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Note

Effect of salinity stress on biochemical constituents and ArHsp22 gene expression in *Artemia franciscana*

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ABSTRACT

The present study analysed the effect of salinity stress on survival, biochemical constituents such as soluble protein, amino acid, trehalose and fatty acid, as well as real time expression of the *Artemia* heat-shock protein 22 (ArHsp 22) gene in *Artemia franciscana*. Results of the study revealed that *Artemia* can withstand sudden salinity increase up to 200 ppt without any mortality up to 6 h. Significantly higher mortality percentage was recorded at 24 h of incubation at 200 ppt. Short-term exposure to hypersaline conditions significantly reduced the protein content in *Artemia* while the protein biosynthesis enhanced after 24 h of incubation at 150 ppt salinity. Long term exposure to 200 ppt salinity did not show any further increase in amino acid content as observed at 100 and 150 ppt conditions, indicating reduced rate of amino acid metabolism and stress. Salt stress induced the synthesis of total polyunsaturated fatty acids (PUFA) in *Artemia* especially the 22:6n-3 and 20:5n-3 after 24 h of incubation at 150 ppt . Exposure to higher salinity induced trehalose production which indicated its vital role in combating salt stress in *Artemia*. The present study also indicated that short-term salt stress can significantly enhance the ArHsp22 gene expression in *Artemia* adults.

Keywords: Amino acid, ArHsp22 gene, Carotenoid, Fatty acid, Salt stress, Trehalose

Aquatic animals are more susceptible to sudden variations in the living medium than the land animals as it is essential for them to maintain osmotic balance. Both, marine and freshwater organisms possess different mechanisms to cope up with the varying environmental conditions. The brine shrimp, *Artemia* are distributed along the hypersaline, inland salt lakes and salt pans around the globe (Vikas *et al.*, 2012; 2014) They are rarely found in waters with salinity lower than 45 ppt, although physiologically they thrive in seawater and even in brackishwater. Salinity is without any doubt the predominant abiotic factor determining the presence of *Artemia* and the natural defense mechanism against predators, consequently limiting its geographical distribution. In *Artemia*, different adaptation mechanisms are prominent to overcome the extreme environmental conditions mainly through the biosynthesis of osmotically active compounds, cryoprotectants/thermoprotectants, polyols, amino acids, quaternary ammonium salts and disaccharides (Yancey *et al*., 1982). During stress, molecular chaperones interact with other proteins to modulate folding, cell localisation and functionality and to protect against irreversible denaturation, and the small heat shock proteins (sHsp's) are known for their protective capability, representing the first line of defense against physiological and environmental stress. Gene expressions of sHsp are either stress induced or in specific cells and tissues, independent of or concomitant with, stress exposure. The objectives of the present study was to understand the effect of salinity stress on the survival rate, biochemical constituents such as soluble protein, amino acid, trehalose as well as fatty acid and to examine the real time expression of ArHsp 22 in *Artemia franciscana. Artemia* cysts were collected from the hypersaline habitats of Kelambakam, Tamil Nadu ($12^0$47'N; $80^0$13'E) during June 2010. The samples were brought to the wet laboratory of ICAR-Central Marine Fisheries Research Institute (ICAR-CMFRI), Kochi, India, suitably cleaned, processed by bipartial floatation technique with brine and freshwater and stored under refrigeration until further use. Decapsulation and hatching o*f Artemia* cysts (strain designation CKF) was performed following Sorgeloos *et al*. (1986) with suitable modifications. Freshly hatched nauplii were harvested and stocked in fiber reinforced plastic (FRP) tanks (1 t) holding seawater of salinity 50‰ (temperature 23±1^0C). Ten percent of the culture medium was exchanged daily with fresh seawater and the nauplii were fed *ad libitum* for 16 days with *Isochrysis galbana*.

Experiments were designed to study the biochemical and genetic variations in *Artemia* subjected to salt stress at four different saline conditions (50, 100, 150 and 200 ppt) for 0, 1, 6 and 24 h durations. Experimental rearing medium at different salinity concetrations were prepared by diluting 6 M sodium chloride (Sigma) solution in distilled water and stocked with *Artemia*. Adult *Artemia* (1000 males and 1000 females) were randomly harvested from the FRP tanks and stocked (@ 1 individual per 10 ml) in glass tanks holding saline solution (20 l) at concetrations of 50 (T1), 100 (T2), 150 (T3) and 200 ppt (T4), each in triplicates. *Artemia* maintained at 50 ppt (T1) was considered as control.

