International Study on Artemia*. XLI. Influence of culture conditions and specific diapause deactivation methods on the hatchability of Artemia cysts produced in a standard culture system

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ABSTRACT: Using cyst material produced in an indoor cyst production system from 4 Artemia strains belonging to the species A. franciscana (San Francisco Bay, California, USA), A. maru (Macau, China), A. salina (Great Salt Lake, Utah, USA) and A. parthenogenetica (Lavandou, France), different factors were studied that affect dormancy in brine shrimp cysts. Preliminary experiments under varyingiotic culture conditions revealed an interaction between environmental conditions and hatching characteristics. Results obtained under better-defined conditions demonstrated a significant positive correlation between the amount of food available to the adult Artemia populations and the hatchability of their encysted offspring. Specific diapause deactivation treatments, e.g. hibernation of the cysts at -2°C or soaking in a 3% H₂O₂ solution, performed on one batch of Great Salt Lake cysts produced under well-defined optimal conditions, resulted in drastic increases in the hatching percentage of these cysts. Results provide indications that the variation in cyst diapause deactivation may be relevant for the stability of brine shrimp populations in their specific biotypes, e.g. inland lakes versus coastal saltworks. Possible hypotheses for differential adaptation to dormancy terminations factors are discussed.

INTRODUCTION

One of the fascinating ecological adaptation mechanisms of brine shrimp Artemia** is their ability to assure survival of the species in unstable environments by the production of dormant offspring. These encapsulated embryos can resist extreme environmental conditions, e.g. of temperature, salinity, desiccation. They remain in a state of diapause, i.e. complete arrest of metabolism, initiated by internal factors before the environment turns unfavorable, until a deactivation process allows the embryo to resume its metabolism and further embryonic development when submitted to optimal hatching conditions. The exact biochemical mechanisms involved in this diapause process are not yet fully understood (Clegg & Conte 1980).

Although dehydration is probably one of the most effective treatments in terminating dormancy, it appears from the literature on this subject (see summary in Table 1; see review by Lavens & Sorgeloos 1986a) that several other treatments can also be effective, e.g. pre-incubation at low temperatures or desiccation have been reported to initiate quiescence in resting eggs from various crustaceans and rotifers. However, the literature on diapause inhibition is often very confusing and even contradictory. This is mainly because the experimental cyst material was not standardized. i.e. details about its origin were not known.

* International Interdisciplinary Study on Artemia strains, coordinated by the Artemia Reference Center, State University of Ghent, Belgium
** Species definition in the genus Artemia has become unclear, especially in view of the important genetic differences between parthenogenetic strains of brine shrimp (Abreu-Grobois & Beadmore 1980, Abreu-Grobois 1986). It was confirmed at the First International Symposium on the Brine Shrimp Artemia that, unless the exact sibling species can be identified, and until specimens in brine shrimp is better understood, only the genus designation Artemia should be used (Persoon et al. 1980)
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Table 1. Effect of different diasparse inhibition methods on testing eggs of different crustacean and rotifer taxa (compiled from Lavens & Sorgeloos 1986a). Presence of a red indicates a diasparse terminating effect for the technique applied.

<table>
<thead>
<tr>
<th>Diasparse deactivation technique</th>
<th>Artemia (Artemia spp.)</th>
<th>Arionulina (Arionula monogona)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehydration</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Rapid dehydration/</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Hydration cycles</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Precipitation at</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Low temperatures</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Illumination</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>U.V. irradiation</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Cytotoxic radiation</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Magnetic fields</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Osmotic shock</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Organic solvents</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Peroxide treatment</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Decapulation</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Manipulation of internal pH</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Anaerobiosis</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Water depth</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Freshwater</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

such as time of deposition of the dormant gastrulae, environmental conditions under which they were produced, and possible exposures to dehydration-hydration cycles. As a result, the study material used could already have been deactivated, thus remaining in a state of quiescence with an environmental, and no longer an endogenous, control of their metabolism and development. In this way possible effects of specific diasparse deactivation techniques could have been masked.

For this study standard cyst material has been produced in a cyst production system that allows control of the abiotic and biotic culture conditions. Moreover, the offspring can be harvested within 24 h after deposition. Different culture conditions and diasparse inhibition techniques have been tested for their influence on the hatchability of the cysts.

MATERIALS AND METHODS

Details on the geographical origin and the batch identification of the Artemia strains used in this study as inoculum for the standard cyst production trials are given in Table 2. Details on the controlled cyst production trial and on the culture conditions are outlined in Lavens & Sorgeloos (1984). The following parameters were kept constant unless otherwise indicated.

Animal density: approximately 5,000; Artemia adults 1 g; total number of individuals used per experiment was 250,000.

Artificial seawater (Lavens et al. 1985); 25°C; 90 ppm.

