Elevated salinities may enhance the recovery of hydrated heat-shocked *Artemia franciscana* cysts (International Study on *Artemia*. LXV)

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ABSTRACT. Hydrated encysted embryos of the crustacean *Artemia franciscana* Kellogg 1906 were incubated at 42ºC and 44ºC for 48 h at elevated salinities. They exhibited better recovery when incubation took place in a high salinity environment. The recovery and/or protection by salinity of *A. franciscana* encysted embryos depend on the severity of the heat shock. Cysts heat shocked at 44ºC and different salinity media (35, 50 and 70 ppt), upon return to optimal conditions, gave similar hatching percentages of less than 5%. When heat shock took place at 80 ppt, a significant increase in hatching was observed (i.e. from 5% to 22%). Concentration of glycerol and translocation of a major protein of about 26 kD (p26) in *Artemia* encysted embryos were studied to reveal the potential protective role of these two factors against elevated temperatures. Increased glycerol levels were scored when cysts were incubated in elevated salinities within the first 4 h and prior to heat shock treatment (i.e. a 38% and 49% increase at the salinities of 70 ppt and 80 ppt, respectively). SDS-PAGE of proteins extracted from control and heat-shocked cysts suggested that the intracellular translocation of p26 was enhanced as the salinity increased. It is proposed that salinity-dependent heat shock seems to be at least partly responsible for i) the significant increase of glycerol and ii) the degree of translocation of p26, a suspected molecular chaperone described previously. These two factors along with others may contribute to the better recovery of heat-shocked *Artemia* encysted embryos. This phenomenon, which may be attributed to increased thermostability of proteins, is reflected as different cyst hatching ability.

KEY WORDS : *Artemia franciscana*, salinity, heat shock, glycerol, p26.

INTRODUCTION

The brine shrimp *Artemia* (Crustacea, Anostraca) is the main zooplanktonic organism that inhabits hypersaline environments all over the world (TRIANTAPHYLLIDIS et al., 1998). This branchiopod has acquired extremely capable adaptive mechanisms to survive and evolve in habitats with extensive and often abrupt fluctuations in salinity, UV irradiation, temperature, and oxygen concentration (PERSOONE & SORGELOOS, 1980). These mechanisms are poorly understood, although several studies showed the ways that *Artemia* responds under various abiotic conditions prevailing in its natural habitats (for extensive review see ABATZOPOULOS et al., 2002 and references therein). MILLER & MCLENNAN (1988a) showed that metabolizing encysted embryos are more thermotolerant compared to 24 h-larvae, while thermotolerance can be induced in adult *Artemia* by the application of a sub-lethal heat treatment or a short period of heat shock, which results in the synthesis of heat shock proteins and heat shock related proteins (MILLER & MCLENNAN, 1988b). However, prolonged heat shock can be lethal (MILLER & MCLENNAN, 1988a).

SORGELOOS et al. (1976) first demonstrated that if fully hydrated cysts remain for 24 h at 40ºC, the embryos stop developing, but without any significant decrease in their survival upon return to a favourable temperature of 28ºC. However, repeated cycles of 8 h at 28ºC followed by 24 h at 40ºC lead to considerable decrease in the hatchability and viability of the embryos (SORGELOOS et al., 1976). CLEGG et al. (1994) as well as ABATZOPOULOS et al. (1994) gave new insights into the effects of prolonged anoxia and heat shock (42ºC) on the viability of the encysted embryos. They demonstrated that diapause can be a reversible phenomenon since some of the cysts do not lose their viability after heat shock or anoxia, but return to a state of diapause, which can be terminated by the application of a diapause-deactivation treatment (i.e. desiccation – ABATZOPOULOS et al., 1994). Although there is evidence that high temperature and lack of oxygen, two very important abiotic factors, «push» metabolically active cysts back into diapause, little is known about the interaction with salinity, another abiotic factor with very significant role in the life history of *Artemia* (ABATZOPOULOS et al., 2002). The interactions among salinity, trehalose breakdown, accumulation of free glycerol in the cyst, internal osmotic pressure and osmotic rupturing of

the chorion, i.e. the necessary steps towards excystment, have been described by CLEGG (1962, 1964).

