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Heat shock protein 70 (Hsp70) response to elevated temperatures in the endemic Baikal sponge *Lubomirskia baicalensis*

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ABSTRACT

A mass bleaching event affecting an endemic Baikal sponge points out the need to study stress responses in these organisms. To get the first data about Hsp70 content changes in endemic Baikal sponge *Lubomirskia baicalensis* during exposure to elevated temperatures which can occur in its natural habitat, we investigated the effect of two elevated temperatures on Hsp70 levels in the *L. baicalensis* and its cell aggregates (primmorphs). After increasing the water temperature from ambient 4 to 9 °C Hsp70 levels increased to protect against temperature stress. Incubation at 13 °C for one month resulted in bleaching and death of the sponges. The decreased level of HSP70 was recorded both in the case of bleaching after exposure to 13 °C, and in diseased individuals from the Lake Baikal. The *L. baicalensis* primmorph temperature stress response was not consistent with that of the sponge. In primmorphs, the Hsp70 level initially decreased at 4 °C and at 13 °C it increased compared with the levels observed in the sponge. Thus, our results show that primmorphs are not a good model system for the study of expression of stress proteins in sponges. Since the temperatures 9–13 °C were observed at *L. baicalensis* habitats, climate warming may increase vulnerability of this sponge to bleaching.

1. Introduction

Sponges (Porifera) constitute an important component of marine and freshwater ecosystems because of their species richness, abundance, and key functional roles (Diaz and Rutzler, 2001; Bell, 2008; Van Soest et al., 2012; Webster and Taylor 2012; Bell et al., 2017). Marine sponges, along with corals, are very sensitive to temperature changes and several sponge mortality events have been correlated with climatic changes (Vicente, 1989; Cerrano et al., 2000; Cebrian et al., 2011). In the Great Barrier Reef sponge *Rhopaloeides odorabile*, even short-term temperature increases cause stress-related physiological responses in adults (Webster et al., 2013). Sponges are benthic filter-feeding organisms that can accumulate water pollutants from the environment in the body. Therefore, sponges are subjected to stronger stress effects than other organisms and are regarded as sensitive bioindicators (Schröder et al., 1999; Cebrian et al., 2007; Bell et al., 2017), which highlights the importance of ecological and physiological studies on sponges.

Lake Baikal is the world's oldest and deepest lake. It is an estimated 35 million years old, and its maximum depth is 1647 m (Timoshkin, 1995). Endemic species have evolved in the lake in a stable environment for millions of years and formed special adaptive mechanisms. The Lake Baikal endemic sponge family Lubomirskiidae constitutes the bulk of the benthic biomass and inhabits the lake at all depths. There are thirteen described Lubomirskiidae species and two subspecies (Efremova, 2001, 2004; Itskovich et al., 2017). *Lubomirskia baicalensis* (Pallas, 1773) inhabits depths of 3–120 m and forms underwater forests. This species, together with *Baikalospongia intermedia* Dybowsky, 1880 dominates the lake's littoral zone (Masuda, 2009).

The heat shock protein (Hsp) response mechanism maintains protein homeostasis while organisms adapt to variable environmental conditions (Bosch et al., 1988; Miller and McLennan 1988a,b; Sanders, 1993; Kültz, 2005). Hsps play an important ecological and evolutionary role in thermal adaptation and are commonly used as heat stress biomarkers (Parsell and Lindquist 1993; Feder and Hofmann, 1999; Tomanek, 2010; Madeira et al., 2014). Hsps act as chaperones and play an important role during exposure to stress by either folding or unfolding nascent polypeptides, repairing molecular structures, and removing damaged protein molecules (Gelthing and Sambrook 1992; Parsell and Lindquist 1993; Morimoto et al., 1994). The *Hsp70* multigene family includes the cytoplasmic stress-inducible Hsp70 and the constitutively expressed heat shock cognate protein Hsc70 (Lindquist

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and Craig 1988; Feder and Hofmann 1999; Cottin et al., 2010). Transcriptional and translational Hsp70 and Hsc70 expression can be induced by many types of stress, such as exposure to extreme temperatures, organic pollutants, and the presence of heavy metals (reviewed in Craig, 1985; Parsell and Lindquist 1993; Feder and Hofmann 1999; Kültz, 2005; Urian et al., 2011). Investigations into heat-shock responses are, therefore, important to determine species optimum temperature and to better understand their evolutionary patterns.

