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# Hatching and nutritional quality of *Artemia* cysts progressively deteriorates as a function of increased exposure to hydration/dehydration cycles

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**Abstract** Nauplii hatching from *Artemia* cysts are crucial in larviculture nutrition. *Artemia* cysts may be exposed to repeated hydration/dehydration (H/D) cycles pre-harvesting or during processing and storage. To observe the effect of these cycles on cyst quality, *Artemia franciscana* cysts were exposed to a comprehensive set of various H/D treatments, differing in the number of cycles (1, 2, or 3) and the duration of the freshwater hydration period (2 or 4 h). Cyst quality was assessed using the criteria of immediate relevance for aquaculture use, such as hatching percentage directly after H/D treatment and after −18 °C storage up to 1 month, longevity of axenically hatched starved nauplii, cyst and naupliar energy content, and (for the most extreme H/D treatment) cyst and naupliar fatty acid and vitamin C content. Repeated H/D cycles resulted in significantly ($P < 0.05$) decreased cyst hatching, reduced starved naupliar longevity and individual energy content, loss in vitamin C and fatty acid content, and moreover a close correlation between these parameters as a function of progressive H/D treatments. This is of immediate relevance for aquaculture nutrition, as commercial *Artemia* cysts may have gone through an unknown sequence of H/D cycles in nature or in the processing line, which affects the nutritional quality of the nauplii used in larviculture operations.

**Keywords** *Artemia* · Hydration/dehydration cycles · Energy content · Quality control · Live food · Fatty acid

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## 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 abiotic conditions such as salinity, UV irradiation, temperature, and oxygen concentration (Persoone and Sorgeloos 1980). These mechanisms are poorly understood, although several studies have shown the ways that *Artemia* responds to varying abiotic conditions prevailing in its natural habitats (for review, see Abatzopoulos et al. 2002 and references therein). To survive environmental stress, *Artemia* has developed two different reproductive patterns, with females releasing either swimming larvae (nauplii) or encysted gastrulae (cysts) (MacRae 2003). When cysts are produced, the embryo enters diapause, a reversible physiological condition during which metabolism is greatly reduced and stress tolerance is increased (Drinkwater and Clegg 1991; Clegg 1997; MacRae 2003, 2005). Exposure to habitat-specific environmental stimuli, such as desiccation and/or low temperature, promotes resumption of cyst development and metabolism (Drinkwater and Crowe 1987; Van Der Linden et al. 1988; Drinkwater and Clegg 1991; Nambu et al. 2008).

The first use of *Artemia* nauplii, hatched from cysts, is known from the 1930s when this zooplankton organism was used as a suitable food source for fish larvae in the culture of commercially important species (Sorgeloos 1980; Léger et al. 1986). Since then, *Artemia* has been found to be a suitable food for diverse groups of organisms of the animal kingdom, especially for a wide variety of marine and freshwater crustaceans and fishes (Sorgeloos 1980).

Cyst hatching is determined by a variety of factors, including genetic factors, the degree of diapause termination, ambient conditions before and during harvesting, processing and storage procedures, and ambient conditions during the hatching incubation process itself. One of the most effective methods for deactivating diapause in cysts of the San Francisco Bay (SFB)-type *Artemia franciscana* (Kellogg 1906) in laboratory conditions is dehydration or well-controlled consecutive hydration/dehydration (H/D) cycles (Sorgeloos et al. 1976; Vanhaecke and Sorgeloos 1982; Lavens et al. 1986). Quiescent cysts (out of diapause) on the other hand may go through H/D cycles when being exposed to ambient conditions pre-harvest in natural habitats or during processing procedures. This may result in variable quality loss exemplified by reduced and/or delayed hatching especially after storage (Vanhaecke and Sorgeloos 1982; Lavens and Sorgeloos 1987). Though some H/D exposure is, to a certain extent, an almost unavoidable element in the history of any commercial cyst sample from pre-harvesting until marketing, and though the related decline in hatching can be substantial, no systematical research has been done in this respect.

This study assumed that the loss of hatching quality as a consequence of one or more H/D cycles would be proportional to the magnitude of the exposure and the duration of subsequent storage. It further assumed that this treatment would also result in deterioration of other quality characteristics relevant for the use of brine shrimp as live food in larviculture. For this purpose, cysts of two *A. franciscana* strains (Great Salt Lake, USA, and the San Francisco Bay-type Vinh Chau, Vietnam) that are of prime importance for global cyst supply were subjected to different treatments each including one or more H/D steps in well-defined experimental conditions. The quality of the resulting embryos was assessed using practical criteria relevant for their use in aquaculture: hatching quality, longevity of starved nauplii and nutritional quality measured as energy content, HUFA, and vitamin C levels of cysts and nauplii.

## Materials and methods

### Cyst samples

Experiments were performed with two strains of *A. franciscana*: one commercial dry sample (water content 4.5 ± 0.2 %) originating from Great Salt Lake (GSL), Utah, USA (INVE Aquaculture Belgium, Type EG (batch number: 21425), and a second sample from Vinh Chau (VC) salt fields, Vietnam (ARC code 1718), supplied by Can Tho University, Vietnam, being dehydrated and stored in saturated NaCl brine (water content 34.8 ± 1.8 %). Both samples had been stored at +4 °C since their arrival at the Laboratory of Aquaculture & *Artemia* Reference Center. Water content of cysts was determined by drying a subsample of raw cysts in an oven for 4 h at 103 °C to a constant weight.

### Hydration/dehydration cycles

Cysts were exposed to successive hydration/dehydration (H/D) cycles by incubating 1.6 g of cysts of each strain in a 1-l cylindroconical glass cone containing 800 ml of medium (freshwater for the hydration step and NaCl-saturated brine (280–300 g $l^{-1}$) for the dehydration step) at 28 °C under strong aeration. A first group of three cones was set up; the cysts in the first cone were exposed to one H/D cycle (2-h hydration and 24-h dehydration), the second one to two cycles, and the third one to three (named A1, A11, and A111, respectively). In parallel, for each strain, three other cones went through a similar setup, but with each hydration period lasting for 4 h (the corresponding treatments named A2, A22, and A222). After the H/D steps, the cyst samples were immediately stored in +4 °C in NaCl-saturated brine (280–300 g $l^{-1}$) until use for any of the tests described under 2.4, 2.5, 2.6, and 2.7.

### Determination of hatching percentage (H %)

For the determination of H % of hydrated/dehydrated cysts, the above procedure was performed in triplicate for each treatment; determination of the hatching percentage was accordingly performed in triplicate. Each sample having gone through the H/D cycles (and a control, not exposed to H/D) was incubated in 800 ml Instant Ocean® solution of 32 ± 1 g $l^{-1}$ in 1-l cylindroconical glass cones under continuous illumination (2000 lux) at 28.0 ± 0.5 °C (Lavens and Sorgeloos 1996). Aeration was provided from the bottom to keep all the cysts in suspension.