Trehalose and glycerol may act as protectors inducing tolerance to heat and/or salt shocks; this is well documented in *Saccharomyces cerevisae* (CARVALHEIRO et al., 1999). Glycerol has also been implicated in the thermoprotection of proteins and whole cells (CARVALHEIRO et al., 1999 and references therein). The combination of osmotic pressure (expressed by NaCl concentration) and temperature enclosed a synergistic effect (CARVALHEIRO et al., 1999). NaCl contributed with an effect fivefold that of temperature on the synthesis of both trehalose and glycerol. SUN et al. (1999) revealed that glycerol stabilized an enzyme, glutamate dehydrogenase, against thermoinactivation. Moreover, their results showed that stabilization, which was induced by glycerol, increased with temperature. Glycerol had the same effects in thermostabilization as pressure (SUN et al., 1999) while it appears to be excluded from protein surfaces by the solvophobic effect (SUN et al., 1999 and references therein). However, glycerol is not completely absent from the surface of the proteins. In fact, glycerol has an affinity for polar regions of proteins (SUN et al., 1999 and references therein) and binds to proteins in direct proportion to its concentration. Glycerol has been shown to reduce the apparent specific volume of proteins in aqueous solutions and to decrease both the volume and compressibility of protein interiors. Compression of the protein structure has been rationalized through the reduction of voids or cavities within the protein (SUN et al., 1999). Also, according to CHEN et al. (2000), glycerol provided protection to IgG proteins from high temperatures by preventing denaturation or unfolding.

Another important factor that may have a similar role to glycerol is p26, a potential chaperone which is a small heat shock/α-crystallin protein and has been described in *Artemia* embryos (LIANG & MACRAE, 1999). A possible mechanism for chaperone action is to prevent irreversible aggregation and allow proteins to refold either spontaneously or with the assistance of other chaperones, through the interaction with exposed hydrophobic regions (see LIANG & MACRAE, 1999 and references therein). The association of p26 with the nucleus and its ability to protect organisms from thermal stress may have a key role during encystment, diapause and quiescence in *Artemia* embryos (LIANG & MACRAE, 1999).

Unfortunately, there is no published work on measurements of cyst temperatures under natural conditions, although it can be safely assumed that cysts (desiccated or hydrated) encounter thermal stresses when they are washed onto shore and exposed to sunlight warmth or to freezing temperatures (CLEGG & TROTMAN, 2002). MILLER & MCLENNAN (1988a) showed that hydrated cysts from Great Salt Lake, Utah (i.e. *A. franciscana*) were reasonably tolerant to high temperatures (LT$_{50}$ was 49ºC for 1 hour exposures); they also demonstrated the presence of several isoforms of Heat Shock (HS) proteins belonging to HSp-70 and -90 families in cysts as well as a small HS protein (MILLER & MCLENNAN, 1988b). When hydrated cysts were exposed to temperatures in the range of 42-50ºC, a nuclear translocation of p26 took place (ABATZOPOULOS et al., 1994; CLEGG et al., 1999).

CLEGG et al. (2001) reported on the thermal tolerance and the HS proteins in *Artemia* cysts from widely different thermal habitats; they found that hydrated SFB cysts cannot stand 50ºC for more than 60 minutes (hatching percentage dropped to 0) while the higher thermal tolerance was observed in Vietnam *A. franciscana* cysts (hatching percentage dropped to 0 when these cysts were incubated at 50ºC for 90 minutes). Maximum water temperatures in San Francisco Bay during summer and spring very rarely exceed 24ºC while the Vietnamese *A. franciscana* strain experiences daily water temperatures near to 38ºC for most of the growing season (CLEGG et al., 2001 and references therein).