Prior to the experiment, *Artemia* individuals were acclimatised to experimental conditions by progressively increasing (in 60-70 min) the salinity to desired levels in all treatments. All the treatments (T1 to T4) were provided with optimum aeration, light (1500 Lux), temperature (23±1^{0}C) and the *Artemia* were fed *I. galabana* (45x 10^6 cells ml^{-1}).

The survival of *Artemia* in different treatments (T1 to T4) were checked after 6 and 24 h of incubation. The survival percentages in all treatments were calculated using the formula:

Survival percentage = [Total number of live animals]/(Total number of live animals + Total number of dead animals)] X 100.

Artemia samples were harvested from all treatment tanks after 6 and 24 h of incubation for various analyses. Samples for the biochemical estimations were harvested, lyophilised (Martin Christ Alpha-1-4 LD freeze-drier, Osterode, Germany) and stored at -80^0 until analysed. Samples for gene expression study (30 individuals) were preserved in separate vials with RNA later solution (0.6 ml) (Sigma Aldrich, USA). All analyses were carried out in triplicates.

Soluble protein content of the lyophilised *Artemia* samples (100 mg) were estimated as per Lowry *et al.* (1951). Total carotenoid content of each lyophilised samples (100 mg) were determined following Tolasa *et al.* (2005) and Olson (1979). Amino acid content of the *Artemia* samples was estimated following the Pico-Tag method (Heinrikson and Meredith, 1984). Trehalose content was estimated following established methods (Jermyn, 1975, Carpenter and Hand, 1986), with suitable modifications. Total lipid (500 mg) was extracted with $CHCl_3$/MeOH (60 ml, 2:1, v/v) and water (20 ml) (Bligh and Dyer 1959). The $CHCl_3$ layer was processed to recover triglycerides and saponified with alkaline reagent (3 ml, 0.5 N KOH/MeOH). The saponified materials were reacted with the methylating mixture (14% BF_3/CH_3OH) to yield methyl esters (FAME) and subsequently extracted with *n*-hexane/H_2O (1:2, v/v) mixture (Metcalfe *et al.,* 1966). Aqueous layer was removed and the *n*-hexane layer was recovered and concentrated in vacuum, reconstituted in petroleum ether and stored at -20°C. Fatty acid composition was analysed using a Perkin Elmer Auto System XL, Gas chromatograph (Perkin Elmer, USA) equipped with a flame ionisation detector.

Total RNA from individual adult *Artemia* were extracted using the GenElute™ Mammalian Total RNA Miniprep kit (Sigma Aldrich, USA), following manufacturer's instructions, and stored at-80°C. Real time RT-PCR was carried out to study the expression of ArHsp22 along with expression of the house keeping genes β actin and GADPH from the cDNA using the gene specific primers.

Statistical analysis of the data was carried by analysis of variance (ANOVA) and the means of all parameters were compared using Duncan's multiple range tests. All statistical analyses were performed with SPSS programme ver. 13.0 (SPSS Inc, Chicago, USA).

The present study revealed that *Artemia* can withstand sudden increase in salinity up to 150 ppt (T3) without any mortality up to 6 h. Low percentage of mortality was noticed during further incubation at 150 ppt up to 24 h.. But at 200 ppt (T4), it was observed that they can survive with low percentage mortality up to 6 h incubation, while significantly higher mortality percentage was recorded at 24 h of incubation (Fig. 1). Though *Artemia* are naturally present in hypersaline environments (above 200 ppt), sudden increase in salinity can act as a stress factor which in turn can make them unfit to survive.