After 24 h of incubation, six subsamples of 250 µl each were taken from each cone with a micropipette and placed in a small vial. Nauplii were fixed by adding a few drops of lugol solution and tap water. The nauplii as well as the umbrellae were counted under the microscope. The unhatched cysts were subsequently decapsulated by adding a few drops of NaOCl and NaOH solution to each vial (Bruggeman et al. 1980), and the orange-colored embryos were counted, according to the procedure described by (Lavens and Sorgeloos 1996). The hatching percentage was calculated as follows:

$$H\,\% = N/(N + U + E) \times 100$$

where $N$ = number of nauplii, $U$ = number of umbrellae, $E$ = number of embryos.

The mean hatching value per cone was recorded, and the overall mean hatching percentage and standard deviation for the three replicate cones were calculated. H % was

determined at day 0 (= immediately after the H/D treatment), after 1 week, and 1 month of storage at −18 °C. The stored samples were placed at room temperature (±22 °C) for one day before H % testing.

The hatching rate was only determined for non-treated cysts (control), by the determination of the hatching percentage obtained after a hatching incubation period of 10, 12, 14, 16, 18, 20, 22, 24, and 48 h.

## Axenic *Artemia* culture

Axenic *Artemia* nauplii of each strain were obtained following decapsulation of samples of hydrated/dehydrated cysts (obtained according to the procedure described above) and subsequent hatching procedures described by Marques et al. (2004). A few grams of hydrated/dehydrated cysts were hydrated in 90 ml tap water for 1 h with strong aeration in non-axenic conditions. The recipient with the cysts was then transferred to a laminar flow hood, where decapsulation was performed using autoclaved and sterile tools. Aeration of the *Artemia* cysts was pumped through a 0.22-μm filter. Then, 50 ml of cold NaOCl containing 15 % (w/v) active chlorine and 3.3 ml of 32 % (w/v) NaOH was added to the hydrated cysts. The reaction was stopped after 150 s by adding 70 ml of sterile $Na_2S_2O_3{\cdot}5H_2O$ (10 mg $l^{-1}$). Decapsulated cysts were washed several times carefully with filtered autoclaved seawater (FASW) and collected over a 50-μm sterile sieve. A few mg of these cysts were then transferred to separate, sterile 50-ml falcon tubes (four replicates per H/D treatment) containing 30 ml of FASW and capped. For hatching incubation, the tubes were placed on a rotor at 4 cycles/min to prevent clogging and sedimentation of the cysts. Cysts were kept at 28.0 ± 0.5 °C and exposed to constant incandescent light (2,000 lux). After 18–20 h, 20 hatched nauplii were picked and transferred to new sterile 50-ml falcon tubes containing 30 ml of FASW, which were mounted on the rotor and incubation was continued. After 12, 24, 36, and 48 h, during which the larvae were not fed, swimming larvae were counted and survival percentage was calculated, as described by Baruah et al. (2010).

Axenity of decapsulated cysts and *Artemia* culture at the end of each experiment was checked by plating 100 μl of the culture medium on marine agar 2216 in two replicates (Difco, Detroit, USA) and incubation for five days at 28.0 °C. In case of contamination, cultures were discarded and the treatment was repeated.

## Energy content determination

A subsample of a few g of decapsulated cysts of each H/D treatment was washed carefully with sterile distilled water over a sterile net (50-μm pores); a few hundred mg of these cysts was then oven-dried at 60.0 °C for 24 h. The remaining cysts were transferred to sterile 500-ml hatching bottles containing 400 ml of FASW. The bottles were incubated at 28.0 ± 0.5 °C and constantly exposed to light. After 24 h, hatched larvae were harvested and oven-dried at 60.0 °C for 24 h. Energy content of decapsulated cysts and nauplii was analyzed on one replicate sample of approximately 0.5 g dry material per treatment group using a bomb calorimeter (C-7000, Ika, Heitersheim, Germany) at the Particle and Interfacial Technology Group, Faculty of Bioscience Engineering, Ghent University, Belgium. In order to calculate the individual cyst energy content, the number of cysts per gram dry weight was determined by counting the cysts under the microscope for three replicate samples of 1 mg.

### Fatty acid analysis

To reduce analytical costs, for each strain, only the raw sample and the A222 treatment (which corresponded with the most extreme H/D treatment) were subjected to fatty acid analysis of decapsulated cysts and instar I nauplii (analysis run in one replicate). Fatty acid composition was determined by gas chromatography according to a modified procedure of Lepage and Roy (1984). This method involves direct acid catalyzed transesterification of dry samples of 10–150 mg without prior extraction of total fat. Ten percent of an internal standard 20:2(n-6) was added before the reaction. Fatty acid methyl esters (FAME) were extracted with hexane. After evaporation of the solvent, the FAME was prepared for injection by redissolving it in isooctane (2 mg $ml^{-1}$). Quantitative determination was done by a Chrompack CP9001 gas chromatograph equipped with an autosampler and a temperature programmable on-column injector. Identification was based on standard reference mixtures (Nu-Chek-Prep, Inc., USA). Integration and calculations were done using the software program Maestro (Chrompack).

### Vitamin C analysis

Vitamin C analysis was performed on the same limited set of samples as used for fatty acid analysis. Vitamin C was determined by a paired-ion, reversed-phase, high-performance liquid chromatography (HPLC) procedure coupled with electrochemical detection and internal standard quantisation based on isoascorbic acid (IAA). The HPLC apparatus consisted of a Varian 8500 pump (Varian Assoc., Palo Alto, CA, USA), an N60 valve injector fitted with a 20-µl loop (Valco, Houston, TX, USA), and a Coulochem 5100A electrochemical detector (ESA, Inc., Bedford, MA, USA) equipped with a model 5010 or 5011 analytical cell.

### Statistical analysis

Hatching and survival percentages data were Arcsin-transformed, and normal distribution and homocedasticity requirements were tested (using Levene's test) before further statistical analysis. For each strain and different duration of storage, the data of hatching percentage for 24 h were subjected to one-way ANOVA to detect an effect of the hydration/dehydration treatments. Similarly, for each strain and hydration/dehydration treatment, the data of hatching percentage for 24 h were subjected to one-way ANOVA to detect an effect of the storage period. Additionally, for each strain, survival data after 12, 24, 36, and 48 h of starvation of metanauplii were each subjected to a one-way ANOVA to detect an effect of the hydration/dehydration treatments. Finally, for each strain, also the values for the number of cysts per gram were subjected to a one-way ANOVA to detect an effect of the hydration/dehydration treatments. For all one-way ANOVA's, $P < 0.05$ was considered as significant. A two-factor ANOVA test (SPSS, version 12.0) was used to detect significant interactions between the duration of the hydration period (2 or 4 h) and the number of H/D cycles (1, 2, and 3 cycles) for hatching and survival percentages, and $P < 0.05$ was considered as significant. Tukey's test was used to detect significant differences between the experimental sample means, and $P < 0.05$ was considered as significant. Linear regression was used to determine the relationship between parameters in the H/D experiment and $P < 0.05$ was considered as significant. For this analysis, hatching percentages after different period of storage were linearly regressed against the energy content of the cysts exposed to various H/D cycles using a scatterplot in Microsoft Excel.