The correlation between heat shock and salinity has not been studied in *Artemia*. This paper describes how salinity affects the response of *Artemia* encysted embryos to prolonged heat shock and discusses the way salinity protects these early developmental stages by enhancing their thermotolerance or thermostability.

MATERIAL AND METHODS

Cysts of the bisexual species *A. franciscana* (from San Francisco Bay – SFB – USA, batch no. 65034, ARC code no. 1258) were used since they exhibited high hatching percentage and good hatching synchrony (ABATZOPOULOS et al., 1994). The number of dehydrated cysts per gram for this specific strain was 275,000 approximately.

Experimental design

The rationale for the experiments to follow (step by step) will be helpful to the reader. The experimental design included the following steps : (a) hydration of the cysts at optimal conditions for $1^{1}/_{2}$ h, (b) transfer of fully hydrated cysts to different salinities for $2^{1}/_{2}$ h under optimal conditions, (c) incubation of hydrated cysts in all different salinities at 42ºC and 44ºC, (d) cysts hatching under optimal conditions. During step (a), complete hydration of cysts is accomplished. In step (b), cysts are allowed to build up different glycerol levels/concentrations due to incubation in elevated salinities (CLEGG, 1962; 1964). During steps (a) and (b) the encysted embryos are in full metabolical stage (for determination of metabolism see ABATZOPOULOS et al., 1994). In this fully metabolizing condition the cysts were heat shocked for 48 h (in different salinity media – step (c)). In step (d), hatching ability of the cysts was estimated i.e. the percentage of cysts that hatched (cysts in quiescence) *versus* cysts in diapause. Determinations of water content, glycerol level, hatching under different conditions and translocation of p26 were performed.

Hydration of the cysts

Dry cysts were weighed out (0.2 g for determination of hatching percentage, 0.15 g for water content estimation and 0.5 g for glycerol determination; these weights were used for each replicate for each treatment – see below) hydrated for 90 min at 30 ppt medium prepared by diluting filtered brine of 140 ppt from the nearby saltworks of M. Embolon (Thessaloniki, Greece); maximum hydration for this SFB cyst material is achieved within this period.

Fully hydrated cysts were harvested on a sieve and placed in small cylindroconical glass tubes that contained 50 ml filtered water of various salinities i.e. 35, 40, 45, 50, 55, 60, 65, 70, 75, 80 and 100 ppt (three replicates for each salinity and heat shock temperature). For the next $2^{1}/_{2}$ h, the temperature was 28ºC while light intensity 2000 lux and aeration were provided for the rest of the experiment. Then, the glass tubes containing the cysts were placed in water baths at 42±0.2ºC and 44±0.2ºC for 48 h while continuous aeration was applied in each tube. All tubes were carefully closed with parafilm and were frequently monitored for salinity, which remained constant.

Cysts water content

Once the prolonged heat shock treatment was completed, the cysts were harvested by rapid filtration and rinsed thoroughly with deionised water to remove salts; rinsing was a very rapid procedure i.e. no more than 10 sec; in this way, water uptake by the cysts was avoided. Then, 0.15 – 0.20 g of the heat-shocked cysts were placed in Eppendorf tubes (three or four for each of the scored salinities) bearing tiny holes at the bottom and centrifuged for 5 min at 13,000 rpm in order to remove water. In this way the cyst surface was free from salts or water. Afterwards the cysts were placed in pre-weighed cups and oven dried at 58±1ºC for 48 h.

