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# Expression of 90 KDa heat shock proteins in the brine shrimp *Artemia* Leach, 1819 (Crustacean: Anostraca) in response to high salinity stress

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## Abstract

The brine shrimp *Artemia* is a primitive crustacean that tolerates the widest ranges of high salinities. Survival under extreme (osmotic) conditions is possible because *Artemia* has special adaptations against environmental stresses. Heat shock proteins are a class of functionally related proteins which their expression is increased when cells are exposed to strong stresses. Although the HSP90s constitute one of the widely studied and multifunctional stress proteins in cells, but little genetic information in aquatic invertebrate, especially in the *Artemia,* was found about. To evaluate the expression of Hsp90 in this genus, four *Artemia* populations from Iran (three bisexual and one parthenogenetic *Artemia*) were exposed to high salinities. The cysts of bisexual *Artemia urmiana* were obtained from Lake Urmia (harvested in 1998, 2000 and 2003). The cysts of the parthenogenetic *Artemia* population were harvested from the Lagoons at vicinity of Lake Urmia. The nauplli reared to maturity at high salinity 175g.l$^{-1}$ and the growth, survival and expression of Hsp90 in adult survived *Artemia* was determined by SDS-polyacrylamide gel electrophoresis and RT-PCR. Using RT-PCR, we explored an up regulation of Hsp90 gene especially among the populations which had better growth performance ($p<0.05$). The data was confirmed by SDS-Page gel electrophoresis and total RNA extraction. This data suggests the existing of two coding regions for Hsp90 in *Artemia* where a dose-dependent expression pattern in α subunit in response to high salinity stress was found.

Key words: *Artemia*, Hsp90, gene expression

## Introduction

Homeostasis of animals is always treated by internal or external stress. These stresses stimulate some physiologic responses in organisms which are under stressful conditions. Various methods for quantification of stress in crustaceans have been developed so far. Heat shock proteins (HSPs), occur in all organisms from bacteria to humans, and play critical roles in protein metabolism, folding, translocation, and refolding of denatured proteins under both stressful and non-stressful conditions. These proteins can be grouped into several families, and are named according to their molecular weights, namely HSP110, HSP90, HSP70, HSP60 and some small HSPs (Lindquist and Craig, 1988; Gupta, 1995; Csermely and Kahn, 1991; Li and Srivastava, 2004).

Heat shock proteins (HSPs) are a class of functionally related proteins whose expression is increased when cells are exposed to elevated temperatures or other stresses (De Maio, 1999). The dramatic up-regulation of the heat shock proteins is a key part of the heat shock response and is induced primarily by heat shock factor (HSF) (Wu, 1995). The Hsp90 constitutes one of the most important, widely studied and multifunctional stress proteins in the cell.

Up to the present, most studies of HSP90s are focused on mammals and typical model organisms. Key studies have included human (*Homo sapiens*) Hsp90α and Hsp90β in cytoplasm, Hsp86 and Hsp84 in mice (*Mus musculus*), and have also incorporated non mammalian studies such as Hsp83 in fruit fly (*Drosophila melanogaster*), high-temperature protein G (HtpG) in *Escherichia coli*, and many others (Young et al., 2001; Bracher and Hartl, 2005). However, in aquatic invertebrates, relatively little gene information regarding HSP90s has been obtained, including the scallops *Chlamys farreri* (Gao et al., 2007) *Argopecten irradians* (Gao et al., 2008), the oyster *Crassostrea gigas* (Choi et al., 2008), the abalone *Haliotis tuberculata* (Farcy et al., 2007), the mussel *Mytilus galloprovincialis* (Pantzartzi et al., 2009), the prawns *Metapenaeus ensis* (Wu and Chu, 2008) and *Penaeus monodon* (Jiang et al., 2009) and the marine crab *Portunus trituberculatus* (Xiao-Yan et al., 2009).