Similarly, survival percentages after different periods of starvation of metanauplii were linearly regressed against the energy content of instar I nauplii after exposure of cysts to various H/D cycles. Per strain and storage period, pooled standard error of means (PSEM) of hatching percentage was calculated using the formula $PSEM = \sqrt{MSE/n}$ (whereby MSE is mean square of groups, and n is number of observations), pooling the values obtained after different H/D cycles. Similarly, for each strain, PSEM was calculated for the survival percentages found after a starvation period of 12, 24, 36, and 48 h.

## Results

### Hatching characteristics

The hatching percentage of the raw material was 90.1 ± 0.3 % for GSL and 95.1 ± 1.2 % for VC. From the hatching curve (Fig. 1), $T_0$ (time of first cyst hatching) and $T_{10}$ (time of 10 % hatching), derived graphically, were 10 and 11 h for GSL and 10 and 10.5 h for VC, respectively. The cysts showed 90 % of their maximum hatchability ($T_{90}$) at 24 h for GSL and 20 h for VC. The hatching synchrony ($T_S = T_{90} - T_{10}$) was different between the strains (13 h for GSL and 9.5 h for VC) (Fig. 1).

Successive hydration/dehydration cycles increasingly affected the hatching percentage. For GSL cysts of the A1 group, H % was 84.8 % on day zero, 80.6 % after 1 week, and 79.6 % after 1 month of storage. H % values for the equivalent VC samples were very similar (Table 1). For each strain, H % of the untreated cysts was significantly higher than that of H/D cysts regardless of the number and duration of H/D cycles and the duration of storage, except for A1/day 0 GSL cysts and A1/1 month VC cysts, where the difference with the untreated cysts was not significant. For GSL cysts, hatchability significantly decreased (Table 1; Fig. 2a) with increasing hydration time and H/D cycles; this decrease was much more prominent for the samples of the A2 series than for the A1 samples, finally resulting for the A222 group in hatching percentages of 42.7 % on day zero, 41.6 % after 1 week, and 36.4 % after 1 month of storage. Interaction between the duration of hydration and the number of H/D cycles was significant, when analyzing the hatching percentage values before storage, after 1 week, and 1 month of storage ($P < 0.05$). The results for the VC cysts showed a similar trend (Table 1; Fig. 2b). Storage always resulted in loss of hatching, though the decrease was generally not significant (Table 1).

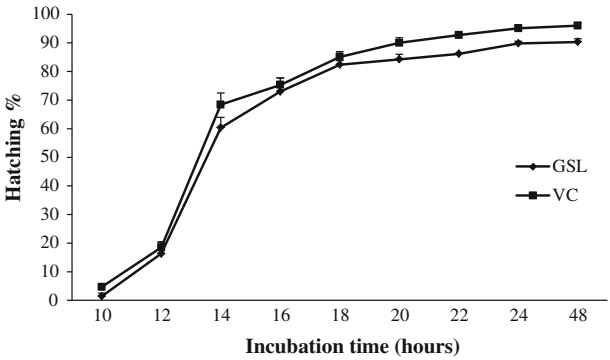


**Fig. 1** Hatching curves of untreated cysts from Great Salt Lake (GSL) and Vinh Chau (VC). Mean values and standard deviation (error bars) of three replicates

**Table 1** Hatching percentage (after 24-h hatching incubation) of cysts from GSL and VC strains previously exposed to different duration of the hydration period (2 or 4 h) and different number of H/D cycles (1, 2, and 3 cycles)

| Strain | Treatment | Hatching percentage after storage at −18 °C for different durations | | |
|---|---|---|---|---|
| | | Day 0 (prior to storage) | 1 week | 1 month |
| GSL | Control | $90.1 \pm 0.3^{aA}$ | $89.1 \pm 1.1^{aA}$ | $87.7 \pm 2.2^{aA}$ |
| | A1 | $84.8 \pm 0.2^{abA}$ | $80.6 \pm 1.8^{bB}$ | $79.6 \pm 2.5^{bB}$ |
| | A11 | $79.1 \pm 2.6^{bA}$ | $78.9 \pm 3.3^{bA}$ | $78.7 \pm 0.8^{bA}$ |
| | A111 | $77.3 \pm 2.5^{bA}$ | $76.0 \pm 1.7^{bA}$ | $75.5 \pm 1.4^{bA}$ |
| | A2 | $66.7 \pm 2.5^{cA}$ | $64.0 \pm 1.2^{cA}$ | $61.9 \pm 2.2^{cA}$ |
| | A22 | $55.8 \pm 1.3^{dA}$ | $54.1 \pm 1.4^{dA}$ | $52.9 \pm 2.3^{dA}$ |
| | A222 | $42.7 \pm 5.9^{eA}$ | $41.6 \pm 3.8^{eA}$ | $36.4 \pm 5.7^{eA}$ |
| | Pooled SEM* | ±1.6 | ±1.3 | ±1.6 |
| | Interaction** | $P = 0.010$ | $P = 0.001$ | $P = 0.000$ |
| VC | Control | $95.1 \pm 1.2^{aA}$ | $92.7 \pm 0.8^{aAB}$ | $91.2 \pm 1.1^{aB}$ |
| | A1 | $88.9 \pm 0.2^{bA}$ | $87.6 \pm 1.7^{bA}$ | $87.5 \pm 1.7^{abA}$ |
| | A11 | $85.0 \pm 1.9^{cA}$ | $83.9 \pm 1.4^{cA}$ | $83.2 \pm 1.0^{bcA}$ |
| | A111 | $83.8 \pm 0.8^{cA}$ | $82.3 \pm 0.9^{cAB}$ | $80.9 \pm 1.1^{cB}$ |
| | A2 | $64.2 \pm 1.2^{dA}$ | $61.8 \pm 1.7^{dAB}$ | $60.0 \pm 1.3^{dB}$ |
| | A22 | $52.8 \pm 0.8^{eA}$ | $50.8 \pm 1.2^{eA}$ | $50.6 \pm 1.1^{eA}$ |
| | A222 | $42.9 \pm 0.8^{fA}$ | $40.9 \pm 0.1^{fA}$ | $35.7 \pm 3.1^{fB}$ |
| | Pooled SEM* | ±0.6 | ±0.7 | ±0.9 |
| | Interaction** | $P = 0.000$ | $P = 0.000$ | $P = 0.000$ |

