This increase results in polar ice melting and in the reduction of the available time for the polar water bodies to freeze. It also alters several physicochemical parameters like salinity, heavy metals, biological oxygen demand (BOD) and chemical oxygen demand (COD), etc. of the aquatic environment (Ficke et al. 2007; de Bij et al. 2006). The existence of multicellular life is limited to a narrow thermal range (Guderley and St-Pierre 2002). Environmental temperatures are expected to rise by 1.4–5.8 °C by the end of this century as a result of global warming (Parry et al. 2007). The exposure of organisms to temperature stress activates

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expression of different heat shock proteins (HSPs) (Piano et al. 2002; Dieterich et al. 2013). HSPs are ubiquitous chaperones that assist refolding of stress-induced denatured proteins (Nollen and Morimoto 2002; Wang et al. 2013). HSPs are regarded as important biomarkers in organisms undergoing pollution or thermal stress in nature (Hofmann et al. 2000; Metzger et al. 2012; An et al. 2014). Amongst these, the highly conserved and temperature-responsive 70-kDa heat shock protein (Hsp70) family is best correlated to both innate and acquired thermal tolerance (Voellmy and Rungger 1982). Moreover, heat-induced generation of reactive oxygen species (ROS, i.e., superoxide, hydrogen peroxide, and hydroxyl radicals) has been implicated in stress-related molecular response and acclimatization (Verlecar et al. 2008). Superoxide dismutase (SOD) is the chief regulator of ROS and a producer of $H_2O_2$ from $O_2^{-}\cdot$ (Fridovich 1995; Landis and Tower 2005). Both of these proteins are primitive and universal in their structure and actions in organisms (Monserrat et al. 2007). Hsp70, being a molecular chaperone help in proper folding of proteins in order to maintain stability and thereby, acts as an important mitochondrial transporter protein (Hofmann et al. 2000). However, manganese superoxide dismutase (MnSOD) is a scavenger of superoxide which is a commonly occurring free radical in oxidative stress, especially in the presence of heat. Scavenging of superoxide is one of the most important stress adaptations because the presence of $O_2\cdot^{-}$ and $H_2O_2$ can also lead to the formation of the highly reactive and damaging hydroxyl radical (OH·) (Halliwell and Gutteridge 1986). The free radicals can perturb structures of all cellular components such as membranes and resulting mutagenic DNA breakage (LaVerne and Pimblott 1993). Mitochondrial cytochrome oxidase in complex IV has facultative MnSOD activity. This complex catalyzes $O_2$ reduction at the terminal point of $e^{-}$ transport chain that accelerates during heat stress and produces more superoxide and induce facultative SOD activity of cytochrome oxidase (Halliwell and Gutteridge 1986). Apart from this, the cytosolic SOD is also induced in temperature stress (Yu et al. 2011).

The crucial balance between the exogenous pro-oxidant factors and the antioxidant defense system of an organism may be evaluated to assess the impact of the environmental stressors on the ecological system (Andersen et al. 2006). SOD and Hsp70 have been established to be up-regulated by the ROS, developed after being exposed to thermal stress (Liu and Post 2000; Banerjee et al. 2009). The combined effects of water temperature and other environmental factors have been shown to influence on the expression of Hsp70 (Wang et al. 2012; Izagirre et al. 2014). In addition, sublethal concentrations of pesticides, fertilizers, detergents, heavy metals, and hydrocarbons resulting from human activities are the causes of contamination of the aquatic environment (Klumpp et al. 2002). Adaptive biochemical changes in certain bioindicator organisms may identify important biomarkers in order to evaluate the long-term impact of these pollutants on the environment (Schwarzenbach et al. 2006).

In the present study, a fresh water molluscan species, *Bellamya bengalensis* (Lamark 1882) (Gastropoda:Viviparidae) was selected as a test animal to evaluate the impact of the physicochemical properties of water, environmental pollution, and heat stress in two wetlands—one ecorestored, devoid of human interference (SI), and the other a human impacts wetland (SII). Besides, biomarker (SOD and Hsp70) expression studies, the survivability, mortality, and stress adaptability in this experimental species were evaluated during summer and winter seasons. The present study has also attempted to have improved the understanding of the use of suitable biomarkers in a commonly occurring species in order to address the impact of global environmental stresses.

