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Evolutionary and ecological aspects of crustacean diapause

Effects of hydrogen peroxide treatment in *Artemia* cysts of different geographical origin

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With 6 figures and 1 table

Abstract: Short-term treatment with peroxide solutions has been tested as a chemical way to break the diapause of *Artemia* cysts of different species and geographical strains. The methodology was further standardized by analyzing the impact of the cyst hydration level and the application conditions (concentration, duration, temperature) on the hatching percentage. The peroxide treatment was most effective in deactivating diapause when applied on fully hydrated cysts, and showed a quantitative nature: a maximal positive effect was obtained for specific combinations of peroxide concentration and exposure time. The peroxide effect was preserved after processing and storage of treated cysts. The biochemical mechanisms possibly involved in induction and termination of diapause are discussed.

Introduction

The worldwide distribution of the brine shrimp *Artemia* in isolated habitats (about 500 natural salt lakes and man-made salterns) with specific ecological conditions has resulted in numerous geographical strains, and genetically different populations. Among these strains a high degree of genetic variability as well as a unique diversity in various quantitative characteristics have been observed (BROWNE et al. 1991). Some of these are phenotypical (e.g. the nutritional composition of the cysts; LÉGER et al. 1986), and change from batch to batch. Others (e.g. cyst diameter, resistance to high temperature) are considered strain-specific and remain relatively constant (VANHAECKE & SORGELOOS 1980).

The phenomenon of diapause in *Artemia* cysts has been the subject of several studies and reviews (CLEGG & CONTE 1980, DRINKWATER & CROWE 1987, LAVENS & SORGELOOS 1987, DRINKWATER & CLEGG 1991). The study of the mechanisms of diapause induction and deactivation can help to maximize the hatching yield of the commercially important *Artemia* cysts. The termination of diapause is a complex process, influenced by multiple and mutually interfering genotypical and environmental factors (LAVENS & SORGELOOS 1987, DRINKWATER & CLEGG 1991).

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The triggering mechanism for the induction of the diapause state is not yet known. Generally, *Artemia* embryos released as cysts in the medium are or soon become diapausing (CLEGG et al. 1996). Upon the deactivation of diapause by environmental factors, cysts enter the stage of quiescence and metabolic activity can be resumed when they are exposed to favourable conditions in terms of temperature, oxygen and light, eventually resulting in hatching (DRINKWATER & CROWE 1987). Several studies indicate that diapause or a similar state can be re-induced by exposure to anoxia (DRINKWATER & CLEGG 1991, CLEGG 1993, CLEGG & JACKSON, this volume) or by a well-dosed heat shock (ABATZOPOULOS et al. 1994).

In response to dehydration by storage in a highly saline medium or by air-drying, San Francisco Bay-type (SFB) cysts are gradually released from diapause (VERSICHELE & SORGELOOS 1980, DRINKWATER & CROWE 1987, LAVENS & SORGELOOS 1987, VU DO QUYNH et al. 1987, DRINKWATER & CLEGG 1991). Various other processes have proven successful in terminating diapause (review by LAVENS & SORGELOOS 1987). A H_2O_2-treatment, for example, was applied with variable success by several authors, but without much uniformity in methodology (MATHIAS 1937, BOGATOVA & SHMAKOVA 1980, BOGATOVA & EROFEEVA 1985). In additional studies (LAVENS et al. 1986, VU DO QUYNH et al. 1987) different strains, and various concentrations and treatment periods were used; the effect of the treatment was variable as well, but was in most cases promising. Despite the variability, a variable portion of diapausing cysts could be activated in all cases.

Our main purpose was the further standardization of the H_2O_2-technique for *Artemia* cysts of diverse geographical origin in order to reduce the variability of the hatching success. Different factors were considered: hydration level of the cysts at the moment of H_2O_2-treatment ("prehydration time"), treatment time, concentration and temperature. The hatchability of H_2O_2-treated cysts after subsequent drying and storage was also studied. By subjecting samples of different brine shrimp strains (and species) to the H_2O_2-treatment, we attempted to gain more insight in the strain-specificity of diapause deactivation. By optimizing and standardizing the technique we also hoped to contribute to a better understanding of the biochemical mechanisms involved.

Material and methods

General

Batches of *Artemia* cysts from various geographical sites were selected (Table 1) according to the following criteria: availability in the laboratory, predominance of the diapause state, and sufficient information on the production and processing conditions. The water content of the samples was analyzed according to SORGELOOS et al. (1986). The hydration level of the experimental cysts was determined according to the respective storage conditions (dried, brine, pond water: Table 1). Upon arrival in the laboratory, cysts were stored at room temperature (20–21 °C) in darkness and kept in conditions preventing further dehydration: dry samples were vacuum-packed in polyethylene bags; wet samples were stored in a closed container with the original medium. During storage, several samples exhibited an increase in hatching. Whenever a treatment was applied or the hatching of a stored sample evaluated, the hatching of the stored and untreated sample was evaluated as well.

The breaking of the diapause state was evaluated by the hatching percentage (H%), which determines the number of nauplii hatching out of 100 full cysts. Unless otherwise stated, the hatching was evaluated after 48-h incubation in hatching conditions as outlined in SORGELOOS et al. (1986).

Table 1. Overview of experimental cyst samples (water content values (%) between brackets are indicative values).

