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Fish larval nutrition and feed formulation: knowledge gaps and bottlenecks for advances in larval rearing

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Abstract

Despite considerable progress in recent years, many questions regarding fish larval nutrition remain largely unanswered, and several research avenues remain open. A holistic understanding of the supply line of nutrients is important for developing diets for use in larval culture and for the adaptation of rearing conditions that meet the larval requirements for the optimal presentation of food organisms and/or microdiets. The aim of the present review is to revise the state of the art and to pinpoint the gaps in knowledge regarding larval nutritional requirements, the nutritional value of live feeds and challenges and opportunities in the development of formulated larval diets.

Key words: enrichment, fish larvae, formulated diets, live feed, nutrient requirements.

Introduction

The major objectives of this review are (i) to analyse the current knowledge, research trends and efforts; and based on this analysis (ii) to identify the gaps and bottlenecks that need to be tackled in future research for the advanced and more efficient production of fish larvae.

Marine fish larvae are very vulnerable during the first stages of development and have strict requirements for biotic and abiotic conditions to survive, develop and grow properly. There are several recent reviews that cover different aspects of larval nutrition and show the advances in knowledge from different perspectives (see reviews in Holt 2011). In spite of the variety of conditions that a developing larva may face in nature, the current knowledge of nutrition in early stages has been based mainly on laboratory studies carried out following reductionist approaches under artificial conditions based on limited prey types and under relatively constant abiotic and biotic conditions. Another aspect to take into account is the variety in ontogeny, feeding physiology and nutritional requirements among species, even within the same family. Consequently, many specific processes cannot directly be extrapolated from findings obtained in model species and require specific studies.

Obviously, a good knowledge of the larval nutritional requirements throughout development would contribute to optimize diets and feeding protocols, and thereby improve larval and juvenile quality. Nevertheless, considering the vulnerability of fish larvae, it is always difficult to identify and meet nutritional requirements when several physiological and metabolic constraints are linked and each of them may prevent growth or an appropriate development. An integrated understanding of the different factors and events interacting in the food acquisition and processing is necessary for designing larval diets that meet the larval requirements for optimal ingestion, digestion and absorption of these diets. This review, which covers the gaps in knowledge on fish larval nutritional requirements, should therefore be read together with the review by Rønnestad *et al.* (in press) that covers the aspects of appetite, feed acquisition and digestive physiology.

Considering all these limitations and based on the analysis of the current information available in marine fish nutrition, the present review attempts to identify the most burning gaps to be addressed in future research to achieve a more efficient production of high quality fish larvae.

Larval nutrition

What are the larval nutrient requirements?

We know very little about the nutritional requirements of marine fish larvae (Holt 2011). Both qualitatively and quantitatively they may differ from those of juveniles or adult fish, since fish undergo dramatic morphological and physiological changes, including metamorphosis, during ontogenesis. Moreover, fish larvae grow extremely rapidly, feed continuously and, therefore, the total ingestion of nutrients must be high. In cod larvae, for example, growth rates of up to 30% per day have been measured (Otterlei *et al.* 1999), while some species such as African catfish (*Clarias gariepinus*) may grow up to 100% per day (Conceição *et al.* 1998a).

The requirement for a particular nutrient can be defined from a physiological point of view as the nutrient intake needed to fulfil a physiological role (Izquierdo & Lall 2004). However, the design and formulation of diets requires translation of the nutritional requirements into the nutrient content in the diet (Kolkovski *et al.* 2009). Micronutrient requirements, but also requirements for protein/amino acids, fatty acids and so forth, are often given as dietary concentrations/fractions, and, expressed in this way requirements do not always increase under demanding conditions, such as high growth rates and metamorphosis. However, if food intake increases, the absolute intake of each single nutrient will also increase under constant dietary composition. The reason for stressing this argument is to differentiate between requirements for a certain volume of feed and the requirement for a balanced diet, where the different nutrients may be required in different ratios to each other, dependent on the developmental stage and the growth rate of the animal.

Nutritional requirements are frequently defined as the 'requirement for maximal growth and/or survival' where the relation fish-diet-feeding has an important effect in the determination of the quantitative needs (Izquierdo & Lall 2004), but they can be also defined as a 'requirement for body maintenance' as the minimum rate of nutrient expenditure needed to keep the animal alive, 'requirement for least cost production' or 'requirement for fish health'. For instance, vitamin C (ascorbic acid, Asc) and E (α-tocopherol, α-TOH) concentrations far above the requirements established for fish (NRC 2011) may stimulate immunity and stress-resistance both in juveniles and larvae (Hamre 2011). However, the number of studies on Asc and α-TOH are very few, so there is a limited basis for the often-accepted idea that fish larvae require higher concentrations of micronutrients than juvenile and adult fish. In order to clarify this, direct requirement measurements are needed. On the other hand, certain nutrients, particularly fat-soluble vitamins, induce pathological effects when provided at high concentrations. Thus, hypervitaminosis A is known to cause skeletal deformities and other malformations (Dedi *et al.* 1997; Fernández *et al.* 2008, 2009; Fernández & Gisbert 2010).

Studies of nutrient requirements using direct and indirect measurement methods will be described below. The main reason that studies that use direct methods are scarce is the difficulty in designing experiments with full control of nutrient composition and environmental factors (i.e. fish density, water quality and renewal, light conditions and so on) in all the experimental tanks. Formulated diets have variable leaching and stability problems (see below), resulting in an unknown difference in nutrient composition between the formulated and ingested diet, while live feed nutrient concentrations may be difficult to control due to the prey organism's own metabolism (see below). As described later, some solutions to these problems have recently been found. In addition to a few direct measurements using dose–response designs (see below), a range of indirect methods (see below) have been applied to try to estimate the nutritional requirements of marine fish larvae. A summary of the different studies is given below.

Direct measurements of larval requirements for example dose response

Macronutrients

Direct investigations on the optimum composition of macronutrients for fish larvae are complicated when using live feed due to the feed organism's own metabolism and nutrient composition. However, Morais *et al.* (2005a,b) used oleic acid (OA) enriched and unenriched *Artemia* for Senegalese sole (*Solea senegalensis*) and found that the unenriched *Artemia* gave better growth in one case (Morais *et al.* 2005a) and a trend of better growth in the other case (Morais *et al.* 2005b). This was probably an effect of the higher protein to lipid ratio in the unenriched *Artemia*, since the non-enrichment was unlikely to change the fatty acid composition in *Artemia* in any favourable way.

The use of experimental microdiets is likewise complicated because of the poor acceptability of most inert diets, and in particular semi-purified ones, by the generality of species. The deficiencies in some specific nutrients may also mask the results. In spite of this, several attempts to advance this issue have been made. Yúfera *et al.* (2005) fed microdiets formulated with two protein levels (55% and 62%) to Senegalese sole larvae and found that the larvae fed with the higher protein content grew and survived just slightly better, but exhibited a clear faster rate of eye migration. To our knowledge, real dose–response studies, using more than two levels of variation of macronutrient composition for fish larvae, are lacking.

On the other hand, the experimental microdiets offer the possibility of testing different dietary macronutrient contents to explore potential macronutrient preferences. Juvenile and adult fish are able to select the appropriate composition from a variety of diets in relation to their requirement for macronutrients (Sánchez-Vázquez *et al.* 1999, Rubio *et al.* 2003). Nevertheless, experiments carried out on gilthead seabream (*Sparus aurata*) larvae using marked food microparticles (Manuel Yúfera, Maria Sánchez-Amaya and Kristin Hamre, unpubl. data, 2011) were not conclusive, suggesting that the capacity for selecting macronutrients is not yet developed in the larval stage. These results may be related to feeding behaviour and the gut development status. As many altricial larvae, gilthead seabream larvae feed continuously without apparent satiation while the food is available. Only when the stomach is formed and functioning as a food reservoir and a more efficient acidic proteolysis is developed, the gut transit starts to be better regulated and consequently the nutrient digestion and absorption become more efficient (Rønnestad *et al.* in press).

As illustrated above, very little work has been done to determine the optimum composition of macronutrients for marine fish larvae. The tendency of fish larvae to feed continuously when feed is available will affect the gut passage time and thereby probably the availability of nutrients and possibly the optimum macronutrient composition. The optimal composition will also be dependent on the form in which the nutrients are given. We are only starting to build an understanding of these issues.

Protein and amino acids

The quality of the dietary protein has a primary relevance. Inclusions of low to medium levels of hydrolysed protein in weaning diets to larval fish have been shown to improve survival and growth. In carp (*Cyprinus carpio*) and European seabass (*Dicentrarchus labrax*) larvae, substitution of 60 and 250 g kg^{-1}, respectively, of the dietary protein with hydrolysed protein was found to be optimal (Cahu *et al.* 1999; Carvalho *et al.* 2004). In an experiment with cod (*Gadus morhua*), supplementation of pepsin hydrolysed protein up to 400 g kg^{-1} protein improved survival rates compared with lower levels of supplementation, while a similar experiment with Atlantic halibut (*Hippoglossus hippoglossus*) did not give improved performance with hydrolysed protein supplementation (Kvåle *et al.* 2009). Inclusion levels above 500 g kg^{-1} of the protein seem to be detrimental to several fish species (gilthead seabream, Kolkovski & Tandler 2000; carp, Carvalho *et al.* 1997; *Dicentrarchus labrax*, Cahu *et al.* 1999; although not to all (*Solea solea*, Day *et al.* 1997; turbot, *Psetta maxima*, Oliva-Teles *et al.* 1999). The different optima found for different fish larvae may be explained by differences in digestive capacity, but a confounding factor is the high leaching rate of water soluble protein from formulated diets (Hamre 2006; Kvåle *et al.* 2006) and differences between fish species in feed ingestion rates.

The aminosulphonic acid, taurine, is formed from methionine or cysteine via decarboxylation of cysteine sulphinic acid to hypotaurine with subsequent oxidation of the latter. Taurine has been shown to be an essential nutrient for cats, and probably also for primates, especially during early development (Sturman 1993). Taurine is not built into protein, but resides in the free amino acid pool and is used for cell volume regulation and bile salt synthesis, among other functions. Taurine enrichment of rotifers or supplementation in formulated diets improves growth in marine fish larvae (Chen *et al.* 2004, 2005; Pinto *et al.* 2010). In Senegalese sole, taurine also led to increased retention of protein (^{14}C-labelled live prey) in the larval body and increased the metamorphosis rate (Pinto *et al.* 2010). Chen *et al.* (2005) fed three levels of taurine to Japanese flounder (*Paralichthys olivaceus*) and found an increase in growth when taurine was increased from 0.5 to1.7 g kg^{-1} dry weight of rotifers. A further increase to 3.0 g kg^{-1} did not give any further increase in growth. There was also a positive effect of taurine on larval morphological development.

Lipid class composition

There is a large body of research on lipid requirements in fish larvae, including both essential fatty acids and the ratio of phospholipids (PL) to neutral lipids (NL). However, studies aimed at determining the quantitative requirements for these nutrients with dose-response, including at least five dietary levels, are very scarce.

Fish larvae fed formulated diets where the lipid is added solely as tri-acyl glycerol (TAG), show poor growth and survival and accumulate lipid droplets in intestinal tissue and in the liver. This is relieved by adding PL to the diet (Fontagné *et al.* 1998). Dietary PL has been found to be required for the growth and survival of a range of species since the early 1980s (Tocher *et al.* 2008; Izquierdo & Koven 2011). Phospholipids are structural constituents of biomembranes and therefore highly demanded in the fast growing larvae. Phospholipids are also involved in the digestion, absorption and transport of lipids from the intestine to the rest of the body. There are several indications that fish larvae are unable efficiently to synthesize PL in a rate fast enough to cover their high demand and therefore PL need to be included in the diet (Izquierdo & Koven 2011). Indeed, the first feeding larvae enterocytes are poorly developed and organelles in which PL synthesis occurs in fish, the rough and smooth endoplasmic reticulum (Sire *et al.* 1981; Caballero *et al.* 2006b) are scarce. Deplano *et al.* (1991) and Caballero *et al.* (2002, 2006a) isolating enterocyte microsomes demonstrated that in gilthead seabream, PL synthesis occurs mainly through the glycerol-3-phosphate pathway, whose activity is modulated through dietary lipids. Moreover, restrictions in the rate of PL synthesis may constrain lipoprotein synthesis (Liu *et al.* 2002). Thus, the addition of 20 g kg^{-1} dry weight of diet (DM) soybean lecithin in microdiets for 15 dph gilthead seabream containing 220 g kg^{-1} DM lipids, significantly increased the occurrence of lipoprotein particles in the lamina propria, promoting transport and utilization of dietary lipids and promoting growth (Liu *et al.* 2002). Phospholipid requirements have been found to be related to the larval age and the degree of digestive system development (Kanazawa 1993; Izquierdo & Koven 2011). Thus, several authors have found that gilthead seabream growth depression related to low dietary PL contents (i.e. PL 71 g kg^{-1} DM, total lipids 220 g kg^{-1} DM in (Liu *et al.* 2002) is relieved when larvae have almost completed the digestive system development (26–36 dph) (Koven *et al.* 1993; Liu *et al.* 2002). Insufficient levels of dietary PL (PL 23.7 g kg^{-1} DM, dietary lipids 178.5 g kg^{-1} DM, Salhi *et al.* 1997) increase the accumulation of lipid droplets in the enterocytes of marine larvae fed a PL deficient diet (Salhi *et al.* 1999; Morais *et al.* 2005b), depending on the type and amount of dietary PL and its relation to the dietary NL content. In tube-fed Atlantic herring (*Clupea harengus*) larvae, a reduction of the ^{14}C-TAG introduced into the digestive tract increased lipid absorption and decreased the evacuation of ^{14}C (Morais *et al.* 2005c).

Besides their importance for growth and dietary lipid utilization, dietary PL improve stress resistance, skeleton development and flatfish metamorphosis and pigmentation (Kanazawa *et al.* 1981; Kanazawa 1993; Fontagné *et al.* 2000a,b; Weirich & Reigh 2001; Koven 2003; Zambonino-Infante & Cahu 2007; Hamza *et al.* 2008; Ebrahimnezhadarabi *et al.* 2011). Since PL constitute a wide group of compounds formed by different bases and types of fatty acids, their effect in the larvae may depend on the particular components of the dietary PL source used. For instance, phosphatidylcholine (PC), as the main product of PL synthesis in fish enterocytes (Caballero *et al.* 2006a), induces apolipoprotein B synthesis to a higher extent than other PL types (Field & Mathur 1995). Enhanced lipoprotein synthesis can be responsible for the growth promotion effect of PC by increasing energy flux from the intestinal mucosa into the blood (Seiliez *et al.* 2006). Thus, PC has been found to promote growth better than other PL classes in several species (Geurden *et al.* 1997, 1998; Hadas *et al.* 2003) and enhances feeding activity (Koven *et al.* 1998). Phosphatidylinositol (PI) has a rich diversity of forms and functions within the cell besides being a structural component of biomembranes. Thus, PI acts as a precursor of second messengers (inositol 3 phosphate, IP3) regulating the entry of calcium ions into the cell from the endoplasmic reticulum (Cahu *et al.* 2003b; Tocher *et al.* 2008). It is also a membrane anchor for a great variety of cell surface proteins. Therefore, PI is involved in a signalling system controlling biological processes in early development in vertebrates (Berridge & Irvine 1989). The effect of PI on larvae development is not yet well understood. Sandel *et al.* (2010) tested the effect of four microdiets (MD) differing in the ratio of PC to PI and one commercial reference diet (100% enriched *Artemia* ration) fed to 20–34 dph gilthead seabream larvae. Developmental performances in 40 dph larvae (growth rate) and 67 and 141 dph juveniles (fish survival, growth and malformation rate) were analysed. The four gelatin based MD replaced 75% of the normal *Artemia* ration (mg DM). The highest PC/PI ratio diet (lowest PI) was considered as the control. However, the results did not show clear positive effects of the PC/PI ratio. Cahu *et al.* (2003a) reviewed the effects of some nutritional components on skeletal development in marine fish larvae and found that in sea bass, a level of 16 mg kg^{-1} DM PI in diet used from first feeding is convenient for preventing deformities during development.

Another aspect of discussion has been the effect of the type of fatty acids binding to the PL, since marine fish larvae natural preys such as copepods have a high content of PLs rich in n-3 highly unsaturated fatty acids. For instance, in larval ayu (*Plecoglossus altivelis*) PL from bonito eggs rich in n-3 highly unsaturated fatty acids improve growth and survival more effectively than PL from a vegetal source. A possible explanation of the results is the different content of essential fatty acids. Salhi *et al.* (1999) fed 11 dph gilthead seabream larvae until 28 dph with microdiets having the same total lipid (178–171 g kg^{-1} DM), PL (24–28 g kg^{-1} DM) and essential fatty acids contents (DHA 9 g kg^{-1} DM, EPA 6 g kg^{-1} DM, ARA 0.5 g kg^{-1} DM), but containing either soybean lecithin or squid PL. They found a significantly higher length and weight when larvae were fed PL rich in n-3 highly unsaturated fatty acids. More recently, dietary substitution of 2 g kg^{-1} DM of soybean lecithin (lipids 213 g kg^{-1} DM, PL 25 g kg^{-1} DM, DHA 24 g kg^{-1} DM) by krill PL (lipids 208 g kg^{-1} DM, PL 27 g kg^{-1} DM, DHA 35 g kg^{-1} DM) improved larval seabream growth in terms of weight and length, enhanced hepatic utilization of dietary lipids and improved gut health (Betancor *et al.* 2012).

Nevertheless, despite the wide range of studies denoting the importance of PL, only few of them have aimed to determine the quantitative requirements by performing dose–response studies with several levels of dietary PL. Cahu *et al.* (2003b) ran a dose–response study with European seabass larvae, using five levels of PL at a constant dietary lipid level (PL, 27–116 g kg^{-1} DM, total lipids 256 g kg^{-1} DM). They found that the diet with the highest dietary PL gave the best larval performance and lower skeletal malformation rates. A similar result was found by Hamza *et al.* (2008) for pikeperch (*Sander lucioperca*) larvae, which also showed the best growth with the diet highest in PL (90 g kg^{-1} DM). On the contrary, an excess of dietary PC was found to decrease survival and to increase the malformation rate in carp larvae (Geurden *et al.* 1998).