In genus *Artemia*, almost all information regarding to the nature of HSP90s has been obtained. In this genus the most studied HSPs are p26, p70 and artemin. Also heat-shocking of embryos causes the same response as long-term anoxia in terms of p26 translocation to nuclei and of the lack of nuclear translocation for artemin and Hsp70 (Clegg et al., 1999). In this study possible involvements of p26, artemin and Hsp70 in the thermotolerance of dried cysts have been studied, but with little insight into the details of their participation. However, no information was found about the nature of HS proteins (especially Hsp90) of *Artemia urmiana* Günther, 1890 and also parthenogenetic *Artemia* populations from Iran.

Regarding the conditions of Lake Urmia (the main habitat of *Artemia urmiana* located in North West of Iran), it should be noted that in recent years because of water inflow deficiency into the Lake mainly induced by precipitation shortages during last decade, the Lake has been saturated with brine. More than 5 meters decline in water levels by 2008, average 250-300 ppt of water salinity and hundreds of meters retreating (especially in shallow coasts) in northeast and south of the Lake were among the results of recent climate changes (Fig. 1). These new conditions of the lake as well as the lack of food for the remaining *Artemia* populations in Urmia Lake, as an assumption, have changed the physiologic responses, protein profiles and expression levels of mRNA of the existing *Artemia*.

We believe that new conditions of Lake Urmia have provided an opportunity to survey on physiology of *A. urmiana* with regard to the effects of HSP. In this study, we aimed to focus on HSP90s of the genus *Artemia,* especially whilst the adult animals were exposed to high salinities.

## Materials and Methods

### Sample preparation

In the present study, we selected four populations of *Artemia* (one parthenogenetic population and three bisexual *A. urmiana* populations). We obtained the cysts samples from cysts

bank of *Artemia* and Aquatic Animals Research Institute, Urmia University, Iran. The *A. urmiana* cysts had been harvested previously in different climate conditions of the Lake (1998, 2000 and 2003). The parthenogenetic *Artemia* cysts had been harvested in 2005 from the lagoons which located at the vicinity of Urmia Lake. The floated parthenogenetic cysts were sieved over the lagoons. Before the experiments, the hatching percentages of the cysts were checked to be surely more than 80%.

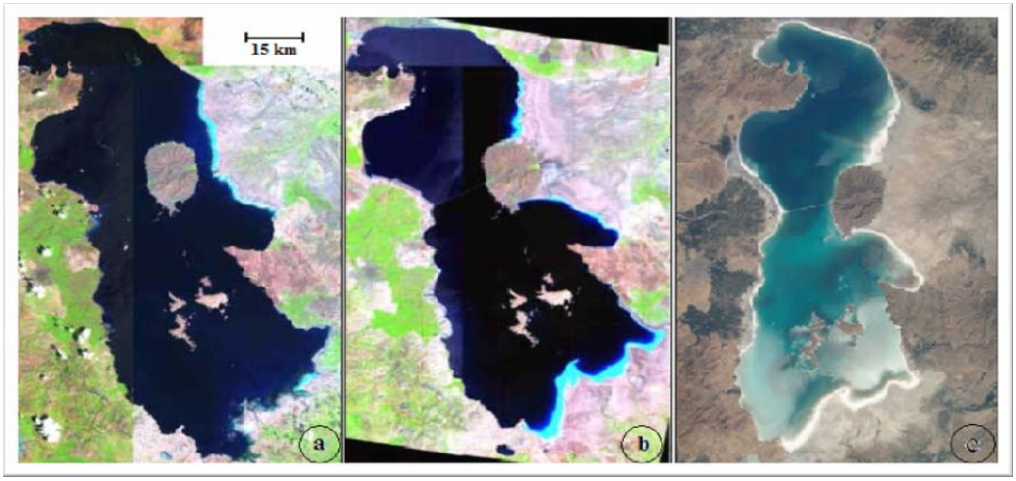


Fig. 1. Satellite image of Lake Urmia in August 1998 (a), August 2001 (b) (Alesheikh et al., 2004) and in 2002 (c), in which the very shallow area edging the lake is shown as a white lining