Origin	species	date of harvest	production and processing conditions	code	water content at arrival (%)	H% at arrival (no treatment) (mean ± st.dev.) 48 h incubation
Kazakhstan	*A. sp.*	1986	natural production: cysts processed and sun dried	KAZ	5.2	0.3 ± 0.5
Vung Tau, Vietnam	*A. franciscana* (GSL strain)	1986	pond production of inoculated GSL strain			
1		March	105 ppt; processed and sun dried	VT1	11.1	4.6 ± 2.2
2		May-July	? ppt, processed and sun dried	VT2	(5-15)	49.5 ± 8.5
Piura, Peru	*A. franciscana* (local strain)	1986	pond production			
1			120 ppt; unprocessed; stored in brine	P1	(15-20)	15.0 ± 4.1
2.			50 ppt; unprocessed; stored in brine	P2	(15-20)	20.1 ± 5.3
San Francisco Bay, CA, USA	*A. franciscana* (SFB strain)	April-May '87	natural production, unprocessed, stored in pond water	SFB	(50-70)	75.9 ± 5.5
Lake Urmiah, Iran	*A. urmiana*	winter '94/'95	Several samples, stored in pond water (170-180 ppt)	URM	60.2-68.5	17.3 ± 2.7 - 33.4 ± 3.4

The stock H_2O_2-solution (Union Chimique Belge NV/SA, Brussels, Belgium, 6171; stabilized by the manufacturer by means of phosphate additives) had a constant concentration of 27% w/w. The experimental concentrations (w/w basis) were prepared by dilution with deionized water, immediately before application. By titration with $KMnO_4$ (Anonymous 1969) it was confirmed that the concentration of the H_2O_2-dilution was not affected by the experimental conditions (illumination, aeration) and remained constant during cyst treatment. The experimental dilutions were discarded after single use.

Before treatment with peroxide, the cyst samples were cleaned according to the method described in SORGELOOS et al. (1978). The cysts were rinsed with tap water before treatment in a 100-ml glass cone filled with the H_2O_2-test solution, in conditions identical to standard hatching conditions (SORGELOOS et al. 1986). After a specific treatment time, the cysts were collected on a sieve, rinsed thoroughly with tap water in order to remove traces of H_2O_2, and incubated in seawater under standard hatching conditions. This basic methodology was standardized in a series of tests varying crucial experimental conditions.

Hydration level of cysts and temperature of H_2O_2-solution

In a first set of tests we studied the influence of the hydration level of cyst samples on the effectiveness of the H_2O_2-treatment. The SFB, P1 and P2 samples were subjected to different hydration periods in tap water prior to the treatment with a 5%- H_2O_2-solution for 5 min. These treatment conditions were based on preliminary results and on literature data. The untreated, raw sample was included as control. Based on the results of our trials a "prehydration" period of 2 h in tap water was chosen as a standard procedure for further experiments.

The effect of temperature of the H_2O_2-solution (5%, 5 min.), in combination with different prehydration times, was tested with the VT1 and VT2 samples. Treatment temperatures varied in the range 4–25°C. The untreated, raw sample was included as control. Based on the results of these tests, in further experiments the H_2O_2-treatment was always performed at room temperature (20–21°C).

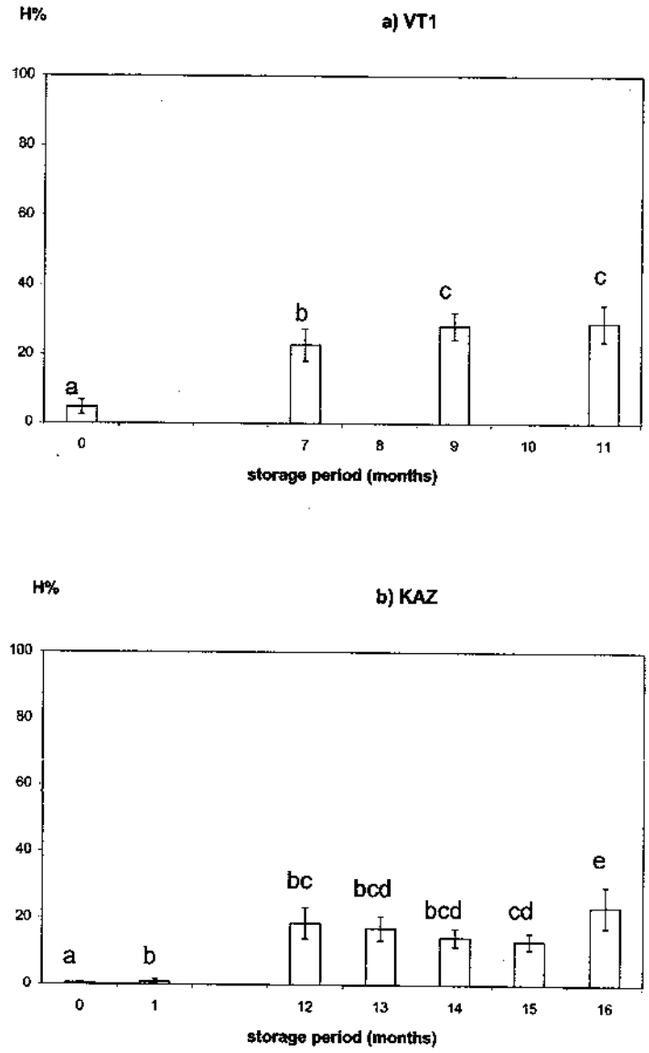

Fig. 1. Hatching percentage of experimental cyst samples during long-term dry and vacuum storage at room temperature (bars sharing the same letter(s) are not statistically different; Duncan, $p < 0.05$).

