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Hatching characteristics and cold storage of nauplii of brine shrimp *Artemia* KKT1 from Thamaraikulam, India

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The brine shrimp *Artemia* has been used widely as a live feed for finfish and shrimp/prawn larvae. An *Artemia* population found in a saltpan of Thamaraikulam (India) is investigated for its hatching characteristics. The hatching percentage was 69.2% while hatching efficiency, hatching synchrony and hatching output of the cysts were found to be 230600 nauplii/g of cysts, $t_s = 12$ h and 661 mg/g cysts, respectively. Cyst decapsulation improved significantly (7%) the hatching percentage. The effects of temperature, salinity and pH on hatching were also studied. It was revealed that temperature had more influence on hatching. The optimum conditions for maximum hatching were found to be 29 ± 1°C, 35 ppt and pH = 8. Cold storage studies revealed that the freshly hatched instar-I nauplii could be maintained at 2-4°C, up to 48 h with >90% survival. No significant dry weight loss was noticed during this storage period. Furthermore, it was demonstrated that the instar-I nauplii can tolerate -1°C for 24 h after treatment with a cryoprotectant.

Key words: *Artemia*, cyst, decapsulation, hatching, cold storage, cryoprotectant.

INTRODUCTION

Artemia has been shown to be an irreplaceable live feed for marine fish and shrimp/prawn larvae (Leger & Sorgeloos, 1992; Sorgeloos *et al*., 2001). Demand for *Artemia* cysts is increasing exponentially in response to the expansion of hatcheries, requiring over 2000 metric tonnes annually on a global scale. Unfavourable weather conditions in Great Salt Lake caused poor yield of cysts during the last decade (Sorgeloos & Van Stappen, 1995; Stephens, 1998), and resulted in severe cyst shortage. The unpredictable fluctuation of *Artemia* cyst productions from "traditional" sites (such as Great Salt Lake or San Francisco Bay, USA) has forced producers to explore new potential sites for cyst harvesting (e.g. Lake Urmia, Iran and Aibi Lake, China) (Dhont & Sorgeloos, 2002). It has been reported that 10% of the world demand for cysts could be provided by a number of natural *Artemia* habitats (Lavens & Sorgeloos, 2000), although these sites cannot provide substantial quantities (Dhont & Sorgeloos, 2002). Also, it has been considered that the local availability of cysts would be an important asset for the development of a viable local aquaculture (Bengtson *et al*., 1991).

Hatching characteristics can reveal the quality of *Artemia* cysts and were reported to be highly variable among not only various strains, but also various batches of the same strain from the same location (Sorgeloos & Persoone, 1975; Smith *et al*., 1978; Lavens & Sorgeloos, 2000). It has been demonstrated that decapsulation of *Artemia* cysts could improve the hatching characteristics (Bruggeman *et al*., 1980). Several authors have revealed that temperature, salinity and pH significantly influence the cyst hatchability (Von Hentig, 1971; Sorgeloos, 1975; Sorgeloos *et al*., 1986; Royan *et al*., 1987).

Freshly hatched instar-I *Artemia* nauplii are com-

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monly used as live feed for fish larvae. Later nauplia r stages (i.e. instar-II, -III or metanauplii) are not suitable for larval feeding; they are by 50% larger in length, swim faster and loose their nutritional value (Watanabe *et al*., 1978; Dye, 1980; Sorgeloos *et al*., 2001). For this reason, daily hatching of cysts and harvesting of nauplii have to be carried out. To reduce this labour as well as time and cumbersome process of hatching and harvesting, cold storage of *Artemia* nauplii was practiced in different *Artemia* strains (Baust & Lawrence, 1980a,b; Leger *et al*., 1983).

In the present paper, we report on the hatching characteristics of an *Artemia* strain from Thamaraikulam (KKT1), i.e. hatching percentage, hatching efficiency, hatching rate and hatching output of the cysts along with the effects of decapsulation, temperature, salinity and pH on hatching. The storage of nauplii at low temperature and the tolerance of nauplii to freezing temperatures (with and without a cryoprotectant) is also described.