For each strain and different duration of storage, *small superscripts* in each column show significant difference between different hydration/dehydration treatments (one-way ANOVA). For each strain and each hydration/dehydration treatment, *capital superscripts* in each row show significant differences between different duration of storage (one-way ANOVA). ** Interaction between duration of hydration (2 or 4 h) and number of H/D cycles (1, 2, or 3 cycles) (two-way ANOVA). Data are mean value ($n = 3$) ± standard deviation and * pooled standard error of means (pooled SEM). Significance level was set at $P < 0.05$

*GSL* Great Salt Lake, *VC* Vinh Chau. A1 = 2-h hydration + 24-h dehydration (1 cycle). A2 = 4-h hydration + 24-h dehydration (1 cycle), A11 = 2-h hydration + 24-h dehydration (2 cycles). A22 = 4-h hydration + 24-h dehydration (2 cycles), A111 = 2-h hydration + 24-h dehydration (3 cycles). A222 = 4-h hydration + 24-h dehydration (3 cycles)

## Survival of starved nauplii under axenic conditions

At the first observation (12-h post-hatching), nauplii of the control group (both for GSL and VC) showed the highest survival (78.8 and 86.3 %, respectively) (Table 2); in the treatment groups, survival was inversely proportional to the number of H/D cycles and especially to the duration of the hydration period (2 vs. 4 h). Interaction between the duration of the hydration period (2 h and 4 h) and the number of H/D cycles was only significant ($P < 0.05$) for the survival at 12 h of the GSL strain. All groups of nauplii showed increasing mortality throughout the 48-h observation period. This resulted in a significantly lower ($P < 0.05$) GSL and VC survival for all A2 treatments as compared to the control for any moment of observation. Also, for the A1 treatments, the divergence from the control value grew as the number of H/D cycles increased. Overall, the

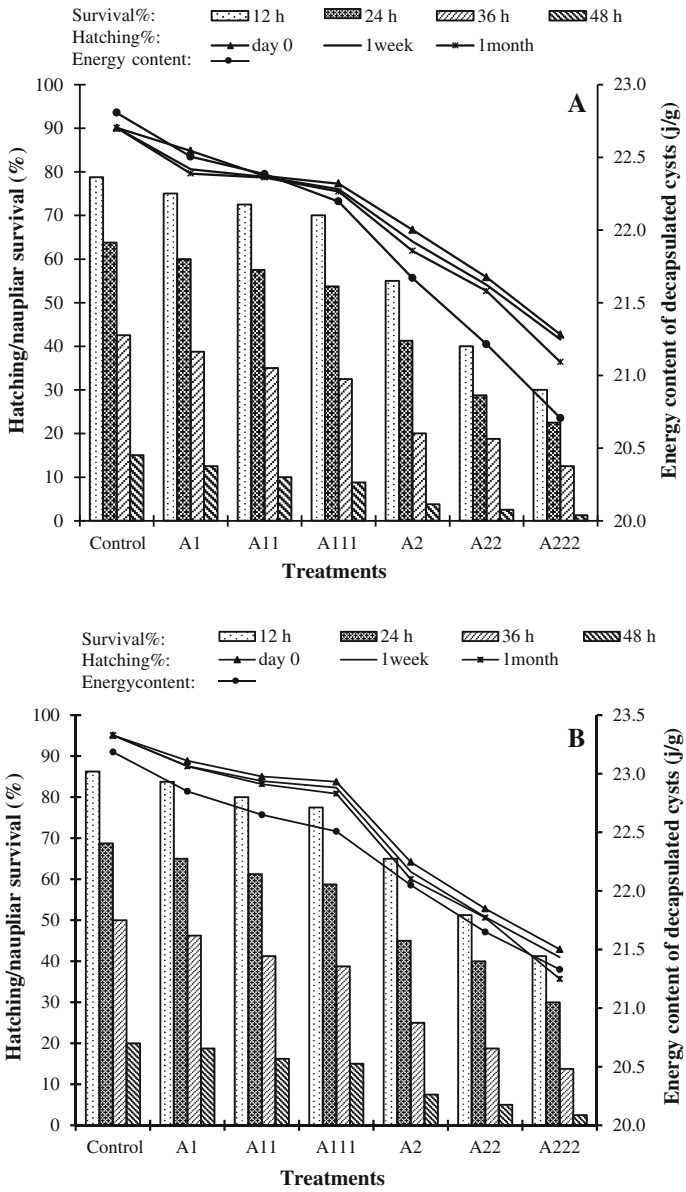


**Fig. 2** **a** Hatching percentage (on day 0, after 1 week, and 1 month storage, respectively), energy content (*line graphs*), and percentage survival of the corresponding starved newly hatched nauplii (at 12, 24, 36, and 48 h, respectively, *bar graph*) of decapsulated GSL cysts previously exposed to various H/D cycles. For abbreviations, see Table 1. **b** Hatching percentage (on day 0, after 1 week, and 1 month storage, respectively), energy content (*line graphs*) and percentage survival of the corresponding starved newly hatched nauplii (at 12, 24, 36, and 48 h, respectively, *bar graph*) of decapsulated VC cysts previously exposed to various H/D cycles. For abbreviations, see Table 1

**Table 2** Survival (%) over a 48-h period of starved nauplii hatched under axenic condition from GSL and VC cysts previously exposed to different duration of the hydration period (2 or 4 h) and different number of H/D cycles (1, 2, and 3 cycles)