Some of the bottlenecks for the determination of quantitative PL requirements have been noted (Tocher *et al.* 2008): (i) pelleted diets are not suitable for most marine fish larvae, and the traditional diets (live feeds) are unsuitable as they contain PL and further enrichment can be difficult; (ii) both pelleted feeds or microdiets can have problems of low palatability as the formulations must be either fish meal-free or use defatted fish meal; (iii) although commercial PL preparations (lecithins) are available, they are not pure products, and they can vary greatly in purity, and have highly variable PL contents and class compositions, as well as very different fatty acid compositions; (iv) the alternative use of pure PL species (classes) has limited availability and is costly, although it is possible to purify individual PL classes in the laboratory; and (v) replacement of one lipid class with another and comparing different PL preparations will invariably alter the fatty acid composition of the diets and this can be difficult to control fully.

Therefore, despite the research effort made by PL studies in fish larvae, several gaps remain to be covered: (i) to define the quantitative requirements for most species, considering larval age and gut development as well as PL source and its content of essential fatty acids; and (ii) to determine the qualitative requirements in terms of the type of PL and their optimum dietary ratios.

Essential fatty acids

There are numerous studies on the effects of essential fatty acids on growth, survival, behaviour and biological functions and processes in marine fish larvae, but few studies quantify the requirements in the different species and in developing larvae. It should be taken into consideration that the relative importance of each fatty acid differs among the species (Dantagnan *et al.* 2010).

Dietary n-3 highly unsaturated fatty acids (HUFA) in rotifers, *Artemia* or microdiets affect larval survival rate and/or growth, as has been found in numerous species including turbot (Gatesoupe & Le Milinaire 1985), red sea bream (*Pagrus major*; Izquierdo *et al.* 1989), gilthead seabream (Koven *et al.* 1990; Rodríguez *et al.* 1994; Salhi *et al.* 1994) and red porgy (*Pagrus pagrus*; Roo *et al.* 2009) as well as swim bladder inflation in gilthead seabream (Koven *et al.* 1990). They have also been found to increase survival after handling stress ('activity test') in several species such as red sea bream (Izquierdo *et al.* 1989) or gilthead seabream (Montero *et al.* 1998). They have an effect on swimming, feeding and escaping behaviour (Izquierdo 1996; Benítez-Santana *et al.* 2007) and water reabsorption in red sea bream (Izquierdo *et al.* 1989; Watanabe *et al.* 1989; Rodríguez *et al.* 1994) and gilthead seabream larvae (Koven *et al.* 1990) on skeleton development (Villeneuve *et al.* 2005a; Roo *et al.* 2009) and on flatfish pigmentation (Rainuzzo *et al.* 1997; Hamre & Harboe 2008b).

Specific studies aimed at determining the quantitative essential fatty acid requirements for marine fish larvae are scarce. Izquierdo *et al.* (1989) conducted a series of trials enriching rotifers and *Artemia* with 6–9 different levels of n-3 HUFA to determine the essential fatty acid requirements of red sea bream. In two trials conducted with rotifers [n-3 HUFA 4.7–19.7% of total fatty acids (TFA), mean total lipids 200 g kg^{-1} DM] they found that the best growth, survival and resistance to stress were obtained with a level of n-3 HUFA of 15% TFA (3.5% n-3 HUFA DM, including DHA 7% TFA and EPA 6.4% TFA; Izquierdo *et al.* 1989). When larvae were fed *Artemia* (n-3 HUFA 4.2–21.0% TFA, total lipids 129–224 g kg^{-1} DM) the best growth, survival and resistance to stress were obtained with a level of n-3 HUFA of 15.9% TFA (3.8% n-3 HUFA DM, including DHA 2% TFA and EPA 9.7% TFA; Izquierdo *et al.* 1989). In a later study, enriching rotifers with either DHA or EPA, it was found that both fatty acids significantly improved survival, but only DHA promoted a significantly higher growth (Watanabe *et al.* 1989). Testing nine different n-3 HUFA levels in *Artemia* (n-3 HUFA 9–24% TFA, total lipids 197–340 g kg^{-1} DM) showed a requirement for improved growth of Japanese flounder larvae of 13% TFA (n-3 HUFA 3.5% DM, DHA 1% TFA, EPA 11.2% TFA; Izquierdo *et al.* 1992), whereas 9.5% TFA (n-3 HUFA 1.84% DM, DHA 0.1% TFA, EPA 9.2% TFA) was enough to maintain good survival. In striped jack (*Longirostris delicatisimus*), feeding five levels of n-3 HUFA in rotifers (3.8–22.8% TFA, total lipids 137–165 g kg^{-1} DM), these authors found that growth and survival was increased proportionally by dietary n-3 HUFA levels without reaching any plateau to determine the requirements (Izquierdo 1988) that were superior to

22.8% TFA (n-3 HUFA 3% DM, DHA 9.3% TFA, EPA 10.5% TFA). A series of studies feeding gilthead sea bream with four different levels of n-3 HUFA either in rotifers or in microdiets demonstrated that with adequate DHA/EPA ratios (>1.3) the n-3 HUFA requirements for this species are 1.5–2% DM (Rodríguez *et al.* 1993, 1994, 1997, 1998; Salhi *et al.* 1997). For instance, feeding microdiets containing four n-3 HUFA levels (n-3 HUFA 9.6–24.5% TFA, total lipids 174 g kg^{-1} DM) significantly best growth was obtained with a level of n-3 HUFA of 18% TFA (2% n-3 HUFA DM, including DHA 11.4% TFA and EPA 6.6% TFA; Salhi *et al.* 1994).

The arachidonic acid (ARA) requirements were also determined for gilthead seabream feeding 17 dph larvae in two trials with seven different diets containing graded levels of ARA, but constant n-3 HUFA and DHA/EPA ratios (Bessonart *et al.* 1999). Significantly best growth was obtained with 7.8% TFA (ARA 1% DM, total lipids 166 g kg^{-1} DM, including DHA 11% TFA and EPA 6.3% TFA). Results recently obtained by Atalah *et al.* (2011a,b) feeding microdiets with five different levels of ARA and EPA confirmed this ARA requirement for gilthead seabream and recommended an EPA/ARA ratio of 4. Similar studies recommended at least 1.2% ARA for European seabass larvae at an EPA/ARA ratio of 4 (Atalah *et al.* 2011a,b).

Typically a dietary DHA/EPA ratio of 2:1 is found in marine species and thus, it has been suggested as adequate for larval nutrition (Sargent *et al.* 1997). Hernandez-Cruz *et al.* (1999) fed rotifers with three levels of EPA and DHA to larval red porgy and obtained the best growth using rotifers containing 2.73% DHA in DM. For other species, such as red sea bream (Izquierdo *et al.* 1989), common dentex (*Dentex dentex*; Mourente *et al.* 1999a,b), gilthead seabream (Izquierdo 2005) or striped trumpeter (*Latris lineate*; Bransden *et al.* 2005) the minimum DHA requirement for optimum growth was found to be 1.2%, 2.3%, 0.8% and 2.0% DM, respectively.

Villeneuve *et al.* (2005b) fed European seabass larvae with microdiets with a different source and concentration of HUFA and found a consistent diminution of deformities (6.6% vs. 50%) in fish fed 1.1% EPA + DHA in the PL fraction. In red porgy, Roo *et al.* (2009) found a reduction (about 50%) in the incidence of skeleton deformities when DHA was increased in rotifers from 9.68%TFA (DHA 1.8% DM, total lipids 220 g kg^{-1} DM) to 20.52% TFA (DHA 4.7% DM, total lipids 273 g kg^{-1} DM), together with an improvement in survival at 25 dph, demonstrating the important role of this FA in the prevention of deformities at the rotifer feeding stage.

A deficiency of n-3 HUFA and high levels (see below) of arachidonic acid (ARA, 20:4 n-6) cause pigmentation disorders in flatfish (Estévez & Kanazawa 1995; Sargent *et al.* 1997; Næss & Lie 1998; Estévez *et al.* 1999; Shields *et al.* 1999; Copeman *et al.* 2002; Villalta *et al.* 2005a; Hamre *et al.* 2007). The data indicate that eicosanoids are involved in the development of pigmentation in flatfish, since increasing ARA, which is the precursor of eicosanoids of the 2-series, gives a dramatically reduced rate of normal pigmentation in several flatfish species (McEvoy *et al.* 1998; Estévez *et al.* 1999; Copeman *et al.* 2002; Villalta *et al.* 2005a). In Senegalese sole, a whole body EPA:ARA ratio of 2.0 gave almost 100% normal pigmentation, while ratios of 0.2–0.4 gave normal pigmentation rates of 16–40% (Villalta *et al.* 2005a). The larvae had been fed differently enriched *Artemia* (EPA:ARA ratios of 14, 0.33 and 0.17) for 37 days at sampling and the ARA levels in the whole body were 1.3–7.6% of TFA. McEvoy *et al.* (1998) showed that dietary ARA and eicosapentaenoic acid (EPA, 20:5 n-3) had opposite effects on pigmentation in turbot and Atlantic halibut and found that a brain EPA:ARA ratio of 4:1 gave the best ratio of normal pigmentation while a ratio of <1:1 would give 100% malpigmentation. However, the best percentage of normal pigmentation found in Atlantic halibut was only 25%, while in turbot the best percentage was 86%. This may have to do with differences in the requirement of DHA between the two species. Hamre and Harboe (2008b) found that a whole body level of DHA of 11.3% of TFA in Atlantic halibut (concentration in *Artemia* 12.5% of TFA, fatty acid methyl esters (FAME) 11 mg g^{-1} DM) at an EPA:ARA ratio of 3.5, gave 77% normal pigmentation, while at a whole body concentration of DHA of 7.4% TFA and an EPA:ARA ratio of 3.0, the pigmentation rate was 48%. The level of total fatty acids in the *Artemia* was 100–120 g kg^{-1} DM and the larvae were fed the two diets for 45 days after first feeding. The DHA requirement for normal pigmentation in turbot is not known, but it can be speculated that it is lower than in halibut, because of the more temperate habitat of the species. In Senegalese sole, a low level of DHA (0.0% of TFA in *Artemia* and 1.5% of TFA in whole larvae after feeding for 36 days after hatching) did not seem to inhibit pigmentation (Villalta *et al.* 2005b). The fatty acid composition needed to ensure normal development of fish larvae may be different from that needed for good growth and survival. The EPA:ARA ratios giving different pigmentation rates in the study of Villalta *et al.* (2005a) did not affect the growth and survival of the larvae, while the whole body DHA needed for good survival of Atlantic halibut larvae appeared to be only 6.6–6.8% of TFA (dietary level 7.8% of TFA, dietary FAME 140 mg g^{-1} DM; Hamre & Harboe 2008a), for example much lower than that needed for normal pigmentation.

Despite the importance of essential fatty acids for fish larvae development and abundant literature regarding these nutrients, quantitative requirements have yet to be defined in most European fish larvae in terms of ARA, EPA, DHA and total HUFA optimum dietary contents in live preys and larval inert diets, as well as DHA/EPA and EPA/ARA ratios. Besides, biotic and abiotic factors that may affect essential fatty acid requirements, including salinity, temperature, green water addition, intensiveness of the culture system, interrelations with other nutrients such as antioxidants and other vitamins, should be considered in dose–response studies with at least five nutrient levels. Moreover, in view of the range of physiological effects of the different fatty acids, requirements should be determined not only in relation to growth and survival, but also considering development of biological functions and processes, such as pigmentation, muscle-skeleton and neural system development.

Vitamins

Only a few dose–response studies have been performed to obtain quantitative vitamin requirements in marine fish larvae, the vitamins studied being vitamin A, C, D, E and K. Some of these studies use only two or a few levels of vitamins.

Vitamin A is involved in vision, growth, bone development, reproduction and normal maintenance of epithelial tissues. The studies on vitamin A in fish larvae are largely focused on the effects on skeletal development. An increasing number of malformations were found in the caudal region and vertebrae of Japanese flounder (Dedi *et al.* 1997) and in the vertebrae of turbot (Estévez & Kanazawa 1995) fed increasing dietary levels of vitamin A palmitate during metamorphosis. Villeneuve *et al.* (2005a) fed European seabass larvae of 7–42 dph five isoproteic and isolipidic compound diets with graded levels of retinyl acetate (RA; RA0, RA10, RA50, RA250 and RA1000, containing 0, 10, 50, 250 and 1000 mg RA kg^{-1} DM, respectively). The analysed dietary levels were 12, 13, 31, 62 and 196 mg all-*trans* retinol kg^{-1} DM. Using malformation rate as an indicator, the optimum level of retinol was found to be around 31 mg kg^{-1} DM. Mazurais *et al.* (2009) fed sea bass larvae of 9–45 dph microparticulate diets with 0, 5, 10, 15, 25, 35 and 70 mg of retinol kg^{-1} added as retinyl acetate. The analysed levels were 0.9, 2.8, 6,2, 10, 10, 21, 28 and 47 mg kg^{-1} DM (1 IU = 0.3 μg of retinol). As far as general larval performance (weight and survival) is concerned, 5–10 mg kg^{-1} added retinol seemed to give the best results, for example lower than the optimal dose found by Villeneuve *et al.* (2005a). Therefore, if the requirement is deduced from the growth data in the Mazurais study, the results are in agreement with the optimum level found by Moren *et al.* (2004; 2.4 mg kg^{-1} DM), who worked with Atlantic halibut juveniles, and only slightly above the minimum requirement given for juvenile and adult fish (0.75 mg kg^{-1}) by NRC (2011).

Merchie *et al.* (1997) found that 20 mg kg^{-1} Asc was sufficient for normal growth and survival of post-larval turbot and sea bass, when using formulated diets, while the Asc requirement for maximum growth in common carp larvae was 45 mg kg^{-1} (Gouillou-Coustans *et al.* 1998), both in agreement with NRC (2011) requirement assessments for fish. The Asc concentrations found in live feed prior to enrichment (4–600 mg kg^{-1}), were sufficient for several fish and shrimp species (Merchie *et al.* 1997). However, boosting the live feed organisms with Asc, up to 2500 mg kg^{-1} improved stress resistance. Kolkovski *et al.* (2000) also found improved stress resistance and survival of freshwater walleye fed high levels of long-chain n-3 PUFA by *Artemia* boosted with Asc from 300 to approximately 1500 mg kg^{-1} DM.

Atalah *et al.* (2008) used 1.5 and 3.0 g kg^{-1} dry diet of α-TOH for gilthead seabream and sea bass, and suggested an optimal level of 3 g kg^{-1} because this high level reduced mortality after stress, but not the mortality under standard rearing conditions. In the same study, the high α-TOH level also alleviated muscular lesions caused by excessive dietary DHA (Betancor *et al.* 2011). The results of these studies are therefore in line with the general opinion that vitamins C and E in larval diets should be above 1 g kg^{-1}, while the requirements given by NRC (2011) for juvenile fish are 30 and 50 mg kg^{-1} for vitamins C and E, respectively. However, when stress and immune responses are used to measure requirements of these vitamins, higher estimations of dietary optima are most often found, also in juvenile and adult fish (Hamre 2011).

Darias *et al.* (2010) fed European seabass larvae with graded levels of vitamin D_3 and found that 19 IU g^{-1} diet (0.5 mg kg^{-1}) was necessary to obtain normal growth and development of the digestive system and the skeleton. This is considerably higher than the requirements measured in fish (0.25–2.4 IU g^{-1}; NRC 2011). Addition of vitamin D_3 or $1{,}25(OH)_2D_3$ to the embryo medium increased bone mineralization in developing yolk-sac larvae of zebrafish (*Danio rerio*) in a dose-dependent manner (Fleming *et al.* 2005). When a diet supplemented with excess of vitamin D_3 compounds was administered during vertebral morphogenesis, vertebral deformities were apparently introduced in some species and not in others (Haga *et al.* 2004). The inconsistency of these and other data justifies the hypothesis of size- and temperature-dependence effects of vitamin D hypervitaminosis.

To our knowledge, almost no published studies on the effect of vitamin K on larval fish are available. However, Udagawa (2001) found that when mummichog (*Fundulus heteroclitus*) larvae were fed with different doses of phylloquinone (PK) or menadione sodium bisulphite (MSB), the effect on vertebrae formation differed according to the vitamin K source. The supply of massive doses of MSB, and not of PK, was harmful to bone development in this species. The lowest rate of fish with malformed vertebrae was found in fish fed PK at 1 mg kg^{-1} (50.7%; 51.4%) and 100 mg kg^{-1} (47.3%, 55%), and MSB at 25 mg kg^{-1} (53.6%; 54.8%), whilst significant larger incidences were observed in fish fed with MSB at 2500 mg kg^{-1} (65.6%–66%), including higher incidences of fusion, deformity and row irregularity of the vertebrae.

In conclusion, the most studied vitamin in fish larvae is vitamin A, but the focus has often been on toxic effects and not so much on requirements. Nevertheless, the study by Mazurais *et al.* (2009) indicates a larval requirement for optimal growth and survival is in the range of 1–10 mg kg^{-1}, which is in line with requirements in juvenile and adult fish (Moren *et al.* 2004; NRC 2011). The maximal non-toxic level of vitamin A for fish larvae is still unknown. Requirements of the other vitamins in marine fish larvae are largely unknown.

Minerals

Research on mineral requirements in fish larvae only started after 2005 and the number of publications is quite small. Nguyen *et al.* (2008) enriched *Artemia* with zinc, manganese or zinc + manganese. Increasing dietary Mn concentration from 12 to approximately 40 mg kg^{-1} DM gave a significant increase in the growth of red sea bream larvae, from 15 to 30 dph. All Mn, Zn and Zn + Mn enrichment gave a reduction of skeletal deformities, from 53% deformed fish in the control group to 39–41% in the treatment groups.

Hamre *et al.* (2008b) found that rotifers have quite low levels of minerals compared with copepods. Selenium in rotifers was even below the requirement given by NRC (2011) for fish. Furthermore, cod larvae fed on copepods cultured in a pond in Northern Norway contained much higher levels of minerals than cod larvae cultured on rotifers (Busch *et al.* 2010). *Artemia francicella* from Great Salt Lake, USA, contained high levels of most minerals, with the exception of iodine and zinc (Hamre *et al.* 2007). The low levels of some minerals in live prey have prompted some requirement studies to check if the feed has a sufficient mineral status.