Despite the ecological importance of Porifera, studies on sponge stress responses are rare. Hsp70 expression was first shown in the freshwater sponge *Ephydatia fluviatilis* Linnaeus, 1759 (Müller et al., 1995). Further investigation has revealed that Hsp70 can be induced in sponges by either thermal or osmotic shock, pH stress, heavy metals, and phenols (Koziol et al., 1996; Wiens et al., 1998; Webster et al., 2013; Guzman and Conaco, 2016). In thermally stressed *Spongilla lacustris* Linnaeus, 1759, stress protein level patterns resemble those of gene expression patterns and exhibit an even greater intensity and sensitivity (Schill et al., 2006). In gemmules (resting bodies that contain totipotent cells) of the freshwater sponge *S. lacustris*, increased levels of cellular Hsp70 and *Hsp70* mRNA likely allow the gemmules to stabilize their proteins and membranes when the water temperature changes (Schill et al., 2006). The dynamics of Hsp70 in marine sponges has been studied extensively to elucidate global warming-induced heat stress. Bleached sponges have high Hsp70 levels, and increases in water temperature can result in coral reef sponge population decline (López-Legentil et al., 2008; Pantile and Webster, 2011; Webster et al., 2013). During the first transcriptome-wide survey of the shallow water sponge *Haliclona tubifera* response to thermal stress the potential mechanisms enabling *H. tubifera* to survive conditions of thermal stress were identified (Guzman and Conaco, 2016). The immediate stress response of *H. tubifera* affects expression patterns of heat shock proteins, antioxidants, and genes involved in signal transduction and innate immunity pathways (Guzman and Conaco, 2016).

Increased Hsp70 level was detected in the Lake Baikal sponges *L. abietina* Swartschewsky, 1901 and *B. intermedia* after exposure at the elevated temperature of 20 °C and waste water from the Baikal Pulp and Paper Mill (Efremova et al., 2002; Schröder et al., 2006). A mass sponge bleaching event in Lake Baikal has been detected recently (Kaluzhnaya and Itskovich 2015; Timoshkin et al., 2016). It points out the need to study stress responses in these organisms. The aim of this study was to analyze the effect of increasing temperatures which can occur in its natural habitat on Hsp70 levels in adults and primmorphs (sponge cell aggregates) of the endemic Baikal sponge *L. baicalensis*. Also our aim was to analyze changes in the level of HSP70 occurring in sponges during the development of the bleaching syndrome.

2. Methods

Lubomirskia baicalensis samples were collected by SCUBA in August 2013 in the Southern basin of Lake Baikal near the village of Bolshie Koty at 10 m depth (Figs. 1 and 5). Immediately after transfer to the laboratory, sponge specimens (n = 3) were placed into an aquarium with Lake Baikal water and were kept at 4 °C at a 12 h:12 h light:dark cycle with aeration for 14 days to acclimatize.

For the temperature experiment, 5 cm of each sponge was maintained in a 6 L aquarium at 9 and 13 °C with daily exchange of two liters of water. The controls were kept at 4 °C for the month.

Temperatures of 9 and 13 °C were chosen according to data on variation in fatty acid composition of total *L. baicalensis* lipids during aquarium cultivation at these temperatures (Glyzina and Glyzin 2014) and data on water temperatures at 10 m depth (Timoshkin et al., 2009). The incubation times were 2 h, 15 h, and 7 days. Small pieces (in triplicate samples) of each sponge were subsequently frozen in liquid nitrogen and another piece was kept at 4 °C to recover for 2, 7, 20, and 36 h. The other samples were incubated at 13 °C for one month.

