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Manipulation of dietary lipids, fatty acids and vitamins in zooplankton cultures

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SUMMARY

1. A wide range of species that are cultivated in commercial mariculture are planktonic during at least part of their life cycle; for example, the larval stages of shellfish (shrimp and molluscs) and the live feeds (rotifers, brine shrimp, copepods) used in the larviculture of marine fish and shellfish. Over the last decades various techniques have been developed to deliver nutrients to these zooplanktonic organisms either through artificial diets or by manipulating the composition of the live prey fed to the carnivorous stages. This paper reviews the methodology that has allowed aquaculturists to gain knowledge of nutritional requirements and may offer interesting opportunities for ecologists to verify the importance of key nutrients in the natural food chain of marine as well as freshwater ecosystems.
2. Live micro-algae can be replaced partially or completely in the diet of filter-feeders such as rotifers, *Artemia*, shrimp larvae and bivalves, by various types of preserved algae, micro-encapsulated diets and yeast-based diets, whereas lipid emulsions and liposomes may be utilized to supplement specific lipid-and water-soluble nutrients, respectively. Microbound and micro-encapsulated diets have been designed to supplement live feed in the culture of micro-predators such as fish and shrimp larvae.
3. Live prey organisms, in particular rotifers and *Artemia*, can be 'bio-encapsulated' with a variety of enrichment diets to manipulate their content in certain nutrients, including ω3 highly unsaturated fatty acids (FA) and the vitamins C, A and E. Nevertheless, the enrichment techniques are not applicable for all nutrients and prey organisms. Phospholipid composition is difficult to manipulate through the diet of live feed and the enrichment of the essential FA docosahexaenoic acid (DHA) is hampered in most *Artemia* species due to the catabolism of this FA following enrichment.

Introduction

Commercial mariculture comprises a wide range of species that are planktonic during at least part of their life cycle, i.e. the larval stages of cultivated species of fish and shellfish, and a few selected species of zooplankton (copepods, rotifers, brine shrimp) that are used as live feed for the former. The natural diet of these planktonic organisms, feeding either as filter-feeders or micropredators, consists of a wide diversity of bacteria, detritus, phytoplankton and/or smaller zooplankton (Omori & Ikeda, 1994). This high diversity of food organisms of different sizes and biochemical composition provides good chances for meeting all the nutritional requirements of the larvae. Larviculture nutrition, more particularly for start-feeding early larval stages, appears to be one of the major bottlenecks for the industrial upscaling of the culture of many species of marine fish and shellfish. Collecting and feeding natural plankton is not practical on an industrial scale and natural food organisms are not consistently available. Over the past decades, trial and error approaches have resulted in the adoption of selected larviculture diets, various species of

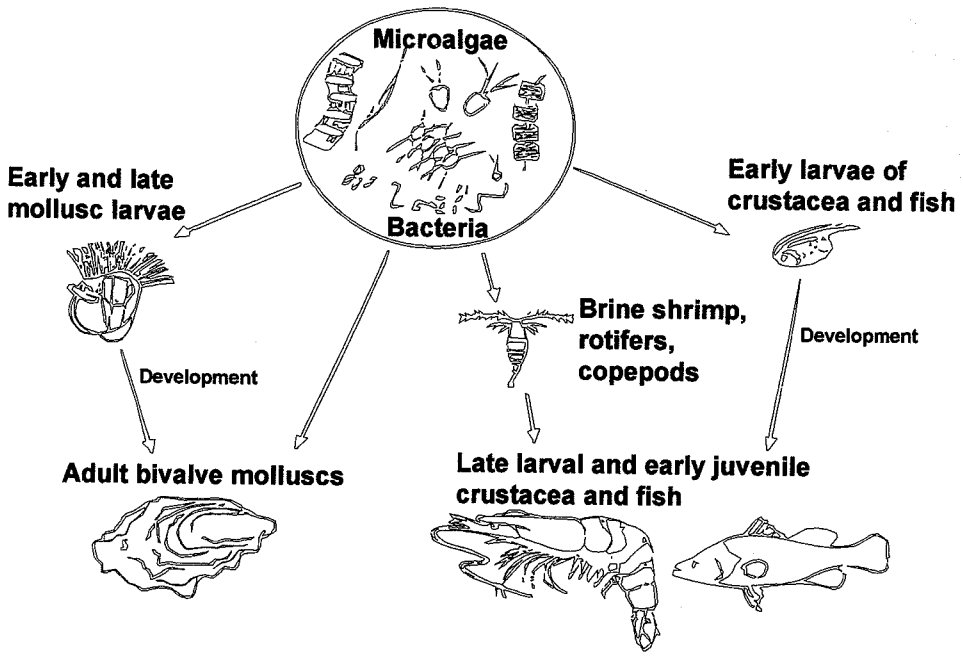

Fig. 1 Live food chains used in marine aquaculture to feed larval and post-larval stages of fish, crustaceans and molluscs (modified from Brown *et al.*, 1989).