## Culture conditions

Almost 1.5gr cysts of the each population were incubated in artificial 0.45 µm filtered medium (salinity: 35 $g.1^{-1}$) for hatching under optimal conditions (Sorgeloos et al., 1986). After hatching, as an each replicate, 500 individuals of instar-I nauplii were transferred directly into the 1l cylindroconical vials at an initial density of 2 nauplii/ml of 175 $g.l^{-1}$ culture medium. Each population consisted of four replicates (over all 2000 nauplii individuals per population). The animals were fed on a mixed diet of yeast-based formulated feed LANSY PZ (INVE Aquaculture SA, Belgium) and *Dunaliella salina*, Butcher (Coutteau et al., 1992; Triantaphyllidis et al., 1995). The density of the animals was reduced to one individual per 4 ml after 8 days. The temperature was arranged at 27±1°C and mild aeration was applied from the bottom of the tubes which were covered with perforated Petri dishes to minimize evaporation. The photoperiod provided by fluorescent light tubes was 12:12 light: dark. Survival and total length of *Artemia* in each group was determined at each water renewal i.e. on days of 3, 7, 11 and 15 after hatching. The survived adult individuals were used as material for the next molecular analyses. Total length of the individuals in the each renewal days were measured according to Triantaphyllidis et al. (1995) and Baxevanis et al. (2004). The total length measurement was performed with 20 individuals from each replicate on the all renewal days.

## Sample preparation for SDS–PAGE

The electrophoretic method of SDS-PAGE was used for total protein analyses of *Artemia* samples (Laemmli, 1970). Whole body of adult *Artemia* individuals (which were survived from previous step of experiment) were prepared for SDS-PAGE in liquid nitrogen and pulverized in 1.5 ml micro tube by appropriate tips. 100 mg of the resulting powder, consisting of 10-15 adults *Artemia* of both genders, was placed into 500 µl of protein extraction buffer (buffer K). The extraction buffer for SDS-PAGE contained: (5 mM $MgCl_2$, 5 mM $NaH_2PO_4$, 40 mM Hepes, 70 mM Potassium gluconate, 150 mM Sorbitol, pH 6.5 containing a protease inhibitor

cocktail (Invitogene™ Mini from Roche Diagnostics GmbH) (Clegg et al., 1994). Protein concentration was determined by the Bio photometer apparatus (Eppendorf). Samples were heated for 5 min at 95 C and subsequently cooled to room temperature in tap water. After low speed centrifugation (1600 g, 5 min) to remove insoluble fragments, supernatants were electrophoresed.

### Electrophoresis (SDS-PAGE)

Flat bed SDS-PAGE was performed with a vertical BioRad System. We prepared a 100×70×0.5 mm dimensions and 10% total acrylamide gel. The samples (30 µl) were loaded on the each gel track. The running buffer was prepared according to Garfin, 1990. The buffer system in the strips formed a discontinuous buffer system together with the gel buffer. High molecular weight Ladder of 250 KD (Fermentas) was used for detection of protein bonds on the gel. Electrophoresis was performed at constant current of 50 mA in BioRad electrophoretic apparatus with power supply, set at 150V for 1 hour. The gel was stained with Coomassie blue-G250 (500 mg coomassie Blue 500 ml resolved in one litter of methanol: acetic acid: H2O (40:50:10). In these SDS-PAGE studies we applied aliquots equivalent to the same mass of *Artemia* per slot. Furthermore, details about all these methods are given in (Clegg et al., 1994).

