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Realtime expression of sHSPs and estimation of Trehalose as a Cyst Quality Index in *Artemia*

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ABSTRACT

The physical and biological conditions of the ecosystem are in a dynamic state, and there is a nature-defined limit where the animals can live naturally. The ability of hypersaline marine invertebrates to withstand salt injuries in oceanic habitats suggests that their cells are equipped with biochemical and genetic mechanisms providing competitive advantages to stress factors. Artemia, universal live feed, is used as live feed for over 85% of marine aquaculture species. The study's objective is to analyze the real-time expression of small heat shock protein (Hsp) 22 in the different developmental stages of Artemia and determine the differential expression of trehalose in the different cryptobiotic Artemia. To develop a biomarker to analyze the quality/hatching percentage of the Artemia cyst. The study revealed the sHSP22 expression in all developmental stages, and the animal is "being prepared for stress" resistance by expressing the sHSP22 for a lifetime. Trehalose, an alpha-linked disaccharide formed by an α, α-1, 1-glucoside bond between two α-glucose units, also protects biological materials against dehydration and desiccation. Artemia cysts survive severe stress, such as long-term anoxia, salinity, and heat, all hallmarks of the Artemia environment due to its rigid chitinous cyst wall permeable and the disaccharide trehalose stabilizing proteins and membranes. Pearson correlation analysis revealed a positive correlation (0.781) between the hatching percentage and trehalose content ($P<0.5$). The result indicates the protective role of trehalose over the embryos leading to enhanced hatching. The trehalose content of the cyst could be a potential biomarker index to assess the hatching quality of the *Artemia* cyst. Artemia Cyst Quality Index (CQI) can be developed, which could be used as a unique standard for determining the quality of *Artemia* cysts.

Key words : *Artemia, Quality index, Trehalose*

Introduction

The ecosystem's physical and biological state is dynamic, and there is a nature-defined limit where the animals can live (Gorshkov, 2012) naturally. The state at which the limit crosses the dynamic equilibrium of the ecosystem leads to the destruction of fauna and flora, and the phenomenon is termed "stress" (Steinberg, 2012). The ability of hypersaline marine invertebrates to withstand salt injuries in oceanic habitats suggests that their cells are equipped with biochemical and genetic mechanisms providing competitive advantages to stress factors. *Artemia*, an invertebrate of a hypersaline marine eco-

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system, was reported till the Jurassic era and therefore has been selected as a model to study the genetic and biochemical adaptation mechanism against salinity stress (Vikas *et al.,* 2016). *Artemia*, universal live feed, is used as live feed for over 85% of marine aquaculture species (Dhont *et al.*, 2013). The requirement of an *Artemia* cyst in India is 150 tones, and it shall cross 200 tones by 2025, costing Rs.1.2 billion for import. In India, *Artemia* is reported from the Salinas of Tamilnadu, Gujrat, Rajasthan, and Maharashtra *Artemia* (Vikas *et al.*, 2012) is eurythaline and eurythermal and withstand the extreme level of salinity and temperature. Encysted gastrula embryo (cyst) is the most resistant of all animal life history stages to environmental stress. The differential composition of vital indicators, viz., trehalose and heat shock proteins, at differet life stages, are essential to study their functional role under stressed conditions. Trehalose, an intracellular protective agent reported to mediate defense against many stresses.

Trehalose, a nonreducing disaccharide, is widely distributed in nature and is reported to be important in tolerance to multiple stresses in numerous species. Trehalose accumulates under stress in many species, such as *Salmonella enterica* under a hypersaline environment, *Listeria monocytogenes* under thermal stress, *Saccharomyces cerevisiae* under cold stress, and *Candida albicans* under anoxia.

