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EFFECTS OF HYDROGEN PEROXIDE, COLD STORAGE AND DECAPSULATION ON THE HATCHING SUCCESS OF *ARTEMIA* CYSTS

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Key words: *Artemia*, cold storage, decapsulation, hatching rate, hydrogen peroxide

Abstract

The effects of hydrogen peroxide, cold storage and decapsulation on diapause in parthenogenetic *Artemia* cysts from the Izmir Çamalti saltern were tested. To test the effect of hydrogen peroxide, cyst samples were incubated for 5 min in a 2% or 5% H_2O_2 solution. In the cold storage experiments, the cysts were divided into three groups. One group was stored in saturated saline, the second in a vacuum and the third in air for 15 days or one month. Decapsulation was tested after a 2-hour prehydration period. The hatching rate of the control group after 24 h in cysts harvested in November was 0, while in cysts harvested in March it was 19.2%. All the treatments for diapause deactivation resulted in increased hatching during the first 24 h but the final hatching rates (at 72 h) of all treated groups were similar to or slightly lower than that of the control.

Introduction

The worldwide distribution of the brine shrimp *Artemia* in isolated habitats with specific ecological conditions has resulted in the development of many distinct geographic strains of genetically different populations. Among the strains, a high degree of genetic variability has been observed in characteristics such as cyst diameter and resistance to high temperature (Browne et al., 1991), which are considered strain-specific and relatively constant (Vanhaecke and Sorgeloos, 1980). But the hatching rate or percentage may vary from batch to batch or between populations (Vanhaecke and Sorgeloos, 1983).

The ability of an organism to create a cryptobiotic state during its lifespan is a major survival mechanism in populations inhabiting biotopes with unsuitable environmental conditions that may threaten the survival of the individual. As a representative of such a group, *Artemia* ensures its survival during periods of, e.g., desiccation or extreme temperature and salinity by producing diapause embryos. These can resist extremely unfavorable con-

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ditions and become the origin of a new population when abiotic and biotic parameters in the habitat again become favorable (Lavens and Sorgeloos, 1987).

The diapause of the *Artemia* cyst has been the subject of several studies and reviews (Dutrieu, 1960; Anderson et al., 1970; Drinkwater and Crowe, 1986; Crowe et al., 1987; Lavens and Sorgeloos, 1987; Clegg et al., 1996). Diapause is a complex process, influenced by genetic factors (Vanhaecke and Sorgeloos, 1982), culture conditions (Versichele , 1980), food availability (Lavens et al., 1986) and salinity (Versichele and Sorgeloos, 1980).

Various processes interrupt diapause, including dehydration (Voronov, 1974), repeated dehydration/hydration cycles (Browne, 1980; Browne et al., 1984), cold storage (Lavens et al., 1986), hydrogen peroxide (Mathias, 1937; Bogatova and Shmakova, 1980; Bogatova and Erofeeva, 1985; Van Stappen et al., 1998), acetone, n-butanol, ethylesther, xylene (Tazawa and Iwanami, 1974) and hypochloride (Bruggeman et al., 1980). These treatments have been applied with varying success by several authors. Despite the variability, a proportion of diapausing cysts was activated in all cases.

Artemia cysts and nauplii are used as food for cultured marine and freshwater fish and crustacea. Izmir Çamaltı Saltern is commercially important as a source of *Artemia* cysts (Kocatas et al., 1991; Koru and Cirik, 1997; Koru and Cirik, 2001) which are harvested and marketed to local hatcheries. The hatching rate strongly depends on the diapause status of the cysts (and can be strain-specific). Harvest and storage conditions, and the length of time the cysts are stored, can also influence the hatching rate. Most of the commercial *Artemia* strains begin to hatch after 15-20 h of incubation and 90% of the hatching occurs within the first 20-30 h (Vanhaecke and Sorgeloos, 1983). The hatching rate and synchrony are important parameters because the energy content of the nauplii decreases as hatching advances. Therefore, lengthy incubation causes a serious loss of energy in the nauplii (Vanhaecke et al., 1983). In comparison with other strains, hatching is moderate in Çamaltı cysts and requires a longer period (Saygı, unpubl. data). Therefore, in this study, the effects of hydrogen peroxide, cold storage and decapsulation were tested as techniques for deactivating the diapause in Çamaltı *Artemia* cysts harvested in two different periods.

Materials and Methods

Test animals. Parthenogenetic *Artemia* from the Izmir Çamaltı Saltern were harvested in November 2000 and March 2001 by the Tekel Salt Company. After harvesting, the cysts were processed in the Tekel establishment; they were cleaned with tap water and dried at 25-28°C room temperature under non-controlled conditions. Prior to the experiments in May 2001, the hatching rate (control) was determined following methods of Lavens and Sorgeloos (1996) after 24, 48 and 72 h incubation. Hatching was carried out in funnel-shaped glass tubes using natural sea water (35 ppt) at a temperature of 28±1ºC with continuous illumination of 1000 lux. The cysts were kept in suspension by gentle aeration from the bottom of the hatching apparatus. Each treatment was repeated three times. Six samples from each replicate were taken after 24, 48 and 72 h, and the number of nauplii in the embryo and umbrella stages were counted under a microscope.