micro-algae, the rotifer *Brachionus plicatilis*, and the brine shrimp *Artemia* (Fig. 1). These live feeds are preferred over natural plankton because of their availability, cost-efficiency and simplicity, as well as versatility in application (Brown, Jeffrey & Garland, 1989; Sorgeloos & Léger, 1992; Lavens *et al.*, 1995b). Nevertheless, cultivation and management of live feeds in hatcheries of fish and shellfish remain costly. Often, only suboptimal nutrition is provided, as can be expected from the significant differences in physical and biochemical characteristics compared with the natural diet. This has resulted in a continuous search for improving live feeds and has encouraged the development of cheaper formulated diets, which can supplement or, in some cases, completely replace the live diets (Sorgeloos & Léger, 1992; Jones, Kamarudin & Le Vay, 1993).

A range of techniques has been developed to manipulate and/or supplement live food organisms. This improved nutrition has resulted in significant progress in larval culture performance for many cultured species (Sorgeloos & Léger, 1992). Some of the techniques that allow the aquaculturist to gain knowledge of nutritional requirements may offer interesting opportunities for the ecologist to verify the importance of key nutrients in the natural food chain. Rather than review the nutritional requirements of larval stages of aquaculture species (see reviews by Léger *et al.*, 1986; Bengtson, Léger & Sorgeloos, 1991; Coutteau, Geurden, Camara et al., 1997; Merchie, Lavens & Sorgeloos, 1997; Rainuzzo, Reitan & Olsen, 1997; Sorgeloos *et al.*, 1997), this paper covers the various tools and approaches which are utilized in marine aquaculture to deliver nutrients to zooplanktonic stages. The various types of artificial diets that have been developed to supplement and/or replace live feed organisms are reviewed and their advantages and disadvantages discussed. Furthermore, an overview of the progress made and the limitations encountered in dietary manipulation of lipids and vitamins in zooplankton for use in larviculture nutrition is presented.

Artificial diets for the delivery of nutrients to zooplankton

The high production costs as well as temporal variations in availability and food value of live food still pose problems for any aquaculture operation that depends on mass-cultures of unicellular algae, rotifers and/or *Artemia*. In order to overcome or reduce these limitations, various investigators have attempted to replace live food by using artificial diets, either as a supplement or as the main food source (see review by Jones *et al.*, 1993). Optimal food particle size for efficient ingestion is species-and stage-specific in the range of 5–100 μm and 40–1000 μm for filter-feeders and carnivorous zooplankton, respectively (Jones & Gabbott, 1976). Furthermore, artificial diets must comply with the basic criteria of aquafeeds: maximum availability in the water column, minimal leaching of nutrients, palatability for predators, acceptable digestibility and balanced nutritional composition. The replacement of live micro-algae in the cultivation of mostly herbivorous zooplankton, i.e. filter-feeders such as rotifers, *Artemia*, larvae of crustaceans and bivalves, has been achieved with variable success by the supplementation of preserved algae (algal paste: Donaldson, 1991; Nell & O'Connor, 1991; spray-dried heterotrophic algae: Biedendach, Smith & Lawrence, 1990; Laing, Child & Janke, 1990; Gladue, 1991), micro-encapsulated diets (Langdon & Siegfried, 1984; Laing, 1987), and yeast-based feeds (Coutteau *et al.*, 1992, 1994a). Live feeds have been substituted in primarily carnivorous planktonic stages of shrimp and prawn by microbound (Teshima, Kanazawa, & Sakamoto, 1982) and micro-encapsulated diets (Levine, Sulkin & Van Heukelem, 1983; Jones, Kurmaly & Arshard, 1987). The advantages and disadvantages of the various types of diets for the delivery of nutrients to zooplankton are discussed in the following section.

