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Use of the brine shrimp, *Artemia* spp., in marine fish larviculture

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Abstract

Since no artificial feed formulation is yet available to completely substitute for *Artemia*, feeding live prey to young fish larvae still remains essential in commercial hatchery operations. The nutritional quality of commercially available *Artemia* strains being relatively poor in eicosapentaenoic acid (EPA, 20:5*n*-3) and especially docosahexaenoic acid (DHA, 22:6*n*-3), it is essential and common practice to enrich these live prey with emulsions of marine oils.

In *Artemia*, the most commonly applied boosting technique is a 24-h enrichment period after hatching. However, the variability of enrichment studied in one *Artemia* strain (Great Salt Lake, Utah, USA) by the ICES Working Group on Mass Rearing of Juvenile Fish, showed a high variability in fatty acid bioaccumulation under laboratory or commercial conditions. To avoid the variation originating from differences in commercial preparations, standardized ICES emulsions with different HUFA and DHA/EPA ratios have been formulated and are available for research purposes. It should be emphasized, however, that the enrichment technique has limitations as *Artemia* are selectively catabolizing some of the nutrients such as DHA and phospholipids. Research on the kinetics of DHA catabolism in various *Artemia* strains has shown that DHA catabolism is strain-dependent and could partially be overcome by the use of strains of different geographical origin.

Nowadays, various enrichment emulsions have been formulated differing in the fatty acid composition of their triglycerides. In this respect, the traditional formulations rich in EPA have been replaced by new products rich in DHA and arachidonic acid. To reduce the risks for oxidation of these fatty acids, higher concentrations of vitamin E are incorporated into the emulsions. Also, vitamin C has been incorporated in booster formulations that increase the level of ascorbic acid in *Artemia* to 2000 ppm.

All these changes in the formulation of the enrichment diets offer more possibilities to cover the needs of different species and help to reduce problems related to diseases, stress resistance, malformation, and pigmentation in numerous fish species.

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Although continuous disinfection of *Artemia* during hatching and enrichment is becoming a routine operation in many hatcheries, the interference of bacteria in hatching and enrichment remains an important study object for which probionts might also give some solutions.

As more attention is given to the use of on-grown *Artemia* as a cheaper alternative to the use of nauplii, simple cost-effective production techniques have been developed. The use of the right size of on-grown *Artemia* for feeding ensures a better energy balance in food intake and assimilation, thereby improving the performance of the fish. Furthermore, its palatability induces a good and fast feeding response. These characteristics, coupled with the use of bioencapsulation techniques to enhance the quality of the on-grown *Artemia*, make this organism an optimum diet for nursery of the fish.

Keywords: Live food; Brine shrimp; Hatching; Enrichment; HUFA; Vitamin C

1. The dependence of marine fish culture on *Artemia*

Since the development of commercial marine fish culture in the late 1970s, the demand for *Artemia* cysts has gradually increased from a few metric tons to approximately 800 metric tons per annum, representing approximately 40% of the total aquaculture demand for feeds for early stages. During the last 25 years, the Great Salt Lake (GSL) has been the premier supplier of *Artemia* cysts to the world aquaculture market and the subject of numerous speculations regarding its capacity to sustain a growing aquaculture industry (Lavens and Sorgeloos, 2000).

Major problems in *Artemia* production began after an intense El Niño in 1982–1984, causing heavy snowfall and a huge amount of melting water from the bordering mountains and resulting in a considerable drop in salinity. The north arm, which is not in direct contact with rivers or supplied by melting water, was separated from the south arm by a causeway in the 1950s and was thus less exposed to dilution and could at that time be used as the main source of *Artemia* cysts. After the historic lake height in 1987, 6 years of decline began in the lake elevation which brought the salinity of the south arm back to ranges suitable for *Artemia* cyst production. The recent El Niño phenomenon would again be the origin of the present salinity decrease. The current salinity of the south part of the lake is now in the 70–80 ppt range, while the optimal salinity for *Artemia* production is in the range of 100–150 ppt. Because of hardly any water exchange with the north arm, the latter has remained close to saturation level.