MATERIALS AND METHODS

Cysts were collected from the condensers of Thamaraikulam saltworks (India) and processed according to John *et al*. (2004). For more details on KKT1 strain, its habitat and cyst hatching procedure, see John *et al*. (2004).

Cyst hatching experiments

The hatching characteristics such as hatching percentage, hatching efficiency, hatching rate (and synchrony) and hatching output were analysed according to Bruggeman *et al*. (1980), Sorgeloos *et al*. (1978), Vanhaecke & Sorgeloos (1982) and Vanhaecke & Sorgeloos (1983), respectively.

Effect of decapsulation on hatching percentage

The *Artemia* KKT1 cysts were decapsulated using a NaOCl solution as described by Sorgeloos *et al*. (1986). Hatching percentage was determined for the decapsulated cysts in order to evaluate the potential improvement following decapsulation (t-test was applied for statistical analysis).

Effects of temperature, salinity and pH on hatching

Artemia KKT1 cysts were hatched at different temperatures (20, 25, 30 and 35°C) and salinities (5, 10, 20, 30, 40, 50 and 60 ppt). The intensity of the light was 1000 lux and the pH was 8.0. Temperature was monitored and culture media with different salinities were prepared as described by John *et al*. (2004). Data were subjected to two-way analysis of variance (ANOVA) as described by Zar (1974). The hatching percentages of the cysts at different pH (6, 7, 8, 9 and 10) were also determined by maintaining temperature at 29 ± 1°C, salinity at 35 ppt and illumination = 1000 lux. The ranges for temperature, salinity and pH were selected according to the abiotic parameters prevailing in Thamaraikulam saltpan.

Cold storage effect on Artemia *KKT1 nauplii*

A homogeneous population of *Artemia* nauplii (instar-I) was harvested from the hatching tank, rinsed with chilled seawater (2-4°C), and transferred into a cylindroconical tube at a density of 1000 nauplii per ml seawater (total volume was 100ml). The tube was kept in a refrigerator and mild aeration was applied constantly. As a control, another set of *Artemia* (same strain) was kept at room temperature (27 ± 1°C). The survival percentage was calculated at 12, 24, 36 and 48h. The data were subjected to two-way ANOVA.

Dry weight analysis of the nauplii was carried out according to Vanhaecke & Sorgeloos (1980a). Five replicates were used and the data were subjected to non-parametric statistical analysis (Kruskal-Wallis test).

Freezing tolerance test

To study the pre-freeze tolerance of *Artemia* KKT1, the nauplii collected at 0, 4, 8, 12 and 24h post-hatching were transferred into an ice bath (-1°C) at a density of 20 nauplii per ml. The survival percentage was recorded at 10, 30 and 90 min. A control was also kept at the room temperature (27 ± 1°C). Five to six replicates were maintained.

Effect of a cryoprotectant

The *Artemia* nauplii (density was 20 individuals per ml) of different developmental stages (0, 4, 8, 12 and 24 h) were first incubated with a cryoprotective agent, 3 M glycerol (Baust & Lawrence, 1980a) for 20 min at -1°C and then kept in a beaker containing ice. The freezing temperature was adjusted to -1, -5 or -10°C slowly at a rate between 0.1 and 1°C per min. Mild aeration was provided. After 24 h, actively swimming nauplii were counted and recorded. Five to six replicates were maintained.

RESULTS

Hatching characteristics of Artemia *KKT1 cysts*

The hatching characteristics of *Artemia* KKT1 cysts are presented in Table 1. The hatching curve indicates that the hatching started after 18 h and reached T_{10} and T_{90} at 24 and 36 h, respectively (Fig. 1).

Effect of decapsulation on cyst hatching

The hatching percentage of cysts without decapsulation was 69.2%. It increased to 76.2%, with a difference of 7% ($t = 3.34$; $p < 0.05$), when the cysts were decapsulated.