Because of its suitable particle size and high stability in the water column, yeast can easily be removed from suspension and ingested by filter-feeding organisms. Furthermore, as opposed to most of the other alternatives for live algae, yeasts can be mass produced at a relatively low cost. The successful application of yeast-based diets as algal replacement diets has been demonstrated for rotifers (Fukusho, Arakawa & Watanabe, 1976; Olsen *et al.*, 1993; Lavens *et al.*, 1995b) and some species of penaeid shrimp (Léger & Sorgeloos, 1992). Nevertheless, a low nutritional value of yeast has been reported for various species of filter-feeders because of nutritional deficiencies and/or indigestible cell walls (rotifers: Hirayama & Funamoto, 1983; *Artemia*: Johnson, 1980; bivalves: Epifanio, 1979; penaeid shrimp: Mock, Revera & Fontaine, 1980). Coutteau, Lavens & Sorgeloos (1990) demonstrated that the digestibility of baker's yeast could be improved for *Artemia* by treating the yeast with sulfhydryl compounds, which render the yeast cell wall more permeable to the digestive enzymes of the brine shrimp. Using ^{14}C tracer techniques, it was shown that the assimilation efficiency of *Artemia* fed at saturating concentrations of baker's yeast, increased from 23.8 to 76.4% because of such a treatment (Coutteau, 1992).

The main problems arising from the use of microparticulate feeds are particle settling, clumping and bacterial degradation, low digestibility and excessive nutrient leaching promoted by the high surface area to volume ratio of the particles. Microbound diets, consisting of dietary ingredients embedded in a gelled hydrocolloid matrix such as carrageenan or alginate, have been used in various nutritional studies with larval shrimp (see review by Teshima, Kanazawa & Koshio, 1993). Their stability while suspended in water appears to be limited (Lopez-Alvarado *et al.*, 1994). Through micro-encapsulation techniques, dietary ingredients can be encapsulated within digestible capsules and delivered to suspension-feeders without losses of nutrients to the aqueous medium (Teshima *et al.*, 1982; Jones, Holland & Jabborie, 1984; Langdon, Levine & Jones, 1985; Fig. 2). Macromolecular compounds such as proteins, carbohydrates and water-insoluble particles can be incorporated and effectively retained in protein-walled microcapsules (Gabbott, Jones & Nichols, 1976; Jones *et al.*, 1976; Langdon, 1983) and calcium alginate microgel particles (Levine *et al.*, 1983; Langdon & Bolton, 1984) made by interfacial crosslinking of protein in an organic solvent and ionic gelation of plant hydrocolloids, respectively. However, due to the permeability of the latter capsule walls, low-molecular weight compounds are rapidly released upon exposure to water (Lopez-Alvarado *et al.*, 1994). Only lipid-walled microcapsules (LWC), consisting of an aqueous core encapsulated within a wall of solidified lipid, are capable of retaining hydrophilic compounds such as water-soluble vitamins, free amino acids and minerals (Langdon & Siegfried, 1984; Chu *et al.*, 1987). Alternatively, gelatine-acacia microcapsules (GAC) can be filled with oil-

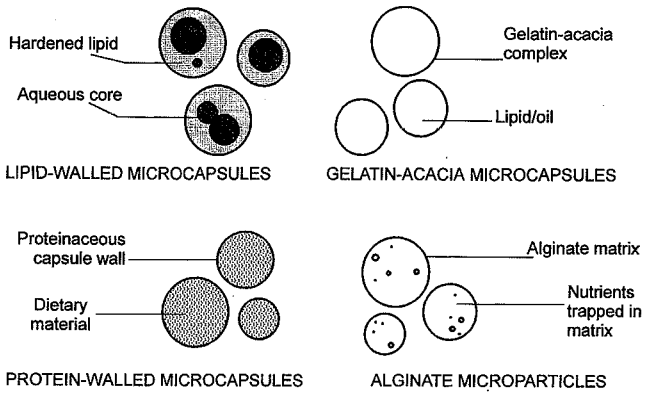

Fig. 2 Various types of artificial diets for zooplanktonic organisms : three types of micro-encapsulated diets differing in composition of the capsule wall (solidified lipid, gelatine-acacia and cross-linked protein) and encapsulated material (hydrophilic phase, lipophilic phase and complete diet mixture); and a microbound diet consisting of nutrients entrapped in an alginate matrix (modified from Southgate, 1988).

soluble nutrients and particulate material (Langdon & Waldock, 1981; Chu *et al.*, 1987; Planas *et al.*, 1990). Although LWC and GAC are not suitable to deliver complete diets, they can be used to feed specific supplements of water-and lipid-soluble nutrients, respectively. Maximal impermeability to avoid nutrient leaching and low susceptibility to bacterial attack versus high digestibility and the capability to release feeding stimulants are conflicting requirements for a capsule wall (Langdon & DeBevoise, 1990). Recently, complex microcapsules have been synthesized in which LWC containing the water-soluble micronutrients are incorporated along with the macronutrients in alginate-gelatine microparticles (Villamar & Langdon, 1993) or protein-walled microcapsules (Ozkizilcik & Chu, 1996). The latter techniques allow the release of low-molecular weight attractants while water-soluble nutrients are retained in the LWC.