Food, in the first set of experiments different monospecies or mixed diets were used, consisting of spray-dried Spirulina (SPR), a byproduct of rice starch extraction (ARP), and micronised waste products from agricultural crop e.g. rice bran (RB), corn (YMOO and UNCA). A mixture of RB and YMOO was used in the other culture tests.

Artemia strain: only 2 strains (Lavalud, Macau, and Great Salt Lake) were used in the first experiment; Lavalud and San Francisco Bay material was tested, for evaluating the influence of food quantity given to the parental animals on the hatching characteristics of the produced cysts, in the third experiment only Great Salt Lake Artemia was used.

Upon harvest the cysts produced were stored in brine and eventually processed following the procedure of Sorgeloos et al. (1985). Repeated dehydration-hydration cycles were carried out by interrupting the normal hatching process after 3 h incubation followed by transfer of the cysts for 24 h to saturated brine. The

<table>
<thead>
<tr>
<th>Source of cysts</th>
<th>Abreviation</th>
<th>Batch number or year of harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. franciscana</td>
<td>SFB Magee</td>
<td>No. 2596</td>
</tr>
<tr>
<td>Great Salt Lake</td>
<td>GSL</td>
<td>No. 185.6</td>
</tr>
<tr>
<td>Macau, Brasil</td>
<td>MAC</td>
<td>1979</td>
</tr>
<tr>
<td>A. parthenogenetica</td>
<td>LVD</td>
<td>1979</td>
</tr>
</tbody>
</table>

Table 2. Artemia strains and batches used as inoculum for the lab culture experiments.
peroxide treatment consisted of soaking the processed,
dehydrated cysts in a 3% HCl solution in the same way as for standard hatching (Vanhaecke & Sorgeloos
1982). After the required period, cysts were collected
on a sieve, rinsed with tap water, and incubated again
in seawater.

Hatching percentages were determined according to
the procedure outlined in Bruggeman et al. (1989). The
tests of different diapause deactivation techniques on
the hatchability were designed to enable statistical
interpretation by means of a 1-way analysis of var-
iance. Model I (Fosk et al. 1969). The hatching data
were normalized through an arc sin percent transfor-
mation. The test of Duncan was used to calculate
significant differences.

**RESULTS**

The first batches of cysts, originating from 3 Artemia
strains and produced in a controlled cyst production
system, revealed very important differences in hatch-
ing percentages (see data in Table 3). Although
analogous culture conditions were applied, hatchability
of Lavalduc offspring varied significantly from 5 to
21%; an extra 10% increase was noted when
Spirulina was added to the diet; a similar effect was
observed with Macau Artemia for which hatching per-
centages even doubled (from 35 to 71%). These results
indicate that the culture conditions under which the
cysts were produced were not yet fully standardized as
was previously accepted, and thus that other, neg-
ligated parameters were still interfering. This is further
confirmed by the varying hatching results (1 to 46%)
of the cysts from Great Salt Lake, which were produced
under varying culture conditions (food quality and
salinity).

In an attempt to improve control of the culture para-
eters, more attention was paid to the feeding condi-
tions in the second set of experiments. The results of
these tests revealed an obvious interaction between
the amount of food available to the parental cultures
and the hatching characteristics of the cysts produced
(see hatching results for a parthenogenetic and a
zygogenetic strain in Fig. 1). Cyst hatchability
increased significantly (p<0.05) when the adults
received more food, i.e. optimal levels versus sub-
optimal food quantities. The effect was even more

<table>
<thead>
<tr>
<th>Strain</th>
<th>Culture conditions</th>
<th>Hatching percentage</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVD</td>
<td>50 ppt 10,000 ind l⁻¹ RB</td>
<td>5.0</td>
<td>2.6</td>
</tr>
<tr>
<td>LVD</td>
<td>50 ppt 10,000 ind l⁻¹ RB</td>
<td>20.8</td>
<td>1.9</td>
</tr>
<tr>
<td>LVD</td>
<td>50 ppt 10,000 ind l⁻¹ RB</td>
<td>31.0</td>
<td>14.0</td>
</tr>
<tr>
<td>MAC</td>
<td>90 ppt 3,000 ind l⁻¹ UNICA</td>
<td>35.1</td>
<td>4.5</td>
</tr>
<tr>
<td>MAC</td>
<td>90 ppt 3,000 ind l⁻¹ UNICA/SPIR</td>
<td>71.1</td>
<td>7.9</td>
</tr>
<tr>
<td>GSL</td>
<td>50 ppt 10,000 ind l⁻¹ RB</td>
<td>3.9</td>
<td>3.8</td>
</tr>
<tr>
<td>GSL</td>
<td>50 ppt 10,000 ind l⁻¹ RB/YMNDO</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>caSL</td>
<td>35 ppt 5,000 ind l⁻¹ AIP</td>
<td>46.1</td>
<td>5.0</td>
</tr>
</tbody>
</table>

**Fig. 3. Artemia. Hatching (%) of lab-produced cysts in
relation to the feed quantity available to parental cultures.**

Standard deviations of means given by error bars.