sHSPs are the first line of defense against physiological and environmental stresses sHSPs interact with other proteins to modulate folding, cell localization, and functionality and to protect against irreversible denaturation. sHSPs synthesis enables the diapausing organisms to achieve reversible dormancy and increased stress tolerance Sun *et al*.. (2005). In *Artemia*, small heat shock protein (sHSP) constitutes approximately 10% of cyst nonyolk protein and for which the amino acid sequence has been determined since they act as a molecular chaperone that promotes cell survival by preventing irreversible protein aggregation (Liang, 1997). sHSPs represent an early line of defense against stress within cells, binding partially denatured proteins by energy-independent processes, preventing irreversible denaturation, and, in cooperation with other molecular chaperones, promoting protein renaturation or protein destruction (Haslbeck, 2005). Existing the fact that protein synthesis is developmentally regulated and does not occur in response to stress (Jackson, 1996). The study's objective is to analyze the real-time expression of small heat shock protein (Hsp) 22 in the different developmental stages of *Artemia* and determine the differential expression of trehalose in the different cryptobiotic *Artemia* to develop a biomarker to analyze the quality/hatching percentage of the Artemia cyst.

Materials and Methods

Artemia samples were collected from the hypersaline habitats of Kelambakam (CKF), Vedaranyam (VDA), Tuticorin (TTJ), Mithapur (GMJ), Tamaraikulam (TNM), and Marakanam (TMM) of India.

Analysis of the real-time expression of the sHSP22 using the gene transcription assay

Total RNA was isolated using Sigma's total RNA Isolation kit. cDNA was reversely transcribed using the BioRad RT reagent kit with oligo d(T)18 as a primer. The PCR amplified hsp22 gene was carried out for confirmation (Fig. 1), and the sequences were deposited in NCBI (GU377282 & GU377283) sHSPs interact with other proteins to modulate folding, cell localization, and functionality and to protect against irreversible denaturation

Real-time quantitative PCR

Real-time quantitative PCR amplifications were conducted in IQ5 Thermal cycler using SYBR supermix.

Lane 1: Artemia cyst Hsp 22
2: Artemia nauplii Hsp 22
3: Artemia adult Hsp 22
4: cyst β actin
5: nauplii β actin
6: adult β actin
7 100 bp DNA ladder.

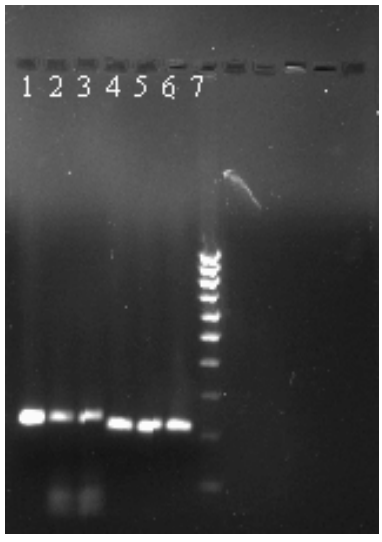

Fig. 1. Agarose gel image

Estimation of Trehalose and hatching percentage of *Artemia*

Trehalose content of the *Artemia* cyst, nauplii, and adult of different origins was estimated using the modified method of Hedge and Hofriter, (1962). Trehalose content of the cyst and the Hatching percentage values were subjected to Pearson correlation using the SPSS13 Package, USA.

Results and Discussion

The relative expression ratio of target mRNAs was calculated using the IQ 5 Multicolor Real-Time PCR software β-actin was used as a housekeeping gene (Fig. 2). Data analysis of real-time quantitative PCR Target RNA concentrations and CT values are inversely related (Fig. 3).

Dissociation curve (-dR/dT vs. T) (R = normalized fluorescence; T = temperature) shows single independent dissociation peaks for *Artemia* cyst, nauplii and adult.

NTC reaction is flat showing no templates present in the reaction mix.