The use of lipid vesicles to supplement specific nutrients has several advantages over using other synthetic microparticles; for example, their near-neutral buoyancy; suitable size range for efficient filtration by filter-feeders; and composition of non-toxic, digestible materials (Parker & Selivonchick, 1986). The protection from leaching of entrapped compounds makes liposomes particularly interesting for the delivery of water-soluble compounds to filter-feeding organisms (Fig. 3). Recent studies have confirmed the uptake by *Artemia* nauplii of maltose, glycine and water-soluble antibiotics entrapped in liposomes (Hontoria *et al.*, 1994; Ozkizilcik & Chu, 1994; Touraki, Rigas & Kastritsis, 1995). Lipid microspheres/emulsions consisting of emulsified lipid droplets provide a maximal amount of lipid per particle and constitute effective carriers for lipid-soluble nutrients. Lipid microspheres have been used extensively to manipulate the fatty acid (FA) profile of live-feed organisms such as *Artemia* and *Brachionus* (see below) and, more recently, to supplement essential FA to live algae in various life stages of bivalves (larval *Ostrea edulis* and *Mercenaria mercenaria*: Coutteau *et al.*, 1994b; juvenile *Placopecten magellanicus*: Coutteau *et al.*, 1996; adult oysters: Heras, Kean-Howie & Ackman, 1994). Furthermore, the recent findings of the importance of dietary ω3 highly unsaturated fatty acid (HUFA) in zooplankton organisms, that is, growth of *Daphnia galeata* (Müller-Navarra, 1995a, b) and reproduction of the copepod *Tisbe holothuriae* (Norsker & Støttrup, 1994), open new perspectives for the use of lipid microspheres in the study of the lipid requirements of zooplankton (Weers & Gulati, 1997; DeMott & Müller-Navarra, 1997).

Live feed manipulation

Lipid and fatty acid composition

In the late 1960s and early 1970s, several authors reported problems in larviculture success with shrimp, prawn, lobster and crab species when using *Artemia* from sources other than San Francisco Bay (SFB, California, U.S.A.) (for review see Léger *et al.*, 1986). High doses of toxic compounds, chlorinated hydrocarbons and heavy metals were initially suspected to be the cause of the poor nutritional value of *Artemia* from the Great Salt Lake (GSL, Utah, U.S.A.) and the People's Republic of China. A comparative study of

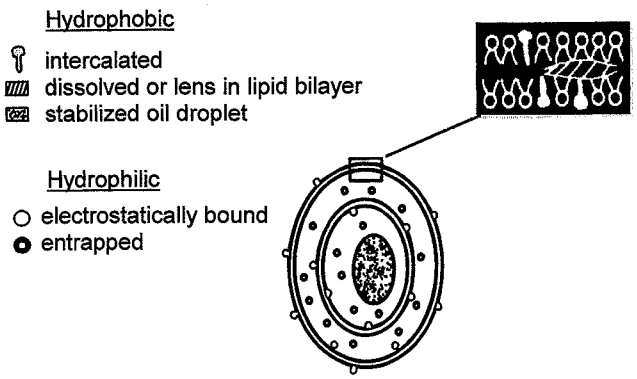

Fig. 3 Entrapment of lipid-and water-soluble compounds in liposomes. Detail shows membrane consisting of phospholipid bilayer (modified from Arnaud, 1993).

eight strains of *Artemia* spp. using crab and mysid shrimp as predator test species confirmed the important nutritional variation among *Artemia* sources (Johns, Peters & Beck, 1980; Léger & Sorgeloos, 1984). Léger *et al.* (1985) documented the nutritional variability in eleven batches of SFB *Artemia* nauplii for the mysid shrimp *Mysidopsis bahia*. Similar to findings in marine fish by Watanabe *et al.* (1978) and Kanazawa, Teshima & Ono (1979), Léger *et al.* (1987a) concluded that the main factor affecting the nutritional value of *Artemia* for marine shrimp larvae was the content of the highly unsaturated fatty acid (HUFA) eicosapentaenoic acid 20 : 5ω3 (EPA). The level of this essential FA for marine organisms in *Artemia* nauplii depends on the maternal diet and is thus largely fluctuating with the natural conditions during cyst production (Lavens, Léger & Sorgeloos, 1989). Since brine shrimp nauplii that have moulted into the second instar stage (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 'bio-encapsulation', also called *Artemia* enrichment or boosting, is widely applied in marine fish and crustacean hatcheries for enhancing the nutritional value of *Artemia* with essential FA. British, Japanese and Belgian researchers developed enrichment products and procedures using selected microalgae and/or micro-encapsulated products, yeast and/or emulsified preparations, self-emulsifying concentrates and/or micro-particulate products (reviewed by Léger *et al.*, 1986).