Effect of temperature and salinity on cyst hatching

Artemia KKT1 cyst hatching at different temperatures (20, 25, 30 and 35°C) and salinities (5, 10, 20, 30, 40, 50 and 60 ppt) is presented in Fig. 2. At 20°C, hatching percentage was low. It increased to a maximum at 30°C and then decreased with the temperature elevation. For instance at 40 ppt, the hatching

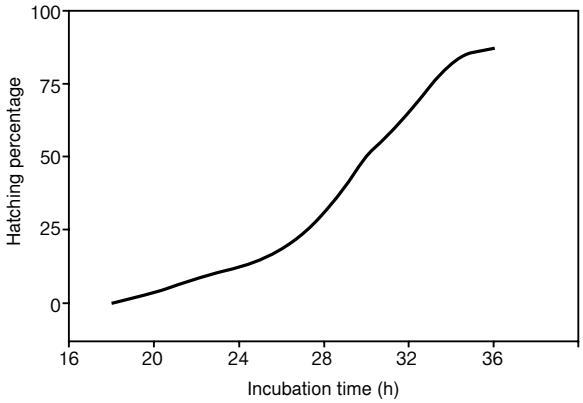

FIG. 1. Hatching curve for the *Artemia* KKT1 cysts.

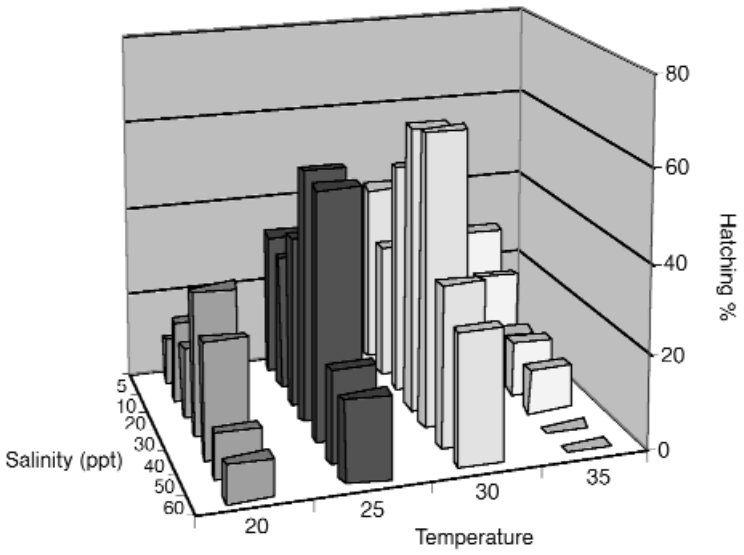

FIG. 2. Effect of temperature and salinity on the hatching percentage of *Artemia* KKT1 cysts.

TABLE 1. Hatching characteristics of *Artemia* KKT1 cysts

	Range	**Mean**	**SD**
Hatching percentage	64 – 73	69.2	3.56
Hatching efficiency (nauplii per g cyst)	213312 – 243309	230673	11877
Hatching output (mg biomass per g cyst)	612.21 – 698.30	661.95	34.086
Hatching rate (in h)			
	T_0 = 18		
	T_{10} = 24		
	T_{90} = 36		
	T_S ($T_{90} - T_{10}$) = 12		

T_0: time until appearance of the first nauplii; T_{10}: time until 10% hatching is attained; T_{90}: time until 90% hatching is attained; T_S (T_{90}-T_{10}): determination of hatching synchrony

TABLE 2. Two-way analysis of variance (ANOVA) for the influence of temperature and salinity on hatching percentage of *Artemia* KKT1 cysts

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	Variance ratio	P level
Temperature variance	3	5464.29	1821.43	26.44	< 0.001
Salinity variance	6	2419.21	403.20	5.85	< 0.005
Error variance	18	1240.21	68.90	—	—
Total variance	27	9123.71	—	—	—

$P < 0.05$ is statistically significant

percentage of 25% achieved at 20°C increased to 65% at 30°C and then dropped to 10% at 35°C. It is obvious that temperatures between 25 and 30°C support maximum hatching percentage. The lowest level of hatching observed between 50-60 ppt and at 35°C. The salinities of 30 and 40 ppt supported maximum hatching at all temperatures.