Treatment time and H_2O_2-concentration

Thirty subsamples of the KAZ sample were subjected to different H_2O_2-exposure treatments: cysts were incubated for 1, 5, 10, 15, 20 or 30 min. to H_2O_2-solutions of 1, 5, 10, 20 or 27%. With these 6 x 5 separate treatments we tried to assess the range of maximal responsiveness of the KAZ cysts to peroxide as a diapause terminating agent. The untreated, raw sample was included as a control. In a separate test

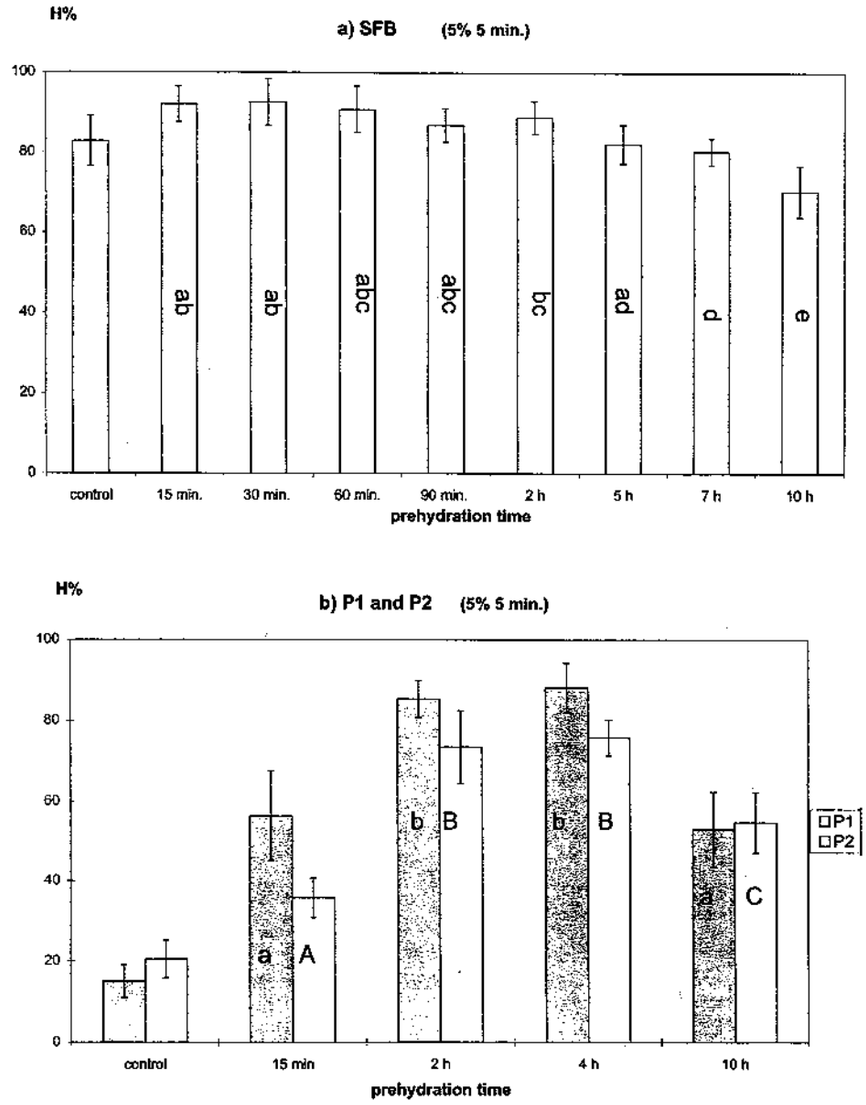

Fig. 2. Effect of prehydration time on diapause deactivation of experimental cyst samples, treated with H_2O_2 (for each separate sample, bars sharing the same letter(s) are not significantly different; Duncan, $p < 0.05$).