Hydrogen peroxide, decapsulation and cold storage treatment. A 27% constant concentration of H_2O_2 solution was maintained during the H_2O_2 treatments. The experimental concentrations (2% and 5%) were prepared immediately before use by diluting the H_2O_2 with deionized water. Dehydrated cyst samples were rinsed with tap water before treatment in a 100 ml glass cone filled with the test solution. After five minutes, the cysts were collected on a sieve, rinsed thoroughly with tap water to remove traces of the H_2O_2 and incubated in sea water under standard hatching conditions.

For the cold storage treatments, cysts (not treated with peroxide) were deep frozen (-25ºC) in air, a vacuum or saturated brine. After 15 days or one month storage, the cysts were incubated in standard conditions.

Decapsulation was tested on the November 2000 samples after a 2-hour pre-hydration period according to Bruggeman et al. (1980).

Statistical analysis. Hatching results were analyzed statistically with a one-way ANOVA to find the overall effects of the treatments. The Post Hoc LSD comparison test was used to detect significant differences between experimental group means at a significance level of $p<0.05$ after data were normalized by arcsin transformation (Sokal and Rohlf, 1981).

Results

The effects of the H_2O_2 treatment were influenced by the concentration (Tables 1, 2). One-way ANOVA showed a significant effect ($p<0.05$) of both treatments (2% and 5% H_2O_2) in both samples (November and March) on the hatching rate at 24 h. In the November control group, no cysts hatched within the first 24 h whereas the hatching rate was 19.2% in March. The 5% H_2O_2 application had a lethal effect on the cysts in the March sample at 72 h; a similar effect was also observed for the November samples. Decapsulation significantly improved the hatching rate of the November samples at 24 h. Cold storage also significantly improved the hatching rate at 24 h. In the November group, the highest 24-h rate was obtained with storage in saline at -25ºC for 15 days while best results in the March group were obtained with storage in saline at -25ºC for 15 days or one month. Long-term storage (1 month) negatively influenced the final hatching rate in most treatments.

Table 1. Mean hatching rate and standard deviation of *Artemia* harvested from Izmir Çamaltı Salterns in November 2000 (n= 18 for each treatment).

	Hatching rate (%)		
Treatment	*24 hour*	*48 hour*	*72 hour*
Control	0.00 ± 0.00^{a}	27.42 ± 4.72^{cd}	38.91 ± 4.69^{acd}
2% H_2O_2/5 min	5.03 ± 1.60^{bc}	28.43 ± 5.50^{cd}	39.14 ± 6.32^{acd}
5% H_2O_2/5 min	3.97 ± 2.15^{b}	31.12 ± 6.41^{a}	33.74 ± 4.66^{bc}
Air/-25°C/15 days	3.51 ± 1.43^{b}	31.83 ± 2.83^{a}	42.73 ± 10.05^{ad}
Air/-25°C/1 month	0.00 ± 0.00^{a}	30.9 ± 3.86^{a}	36.53 ± 9.15^{abc}
Saline/-25°C/15 days	11.93 ± 4.43^{d}	33.29 ± 5.95^{a}	36.49 ± 4.80^{abc}
Saline/-25°C/1 month	5.55 ± 1.96^{bc}	28.45 ± 6.30^{cd}	30.73 ± 4.06^{b}
Vacuum/-25°C/15 days	3.98 ± 1.98^{b}	29.97 ± 4.38^{c}	32.90 ± 6.77^{bc}
Vacuum/-25°C/1 month	4.07 ± 2.12^{b}	32.11 ± 5.99^{a}	34.57 ± 4.00^{abc}
Decapsulation	6.88 ± 5.80^{c}	24.60 ± 9.29^{bd}	44.81 ± 9.45^{de}

Values in a column sharing the same letter are not significantly different (Post Hoc LSD, $p<0.05$).

Table 2. Mean values and standard deviation of hatching rate of *Artemia* harvested from Çamaltı Salterns on March 2001 (n = 18 for each treatment).