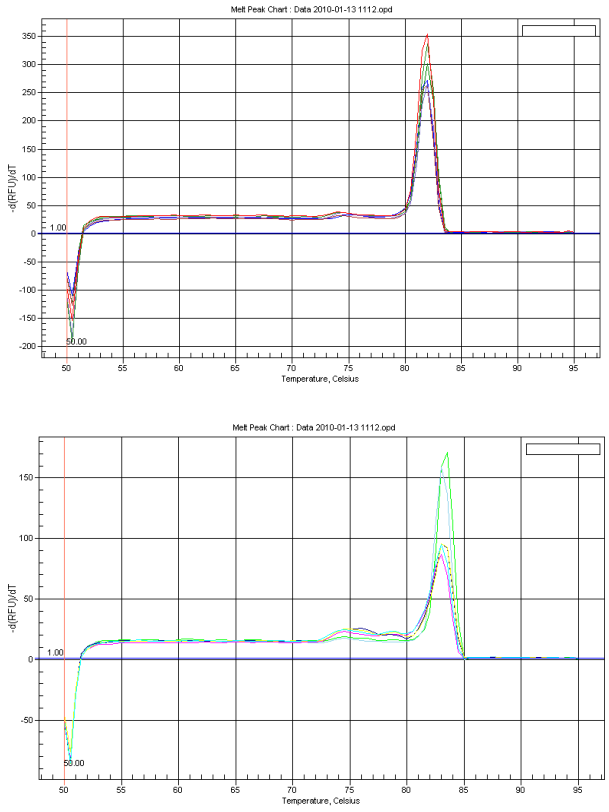

Fig. 2. Melt curve Analysis

Ct—threshold cycle where amplification plot cuts the threshold level.

At the end of the entire PCR reactions reached approximately the same level of fluorescence (dotted box).

The real time expression of â actin revealed the constant level of expression in the different developmental stages such as cyst, than the nauplii and adult (Fig. 4 & Table 1).

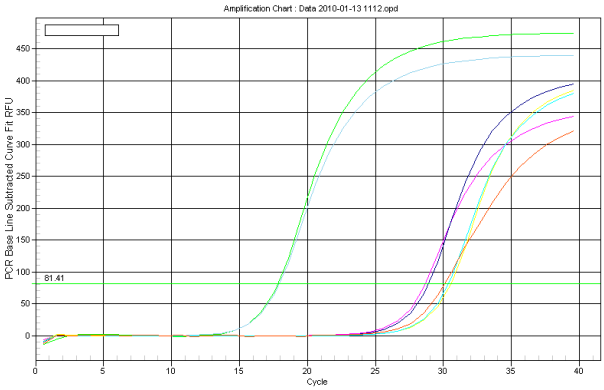

Fig. 3. Amplification plots of sHsp 22 for *Artemia* cyst, nauplii and cyst sample in duplicates.

The real-time expression of sHSP22 revealed the highest expression of sHSP22 in the cryptobiotic cyst stage. Earlier immunoglobin of western blot results postulated that the sHSP22 expression is stress-induced and absent in the other stages, and an external heat shock stress is required to activate the gene. The present results showed the action of the sHSP22 in all life forms without stress, especially in adults and Instar II nauplii. The higher expression in the cyst stage may be due to activation of sHSP22 prior to the diapausing stage, and it persists for a rela-

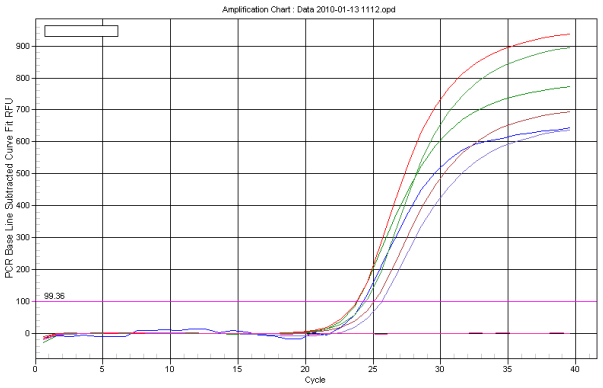

Fig. 4. Amplification plots of sHsp 22 for Artemia cyst, nauplii and cyst sample in duplicates.

Table 1. Melt curve values and threshold values of Real time PCR

Sample ID		Melt Temp.	Threshold Cycle (Ct)	Ct Mean	Ct Std. Dev
hsp22	Cyst 1	82.00	17.83	17.87	0.068
	Cyst 2	82.00	17.92	17.87	0.068
	Nauplii 1	82.00	28.69	28.80	0.158
	Nauplii 2	82.00	28.91	28.80	0.158
	Adult 1	82.00	30.57	30.47	0.145
	Adult 2	82.00	30.36	30.47	0.145
â actin	Cyst 1	83.50	23.80	24.03	0.322
	Cyst 2	83.00	24.26	24.03	0.322
	Nauplii1	83.00	25.07	25.35	0.402
	Nauplii 2	83.00	25.64	25.35	0.402
	Adult1	83.00	24.46	24.10	0.509
	Adult 2	83.00	23.74	24.10	0.509

tively short time during post-diapauses development. Our findings suggest that the shsp22 expression is persistent even in adults, and this extended expression of sHSP22 confers a thermo tolerance to the adult animal and thereby making the candidate strain more thermoresistant