### RNA extraction and cDNA synthesis

Reverse transcription PCR was used to study the expression of Hsp90 in adult individuals. Total RNA was isolated from adult *Artemia* samples on day 15 of experiments using Trizol reagent (Invitrogen). RevertAid™ First Strand cDNA Synthesis Kit, (Fermentase) was used to study expression after culturing in high salinity on RT-PCR level. The cDNA fragment was amplified using Hsp90 primer combination as forward primer; 5'-TTACGA GTC CAG ATG GGC TT-3' and reverse; 5'-ATC CGT TAT GAA TCC CTG ACT GA-3'. These primers previously had been developed successfully for Hsp90 *Daphnia* (Kotov et al., 2006), 700bp fragment of Hsp90 with two introns. PCR thermal cycling parameters for these specific primers were somewhat modified as: 35 cycles of 95°C for 30 seconds, 40°C for 30 seconds, and 72°C for 80 seconds with final extension of 72°C for 5 minute with BioRad thermal cycler. After amplification, all products were run on 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized in a UV- transilluminator contained CCD camera.

### Statistical analysis

The results of survival were expressed as percentage. One-way ANOVA, Duncan's test of SPSS 16 software were used to identify differences among means and significances were accepted at $p<0.05$ (Triantaphyllidis et al., 1995; Baxevanis et al., 2004).

## Results

### Survival and total length

Survival rates and percentage of animals attaining adulthood were measured over a period of 15 days. Results indicated that both sexual and parthenogenetic *Artemia* have been affected by high salinities at different ways. While the samples *A. urmiana* 2003, 2000 showed significant low survival rate, the samples of *A. urmiana* 1998 and parthenogenetic *Artemia* revealed the highest survival rate ($p<0.05$) (Table 1).

At the parameter of total length, two bisexual *A. urmiana* 2003, 2000 had the highest growth values in high salinities compared to the samples of *A. urmiana* 1998 and parthenogenetic *Artemia* (Table 2, ANOVA, $p<0.05$). The maximum total length of *Artemia* was significantly obtained from the parthenogenetic strains (Parth. *Artemia*: 7.70±1.24 mm) and sexual *Artemia* 1998 (*A. urmiana*: 7.25±2.05 mm) while for the samples of *A. urmiana* 2003 and 2000 it was less than 5.97±2.72 and 6.29±3.11 mm respectively.

Table 1. Survival of *Artemia* at salinity 175 g.l$^{-1}$

| *Artemia* populations | Day 3 | Day 7 | Day 11 | Day 15 |
|---|---|---|---|---|
| Parth. *Artemia* | 89.65±0.03 b | 81.95±0.05 c | 74.95±0.04 b | 73.0±0.04 b |
| *Artemia urmiana*, 1998 | 84.25±0.05 b | 73.20±0.14 bc | 62.67±0.15 b | 58.73±0.15 b |
| *Artemia urmiana*, 2000 | 85.00±0.05 b | 64.25±0.10 ab | 40.70±0.15 a | 34.15±0.03 a |
| *Artemia urmiana*, 2003 | 68.00±0.01 a | 50.1±0.02 a | 37.4±0.04 a | 28.50±0.09 a |

Same character in each column indicate insignificant differentiation

Table 2. Total length of *Artemia* at salinity 175 g.l$^{-1}$

| *Artemia* populations | Day 3 | Day 7 | Day 11 | Day 15 |
|---|---|---|---|---|
| Parth. *Artemia* | 1.22±0.08 ab | 2.89±1.36 b | 4.62±1.83 a | 7.70±1.24 b |
| *Artemia urmiana*, 1998 | 1.17±0.09 b | 2.24±0.65 ab | 4.67±1.76 a | 7.25±2.05 ab |
| *Artemia urmiana*, 2000 | 1.27±0.13 a | 2.09±0.42 a | 4.64±1.87 a | 6.29±3.11 ab |
| *Artemia urmiana*, 2003 | 1.28±0.12 a | 2.35±0.55 ab | 4.19±2.16 a | 5.97±2.72 a |

Same character in each column indicate insignificant differentiation

## Expression studies

After total RNA extraction, high levels of total RNA in some samples was found by spectrophotometer analyses (Table 3). The spectrophotometer quantified two samples of parthenogenetic *Artemia* and *A. urmiana* 1998 as having the highest amount of total RNA.