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(Lavens et al.: Influence of culture conditions on Artemia cysta. 1999)
pronounced for the cysts produced in the tests where varying amounts of food were given.

Nonetheless, best hatching results obtained with cysts from these laboratory cultures were still not comparable to the higher yields obtained with cysts from commercial sources (75 and 80% respectively for Lavalduc and San Francisco Bay: Vanheecke & Scoggin 1983).

Specific diapause deactivation treatments were performed with cysts from the Great Salt Lake stream. From Table 4 it appears that prolonged hibernation or pre-treatment of the cysts in a peroxide solution have a drastic effect on the hatchability of the cysts. Minor effects were noted for the U.V. irradiation treatment. On the other hand different dehydration techniques, e.g. storage in NaCl or MgCl₂ and oven drying, or hydration-dehydration cycles, did not result in significant increases in hatchability (Fig. 2).

**DISCUSSION**

The poor hatchability of some commercial batches of *Artemia* cysts has been often blamed on improper processing of the cysts after collection in nature (Van-

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Table 4. Effect of different diapause deactivation treatments on the hatchability of Great Salt Lake cysts produced under laboratory conditions

<table>
<thead>
<tr>
<th>Diapause inhibition treatments</th>
<th>Treatment number</th>
<th>Processed cysts hatching</th>
<th>Treatment code (2 x wk at −25°C)</th>
<th>Treatment code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Storage at ambient temperature</td>
<td>1 mo</td>
<td>0.6</td>
<td>A</td>
<td>38.5</td>
</tr>
<tr>
<td></td>
<td>2 mo</td>
<td>3.7</td>
<td>B</td>
<td>32.4</td>
</tr>
<tr>
<td>Dehydration in MgCl₂</td>
<td>3</td>
<td>2.7</td>
<td>C</td>
<td>43.3</td>
</tr>
<tr>
<td>Drying; 24 h at 35°C</td>
<td>4</td>
<td>11.0</td>
<td>D</td>
<td>55.4</td>
</tr>
<tr>
<td>Hydration/dehydration</td>
<td>1 cycle</td>
<td>1.7</td>
<td>E</td>
<td>46.7</td>
</tr>
<tr>
<td></td>
<td>2 cycles</td>
<td>4.8</td>
<td>F</td>
<td>39.3</td>
</tr>
<tr>
<td></td>
<td>3 cycles</td>
<td>4.7</td>
<td>G</td>
<td>40.1</td>
</tr>
<tr>
<td>U. V. 6 h/30 W</td>
<td>8</td>
<td>4.2</td>
<td>H</td>
<td>48.9</td>
</tr>
<tr>
<td>Hibernation</td>
<td>9</td>
<td>5.6</td>
<td>I</td>
<td>45.6</td>
</tr>
<tr>
<td></td>
<td>10 wk at −4°C</td>
<td>10.9</td>
<td>J</td>
<td>35.0</td>
</tr>
<tr>
<td></td>
<td>12 wk at −25°C</td>
<td>39.3</td>
<td>K</td>
<td>47.3</td>
</tr>
<tr>
<td></td>
<td>12 wk at −25°C</td>
<td>49.1</td>
<td>L</td>
<td>42.1</td>
</tr>
<tr>
<td></td>
<td>24 wk at −25°C</td>
<td>42.1</td>
<td>M</td>
<td>44.3</td>
</tr>
<tr>
<td>Peroxide</td>
<td>15 min/3% H₂O₂</td>
<td>18.6</td>
<td>N</td>
<td>56.6</td>
</tr>
<tr>
<td></td>
<td>30 min/3% H₂O₂</td>
<td>17.4</td>
<td>O</td>
<td>55.6</td>
</tr>
<tr>
<td></td>
<td>60 min/3% H₂O₂</td>
<td>17.4</td>
<td>P</td>
<td>55.6</td>
</tr>
</tbody>
</table>
Lavens et al.: Influence of culture conditions on Artemia cysts

This study also provides evidence that specific diapause deactivation methods may affect the hatchability of freshly released cysts. At least in some strains of Artemia. Pre-incubation at low temperatures (−23°C) for extended periods and treatment with 3% (v/v) acetic acid solution for a maximum of 1 h can especially efficient dormancy terminating methods. The combination of both diapause deactivation methods yielded cysts of Great Salt Lake Artemia with a hatching quality as high as 86%, which is higher than the best figure of 75% obtained in cysts collected from the Great Salt Lake biotope (Vanhaecke & Sorgeloos 1988).