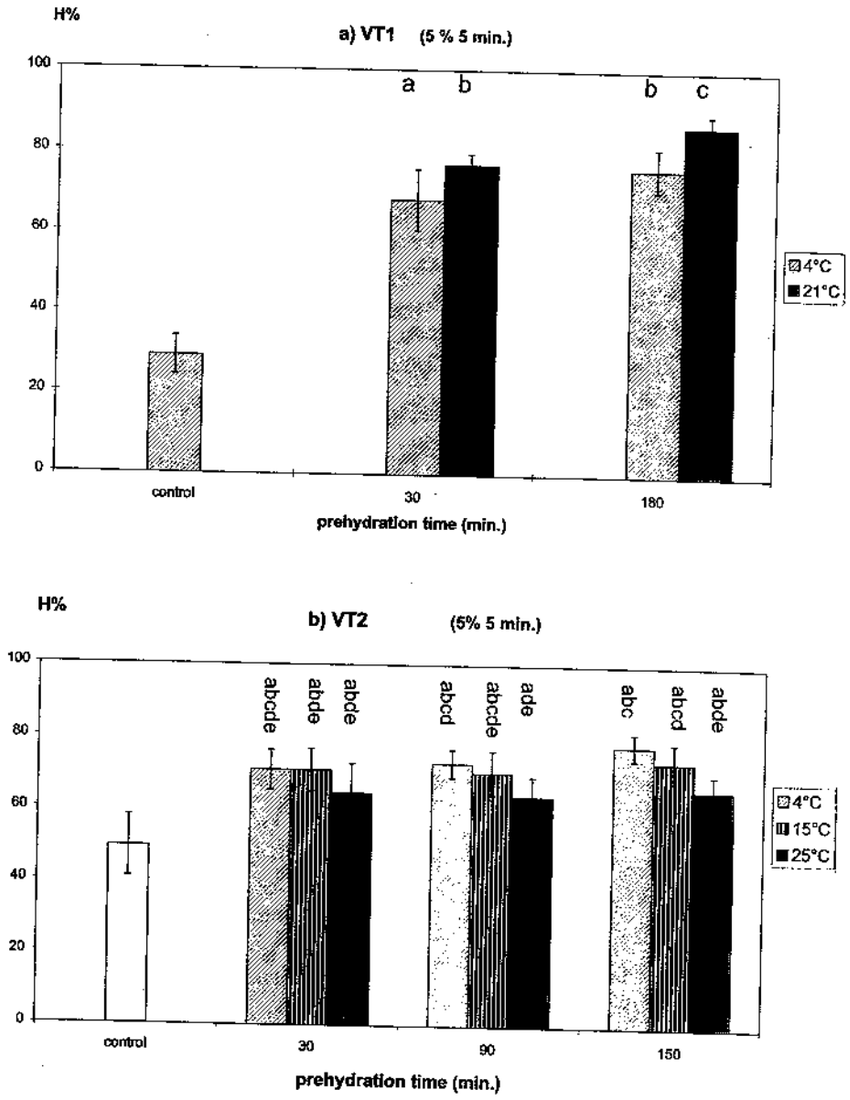

Fig. 3. Effect of prehydration time and H_2O_2-treatment temperature on diapause deactivation of experimental cyst samples, treated with H_2O_2 (bars sharing the same letter(s) are not statistically different; Duncan, $p < 0.05$).

aiming to maximize the hatching of four different URM samples, each sample was subjected to two different H_2O_2-treatments (3%-3 min. and 5%-5 min.; these conditions were based on preliminary tests that showed maximal sensitivity of *A. urmiana* within this exposure range). The untreated, raw sample was included as control and the H% was evaluated both after 24 h and 48 h hatching incubation.

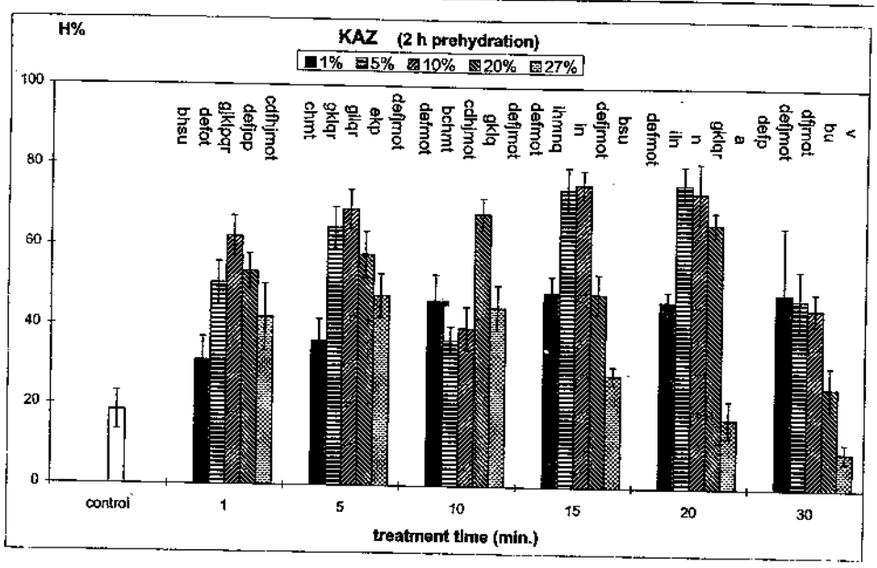

Fig. 4. Effect of treatment time and concentration on diapause deactivation of experimental cyst sample, treated with H_2O_2 (bars sharing the same letter(s) are not significantly different; Duncan, $p < 0.05$).

Long-term persistence of the peroxide effect

To test for the long-term preservation of the activating peroxide effect, VT1 cysts were first activated with H_2O_2 (5%–5 min.) and subsequently used in a storage experiment with varying dehydration levels and storage temperatures. The H_2O_2-treated sample was subdivided into fractions, these fractions were either dehydrated in saturated NaCl brine, or layer dried in an oven at 35 °C (resulting in 15–20 % and 3–5 % water content, respectively; SORGELOOS et al. 1986). The dried subsamples were subdivided again into two fractions: one stored in vacuum, the other in air. Each dehydrated sample (brine, dried + vacuum, dried + air) was again subdivided into 2 fractions: one stored at –25 °C, the other at +20 °C. The H% was evaluated after one, three and six months of storage. These values were compared to the hatching of the raw sample (no peroxide treatment) before storage and after one and three months of storage in 'aging' conditions (room temperature, darkness, prevention of dehydration), and to the hatching of the initial H_2O_2-activated sample, before dehydration and storage.