| Strain | GSL | | | | VC | | | |
|---|---|---|---|---|---|---|---|---|
| Treatment | 12 h | 24 h | 36 h | 48 h | 12 h | 24 h | 36 h | 48 h |
| Control | $78.8 \pm 6.3^{a}$ | $63.8 \pm 6.3^{a}$ | $42.5 \pm 8.7^{a}$ | $15.0 \pm 4.1^{a}$ | $86.3 \pm 8.5^{a}$ | $68.8 \pm 11.1^{a}$ | $50.0 \pm 8.2^{a}$ | $20.0 \pm 5.8^{a}$ |
| A1 | $75.0 \pm 4.1^{a}$ | $60.0 \pm 7.1^{a}$ | $38.8 \pm 7.5^{a}$ | $12.5 \pm 2.9^{a}$ | $83.8 \pm 4.8^{a}$ | $65.0 \pm 10.8^{ab}$ | $46.3 \pm 6.3^{a}$ | $18.8 \pm 4.8^{ab}$ |
| A11 | $72.5 \pm 2.9^{a}$ | $57.5 \pm 6.5^{a}$ | $35.0 \pm 5.8^{a}$ | $10.0 \pm 4.1^{ab}$ | $80.0 \pm 4.1^{a}$ | $61.3 \pm 11.1^{abc}$ | $41.3 \pm 6.3^{a}$ | $16.3 \pm 4.8^{abc}$ |
| A111 | $70.0 \pm 5.8^{a}$ | $53.8 \pm 6.3^{ab}$ | $32.5 \pm 6.5^{ab}$ | $8.8 \pm 2.5^{abc}$ | $77.5 \pm 2.9^{ab}$ | $58.8 \pm 9.5^{abc}$ | $38.8 \pm 4.8^{a}$ | $15.0 \pm 4.1^{abc}$ |
| A2 | $55.0 \pm 7.1^{b}$ | $41.3 \pm 4.8^{bc}$ | $20.0 \pm 4.1^{bc}$ | $3.8 \pm 2.5^{bcd}$ | $65.0 \pm 4.1^{bc}$ | $45.0 \pm 4.1^{bcd}$ | $25.0 \pm 4.1^{b}$ | $7.5 \pm 5.0^{bcd}$ |
| A22 | $40.0 \pm 8.2^{c}$ | $28.8 \pm 8.5^{cd}$ | $18.8 \pm 7.5^{bc}$ | $2.5 \pm 2.9^{cd}$ | $51.3 \pm 7.5^{cd}$ | $40.0 \pm 9.1^{cd}$ | $18.8 \pm 6.3^{b}$ | $5.0 \pm 7.1^{cd}$ |
| A222 | $30.0 \pm 5.8^{c}$ | $22.5 \pm 5.0^{c}$ | $12.5 \pm 2.9^{c}$ | $1.3 \pm 2.5^{c}$ | $41.3 \pm 8.5^{d}$ | $30.0 \pm 9.1^{d}$ | $13.8 \pm 4.8^{b}$ | $2.5 \pm 2.9^{d}$ |
| Pooled SEM* | ±3.0 | ±3.2 | ±3.2 | ±1.6 | ±3.1 | ±4.8 | ±3.0 | ±2.5 |
| Interaction** | $P = 0.031$ | $P = 0.236$ | $P = 0.826$ | $P = 0.885$ | $P = 0.102$ | $P = 0.724$ | $P = 0.870$ | $P = 0.961$ |

For abbreviations, see Table 1

For each strain, different hydration/dehydration treatment, and different starvation period (12, 24, 36, and 48 h), small superscripts in each column show significant difference between different treatments (one-way ANOVA). ** Interaction between duration of hydration (2 or 4 h) and number of H/D cycles (1, 2, or 3 cycles) (two-way ANOVA). Data are mean ($n = 4$) ±standard deviation and * pooled standard error of means (pooled SEM). Significance level was set at $P < 0.05$

**Table 3** Energy content of decapsulated GSL and VC *Artemia* cysts previously exposed to different duration of the hydration period (2 or 4 h) and different number of H/D cycles (1, 2, and 3 cycles), and of their corresponding instar I nauplii; percentage loss of individual energy during the H/D cycles as compared to the control, and number of cysts per gram dry weight

| Strain treatment | GSL | | | | | VC | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Energy content of decapsulated cysts (J $g^{-1}$ DW) | Energy content of instar I nauplii (J $g^{-1}$ DW) | Individual energy content (mj $cyst^{-1}$) | Energy loss (%) | Number of cysts ($g^{-1}$ DW) | Energy content of decapsulated cysts (J $g^{-1}$ DW) | Energy content of instar I nauplii (J $g^{-1}$ DW) | Individual energy content (mj $cyst^{-1}$) | Energy loss (%) | Number of cysts ($g^{-1}$ DW) |
| Control | 22.807 | 22.297 | 84.1 | 0.0 | $271{,}070 \pm 805^{g}$ | 23.186 | 22.631 | 67.8 | 0.0 | $342{,}100 \pm 900^{g}$ |
| A1 | 22.504 | 21.956 | 81.9 | 2.6 | $274{,}693 \pm 606^{f}$ | 22.851 | 22.330 | 66.2 | 2.3 | $345{,}180 \pm 815^{f}$ |
| A11 | 22.385 | 21.750 | 81.9 | 3.7 | $276{,}133 \pm 1000^{e}$ | 22.651 | 22.010 | 65.3 | 3.7 | $347{,}147 \pm 938^{e}$ |
| A111 | 22.196 | 21.549 | 79.8 | 5.2 | $278{,}147 \pm 531^{d}$ | 22.508 | 21.831 | 64.3 | 5.2 | $350{,}113 \pm 821^{d}$ |
| A2 | 21.669 | 20.896 | 76.3 | 9.3 | $284{,}023 \pm 921^{c}$ | 22.050 | 21.470 | 61.8 | 8.9 | $357{,}077 \pm 936^{c}$ |
| A22 | 21.215 | 20.427 | 73.3 | 12.8 | $289{,}143 \pm 535^{b}$ | 21.651 | 21.231 | 59.8 | 11.8 | $362{,}270 \pm 661^{b}$ |
| A222 | 20.707 | 19.99 | 70.3 | 16.4 | $294{,}407 \pm 472^{a}$ | 21.331 | 20.791 | 57.9 | 14.6 | $368{,}410 \pm 618^{a}$ |

For abbreviations, see Table 1

For each strain and number of cysts per gram dry weight, small superscripts in each column show significant difference between different treatments (one-way ANOVA). Energy values correspond with one measurement of energy content per sample; data for the number of cysts are mean value ± standard deviation ($n = 3$). Significance level was set at $P < 0.05$. DW = dry weight

**Table 4** Fatty acid methyl esters (FAME) composition (mg $g^{-1}$ DW) and vitamin C contents (mg $g^{-1}$ DW) in control and A222 decapsulated cysts and in the corresponding instar I nauplii of GSL and VC *Artemia*