In a pilot study, Hamre *et al.* (2008a) fed cod larvae with rotifers enriched with iodine and selenium combined and found a significantly higher survival than in the control group. The added selenium was transferred to the larvae, but not the iodine. The lack of transfer of iodine was probably caused by the low retention of NaI in rotifers during storage (Srivastava *et al.* 2012). In an unpublished study, enrichment of *Artemia* with iodine had no or minor effects on Atlantic halibut larval performance (Mari Moren, pers. comm., 2004). Furthermore, in an experiment with cod (Samuel James Penglase *et al.* submitted, 2012), also using NaI enriched rotifers but taking measures to keep the concentration of iodine high until the rotifers were fed to the larvae, the control rotifers appeared to have sufficient iodine (0.6 g kg^{-1} DM) to cover the larval requirement. On the other hand, Ribeiro *et al.* (2011) detected goitre, lowered growth and effects on thyroid hormone metabolism in Senegalese sole larvae fed control rotifers and *Artemia*, compared with larvae reared on iodine enriched live feeds. An important difference between the three studies is that the Atlantic halibut and cod larvae were reared in a flow-through system, while the Senegalese sole was reared in a recirculation system. Ozone used for disinfection of the water during recirculation will probably oxidize bioavailable iodide in sea water to the unavailable form IO_3^-. Furthermore, a build-up of nitrate in recirculation units may block the uptake of iodide in fish (Morris *et al.* 2011). Analyses of a range of different rotifer samples (Kristin Hamre, unpubl. data, 2008–2012) also show that iodine may drop below the minimum requirement for fish (NRC 2011). In the study by Samuel James Penglase *et al.* (submitted, 2012), iodine was taken up in cod larvae in a dose dependent manner, and 129 mg kg^{-1} DM iodine in rotifers gave a mild colloid goitre in the larvae, indicating mild toxicity, whereas copepods can contain up to 350 mg kg^{-1} iodine (Solbakken *et al.* 2003). Why copepods are not toxic to fish larvae may be explained by differences in bioavailability and/or toxicity between different forms of iodine. A confounding effect is that iodine has bactericide properties that may reduce the bacterial load in live prey cultures during enrichment and thereby increase larval survival. The data thus far therefore suggest that rotifer enrichment diets should contain some iodine to take into account possible low basic levels in rotifers and depletion of iodine in larvae reared in recirculation systems. A safe level of iodine in rotifers is 26 mg kg^{-1} DM (Samuel James Penglase *et al.*, submitted, 2012).

Penglase *et al.* (2010) fed rotifers enriched with selenium up to 3 mg kg^{-1} dry weight selenium to cod larvae. They found only minor effects on growth and survival, but gene expression and activity of the glutathione peroxidases were enhanced by the enrichment, indicating a requirement of selenium above the control level. Control rotifers in this experiment contained 0.7 mg kg^{-1} selenium, while in other experiments, selenium concentrations down to 0.04 mg kg^{-1} dry weight have been found in rotifers (Samuel James Penglase *et al.*, unpubl. data, 2012). The requirement for selenium in juvenile and adult fish is around 0.35 mg kg^{-1} (NRC 2011).

As illustrated above, direct measurements of mineral requirements in fish larvae are fragmented and scarce, as each nutrient is measured only in a few species or not at all. Mineral requirement studies are also complicated by the presence of mineral in seawater and by the fact that different forms of minerals may have different bioavailabilities. More work is therefore needed to conclude on whether mineral requirements in fish larvae are different from those in juvenile and adult fish.

Indirect measurements

Nutrient utilization during the yolk sac period

It has been suggested that composition, utilization and larval retention of the yolk nutrients can provide a good estimate of the larval nutrient requirements, particularly in the first feeding stages (Heming & Buddington 1988; Izquierdo 1996). This is based on the presumption that good quality larvae have undergone complete embryogenesis, and grown from a single cell to a complete free swimming individual based on a feed with good/optimal nutrient composition (=yolk). By measuring the absorption of different nutrients from the yolk to the larvae (=endogenous feed intake) and analyse their retention in fish larvae, the requirement can be qualitatively and quantitatively calculated. This model does not take bioavailability and digestion into account but merely discuss the requirement at the tissue level. The approach also is based on an extrapolation of the requirements in the yolk-sac stage into the first feeding stages and possibly beyond. When such studies are considered, attention should be paid also to the fact that the composition of the eggs reflects that of the maternal diet: Lie (1993) found that levels of DHA and EPA in cod eggs from cultured broodstocks were lower than those found by other authors (Tocher & Sargent 1984; Fraser *et al.* 1988) in eggs from wild fish. Despite these uncertainties, this approach gives valuable information.

There are several studies that use this approach for discussing utilization and retention of dry matter, energy and macronutrients (Blaxter & Hempel 1966; Cetta & Capuzzo 1982; Heming & Buddington 1988; Rønnestad *et al.* 1992a,b, 1993; Finn 1994; Faleiro & Narciso 2010), while similar analysis of vitamins, minerals and trace elements are scarce (Rønnestad *et al.* 1997, 1999; Mæland *et al.* 2003). At present there is no holistic understanding of the utilization of the yolk matter by the growing fish larvae. As pointed out by Rønnestad *et al.* (1995), this approach must be based on data calculated in absolute terms (e.g. moles per individual), before discussing aspects of utilization, synthesis, bioconversion, selective retention or catabolism of various components in developing eggs and larvae. Presenting relative data can lead to erroneous conclusions, since proportional numbers most often are related to a component that also changes per individual with development.

There are several studies, particularly in marine species, that describe the energy metabolism in the early stages, including utilization of substrates in the yolk and the energetic role of (an) oil droplet(s) in the egg. Free amino acids (FAA), proteins and lipids seem to be key factors for energy metabolism (Cetta & Capuzzo 1982; Vetter *et al.* 1983; Quantz 1985; Tocher *et al.* 1985a,b; Heming & Buddington 1988; Rainuzzo *et al.* 1992; Rønnestad *et al.*1992a,b, 1993; Finn *et al.* 1995, 1996; Conceição *et al.* 1998b; Yúfera *et al.* 1999a; Parra & Yúfera 2001; Buentello *et al.* 2011).

Amino acids.

In newly spawned pelagic eggs, free amino acids (FAA) account for 20–40% of total amino acids while in benthic eggs it is only 2–4%. In Atlantic halibut, FAA and protein represent 12% and 57% of egg dry weight, respectively. Thus, in this pelagic egg FAA comprise a similar amount to the total lipids. The timing and sequential utilization of yolk protein and FAA vary within teleosts (Rønnestad *et al.* 1993; Ohkubo *et al.* 2008; Hastey *et al.* 2010). It is presumed that yolk proteins are hydrolysed as part of the absorption process and that at the cellular level both FAA and proteins originating from the yolk are presented as FAA. In eggs with no oil globules, amino acids, mainly from the free pool, represent 40–90% of the energy substrates from the onset of first feeding, while in eggs that contain oil globule(s) amino acids supply about 10% of the energy at first feeding. The remainder will be from different lipid sources (Finn *et al.* 1995, 1996; Rønnestad *et al.* 1995). These numbers can be used as an approximation of the requirement for amino acids (AA) for energy metabolism in first feeding larvae. Moreover, amino acids are primarily required as building blocks for body proteins, and are also precursors of other molecules with important physiological functions, such as purines, hormones and neurotransmitters. Therefore, the total requirement for amino acids will depend on the cumulative requirements for net protein accretion, energy and synthesis of other molecules, and will need to take into account potential buffering effects of synthesis and turnover of different proteins (Conceição *et al.* 2003b), but so far no quantitative model using this approach has been developed. Also, the physiological role of individual amino acid varies, as does the utilization of the amino acid from the yolk. Amino acids that are handled very differently from the others include taurine, which remains constant and everything present in the yolk is retained in the larva, as well as phosphoserine for which there is a net synthesis in the free pool (Rønnestad *et al.* 1995).

The decline in individual amino acid contents of whole yolk-sac larvae, may be used as an index of amino acid requirement for energy production. However, amino acid transaminations, or synthesis of other N-containing molecules, may also affect such amino acid depletion rates. Moreover, changes in amino acid depletion rates can be, at least partly, explained by differences in the larval and yolk amino acid profiles, and also by changes in AA profile during ontogeny. The high apparent depletion rates of the branched chain amino acid (Leu, Ile, Val) were associated by Conceição *et al.* (1998b) with a decrease in their contribution to the larval AA profile and/or with an excess of these AA in the yolk compared with the larval AA profile. In turn, the low yolk content of African catfish larvae of phenylalanine, lysine, cysteine and tyrosine compared with the larval AA profile were associated with relatively low depletion rates for these AA.

Lipids.

The lipid content of eggs varies between species. It accounts for 8–26% of DM (Heming & Buddington 1988) and is the second largest DM component. The composition of lipids (total lipids, lipid classes and fatty acids) also varies between species (see above). The same is also the case for the utilization of lipids and fatty acids until first feeding. Some of these differences seem to be related to habitat water temperatures. In halibut, a detailed study (Rønnestad *et al.* 1995) revealed that lipids in the yolk accounted for approximately 11% of DM. Of the total yolk lipids phosphatidylcholine (PC) accounted for 57%, while phosphatidylethanolamine (PE), triacylglycerol (TAG), cholesterol and sterol ester (SE) accounted for 12%, 12%, 9% and 6%, respectively. The main fatty acids in the PC fraction were 22:6n-3, 16:0 and 20:5n-3. During development there were some, but relatively minor, changes in the relative composition of lipids in the yolk, indicating a non-selective endocytotic bulk uptake of lipids from the yolk. Towards first feeding, however, there was a selective catabolism of PC and a net synthesis of PE in the developing body, resulting in a shift in the lipid class composition in the body compared with that of the yolk (Rønnestad *et al.* 1995).

The fatty acids released from lipid hydrolysis were mainly used as energy substrates by the growing halibut larvae and it is interesting to note that 22:6n-3 was quantitatively one of the most important fatty acid fuels. At the same time, 38% and 23% of the 22:6n-3 released from PC was retained by the PE and neutral lipids in the growing larval body, respectively. Except for 20:5n-3 (2%, 14%) no similar retention was seen in any of the other fatty acids. In developing larvae of Atlantic halibut, there was a net synthesis of PE concomitant with an increase in the fraction of 22:6 n-3 in it from 28% of total fatty acids at hatching to 45% at 200 days post hatch (Rønnestad *et al.* 1995). This indicates important functions for PE in the developing larval body.

Also in other species there seems to be some conservation of specific fatty acids, such as the selective retention of PUFAs in Atlantic cod, plaice and turbot (Rainuzzo *et al.* 1992). There are more studies that describe the utilization of fatty acids and lipid classes in variable detail (Finn *et al.* 1995, 1996; Mourente *et al.* 1999a,b; Ohkubo *et al.* 2008; Samaee *et al.* 2009). However, a compilation of such data into a model for requirement at first feeding is still lacking.

Vitamins.

Also for vitamins the few available data indicate species differences in the utilization before onset of first feeding. A study in halibut (Rønnestad *et al.* 1999) revealed that Asc and α-TOH levels in whole larvae were constant during the yolk sac stage, suggesting no loss or utilization of these vitamins. At hatching about 80% of the Asc and 97% of the α-TOH were contained within the yolk-sac compartment. With development, Asc and α-TOH levels in the yolk decreased, although at different rates. At first feeding >95% of Asc but only <30% of α-TOH in the yolk at hatching had been transferred to the larval body. The transfer of α-TOH was completed when the yolk was absorbed completely.

For vitamin A the mass budget during endogenous feeding is more complicated (Rønnestad *et al.* 1998), since there are several forms in the yolk and in the larvae of the vitamin (all *trans* retinol, 13 *cis* retinol and all *trans* retinal) as well of its carotenoid precursors (lutein, zeaxanthine, α-carotene, β-carotene). The conversion of the precursors to vitamin A has been studied in juveniles. All carotenoids tested were converted to vitamin A, but to different degrees (Moren *et al.* 2002). The efficiency of conversion during the important phase when the eyes become functional before first feeding is not known.

A study of vitamin B_6 revealed that about 25% of the vitamin present in the yolk of halibut had been lost at first feeding (Rønnestad *et al.* 1997). The net consumption of B_6 was slow at first and then increased steadily during the yolk-sac stage when the DM of the embryo increased. Also in rainbow trout (Sato *et al.* 1987), a decline during endogenous feeding was shown, representing a loss of 45% of B_6. Calculation of the data of Rønnestad *et al.* (1997) acquired during the endogenous feeding phase support a review of Woodward (1994) who postulated that there is a uniform B_6 requirement among fishes of 3 μg g^{-1} DM weight gain. Thus, it is important to take into account that B_6 is used and must be replenished through the diet.

Measurements of the utilization of nutrients from the yolk can give a good estimate of the amount of individual nutrients needed to build the organism, but fails to include possible limitations resulting from inefficient digestion and absorption of the nutrients. New knowledge may be extracted from the data as they appear in the literature, by recalculation of the rate of uptake from the yolk per unit of larval growth. These figures can be compared with requirements measured by direct methods. Since the developing larvae during endogenous feeding represent a (almost) closed system, these data could also be utilized better by building mass balance based kinetic models of the transfer of nutrients from yolk to the body that could provide a better understanding of the nutritional requirements of the growing larvae.

Nutrient composition of copepods

Although fish larvae in the oceans may feed on algae and different plankton organisms, copepods are the main feed for wild fish larvae (Arthur 1976), and it can be assumed that this group of prey organisms will cover the larval nutrient requirements. Copepods are very rich in protein, n-3 fatty acids, polar lipids, certain vitamins and microminerals (Tables 1–3; Mæland *et al.* 2000; Hamre *et al.* 2008b, 2002; van der Meeren *et al.* 2008), when compared with given nutrient requirements for fish (NRC 2011) and it can be speculated that fish larvae have adapted to ingesting and digesting these prey organisms during evolution and therefore accordingly have high requirements. However, it is also possible that the actual requirements of the larvae are less or even much lower than what they get through their natural feed. Nutrient concentrations in copepods may vary according to species, food supply and environmental conditions, and copepods cultured in a pond with ample food supply have higher levels of micronutrients than copepods harvested from the sea (Kristin Hamre, unpubl. data, 2009). Variation may also occur between copepods from different geographical areas/latitudes, while most of the analytical work has been done on copepods from the Northern hemisphere, which are the main source of the information presented here.

van der Meeren *et al.* (2008) measured protein bound and free amino acids in copepods, rotifers and *Artemia* and the sum amounted to 450–500, 260 and 320–350 mg g^{-1} DM total amino acids, respectively. This total amino acid content of rotifers probably represents an underestimation, since it should be 380–450 mg g^{-1} DM (Øie *et al.* 1997; Srivastava *et al.* 2006; Helland *et al.* 2010). However, the relative differences should be representative, and by calculation, copepods should contain about 660–730 mg g^{-1} total amino acids. This is in accordance with the total amino acid content of copepods given in Table 1 of 634 ± 89 mg g^{-1} DM and slightly higher than data from Perumal *et al.* (2009). These authors analysed two species of copepods, *Acartia spinicauda* and *Oithona similis*, from three stations along the southeast coast of India. The protein levels analysed as N*6.25 were similar at 590–700 mg g^{-1} DM for *O. similis* and at 670–750 mg g^{-1} DM for *A. spinicauda* at the three stations. Recalculated using a protein to nitrogen factor of 5.30 (Table 3), the levels would be 500–594 and 568–636 mg g^{-1} DM, respectively. It is expected that the total amino acid content within the same stage and species of copepods will be quite stable because it is largely genetically determined, but there may be variation between different species. Copepods contain a larger fraction of free amino acids than rotifers and *Artemia* (Table 1; van der Meeren *et al.* 2008), while live feed in general contains a large fraction soluble protein (50–70%, Carvalho *et al.* 2004, 2003; Tonheim *et al.* 2007).

The fat content of the stages of copepods that are eaten by marine fish larvae, for example nauplii and copepodites, is 6–16% of DM, according to van der Meeren *et al.* (2008). Perumal *et al.* (2009) found total lipid levels in the range 120–180 mg g^{-1} DM in *A. spinicauda* and 99–180 mg g^{-1} DM in *O. similis*. Lipid in copepods is characterized by a high level of n-3 fatty acids (20–56% of total fatty acids; van der Meeren *et al.* 2008; Shields *et al.* 1999; Table 2), a low level of ARA (<1.6%) and a large fraction of polar lipid (56–63% of total lipid and 54–63 mg g^{-1} dry weight). The polar lipids are present mainly in biological membranes, so excess polar lipid cannot be stored in the body and the level expressed as mg g^{-1} DM should be quite stable. Glycogen in *O. similis* and *A. spinicauda* from the southeast of India varied between 34–66 and 40–80 mg g^{-1} DM, respectively, and ash between 30 and 48 mg g^{-1} DM in for both species. The glycogen levels were approximately 10 times higher and the ash levels were lower than measured in copepods from a fertilized seawater pond in western Norway (Table 1).

Table 1 shows that several micronutrients are present at much higher concentrations in copepods than the ranges of requirements given for fish by NRC (2011). Copepods do not contain vitamin A (Rønnestad *et al.* 1998; Moren *et al.* 2005), but marine fish larvae probably convert astaxanthin present at high levels in copepods, to vitamin A, as is the case for Atlantic halibut juveniles (Moren *et al.* 2002).