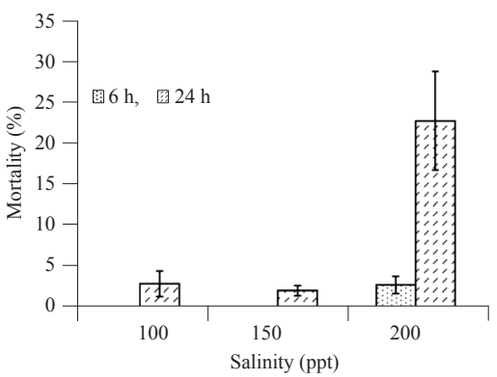

Fig. 1. Mortality percentage of *Artemia* exposed to different saline conditions after 6 and 24 h of incubation

Short term exposure under hypersaline conditions (150 ppt and above) significantly reduced the protein content in *Artemia* while the protein biosynthesis enhanced after 24 h of incubation in *Artemia* maintained at 150 ppt salinity. The initial reduction in protein content may be due to the increasing activity of acid and alkaline proteases (Parida *et al*., 2004). The enhanced production of soluble protein after long term exposure could be attributed to over production of small heat shock proteins (sHsp 21, Hsp 22 and p 26) in *Artemia* to overcome the stressed environment which is

the characteristic mechanism exhibited by the diapausing organisms for achieving reversible dormancy (Qiu and Macrae, 2008). Protein degradation in animal tissues may vary individually due to the physical conformation and other properties of the protein complex. The large proteins are more susceptible to degradation than smaller ones and also the rate of degradation will be more in acidic proteins than neutral or basic proteins (Dice *et al*., 1979). In *Artemia*, sHsp's form distinctive molecular chaperones characterised by monomers ranging from 12 to 43 kDa. sHsp's have well defined role in preventing irreversible protein denaturation, apoptosis resistance, modulation of actin assembly as well as microfilament stability, conferring thermo tolerance on cells. This was found to be a well known phenomenon in plants also where the salt stress induces quantitative and qualitative changes in protein content of the cells (Wimmer, 2003).

The present study revealed the amino acid synthesis ability of *Artemia* to overcome salt stress (Fig. 2.) Accumulation or overexpression of nitrogen containing compounds (proline, other amino acids, quaternary amino compounds and polyamines) and hydroxyl compounds metabolites in plants, algae, fungi and bacteria under salt stress are common (Csonka, 1989). Long-term exposure at 200 ppt salinity did not show any further increase in amino acid content as observed at 100 and 150 ppt which indicates reduction in amino acid metabolism due to sudden exposure to hypersaline condition.

The key aspect of fatty acid dynamics in *Artemia* and other zooplankton is whether they are able to modify the dietary fatty acids obtained from the exogenous dietary source. Salt stress induced the synthesis of total polyunsaturated fatty acids (PUFA) in *Artemia* especially the 22:6n-3 and 20:5n 3 after 24 h incubation at 150 ppt (Table 1).

Conversion of 18: 2n-3 to 20: 5n-3 in *Artemia* is clearly evident from the fatty acid data under different salinities, where the 18: 2n-3 content reduced and 20: 5n-3 increased