Table 3. Total RNA extraction of *Artemia* samples cultivated at salinity 175 g.l-1

| *Artemia* populations | Total RNA (mg.ml$^{-1}$) |
|---|---|
| Parth. *Artemia* | 159.0 ±51.19 a |
| *A. urmiana*,1998 | 131.3±33.87 b |
| *A. urmiana*, 2000 | 87.4±9.62 c |
| *A. urmiana*, 2003 | 49.4±15.84 d |

Same character in each column indicates insignificant differentiation

The SDS-PAGE electrophoresis released a significant level of expression of a 90KDa protein from two experimental populations as parthenogenetic *Artemia* and *A. urmiana* 1998. The up regulated gene expression were related to the samples which depicted better survival and total length performance. These results emphasized that, after stimulation of the animals with high salinity, the expression level of this protein was enhanced especially in the populations that could pass the stressful conditions successfully (Fig. 2).

The PCR amplification was successful in two populations (parthenogenetic *Artemia* and *A. urmiana* 1998). Almost 680 bp fragment, which was identical to the Hsp90 size in *Daphnia*, was found. By contrast, any PCR product of Hsp90 was not obtained from rest of the samples with different PCR protocols (Fig. 3).

## Discussion

We have recently focused on climate changes of the Lake Urmia basin which have occurred in the last 12 years. Dramatic increasing of salinity during 1998 to 2003 (personal communications, data not shown), is thought, has decreased the phytoplankton as the primary food source for *Artemia* (particularly *Dunaliella* spp.). Lower food sources and higher

salinity, apparently, have changed the tolerance threshold of *A. urmiana* against environmental stresses.

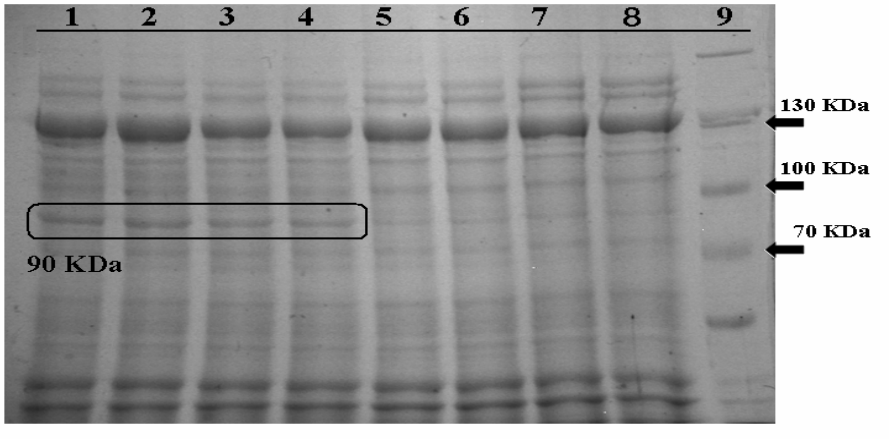


Fig. 2. SDS-PAGE of the survived adult *Artemia* in high salinity 175 g.l$^{-1}$, Lane 1-2: parthenogenetic *Artemia*, lane 3-4; *A. urmiana* 1998; lane 5-6 *A. urmiana* 2000; lane 7-8 *A. urmiana* 2003; lane 9 250 KD protein ladder.