Determination of hatching percentage after heat shock treatment

Cysts that were heat shocked at 35, 50, 70, 80 and 100 ppt (0.08 g per cylindroconical tube) were incubated under optimal conditions and the hatching percentage was determined according to SORGELOOS et al. (1986) at the end of 48 h (three samples of 250 µl each, were taken from each replicate and for each salinity treatment and in each of the two HS temperatures); although our final hatching data are reported for 48 h, we observed no increase in hatching levels after five days of incubation. Fully hydrated (non heat-shocked) cysts were incubated under optimal conditions at five different salinities (i.e. 35, 50, 70, 80 and 100 ppt) for 240 h (10 days) in order to exclude the case of a delayed hatching rate (ABATZOPOULOS et al., 1994).

Glycerol

Fully hydrated cysts (0.5 g) were placed at 35, 70 and 80 ppt (for each salinity, three samples were taken). Glycerol determination was performed according to the calorimetric method of BURTON (1957) and following the procedures described by CLEGG (1962, 1964).

SDS-PAGE

Denaturing polyacrylamide slab gel electrophoresis (SDS-PAGE) was performed following ABATZOPOULOS et al. (1994). Control and heat-shocked (at 44ºC) cysts were homogenized (0ºC) in buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 0.1 mM phenylmethylsulfonyl fluoride, 40 mM Hepes, pH 7.55) using glass homogenizers (100 mg wet weight embryos/ml buffer K) and the homogenate centrifuged at 1630 g (4ºC) for 5 min following CLEGG et al. (1994). The

resulting pellets (which contained nuclei, yolk platelets and shell fragments) were washed with 50 times their volume in buffer K and resuspended to their original volume. Aliquots of pellets and supernatants were added to 2x sample buffer (LAEMMLI, 1970) heated at 100ºC for 5 min, and centrifuged at 10,000 g for 10 min. The supernatants were analyzed on 15% SDS polyacrylamide gels and proteins stained with Coomassie brilliant blue G-250 (CBB : 0.25 w/v in methanol 40% v/v and acetic acid 8% v/v). Quantification of relative amounts of p26 was evaluated densitometrically.

Statistical analysis

The variables analysed were : a) the cyst water content in various salinities, b) the hatching percentage of heat-shocked cysts at 42ºC and different salinities, c) hatching percentage of heat-shocked cysts at 44ºC and different salinities. Statistical analysis was performed using parametric (ANOVA) and non-parametric (Kruskal-Wallis) statistical tests. The selection of the statistical test was based on the assumption that the variances were homogeneous (SOKAL & ROHLF, 1981). Bartlett's, Hartley's, Cochran's and Levene's tests were applied to determine the homogeneity of the variance. STATISTICA 5.1 and STATGRAPHICS 5.0 were used for the analyses.

RESULTS

Water contents of cysts in various salinities after incubation for 48 h at 42ºC are presented in Table 1. The water content varied from about 62% at 35 ppt to about 54% at 100 ppt. Comparison of the cyst water contents revealed that there were significant differences among the examined salinities (ANOVA, F = 3.207, df = 10, 29, $p < 0.05$). Salinities between 35 ppt and 70 ppt resulted in similar cyst water content while apparent differences existed between the water content in 35 ppt and 75 ppt onwards (Duncan's multiple range test, $p < 0.05$ – see Table 1). The same battery of experiments performed at 44ºC, exhibited no differences in cyst water content compared to those at 42ºC (Kruskal-Wallis, $p > 0.05$) and, therefore, are not presented here.

TABLE 1

Water content in *A. franciscana* cysts (expressed as percent of the dry cysts, w/w) after 48 hours incubation at 42˚C in various salinities. Results that share the same letters are not significantly different (Duncan's multiple range test, $p < 0.05$). n : number of samples.