As the salinity continues to decline, the brine shrimp industry has been faced with a drastic decline in harvest figures. Moreover, a much higher percentage of empty shells and lysed cysts were gathered, which in turn decreased recovery yields to half and even one third. The 1999–2000 harvest was a record low. Total GSL harvest will not even satisfy 20% of the global demand and previous year's stocks have been sold.

The situation at GSL will affect the aquaculture industry as a whole. It has also caused a considerable burden to the brine shrimp industry, and several harvesting companies have had to quit. Before this, close to 40 companies were fishing with a fleet in excess of 200 boats for landing too little product. Because of investing more in harvesting for fishing fewer tons at the end, costs per unit had escalated tremendously. Although warnings about the risk of dependency on one single natural (and unpredictable) resource had been issued at repeated occasions (Bengtson et al., 1991), it was

only as of 1994 that the harvesting companies became concerned about the variable yields from the Great Salt Lake, and started to diversify their activities in *Artemia* harvesting by setting up new *Artemia* exploitations all over the world. The increased production and investment cost for exploring new locations have boosted the *Artemia* prices, but despite this price increase, *Artemia* cysts remain scarce and cyst producers can hardly cope with the constantly increasing demand of the rapidly growing aquaculture industry.

Aside from the extensive efforts that need to be carried out in the field of diversification of *Artemia* sourcing and harvest efficiency, its is obvious that an at least equal amount of attention has to be dedicated to a more efficient use of the available *Artemia* sources as well as to non-*Artemia* alternatives. The search for complete substitutes for *Artemia* has been extensive and is still ongoing. Total replacement will certainly be achieved before the end of the decade for numerous marine fish species, but probably at the cost of culture time, yield, health and quality that will eventually affect farm economics and sustainability.

For the time being, the dependency on *Artemia* in hatcheries could be alleviated by a delay in *Artemia* feeding (e.g., by extended rotifer feeding) and a maximum substitution with inert diets. Although *Artemia* will, in the future, undoubtedly be further replaced by formulated diets, it is obvious that the use of freshly hatched nauplii will continue to be market-driven for at least a few more years, and that record harvests at Great Salt Lake (as happened already during the 2000–2001 harvest season, i.e., some 9,000 metric tons of fresh product) and new locations might very quickly reverse the actual trends.

2. Production and use of freshly hatched nauplii

Although using *Artemia* cysts appears to be simple, several factors are critical for hatching the large quantities needed in larval fish production. These include cyst disinfection or decapsulation prior to incubation, and hatching under the following optimal conditions: constant temperature of 25–28 °C, 15–35 ppt salinity, minimum pH of 8.0, near saturated oxygen levels, maximum cyst densities of 2 g/l, and strong illumination of 2000 lx (Lavens and Sorgeloos, 1996). All these factors will affect the hatching rate and maximum output, and hence, the production cost of the harvested *Artemia* nauplii. Especially now that the availability of Great Salt Lake *Artemia* is not stable, *Artemia* harvested from other locations will be subjected to variable quality. Attention should be paid to select *Artemia* cyst lots with good hatching synchrony (less than 7 h between hatching of first and last nauplii) and high hatching efficiency (more than 200,000 nauplii per gram product), as considerable variation has been demonstrated for cysts of various origin, and even among batches from the same strain (Lavens and Sorgeloos, 1996). Nowadays, slower hatching batches of *Artemia* cysts from RH® (INVE Aquaculture, Belgium) are in the market but with perfect synchrony in hatching, which make harvesting, enrichment and feeding procedures very easy to control and result in a homogenous and high enrichment.

After hatching, and prior to feeding them to the larvae, *Artemia* nauplii should be separated from the hatching wastes. After switching off the aeration in the hatching tank, cyst shells will float and nauplii will concentrate at the bottom of the tank. They should

be siphoned off within 5–10 min and thoroughly rinsed with seawater or freshwater, preferentially using submerged filters (Sorgeloos and Léger, 1992) to prevent physical damage to the nauplii. On a commercial scale, the separation of nauplii from cyst shells is performed with a standpipe perforated a few centimeters from the bottom. The free-swimming nauplii on top of the unhatched cyst are evacuated through the perforation, while the unhatched cysts are kept out of the turbulent area. The nauplii are further concentrated in a concentrator rinser and separated from the last cysts on a double screen. When decapsulated cysts are used, the membranes are generally skimmed off by the use of high performant airstones.