## Materials and methods

An ecorestored and un-interfered wetland having an area of 40,200 $m^2$ and maximum depth of 5 m located at Gurguripal (SI) about 10 km away from Midnapore city of southwestern part of West Bengal, India, has been selected as one study site. This wetland has been ecorestored through watershed management of the degraded forest on undulating lateritic tracts through the concerted efforts of local people by the way of afforestation. Such environmental strategies promoted the conversion of a degraded seasonal wetland to a perennial wetland having normal biotic and abiotic components. Another water body (SII) having an area of 1500 $m^2$ and maximum depth of 4 m located in Midnapore city, used for all domestic works, has been selected as an ecologically stressed habitat of the species understudy. The SI supported a good number of macrophytic species along the bank which includes *Scirpus articulatus*,

*Nymphoides cristatum*, *Utricularia* sp., *Brachiaria sp*., *Cardanthera difformis*, *Cyanotis axillaris*, *Eriocaulon* sp., and *Hydrilla* sp., while the SII is almost free from any macrophytic coverage except *Eichornia sp*.

Physicochemical parameters including temperature, dissolved oxygen (DO), pH, turbidity, alkalinity, total suspended solids (TSS), total dissolved solids (TDS), biochemical oxygen demand (BOD), chemical oxygen demand (COD), and nutrients—total Kjeldahl nitrogen (TKN) and total Kjeldahl phosphorus (TKP) were measured at regular monthly intervals during morning hours from three separate subsites within each study site following standard methods (Eaton 2005; Trivedy and Goel 1984).

Adult specimens of *B. bengalensis* were collected from both water bodies (SI and SII) during the summer (April–May) and winter (December–January). This species was selected for its availability and demand as high protein food (Baby et al. 2010). After the collection from the natural environment, specimens were kept in plastic trays in the laboratory temperature at 25 °C and fed with leaves of spinach, lettuce, and green algae.

After collection, twenty individuals of *B. bengalensis* per tray were kept in three trays and incubated for 24, 48, or 72 h maintaining the desired temperature viz. 32, 36, 38, or 40 °C with adequate food and water in both summer and winter. Three such replicates were used to study the mortality rate of studied species.

At the end of each treatment period, digestive glands were collected from live representative animals and homogenized in lysis buffer containing 50 mM Tris-Hcl, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM $NaOV_4$, (*v*/*v*) $NP_{40}$, and 1 mM PMSF and centrifuged at 10,000 rpm for 30 min. Supernatants were utilized for protein measurement (Bradford 1976). These were kept in aliquot at −20 °C until use. Proteins (20–50 μg) from cytosolic fraction were separated by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were incubated overnight at 4 °C with respective primary antibodies after blocking with 5 % bovine serum albumin (BSA). Interspecies cross-reactive antibodies against Hsp70 (cat. no. 610607, BD Pharmingen, against amino acids 429–640 of human Hsp70) and manganese superoxide dismutase (MnSOD) (cat. no. 611580, BD Pharmingen) were used in this investigation. The β-actin antibody was purchased from Imgenex (Cat. no. IMG-5142A). The membranes were incubated with 1:5000 dilutions of the appropriate peroxidase-conjugated secondary antibodies and/or alkaline phosphatase-conjugated antibodies and developed for detection by chemiluminescence or colorimetry. Western blots were scanned and densitometric analysis of the scans was performed by using ImageJ software. All biochemical experiments were run in triplicate from independent set of experiments, and the representative data/pictures are presented in this study.

## Results and discussion

The mortality rate of *B. bengalensis* from SII was found to be always less from that of SI animals in similar experimental condition. No mortality of the test specimen was found up to 32 °C in both study sites and seasons (winter and summer). Mortality was also not recorded up to 36 °C in the summer for both of the study sites. However, mortality was greater for *B. bengalensis* collected from SI compared to SII in winter. The SI animals displayed a higher and similar mortality at 38 °C (in winter and summer) with comparison to SII animals. SII animals showed a very nominal mortality in summer than that of winter at this temperature. At 40 °C temperature, specimens collected from both SI and SII have experienced less than 50 % mortality at 24 h in summer, which increased to ~70–75 % mortality in winter. The mortality rate was found to be ~100 % at 48 and 72 h in both the seasons and sites (Fig. 1). In general, the effect of stress on mortality rate was markedly higher at 40 °C without exhibiting any prominent distinctions between sites and seasons. All potential stressors were higher at SII (Fig. 2) where most of the parameters exhibited their higher values from May to September.