Salinity (ppt)	Water content (%)	n
35	61.69 ± 0.85 [a]	4
40	60.89 ± 1.04 [a,b]	3
45	60.27 ± 1.11 [a,b]	4
50	59.02 ± 1.94 [a,b]	3
55	59.07 ± 1.49 [a,b]	4
60	59.29 ± 1.01 [a,b]	3
65	59.51 ± 1.14 [a,b]	4
70	58.33 ± 2.23 [a,b]	4
75	57.27 ± 4.71 [b,c]	4
80	56.92 ± 2.44 [b,c]	3
100	54.07 ± 3.21 [c]	4

Hatching percentages (HP) of the heat-shocked cysts (42°C) in each salinity presented significant differences (ANOVA, F = 20.78, df = 4, 14, p < 0.05 – see Table 2). A significant drop in the apparent hatching capability occurred in the heat-shocked cysts (42°C) at salinities below 70 ppt (i.e. from 92.5% in control material, decreased to below 70%, Scheffé test, p < 0.05, see Table 2). Cysts that were heat shocked at 44°C in the examined salinities presented significant differences in their HP upon their return to optimal hatching conditions (ANOVA, F = 343.86, df = 4, 14, p < 0.05 – see Table 2). The drop of the HP at this temperature was dramatic (i.e. from 92.5% – see heading of Table 2 – decreased to less than 5% when HS salinity was 70 ppt, Scheffé test, p < 0.05, see Table 2). It seems that at 44°C there was a threshold between the salinities of 70 ppt and 80 ppt; cysts that were heat shocked at the salinity 80 ppt and above showed a higher level of recovery (Scheffé test, see Table 2). Cysts that were incubated in 100 ppt during heat shock at 44°C, resulted in a hatching percentage 25 times higher than those incubated in 35 ppt during heat shock (i.e. 41.72% compared to 1.67%, Scheffé test, p < 0.05, Table 2). Therefore, at 44°C the hatching percentage of heat-shocked cysts in the respective salinities (Table 2), revealed significant differences upon return to favourable conditions. In this way, they exhibited a clear trend of higher recovery capability as the salinity, in which the prolonged heat shock took place, rose.

TABLE 2

Hatching percentage (HP) of *A. franciscana* cysts after 48 h incubation at 42°C and 44°C in 5 different salinities and returned to optimal conditions (HP was determined after 48 h). Results that share the same letters in each column are not significantly different (Scheffé test, p < 0.05). The number of samples measured for each treatment (temperature and salinity) was 3 (3 subsamples were taken per replicate). Cysts incubated at optimum conditions (S : 35±2 ppt, T : 27±1°C, pH = 8.75) without a prior heat shock (control) resulted in a hatching percentage of 92.50±1.88%.

Salinity (ppt)	Hatching percentage (HP, %)	
	After heat shock at 42°C	After heat shock at 44°C
35	66.45 ± 2.90 [a]	1.67 ± 1.07 [a]
50	68.69 ± 2.87 [a]	2.35 ± 1.45 [a]
70	68.91 ± 3.16 [a]	4.09 ± 2.73 [a]
80	70.40 ± 2.31 [a]	22.37 ± 3.64 [b]
100	77.18 ± 2.03 [b]	41.72 ± 4.01 [c]

When cysts not subjected to heat shock were incubated in five different salinities (i.e. 35, 50, 70, 80 and 100 ppt – the rest of the conditions were at optimal levels), the following results were observed : when salinity increased above 70 ppt, there was a delay in hatching rate while cysts incubated in 100 ppt presented less than 5% hatching after 8 or 10 days denoting that a minimum metabolism was taking place (Fig. 1). The rationale of this experiment is discussed in the discussion section.

Fully hydrated cysts placed in different salinities (35, 70 and 80 ppt) revealed different glycerol levels
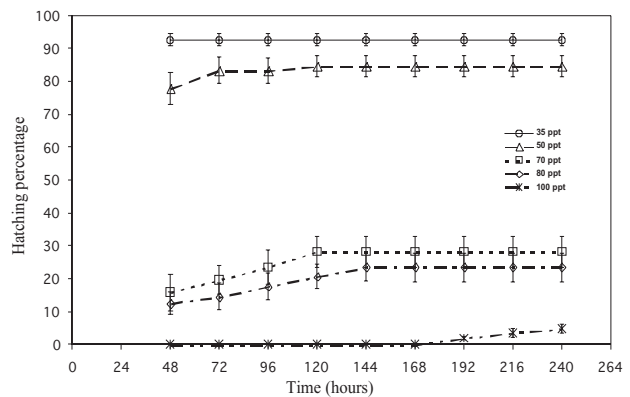