The data presented here also reveal that hibernation is a quantitative effect, i.e., a function of incubation period and temperature. Analogous effects at 4°C were reported for brine shrimp cysts from the Azov sea (USSR) saltworks (Voronov 1973) and from Mono Lake, California, USA (Dana 1981, Thun & Jarrett 1986). In Lake Lavaduc (France) Artemia, maximal hatchability is ensured after 14 d storage of the dormant embryos at −23°C (Sorgeloos 1979, Godeluck 1980). Since the latter Artemia strains all occur in temperate regions, it is very likely that they display hibernation as an essential adaptation to the local environment, i.e., not only can encrypted offspring produced during summer be saved from unfavourable conditions in winter but, furthermore, this hibernation effect will ensure that in spring all cysts are quiescent, resulting in spring hatching and fast start-up of the new population. A similar adaptation has been reported by Marcus (1979, 1980) for the calanoid copepod Leidicicora astatica.

Differences between Artemia strains in diapause deactivation sensitivity might be related to variations in habitat conditions. This might explain why dehydration or dehydration-hydration cycles do not affect Great Salt Lake cysts but do deactive diapause in (sub-)tropical populations. Although still a hypothesis, such climatic adaptations might have resulted in long-term adaptations and even in strain-specific differences among Artemia belonging to the same sibling species but living in widely different habitats, e.g., A. franciscana from San Francisco Bay and Great Salt Lake. In fact, this should not be surprising in Artemia since this differential adaptation of geographically separated populations has been well documented in various other insect larvae (see review by Lavens & Sorgeloos 1986a).

The peroxide treatment is very effective and totally suppresses the need for hibernation. Its effect on brine shrimp hatching was reported by Bogatova & Schmakova (1980) who claimed that the reactive oxygen atoms catalyse the oxidation-reduction process whereby trehalose is converted into glycerol and glycogen during embryogenesis (Muramatsu 1960, Emer-

Table 3. Variance analyses and Duncan tests on data presented in Table 4. Underlined letters indicate no significant difference at the 0.05 level

<table>
<thead>
<tr>
<th>Treatment code</th>
<th>A E C B H G F I D M J K L O N</th>
<th>F = 0.565</th>
<th>Cv = 10.291</th>
</tr>
</thead>
<tbody>
<tr>
<td>J K S R P T V U Q W X Z Y</td>
<td>F = 96.529</td>
<td>Cv = 7.922</td>
<td></td>
</tr>
</tbody>
</table>

Vanhaecke & Sorgeloos 1982). The results presented in this paper, however, clearly demonstrate that the culture conditions under which the cysts are produced, as well as specific diapause deactivation techniques, can significantly influence the hatching characteristics of dormant eggs. This may also explain the confusion existing in the literature since either of these parameters may have masked the effects of experimental conditions.

Food availability to the parents appears to be the factor of primary importance in determining the hatching quality of the cysts produced. The great variation in hatching percentages of the cysts produced in the first set of experiments might also be explained by sub-optimal feeding levels, in fact it was only in the second set of tests that maximal (i.e. optimal) feeding levels of 12% of population wet weight biomass d−1 (Lavens & Sorgeloos 1986b) were applied. This correlation between food quantity and hatchability of Artemia cysts has recently been confirmed by Tacke et al. (1986) using Dunaliella spp. as a food source for hibernation studies in batch culture systems with different Artemia strains.

The interaction between food availability in adult cultures and the hatchability of their encysted, dormant eggs is unclear. It may interfere with the state of dormancy itself, e.g., resulting in a variable sensitivity for diapause deactivation treatments, or it may affect the embryogenesis of the cysts, e.g., by changing the carbohydrate levels which are critical for an optimal functioning of the trehalose-glycerol hyporomatic regulatory system (Conte et al. 1977). Preliminary analyses of sugar content of laboratory-produced cysts revealed no correlation between trehalose or glyceral concentrations in the cyst and their hatchability or parental food availability (Lavens unpubl.).
son 1963). In this regard the observation of Van Der Linden (pers. comm.) that the light triggering effect in hydrated Artemia cysts (Sorgelos & Bengtson 1973) decreases in cysts that have been pre-incubated in peroxida may be of interest, especially since this illumination energy normally seems to catalyse oxidation-reduction pro-
cesses via excitation by haem pigments (Van Der Lin-
den et al. 1986).

In conclusion, our findings confirm the urgent need for standardized cyst material of well-known pro-
tection origin. This is essential for the further study of the basic mechanisms involved in the diapauser deactiva-
tion, and consequently the hatching metabolism in
Artemia cysts. This research might not only result in a
better understanding of the specific ecological adapta-
tions of this organism to its environment but, moreover, will contribute to a more economical use of this pre-
cious live food in aquaculture.

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