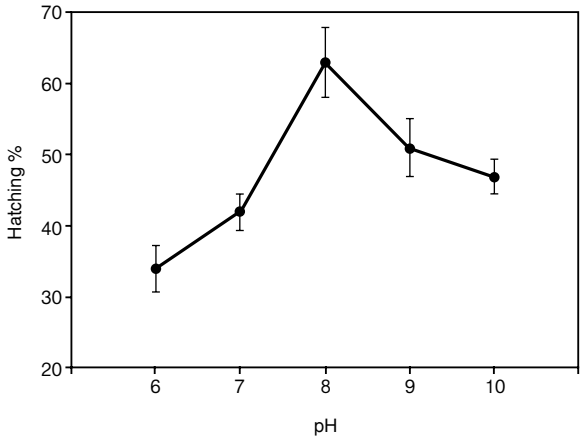

FIG. 3. Effect of pH on hatching percentage of *Artemia* KKT1 cysts.

As far as the combined effect of temperature and salinity is concerned, although both temperature and salinity influenced the hatching percentage, temperature had a greater impact [F(1)3, 18 = 26.44] than salinity [F(1) 6, 18 = 5.85] (Table 2).

Effect of pH on hatching

The hatching percentage of *Artemia* KKT1 cysts at different pH values (6, 7, 8, 9 and 10) is shown in Fig. 3. pH = 8 seems to correspond to the optimum value, since it results in maximum hatching (63%).

Survival of Artemia *KKT1 nauplii stored at 2-4°C*

The survival of *Artemia* nauplii stored at 2-4°C and compared with the survival of a control kept at 27 ± 1°C was studied. One hundred percent survival could be achieved initially for a period of 12 h at 2-4°C. But later on, the percentage of survival was gradually decreased to 98, 95 and 93% at 25, 36 and 48 h, respectively (Fig. 4A). Two way ANOVA revealed that storage temperature has less significance [F(1)1, 3 = 3.33; p > 0.05] than time [F(1)3, 3 = 10.50;

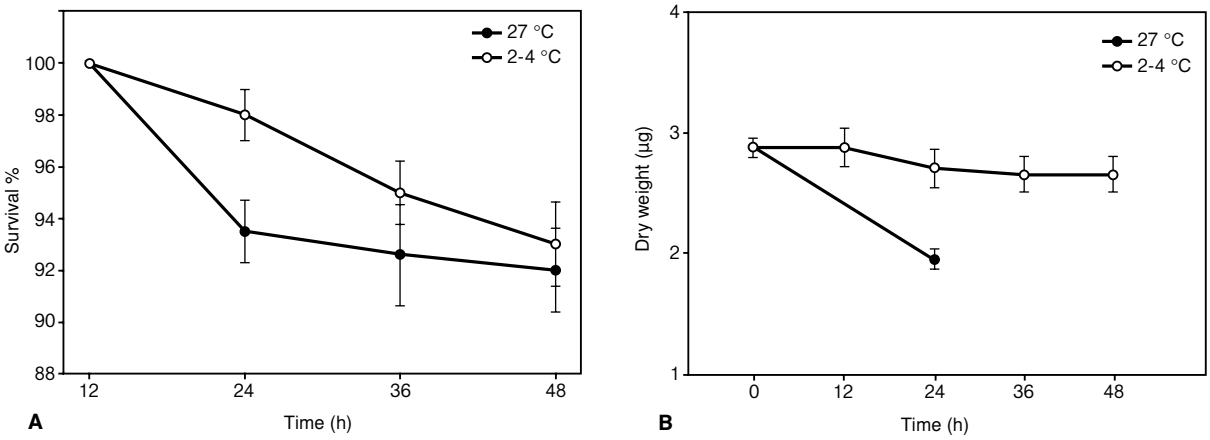

FIG. 4. A. Survival of *Artemia* KKT1 nauplii stored at 2-4°C and 27°C for 48 h. B. Dry weight (µg) changes in *Artemia* KKT1 nauplii stored at 2-4°C and 27°C for different hours.