In summary, there is quite a lot of information on the nutrient composition of copepods, but the work is mainly based on copepods cultured in seawater ponds in Norway, in a nutrient rich environment that favours a low number of species. Copepods from the open sea and from other latitudes may have a different composition from

Table 1 Basic levels of macronutrients, vitamins and minerals in unenriched rotifers, *Artemia* nauplii (EG-type, Great Salt Lake UT, USA, INVE Aquaculture) ongrown *Artemia* and zooplankton, mainly copepods, harvested from a fertilized seawater pond in western Norway (Svartatjønn). The ranges of requirements in juvenile and adult fish given by NRC (2011) are listed for comparison

	Rotifers†	*Artemia*‡	*Ongrown Artemia*‡	Copepods§	NRC (2011)
Macronutrients (g kg^{-1} DM)					
Total amino acids (TAA)	396 ± 12	471–503	596 ± 59	634 ± 89	–
Nitrogen	89 ± 2	85–102	101 ± 10	119 ± 5	–
Protein/nitrogen factor	4.46	4.95–5.57	5.79 ± 0.85	5.30 ± 0.44	–
Soluble AA (% of TAA)	44–61¶	54 ± 4¶	na	na	–
FAA (% of TAA)	5–7	9–10	na	12–13	–
Lipid (TL)	95–110	102	178 ± 34	156 ± 31	–
PL (% TL)	34	31	33 ± 2	50 ± 12	–
NL (% TL)	66	69	67 ± 2	50 ± 12	–
Total fatty acids	90 ± 21	119	84 ± 8	na	–
Glycogen	na	74–96	21 ± 1	5 ± 2	–
Ash	96	90	197 ± 12	95–104	–
Vitamins (mg kg^{-1} DM)					
Vitamin C	117–190	798	400–1000	500	50
Riboflavin	22–44	37	27–60	14–27	4–7
Thiamine (B1)	2.0–57	4.2	3–12	13–23	1
Folic acid	4.0–57	14	6–11	3–5	1
Pyridoxine (B6)	20–25	28	2–33	2–6	3–6
Biotin	1.6–1.8	4.5	2–5	0.6–0.9	0.15–1
Cobalamin (B12)	23–43	0.00	2–5	1–2	0.02
Niacin	191–249	159	160–250	100–150	10–28
Vitamin E	85–294	70	64–500	110	50
Carotenoids	24	630–750	650–750	630–750	
Vitamin A	0.00	0.00	0.00	0	0.75
Minerals (g kg^{-1} DM)					
Phosphorus	9.4 ± 0.7	12–19	na	12.4–15.0	3–8
Calcium	1.9 ± 0.2	1.9–2.0	na	1.1–2.4	nd
Magnesium	4.8 ± 0.5	2.0–5.0	na	2.4–3.1	0.4–0.6
Minerals (mg kg^{-1} DM)					
Iodine	3.2–7.9	0.5–4.6	2.2 ± 0.4	50–350	0.6–1.1
Manganese	3.9–5.1	4–30	na	8–25	2–12
Copper	2.7–3.1	7–40	na	12–38	3–5
Zinc	62–64	120–310	na	340–570	15–37
Selenium	0.08–0.09	2.2	na	3–5	0.15–0.25
Iron	84–114	63–130	na	85–371	30–150

na, not analysed; nd, not determined.
†The rotifers were grown on yeast and cod liver oil, yeast and Algamac™ (Aquafauna Bio-marine, Inc., CA, USA) or yeast and Chlorella (Chlorella Industry Co. Ltd, Tokyo, Japan) for 4 days. Data from Srivastava *et al.* (2006), van der Meeren *et al.* (2008) and Hamre *et al.* (2008b).
‡The *Artemia* were either newly hatched or grown on micronized fish meal for 4 days after hatching. Data from Hamre *et al.* (2002, 2007), van der Meeren *et al.* (2008) and Hamre and Harboe, unpubl. data.
§Data on copepods are from Hamre *et al.* 2005, 2008b and Rønnestad *et al.* 1995.
¶Carvalho *et al.* (2003).

those referred in the present review. As stressed in the introduction to this chapter, copepod nutrient levels are not equivalent to larval requirements but can be used as an estimate of the composition of the larval diet in nature.

Larval body composition

The indispensable AA (IAA) profile of fish larvae whole body has been proposed as a good index of the IAA requirements (Tulli & Tibaldi 1997; Conceição *et al.* 2003b). In order to verify to what extent AA requirements are met by the prey organisms and microdiets currently used to feed fish larvae, a first approximation can be to compare the dietary and larval AA profiles.

Rotifers seem to have an unbalanced AA profile for marine fish larvae. Rotifers seem deficient in histidine, arginine and lysine for gilthead seabream larvae

Table 2 Composition of total amino acids (% of protein) in rotifers, *Artemia* (EG- type, Great Salt Lake UT, USA, INVE Aquaculture) and copepods harvested from a fertilized seawater pond in western Norway (Svartatjønn)

	Rotifers	*Artemia*	Copepods
Leu†	8.2 ± 0.1	8.1 ± 0.1	7.6 ± 0.1
Lys†	7.4 ± 0.2	8.3 ± 0.1	7.4 ± 0.2
Arg†	6.0 ± 0.0	7.4 ± 0.1	7.5 ± 0.3
Val†	5.6 ± 0.0	5.4 ± 0.0	5.3 ± 0.1
Ile†	5.2 ± 0.1	5.0 ± 0.0	4.4 ± 0.1
Phe†	5.2 ± 0.0	4.7 ± 0.0	4.1 ± 0.2
Tyr†	4.5 ± 0.1	4.6 ± 0.0	4.6 ± 0.3
Thr†	4.4 ± 0.1	4.9 ± 0.0	4.9 ± 0.1
Cys†	2.4 ± 0.3	na	na
His†	2.1 ± 0.1	1.9 ± 0.1	2.2 ± 0.4
Met†	1.9 ± 0.1	2.1 ± 0.1	2.3 ± 0.1
Trp†	1.4 ± 0.1	na	na
Glu	13.4 ± 0.1	13.9 ± 0.1	13.6 ± 0.3
Asp	10.4 ± 0.1	9.3 ± 0.1	9.6 ± 0.1
Ser	6.3 ± 0.2	5.2 ± 0.0	5.3 ± 0.2
Ala	5.6 ± 0.1	6.8 ± 0.0	7.1 ± 0.1
Pro	5.6 ± 0.1	5.2 ± 0.1	5.3 ± 0.3
Gly	4.3 ± 0.1	5.2 ± 0.0	7.5 ± 0.3
Tau	0.08 ± 0.04	2.1 ± 0.1	1.5 ± 0.2
EAA†	47.3	42.4 ± 0.1	40.2 ± 0.5
NEAA	52.7	57.6 ± 0.1	59.8 ± 0.5
Total AA	100	100	100

EAA, essential amino acids; NEAA, non-essential amino acids. na, not analysed. Data from Srivastava *et al.* (2006) and recalculated from Hamre *et al.* (2002).
†Indispensable amino acids.

Table 3 Composition of free amino acids (% of total free amino acids) in rotifers, *Artemia* (EG- type, Great Salt Lake UT, USA, INVE Aquaculture) and copepods harvested from a fertilized seawater pond in western Norway (Svartatjønn)

	Rotifers	*Artemia*	Copepods
Arg†	8.3 ± 0.3	9.8 ± 0.2	11.9 ± 0.5
Lys†	7.8 ± 0.2	9.2 ± 0.1	8.7 ± 0.3
Leu†	7.7 ± 0.3	6.3 ± 0.3	7.5 ± 0.2
Phe†	5.7 ± 0.2	3.8 ± 0.1	3.7 ± 0.3
Tyr†	5.4 ± 0.2	4.4 ± 0.1	3.9 ± 0.8
Val†	4.9 ± 0.0	4.7 ± 0.1	5.2 ± 0.2
Ile†	4.9 ± 0.0	2.4 ± 0.1	3.1 ± 0.5
Thr†	4.1 ± 0.0	3.4 ± 0.1	3.6 ± 0.1
His†	2.0 ± 0.1	2.2 ± 0.1	2.0 ± 0.1
Met†	1.9 ± 0.2	2.6 ± 0.1	3.1 ± 0.1
Trp†	1.4 ± 0.1	1.0 ± 0.1	1.1 ± 0.3
Orn	nd	0.5 ± 0.1	0.1 ± 0.0
Glu	14.1 ± 0.6	7.2 ± 0.3	5.9 ± 1.4
Asp	10.5 ± 1.0	1.4 ± 0.1	2.1 ± 0.4
Ala	8.6 ± 0.4	11.2 ± 0.2	8.2 ± 0.4
Ser	6.2 ± 0.4	5.3 ± 0.1	4.4 ± 0.2
Pro	3.5 ± 0.3	6.8 ± 0.6	7.3 ± 0.7
Gly	2.8 ± 0.5	3.5 ± 0.2	10.6 ± 1.2
Tau	1.4 ± 0.7	7.9 ± 0.4	4.4 ± 0.4
Gln	nd	6.4 ± 0.3	3.2 ± 0.5
Orn	nd	0.5 ± 0.1	0.1 ± 0.0
Hyp	nd	0.1 ± 0.0	nd
SUM	100	100	100

Data from Srivastava *et al.* (2006) and recalculated from Hamre *et al.* (2002).
nd, not detected.
†Indispensable amino acids.

(Aragão *et al.* 2004c), and unbalanced in histidine, arginine, lysine, threonine and cysteine for both white seabream (Saavedra *et al.* 2006) and sharpsnout seabream (Saavedra *et al.* 2007). Histidine is probably the first-limiting AA when rotifers are fed to any of these three Sparid species. The IAA profile of rotifers also seems to be deficient in leucine, arginine and methionine for 6-day-old turbot larvae, and in leucine and threonine at 11-day-old larvae (Conceição *et al.* 1997). The same authors report that the IAA profile of *Artemia* seems to be deficient in leucine and methionine for 23-day-old turbot larvae.

Variations in AA profile during larval development may indicate changes in larvae requirements at different ages (Conceição *et al.* 2003b). Such ontogenetic changes in AA profile are associated with larval allometric growth (Oikawa & Itazawa 1984; Osse & Boogaart 1995), as different organs and tissues develop at varying rates. The magnitude of these developmental changes in AA profile vary among species, depending on their developmental pattern; in species with a marked metamorphosis, such as the flatfish Senegalese sole, changes in the AA profile during ontogeny are more pronounced than in species that have a smoother metamorphosis, such as the sparid gilthead seabream (Aragão *et al.* 2004b).

Still, the larval IAA profile is a rough indicator of the AA requirements. Several factors may reduce (or amplify) the impact of the dietary imbalances. As AA are an important energy source for fish larvae, there is an obligatory AA loss independent of the AA profile of the diet, as some AA will always be used for energy production even when there is a perfect match between dietary and larval AA profiles. However, at least in fast growing fish larvae these obligatory AA losses are probably much smaller (in % of absorbed AA) than in slower growing larger fish (Conceição *et al.* 2003b). Furthermore, differential absorption and selective catabolism of individual AAs (Saavedra *et al.* 2008a,b) may reduce (or amplify) the impact of the dietary imbalances on AA losses. Therefore, when using the larval IAA profile as an indicator of AA requirements in fish larvae, a correction for the bioavailability of the individual AA should be performed. Methods using tracers have been employed to assess the relative bioavailability of individual AA in fish larvae (Conceição *et al.* 2003a; Saavedra *et al.* 2007).

Tracer studies

Tracer studies have been used occasionally to determine food intake, digestion, absorption and utilization of nutrients since the 1960s, and more intensively in recent years. These studies have advantages since they enable controlled dose–response studies, and quantification of feed ingestion and depending on nutrient, also assessment of digestibility. This technique has also been proposed to estimate the nutrient requirement based on the oxidation method (Morais & Conceição 2009). However, tracer studies are short-term evaluations and with other known limitations (Rønnestad *et al.* 2001; Conceição *et al.* 2007), and therefore interpretation of the results should be done with care. In particular, the main results should be validated in long-term trials. Most tracer studies consist of a mass balance that follows the compartmental distribution of a tracer, that could be radioactive isotope (e.g. ^{14}C, ^{35}S, ^{3}H) or a stable isotope (e.g. ^{13}C, ^{15}N), that has been fed to larvae as part of a meal or given directly into the digestive tract by tube-feeding (Conceição *et al.* 2007). This tracer nutrient (normally ^{14}C-labelled) is then quantified in different compartments – faeces, retention in body tissues and catabolized – after a given time. Such studies have been used to assess the relative digestion/absorption capacity of protein, individual AA, fatty acids and lipid classes, as well as their relative utilization for energy production (Conceição *et al.* 2007, 2010a; Rønnestad and Conceição *et al.* 2012).

Digestibility. Using ^{14}C-labelled proteins it has been shown that fish larvae absorb faster and retain more efficiently FAA than protein (Rønnestad *et al.* 2000; Rojas-García & Rønnestad 2003) and hydrolysed protein is also absorbed faster than an intact complex protein (Tonheim *et al.* 2005). In addition, Tonheim *et al.* (2005) demonstrated that digestion capacity of a complex protein increases with Atlantic halibut larval age and size. Such studies suggest that fish larvae have problems in digesting diets based on complex proteins. However, Morais *et al.* (2004a,b) and Engrola *et al.* (2010) reported that Senegalese sole and Atlantic herring larvae have a good capacity to digest *Artemia* protein (57–83% of total *Artemia* intake). At the same time, more than 50% of *Artemia* proteins are water-soluble and should therefore be highly digestible (Carvalho *et al.* 2003, 2004). This shows that fish larvae may realize their tremendous growth potential when the right protein quality is provided.

Tube-feeding may also be used to study the absorption efficiency of individual amino acids; in Senegalese sole and Atlantic halibut larvae lysine, arginine, glutamate and alanine were all absorbed with a very high efficiency; average 97.5% and 94%, respectively (Rønnestad *et al.* 2001; Applebaum & Rønnestad 2004). In white seabream (*Diplodus sargus*) larvae, methionine and arginine were shown to be better absorbed than tryptophan, tyrosine and especially lysine (Saavedra *et al.* 2008a,b). It is likely that the absorption efficiency will vary with administered dose and gut residence time, although Applebaum and Rønnestad (2004) failed to demonstrate saturation of transport at luminal amino acid concentrations below 20 mM.

Feeding regime and diet type have also been shown to affect the digestibility of protein from ^{14}C-labelled *Artemia* (Engrola *et al.* 2009, 2010). When replacing *Artemia* by an inert diet for Senegalese sole larvae, a low *Artemia* replacement (20% DM of total diet) had no effect on protein digestibility (Engrola *et al.* 2009), in contrast to a high *Artemia* replacement level (58% of total diet), where *Artemia* protein digestibility decreased to 69% during metamorphosis (Engrola *et al.* 2010), compared with a value of 78% in the group fed *Artemia* alone.

The form in which a fatty acid is supplied in the diet (free or esterified to TAG or PL) affects its digestion and absorption (Morais *et al.* 2005a–c). Apparently, the digestibility of dietary FA is higher when supplied in the form of PL rather than TAG. The digestibility of OA in its free form or as a PL is also higher when compared with supplying it in a TAG (Morais *et al.* 2005b,c, 2006). Moreover, lipid absorption in larval Atlantic halibut has been shown to decrease with increasing lipid complexity (Mollan *et al.* 2008). The larval faecal evacuation after tube feeding ranged from 66 ± 20% of TAG to 9 ± 6% of MAG. DAG was intermediate with 52 ± 21%. This led to the hypothesis that hydrolysation of the lipid would improve lipid utilization. In order to test this hypothesis cod larvae were fed formulated diets supplemented with radiolabelled hydrolysed and intact TAG and hydrolysed and intact PC (Hamre *et al.* 2011). The four diets had the same lipid level and composition, and the radioactive label was placed on the different lipids. Less than 16% of the label was evacuated during a period of 10 h after a single meal and there were no differences between the diets. Absorption of the tracer was dose dependent, larvae eating less lipid than 0.5% of whole body DM absorbed close to 100% of the label, while at higher feed intakes, absorption efficiency decreased accordingly. PC seemed to be retained both in the digestive tract and carcass tissue, while TAG was rapidly absorbed from the gut into the body and catabolized. However, there was no effect of hydrolysation on absorption and utilization of the lipids. The different results from the tracer studies with lipids may be due to species dependent differences in lipid digestion, absorption and metabolism, or to differences in methodology. Feeding regime and diet type have also been shown to affect lipid digestibility. Mai *et al.* (2009) observed that *Artemia* replacement strategy in Senegalese sole larvae directly affected lipid digestibility.

Moreover, using ^{14}C-labelled microdiets it has been shown that the absorption of dietary FA is enhanced by the inclusion of dietary lecithin and PC (Koven *et al.* 1993; Hadas *et al.* 2003). These findings are in line with the hypothesis that fish larvae have a dietary requirement for PL, in order to maintain acceptable rates of lipoprotein synthesis and export of the absorbed lipids from the enterocytes into the body.

Amino acids. Tube-feeding of ^{14}C-labelled amino acids have shown that dispensable amino acids are used preferentially for energy in the larval stages of Atlantic herring, Senegalese sole and Atlantic halibut, while indispensable amino acids are preferentially spared for growth (Rønnestad *et al.* 2001; Conceição *et al.* 2002; Applebaum & Rønnestad 2004). Furthermore, aromatic amino acids (phenylalanine and tyrosine) were preferentially retained during metamorphosis climax in Senegalese sole larvae, while no ontogenetic changes were clear in gilthead seabream larvae (Pinto *et al.* 2009).

The tube-feeding technique may also be used to study short-term effects of amino acids supplementation in fish larvae. Amino acid retention has been shown to increase in the larval Senegalese sole fed diets with balanced amino acids profiles (Aragão *et al.* 2004c). In white seabream larvae such studies have shown that the catabolism of individual indispensable amino acids varies (Saavedra *et al.* 2008a,b), being higher for tyrosine, intermediate for methionine, tryptophan and lysine, and lower for arginine, which may have consequences in terms of amino acid requirements.

A method combining high-resolution ^{13}C-NMR spectroscopy and the use of ^{13}C-labelled live food has been used to show that the relative bioavailability of individual amino acids is variable in gilthead seabream larvae (Conceição *et al.* 2003a). Saavedra *et al.* (2007) performed a similar study in sharpsnout seabream (*Diplodus puntazzo*) larvae, using a more sensitive and simpler combination of ^{15}N-enriched rotifers and GC-IRMS. These methods allow the study of the qualitative amino acids requirements of fish larvae by considering the differences in bioavailability of individual amino acids. Estimation of the relative bioavailabilities of individual amino acids can then be used to correct the larval indispensable amino acids profile, enabling an estimation of the ideal dietary amino acid profile. For instance, Saavedra *et al.* (2007) showed that when amino acid profiles of snarpshout seabream larvae were corrected with bioavailability data, a dietary deficiency of lysine, methionine and tyrosine occurred at certain developmental stages, contrary to what was apparent by using the larval indispensable amino acid profile alone.

Fatty acids. Studies conducted using radiolabelled fatty acids, supplied either in the microdiet (Izquierdo *et al.* 2001) or tube-fed to the larvae (Morais *et al.* 2005c), have shown that individual FA are metabolized differently. Long chain polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), tend to be mostly retained in the body tissues, while OA is mostly incorporated into TAG and preferentially catabolized as an energy source (Izquierdo *et al.* 2001; Morais *et al.* 2005c).

The tube-feeding technique has also been proposed to estimate nutrient requirements (Morais & Conceição 2009). These authors suggested that increasing dietary supply of DHA above the larval requirement level results in increased oxidation for energy purposes. Tracer studies can thereby be used in conjunction with dose–response studies to acquire more precise requirement estimates.