Fig. 1. – Hatching percentage (HP) of *Artemia franciscana* cysts in various salinities (all other hatching conditions were kept optimal).
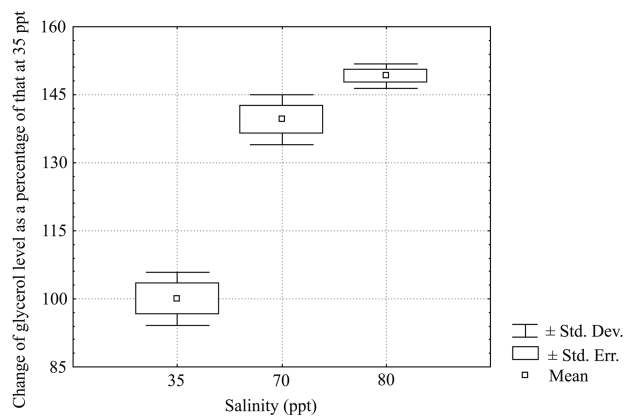

Fig. 2. – Changes in glycerol levels with increasing salinity. At 35 ppt, glycerol levels were arbitrarily considered as 100%.
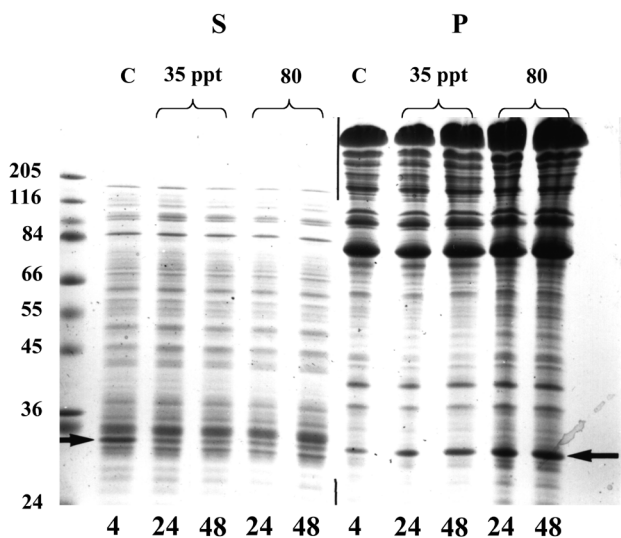

Fig. 3. – SDS-PAGE profiles of fractions from hydrated but non-heat-shocked cysts of *Artemia franciscana* (4), and after 24 h and 48 h at 42°C in 35 ppt and 80 ppt. Supernatant (S) and pellet (P) fractions were analyzed (see Materials and Methods). Molecular weight standards are in kiloDaltons (kD). The arrow denotes the 26 kD protein.

after $2^1/_2$ h (Fig. 2). Glycerol levels at 35 ppt were arbitrarily considered as 100 ($\pm$5.96)%. When salinity increased from 35 ppt to 70 ppt, the glycerol concentration increased by 39 ($\pm$5.49)%, while in 80 ppt the increase was nearly 50% (49.5$\pm$2.73%). These differences in glycerol levels were obviously significant (Kruskal-Wallis, H = 7.2, n = 9, p < 0.05).

SDS-PAGE of hydrated *Artemia* cysts not exposed to heat shock treatment and heat shocked at 44ºC for 24 and 48 h in 35 ppt and 80 ppt revealed very similar protein profiles (see Fig. 3). There is, however, one exception : a protein of about 26 kD (p26) appears to be translocated from the supernatant (S, soluble fraction) to the pellet (P, mainly nuclei) as a result of heat shock. Moreover, the amount of p26 in the pellet fraction from 80 ppt was fairly increased compared to that from 35 ppt.