| Strain | GSL | | | | VC | | | |
|---|---|---|---|---|---|---|---|---|
| | Cysts | | Nauplii | | Cysts | | Nauplii | |
| FAME | Control | A222 | Control | A222 | Control | A222 | Control | A222 |
| 14:00 | 1.3 | 1.1 | 0.9 | 0.9 | 3.3 | 3.0 | 3.0 | 2.9 |
| 14:1n-5 | 1.7 | 1.6 | 1.7 | 1.6 | 1.1 | 1.0 | 1.1 | 1.1 |
| 15:00 | 0.3 | 0.3 | 0.2 | 0.2 | 6.1 | 5.7 | 5.6 | 5.6 |
| 15:1n-5 | 0.7 | 0.3 | 0.8 | 0.3 | 0.6 | 0.6 | 0.6 | 0.6 |
| 16:00 | 17.0 | 16.5 | 16.2 | 15.7 | 19.4 | 18.0 | 18.9 | 18.3 |
| 16:1n-7 | 3.8 | 3.7 | 3.2 | 3.0 | 17.0 | 15.8 | 17.0 | 16.9 |
| 17:00 | 1.1 | 1.0 | 1.1 | 1.0 | 6.7 | 4.8 | 7.0 | 5.4 |
| 17:1n-7 | 1.4 | 1.3 | 1.3 | 1.2 | 2.1 | 1.7 | 2.0 | 1.9 |
| 18:00 | 7.1 | 6.7 | 5.6 | 7.8 | 4.8 | 4.4 | 5.4 | 5.1 |
| 18:1n-9 | 27.5 | 25.8 | 27.3 | 26.2 | 20.9 | 19.2 | 22.8 | 20.7 |
| 18:1n-7 | 8.5 | 8.2 | 8.8 | 8.7 | 14.5 | 13.3 | 15.1 | 15.6 |
| 18:2n-6$^{t}$ | 0.4 | 0.4 | 0.4 | 0.4 | 0.3 | 0.1 | 0.4 | 0.4 |
| 18:2n-6$^{s}$ | 9.2 | 9.0 | 10.0 | 9.9 | 4.4 | 4.1 | 4.8 | 4.7 |
| 19:00 | 0.1 | 0.1 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 1.5 |
| 18:3n-6 | 1.3 | 1.3 | 1.4 | 1.4 | 1.3 | 1.3 | 1.3 | 1.3 |
| 19:1n-9 | 0.5 | 0.4 | 0.5 | 0.5 | 1.3 | 1.2 | 1.5 | 1.5 |
| 18:3n-3 | 43.6 | 43.4 | 51.4 | 51.8 | 3.8 | 3.6 | 4.4 | 4.3 |
| 18:4n-3 | 9.8 | 9.8 | 11.5 | 11.7 | 1.6 | 1.4 | 1.5 | 1.4 |
| 20:00 | 0.1 | 0.1 | 0.2 | 0.2 | 0.4 | 0.1 | 0.3 | 0.1 |
| 20:1n-9 | 0.7 | 0.6 | 1.0 | 0.9 | 1.4 | 1.2 | 0.8 | 1.2 |
| 20:1n-7 | 0.1 | 0.1 | 0.2 | 0.1 | 0.2 | 0.2 | 1.9 | 0.2 |
| 20:3n-6 | 0.2 | 0.1 | 0.2 | 0.2 | 0.5 | 0.4 | 0.5 | 0.5 |
| 20:4n-6 | 0.4 | 0.4 | 0.3 | 0.3 | 6.0 | 5.6 | 6.7 | 6.5 |
| 20:3n-3 | 1.5 | 1.4 | 2.2 | 2.2 | 0.1 | 0.1 | 0.2 | 0.1 |
| 20:4n-3 | 1.6 | 1.6 | 2.2 | 2.2 | 0.7 | 0.6 | 0.6 | 0.9 |
| 22:00 | 0.3 | 0.3 | 0.6 | 0.5 | 0.3 | 0.1 | 0.4 | 0.4 |
| 20:5n-3 | 1.6 | 1.6 | 1.2 | 1.2 | 17.6 | 17.0 | 19.9 | 19.6 |
| 22:6n-3 | 0.0 | 0.0 | 0.1 | 0.0 | 0.2 | 0.0 | 0.2 | 0.0 |
| Total (n-3) | 4.9 | 4.8 | 6.1 | 5.8 | 19.0 | 18.0 | 21.6 | 21.0 |
| Total (n-6) | 11.5 | 11.2 | 12.3 | 12.2 | 12.7 | 11.6 | 13.9 | 13.5 |
| Total (SFA) | 27.3 | 26.1 | 24.9 | 26.4 | 41 | 36.1 | 40.6 | 39.3 |
| Total MUFA | 44.9 | 42.0 | 44.8 | 42.5 | 59.1 | 54.2 | 62.8 | 59.7 |
| Total PUFA | 69.6 | 69.0 | 80.9 | 81.3 | 36.5 | 34.2 | 40.5 | 39.7 |
| Total FAME | 153.3 | 149.3 | 165.8 | 162.9 | 171.9 | 166.5 | 182.1 | 173.7 |
| Vitamin C | 0.367 | 0.112 | 0.803 | 0.724 | 0.289 | 0.111 | 0.676 | 0.611 |

Values correspond with one single analysis. For abbreviations, see Table 1

discrepancy between the treated groups and the control grew as the nauplii grew older. Overall performance of the VC nauplii (control and treatments) was better than for the GSL sample (Table 2; Fig. 2a, b).

## Energy content

The number of cysts per gram and the energy content of hydrated/dehydrated decapsulated cysts and instar I nauplii (J/g dry weight) were higher for VC than for GSL, but the individual cyst energy content was lower in VC cysts, which are smaller than GSL cysts (Table 3). Moreover, in both strains, the individual energy content was higher in the control than in the hydrated/dehydrated cysts, with a gradual decrease in energy content as the number of H/D cycles and the duration of the hydration period increased, illustrating that the cysts consume energy while being hydrated. The rate of energy loss during exposure to the various H/D treatments was similar for both strains (Table 3; Fig. 2a, b). In both strains, cysts lost more energy when being hydrated for 4 h, than when being hydrated twice for 2 h (e.g., 8.9 vs. 3.7 % for A2 and A11, respectively, VC cysts). In both strains, a double or a triple H/D cycle did not result in a two- or threefold decrease in individual energy content.

## Fatty acid composition

A total of 28 fatty acids were recorded (Table 4). The saturated fatty acids (SFA) were dominated by 16:0 and 18:0 in the two strains. Among the monounsaturated fatty acids (MUFA), 18:1n-9 was the most abundant in the two strains with values in the range 19.2–27.5 mg $g^{-1}$ DW; this fatty acid showed higher levels in the GSL control and A222 cysts and nauplii than in the corresponding VC samples. Of all HUFAs, 18:3n-3 dominated in the GSL samples (43.4–51.8 mg $g^{-1}$ DW in cysts and nauplii), whereas in VC 20:5n-3 was the most abundant HUFA 17.0–19.9 mg $g^{-1}$ DW in cysts and nauplii.

Though variations were found among the individual fatty acids, in general total (n-3), total (n-6), and total fatty acids were higher in the nauplii than in the corresponding cyst samples. In addition, total fatty acid levels of the samples exposed to H/D cycles were slightly lower than in the control in both cysts and nauplii, but there was no effect of the H/D cycles on the levels of highly unsaturated fatty acids, such as eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3), arachidonic acid (ARA, 20:4n-6), or linolenic acid (18:3n-3), illustrating that net breakdown of HUFAs during the hydration/dehydration process is limited (Table 4).

## Vitamin C content

Considerably higher vitamin C values, expressed as ascorbic acid, were found in the control nauplii (0.676 and 0.803 mg $g^{-1}$ DW) than in the control cysts (0.289 and 0.367 mg $g^{-1}$ DW), for VC and GSL, respectively. In both strains, three H/D cycles with 4-h hydration decreased the vitamin C content with 62–69 % in cysts and about 10 % in nauplii (Table 4).