This unique feature found in the native strain is a novel report regarding the extension of expression of sHSP22 beyond the nauplii stage with a regular decline compared to the cyst stage. In order to confirm this new finding, qualitative detection of these proteins at the protein level by western blotting is being suggested. Our study revealed the sHSP22 expression in all developmental stages, and the animal is "being prepared for stress" resistance by expressing the sHSP22 for a lifetime.

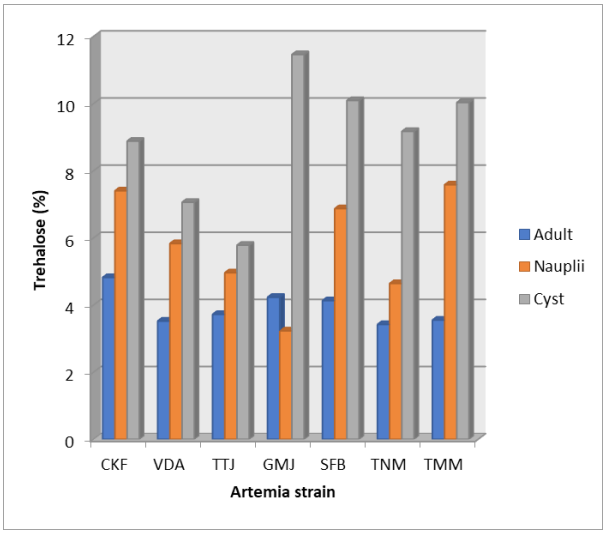

Fig. 5. Trehalose (%) in the developmental stages of *Artemia*

Development of a Biomarker to assess the hatching of the *Artemia* cyst

Trehalose, an alpha-linked disaccharide formed by an α, α-1, 1-glucoside bond between two α-glucose units, also protects biological materials against dehydration and desiccation.

Artemia cysts survive severe stress, such as long-term anoxia, salinity, and heat, all hallmarks of the Artemia environment due to its rigid chitinous cyst wall permeable and the disaccharide trehalose stabilizing proteins and membranes. Trehalose was high in cysts than nauplii and adult (Fig. 5).

Artemia Cyst Quality Index

Pearson correlation analysis revealed a positive correlation (0.781) between the hatching percentage and trehalose content (P<0.5) (Fig. 6). The result indicates the protective role of trehalose over the embryos leading to enhanced hatching. It is proposed

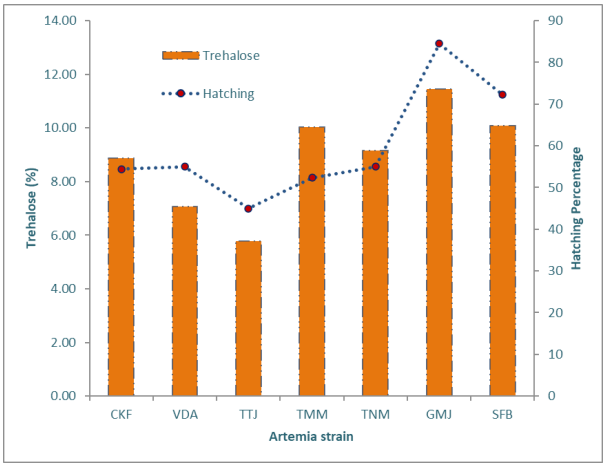

Fig. 6. Correlative response of the cyst trehalose and hatching percentage of different *Artemia* strain

that the trehalose content of the cyst could be a potential biomarker index to assess the hatching quality of the *Artemia* cyst. *Artemia* Cyst Quality Index (CQI) can be developed, which could be used as a unique standard for assessing the quality of *Artemia* cysts.

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