DISCUSSION

Incubation of fully hydrated cysts in various salinities at 42 or 44ºC for 48 h and immediate transfer to optimal conditions for determination of hatching percentage, showed a better recovery of the cysts that were heat shocked in a high salinity environment. There is a gradual increase in hatching percentage as salinity elevates. Even a small increase in salinity of the order of 10 ppt (i.e. from 70 ppt to 80 ppt) seems to offer the embryos a substantial advantage against the stressor of 44ºC (i.e. hatching percentage was increased from 4.09% to 22.37% – Table 2). This advantage offered by the elevated salinity to *Artemia* cysts, raises several questions regarding the mechanisms that induce this phenomenon. One explanation would be that the various salinities affect the hydration level of the cysts, hence, their metabolic status and sensitivity to various shocks. This seems not to be valid since in this work, the results from the water content determination (see Table 1) suggested that the hydration level of the cysts was sufficient to sustain metabolism in the encysted embryos for nearly all salinities tested with the exception of 100 ppt, as referenced by the hatching response (see Fig. 1). These results are consistent with the opinion that above salinity levels of 90 ppt, which is a potential "threshold" for most *Artemia* populations, water is absorbed in insufficient quantities to support the metabolism of the embryos and consequently they remain in quiescence (LAVENS & SORGELOOS, 1987) or exhibit a very retarded hatching (see incubation at 100 ppt – Fig. 1).

It is well known that salinity is one of the major factors that influences the hatching ability of *Artemia* cysts by affecting the osmotic potential of the embryo (CONTE et al., 1977) and special biochemical activities such as that of the hatching enzyme (SATO, 1967). The salinity of the incubation medium affects the water content of the cysts and thus the accumulation of free glycerol (CLEGG, 1964); gradual accumulation of the glycerol in the cyst causes a corresponding increase in the internal osmotic pressure. This enhances the ability of the cyst to imbibe water and therefore to continue its development; then the chorion of the cyst ruptures osmotically only if the increased internal osmotic pressure eventually exceeds that of the environment (CLEGG, 1964).

As the water content is obviously not the only parameter responsible for the metabolic status of the encysted embryos or their behaviour during incubation at high salinities and temperatures, the question arises about the exact role of salinity in the recovery of cysts from heat shock. Three other factors may play an important role in cyst thermostability : glycerol, p26, trehalose or combinations of these (CLEGG & TROTMAN, 2002). Only two of them (i.e. glycerol and p26) are involved in heat protection of hydrated cysts, while trehalose is related to desiccated cysts (CROWE et al., 2001 and references therein). Therefore, the question addressed above may be reconstructed as follows : In what way does salinity enhance the tolerance of fully hydrated cysts to heat shock temperatures? Is it due to glycerol concentration and/or translocation of p26?

Glycerol concentration

The heat-shocked cysts at 42ºC presented a slight but statistically different reduction of the hatching percentage compared to the control cysts; this was probably due to the percentage of the cysts re-entering diapause (ABATZOPOULOS et al., 1994). There were no differences in the hatching percentages of the heat-shocked cysts in different salinities with the exception of the outcome in 100 ppt (see Table 2). At 44ºC, the hatching percentage of the heat-shocked cysts reduced significantly compared to the control cysts, while a relationship between the elevation of salinity and the hatching percentage appeared (hatching percentage of the heat-shocked cysts in 100 ppt was 25 times higher than the heat-shocked cysts in 35 ppt – see Table 2). The dramatic drop in hatching percentage at the low salinity treatment must be attributed to cysts that either enter a deep diapause (from which we cannot recover them with the empirical techniques presently at our disposal, see VAN STAPPEN et al., 1998) or die.