Conclusion. In summary, tracer studies may be instrumental for improving the understanding of nutritional requirements of fish larvae. They allow the assessment of short-term effects on digestion, absorption capacity, catabolism and retention for proteins, amino acids, fatty acids and lipid classes.

However, the results obtained using tracer studies do not necessarily represent the digestive and metabolic performance of an undisturbed larvae feeding *ad libitum* in a culture system or in the open ocean (Conceição *et al.* 2007, 2010a). Their main findings should be confirmed through long term growth trials. Still, tracer studies can be very important tools to study developmental changes and to compare relative performance under different conditions. Furthermore, tracer studies can be very useful for screening the main effects, refining experimental designs and reducing the number of treatments to be tested in growth trials.

Extrapolation from juveniles

Using multivariate designs, Hamre *et al.* (2003) and Hamre and Mangor-Jensen (2006), measured the optimal composition of macronutrients in diets for Atlantic halibut and Atlantic cod, with initial weights of 0.2 and 0.5 g, respectively (Fig. 1). Atlantic halibut had a very low tolerance for carbohydrates, since dietary levels above 50 g kg^{-1} led to an accumulation of glycogen in the liver, dramatically increased liver weights and ultimately reduced growth. Exchange of protein with lipid at low carbohydrate levels had little impact on growth, but led to accumulation of lipid in the liver. This last result was confirmed by Hamre *et al.* (2005). The protein require-

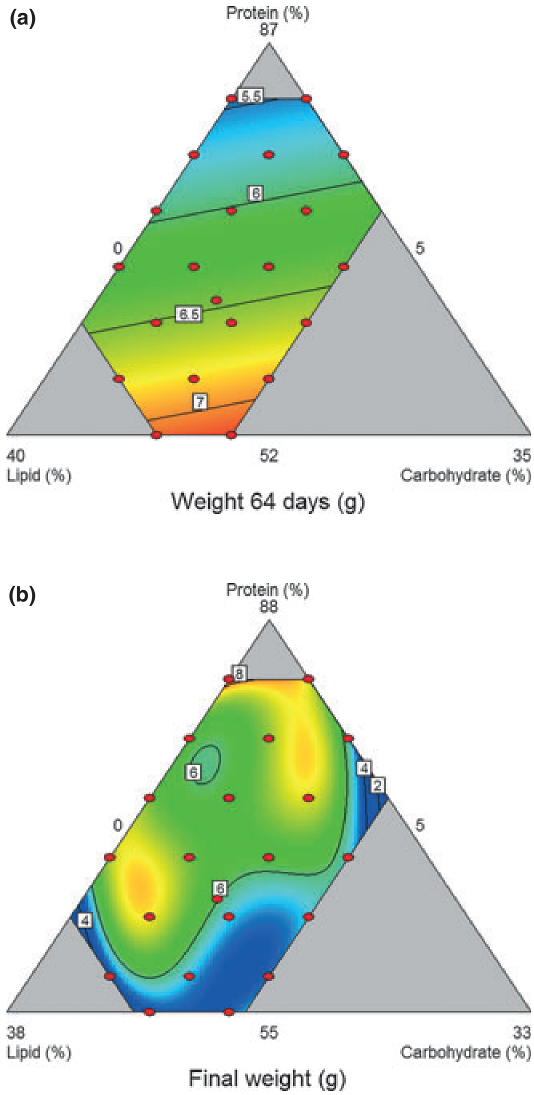

Figure 1 Final weight of (a) Atlantic cod (weight range of 5.2–7.3 g) and (b) Atlantic halibut juveniles (weight range of 4.5–8.5 g) grown for 2 months, from 0.26 and 0.5 g, respectively, on diets differing in macronutrient composition. The triangle represents all possible combinations of the three nutrients, while the red dots give the composition of the different diets.

ment for maximum growth in halibut appears to be in the range of 580 g kg^{-1} dry diet. On the other hand, Atlantic cod showed increased growth with increasing levels of both lipid and carbohydrate (up to 300 and 150 g kg^{-1} dry diet, respectively), and reduced growth at protein levels above 600 g kg^{-1} (Hamre & Mangor-Jensen 2006). The protein requirement for maximal growth in cod seems to be approximately 400 g kg^{-1} (Åsnes 2006). The data thus indicate that Atlantic cod and Atlantic halibut differ substantially in their requirements for macronutrients.

Moren *et al.* (2004) fed six diets with graded levels of vitamin A from 0 to 250 mg kg^{-1} DM to Atlantic halibut juveniles, start-weight 0.4 g. Fish fed <0.75 mg kg^{-1} retinyl equivalents, showed skin haemorrhages and reduced lengthwise growth. A dietary level of 2.5 mg kg^{-1} was needed for maximum activity of intestinal brush border enzymes and minimum enterocyte proliferation, both used as indicators of a differentiated gut epithelium, under the assumption that proliferation and differentiation of cells are modulated by retinol. The enzyme activity data also indicate that a concentration of 25 mg kg^{-1} dietary retinyl equivalents is too high. The minimum requirement measured in this study is thus similar to that given by NRC (2011) of 0.75 mg kg^{-1}, while the optimum level seems to be slightly higher. This is also in line with the requirement measured in European seabass larvae by Mazurais *et al.* (2009).

Low (5 g kg^{-1} DM, compared with 12 g kg^{-1} DM in the control feed) dietary phosphorus gave a higher incident of skeletal deformities and lower haematocrit, bone ash and bone phosphorus in Atlantic halibut of 4.6 g initial weight fed the experimental diets for 14 weeks (Lewis-McCrea & Lall 2010).

The question arises to what extent it is correct to extrapolate requirement data obtained from juveniles to fish larvae of the same species. Using vitamin A as a case study is illustrative of the difficulties faced by this approach. The fact that carotenoids is the main source of vitamin A in live feed complicates extrapolation for this vitamin. However, similar whole body vitamin A levels (wet weight) were found in larvae fed *Artemia* or zooplankton as in juveniles fed diets with 0.75 and 2.4 mg kg^{-1} DM retinyl equivalents, indicating that both feed organisms covered the requirement. Other studies have shown that vitamin A levels at the minimum requirement for fish in rotifers (0.77 mg kg^{-1} DM), which contain very little carotenoids, produce larvae without visible vitamin A deficiency symptoms and similar whole body vitamin A levels as juveniles fed 0.75–2.4 mg kg^{-1} retinyl equivalents (Moren *et al.* 2004). Moreover, when it comes to optimizing the dietary macronutrient composition for fish larvae, one must take into account that larvae and juveniles have a totally different feeding behaviour, partly due to the difference in the development of the stomach. As previously mentioned, fish larvae tend to eat when feed is available with few signs of satiation, and the gut transit time and nutrient uptake efficiency decrease with increased feed availability (Øie *et al.* 1997). Fish juveniles will eat until the stomach is full and then stop. This may affect digestion and absorption of the macronutrients differently so that the optimum dietary composition becomes different in larvae and juveniles. It could also be that a too fast gut transit prevents critical and slowly digestible and absorbable nutrients to be efficiently absorbed (Harboe *et al.* 2009).

Feed formulation

Live feed enrichment

Basic levels of nutrients in rotifers and Artemia

Data from the literature concerning the protein content of live feeds are highly variable (e.g. 24–67% protein of dry weight in rotifers; Lubzens *et al.* 1989; Dhert *et al.* 2001; van der Meeren *et al.* 2008). However, the protein content expressed as a fraction of the wet weight in one species should be relatively constant, since it is highly dependent on the genetic code of the animal, supported by the fact that rotifers cultured on diets with different protein contents had similar contents of body protein (Srivastava *et al.* 2006). The variation in reported levels therefore seems to depend primarily on the differences in analytical methods. Colorimetric methods (Lowry *et al.* 1951; van der Meeren *et al.* 2008) have a tendency to underestimate protein because they are intended for analyses of water-soluble proteins, while live feed organisms also contain proteins that are insoluble in water. Protein can also be determined by first measuring the nitrogen content of the sample and then multiplying by a particular factor. A factor of 6.25 is usually employed, but in fact, each individual organism has its own factor, which needs to be determined by measuring the total nitrogen and the total amino acid content. This has been done for rotifers, which had a factor of 4.2–4.46 (Øie *et al.* 1997; Srivastava *et al.* 2006). Table 1 gives protein to nitrogen factors based on nitrogen and total amino acid analyses for unenriched rotifers which had been cultured on basic ingredients such as yeast, cod liver oil, *Chlorella* and Algamac, unenriched *Artemia* nauplii, *Artemia* ongrown for 4 days on micronized fish meal (unenriched) and for copepods harvested from a seawater pond in Western Norway. For the total amino acid content there is a clear trend for levels below 400 mg g^{-1} DM in rotifers, just below 500 mg g^{-1} DM in *Artemia* and 550–700 mg g^{-1} DM in ongrown *Artemia* and copepods. Rotifers have a very low protein to nitrogen factor because they contain large amounts of non-protein nitrogen. Compared with the protein requirements of juvenile and adult fish, which fall between 30% and 60% of feed dry matter (NRC 2011), it seems that especially rotifers may be too low in protein. The protein content per individual prey organism primarily depends on its size, however, rotifers may lose as much as 30% of their body weight, including protein content, during 24 h starvation, for example when present in the larvae tank. Conversely they may increase their body weight and individual protein content during intensive feeding and growth periods (Lubzens *et al.* 1989; Øie *et al.* 1997).

The contents of FAA may be more than 12–13% of total amino acids in copepods, approximately 6% in rotifers and 9–10% in *Artemia* (Table 1). Rotifers and *Artemia* contain quite high levels of water-soluble protein (approximately 50%, Table 1), which will probably be the case also for copepods. The amount of FAA and soluble protein seems to be quite constant within the same species when grown on different feeds (Srivastava *et al.* 2006), but the fraction of soluble nitrogen was higher in fed compared with starved rotifers (61% and 46% of total N, respectively; Carvalho *et al.* 2003). Free amino acids are easily available for the fish larvae, since they are directly absorbed from the digestive tract without prior digestion. Furthermore it is hypothesized that water-soluble protein is more digestible than insoluble protein (Carvalho *et al.* 2003; Tonheim *et al.* 2007), making the protein in live prey more bioavailable than that in formulated diets. The composition of total amino acids is also relatively constant and independent of culture conditions within the same live prey species (Lubzens *et al.* 1989; Dhert *et al.* 2001; Aragão *et al.* 2004a; Srivastava *et al.* 2006). On the other hand, the composition of the FAA pool can be influenced by the amino acid composition of the diet (Aragão *et al.* 2004a).

Where the protein content and amino acid composition of live prey are relatively constant and dependent on the metabolism and the genetic code of the organism, the lipid level and fatty acid composition are highly dependent on the diet. The lipid level of unenriched rotifers is dependent on the lipid level of the culture diet (Table 1, Ashutosh Srivastava *et al.*, unpubl. data, 2006), and both rotifers and *Artemia* can be enriched with lipid and obtain levels of 20–30% of dry weight. The fatty acid composition of rotifers largely mirrors the fatty acid composition of the diets (Table 4, Srivastava *et al.* 2011; Olsen 2004; Srivastava *et al.* 2006). To obtain a good fatty acid profile in rotifers, they can be cultured on a diet containing a synthetic oil enriched in EPA and DHA (Table 4). Then one can focus on increasing the levels of protein and micronutrients during enrichment. *Artemia*, on the other hand, has a fatty acid profile with no DHA and limited and variable amounts of EPA upon hatching (Table 4) and must therefore be enriched with these fatty acids. *Artemia* and rotifers typically contain approximately 30% polar and approximately 70% neutral lipid, whereas copepods may have a ratio of approximately 50:50 (Table 1). Moreover, there are difficulties in achieving high levels of DHA and a correct balance between DHA and EPA in *Artemia*, given its natural tendency to retroconvert DHA into EPA (Navarro *et al.* 1999). Also, it is difficult to achieve a balanced PUFA composition in

Table 4 Fatty acid profiles of unenriched rotifers grown on yeast and cod liver oil (CLO) or yeast and EPAX 2010 (a synthetic oil from Pronova, Norway with 50% DHA and 10% EPA of total fatty acids), unenriched *Artemia* (EG- type, Great Salt Lake UT, USA, INVE Aquaculture) and copepods harvested from a fertilized seawater pond in western Norway (Svartatjønn)

Fatty acids (% TFA)	Rotifers/CLO	Rotifers/ EPAX 5010	*Artemia* nauplii unenriched	Copepods
14:0	2.7 ± 0.3	1.1 ± 0.2	0.7	5.9 ± 1.8
16:0	9.7 ± 0.5	6.9 ± 0.2	10.8	13.2 ± 1.5
16:1n-9	1.1 ± 0.2	1.1 ± 0.1	1.2	0.5 ± 0.3
16:1n-7	8.9 ± 1.3	9.6 ± 1.8	2.6	5.2 ± 3.1
18:0	3.2 ± 0.4	2.4 ± 0.6	4.6	1.9 ± 0.6
16:3n-3	1.2 ± 0.0	0.0 ± 0.0	0.0	0.7 ± 0.9
18:1n-11	2.4 ± 0.3	0.5 ± 0.3	0.0	0.0 ± 0.0
18:1n-9	22.2 ± 0.1	13.2 ± 1.2	17.3	1.5 ± 0.4
18:1n-7	3.4 ± 0.2	1.9 ± 0.2	7.2	2.5 ± 1.2
18:2n-6	5.9 ± 0.3	5.9 ± 0.7	6.3	2.7 ± 2.5
18:3n-3	0.9 ± 0.0	1.7 ± 0.5	30.3	2.2 ± 0.7
20:1n-11	1.4 ± 0.2	0.2 ± 0.3	0.0	0.0 ± 0.0
20:1n-9	7.0 ± 0.9	2.5 ± 1.1	0.5	0.2 ± 0.1
18:4n-3	0.0	0.0	0.0	4.0 ± 1.7
20:4n-6	0.6 ± 0.0	1.5 ± 0.4	1.6	0.7 ± 0.2
22:1n-11	4.0 ± 0.8	1.1 ± 0.7	0.0	0.1 ± 0.1
22:1n-9	1.5 ± 0.2	0.9 ± 0.4	0.0	0.0 ± 0.0
20:4n-3	1.3 ± 0.1	1.0 ± 0.0	0.8	0.5 ± 0.1
20:5n-3	5.1 ± 0.3	9.2 ± 1.7	2.2	18.6 ± 3.2
24:1n-9	0.5 ± 0.0	0.4 ± 0.2	0.0	0.3 ± 0.1
22:5n-3	1.5 ± 0.1	5.6 ± 0.6	0.0	0.9 ± 0.2
22:6n-3	8.3 ± 0.7	21.4 ± 1.7	0.0	28.5 ± 4.8
Saturated	16.8 ± 0.8	11.1 ± 0.5	16.9	22.5 ± 1.0
Monoenes	52.6 ± 0.9	31.4 ± 2.4	29.2	10.4 ± 4.0
n-3	19.0 ± 0.7	39.3 ± 3.3	39.3	55.9 ± 4.8
n-6	6.8 ± 0.2	7.9 ± 0.4	7.9	4.3 ± 3.3
Polyenes	25.8 ± 0.8	47.2 ± 3.0	47.2	60.9 ± 5.1
n-3/n-6	2.8 ± 0.0	5.0 ± 0.7	5.0	19.7 ± 11.5
DHA/EPA	1.6 ± 0.2	2.4 ± 0.5	0	1.6 ± 0.4
EPA/ARA	9.1 ± 0.1	6.9 ± 2.7	1.4	29 ± 10

Data recalculated from Hamre *et al.* (2002), van der Meeren (2008) and Srivastava *et al.* (2011, 2012).

Artemia phospholipids as discussed elsewhere (Conceição *et al.* 2010b).

If we use copepod composition and the requirement of juvenile and adult fish (NRC 2011) as references, most of the water-soluble vitamins are present in adequate amounts in both unenriched *Artemia* and rotifers. Exceptions are Asc in rotifers, thiamine in rotifers, *Artemia* nauplii and ongrown *Artemia* and cobalamine in unenriched *Artemia* nauplii (Table 1). Of the lipid soluble vitamins, vitamin A and E are well characterized, together with carotenoids which is the pro-vitamin A form in live prey (Table 1; Moren *et al.* 2002). Unenriched live prey normally does not contain vitamin A, however, *Artemia* contains large amounts of cantaxanthin that can be converted to vitamin A in fish (Moren *et al.* 2002). Rotifers contain much less carotenoids than *Artemia* and copepods and there may be a risk of vitamin A deficiency in fish larvae fed rotifers. However, cod larvae fed rotifers enriched without vitamin A seemed to have adequate whole body vitamin A levels (Kristin Hamre, unpubl. data, 2009). α-TOH may fall to very low levels in rotifers cultured on a diet deficient in this vitamin, while *Artemia* have levels of α-TOH which are above copepod levels (Table 1). To our knowledge, there are no data on typical levels of vitamin D and K in unenriched live prey, however enriched rotifers and *Artemia* contained 0.9–1.8 $\mu g\ g^{-1}$ vitamin D, while there was no detectable vitamin D in copepods (van der Meeren *et al.* 2008).

Of the macrominerals, rotifers may have slightly too low phosphorus levels, based on the level found in copepods. Otherwise, phosphorus in *Artemia* and calcium and magnesium in both rotifers and *Artemia* seem to be adequate (Table 1). The trace elements with exception of iodine and zinc are adequate in *Artemia*, however, in rotifers, all trace elements except iron are lower than in copepods. Selenium is also lower in rotifers than the requirements in juvenile and adult fish given by NRC (2011) (Table 1). Further studies have given selenium levels in unenriched rotifers between 0.04 and 0.7 $\mu g\ g^{-1}$ DM (Penglase *et al.* 2011; Samuel James Penglase *et al.*, unpubl. data, 2012), showing that selenium in rotifers can fall well below the requirement in juvenile and adult fish (NRC 2011).