The highest enrichment levels were obtained in *Artemia* when using emulsified concentrates. To prepare these, freshly hatched nauplii are transferred to the enrichment tank at a density of 100–300 nauplii ml^{-1} (for enrichment periods > 24 h or < 24 h, respectively, with the longer enrichment period requiring lower nauplii densities) (Léger, Naessens-Foucquaert & Sorgeloos, 1987b; Merchie, 1996). The enrichment medium consists of hypochlorite-disinfected and neutralized seawater maintained at 25 °C. The enrichment emulsion is added in doses of 0.3 g l^{-1} every 12 h. Strong aeration using airstones or pure oxygen is required to maintain dissolved oxygen levels above 4 mg l^{-1}. Enriched nauplii are harvested after 24 or 48 h, thoroughly rinsed and stored at < 10 °C in order to assure that the HUFAs are not metabolized during storage. Enrichment levels of 50–60 mg g^{-1} DW ω3 HUFA are obtained after 24 h enrichment with the emulsified concentrates. Nauplii should be transferred or exposed to the enrichment medium as early as possible before they begin feeding, that is, immediately after the opening of the alimentary tract in the instar II stage. Thus, the increase of nauplius size during enrichment can be minimized, that is, after 24 h enrichment GSL *Artemia* nauplii will attain a length of 660 mm, and after 48 h enrichment about 790 mm. *Artemia* juveniles, originating from intensive or super-intensive production systems such as ponds and tanks, can be enriched with ω3 HUFA in the same way as nauplii by adding ω3 HUFA-rich diets (emulsions or powdered diets) during or at the end of the culture period (Dhont & Lavens, 1996).

Improvement of the FA profile of the rotifer *B. plicatilis* for use as food for marine fish larvae has been achieved by either long-term or short-term enrichment techniques, depending on whether the rotifers are exposed to the enrichment diet during or

following their cultivation (Olsen *et al.*, 1993; Lavens *et al.*, 1995b). Long-term enrichment results in rotifers with similar FA profiles to their culture diet consisting of algae (Watanabe *et al.*, 1983), mixtures of fresh baker's yeast and crude or emulsified oil (Olsen *et al.*, 1993), or commercially available diets for combined culture and enrichment (Lavens *et al.*, 1994). Short-term enrichment of rotifers involves exposure of rotifer suspensions (200–500 ml^{-1}) to high concentrations (100–250 mg l^{-1}) of ω3-HUFA-rich diets during short time periods (<8 h), and this allows a drastic increase of the content of lipid (> 300 mg g^{-1} DW; Olsen *et al.*, 1993) and ω3-HUFA (> 100 mg g^{-1} DW; Dhert, Sorgeloos & Devresse, 1993).

Feeding ω3-HUFA-enriched live feed has resulted in better performance of the larval and post-larval stages of several commercial species of fish and shrimp in terms of increased survival, growth, and resistance to salinity stress and/or a more precocious and more synchronous metamorphosis (reviewed by Bengtson *et al.*, 1991; Izquierdo, 1996; Rainuzzo *et al.*, 1997; Sorgeloos *et al.*, 1997). Although the previous studies provided convincing evidence of the importance of the ω3-HUFA contents in *Artemia* and rotifers when used as food for marine larvae, quantitative dietary requirements, as well as the relative importance of various essential FA (EPA; docosahexaenoic acid 22 : 6ω3, DHA; arachidonic acid 20 : 4ω6, ARA) remain to be explored. Recent studies with various species of marine fish have revealed that DHA is more important than EPA for various physiological functions, including survival, growth, and pigmentation (Kanazawa, 1993; Watanabe, 1993). Furthermore, the dietary ratio DHA : EPA rather than the content of DHA plays a major role in the pigmentation of turbot (Reitan, Rainuzzo & Olsen, 1994). In order to investigate the role of DHA for different species, emulsions were prepared with DHA : EPA ratios varying from 0.6–4.0 to prepare live prey with constant HUFA levels but varying DHA : EPA ratios (ICES, 1991). However, DHA : EPA ratios obtained immediately after enrichment with DHA-rich emulsions are considerably lower in most *Artemia* species (including the most-commonly used species, *A. franciscana*) than those obtained in rotifers (Dhert *et al.*, 1993). Contrary to rotifers, the enrichment of *Artemia* with DHA is difficult because of the inherent breakdown of this FA upon enrichment (Evjemo *et al.*, 1997). This makes *Artemia* a less suitable experimental live food to study quantitative DHA