The variability in the protein levels of Hsp70 and MnSOD as observed by the immunoblotting method is presented in Fig. 3. In winter (Fig. 3a), *B. bengalensis* inhabiting SI displayed an almost 6-fold increase of Hsp70 levels when they were subjected to external heat shock of 36 °C (72 h). These elevated levels of Hsp70 were further increased by an exposure to a higher temperature of 38 °C (72 h). The MnSOD expression also increased by ~7-fold at 36 and 38 °C after an exposure of 48-h duration for snails collected from SI in the winter. However, that expression rate was either inconsistent or decreased at higher temperature (38 °C, 72 h). Both the Hsp70 and MnSOD levels also showed no

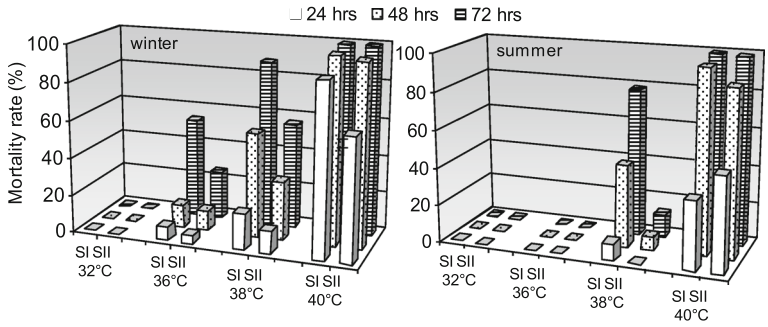


**Fig. 1** Mortality rates for *Bellamya bengalensis* in summer and winter seasons from site I (SI) and site II (SII) exposed to different heat stresses for different durations. Data are presented as mean±SD of triplicate samples

significant alteration in response to elevated temperature for animals collected from SI during the summer (Fig. 3b) except the Hsp70 expression at 38 °C which was increased by 1.8–2.7-fold at different periods of exposure.

The Hsp70 expression in *B. bengalensis* from SII (Fig. 3c) also demonstrated a gradual increase in Hsp70 levels over the control in winter. However, the up-regulation of Hsp70 in treatment at SII never exceeded by a 2.5-fold increase at both 36 °C (48 h) and 38 °C (48 h). The expression of this protein exhibited higher results with respect to control, but the duration of the dependant response was not prominent. In contrast, snails collected from SII during the summer had demonstrated a significant up-regulation of both Hsp70 and MnSOD and had an almost 4-fold increase (36 °C for a span of 48 h) and a 2.5-fold increase (38 °C of the duration of 48 h), respectively (Fig. 3d). It can be concluded that longer durations at higher temperatures result in reduced protein expression in the present study.

MnSOD levels showed a considerable increase during an exposure of 24 and 48 h over untreated controls for both sites and seasons. MnSOD levels of *Bellamya* exhibited markedly higher levels at SII than at SI in winter. Moreover, the increase of MnSOD was sustained through 72 h of treatment when maximum mortality was observed. In summer, the expression of Hsp70 of studied animals from both the SI and SII did not indicate any