Opportunities and limitations in enrichment of live feed

Biology of feed organisms relevant for enrichment. Characteristics of the live feed organism that are important for the technical process of enrichment are particle size selectivity, gut-filling and -evacuation rates and the volume of the digestive tract in relation to the whole animal. Baer *et al.* (2008) measured these traits using *Brachionus* 'Cayman' (168 μm lorica length) fed with latex beads and found that this rotifer had the ability to ingest particles of 1.6–10 μm. Particles of 12 and 14 μm were captured, but not swallowed. The latex beads of different sizes were given at a constant bead volume per volume of water, i.e. the number of beads decreased with increasing bead size and the rotifers ingested similar volumes of beads regardless of bead size up to 10 μm. This indicates that the ingestion was a function of the amount of filtered water and that the rotifers did not prefer any bead size within this range. The reason for this could be that rotifers do not feed actively on latex, but rather filter a given water volume per time unit depending on the swimming speed, which in turn is modulated by the food particles concentration (Yúfera 2007).

The gut in the experiment above was filled in 35 min and the gut evacuation time was dependent on the temperature; 140 min at 26°C and more than 18 h at 4°C. Dhert (1996) reported that rotifers evacuated their guts in 20–25 min at 25°C. Furthermore, gut transit time is reduced if new particles are available for ingestion (Lindemann & Kleinow 2000). This means that rotifers fill their guts in a short period of time and can be boosted with nutrients just prior to being fed to the fish larvae. The total gut volume seems to be relatively small although the reported values vary in the different studies probably due to differences in the *Brachionus* strain, type of food and methodology. Baer *et al.* (2008) estimated as 18 pL and only 1.2% of body volume in *Brachionus* 'Cayman'. In another strain of *Brachionus* (200 μm lorica length), the gut volume was estimated as 60–120 pL (Kleinow *et al.* 1991). Recently, Romero-Romero and Yúfera (in press) found that in *B. plicatilis* sensu strict (adult: 220–250 μm) the average gut volume can reach 230 pL at high algal concentration. In this study the gut volume varied from 0% to 15% of body volume depending on the algal cell concentration in water, though in terms of dry mass the gut content reached up to 38% of body matter. Thus, there are limitations to the amount of extra nutrients that can be contained in a short-term enriched rotifer and tissue enrichment should be considered where it is possible. Longer-term enrichment may result in greater absorption and assimilation of ingested nutrients; for example, Walford and Lam (1987) found that the highest assimilation of supplemented n-3 HUFAs occurred after 12 h enrichment with microcapsules and Watanabe (1993) recommended an optimal enrichment period of 12 h using lipid emulsions.

Artemia are obtained as cysts collected from natural ecosystems with periods of high salinity, Great Salt Lake in Utah, USA being the most important source. They are hatched and in some cases directly fed to fish larvae. To be enriched, they must be grown to the instar II stage (approximately 8 h after hatching) when they can ingest particles of 1–50 μm (Dhont and van Stappen 2003). To our knowledge, there are no studies on gut filling and evacuation kinetics and the capacity of the gut to store nutrients, in *Artemia*.

Delivery vehicles for water soluble nutrients. The lipid soluble micronutrients can be given in formulated diets or in emulsions. The water-soluble nutrients will not be contained easily in emulsion droplets or within a formulated diet, from which they will leach at a high rate because of the high surface to volume ratio of the very small particles. In both cases, the water-soluble nutrients will be dissolved in the enrichment water, which gives a low enrichment efficiency compared with when the nutrients are contained within a particle (Tonheim *et al.* 2000; Nordgreen *et al.* 2007; Langdon *et al.* 2008; Hawkyard *et al.* 2011). Two methods to deliver water-soluble nutrients embedded in particles to live feed organisms have been developed; the production of lipid spray beads (LSB; Nordgreen *et al.* 2007; Langdon *et al.* 2008; Hawkyard *et al.* 2011) and liposomes (Tonheim *et al.* 2000; Barr & Helland 2007). The LSB are produced from molten lipid mixed with micronutrients and sprayed into a chamber with liquid nitrogen. Liposomes consist of a bilayer of phospholipid surrounding an aqueous core in which water-soluble nutrients can be dissolved. Furthermore, several products on the market contain minerals in forms that are particulate and insoluble or only partly soluble in water and therefore can be given, for example together with an enrichment diet. These include selenium enriched yeast and chelated minerals (Penglase *et al.* 2011; Andreas Nordgreen *et al.*, unpubl. data, 2011).

Enrichment of rotifers. If the composition of copepods is used as a reference, nutritionally well-balanced rotifers should be enriched with protein, phospholipids, EPA and DHA, Asc, thiamine, vitamin A and E and all the microminerals, except iron. The levels of vitamins D and K in unenriched rotifers are still not known. It is quite easy to control the levels of most of the micronutrients within rotifers, since their concentration is directly proportional to the concentration in the rotifer diet (Merchie *et al.* 1997; Srivastava *et al.*, 2011, 2012; Penglase *et al.* 2011; Andreas Nordgreen *et al.*, unpubl. data, 2011). Vitamin A is an exception, since it seems not to be easily taken up by rotifers, at least not by the strain used by Srivastava *et al.* (2011). Iodine given as NaI is assimilated in rotifers in a dose dependent manner, but is totally discarded after 2 h starvation. Enrichment with thymol iodide also gave a dose dependent increase in iodine, but with this compound, iodine was retained during storage of the rotifers (Srivastava *et al.* 2012). All the micronutrients can be enriched during long term culture or during a short enrichment period (e.g. 3 h). However, especially for potentially toxic nutrients such as minerals and vitamin A, short term enrichment is preferred. The microminerals, with the exception of iodine, are retained in the rotifers over time (Penglase *et al.* 2011; Andreas Nordgreen *et al.*, unpubl. data, 2011), while storage experiments with rotifers enriched with vitamins have not been performed. Many of the micronutrients exist in different forms that may have different effects during enrichment as described above for iodine, but the different forms may also have different effects on fish larvae (Samuel James Penglase *et al.*, unpubl. data, 2012).

As described above, rotifers can be enriched to contain up to 20–30% lipid with a defined fatty acid composition.

The fatty acid composition is also easily manipulated to contain DHA and EPA concentrations within the lower range of copepods, through the culture diet (Table 4). This will give rotifers with 10–15% lipid, more in line with the lipid levels of copepods, and with a higher ratio of protein to lipid. Enrichment of rotifers with phospholipid (PL) is more complicated, since it is limited by the volume of the rotifer digestive tract. Rotifers will digest the PL, a process that probably is very fast due to the short gut transit time (Baer *et al.* 2008; Romero-Romero & Yúfera, in press) and the digested fat will be absorbed and stored as TAG. Since PL makes up the membranes of the animal, its concentration will be more or less constant. Enrichment of rotifers with lipids and fatty acids has been reviewed by Lubzens *et al.* (1989), Rainuzzo *et al.* (1997), Lubzens *et al.* (2001) and Olsen (2004).

When trying to enrich rotifers with protein, one will meet the same problem as found with PL. The protein will be digested rapidly to free amino acids and peptides that will be absorbed and built into the rotifer protein by a set of processes determined by the genetic code. Enrichment of rotifers with protein is therefore also limited by the volume of the digestive tract and measures should be taken to avoid evacuation and/or catabolism of the protein-enrichment before the rotifers are fed to the fish larvae. The protein concentration of rotifers has been increased from 38% to 41% (Kristin Hamre, unpubl. results, 2010) or from 48% to 53% (Helland *et al.* 2010) by short-term enrichment with diets containing more than 58% protein. This may be sufficient if the minimum requirement for protein in the fish larvae is 40% of DM, as found for cod by Åsnes (2006). On the other hand, the contents of individual amino acids in rotifers are also of major concern (Aragão *et al.* 2004a; Saavedra *et al.* 2006). These can be increased to a limited extent by short term enrichment, by manipulating the free amino acid pool in the rotifers (Barr & Helland 2007; Saavedra *et al.* 2008a; Helland *et al.* 2010).

Enrichment of Artemia. *Artemia* may contain sufficient levels of protein, but needs to be enriched with fatty acids, and possibly thiamine, zinc and iodine, based on the nutrient composition of copepods (Hamre *et al.* 2007). The concentration of Asc is slightly lower than in copepods, but far above the requirements given for fish (NRC 2011), while the other water-soluble and the fat-soluble vitamins seem to be sufficient (Mæland *et al.* 2000; Hamre *et al.* 2007). The fatty acid profile must be modified by enrichment with lipids rich in n-3 highly unsaturated fatty acids. However, this will often lead to prey organisms with a high lipid content, whereas the fatty acid composition may be difficult to control due to selective metabolism and thereby consumption of DHA in *Artemia* (Navarro *et al.* 1999). The thiamine and iodine concentrations are easily increased just by dissolving the nutrients in the enrichment water. Alternatively they can be delivered by liposomes or lipid spray beads (Langdon *et al.* 2008; Hawkyard *et al.* 2011). Nguyen *et al.* (2008) increased the zinc level in *Artemia* by dissolving a Zn salt in an enrichment emulsion.

Formulated diets

General characteristics of formulated larval diets

One important goal of research on nutrition and feeding in marine fish larvae is to generate knowledge on which to base the development of formulated feeds (microdiets) that can be used as early as possible in the larval phase. This will reduce the need for live feed organisms, which are both labour-intensive and complicated to culture. Reciprocally it is necessary to have reliable feeds with well-known designed formulations for advancing larval nutrition. This was considered as utopian during the 1970s and 1980s, but nowadays its achievement and wide commercial use is closer. In fact, a complete and efficient replacement has been achieved at the experimental scale (Cahu *et al.* 2003b), and overall the results of progressive replacing in different species are highly promising (Yúfera *et al.* 2000, 2005; Seiliez *et al.* 2006; Engrola *et al.* 2009). The design and development of microdiets for fish larvae are focusing on several objectives and uses: advanced weaning onto prepared feeds, co-feeding using live prey and inert diets, replacement of live prey from first-feeding and delivering of some specific compounds in the digestive tract of fish larvae. To be useful for feeding small pelagic larval fish, the microdiets need to comply with several structural and biochemical characteristics. The first one is to be stable enough to prevent the particles disintegration after the immersion in water and to maintain a good retention of hydrosoluble micronutrients. Secondly, the particles should be accessible to larval fish being available in the water column and having an appropriate diameter. Furthermore, the particles have to be identified as a food item to be ingested and also be digestible by the larval digestive system. Finally, the microdiet has to meet properly the energetic and nutritional requirements for larval growth and development (Koven *et al.* 2001; Langdon 2003; Yúfera *et al.* 2003). The achievement of all these requisites is a difficult challenge, particularly to find an equilibrium between stability to prevent excessive leaching and digestibility of food particles.

For the feed particles to be identified as food items, it may be necessary to add attractants to the diet. Some amino acids are potent stimulants for fish. During food search, detection and recognition of these attractants in food particles activate food intake. Furthermore, amino acids stimulate specific digestive hormones, facilitating larval digestion and assimilation (Sandel *et al.* 2010). The amino acids that stimulate olfaction in fish are species-specific, even in mixture, and numbers, kinds and concentrations vary greatly. Kolkovski *et al.* (1997) described how microdiet ingestion rates in gilthead seabream larvae (20 dph) increased up to 120% when the fish were exposed to the visual and chemical stimuli (working synergistically) of various concentrations of *Artemia* nauplii. The chemical stimuli provoking such larval response included some free amino acids (alanine, glycine and arginine) and the compound betaine. These were identified from 14 metabolites found in the *Artemia*-rearing medium and the selection was supported by monitoring the effect (reduction in the MD digestion rate) of the removal of each of them. Further, the degree of influence of these FAAs and betaine on larval ingestion rate was shown to be age-dependent (Kolkovski *et al.* 1997). The effectiveness of alanine, glycine and betaine as feeding stimulants was tested by Koven *et al.* (2001) in 7-day-old gilthead seabream larvae. The amino acids were incorporated into a Cx labelled MD and the ingestion rate was measured in the presence or absence of rotifers. In the presence of rotifers there was a reduction in the ingestion rate of all the MD. However, the ingestion rate in larvae feeding on the diet added the FAA was significantly ($P < 0.05$) better than the other MD treatments, suggesting that the supplementation of the *Artemia* FAA stimulated a feeding response even in the presence of rotifers. Free amino acids and betaine were also found to be effective attractants in salmonids (Hughes 1990), weatherfish, abalone and yellowtail (Harada *et al.* 1987; Harada 1992). All these findings suggest that MD development cannot be based on simply imitating the proximate composition of the live food but, for optimal attraction, digestion and assimilation, attractants should be incorporated in inert feeds. As a consequence, any diet administered to fish larvae should take into account the species-specific sensitiveness and attractiveness of chemical substances present in the diet, but no data are actually available on most of the reared species. Future MD research should continue to define, isolate and understand the interdependence of those factors in live food that stimulate feeding activity visually and chemically, influence larval digestion, assimilation and transport of nutrients, as well the endocrine hormonal control of feeding and digestive enzyme secretion (Koven *et al.* 2001). The species specific optimal mixture of attractants has been identified only for a few species and more work is warranted in this field.

Types of formulated microdiets

Many different types of microparticles have been developed using different methodologies and the terminology for these different technologies is sometimes confusing. It is beyond the scope of this review to describe the preparation methods but basically there are two types of microdiets with complete formulation, microbound and microencapsulated diets (Baskerville-Bridges & Kling 2000; Langdon 2003). In addition, coating is a process that can be done with particles prepared by either of the above methods. On the other hand, lipid beads and liposomes have been used for specific lipid formulation as well as for delivering hydrosoluble micronutrients such as amino acids, vitamins and minerals (Onal & Langdon 2004; Monroig *et al.* 2006). Most of the currently available feeds are microbound, where the components of the feed are bound together by a binder that forms a network with the different ingredients. The final dry mixture is crushed and sieved to obtain the desired particle size. The microbound diet can also be mechanically agglomerated or extruded, as occurs with some commercial starter feeds. With the different microencapsulation technologies the particles are individually formed with a spherical or almost spherical shape. The shell, coating or matrix is constituted by polymers such as alginate, chitosan, gelatine, zein, carboxymethyl-cellulose or cross-linked protein (Kanazawa & Teshima 1988; Kolkovski *et al.* 1993; Yúfera *et al.* 1999b, 2005; Baskerville-Bridges & Kling 2000).

The most advanced experimental development in formulated feeds is the production of complex particles. This type incorporates particles of very small diameter inside a particle of regular diameter. The idea of these complex particles is to better prevent the leaching of water soluble nutrients. For this purpose, both liposomes and LSB can be used (Ozkizilcik & Chu 1996; Langdon *et al.* 2007).

Technical limitations

Leaching. The most complicated technical problem to solve in the design of formulated larval feeds is to prevent the high rate of leaching of watersoluble compounds after the rehydration of the particles distributed into the rearing tanks. This is due to the small size of the food particles when compared with the pellet used in juveniles after the weaning. In the microparticles, the surface/volume ratio is very high and the diffusion distance from the core to the surface very short. The leaching of hydrosoluble components such as amino acids and some vitamins has been studied in different types of microdiets showing in general an important leaching and also high differences among types of microdiets (Lopez-Alvarado *et al.* 1994; Baskerville-Bridges & Kling 2000; Yúfera *et al.* 2002; Onal & Langdon 2004; Kvåle *et al.* 2006; Nicklason & Johnson 2008; Nordgreen *et al.* 2009). According to those studies, microbound diets may lose between 50% and 95% of free amino acids and protein hydrolysates during the first few minutes of rehydration, and can also easily lose between

15% and 30% of the DM in this short time. On the other side, protein cross-linked capsules could retain up to 85% of the total amino acids content during the first hours and practically almost the total dry mass amount in several hours. Nevertheless the cross-linked protein capsules lose part of the encapsulated amino acids with the washing during the preparation process. Besides, some free amino acid or peptides may be incorporated by polymerization to the shell. These differences are only indicative because the properties of a given microdiet may vary with changes in the preparation parameters, in the type of dietary and binding ingredients, the amount of a given hydrosoluble ingredient and the particles size.

An amount as high as 90% of water-soluble vitamins and minerals may also be lost during the first few minutes of water immersion (Nordgreen *et al.* 2008). LSB can retain more efficiently vitamins such as riboflavin (close to 90% after 4 h of immersion; (Onal & Langdon 2004). The complex microdiets, usually formed by small lipid-walled microcapsules incorporated in larger zein-microbound or microencapsulated particles (Ozkizilcik & Chu 1996; Onal & Langdon 2005; Langdon *et al.* 2007), have resulted in better retention capacity and in supplying a more complete and accessible diet for the larvae.

Digestibility. Digestibility is a fundamental condition of the microdiets and is critical for their success in larval rearing. The production process directly affects the digestibility of the shell, of the whole particle or of some dietary ingredients, mainly the protein. On the other hand, the digestion capacity of the larvae improves with development and the same particle can turn from difficult to easily digestible in a few days. The texture, hardness and digestibility of the microparticle can be modulated by changing the elaboration parameters and the concentration of some reactants and ingredients (Nordgreen *et al.* 2008). The protein digestibility of the raw materials may be higher than in the final feed, when protein is polymerized during encapsulation.

The digestibility of the dietary protein source is a decisive factor in determining how efficiently the ingested protein can be utilized by the fish larva. Very little is known about protein digestibility in fish larvae (see recent review by Rønnestad *et al.* (in press), and data based on studies of adult fish are not directly relevant because of the different digestive systems of larvae. In most fish larvae the lack of a stomach at the larval stage means that ingested protein is neither exposed to the denaturing conditions imposed by the gastric acids nor pre-digested by pepsin before entering the mid-gut. Until a gastric digestion is attained (not the case of agastric fish), fish larvae digestion relies mainly on pancreatic enzymes with an optimum of activity at a neutral or alkaline pH, conditioning the range of proteins that fish larvae are able to digest. In larval fish diets and weaning diets, part of the protein fraction is usually supplied in the form of hydrolysate (Carvalho *et al.* 1997; Cahu *et al.* 1999; Kvåle *et al.* 2009; Nankervis & Southgate 2009). This is a way to facilitate digestion and to increase the availability of peptides and amino acids absorbed within the larval gut. Some authors also focus on the solubility of the protein source, under the hypothesis that water-soluble protein is highly digestible by fish larvae (Carvalho *et al.* 2003, 2004; Tonheim *et al.* 2007).