For the experiments with dissociated cells, 1 cm^3 of each sponge (n = 3) was pressed through a 250 μm nylon mesh and the cell suspensions were placed in Petri dishes with 50 mL Lake Baikal water as described in Efremova et al. (2002). After cell conglomerates had formed, the water was changed several times and the primmorphs were then cultivated with daily water changes at the same temperatures as the sponge samples (4, 9, and 13 °C) (Fig. 1). After exposures of 2 h and 7 days primmorphs were frozen in liquid nitrogen. Triplicate samples of each primmorph were used for subsequent protein extraction.

Total protein was extracted from three replicates of each sample frozen in liquid nitrogen immediately after temperature treatment as described earlier (Voinikov et al., 1986). Protein concentration in the samples was determined with a Quant-iT™ Protein Assay Kit (Thermo Fisher Scientific). Thirty-μg of protein from each sample were separated by electrophoresis in 12% SDS-PAGE (Laemmli, 1970); the protein was then transferred onto a nitrocellulose membrane in a mini–Protean III (Bio-Rad, USA) system according to the manufacturer's instructions. Hsp70 concentration was measured by western blotting with primary antibodies (H5147: Sigma, USA) and secondary antibodies conjugated with alkaline phosphatase (Sigma) as previously described (Timmons and Dunbar, 1990). Protein molecular mass was determined by run length (Rf) via standard molecular markers (molecular weight marker kit, Sigma). Hsp70 content in the protein sample was determined from the overall intensity of protein-stain coloration on the membrane. Coloration intensity was determined in ImageJ 1.50i (Rasband W; National Institutes of Health, USA), according to the number and intensity of colored pixels on the membrane after visualization as a percentage of protein spot coloring intensity in the control (=100%, left bar in Figs. 2–4,6 and 7). A typical membrane from each biological replicate was used to determine spot intensity. The Hsp70 spot coloring was normalized according to the color intensity of the total protein staining in the gels using Coomassie R-250 with ImageJ software. Beta-actin, which is commonly used to normalize, could not be used in this case because its content in this sponge is altered by heat shock. Moreover, the band corresponding to beta-actin on immunoblots sometimes separates into two bands of different molecular weight (see Fig. A Additional materials). Therefore signal intensities were normalized to total protein by staining membranes with Coomassie Blue as recommended in previous studies (Eaton et al., 2013; Gilda and Gomes, 2013). Statistical analysis, including mean ± SD, normality test (Shapiro–Wilk), and one-way analysis of variance (ANOVA), was carried out in SigmaPlot V 12.0 (SysStat Software Inc., CA, USA). Statistical significance was tested by the Fisher LSD method.

3. Results

Given the highly conserved structure of Hsp70, it was necessary to confirm that the antibodies used detected Hsp70 proteins in the sponges rather than reacting with Hsp70 from the sponge's microalgal symbionts. Therefore, blotting was performed on Hsp70 proteins from green algae (*Chlorella*) to compare the results to those from the sponge adults and primmorphs. Two immunoreactive bands with similar molecular weights corresponding to the molecular mass of Hsp70 proteins were detected in the sponge samples. The molecular weight of the algae protein was higher than sponge Hsp70 and the degree of interaction by the antibodies used was very weak (Fig. B Additional materials). Thus, we found that the sponge and algae HSP70 proteins had very different molecular weights and were easily distinguished on the immunoblots. On the sponge protein immunoblots, no bands with molecular weights similar to the algae proteins were observed. Therefore, it was concluded that these antibodies only recorded sponge Hsp70.

The experiments were conducted to study the effect of elevated temperatures on the dynamics of Hsp70 accumulation in the endemic Baikal sponge *L. baicalensis*. Constitutive synthesis of Hsp70 was detected in samples immediately after sampling. We did not detect increased Hsp70 levels after acclimatization and maintenance in the aquarium for one month at 4 °C, which indicated appropriate