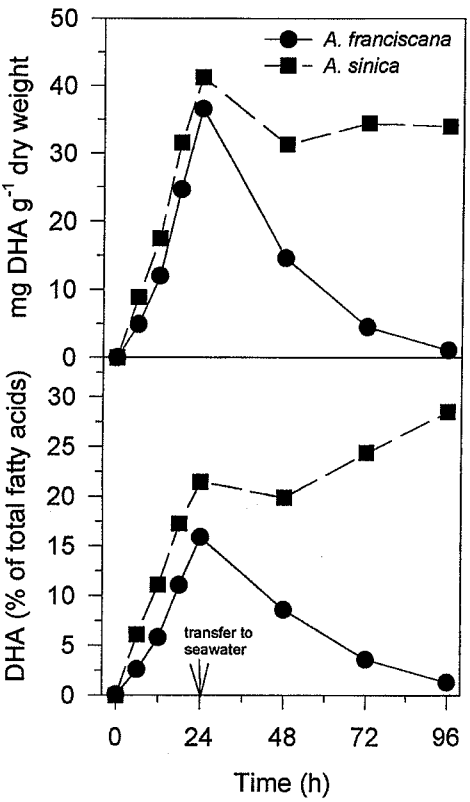

Fig. 4 DHA content during 24-h enrichment (at 28 °C) with a DHA-rich emulsion (ICES 30/4/C/1, containing 24% DHA and 6% EPA; added as two doses of 0.3 g l^{-1} at the start and after 12 h) and subsequent starvation (at 12 °C) in *Artemia franciscana* (Great Salt Lake, Utah, U.S.A.; ●) and *A. sinica* (population from Yimeng, Inner Mongolia, P.R. China; ■) (modified from Evjemo *et al.*, 1997).

requirements. Nevertheless, the capability of some Chinese *Artemia* populations to reach and maintain high DHA levels during enrichment and subsequent starvation (Dhert *et al.*, 1993; Evjemo *et al.*, 1997; Fig. 4), offers new possibilities for providing higher dietary DHA levels and DHA : EPA ratios to fish and crustacean larvae. Marine organisms lacking Δ5 desaturase activity cannot synthesize ARA, which serves as a precursor for a wide variety of biologically active compounds known collectively as eicosanoids (Bell *et al.*, 1995). The suitability of FA profiles in live prey

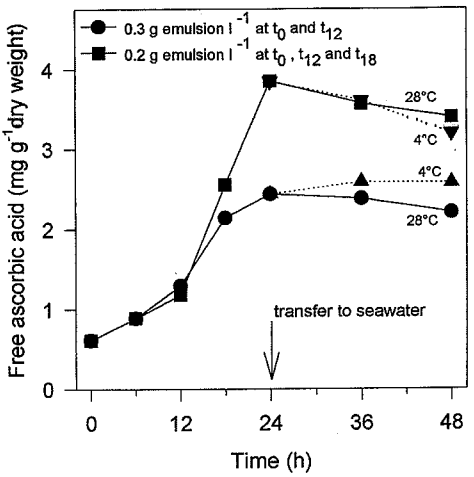

Fig. 5 Ascorbic acid (AA) content during 24-h enrichment of *Artemia franciscana* with an emulsion containing 20% ascorbyl palmitate (AP) and subsequent 24-h starvation at 4 or 28 °C (dotted and continuous lines, respectively). Enrichment emulsion was added either as two doses of 0.3 g l^{-1} (at the start and after 12 h; circles) or as three doses of 0.2 g l^{-1} (at the start, after 12 and 18 h; squares) (modified from Merchie, 1996).

therefore may be better evaluated in terms of the ratio DHA : EPA : ARA rather than only as DHA : EPA (Sargent, McEvoy & Bell, 1997). So far, neither the kinetics of ARA enrichment in live feed nor the importance of the ratio EPA : ARA in larval nutrition has been documented.