Microdiet formulation and nutrition experiments

Both a good knowledge of larval nutrition and an appropriate microparticulation technology are necessary to fulfil all the nutritional and energetic requirements of early larvae. A food microparticle able to admit any kind of compound/ingredient according to the formulation needs would be an ideal tool for advancing in larval nutrition. However, there are some restrictions for the formulation of a microdiet. First, part of the diet mass is constituted by the binder that forms the shell and the matrix of the particle. This binding part can be digestible, partially digestible or indigestible and may vary roughly between 5% and 40% of the total particle mass depending on the particulation technology. For instance, high amounts of casein (35–40% of the total mass) are necessary to obtain stable crosslinked casein-protein microcapsules. Although the casein can be digested in many cases, such a high amount may prevent a balanced formulation of all the necessary nutrients (e.g. levels of some indispensable amino acids). On the other hand, the leaching problems already commented on may change the real proportion of hydrosoluble micronutrients in the diet. In spite of these constraints, the microdiets allow the design of specific formulations.

Experimentation on larval growth and nutrition by changing the microparticulation technology or the dietary formulation is a complicated exercise because there are many potential causes for a lack of the expected response. Such constraints may occur during the preparation of the microdiet, during the water immersion and during the digestion of the food particles. Monitoring a dietary compound is a laborious task that requires its determination in the food particle, in the rearing water and in the larval tissues (Yúfera *et al.* 2003).

In spite of all these limitations, microdiets are being used currently for research into nutrition in larval stages with good results. The relatively good capacity to admit notable changes in the formulation of the dietary ingredients without the interference of metabolic processes, as in live feed, makes microdiets an excellent tool for nutritional studies. For instance, some experiments carried out in larvae of gilthead seabream and white seabream (Aragão *et al.* 2007; Saavedra *et al.* 2009) have shown how an adequate supplementation of crystalline amino acids in the microdiet, in order to obtain a balanced amino acid profile similar to that exhibited by the larval body, may enhance survival and growth, as well as the larval quality. Experiments with larvae of European seabass (Cahu *et al.* 2003b) and gilthead seabream (Seiliez *et al.* 2006; Martins *et al.* 2010) fed on tailored designed microdiets have shown that the level and source of phospholipids differing in its fatty acid composition affect the body composition in lipid classes and growth potential. Experiments carried out with Senegalese sole larvae have demonstrated that an adequate total protein content (Yúfera *et al.* 2005) and the supplementation of taurine (Pinto *et al.* 2010) in the diet contributes to enhance the growth potential and the metamorphosis process. Likewise, the larval growth of Asian seabass (*Lates calcarifer*) was significantly affected by the source of the protein used in the preparation of the formulated microdiet (Nankervis & Southgate 2006).

Early weaning has been the primary target in using microdiets. The pre-conditioning of the developing larvae by the application of co-feeding protocols (live prey + microdiet) and the launching of new commercial and experimental microdiets have allowed the complete removal of the live prey some weeks earlier in some species without relevant losses in larval growth and performance, at least at the experimental level. A much more difficult challenge is to obtain good growth from first feeding in altricial larvae. Feeds with lower leaching rates would offer a better guarantee to satisfy the nutritional requirements of the larvae, and should theoretically result in better growth and survival rates. Nevertheless, such an idea is not clearly supported by the current published growth results. Different results have been obtained with the different microdiets and fish species but, in general, very or relatively good growth and survival have been observed when some live prey is added together the microdiet (microbound or microencapsulated), and poor results when the microdiet is supplied alone from the first feeding (Yúfera *et al.* 1999b; Curnow *et al.* 2006; Seiliez *et al.* 2006). European seabass has been the only marine fish in which good or very good results have been obtained from first feeding in experimental studies (Fontagné *et al.* 2000b; Cahu *et al.* 2003b). These results indicate that microdiets are lacking some nutritional factors and/or characteristics that are essential for both the digestion and availability of nutrients particularly at very early stages.

Gaps and bottlenecks in obtaining knowledge on nutritional requirements of marine fish larvae

The most studied topic in marine fish larval nutrition is polyunsaturated fatty acid metabolism and requirement, and even within this topic quantitative requirements still have to be determined in most European fish larvae. For all other nutrients, requirement studies using dose–response designs and at least five dietary levels are largely lacking. Moreover, the few existing studies have typically been performed in the later larval stages, and requirements in early life are likely to be somewhat different.

The main reason for this scenario is a lack of appropriate diets that can be used for running requirement studies. Nutrient concentrations in live feed may be difficult to control, due to the organisms' own metabolism and formulated feeds have technical limitations, such as high leaching rates and low digestibility. Lately, there has been an improvement in formulated diets and increased knowledge on how to control the nutrient composition of live feed. Therefore, we are now in a better position to do these studies. However, the knowledge on larval diets needs to be improved further in order to increase the quality of nutrient requirement studies.

We also do not know enough about the behaviour of marine fish larvae in relation to feed intake and the consequences this may have for nutrient digestion and absorption, for example the bioavailability of the different nutrients. Studies on topics such as the effects of feeding regimes, feeding intensity, diurnal rhythms and so forth, on gut passage time and the bioavailability of nutrients are needed to build a good framework for how to design and run requirement studies.

Indirect measurements of requirements always leave the question mark of how relevant the results are for the 'real' situation. However, the studies performed so far have given approximate answers, which are necessary when the precise answers cannot be obtained. They also have established a large amount of information about the biological responses of marine fish larvae to different nutritional inputs. When nutrient requirement studies are designed in the future it is important to measure the relevant biological responses in addition to growth and survival, because the requirement for growth can be different from, for example the requirement for optimal innate immune response, normal pigmentation and muscle-, skeleton and neural system development. Another aspect that should be taken into account is the interaction of nutrients with other nutrients and with environmental conditions.

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References

Applebaum SL, Rønnestad I (2004) Absorption, assimilation and catabolism of individual free amino acids by larval Atlantic halibut (*Hippoglossus hippoglossus*). *Aquaculture* **230**: 313–322.

Aragão C, Conceição LEC, Dinis MT, Fyhn HJ (2004a) Amino acid pools of rotifers and *Artemia* under different conditions: nutritional implications for fish larvae. *Aquaculture* **234**: 429–445.

Aragão C, Conceição LEC, Fyhn HJ, Dinis MT (2004b) Estimated amino acid requirements during early ontogeny in fish with different life styles: gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*). *Aquaculture* **242**: 589–605.

Aragão C, Conceição LEC, Martins D, Rønnestad I, Gomes E, Dinis MT (2004c) A balanced dietary amino acid profile improves amino acid retention in post-larval Senegalese sole (*Solea senegalensis*). *Aquaculture* **233**: 293–304.

Aragão C, Conceição LEC, Lacuisse M, Yúfera M, Dinis MT (2007) Do dietary amino acid profiles affect performance of larval gilthead seabream? *Aquatic Living Resources* **20**: 155–161.

Arthur DK (1976) Food and feeding of larvae of three fishes occurring in California current, *Sardinops sagax*, *Engraulis mordax*, and *Trachurus symmetricus*. *Fishery Bulletin* **74**: 517–530.

Åsnes ER (2006) Optimal makronæringsstoffsammensetning i fôr til torskeyngel (*Gadus morhua* L.) og effekt av pepsinprehydrolysert protein i fôr på vekst og overlevelse. University of Bergen, Bergen.

Atalah E, Hernández-Cruz CM, Montero D, Ganuza E, Benítez-Santana T, Ganga R *et al.* (2008) Enhancemnt of Gilthead Seabream and sea bass larval growth by dietary vitamin E in relation to different levels of essential fatty acids. In: *XIII International Symposium on Fish Nutrition and Feeding*, June 1–5, Florianopolis, Brazil.

Atalah E, Hernández-Cruz CM, Benítez-Santana T, Ganga R, Roo J, Izquierdo M (2011a) Importance of the relative levels of dietary arachidonic acid and eicosapentaenoic acid for culture performance of gilthead seabream (*Sparus aurata*) larvae. *Aquaculture Research* **42**: 1279–1288.

Atalah E, Hernández-Cruz CM, Ganuza E, Benítez-Santana T, Ganga R, Roo J *et al.* (2011b) Importance of dietary arachidonic acid for the growth, survival and stress resistance of larval European sea bass (*Dicentrarchus labrax*) fed high dietary docosahexaenoic and eicosapentaenoic acids. *Aquaculture Research* **42**: 1261–1268.

Baer A, Langdon C, Mills S, Schulz C, Hamre K (2008) Particle size preference, gut filling and evacuation rates of the rotifer *Brachionus* 'Cayman' using polystyrene latex beads. *Aquaculture* **282**: 75–82.

Barr Y, Helland S (2007) A simple method for mass-production of liposomes, in particular large liposomes, suitable for delivery of free amino acids to filter feeding zooplankton. *Journal of Liposome Research* **17**: 79–88.

Baskerville-Bridges B, Kling LJ (2000) Development and evaluation of microparticulate diets for early weaning of Atlantic cod *Gadus morhua* larvae. *Aquaculture Nutrition* **6**: 171–182.

Benítez-Santana T, Masuda R, Carrillo EJ, Ganuza E, Valencia A, Hernández-Cruz CM *et al.* (2007) Dietary n-3 HUFA deficiency induces a reduced visual response in gilthead seabream *Sparus aurata* larvae. *Aquaculture* **264**: 408–417.

Berridge MJ, Irvine RF (1989) Inositol phosphates and cell signaling. *Nature* **341**: 197–205.

Bessonart M, Izquierdo MS, Salhi M, Hernández-Cruz CM, González MM, Fernández-Palacios H (1999) Effect of dietary arachidonic acid levels on growth and survival of gilthead sea bream (*Sparus aurata* L.) larvae. *Aquaculture* **179**: 265–275.

Betancor MB, Atalah E, Caballero MJ, Benítez-Santana T, Roo J, Montero D *et al.* (2011) Alpha-tocopherol in weaning diets for European sea bass (*Dicentrarchus labrax*) improves survival and reduces tissue damage caused by excess dietary DHA contents. *Aquaculture Nutrition* **17**: E112–E122.

Betancor MB, Nordrum S, Atalah E, Caballero MJ, Benítez-Santana T, Roo J *et al.* (2012) Potential of three new krill products for seabream larval production. *Aquaculture Research* **43**: 395–406.

Blaxter JHS, Hempel G (1966) Utilization of yolk by herring larvae. *Journal of Marine Biological Association of the United Kingdom* **46**: 219–234.

Bransden MP, Cobcroft JM, Battaglene SC, Morehead DT, Dunstan GA, Nichols PD *et al.* (2005) Dietary 22: 6n-3 alters gut and liver structure and behaviour in larval striped trumpeter (*Latris lineata*). *Aquaculture* **248**: 275–285.

Buentello JA, Pohlenz C, Margulies D, Scholey VP, Wexler JB, Tovar-Ramirez D *et al.* (2011) A preliminary study of digestive enzyme activities and amino acid composition of early juvenile yellowfin tuna (*Thunnus albacares*). *Aquaculture* **312**: 205–211.

Busch KET, Falk-Petersen IB, Peruzzi S, Rist NA, Hamre K (2010) Natural zooplankton as larval feed in intensive rearing systems for juvenile production of Atlantic cod (*Gadus morhua* L.). *Aquaculture Research* **41**: 1727–1740.

Caballero MJ, Obach A, Rosenlund G, Montero D, Gisvold M, Izquierdo MS (2002) Impact of different dietary lipid sources on growth, lipid digestibility, tissue fatty acid composition and histology of rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* **214**: 253–271.

Caballero MJ, Gallardo G, Robaina L, Montero D, Fernández A, Izquierdo M (2006a) Vegetable lipid sources affect *in vitro* biosynthesis of triacylglycerols and phospholipids in the intestine of sea bream (*Sparus aurata*). *British Journal of Nutrition* **95**: 448–454.

Caballero MJ, Torstensen BE, Robaina L, Montero D, Izquierdo M (2006b) Vegetable oils affect the composition of lipoproteins in sea bream (*Sparus aurata*). *British Journal of Nutrition* **96**: 830–839.

Cahu CL, Zambonino-Infante JL, Quazuguel P, Le Gall MM (1999) Protein hydrolysate vs. fish meal in compound diets for 10-day old sea bass *Dicentrarchtus labrax* larvae. *Aquaculture* **171**: 109–119.

Cahu C, Zambonino-Infante JL, Takeuchi T (2003a) Nutritional components affecting skeletal development in fish larvae. *Aquaculture* **227**: 245–258.

Cahu CL, Zambonino-Infante JL, Barbosa V (2003b) Effect of dietary phospholipid level and phospholipid: neutral lipid value on the development of sea bass (*Dicentrarchus labrax*) larvae fed a compound diet. *British Journal of Nutrition* **90**: 21–28.

Carvalho AP, Escaffre AM, Teles AO, Bergot P (1997) First feeding of common carp larvae on diets with high levels of protein hydrolysates. *Aquaculture International* **5**: 361–367.

Carvalho AP, Oliva-Teles A, Bergot P (2003) A preliminary study on the molecular weight profile of soluble protein nitrogen in live food organisms for fish larvae. *Aquaculture* **225**: 445–449.

Carvalho AP, Sa R, Oliva-Teles A, Bergot P (2004) Solubility and peptide profile affect the utilization of dietary protein by common carp (*Cyprinus carpio*) during early larval stages. *Aquaculture* **234**: 319–333.

Cetta CM, Capuzzo JM (1982) Physiological and biochemical aspects of embryonic and larval development of the winter flounder *Pseudopleuronectes-americanus*. *Marine Biology* **71**: 327–337.

Chen JN, Takeuchi T, Takahashi T, Tomoda T, Koisi M, Kuwada H (2004) Effect of rotifers enriched with taurine on growth and survival activity of red sea bream *Pagrus major* larvae. *Nippon Suisan Gakkaishi* **70**: 542–547.

Chen JN, Takeuchi T, Takahashi T, Tomoda T, Koisi M, Kuwada H (2005) Effect of rotifers enriched with taurine on growth in larvae of Japanese flounder *Paralichthys olivaceus*. *Nippon Suisan Gakkaishi* **71**: 342–347.

Conceição LEC, van der Meeren T, Verreth JAJ, Evjen MS, Houlihan DF, Fyhn HJ (1997) Amino acid metabolism and protein turnover in larval turbot (*Scophthalmus maximus*) fed natural zooplankton or *Artemia*. *Marine Biology* **129**: 255–265.

Conceição LEC, Dersjant-Li Y, Verreth JAJ (1998a) Cost of growth in larval and juvenile African catfish (*Clarias gariepinus*) in relation to growth rate, food intake and oxygen consumption. *Aquaculture* **161**: 95–106.

Conceição LEC, Ozorio ROA, Suurd EA, Verreth JAJ (1998b) Amino acid profiles and amino acid utilization in larval African catfish (*Clarias gariepinus*): effects of ontogeny and temperature. *Fish Physiology and Biochemistry* **19**: 43–57.

Conceição LEC, Rønnestad I, Tonheim SK (2002) Metabolic budgets for lysine and glutamate in unfed herring (*Clupea harengus*) larvae. *Aquaculture* **206**: 305–312.

Conceição LEC, Grasdalen H, Dinis MT (2003a) A new method to estimate the relative bioavailability of individual amino acids in fish larvae using C-13-NMR spectroscopy. *Comparative Biochemistry and Physiology B* **134**: 103–109.

Conceição LEC, Grasdalen H, Rønnestad I (2003b) Amino acid requirements of fish larvae and post-larvae: new tools and recent findings. *Aquaculture* **227**: 221–232.

Conceição LEC, Morais S, Rønnestad I (2007) Tracers in fish larvae nutrition: a review of methods and applications. *Aquaculture* **267**: 62–75.

Conceição LEC, Aragão C, Richard N, Engrola S, Gavaia P, Mira S *et al.* (2010a) Novel methodologies in marine fish larval nutrition. *Fish Physiology and Biochemistry* **36**: 1–16.

Conceição LEC, Yúfera M, Makridis P, Morais S, Dinis MT (2010b) Live feeds for early stages of fish rearing. *Aquaculture Research* **41**: 613–640.

Copeman LA, Parrish CC, Brown JA, Harel M (2002) Effects of docosahexanoic, eicosapentaenoic and arachidonic acids on the early growth, survival, lipid composition and pigmentation of yellowtail flounder (*Limanda ferruginea*): a live food enrichment experiment. *Aquaculture* **210**: 285–304.

Curnow J, King J, Bosmans J, Kolkovski S (2006) The effect of reduced *Artemia* and rotifer use facilitated by a new microdiet in the rearing of barramundi *Lates calcarifer* (BLOCH) larvae. *Aquaculture* **257**: 204–213.

Dantagnan P, Borquez A, Hernández A, Izquierdo M (2010) Effect of EPA/DHA ratios on the growth and survival of *Galaxias maculatus* (Jenyns, 1842) larvae reared under different salinity regimes. *Aquaculture Research* **41**: e239–e244.

Darias MJ, Mazurais D, Koumoundouros G, Glynatsi N, Christodoulopoulou S, Huelvan C *et al.* (2010) Dietary vitamin D-3 affects digestive system ontogenesis and ossification in European sea bass (*Dicentrachus labrax*, Linnaeus, 1758). *Aquaculture* **298**: 300–307.

Day OJ, Howell BR, Jones DA (1997) The effect of dietary hydrolysed fish protein concentrate on the survival and growth of juvenile Dover sole, *Solea solea* (L.), during and after weaning. *Aquaculture Research* **28**: 911–921.

Dedi J, Takeuchi T, Seikai T, Watanabe T, Hosoya K (1997) Hypervitaminosis a during vertebral morphogenesis in larval Japanese flounder. *Fisheries Science* **63**: 466–473.

Deplano M, Diaz JP, Connes R, Kentouri-Divanach M, Cavalier F (1991) Appearance of lipid-absorption capacities in larvae of the sea bass *Dicentrarchus labrax* during transition to the exotrophic phase. *Marine Biology* **108**: 361–371.

Dhert P (1996) Rotifers. In: Sorgeloos P, Lavens P (eds) *Manual on the Production and Use of Live Food for Aquaculture*, pp. 49–78. Food and Agriculture Organization of the United Nations, Rome.

Dhert P, Rombaut G, Suantika G, Sorgeloos P (2001) Advancement of rotifer culture and manipulation techniques in Europe. *Aquaculture* **200**: 129–146.

Dhont J, Van Stappen G (2003) Biology, tank production and nutritional value of Artemia. In: Stottrup JG, McEnvoy LA (eds) *Live Feeds in Marine Aquaculture*, pp. 65–121. Blackwell Publishing, Oxford.

Ebrahimnezhadarabi M, Saad CR, Harmin SA, Kamal M, Satar A, AbedianKenari A (2011) Effects of phospholipids in the diet on biochemical factors of sturgeon fish (*Huso huso*) juveniles. *African Journal of Biotechnology* **10**: 8511–8516.