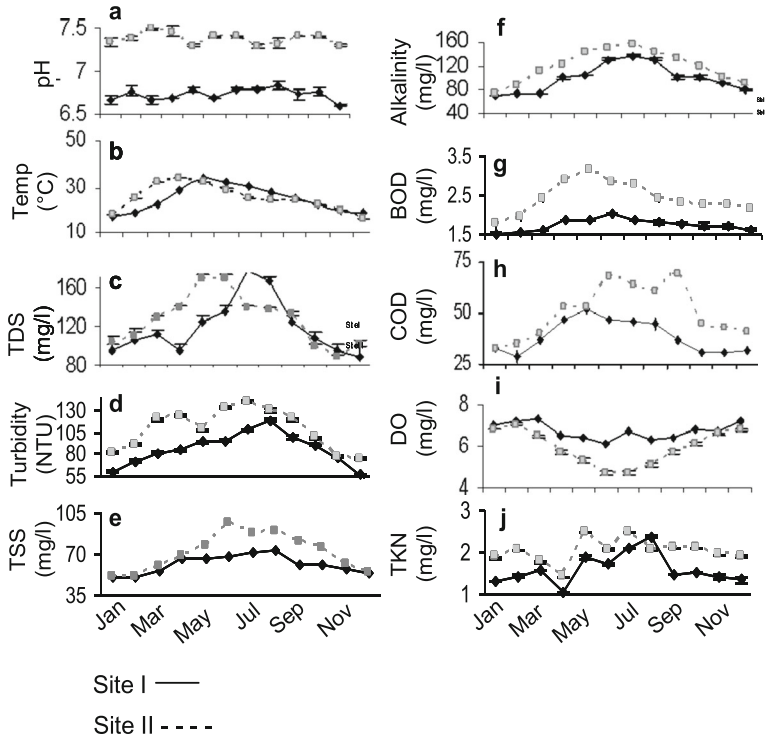


**Fig. 2** Monthly patterns of physicochemical parameters for SI and SII. Data are presented as mean±SD of triplicate measurements

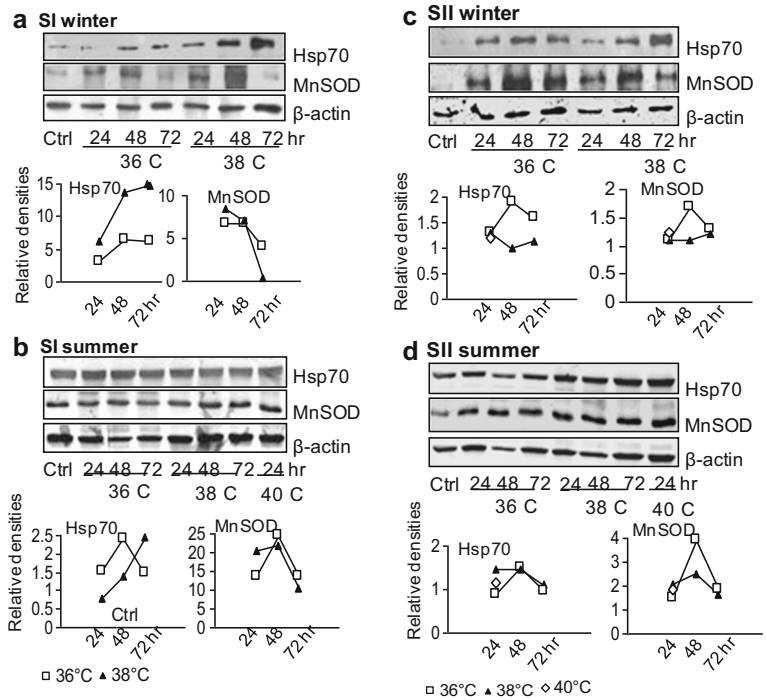


**Fig. 3** Expression of Hsp 70 and MnSOD in *Bellamya bengalensis* from SI in response to heat stress in winter (**a**) and summer (**b**). Expressions of Hsp 70 and MnSOD in *Bellamya bengalensis* from SII in response to heat stress in winter (**c**) and summer (**d**). The *graph plots* represent the normalized values of densitometric data (ImageJ software) of the corresponding band

alteration in protein levels. The basal levels of Hsp70 protein in control (laboratory stress unexposed) snails of both sites were found to be notably higher in summer than that of winter. As a result, a further expression of this protein was found not to be very significant due to the laboratory temperature exposure to these snails. Hence, these results define better the field-derived component (not laboratory derived) represented by the animals. Though these seasonal protein expression variations are mainly attributed by the temperature change, we have to take into account the interactive role of other physicochemical variables of the corresponding environment. Due to the higher incidence of mortality in *Bellamya* after being subjected to elevated temperature at 40 °C in specimens collected in winter, Hsp70 and MnSOD levels could not be measured.