Statistics

Most hatching results were analyzed statistically with a one-way analysis of variance (ANOVA) to find an overall effect of the treatment. A two-way analysis was applied where combinations of treatments had been tested. Duncan's multiple range test was used to detect significant differences between the experimental group means at a significance level of $p < 0.05$. In some experiments the control sample could not properly be included in the ANOVA; in these cases the treated samples were pairwise compared to the control sample in a t-test ($p < 0.05$). Prior to analysis, the data were checked for homoscedasticity and normality using Barlett's test and the Kolmogorov-Smirnov test, respectively. Departure from the assumptions of analysis of variance was rectified by arcsin transformation of the original data (SNEDECOR & COCHRAN 1967, SOKAL & ROHLF 1969).

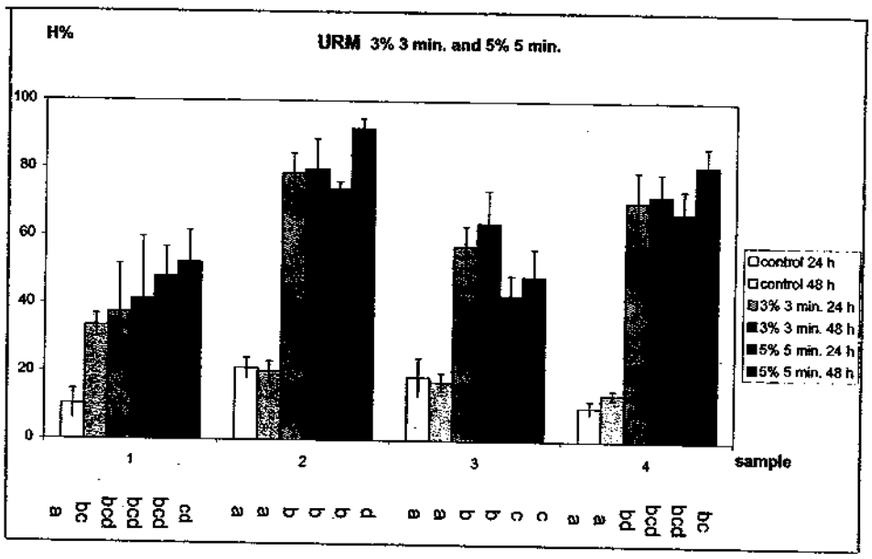

Fig. 5. Effect of two different H_2O_2 -treatments on hatching rate of different experimental batches of *Artemia urmiana* cysts (for each separate sample, bars sharing the same letter(s) are not significantly different; Duncan, $p < 0.05$).

Results

Aging

Fig. 1 shows the H% of the VT1 (Fig. 1a) and KAZ (Fig. 1b) samples, stored dry and vacuum packed, at room temperature and for variable durations. There was an overall significant effect of storage duration on H%, both in the VT1 (ANOVA, $F = 75.30$, $p < 0.05$) and the KAZ samples (ANOVA, $F = 114.26$, $p < 0.05$). Initial values for both samples were very low ($4.6 \pm 2.2\%$ and $0.3 \pm 0.5\%$, respectively) but increased significantly ($p < 0.05$) after prolonged storage ($22.8 \pm 4.7\%$ after 7 months in VT1, $18.4 \pm 4.6\%$ after 1 year in KAZ), showing a gradual release from diapause. During the following months of storage, a further gradual but significant release from diapause ($p < 0.05$) was observed in VT1 cysts ($29.0 \pm 5.3\%$ after 11 months), while for the KAZ sample the pattern was more variable.

Hydration level of cysts and temperature of H_2O_2-solution

Fig. 2 presents the hatching results of SFB (Fig. 2a), P1 and P2 (Fig. 2b) cysts, treated with 5%-5 min. H_2O_2 after different prehydration times. For SFB cysts, analysis of the peroxide treated samples showed a significant effect of the treatment (ANOVA, $F = 23.27$, $p < 0.05$). Pairwise comparison with the untreated control showed significant differences for the samples, prehydrated for 90 min., 5, 7 and 10 h (t-test, $p < 0.05$). The SFB cysts (stored in pond water, 50-70% water content) showed maximal hatching ($92.7 \pm 5.8\%$) after 30 min.

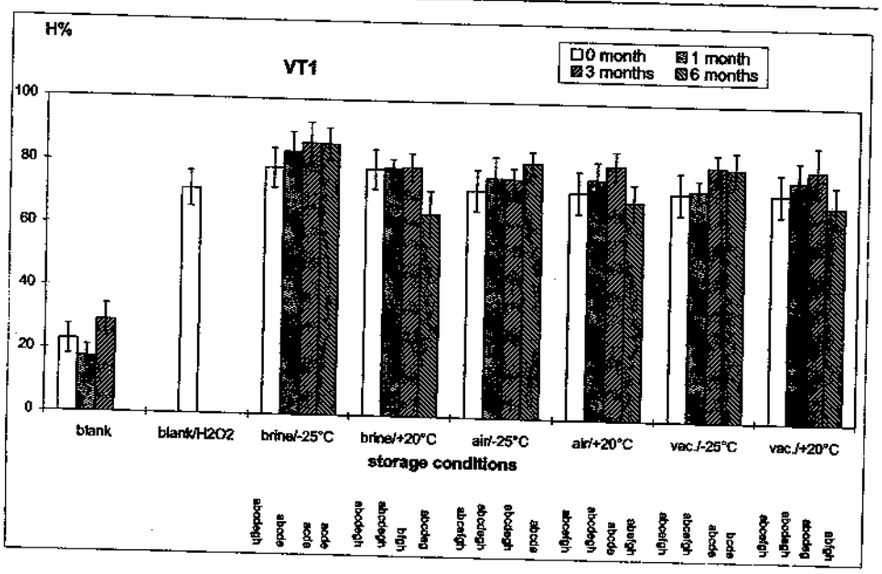

Fig. 6. Effect of storage in different conditions on viability of 5%-5 min. H_2O_2-treated Vietnam 1 cysts (bars sharing the same letter(s) are not significantly different; Duncan, $p < 0.05$).