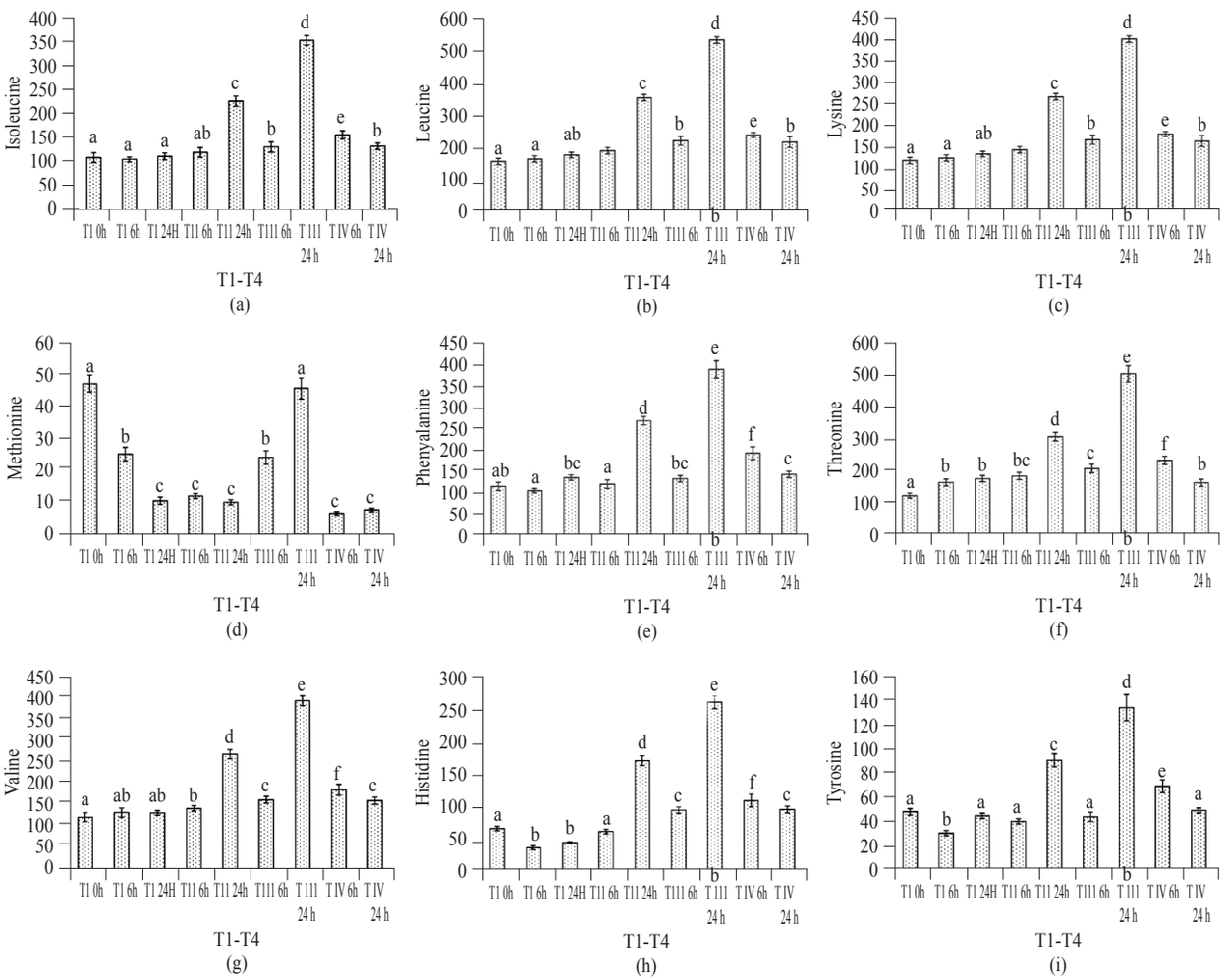

Fig. 2. Indispensible amino acid content in *Artemia* exposed to different saline conditions after 6 and 24 h of incubation