It seems that there is a "threshold" in salinity levels; cysts that were heat shocked in 80 ppt or 100 ppt hatched upon return to optimal conditions (hatching percentage : 22.37$\pm$3.64% and 41.72$\pm$4.01%, respectively). One explanation would be based on the water content of the cysts and glycerol's properties (see Introduction – SUN et al., 1999 and references therein). Heat-shocked cysts in relatively low salinities (35 ppt to 70 ppt) contained more water than those in higher salinities (80 ppt or 100 ppt). This reduction of water content (about 14% in 100 ppt) was one of the parameters that caused the increase of glycerol concentration. The other parameter was the build up of cyst glycerol (for increasing internal osmotic pressure) in elevated salinities towards rupturing of chorion (step b of the Experimental design). It is obvious (see Fig. 2) that the salinity increase is related to the increase of the concentration of glycerol (corresponding to an increase of almost 50% in 80 ppt compared to 35 ppt). Thus, these two incidents must be responsible for the substantial increase of glycerol concentration and most probably for the protection of proteins (see Introduction). As a consequence, heat-shocked cysts in the high salinity treatment showed better hatching compared to those treated in low salinities, since the latter were driven either to re-enter diapause or to "die".

p26

The higher levels of p26 in nuclei that were observed after prolonged heat shock in 80 ppt compared to those in 35 ppt, is an indication that this salinity level activates translocation of the molecular chaperone p26, which protects cysts from heat shock. Previous work of Abatzopoulos et al. (1994) has demonstrated that after 48 h heat shock at 42ºC and 35 ppt salinity, at least some *Artemia* cysts are pushed back into a diapause state. This diapause induction was accompanied by a translocation of a 26 kD protein from the supernatant to the pellet (i.e. from the cytoplasm into nuclei). Clegg et al. (1994) and Liang et al. (1997a,b) demonstrated that anoxia and prolonged incubation at low temperatures (0-20ºC, one month) can also induce a similar translocation, the same as seen in embryos exposed to aerobic heat shock (42ºC) (Abatzopoulos et al., 1994). In this study, a major translocation of p26 was not apparent, although a gradual increase of p26 was recorded in the pellet from 80 ppt and 44ºC. Clegg et al. (1996) showed that encysted embryos in the state of diapause produced in laboratory cultures and collected from field, also share the p26 between the fractions of pellet and supernatant. Clegg et al. (1994) presented evidence that the 26 kD protein is a subunit of a native protein with molecular mass of about 500 kD (or 700 kD according to more recent work – Jackson & Clegg, 1996; Liang et al., 1997a, b), and that its role might be that of a protective molecular chaperone (Jackson & Clegg, 1996). Our data revealed that both salinity and heat shock seem to have an additive effect, since the intensity of the 26 kD protein in the pellet after 24 h and 48 h heat shock at 44ºC and 80 ppt salinity is higher when compared to a similar heat shock in 35 ppt. The above results are in agreement with the hypothesis of Clegg et al. (1994) that the 26 kD protein might be a protective molecular chaperone, since it allows the embryos to withstand elevated temperatures without a tremendous loss of their hatching ability.

Concluding, it is evident that elevated salinities result in increased glycerol concentrations, which, together with increased p26 in nuclei, may prevent the encysted *Artemia* embryos from entering a deep diapause state from which they cannot be recovered by applying empirical techniques. The major role of glycerol as thermostabilizing agent has to be stressed although further experimentation is needed to document this hypothesis.

Attempting to explain an ecological significance of this phenomenon, one may attribute it to the special conditions that cysts face when washed ashore. It has been observed quite frequently and by many teams (personal communication) that cysts – when driven ashore – float in shallow waters (the water column is only few cm) with salinities ranging from 80-200 ppt and water temperature as high as 45ºC or more. Since this situation is very common, this phenomenon may be of high adaptive significance for *Artemia* encysted embryos. These findings have been confirmed in other *Artemia* populations, such as parthenogenetic populations (unpublished data).

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