## Correlations between quality parameters

Linear regression analysis between hatching percentage (on day 0, after 1 week, and 1 month of storage) on the one hand and individual energy content of cysts on the other

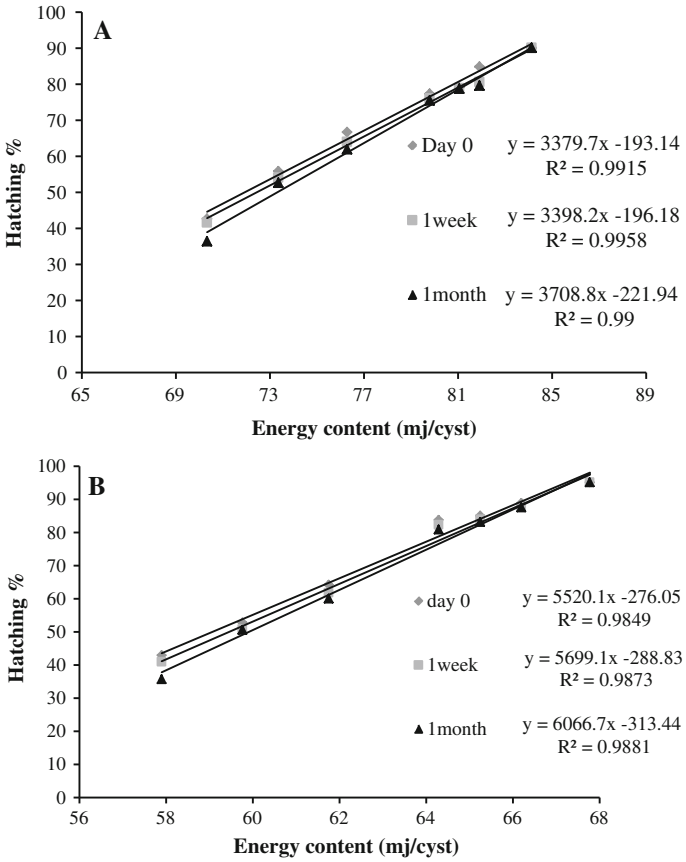


**Fig. 3** **a** Linear regression between hatching percentage (on day 0, after 1 week, and 1 month of storage) and energy content (mj $cyst^{-1}$) in GSL cysts exposed to various H/D cycles. **b** Linear regression between hatching percentage (on day 0, after 1 week, and 1 month of storage) and energy content (mj $cyst^{-1}$) in VC cysts exposed to various H/D cycles

indicated a very strong positive correlation in both GSL and VC strains ($R^2 > 0.99$ for GSL and $R^2 > 0.98$ for VC (Fig. 3a, b), A similar positive correlation was found between survival of the starved nauplii and the energy content (expressed in J $g^{-1}$ dry weight) of instar I nauplii ($R^2 > 0.95$ for GSL and $R^2 > 0.92$ for VC (Fig. 4a, b). There were no significant differences ($P > 0.05$) between the slopes and between the intercepts of the regression lines in Fig. 3a, b, whereas in both Fig. 4a, b all slopes and intercepts of the four regression lines were significantly different ($P < 0.05$) from each other.

## Discussion

The ability of *Artemia* to form cysts accounts in part for its convenience as a larval food source (Léger et al. 1986). *Artemia* cysts have a remarkable shelf life; the ease and simplicity of hatching make brine shrimp one of the most convenient, least labor-intensive live foods available for aquaculture.

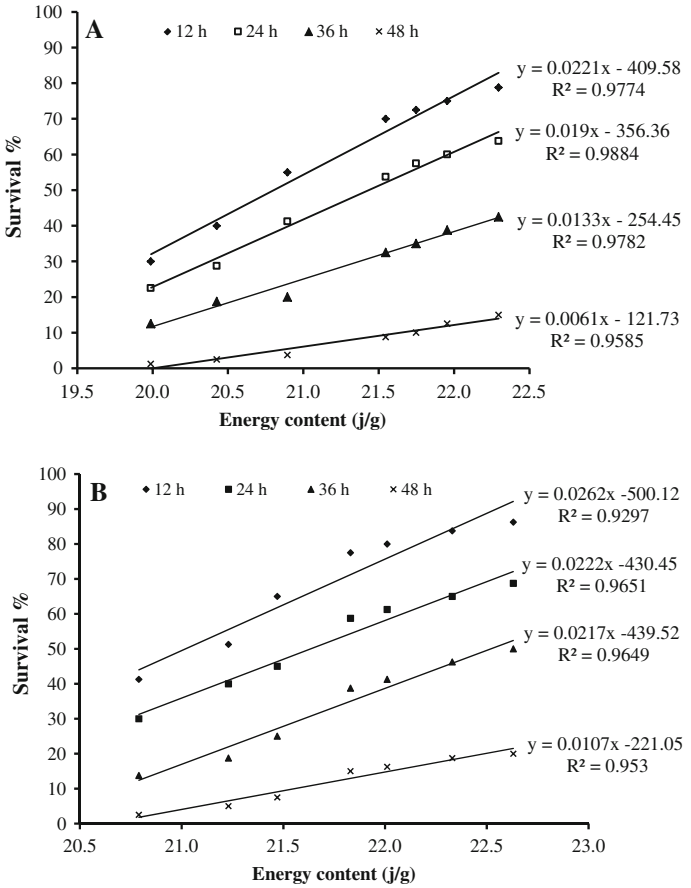


**Fig. 4** **a** Linear regression between survival % (after 12, 24, 36, and 48 h) and energy content (J $g^{-1}$ DW) of GSL instar I nauplii after exposure of the cysts to various H/D cycles. **b** Linear regression between survival % (after 12, 24, 36, and 48 h) and energy content (J $g^{-1}$ DW) of VC instar I nauplii after exposure of the cysts to various H/D cycles

This study aimed to gauge to what extent cyclic H/D exposure of cyst samples from two commercially important *A. franciscana* strains (Great Salt Lake, USA, and the San Francisco Bay-type Vinh Chau, Vietnam) resulted in quality loss as assessed by aquaculture-relevant parameters such as hatching, longevity, and nutritional quality of nauplii.

The untreated cyst samples used in our study were in quiescence, as illustrated by the high hatching percentage (>90 %). The hatching quality of the cysts was negatively affected when cysts were stored after exposure to a succession of H/D cycles, and this effect aggravated as the number of cycles increased from one to three, and as the hydration period, preceding dehydration, was lengthened from 2 to 4 h, finally resulting in a loss of hatching in the range 50–60 % of the control value. The loss as a result of multiple H/D cycles was more marked when a hydration period of 4 h was used, as compared with hydration during 2 h. The difference in water content of the VC and GSL control sample (34.8 vs. 4.5 %, respectively) did not affect these results: after incubation in seawater, cysts absorb water at a fast rate, reaching a maximal water content of 140 % of their dry weight after 1.0–1.5 h at 28.0 °C, as described by (Lavens and Sorgeloos 1987).