Table 1. Fatty acid content of *Artemia* under different salinity conditions

Fatty acid	50‰ 0 h	50‰ 6 h	50‰ 24h	100‰ 6 h	100‰ 24 h	150‰ 6 h	150‰ 24 h	200‰ 6 h	200‰ 24 h
12:00	0.14	1.17	3.90	0.55	0.93	1.95	1.15	2.29	0.55
13:00	0.14	0.32	0.30	0.21	0.17	0.65	0.04	0.18	0.27
14:00	3.87	3.37	4.39	4.71	6.10	6.52	5.24	3.55	4.45
15:00	0.32	7.19	0.90	5.62	0.41	0.28	0.15	0.40	1.03
16:00	18.84	20.59	20.89	18.03	18.98	16.66	15.00	22.17	19.10
17:00	1.21	0.97	3.40	3.19	2.55	0.74	2.30	1.21	3.49
18:00	7.41	8.87	8.60	7.21	8.30	6.89	6.81	9.35	8.15
20:00	1.38	0.78	1.40	0.90	0.87	0.84	0.80	1.12	0.75
22:00	1.03	1.04	0.80	1.80	0.87	0.84	2.07	0.54	0.27
24:00	0.74	0.32	0.20	0.35	0.17	0.47	0.27	1.08	0.21
∑ SFA	35.08	44.62	44.78	42.57	39.35	35.84	33.83	42.00	38.27
14:1n7	3.94	1.10	5.80	0.49	4.53	7.26	4.02	5.76	5.75
16:1n7	6.99	4.14	3.40	4.02	4.47	3.35	3.52	2.92	3.15
18:1n9	18.98	19.95	15.99	14.63	26.12	12.38	14.92	18.89	16.02
17:01	0.50	1.10	1.90	1.18	0.35	1.68	1.19	0.54	0.68
20:1n11	0.50	0.39	0.70	1.04	0.87	1.86	0.99	1.26	2.40
∑ MUFA	30.91	26.68	27.79	21.36	36.34	26.53	24.64	29.00	28.00
18:2n6	4.43	4.34	4.60	4.16	4.24	3.26	4.13	2.56	3.63
18:3n6	3.83	4.08	3.10	4.30	3.89	2.89	4.06	2.11	3.08
18:3n3	0.89	2.07	1.50	1.59	1.22	2.79	1.07	1.80	1.71
20: 2n6	3.90	3.89	1.90	5.48	4.93	2.98	6.16	3.37	4.11
20:3n6	0.92	0.52	0.60	0.97	0.35	1.21	0.92	3.37	1.10
20:4n6	0.67	1.36	1.00	1.53	0.29	1.30	0.96	0.94	1.03
20:5n3	3.55	3.11	3.90	4.58	2.03	4.00	3.63	0.67	4.31
22:5n3	3.02	0.91	2.70	1.66	0.29	2.14	1.19	0.45	0.96
22:6n3	6.10	3.04	4.70	7.63	3.72	12.29	10.14	6.03	6.30
∑ PUFA	27.31	23.32	24.00	31.90	20.96	32.86	32.26	21.00	26.23
∑ n3	13.56	9.13	12.80	15.46	7.26	21.22	16.03	8.95	13.28
∑ n6	13.75	14.19	11.20	16.44	13.70	11.64	16.23	12.35	12.95
∑ C18PUFA	9.15	10.49	9.20	10.05	9.35	8.94	9.26	6.47	8.42
∑ C20PUFA	9.04	8.88	7.40	12.56	7.60	9.49	11.67	8.35	10.55
n3/n6	0.99	0.64	1.14	0.94	0.53	1.82	0.99	0.72	1.03
∑PUFA/∑SFA	0.78	0.52	0.54	0.75	0.53	0.92	0.95	0.50	0.69
EPA/AA	5.30	2.29	3.90	2.99	7.00	3.08	3.78	0.71	4.18
∑DHA/∑EPA	1.72	0.98	1.21	1.67	1.83	3.07	2.79	9.00	1.46

than the control at 100 and 150 ppt. This indicates the increased activity of elongases and Δ5-desaturase at 100 ppt to biosynthesise 20:5n-3 from 18:3n-3.

Exposure to higher salinity induced trehalose production (Table 2) which indicates its vital role in salt stress resistance in *Artemia*. Hare *et al*. (1998) reported that trehalose functions as compatible solute in the stabilisation of biological structures and serve as osmoprotectants under abiotic stress. Trehalose may also stabilise the dehydration of enzymes, proteins and lipid membranes efficiently. Pelah *et al.* (2004) and Czygan (1964) reported that stress can induce the biosynthesis of secondary carotenoids astaxanthin, canthaxanthin and the total secondary carotenoids. Adaptive advantages of *Artemia* for enhanced production and accumulation of carotenoids in tissues have been reported in earlier studies. Soejima *et al.* (1980) reported that the canthaxanthins form the major carotenoid and have specific role in photoprotective function. Carotenoid content of *Artemia* showed increased production when exposed to 100 and 150 ppt and it was down regulated at 200 ppt (Table 2).

ArHsp22 is the third sHsp identified in *Artemia* cysts after ArHsp21 and p26 and it possesses an α crystalline domain flanked by variable amino and carboxyl terminals, all with characteristic sequence properties (Qiu and Macrae, 2008). Though ArHsp22 synthesis is developmentally regulated, heat shock can induce the production in adults, in contrast to ArHsp21 and p26 gene expression. ArHsp22,

Table 2. Trehalose, protein and carotenoid content of *Artemia* exposed to different salinity conditions