3. Size and energy content

In their first stage of development, *Artemia* nauplii do not feed but consume their own energy reserves (Benijts et al., 1976). At the high water temperatures that are applied during cyst incubation, freshly hatched *Artemia* nauplii develop into the second larval stage (Instar II metanauplii) within 6–8 h. It is important to use first-instar nauplii for feeding, rather than starved second-instar metanauplii, which are transparent and less visible. Instar II metanauplii are about 50% larger in length and swim faster than first instars. As a result, they are less acceptable as prey. Furthermore, they contain lower amounts of free amino acids, so they are less digestible and their lower individual dry weights (1.63 versus 2.15 μg in the San Francisco Bay, SFB, strain) and energy content (0.0366 versus 0.0500 J in the same strain) reduce the energy uptake by the predator per unit of hunting effort (Léger et al., 1986). All this will be reflected in reduced larval growth in the face of increased *Artemia* cyst consumption (20–30% more cysts are needed to feed the same weight of starved metanauplii to the predator).

Storing freshly hatched nauplii at temperatures near 4 °C, in densities of up to eight million nauplii per liter for up to 24 h (Léger et al., 1983), will greatly reduce their metabolic rate, i.e., only 2.5% drop in individual dry weight versus 30% at 25 °C, and preclude molting to the second instar stage. This 24-h cold storage economizes the *Artemia* cyst hatching effort (e.g., fewer tanks, larger volumes, a maximum of one hatching and harvest per day) and allows not only a constant supply of a high-quality product but also the possibility of more frequent food distributions. This is beneficial for fish larvae because food retention time in larviculture tanks can be reduced and hence the growth of *Artemia* in the culture tank minimized.

4. Nutritional quality

4.1. Fatty acid enrichment

In the late 1960s and early 1970s, several authors reported problems in larviculture success with marine fish and crustacean species when using *Artemia* sources other than SFB *Artemia* (for reviews see Sorgeloos, 1980 and Léger et al., 1986). High doses of toxic compounds, e.g., chlorinated hydrocarbons and heavy metals, were initially suspected to be the cause of the poor nutritional value of *Artemia* from GSL and the

People's Republic of China. A comparative study with eight strains of *Artemia* spp. using *Pseudopleuronectes americanus* as predator test species confirmed the nutritional variation among *Artemia* sources (Klein-MacPhee et al., 1980, 1982). Léger et al. (1985a) documented the nutritional variability in 11 batches of SFB *Artemia* nauplii for the mysid shrimp *Mysidopsis bahia*. Similar to findings by Watanabe et al. (1978) and Kanazawa et al. (1979) in marine fish, Léger et al. (1985b, 1987a) concluded that the main factor affecting the nutritional value of *Artemia* was the content of the highly unsaturated fatty acid (HUFA) eicosapentaenoic acid, 20:5*n*-3 (EPA).

Taking advantage of the primitive feeding characteristics of *Artemia* nauplii, it is possible to manipulate the nutritional value of HUFA-deficient *Artemia*, e.g., the GSL strain. Since brine shrimp nauplii that have molted into the second instar stage (i.e., about 8 h following hatching) are non-selective particle feeders, simple methods have been developed to incorporate different kinds of products into the *Artemia* prior to feeding to predator larvae. This method of "bioencapsulation", also called *Artemia* enrichment or boosting, is widely applied in marine fish and crustacean hatcheries for enhancing the nutritional value of *Artemia* with essential fatty acids.