prehydration. With longer prehydration durations a gradual decrease in hatching occurred, with a significant reduction of the hatching percentage (70.5 ± 6.4%) between 7 and 10 h prehydration.

A significant overall effect of the treatment was also found for the P1 and P2 cysts (stored in saturated brine, 15–20% water content, Fig. 2a) (ANOVA, F = 52.98 and 73.96 for P1 and P2, respectively, $p < 0.05$). Pairwise comparison showed a significant difference between any treated sample and the respective raw, untreated sample (t-test, $p < 0.05$). Maximal hatching occurred after 4 h of prehydration: 88.2 ± 6.1% in P1 and 75.8 ± 4.4% in P2. The significant negative effect of extended prehydration periods (up to 10 h) was confirmed for both P1 and P2.

Hatching in VT1 and VT2 cysts (dried, water content ±10%, tested in combination with different H_2O_2-treatment temperatures; Fig. 3a and 3b, respectively) revealed a significant overall effect when treated with 5%-5 min. H_2O_2 (VT1: two way-ANOVA, F = 30.11 and F = 26.00 for effect of temperature and prehydration time, respectively; $p < 0.05$; VT2: two way-ANOVA, F = 22.18 and F = 4.32, idem, $p < 0.05$). No significant interaction was detected between treatment temperature and prehydration time. All treatments of VT1 and VT2 resulted in significantly better hatching than the respective controls (raw untreated sample; VT1: 29.0 ± 4.8%; VT2: 49.5 ± 8.5%) (t-test, $p < 0.05$). For the VT1 sample, hatching percentage after 3 h prehydration was significantly ($p < 0.05$) higher than after 30 min for both treatment temperatures tested; they equally showed a significantly higher H% ($p < 0.05$) for H_2O_2-application at 21°C, compared to the 4°C treatment.

Treatment time and H_2O_2-concentration

The effect of the H_2O_2-treatment on hatching in KAZ cysts was influenced by the concentration applied and by the treatment period (Fig. 4). Two-way ANOVA of the KAZ results showed a significant overall effect of each variable ($F = 328.65$ and 107.92 for effect of concentration and incubation time, respectively, $p < 0.05$), and a significant interaction between both ($F = 70.50$, $p < 0.05$). Nearly all treatment combinations resulted in significantly better hatching than in the untreated raw sample (18.4 ± 4.9%, t-test, $p < 0.05$), except for the 27%-30 min. and the 27%-20 min. treatments (significantly lower and no difference, respectively). A maximal hatching (76.1 ±4.6%) was obtained after a 5%-20 min. treatment. Overall, the lowest hatching took place when both variables were either at the lower or higher side of the ranges tested.

Fig. 5 shows the effect of two H_2O_2-treatments (3%-3 min. and 5%-5 min.) on the H% of four different samples of *A. urmiana*, after 24 and 48 h of hatching incubation. Hatching percentages of the non-treated samples ranged between 10.0–22.0% after 24 h of hatching incubation. There was a significant effect of the peroxide treatment for the four samples (2-way ANOVA, $F = 15.53$, 535.44, 193.70 and 74.54, respectively, $p < 0.05$), and a significant effect of the hatching incubation time for samples 1 and 2 ($F = 4.90$ and 10.20, respectively, $p < 0.05$). A significant interaction between treatment and hatching incubation time was found for sample 2 ($F = 15.62$, $p < 0.05$). For samples 2 and 4, the 5%-5 min. treatment resulted in a further significant ($p < 0.05$) hatching increase, when incubated for 48 h.

Long-term persistence of the peroxide effect

Peroxide treatment resulted in a significantly higher hatching (t-test; $p < 0.05$; 71.5 ± 5.6%) than in the control (22.8 ± 4.7%) (Fig. 6). There was a significant effect both of storage conditions and of storage time, and a significant interaction between both (two way-ANOVA, $F = 13.63$, 13.48 and 6.00, respectively; $p < 0.05$). All treated and stored samples hatched significantly better (t-test, $p < 0.05$) than the untreated, raw sample before storage. Except for the sample stored for six months in brine at 20°C, all treated and stored samples hatched as well or significantly better (t-test, $p < 0.05$) than the initial H_2O_2-activated sample, before dehydration and storage.

Discussion

Application of a H_2O_2-treatment lead to activation of a fraction of the diapausing cysts in the experimental conditions; in some tests the increase in H% was considerable (Fig. 2b: 2 h prehydration time; Fig. 5: sample 2). Strains of different geographical origin and of different *Artemia* species seemed at least to some extent susceptible. The effect was dependent on certain parameters like hydration level of the cysts, H_2O_2-concentration and treatment time. In case of long-term storage (months) of raw cysts, 'aging' had a positive influence on the hatching as well.