Artemia culture condition	Protein (%)	Carotenoid (µg g^{-1})	Trehalose (%)
50‰ 0 h	22.56 ± 0.04^{a}	154.84 ± 1.11^{a}	2.50 ± 0.08^{a}
50‰ 6 h	21.89 ± 0.03^{a}	158.71 ± 1.08^{a}	2.49 ± 0.08^{a}
50‰ 24 h	22.02 ± 0.04^{a}	159.65 ± 1.06^{ac}	2.56 ± 0.08^{ab}
100‰ 6 h	24.41 ± 0.1^{a}	126.95 ± 1.50^{b}	4.66 ± 0.35^{b}
100‰ 24 h	22.88 ± 0.02^{a}	199.76 ± 1.09^{c}	4.29 ± 0.77^{cf}
150‰ 6 h	16.75 ± 0.01^{bcd}	151.45 ± 1.03^{d}	3.60 ± 1.18^{d}
150‰ 24 h	23.24 ± 0.01^{a}	200.34 ± 0.50^{e}	4.74 ± 0.92^{e}
200‰ 6 h	18.67 ± 0.01^{cb}	103.76 ± 1.09^{f}	4.24 ± 0.34^{f}
200‰ 24 h	15.24 ± 0.08^{dc}	110.71 ± 1.08^{g}	2.61 ± 0.07^{a}

β actin and GADPH gene expression were analysed by SYBR® Green I based real-time RT-PCR. PCR efficiency of three genes is presented in Fig. 3. Melt curve dissociation analysis showed single peak in ArHsp22, β actin and GAPDH gene indicating the specificity of the reaction (Fig. 4.). The normalised expression of ArHsp 22 gene in *Artemia* showed a gradual increase at 100 ppt salinity after 6 h of incubation. While under 150 and 200 ppt saline conditions, it showed a sharp increase and recorded maximum expression at 150 ppt after the first 6 h of incubation. However the ArHsp22 gene expression was considerably reduced in 100, 150 and 200 ppt saline conditions after 24 h of incubation when compared to the 6 h expression (Fig. 5.). Irrespective of incubation time, the normalised expression of ArHsp22 gene was high in 150 ppt salinity incubated *Artemia*.