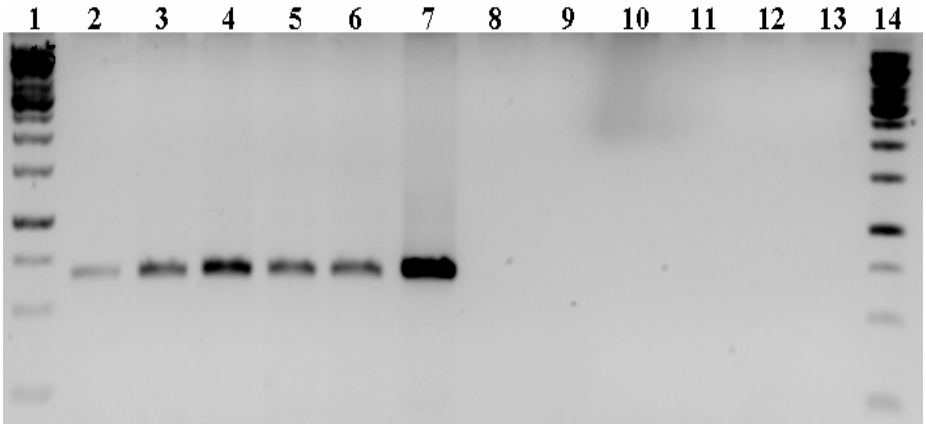


Fig. 3. Semi quantitative RT-PCR gel electrophoresis of Hsp90 expression in various *Artemia* samples. Lane 1 and 14: 1 Kb ladder, lane 2 to 4: parthenogenetic *Artemia*, lane 5 to 7; *A. urmiana* 1998, lane 8 to 10 *A. urmiana* 2000, lane 11 to 13 *A. urmiana* 2003.

In present study we found significant survival and total length which was inversely related to salinity in both sexual and asexual *Artemia*. According to these results, *A. urmiana* 1998 and parthenogenetic *Artemia* could tolerate higher salinities. Indicatively, this population has been harvested in lower salinity and normal phytoplankton availability as food from Urmia Lake. However, high mortality was observed in all treatments in high salinities which was in good agreement with literature. Interestingly, , a significant differentiation was found among the *A. urmiana* samples which were harvested before and after drought period on the life span characteristic.

It should be mentioned that the laboratory culture and maintenance of the genus *Artemia* at salinities higher than 200 g.l$^{-1}$ has always been

difficult (Wear and Haslett, 1986; Wear et al., 1986). Browne and Hoopes (1990) reported only 9% survival at 190 g.l$^{-1}$ and no survival at all at 230 g.l$^{-1}$ in a parthenogenetic *Artemia* from Salin de Giraud (France). Dana and Lenz (1986) studying the bisexual *Artemia* from the Mono Lake, California, USA, found low survival in 159 and 179 g.l$^{-1}$ under laboratory conditions. In this regard, Triantaphyllidis et al. (1995) reported over 80% mortality for both the parthenogenetic *Artemia* from Tanggu area (China) and *A. franciscana* Kellogg 1906 at 180 g.l$^{-1}$ and 25°C over a 23- day culture period.

In the experiments performed by El-Bermawi et al. (2004) on *Artemia* populations from Egypt, 100% mortality was observed in bisexual *Artemia salina* Leach 1819 from Wadi El-Natrun in 150 and 200 g.l$^{-1}$ within 17 days, but the high salinity had little effect on the ability of parthenogenetic populations to survive. Similarly to these findings the study of Agh in 2007 in agreement with the findings of Triantaphyllidis et al. (1995), showed a steady decline in survival when salinity increased from 100 to 170 g.l$^{-1}$. In the contrary, Abatzopoulos et al. (2006) reported very low survival for *A. urmiana* in salinities of 35 and 50 g.l$^{-1}$. But they found high survival at 100, 140 and 180 g.l$^{-1}$.

According to Gilchrist (1960), Triantaphyllidis et al. (1995) and El-Bermawi et al. (2004) growth (total length) was inversely proportional to salinity. Triantaphyllidis et al. (1995) reported significant differences in the growth of *Artemia* especially in the parthenogenetic population from Tanggu (China) cultured at different salinities. According to their experiments parthenogenetic *Artemia* at 180 g.l$^{-1}$ attained only 50% of the length of those at 35, 60 and 100 g.l$^{-1}$ and *A. franciscana* at 180 g.l$^{-1}$ achieved 60% of the length in comparison to animals grown at 35 g.l$^{-1}$ . In contrary to this study El-Bermawi et al. (2004) did not observe significant differences in the growth of parthenogenetic and bisexual populations of *Artemia* from Egypt grown in the laboratory at salinities ranging from 35 to 200 g.l$^{-1}$. Abatzopoulos et al. (2006) found that growth rate of *A. urmiana* was not affected by the increase of salinity. Agh (2007) indicated that growth rate in *Artemia* populations from the Lake Urmia regions (bisexual and parthenogenetic) is inversely related to salinity, supporting the findings of Gilchrist (1960), Triantaphyllidis et al. (1995) and El-Bermawi et al. (2004).