British, Japanese, and Belgian researchers developed enrichment products and procedures using selected microalgae, micro-encapsulated products, yeast, emulsified preparations, self-emulsifying concentrates, and micro-particulate products, either singly or in various combinations (Léger et al., 1986). The highest enrichment levels are obtained from emulsified concentrates: freshly hatched nauplii are transferred to the enrichment tank at a density of 100–300 nauplii/ml for enrichment periods >24 or <24 h, respectively. The enrichment medium consists of hypochlorite-disinfected and neutralized seawater maintained at 25 °C. The enrichment emulsion is added in consecutive doses of 0.3 g/l every 12 h. Strong aeration using airstones or pure oxygen is required to maintain dissolved oxygen levels above 4 ppm. Enriched nauplii are harvested after 24 or 48 h, thoroughly rinsed and stored at temperatures below 10 °C to assure that HUFAs are not metabolized during storage. Enrichment levels of 50–60 mg/g DW *n*-3 HUFAs are obtained after 24-h enrichment with the emulsified concentrates. Nauplii should be transferred or exposed to the enrichment medium as soon as possible before first feeding, so they begin feeding immediately after the opening of the alimentary tract (instar II stage). As a result, the increase of nauplius size during enrichment can be minimized, i.e., after 24-h enrichment, GSL *Artemia* nauplii will reach about 660 μm, and after 48-h enrichment, about 790 μm (Lavens and Sorgeloos, 1996).

In the 1980s, most attention was dedicated to the presence of EPA in *Artemia* as a guarantee for successful production of marine fish larvae (Watanabe et al., 1983; Léger et al., 1985a). Because of this, major emphasis was placed on increasing the EPA levels by using algae, or emulsified and particulate enrichment products (Léger et al., 1986). In the late 1980s and early 1990s, more attention was paid to the level of DHA; good survival appeared to be correlated with EPA, but DHA improved larval quality and growth (Lisac et al., 1986). The importance of DHA, more particularly the requirement for high DHA/EPA ratios in promoting growth, stress resistance, and pigmentation, was revealed (Lavens et al., 1995; Kraul, 1993; Reitan et al., 1994; Mourente et al., 1993). While in the past, satisfactory results were obtained with DHA/EPA ratios of less than 1, the emphasis now is on attaining levels of 2 and higher (Dhert et al., 1993; Sargent et

al., 1993). Since these values are not found naturally in *Artemia* (Dhert et al., 1993; Triantaphyllidis et al., 1995), special formulations and *Artemia* with low DHA-catabolizing activity had to be identified.

Besides the emulsions, spray-dried cells of *Shizochytrium* sp. and phospholipid extracts of DHA-rich algal biomass from *Crypthecodinium* sp. containing 49% DHA and less than 0.5% EPA are available as dry powder (Harel et al., 1998). Enrichment of *Artemia* with these products allowed a significant increase in nauplii–lipid content from 16.3–23.7% DW after 16 h, and 17% of total fatty acids contained DHA.

Contrary to other live feeds, such as rotifers, the enrichment of *Artemia franciscana* with DHA is difficult because of the inherent catabolism of this fatty acid upon enrichment resulting in low DHA/EPA ratios. The capability of some *Artemia* strains to reach high DHA levels during enrichment (Dhert et al., 1993; Velazquez, 1996) and to maintain them during subsequent starvation (Evjemo et al., 1997) offers new perspectives for providing higher dietary DHA levels and DHA/EPA ratios to fish larvae. It is not surprising that since the interest is going towards DHA-rich products, increased interest is also shown in better preservation of DHA in *Artemia* (McEvoy et al., 1995; Southgate and Lou, 1995) or in the use of copepods, which have a high natural DHA content (Nanton and Castell, 1999; Sargent et al., 1998; Shansudin et al., 1997; Støttrup and Norsker, 1998). As a consequence of the high price and low DHA value of *Artemia*, early weaning in combination with a prolonged rotifer feeding is becoming more and more popular.

Recent work performed by Koven et al. (2000) showed that besides DHA not only highly unsaturated fatty acids of the (*n*-3) series are important but that also arachidonic acid ARA (20:4*n*-6) may play a significant role. ARA may improve larval growth and pigmentation in several marine fish species since it provides precursors for eicosanoid production (Castell et al., 1994; Estevez et al., 1997). The requirement of ARA in fish, however, seems to depend on the fish species and larval development, and needs to be dosed with extreme care since it may act in a different way depending on the DHA concentration (Castell et al., 1994; Koven et al., 2000).