TABLE 3. Two-way analysis of variance (ANOVA) for the influence of storage temperature and time on the survival of *Artemia* KKT1 cysts

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	Variance ratio	P level
Time variance	3	70.13	23.38	10.50	< 0.05
Temperature variance	1	7.42	7.42	3.33	> 0.05
Error variance	3	6.68	2.23	–	–
Total variance	7	84.23	–	–	–

P < 0.05 is statistically significant

$p < 0.05$] (Table 3). The nauplii examined after 48 h of storage showed that all remained at instar-I, whereas the nauplii kept at 27˚C passed onto instar-II after moulting.

Dry weight analysis

The dry weight changes of stored live *Artemia* nauplii at different moments are shown in Fig. 4B. The dry weight losses were very small and statistically insignificant ($p > 0.05$). Even after 48 h of storage, only 8% of the dry weight was lost, which is negligible when compared to that of nauplii stored at 27˚C.

Survival of Artemia *nauplii at 27˚C*

The nauplii of *Artemia* KKT1 collected at 0, 4, 8, 12 and 24 h post-hatching, were maintained at 27 ± 1˚C for 24 h and survival was 98.2 ± 0.75%, irrespectively of age (Fig. 5). There was no significant variation ($F = 0.85$, $p > 0.05$) observed on survival percentage among different developmental stages.

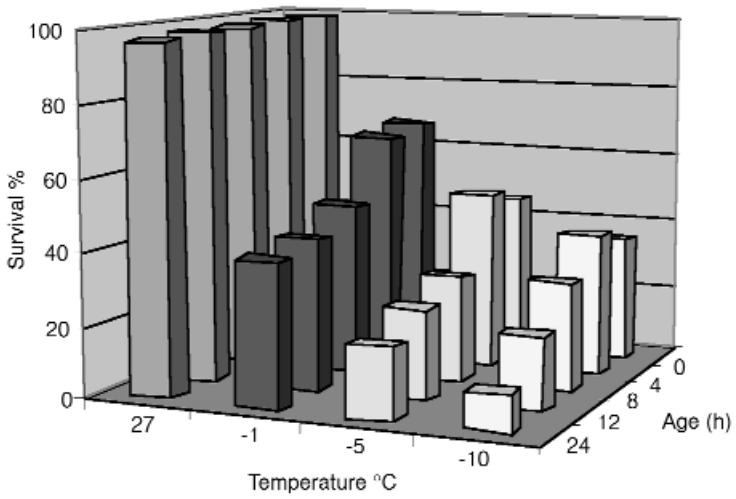

FIG. 5. Pre-freeze survival of *Artemia* KKT1 nauplii of different developmental stage at -1°C.

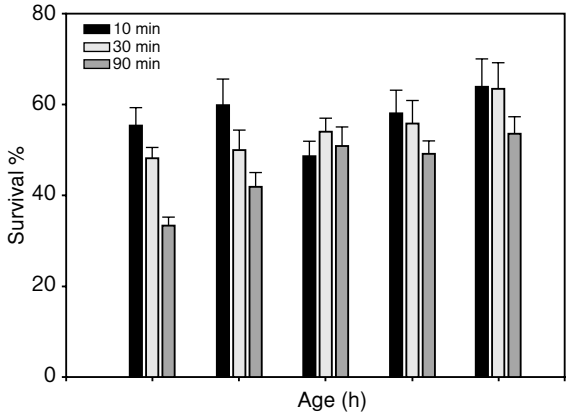

FIG. 6. Survival of *Artemia* KKT1 nauplii of different developmental stages at 27˚C without a cryoprotectant and with a cryoprotectant (i.e. 3 M glycerol) at -10, -5 and -1˚C for 24 h.

Survival of nauplii at -1˚C without a cryoprotectant

To understand the ability of nauplii to survive at -1˚C, different developmental stages as above, were exposed to -1˚C for 10, 30 and 90 min. The data suggest that developmental stage of nauplii do not influence much on the survival percentage (Fig. 6).