Aging

Cysts of Vietnam (VT1) and Kazakhstan (KAZ) populations stored for variable duration under laboratory conditions showed a gradual release from diapause without any particular treatment (Fig. 1). This gradual release from diapause for brine shrimp cysts by rest has been observed by other authors as well (LAVENS & SORGELOOS 1987, VU DO QUYNH et al. 1987). In analogy to 'hibernation' it is called 'aestivation'. Strictly spoken, compared with the conditions in the maternal brood pouch (17 ppt, according to G. CRIEL, pers. comm.), any condition of storage can be considered as a 'treatment', and a real 'blank' sample does not exist. Due to the negative effect of long-term storage on hatching of quiescent cysts, the net effect of rest is largely unpredictable. It is therefore required to constantly re-evaluate the hatching of the control.

Hydration level of cysts

Our results suggest that at intermediate hydration levels of the resting eggs, the positive effect of a certain H_2O_2-treatment may be limited, and that its maximal effect is obtained at full hydration of the cysts (after 2 h in tap water) at an early stage of development (Figs 2, 3). This is in contradiction with the results of BOGATOVA & SHMAKOVA (1980), who recommended a low water content for a maximal H_2O_2-effect. It is difficult to establish an accurate link between cyst water content, phase of pre-emergence development, and H_2O_2-effect, since it is uncertain what embryonic developmental stage is maximally susceptible. Hydration of the very hygroscopic *Artemia* cysts is a rapid process, and within 1–2 h full hydration of the embryo is usually achieved (LAVENS & SORGELOOS 1987). The negative effect after prolonged prehydration (>4 h) can be attributed to reduced susceptibility in function of the developmental stage, and/or to the toxicity of the chemical for the ongoing embryogenesis.

Temperature of H_2O_2-solution

We found an overall significant effect of treatment temperature on the hatching percentage in two Vietnamese *Artemia* populations. Since the action of H_2O_2 is supposed to be of a chemical nature, an effect of the treatment temperature was to be expected. At any temperature tested, H_2O_2 had a significantly positive effect, compared to the raw sample. For practical reasons H_2O_2-treatment at room temperature (20–21 °C) was chosen as a routine procedure.

Treatment time and H_2O_2-concentration

A treatment in the range 5%-5 min. had a marked positive, but possibly not maximal effect on hatching for most cyst strains and batches tested (Figs 2–5). This is in line with the procedure of 3%-15 min. of BOGATOVA & SHMAKOVA (1980). As exposure to this level of H_2O_2-concentration is generally recognized as lethal for any animal cell, one can assume that only a fraction – if any – actually enters the embryo (CLEGG et al. 1996). The embryo, protected by its cyst wall, is out of the reach of non-volatile solutes and ions (CLEGG 1986), moreover, the chemical reactions involved when exposing *Artemia* cysts to H_2O_2 are most probably manifold and complex.

When testing different combinations of H_2O_2-treatment time and concentration for KAZ cysts (Fig. 4), several combinations resulted in a maximal response. Peroxide treatments with very low (e.g. 1%-1 min.) or very high values (27%-30 min.) of either or both variables resulted in suboptimal effects, although the effects of the various combinations were often not significantly different ($p < 0.05$).

An increased hatching after a fixed hatching incubation time may not only be due to the positive effect of H_2O_2 on diapause deactivation, but also to a faster hatching response after treatment. According to D. COLE (pers. comm.), application of H_2O_2 resulted in a 'time gain' of 12 h for cyst batches hatching late. In comparison to the methodology of other authors (CLEGG 1997: up to 30 days for post-anoxic cysts), or for certain batches, a hatching incubation of 48 h is relatively short. The *A. urmiana* tests (Fig. 5) illustrate the differences that can occur in the ratio early/late hatchers, with or without H_2O_2-treatment. The control of sample 1 shows a significantly higher hatching after 48 h hatching incubation, compared to 24 h; H_2O_2-treatment of the control sample gives no significant hatching gain compared to the 48 h value of the control, suggesting that the peroxide treatment has shortened the hatching delay. The untreated samples 2, 3 and 4, on the other hand, have achieved maximal hatching after 24 h hatching incubation. Peroxide application leads to significantly increased hatching output within 24 h. Sample 2 shows a further significant hatching increase for the sample treated with 5%-5 min. H_2O_2, incubated for 48 h.

Long-term persistence of the peroxide effect

All VTI samples that were first treated with H_2O_2 and subsequently stored in different ways, hatched significantly better than the untreated raw sample (Fig. 6). Furthermore, these peroxide-treated and stored samples hatched as well or significantly better than the peroxide-treated sample before dehydration and storage (except for the brine sample, stored for six months at 20°C), suggesting that the peroxide effect persists through storage. According to BOGATOVA & SHMAKOVA (1980), the effect of the cyst activation persisted for 1.5–2 months; longer storage negatively affected cyst hatching. However, in long-term storage tests several other factors may interfere, making interpretation of the results ambiguous: positive effect of aging and diapause deactivation by hibernation, negative effect of long-term storage in presence of oxygen.