In order to explain these variabilities, Browne and Wanigasekera (2000) claimed that differences in the culture conditions, intraspecies and population dependent characteristics could be among the reasons for the variable results obtained by different researchers. In other words, different feedings with various *Artemia* strains could be an indication for strain-specific adaptation patterns of various *Artemia* populations to diverse physical, chemical and biological characteristics of their biotopes.

In order to understand the nature of Hsp90 in adult *Artemia,* this animal was exposed to the high salinity. Results suggested that the Hsp90 might related to living of the animal in stress conditions. Due to the lack of information on the nucleotide sequence and expression of Hsp90 in *Artemia* (especially *A. urmiana*), from these results preliminarily we proposed the effect of Hsp90 in *Artemia* exposed to high salinities. In respect to the role of Hsp90 in other organism, we assume that similar to Hsp70 (the most studied HSPs), Hsp90 is a molecular chaperone (Jakob and Buchner, 1994; Feder and Hofmann, 1999), which plays an important role in the genus *Artemia* to protect its cells against stress.

Despite to the importance of HSP90 in invertebrates, only one Hsp90 gene from the crustaceans *Penaeus monodon* and *Metapenaeus ensis* has been cloned and characterized so far (Wu and Chu, 2008; Jiang et al., 2009). Also in *Anopheles albimanus* and *Mytilus galloprovincialis*, two Hsp90 genes have been obtained but they encoded the same Hsp90 (Benedict et al., 1996; Pantzartzi et al., 2009). In this regard Pantzartzi et al. (2009) reported that there existed a unique cytoplasmic Hsp90, which was encoded by a single or by two gene copies (Pantzartzi et al., 2009). Recently Xiao-Yan et al. (2009) succeeded to isolate two complete Hsp90s (named ptHSP90-1 and ptHSP90-2) genes from *P. trituberculatus* which encoded two distinct Hsp90s. This was also the first report about the cloning and characterization of two

Hsp90s with distinct expression levels in invertebrates.

In present study we found an up-regulation at the level of total RNA in the populations which provided better survival and growth. This up-regulation was also confirmed by successful amplification of a 680bp (Hsp90) segment at the same populations (also by a 90KDa protein bonds which is related to Hsp90). This gene expression is in good agreement with the occurrence of two coding regions for HSP90s (subunit α and β).

Precisely, the better survival and total length were related to the expression of α subunit of Hsp90 gene (which was detected by our primer combinations). We proposed that Hsp90 was appeared at about the time of maturation of *Artemia* especially while the animal has been exposed to environmental stress. These results indicated that Hsp90 was constitutive and inducible expressed and could play a critical role in *Artemia*. In this regard, Quraishi and Brown in 1995 reported the expression of p90 in adult rabbits and Huang et al., (1999, 2002) in porcine.

It should be added that the RT-PCR as one of the most accurate and comprehensive methodfor quantification of gene expression have been noticed for years. In this regard, Qui et al. (2007) reported an up regulation in diapause embryo of *Artemia*. By using semi-quantitative RT–PCR, DNA methyl-transferase 2 Dnmt2 was found to be expressed through all developmental stages and its expression increased during resumption of diapause cysts development (Feng et al., 2007). Also Zhu et al. (2007) considered RT-PCR as one the most useful methods for determination of gene expression of protein kinas subunit α during growth of *Artemia* while exposed to the high salinities.

As a final remark it is needed to note that, the present study is preliminarily proposing the expression of Hsp90 in *Artemia* in respond to high salinity stress and must be confirmed by an accurate method such as cDNA sequencing, real time PCR or western blot.

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