Although the technique to enrich filter-feeding organisms like *Artemia* and *Brachionus* with ω3-HUFA appears to be well standardized, it involves a complex chain of biological processes (ingestion, digestion, assimilation and metabolism), which is influenced by several environmental conditions (dissolved oxygen level, salinity, water quality, mechanical disturbance) as well as the physiological condition of the live food; and the quality, concentration and stability of the diet (Omori & Ikeda, 1984). This is illustrated by the high variation of the (ω3) HUFA content in *Artemia* enriched according to a standard protocol, even if the same person handled the enrichment procedure. A coefficient of variation of up to 31% was observed for ten trials by Lavens, Coutteau & Sorgeloos (1995a). To improve the standardization of the enrichment diets used in nutritional studies with marine fish, a series of experimental emulsions were made available through the International Council for the Exploration of the Sea (ICES)—Working Group on Mass Rearing of Juvenile Fish (ICES, 1994). ICES emulsions are a better alternative for commercial enrichment diets in the study of (ω3) HUFA requirements as they offer a specific range of (ω3) HUFA profiles.

The beneficial effects of dietary phospholipids on survival and growth of various species of fish and crustaceans are well documented (reviewed by Coutteau *et al.*, 1997). However, very limited information is available on the role of phospholipids in start-feeding fish and shrimp larvae because of the difficulty of manipulating the phospholipid content and composition of the live prey organisms; for example, *Artemia* is not a suitable test diet to study phospholipid requirements as enrichment with soybean phosphatidylcholine (PC) was unsuccessful (Tackaert, Camara & Sorgeloos, 1991). Also, Rainuzzo *et al.* (1994) found very similar lipid composition in *Artemia* and *Brachionus* enriched with an emulsion based on either ethyl esters (containing 72.6% of neutral lipids, mainly ethyl esters) or halibut roe (containing 71.2% of polar lipids, mainly PC and phosphatidylethanolamine, PE). Still, limited shifts of the lipid classes, for example, PC : PE ratio in *Artemia* (Rainuzzo *et al.*, 1994), because of dietary inputs of phospholipids are poorly documented in zooplankton and their significance for its nutritional value for the predator is unknown.

Vitamins

Contrary to the vast literature on the manipulation of FA in live food, the content and potential for enrichment of vitamins has been investigated only recently and data are mainly restricted to ascorbic acid (AA). Microalgae are generally rich sources of AA, but the content varies greatly among different species (1.1–16.2 mg g^{-1} DW; Brown & Miller, 1992). Also, cysts of various batches of *Artemia* were found to differ considerably in ascorbic acid 2-sulphate (AAS) content (160–517 mg g^{-1} DW, expressed as AA) (Dabrowski, 1991; Merchie *et al.*, 1995). The amount of AA liberated in the 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 (Dabrowski, 1991; Nelis *et al.*, 1994). Differences in AAS content in the cysts among species, geographical populations

and broods from different years result in variable AA levels in the freshly hatched nauplii. Therefore, their nutritional value for larval fish and crustaceans is not constant. Enrichment techniques have been developed using ascorbyl palmitate (AP), a lipophilic ester of vitamin C, to increase the levels of stable and bioavailable vitamin C in both *Brachionus* and *Artemia* (Merchie *et al.*, 1995). Applying the same enrichment procedure as for FA boosting (Léger *et al.*, 1987b) with experimental self-emulsifying concentrates containing 20% AP, levels up to 2.5 mg free AA g^{-1} DW can be incorporated into brine shrimp nauplii within 24 h (Merchie *et al.*, 1995; Fig. 5). The latter authors also demonstrated that the addition of 30% AP to the diet of *Brachionus* enhanced their AA content from 0.13 to up to 2 mg AA g^{-1} DW over 3 days of culture. These high AA concentrations, which are obtained by a fast conversion of the AP into free AA in the digestive tract of the live food, did not drop when the rotifers and 24-h-enriched *Artemia* nauplii were stored for yet another 24 h in seawater at 25 °C and 28 °C, respectively. The effect of vitamin C enrichment in live food on the larviculture outputs of various species of fish and shrimp has been reviewed by Merchie *et al.* (1997).

For the lipid-soluble vitamins, similar enrichment techniques may be applied as for AP. This can elevate vitamin A levels in *Artemia* nauplii from 1.3 to 1283 IU g^{-1} DW over an 18-h period through the addition of vitamin A palmitate to an egg-yolk-based emulsion (Dedi *et al.*, 1995). Also, high levels of α-tocopherol can be bio-accumulated and maintained in *Artemia* nauplii (P. Lavens & H. Nelis, personal communication). The delivery of water-soluble vitamins besides lipophilic forms of vitamin C has not been studied so far.