4.2. Phospholipids

Although phospholipid requirements are well-documented in juvenile stages for various fish species, only limited information is available on the role of phospholipids in start-feeding stages (reviewed by Coutteau et al., 1997). As shown by Tackaert et al. (1991), *Artemia* does not appear to be a suitable test diet to study phospholipid requirements; i.e., dietary enrichment with phosphatidylcholine (PC) did not enhance the PC content in *Artemia*. Rainuzzo et al. (1994) found similar lipid composition in *Artemia* enriched with an emulsion based on either ethyl esters or halibut roe, containing, respectively, 72.6% neutral lipids (mainly ethyl esters) and 71.2% polar lipids (mainly PC and phosphatidylethanolamine, PE). Still, limited shifts of lipid classes, e.g., PC/PE ratio (Rainuzzo et al., 1994), due to enrichment of *Artemia* are poorly documented and their significance in terms of nutritional value unknown. In an effort to determine the most effective molecular carrier of DHA for *Artemia*, DHA-ethyl esters were compared with DHA-containing phospholipids. Harel et al. (1998) found signifi-

cantly higher absorption of DHA at 10% dietary phospholipid levels compared to 5%, while no further improvement in absorption was obtained at higher phospholipid percentages. In further studies, it was observed that mixtures of phospholipids with DHA sodium salts resulted in maximal absorption of DHA phospholipids in *Artemia* (Harel et al., 1999) and may be used to increase the polar lipid content in larval live food.

4.3. Vitamins

Vitamin C, more specifically ascorbic acid (AA), is generally considered to be an essential dietary component for the various stages of aquaculture organisms (Merchie et al., 1997). Several biological (e.g., skeletal development, growth, survival) as well as physiological functions (e.g., resistance to toxicants and stress, immunoactivity) are enhanced in larvae from supplemental dietary ascorbate (Dabrowski, 1992; Merchie et al., 1996). Ascorbic acid 2-sulphate (AAS), a stable derivative of AA, was discovered in dormant cysts of *Artemia* by Mead and Finamore (1969). Cysts of various batches differed considerably in AAS content: 160–517 μg/g DW, expressed as AA (Dabrowski, 1991; Merchie et al., 1995). The amount of AA liberated in freshly hatched nauplii reflects the AAS reserve present in the cysts and provides evidence for the conversion of AAS to free AA during completion of embryonic development into nauplii (Golub and Finamore, 1972; Dabrowski, 1991; Nelis et al., 1994).

The variation in AAS concentration observed in *Artemia* cysts may reflect adult nutrition during egg production, as was demonstrated for HUFA content (Lavens et al., 1989), and this may explain the differences among batches of the same strain (Merchie et al., 1995). Differences among geographical populations and *Artemia* species and broods from different years may significantly influence the AAS content in the cyst material, and thus the AA levels in freshly hatched nauplii, and consequently, their nutritional value for larval fish.

Tests have been conducted to incorporate extra AA into *Artemia* nauplii in a stable and bioavailable form. Applying a standard enrichment procedure (Léger et al., 1987b) and experimental self-emulsifying concentrates containing 10–20% ascorbyl palmitate (AP), levels up to 2.5 mg free AA/g DW can be incorporated into brine shrimp nauplii within 24 h (Merchie et al., 1995). These concentrations did not drop when the 24-h enriched nauplii were stored for another 24 h in seawater at 28 °C or 4 °C. When vitamin C-enriched *Artemia* were fed to turbot larvae, no differences in growth or overall survival could be detected with the non-enriched live food already containing 500 μg AA/g DW. The larvae of the high AA treatment, however, showed a better pigmentation rate compared to the control group. Evaluation of the physiological condition applying a salinity stress test revealed an improvement by feeding extra AA. Cumulative mortalities after challenge with *Vibrio anguillarum* amounted to 50% for the control versus 40% for the AA-supplemented fish, with a slower onset of mortality for the AA-fed fish (Merchie et al., 1996).

High levels of α-tocopherol can be bioaccumulated and maintained in *Artemia* nauplii, making this live food delivery system useful for studying dietary requirements as well as the antioxydative effects of vitamin E (Huo et al., 1996).

Vitamin A levels in *Artemia* nauplii could be raised from 1.3 to 1283 IU/g DW over an 18-h period through the addition of vitamin A palmitate to an egg-yolk-based emulsion (Dedi et al., 1995).