Strain or batch variability

Peroxide treatment of different strains or of different batches of a same strain did not result in the same degree of diapause deactivation (e.g. Fig. 2b, 5%-5 min. treatment after different prehydration times with P1 and P2 cysts; tests with different URM samples; Fig. 5, application of the 5%-5 min. treatment on different samples). Differences in tolerance and responsiveness to the H_2O_2-treatment may be genetic and thus strain-specific, or due to environmental factors, including processing and storage. In the course of other (unpublished) experiments, for example, some samples exhibited a shift in sensitivity for H_2O_2-treatment after prolonged storage time (few months). These observations may be related to 'aestivation', and illustrate the risks of generalization. Furthermore, the observation of J. CLEGG (pers. comm.), that, at least for cysts from Vietnam (SFB inocula), H_2O_2 becomes less effec-

tive as diapause continues after prolonged storage, offers an interesting perspective for further research. Although general recommendations can be formulated with regard to H_2O_2-concentration and exposure time, a limited screening of different combinations can lead to further optimization when new batches are tested. The *A. urmiana* samples (Fig. 5) also displayed unusual variation for other, supposedly strain-specific characteristics (e.g. biometrics), and possibly originated from mixed populations.

Mechanism of diapause induction and deactivation

Since the tests have been performed with populations of cysts (one hatching percentage accounts for 1000–1500 individual embryos), they suggest that at least at the population level the deactivation of diapause is not an 'on/off' phenomenon, but is of a quantitative nature. If a large fraction of a batch consists of dead cysts, e.g. by mortality due to previous treatments, the hatching and diapause behaviour of the remaining fraction may not be representative for the entire population.

According to VAN DER LINDEN (pers. comm.), H_2O_2 makes the induction of the hatching process by a light stimulus redundant, in the hypothesis that light quanta catalyze oxidation-reduction reactions in the embryos via captation by haem-pigments (VAN DER LINDEN et al. 1986). Alternatively, C. TROTMAN et al. (this volume) assigns a crucial role to the haem-pigments in diapause deactivation via oxidation. Since brine shrimp is capable of direct uptake of O_2 from the environment, the synthesis of these complex oxygen transporters must have a specific metabolic significance, beyond catabolization into the haematine which is in turn incorporated in the cyst wall. According to BOGATOVA & SHMAKOVA (1980), the H_2O_2 catalyzes the oxidation-reduction conversion process of trehalose into glycerol and glycogen. Trehalose is further thought to play a role in the maintenance of the integrity of membranes, and in the stabilization of proteins (CLEGG et al. 1996). It is suggested by J. CLEGG (pers. comm.) that the H_2O_2 molecule might contribute to the increase of the embryo's internal pH by the formation of hydroxylenes. This is in line with the observations of BUSA & CROWE (1983) that NH_4Cl acts as a very efficient tool to break diapause in *Artemia*. It is not clear whether the chemical is consumed during its application, as titration with $KMnO_4$ did not show any detectable decrease in its concentration. It might, however, be used at a rate below the accuracy level of the titration method.

CLEGG et al. (1996) utilize the concept of diapause 'depth', which, at least for SFB cysts, appears to increase as the duration of diapause continues, as illustrated by a delayed resumption of respiration rate after treatment of diapausing cysts with H_2O_2. It is likely that the 'shifts' in sensitivity, or differences between batches, to diapause deactivators such as H_2O_2 may also be related to some extent to the duration of the diapause state in our samples. DRINKWATER & CLEGG (1991) mentioned the influence of environmental conditions during embryo formation on depth of developmental arrest and ease of activation, emphasizing the need to know the antecedents of samples studied for diapause regulation, in order to understand intra- and inter-population differences.

Conclusions

In contrast to environmental factors such as hibernation and dehydration, chemical induction by H_2O_2 has no direct ecological relevance. Nevertheless, of all diapause deactivating factors currently applied, it is the most 'universal': it has at least some effect on most strains and batches of brine shrimp cysts. It is therefore currently used in the laboratory, where the potential hatchability of an unknown cyst batch can be assessed via a fast and inexpensive method. The general applicability of this technique suggests that it interferes directly or indirectly with the basic mechanisms of diapause itself. Nevertheless, the effect of the treatment depends on a number of application factors, such as hydration level of the cysts, treatment temperature, treatment time and concentration. Furthermore, the effect is not entirely predictable: strain and batch differences occur, and more research is needed to relate these to genotypical or phenotypical factors. The H_2O_2-effect is preserved after dehydration and subsequent storage of the activated cyst batches, used in our experiments.

Our results confirm that diapause deactivation can not be considered an "on/off" phenomenon, but has a quantitative nature at the population level. Since it is currently not feasible to study the state of diapause in individuals, all information involves populations of embryos, which can be considered a 'continuum' of metabolic states (DRINKWATER & CLEGG 1991), including individual differences in 'sensitivity' and 'threshold levels'.

The standard H_2O_2-treatment procedure, as worked out in our laboratory, includes a 2 h-prehydration phase in tap water, followed by treatment with the H_2O_2-solution at room temperature in standard hatching conditions (SORGELOOS et al. 1986). For identification of an optimal response from unknown batches, a screening of several treatments, combining concentrations in the range 1-10% and treatment times in the range 1–10 min. is performed. If the positive effect on the hatching percentage is below expectation, other combinations beyond these ranges are tested. If a decrease in hatching is observed, supposedly as a consequence of toxicity, lower concentrations and/or shorter treatment times are applied.

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