A similar effect was found for the axenically hatched nauplii with survival negatively affected by increasing H/D cycles, longer hydration incubation, and prolonged starvation over a 48-h observation period, as energy reserves were gradually depleted (Benijts et al. 1976). Analogously, the individual energy content, being maximal in the control cysts, gradually declined as the number of H/D cycles and the duration of the hydration period increased, corresponding with energy consumption during the repeated hydration process (Morris 1971; Vanhaecke and Sorgeloos 1982; Vanhaecke et al. 1983). In both strains, hydration for 4 h resulted to more than three times (3.5–3.8 times) the energy loss resulting from hydration for 2 h, illustrating that as hydration continues, metabolism intensifies and energy consumption proceeds at a faster rate (Lavens and Sorgeloos 1987). On the other hand, doubling or tripling the H/D cycle did not result in an accordingly double or triple energy loss. This suggests that after the first cycle, a number of metabolic mechanisms have been initiated, which are not repeated during the following cycles. The similarity in energy consumption during the successive H/D cycles, observed in both strains, suggests that the underlying mechanisms are common within the species *A. franciscana* and possibly within the genus *Artemia.* Nevertheless, as energy content in the cysts dropped, cyst hatching and naupliar survival decreased faster in VC than in GSL, as reflected in the slope of the regression curves (Figs 3b, 4b), indicating that VC cysts are more sensitive to improper storage conditions than GSL, which may be linked to their smaller size (generally with a diameter of approximately 225 μm for VC, as compared to about 250 μm for GSL cysts (Vanhaecke and Sorgeloos 1980; Dhont and Sorgeloos 2002).

Comparative literature information on the fatty acid contents of cysts and the nauplii emerging from them is scarce (Garcia-Ortega et al. 1998; Dhont and Sorgeloos 2002), and no information is available on the fatty acid metabolism involved in the hatching process. Our FAME results of control decapsulated cysts and nauplii of the VC and GSL strains, and the differences in dominating fatty acids between both strains, are similar to the literature data published for those strains (Evjemo et al. 1997; Garcia-Ortega et al. 1998; Dhont and Sorgeloos 2002; Ando et al. 2002). The effect of hydration and subsequent dehydration on the fatty acid contents of cysts has not been the subject of systematic studies in the past; in our study, differences between the control and A222 samples were limited, which suggests that net fatty acid breakdown during the hydration/dehydration process is limited. This is also illustrated by the similarity between the data of fatty acid contents reported in the literature by various authors for (GSL or VC) samples, which may have a widely diverging history of exposure to hydration and dehydration prior to analysis.

In general, fatty acid analysis showed that the two populations of *Artemia* from Vinh Chau and from Great Salt Lake are essentially different in fatty acid composition, especially in terms of EPA (20:5n-3) and linolenic acid (18:3n-3) contents. Variations in cyst fatty acid profile are generally linked to the characteristics of the phytoplankton population as food source for the maternal population (Navarro and Amat 1992, Navarro et al. 1992; Zhukova et al. 1998; Thinh et al. 1999, Torrentera and Dodson 2004), though also other environmental parameters, such as ambient temperature, and genetic factors may have an effect (Ruiz et al. 2007, 2008; Nguyen Thi Hong Van, unpublished results).

In order to meet the nutritional requirements of especially marine fish and shellfish larvae, enrichment of *Artemia* metanauplii is a standard procedure in many hatcheries when using the HUFA-deficient GSL strain. HUFA levels post-enrichment thus overwhelm the levels of the freshly hatched nauplius, or of the metanauplius having gone

through some period of starvation. Enrichment is not applied for the VC strain, however, which contains relatively high levels of highly unsaturated fatty acids. Moreover, strict hatching procedures (e.g., harvesting of nauplii after a hatching incubation period of 24 h) generally reduce the risk of starved (meta) nauplii being fed to (shell) fish larvae.

Vitamin C levels were conforming to the conversion of vitamin C from ascorbic sulfate into ascorbic acid during completion of the embryonic development into the nauplius stage (Dabrowski 1991; Golub and Finamore 1972; Nelis et al. 1994) and comparable to the range (162–428 μg $g^{-1}$ DW) reported by (Dabrowski 1991) for cysts. The variation in vitamin C contents found between cysts of different geographical origin (in the range 296–517 μg $g^{-1}$ DW expressed as ascorbic acid), its conversion into free ascorbic acid during the hatching process, and the role of ascorbic sulfate as storage form has been studied in detail (Mead and Finnamore 1969; Merchie et al. 1995). The nutritional quality of the A222 cysts and nauplii was lower than in the control, as shown by reduced fatty acid and vitamin C levels. In the case of vitamin C, this loss amounted in both strains up to 62–69 % for A222 cysts as compared to the control, whereas fatty acid losses were generally in the order of few percentages.

Knowledge of hatching characteristics in *Artemia* samples is important due to the reported variability among batches and strains. The nutritional quality in *Artemia* varies considerably as well, in relationship with its geographical origin (Léger et al. 1986). (Vanhaecke and Sorgeloos 1982) reported that the poor hatchability of commercial batches of *Artemia* cysts can be linked to improper processing of the cysts after collection in nature, and that long-term storage of such material may result in a further substantial decrease in hatching success. In natural conditions, cysts may be exposed to H/D cycles, as they are floating driven by wind and currents, and may temporally or definitively accumulate on the shore where they undergo the fluctuations of atmospheric conditions. Harvesting of good-quality cyst product requires collection of recently produced cyst batches from the open water shortly followed by adequate processing, but these conditions are seldom fulfilled, especially when harvesting natural production in inland lakes, when site accessibility is limited, timing of harvesting is irregular, transport and storage is an issue, and/or overall expertise is lacking. In *Artemia* pond production, such as in the Mekong Delta, Vietnam, frequent harvesting (up to 2–3 times a day) followed by adequate processing is currently done, which contributes to the good quality of the resulting cyst product (Anh et al. 2009).

Our results confirm that cyst metabolism, as initiated after hydration, is to a certain degree reversible and that cysts can be converted from a hydrated, metabolically active mass of cells into a dehydrated, ametabolic state (and vice versa). They also show that repeated H/D cycles not only result in a decreased hatching output, but also in an inferior quality of those nauplii hatching, as quantified in our study by naupliar longevity, energy, FAME, and vitamin C content. These observations are of fundamental importance in understanding the cysts quality and they have significant potential for application in aquaculture.

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