Response to stress is a key factor for survival of an organism (Storey 1996; Gidalevitz et al. 2011). Low-molecular-weight heat shock proteins have been established as stress markers in aquatic organisms such as amphipods and other taxa with a narrow niche breadth (Timofeev et al. 2008; Sanders 1993). Heat shock protein (HSP) expression has also been linked to oxidative stress, heavy metal toxicity, and chemical pollution (Feder and Hofmann 1999; Liu et al. 2014). Heat stress has been correlated to the oxidative damage in different cellular components such as lipids, proteins, and thiols in gold fish and other aquatic animals (Lushchak and Bagnyukova 2006). Altered regulation of MnSOD has been linked to stress due to environmental factors, metabolic stress, and microbial infections in aquatic animals like *Biomphalaria glabrata* and others. This suggests that MnSOD serves as an important marker for cell stress and survival (Zoysa et al. 2009; Fried and Reddy 1999). The cytosolic SOD (Cu-Zn SOD) has also been shown to counteract different stresses (LaVerne and Pimblott 1993).

In the present investigation, *B. bengalensis* collected from both sites was found to be susceptible to mortality by thermal stress (Fig. 1). However, at SII, which has been used regularly for domestic purpose (washing and bathing etc.), results in greater stress adaptation in aquatic animals. Probably for this reason, the water pH, alkalinity, TDS, TSS, BOD, and COD were found to be consistently higher in SII compared to SI. The higher BOD and productivity in SII was likely also responsible for the lower DO concentration in this site. In addition, unlike SI, SII is a closed and isolated water body which does not experience the water recirculation and passage with other water bodies. The time- and temperature-dependant changes in stress responses were less prominent at SII than SI during summer. But the responses at

SI and SII in winter were found to be similar. Both the increase in MnSOD and HSP70 appeared to be time dependant at different temperatures during winter. Since the basal values of both proteins were higher in summer compared to winter, further elevation of temperature in laboratory condition resulted little change in *B. bengallensis* collected during the summer. This observation indicates that natural temperature changes due to seasonal variations in association with the corresponding physicochemical parameters had measurable effects on Hsp70 and MnSOD expressions. The responses generated due to the seasonal variation were similar to the results noticed in laboratory temperature experiments. It reveals the ubiquitous impact of heat stress adaptation in these animals. The increase in both MnSOD and Hsp70 is interpreted as adaptive changes against heat stress.

In summer, there is a comparatively small difference between the ambient water temperatures of both sites and the temperature applied for inflicting thermal stress to *Bellamya*. As a result, the impact of the thermal stress alone is much lower in summer in comparison to that of winter. This was reflected by the lower mortality of *Bellamya* as observed for both SI and SII (Fig. 1). However, SII *Bellamya* specimens exhibited more resistance to mortality in summer. It was further observed that when exposed to elevated thermal stress, the snails did not have mortality probably due to their pre-adaptation to apparently polluted water.

In this context, it can be inferred that the heat shock response can be induced in *B. bengalensis* through induction of Hsp70 when the thermal stress is substantial. At lower thermal stress levels, induction of Hsp70 was found to be at minimum levels, whereas higher Hsp70 levels did not show any relation to survival or adaptation. The MnSOD levels, as an indicator of survival in the studied species, reflect variability in the tolerance level from lower intensity stresses to poor quality water condition. Thus, measurement of MnSOD levels is supposed to serve as the potential biomarker in a freshwater bioindicator species, *B. bengalensis*. The significance of the present study focuses on the dependent biomarkers to predict environmental stresses imprinted on a bioindicator species.

It is noticed in the present experiment that the SII animals were acclimatized and adapted to an array of pollutants and thereby expressed more tolerance prior to their utilization in the heat stress experiments. It can be hypothesized that the pre-adaptation to a certain degree of cumulative stress may provide some resistance against adverse stress condition. In future, the best possible laboratory experimental schedule resembling natural environmental condition may open the further scope for the understanding of natural stress adaptation.

In view of the present scenario for global warming, extrapolation of this research outcomes may provide the groundwork for further studies to explore the strategy of natural and long-term stress adaptation against sustained environmental threats.

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