Survival of nauplii at freezing temperatures with a cryoprotectant

The effect of a cryoprotectant, 3M glycerol, on the survival of *Artemia* KKT1 nauplii was determined by storing groups of nauplii of different developmental stage at freezing temperatures, such as -1, -5 and -10˚C (Fig. 5). At -1˚C, all groups were found after 24 h to survive by over 40%. The cryoprotectant helped the freshly hatched nauplii (0-4 h) to survive by 60%, however, the survival of 8 h and above nauplii was decreased to less than 50%. Further decrease of the freezing temperature to -5 and -10˚C, decreased the survival by ~ 50% (Fig. 5).

DISCUSSION

The parthenogenetic *Artemia* population from Thamaraikulam (India) has been previously characterised by John *et al*. (2004). In the present report, we analysed the hatching characteristics of the same strain. We used in all experiments, cysts collected from the first batch. The maximum hatching percentage was found to be 69.2%. This value of hatching percentage may be due to the exposure of cysts to sub-optimal conditions in nature and to the suspected mortality of the embryos, as suggested by Vanhaecke & Sorgeloos (1983) and Van Ballaer *et al*. (1987). The hatching percentage of *Artemia* KKT1 is lower than that of Greek parthenogenetic strains (Citros: 88%, M. Embolon: 84%; Abatzopoulos *et al*., 1989), but not one of the worst reported since it is higher than the value (36%) of another Indian strain collected from Balamba (Royan *et al*., 1987).

The knowledge of hatching synchrony would be helpful for harvesting nauplii at instar-I stage and high energy content (see also Benijts *et al*., 1976). The hatching synchronies of Citros, M. Embolon are 7.5 and 9 h, respectively, while for KKT1 is 12 h (Abatzopoulos *et al*., 1989). The hatching efficiencies of sexual *Artemia* vary between 60000 (Canada) and 300000 (SFB) nauplii/g cysts, whereas in parthenogenetic strains the efficiency ranges between 70000 and 230000 (Vanhaecke & Sorgeloos, 1980b, 1983; Sorgeloos *et al*., 1986). The hatching efficiency of KKT1 strain was determined to be 230000, which is among the highest recorded for parthenogenetic populations.

The effect of decapsulation on cyst hatching was studied in several *Artemia* strains and the percentage of hatching improvement was reported to vary between 2% (Lavalduc, France) and 132% (Chaplin Lake, Canada) (Bruggeman *et al*., 1980; Vanhaecke & Sorgeloos, 1983). In the KKT1 strain, decapsulation resulted in hatching percentage increase of 7%, which is not very spectacular. Although speculative, this poor result could be attributed either to sub-optimal conditions in nature (i.e. long exposure to high temperatures) or to the deep diapause in which embryos may be locked. Special treatments for deactivation of diapause (i.e. H_2O_2) could have led to better hatching (Van Stappen *et al*., 1998).

Our experiments revealed that 30˚C temperature and 30-40 ppt salinity is the optimum condition for maximum hatching of KKT1 cysts. The optimum pH was found to be 8.0. Similar conditions have also been described by Royan *et al*. (1987) for another parthenogenetic strain from Balamba (India).

Freshly hatched nauplii of several bisexual and parthenogenetic *Artemia* strains have been reported to be suitable for storage with high survival (> 90%) in densities of up to eight million nauplii per litre for 24 h (Leger *et al*., 1983). In the present study, the cold storage of *Artemia* KKT1 live nauplii at 2-4˚C could maintain the survival above 90% even after 2 days, without any significant loss in the dry weight. The developmental stage of nauplii was also stalled at instar-I (Abatzopoulos *et al*., 1989). These data reveal the applicability of this practice in finfish and shrimp/prawn hatcheries.

A preliminary study was performed on the tolerance of *Artemia* nauplii to freezing temperatures using a cryoprotectant. The freshly hatched nauplii (0-4 h post hatching) showed about 60% survival at -1˚C for 24h. It has been recently demonstrated that, *Artemia* nauplii generally are more tolerant to low temperatures while developmental stage and cold tolerance are inversely related (Xian *et al*., 2002). In the present study, also, among the various developmental stages tested, the group 0-4 h nauplii showed the highest tolerance to freezing temperatures.

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