The effectiveness of *Artemia* nauplii as a dietary carrier system could be tested for various other nutritional components, i.e., liposoluble products administered via an emulsion, water-soluble compounds via liposomes (Hontoria et al., 1994), and/or microcapsule delivery (Sakamoto et al., 1982). For each nutrient, however, the usefulness of the *Artemia* bioencapsulation method remains to be verified by chemical analysis.

5. Other forms of *Artemia*

Aside from the most common regime of feeding freshly hatched and/or 24-h-enriched nauplii, the use of dry decapsulated cysts, juveniles and adult biomass is practiced with some freshwater fish species (Dhert et al., 1997; Vanhaecke et al., 1995; Verreth and Den Bieman, 1987). Decapsulated cysts (also called de-shelled or shell-free cysts) can be used in start-feeding; however, the rapid settling of the cysts can make them unavailable for planktonic larvae unless they have been previously dried and float on the water surface. The major advantage here might be, apart from being a directly available off-the-shelf product, that cysts with poor hatching quality can still be used as a food source. Especially in the ornamental fish industry, the product seems to have great potential, where it is producing larvae of superior quality (Dhert et al., 1997).

Depending on the objectives and the opportunities, different culture procedures for super-intensive *Artemia* production may be applied. The final selection of one or another type of installation will be subject to local conditions, production needs, and investment possibilities. Two basic options are: should water be renewed (open flow-through) or not. In the latter case, should a particular water treatment be applied (closed flow-through) or not (stagnant or batch system). Obviously, there are all kinds of transition types, ranging from open flow-through with 0% recirculation to closed flow-through with 100% recirculation (Dhont, 1996). *Artemia* biomass is generally well accepted by all marine fish. Kim et al. (1996) found that coho salmon fry fed adult *Artemia* to excess grew significantly faster than fry fed on other test diets, mainly due to increased food intake. Most of the time, however, juvenile *Artemia* are used instead of adults just before weaning (Olsen et al., 1999; Lee and Litvak, 1996; Duray et al., 1996; Kraul, 1993).

Although the fresh–live form has the highest nutritive value, harvested *Artemia* can also be frozen, freeze-dried or acid-preserved (Abelin et al., 1991; Naessens et al., 1995) for later use, or made into a flakes or other forms of formulated feed.

6. Control on the bacterial input from cysts and nauplii

Chemical decapsulation of *Artemia* cysts using hypochlorite is a widely applied technique in hatcheries. Besides its advantages for zootechnical reasons (easier separa-

tion, prevention of gut obstruction, direct use of decapsulated cysts), it is believed to have a beneficial effect on hatching and provide a complete disinfection of the cysts. Standard decapsulation procedures, as described by Sorgeloos et al. (1977) are giving good results for most commercial *Artemia* strains. Some *Artemia* strains, however, are sensible to this decapsulation procedure and require a modified technique (De Wolf et al., 1998).

Although the treatment with hypochlorite completely disinfects the cysts, these are very quickly recolonized with bacteria during the breaking stage shortly before hatching. At this stage, glycerol is released from the cysts and offers an ideal culture medium for *Vibrio* sp., which may be a threat to the health of the larvae feeding on the *Artemia*. Innovative products and procedures have recently been introduced to disinfect *Artemia* during hatching and/or consequent enrichment, resulting in a 10,000-fold reduction of the *Vibrio*-load compared to traditional methods (Dehasque et al., 1998). The progress in the production of more hygienic *Artemia* also opens new applications for intensification. The cleaner medium results in lower oxygen demands, which permits merging of different handlings; as for instance, hatching and enrichment without intermediate harvesting, resulting in savings in labor, infrastructure and running costs.

Besides the progress in disinfection techniques, the same approach of bacterial manipulation in *Artemia* has been followed as for rotifers (Rombaut et al., 1997). Research, however, is focusing on the microbial control of the culture of *Artemia* juveniles through pre-emptive colonization by selected bacterial strains (Verschuere et al., 1997, 1999). It has been demonstrated that these bacteria prevent the development of the pathogen *V. proteolyticus* through competition for chemicals and available energy (Verschuere et al., 2000) and are harmless for the predator (Verschuere et al., in press).

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