	Hatching rate (%)		
Treatment	*24 hour*	*48 hour*	*72 hour*
Control	19.20 ± 4.25^{a}	62.24 ± 6.38^{cd}	68.49 ± 9.60^{a}
2% H_2O_2/5 min	30.80 ± 5.97^{cd}	63.17 ± 11.2^{ce}	70.50 ± 4.94^{a}
5% H_2O_2/5 min	28.30 ± 5.88^{cd}	48.30 ± 4.09^{a}	49.44 ± 7.65^{b}
Air/-25°C/15 days	33.40 ± 6.68^{d}	55.90 ± 5.34^{ac}	60.20 ± 7.08^{c}
Air/-25°C/1 month	26.50 ± 5.97^{bc}	47.71 ± 7.92^{ab}	51.20 ± 6.27^{b}
Saline/-25°C/15 days	38.50 ± 7.25^{ef}	58.76 ± 6.21^{c}	60.89 ± 7.52^{c}
Saline/-25°C/1 month	35.00 ± 5.30^{e}	60.91 ± 7.87^{c}	61.60 ± 4.39^{c}
Vacuum/-25°C/15 days	24.60 ± 5.42^{b}	55.17 ± 7.19^{ac}	70.50 ± 6.72^{a}
Vacuum/-25°C/1 month	30.10 ± 5.90^{cd}	60.35 ± 9.81^{cde}	60.45 ± 6.50^{c}

Values in a column sharing the same letter are not significantly different (Post Hoc LSD, $p<0.05$).

Discussion

According to Morris (1971), long storage of *Artemia* cysts leads to a lower hatching rate because of the effect of dehydration on embryo development. Further, the presence of oxygen causes formation of highly detrimental free radicals and a loss of energy content which also lower the hatching rate (Crowe, 1971). In the present study, the hatching rate in the March samples was significantly higher than in the November samples. This is probably related to the lengthy storage time, inappropriate storage conditions and/or the diapause phenomenon. The November samples were harvested approximately six months before the test in May. Alpbaz et al. (1993) obtained a hatching rate of 70% of Çamaltı *Artemia* cysts immediately after harvest and the rate reached its maximum after a 25-hour incubation period. They also suggested that inappropriate processing, a long storage period and improper storage conditions caused a serious decrease in the hatchability as cysts were cleaned with tap water, kept in non-standard conditions during the desiccation process and stored in a non-vacuum plastic bag during a lengthy storage period which exposed them to moisture. Koru and Cirik (2001) reported that Çamaltı *Artemia* cysts remained in diapause during December, January and February, when the temperature was below 5ºC. Therefore, in addition to less-than-ideal storage conditions and duration, probably diapause in the cysts led to lower hatchability in our November samples.

Mathias (1937) reported a positive effect of hydrogen peroxide on *Artemia* cysts. Later, this phenomenon was confirmed (Bogatova and Shmakova, 1980; Bogatova and Erofeeva,

1985). The effect of the hydrogen peroxide treatment on hatching in Çamaltı cysts differed with the concentration. During both peroxide treatments, significant stimulation was observed, resulting in significantly better hatching than in the untreated control at 24 h. But final results in the 2%/5 min treatment did not differ from the control group. Although H_2O_2 treatment in the 5%/5 min caused faster response during the first 24 h, the hatching rate at 48 and 72 h evidently decreased compared to the control. This is contrary to the results of the 5%/5 min treatment of Van Stappen et al. (1998). In fact, this concentration of peroxide is generally considered lethal for any animal cell (Clegg et al., 1996). In conclusion, our experiments showed that the peroxide application led to significantly increased hatching within the first 24 h but, after that, peroxide treatment resulted in either no significant gain or a negative effect on cyst hatching.

Bruggeman et al. (1980) and Vanhaecke and Sorgeloos (1983) indicated that the use of decapsulated cysts caused a significant increase in hatchability of *Artemia* cysts. In the present study, decapsulated samples indeed showed higher hatching results after 72 h, compared to the control ($p<0.05$).

Several authors revealed substantial evidence on the effect of cold treatments on diapause deactivation of brine shrimp cysts. Van der Haegen (1981) detected a hatching increase of 10% when dehydrated cysts were frozen at -20ºC. Lavens et al. (1986) used laboratory-produced Great Salt Lake cysts to prove that the effect on hibernation can be quantified, e.g., eight weeks storage at -25ºC can improve hatchability. Sorgeloos (1979) reported that maximum hatchability of Lavalduc (France) *Artemia* was obtained after two months storage at about -30ºC. The present study revealed that Çamaltı cyst samples stored at -25ºC hatched significantly better than the control within 24 h, except in the air/1 month treatment of the November samples. In spite of the faster hatching response, one month storage caused a slight decrease in the hatching rate at 48 and 72 h.

As suggested by Smith et al. (1978), the hatching rate can vary widely. On the other hand, peroxide and other treatments applied on different strains or batches of the same strain did not result in the same degree of diapause deactivation. Differences in tolerance and responsiveness to a treatment may be genetic (strain-specific) or due to environmental factors (Van Stappen et al., 1998). The hatching rate is also sensitive to varying factors prior to and during cyst harvesting, processing and storage (Sorgeloos et al., 1976). According to these, long storage periods decrease the viability of embryos. In the present study, all treatments for diapause deactivation caused a hatching gain during the first 24 h, suggesting that these applications can shorten the hatching delay. Nevertheless, final results (after 72 h) were generally similar or slightly lower than in the control. Thus, the hatching output of Çamaltı *Artemia* cysts might be strain-specific. But the results from the November group revealed that other factors such as storage conditions or time could also significantly affect the hatching percentage.

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