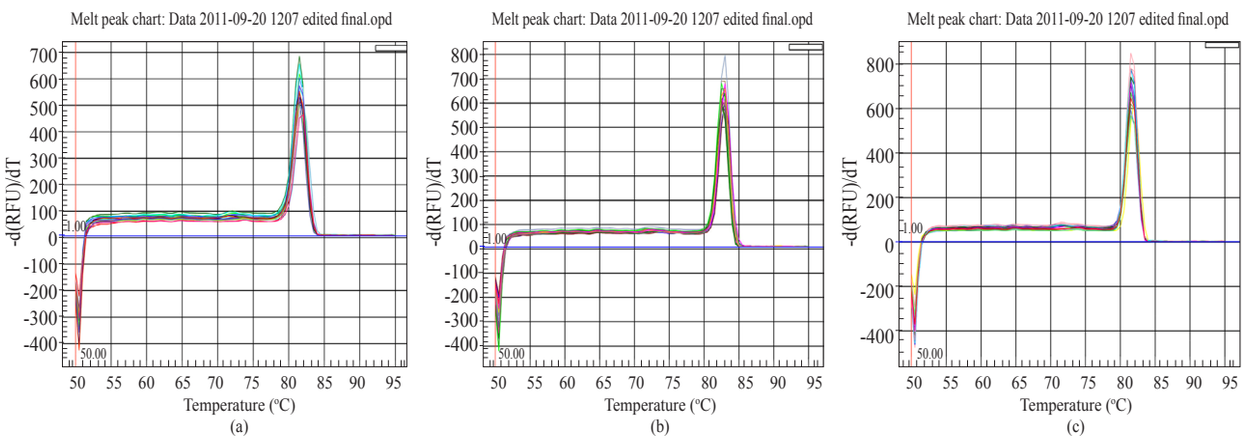

Fig. 3. PCR efficiency of ArHsp22, β actin and GAPDH genes

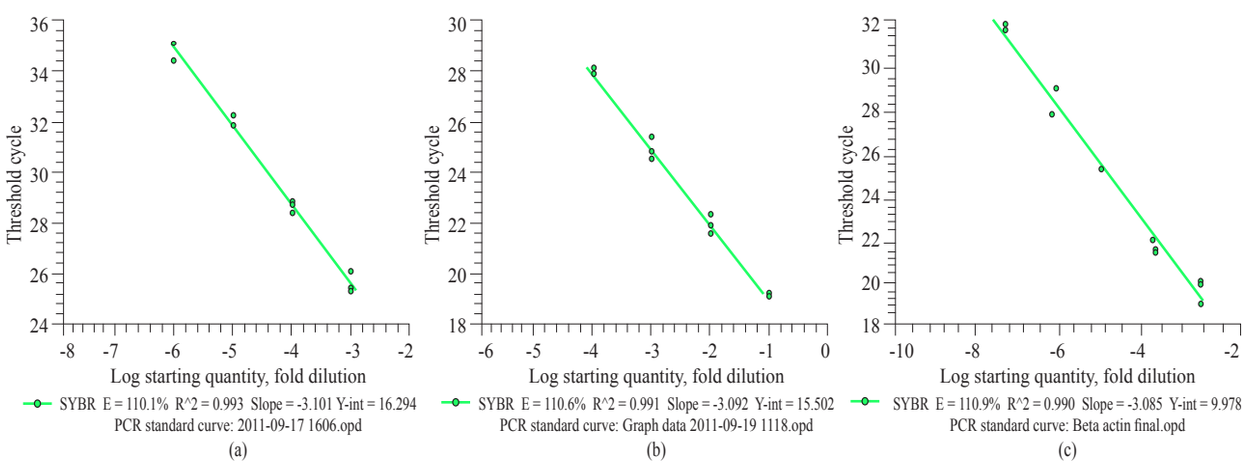

Fig. 4. Melt curve dissociation analysis of ArHsp22, β actin and GAPDH genes

Normalisation of the target gene expression is an essential component of a reliable qPCR assay because this process controls for variations in extraction yield, reverse-transcription yield and efficiency of amplification, thus enabling comparisons of mRNA concentrations across different samples (Bustin *et al*., 2009). According to Huggett *et al.* (2005), the use of reference genes as internal controls

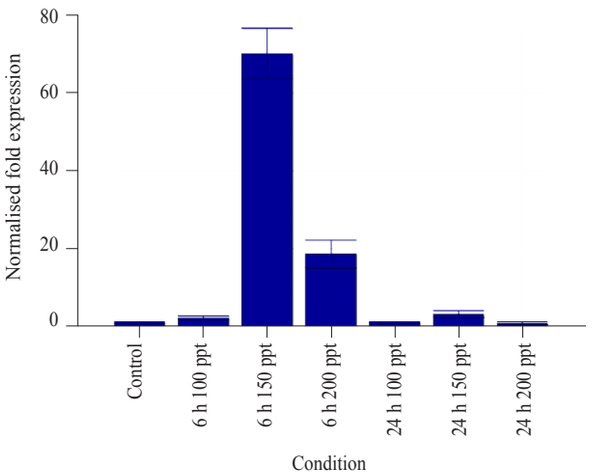

Fig. 5. ArHsp22 gene expression in *Artemia* incubated under different salinity conditions

is the most common and accepted method for the most appropriate normalisation of cellular mRNA data. ArHsp22 gene expression was normalised with the housekeeping gene GADPH and β actin using SYBR® Green I, and the analysis revealed variation in ArHsp22 gene expression with salinity in *Artemia*. Study indicated that short-term salt stress can significantly enhance the ArHsp22 gene expression in *Artemia* adults. The ArHsp22 chaperones prevent irreversible, stress induced protein denaturation and act as the first line of defense against salt stress. Though ArHsp22 lack typical nuclear localisation signal, they migrate into the nucleus by association with proteins possessing a nuclear localisation signal or if retained may preferentially interact with an intranuclear component such as the lamins/nuclear matrix (Qiu and Macrae, 2008).

Results of the present study revealed that *Artemia* can withstand sudden increase in salinity up to 150 ppt without any mortality up to 6 h, and with low levels of mortality up to 24 h incubation. Enhanced expression of biochemical constituents such as protein, trehalose, carotenoid and fatty acid in *Artemia* might have contributed towards protection of cell membranes and biomolecules from the salt stress. Further, ArHsp 22 gene expression might have acted as the first line of defense against the sudden salinity stress in *Artemia*.

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