Conclusions

Rearing of several species of marine fish and shrimp in commercial hatcheries has benefited during the last two decades from improved nutrition of the early life stages. Artificial diets have been designed to supplement the live food chain consisting of microalgae, rotifers and/or brine shrimp nauplii. Furthermore, techniques have been developed to manipulate the nutritional composition of live prey. Artificial diets have been formulated to deliver nutrients to herbivorous as well as carnivorous zooplankton. Complete diet formulations can be incorporated in micro-bound and micro-encapsulated diets. For filter-feeders, microcapsules, liposomes and lipid emulsions have great potential as carriers to study specific nutrients. The requirements of carnivorous stages for certain nutrients, including ω3-HUFA, vitamins C, E and A, can be studied by modifying the content of these nutrients in rotifers and *Artemia* using simple bio-encapsulation techniques. Although the enrichment technique has limited potential for certain nutrients such as phospholipids, further research is needed to explore its applicability for other prey species and nutrients. Some of the techniques used in marine larviculture nutrition may significantly contribute to a better understanding of the nutritional requirements in zooplankton species of greater ecological significance in both marine and freshwater ecosystems.

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Four polymorphic microsatellite markers in the European eel *Anguilla anguilla* (L.)

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Source/description: Clones were obtained from a size-selected library (300–800 bp *Hae*III, *Pvu*II, *Eco*RV-digested inserts), by hybridization with γ^{32}P-labelled $(dG\text{-}dT)_{15}$. The sequences of the loci *Aan01*, *Aan02*, *Aan03* and *Aan04* have been submitted to GenBank/EMBL (acc. nos U67163, U67164, U67165 and U67166).

Primer sequences: Primers were designed with the program PCR Plan from the software package PC Gene (Intelligenetics).

Aan01
forward primer: 5'-GTCTGATCTCATTAACAATCGAGGAGAG-3'
reverse primer: 5'-ATAATTCTTCTTCATTTTGGCTTGCCAT-3'
Aan02
forward primer: 5'-GGATTCCGCACAGCACAACG-3'
reverse primer: 5'-GGTTGCGTTGGCAGACTTTGG-3'
Aan03
forward primer: 5'-GGCAACAAGCTAGCCATGACC-3'
reverse primer: 5'-CCTGGCACAAATGTGCTGTTG-3'
Aan04
forward primer: 5'-TCAGCAGCCTGAGCAAAGCCAGG-3'
reverse primer: 5'-GGCAGTCGTGCAAGTTGAATCATAGGA-3'

Table 1. Number of alleles, size range, observed and expected heterozygosity[1] for four microsatellite loci in glass eels from Italy and Ireland

Sample	*n*	Locus	No. of alleles	Range of alleles (bp)	H_{obs}	H_{exp}
Italy	50	*Aan01*	10	220–244	0·5600	0·7204
	45	*Aan02*	25	174–234	0·5333	0·9446
	49	*Aan03*	5	168–180	0·2857	0·2891
	33	*Aan04*	11	223–234	0·7879	0·8490
Ireland	50	*Aan01*	13	214–250	0·7000	0·7438
	46	*Aan02*	26	176–236	0·7826	0·9537
	49	*Aan03*	3	176–180	0·2727	0·3166
	46	*Aan04*	12	223–235	0·7609	0·8010

PCR conditions: The 10 µl PCR reactions contained: 1 µM non-labelled primers (forward and reverse) and 0·1 µM γ^{32}P-labelled forward primer, 75 µM each dNTP, 1 × PCR reaction buffer (Eurogentec), 1·5 mM $MgCl_2$ (*Aan01*, *Aan02*) or 2·0 mM $MgCl_2$ (*Aan03*, *Aan04*), 0·2 units *Taq* polymerase (Goldstar, Eurogentec) and 10–20 ng genomic DNA. The amplifications were carried out on a Thermocycler (Corbett) or Triobloc (Biometra). The cycling profiles were:

Ana01 & *Aan02*: 95°C for 40 s; 61°C for 35 s; 72°C for 40 s
Aan03 & *Aan04*: 95°C for 40 s; 54°C for 35 s; 72°C for 40 s

30 cycles were performed for both conditions with, at the end of the amplification, an additional elongation step at 72°C for 10 min. PCR products were electrophoresed on 6% denaturing polyacrylamide gels together with an M13 control sequence in order to size the alleles. Gels were dried and exposed to X-ray films (Kodak) to visualize the PCR products.

Mendelian inheritance: Mendelian inheritance could not be established as eels do not reproduce in captivity.

Polymorphism: Genomic DNA of Italian and Irish glass eels was analysed for the four loci. Number of alleles per locus, observed and expected heterozygosity[1] were established (Table 1).

Chromosomal location: Unknown for the four markers.

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