Engrola S, Mai M, Dinis MT, Conceição LEC (2009) Co-feeding of inert diet from mouth opening does not impair protein utilization by Senegalese sole (*Solea senegalensis*) larvae. *Aquaculture* **287**: 185–190.

Engrola S, Dinis MT, Conceição LEC (2010) Senegalese sole larvae growth and protein utilization is depressed when co-fed high levels of inert diet and *Artemia* since first feeding. *Aquaculture Nutrition* **16**: 457–465.

Estévez A, Kanazawa A (1995) Effect of n-3 PUFA and vitamin A *Artemia* enrichment on pigmentation success of turbot, *Scophthalmus maximus* (L.). *Aquaculture Nutrition* **1**: 159–168.

Estévez A, McEvoy LA, Bell JG, Sargent JR (1999) Growth, survival, lipid composition and pigmentation of turbot (*Scophthalmus maximus*) larvae fed live-prey enriched in arachidonic and eicosapentaenoic acids. *Aquaculture* **180**: 321–343.

Faleiro F, Narciso L (2010) Lipid dynamics during early development of *Hippocampus guttulatus* seahorses: searching for clues on fatty acid requirements. *Aquaculture* **307**: 56–64.

Fernández I, Gisbert E (2010) Senegalese sole bone tissue originated from chondral ossification is more sensitive than dermal bone to high vitamin A content in enriched *Artemia*. *Journal of Applied Ichthyology* **26**: 344–349.

Fernández I, Hontoria F, Ortiz-Delgado JB, Kotzamanis Y, Estévez A, Zambonino-Infante JL *et al.* (2008) Larval performance and skeletal deformities in farmed gilthead sea bream (*Sparus aurata*) fed with graded levels of vitamin A enriched rotifers (*Brachionus plicatilis*). *Aquaculture* **283**: 102–115.

Fernández I, Pimentel MS, Ortiz-Delgado JB, Hontoria F, Sarasquete C, Estévez A *et al.* (2009) Effect of dietary vitamin A on Senegalese sole (*Solea senegalensis*) skeletogenesis and larval quality. *Aquaculture* **295**: 250–265.

Field FJ, Mathur SN (1995) Intestinal lipoprotein synthesis and secretion. *Progress in Lipid Research* **34**: 185–198.

Finn RN (1994) *Physiological Energetics of Developing Marine Fish Embryos and Larvae*. University of Bergen, Bergen, Norway.

Finn RN, Fyhn HJ, Evjen MS (1995) Physiological energetics of developing embryos and yolk-sac larvae of Atlantic cod (*Gadus morhua*). 1. Respiration and nitrogen metabolism.. *Marine Biology* **124**: 355–369.

Finn RN, Fyhn HJ, Henderson RJ, Evjen MS (1996) The sequence of catabolic substrate oxidation and enthalpy balance of developing embryos and yolk-sac larvae of turbot (*Scophthalmus maximus* L). *Comparative Biochemistry and Physiology A* **115**: 133–151.

Fleming A, Sato M, Goldsmith P (2005) High-throughput *in vivo* screening for bone anabolic compounds with zebrafish. *Journal of Biomolecular Screening* **10**: 823–831.

Fontagné S, Burtaire L, Corraze G, Bergot P (2000a) Effects of dietary medium-chain triacylglycerols (tricaprylin and tricaproin) and phospholipid supply on survival, growth and lipid metabolism in common carp (*Cyprinus carpio* L.) larvae. *Aquaculture* **190**: 289–303.

Fontagné S, Robin J, Corraze G, Bergot P (2000b) Growth and survival of European sea bass (*Dicentrarchus labrax*) larvae fed from first feeding on compound diets containing medium-chain triacylglycerols. *Aquaculture* **190**: 261–271.

Fontagné S, Geurden I, Escaffre AM, Bergot P (1998) Histological changes induced by dietary phospholipids in intestine and liver of common carp larvae. *Aquaculture* **161**: 213–223.

Fraser AJ, Gamble JC, Sargent JR (1988) Changes in lipid-content, lipid class composition and fatty-acid composition of developing eggs and unfed larvae of cod (*Gadus Morhua*). *Marine Biology* **99**: 307–313.

Gatesoupe FJ, Le Milinaire C (1985) Adaptation de la qualité alimentaire des filtreurs-proies aux besoins nutritifs des larvaes de poissons marins. *Colloques Franco Japonaise du Océanographie Marseille* **85**: 51–63.

Geurden I, Coutteau P, Sorgeloos P (1997) Effect of a dietary phospholipid supplementation on growth and fatty acid composition of European sea bass (*Dicentrarchus labrax* L) and turbot (*Scophthalmus maximus* L) juveniles from weaning onwards. *Fish Physiology and Biochemistry* **16**: 259–272.

Geurden I, Reyes OS, Bergot P, Coutteau P, Sorgeloos P (1998) Incorporation of fatty acids from dietary neutral lipid in eye, brain and muscle of postlarval turbot fed diets with different types of phosphatidylcholine. *Fish Physiology and Biochemistry* **19**: 365–375.

Gouillou-Coustans MF, Bergot P, Kaushik SJ (1998) Dietary ascorbic acid needs of common carp (*Cyprinus carpio*) larvae. *Aquaculture* **161**: 453–461.

Hadas E, Koven W, Sklan D, Tandler A (2003) The effect of dietary phosphatidylcholine on the assimilation and distribution of ingested free oleic acid (18: 1n-9) in gilthead seabream (*Sparus aurata*) larvae. *Aquaculture* **217**: 577–588.

Haga Y, Takeuchi T, Murayama Y, Ohta K, Fukunaga T (2004) Vitamin D3 compounds induce hypermelanosis on the blind side and vertebral deformity in juvenile Japanese flounder *Paralichthys olivaceus*. *Fisheries Science* **70**: 59–67.

Hamre K (2006) Nutrition in cod (*Gadus morhua*) larvae and juveniles. *ICES Journal of Marine Science* **63**: 267–274.

Hamre K (2011) Metabolism, interactions, requirements and functions of vitamin E in fish. *Aquaculture Nutrition* **17**: 98–115.

Hamre K, Harboe T (2008a) *Artemia* enriched with high n-3 HUFA may give a large improvement in performance of

Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae. *Aquaculture* **277**: 239–243.

Hamre K, Harboe T (2008b) Critical levels of essential fatty acids for normal pigmentation in Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae. *Aquaculture* **277**: 101–108.

Hamre K, Mangor-Jensen A (2006) A multivariate approach to optimization of macronutrient composition in weaning diets for cod (*Gadus morhua*). *Aquaculture Nutrition* **12**: 15–24.

Hamre K, Opstad I, Espe M, Solbakken J, Hemre G-I, Pittman K (2002) Nutrient composition and metamorphosis success of Atlantic halibut (*Hippoglossus hippoglossus*, L.) larvae fed natural zooplankton or *Artemia*. *Aquaculture Nutrition* **8**: 139–148.

Hamre K, Øfsti A, Næss T, Nortvedt R, Holm JC (2003) Macronutrient composition in formulated diets for Atlantic halibut (*Hippoglossus hippoglossus*, L.) juveniles – a multivariate approach. *Aquaculture* **227**: 233–244.

Hamre K, Baeverfjord G, Harboe T (2005) Macronutrient composition of formulated diets for Atlantic halibut (*Hippoglossus hippoglossus*, L.) juveniles, II: protein/lipid levels at low carbohydrate. *Aquaculture* **244**: 283–291.

Hamre K, Holen E, Moren M (2007) Pigmentation and eye migration in Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae: new findings and hypotheses. *Aquaculture Nutrition* **13**: 65–80.

Hamre K, Mollan TA, Sæle O, Erstad B (2008a) Rotifers enriched with iodine and selenium increase survival in Atlantic cod (*Gadus morhua*) larvae. *Aquaculture* **284**: 190–195.

Hamre K, Srivastava A, Rønnestad I, Mangor-Jensen A, Stoss J (2008b) Several micronutrients in the rotifer *Brachionus* sp. may not fulfil the nutritional requirements of marine fish larvae. *Aquaculture Nutrition* **14**: 51–60.

Hamre K, Lukram IM, Rønnestad I, Nordgreen A, Sæle O (2011) Pre-digestion of dietary lipids has only minor effects on absorption, retention and metabolism in larval stages of Atlantic cod (*Gadus morhua*). *British Journal of Nutrition* **105**: 846–856.

Hamza N, Mhetli M, Ben Khemis I, Cahu C, Kestemont P (2008) Effect of dietary phospholipid levels on performance, enzyme activities and fatty acid composition of pikeperch (*Sander lucioperca*) larvae. *Aquaculture* **275**: 274–282.

Harada K (1992) Effects of attractant and repellent mixtures on behavior of the oriental weatherfish *Misgurnus-anguillicaudatus*. *Nippon Suisan Gakkaishi* **58**: 1427–1430.

Harada K, Eguchi A, Kurosaki Y (1987) Studies on the feeding attractants for fishes and shells. 14. Feeding attraction activities in the combinations of amino-acids and other compounds for abalone, oriental weatherfish and yellowtail. *Nippon Suisan Gakkaishi* **53**: 1483–1489.

Harboe T, Mangor-Jensen A, Moren M, Hamre K, Rønnestad I (2009) Control of light condition affects the feeding regime and enables successful eye migration in Atlantic halibut juveniles. *Aquaculture* **290**: 250–255.

Hastey RP, Phelps RP, Davis DA, Cummins KA (2010) Changes in free amino acid profile of red snapper *Lutjanus campechanus*, eggs, and developing larvae. *Fish Physiology and Biochemistry* **36**: 473–481.

Hawkyard M, Sæle O, Nordgreen A, Langdon C, Hamre K (2011) Effect of iodine enrichment of *Artemia* sp on their nutritional value for larval zebrafish (*Danio rerio*). *Aquaculture* **316**: 37–43.

Helland S, Oehme M, Ibieta P, Hamre K, Lein I, Barr Y (2010) Effects of enriching rotifers *Brachionus* (Cayman) with protein, taurine, arginine, or phospholipids – start feed for cod larvae *Gadus morhua* L In: *Aquaculture Europe 2010*, October 6–10. European Aquaculture Society, Porto.

Heming TA, Buddington RK (1988) Yolk absorption in embryonic and larval fishes. In: Hoar WS, Randall DJ (eds) *The Physiology of Developing Fish. Part A. Eggs and Larvae*, pp. 407–446. Academic Press, New York, NY.

Hernández-Cruz CM, Salhi M, Bessonart M, Izquierdo MS, González MM, Fernández-Palacios H (1999) Rearing techniques for red porgy (*Pagrus pagrus*) during larval development. *Aquaculture* **179**: 489–497.

Holt J (2011) *Larval Fish Nutrition*, pp. 435. Wiley-Blackwell, Chichester, UK.

Hughes SG (1990) Effects of aqueous amino acid solutions on the feed intake of juvenile Atlantic salmon. *Salmonid* **13**: 13–14.

Izquierdo MS (1988) *Essential Fatty Acid Requirements for Marine Fish Larvae*, pp. 211. Tokyo Suisan Daigaku, Tokyo.

Izquierdo MS (1996) Review: essential fatty acid requirements of cultured marine fish larvae. *Aquaculture Nutrition* **2**: 183–191.

Izquierdo MS (2005) Essential fatty acid requirements in Mediterranean fish species. *Cahiers Options Mediterranéennes* **63**: 91–102.

Izquierdo MS, Koven W (2011) Lipids. In: Holt J (ed.)*Larval Fish Nutrition*, pp. 47–84. Wiley-Blackwell, John Wiley and Sons, Chichester, UK.

Izquierdo MS, Lall S (2004) Experimental design for lipid research. Workshop on Methodologies in Fish Nutrition Research. 2–7 May 2004, Phuket, Thailand.

Izquierdo MS, Watanabe T, Takeuchi T, Arakawa T, Kitajima C (1989) Requirement of larval red seabream *Pagrus major* for essential fatty-acids. *Nippon Suisan Gakkaishi* **55**: 859–867.

Izquierdo MS, Arakawa T, Takeuchi T, Haroun R, Watanabe T (1992) Effect of n-3 hufa levels in *Artemia* on growth of larval Japanese flounder (*Paralichthys olivaceus*). *Aquaculture* **105**: 73–82.

Izquierdo MS, Tandler A, Salhi M, Kolkovski S (2001) Influence of dietary polar lipids' quantity and quality on ingestion and assimilation of labelled fatty acids by larval gilthead seabream. *Aquaculture Nutrition* **7**: 153–160.

Kanazawa A (1993) Nutritional mechanisms involved in the occurrence of abnormal pigmentation in hatchery-reared flounder. *Journal of the World Aquaculture Society* **24**: 162–166.

Kanazawa A, Teshima S (1988) Microparticulate diets for fish larvae. In: *NOAA. Tech. Rep. NMFS*, pp. 57–62. Natl. Mar. Fish. Ser., Seattle, WA.

Kanazawa A, Teshima S, Inamori S, Iwashita T, Nagao A (1981) Effects of phospholipids on growth, survival rate and incidence of malformation in the larval Ayu. *Memoirs of the Faculty of Fisheries, Hokkaido University* **30**: 301–309.

Kleinow W, Wratil H, Kuhle K, Esch B (1991) Electron-microscope studies of the digestive-tract of *Brachionus plicatilis* (Rotifera). *Zoomorphology* **111**: 67–80.

Kolkovski S, Tandler A (2000) The use of squid protein hydrolysate as a protein source in microdiets for gilthead seabream *Sparus aurata* larvae. *Aquaculture Nutrition* **6**: 11–15.

Kolkovski S, Tandler A, Kissil GW, Gertler A (1993) The effect of dietary exogenous digestive enzymes on ingestion, assimilation, growth and survival of gilthead seabream (*Sparus aurata*, Sparidae, Linnaeus) larvae. *Fish Physiology and Biochemistry* **12**: 203–209.

Kolkovski S, Arieli A, Tandler A (1997) Visual and chemical cues stimulate microdiet ingestion in sea bream larvae. *Aquaculture International* **5**: 527–536.

Kolkovski S, Czesny S, Yackey C, Moreau R, Cihla F, Mahan D *et al.* (2000) The effect of vitamins C and E in (n-3) highly unsaturated fatty acids-enriched *Artemia* nauplii on growth, survival and stress resistance of fresh water walley *Stizostedion vitreum* larvae. *Aquaculture Nutrition* **6**: 199–206.

Kolkovski S, Lazzo J, Leclercq D, Izquierdo M (2009) Fish larvae nutrition and diet: new developments. In: Burnell G, Allan G (eds) *New Technologies in Aquaculture Improving Production Efficiency, Quality and Environmental Management*, pp. 315–369. CRC Woodhead, Cambridge, UK.

Koven W (2003) Key factors influencing juvenile quality in mariculture: a review. *Israeli Journal of Aquaculture-Bamidgeh* **55**: 283–297.

Koven WM, Tandler A, Kissil GW, Sklan D, Friezlander O, Harel M (1990) The effect of dietary (n-3) polyunsaturated fatty-acids on growth, survival and swim bladder development in *Sparus aurata* larvae. *Aquaculture* **91**: 131–141.

Koven WM, Kolkovski S, Tandler A, Kissil GW, Sklan D (1993) The effect of dietary lecithin and lipase, as a function of age, on n-9 fatty-acid incorporation in the tissue-lipids of *Sparus aurata* larvae. *Fish Physiology and Biochemistry* **10**: 357–364.

Koven WM, Parra G, Kolkovski S, Tandler A (1998) The effect of dietary phosphatidylcholine and its constituent fatty acids on microdiet ingestion and fatty acid absorption rate in gilthead sea bream, *Sparus aurata*, larvae. *Aquaculture Nutrition* **4**: 39–45.

Koven W, Kolkovski S, Hadas E, Gamsiz K, Tandler A (2001) Advances in the development of microdiets for gilthead seabream, *Sparus aurata*: a review. *Aquaculture* **194**: 107–121.

Kvåle A, Yúfera M, Nygard E, Aursland K, Harboe T, Hamre K (2006) Leaching properties of three different micropaticulate diets and preference of the diets in cod (*Gadus morhua* L.) larvae. *Aquaculture* **251**: 402–415.

Kvåle A, Harboe T, Mangor-Jensen A, Hamre K (2009) Effects of protein hydrolysate in weaning diets for Atlantic cod (*Gadus morhua* L.) and Atlantic halibut (*Hippoglossus hippoglossus* L.). *Aquaculture Nutrition* **15**: 218–227.

Langdon C (2003) Microparticle types for delivering nutrients to marine fish larvae. *Aquaculture* **227**: 259–275.

Langdon C, Clack B, Onal U (2007) Complex microparticles for delivery of low-molecular weight, water-soluble nutrients and pharmaceuticals to marine fish larvae. *Aquaculture* **268**: 143–148.

Langdon C, Nordgreen A, Hawkyard M, Hamre K (2008) Evaluation of wax spray beads for delivery of low-molecular weight, water-soluble nutrients and antibiotics to *Artemia*. *Aquaculture* **284**: 151–158.

Lewis-McCrea LM, Lall SP (2010) Effects of phosphorus and vitamin C deficiency, vitamin A toxicity, and lipid peroxidation on skeletal abnormalities in Atlantic halibut (*Hippoglossus hippoglossus*). *Journal of Applied Ichthyology* **26**: 334–343.

Lie Ø (1993) Changes in the fatty acid composition of neutral lipids and glycerophospholipids in developing cod eggs. In: Walther BT, Fyhn HJ (eds) *Physiological and Biochemical Aspects of Fish Development*, pp. 330–337. University of Bergen, Bergen, Norway.

Lindemann N, Kleinow W (2000) A study of rotifer feeding and digestive processes using erythrocytes as microparticulate markers. *Hydrobiologia* **435**: 27–41.

Liu J, Caballero MJ, Izquierdo M, Ali TES, Hernández-Cruz CM, Valencia A *et al.* (2002) Necessity of dietary lecithin and eicosapentaenoic acid for growth, survival, stress resistance and lipoprotein formation in gilthead sea bream *Sparus aurata*. *Fisheries Science* **68**: 1165–1172.

Lopez-Alvarado J, Langdon CJ, Teshima S-I, Kanazawa A (1994) Effect of coating and encapsulation of crystalline amino acids on leaching in larval feeds. *Aquaculture* **122**: 335–346.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**: 265–275.

Lubzens E, Tandler A, Minkoff G (1989) Rotifers as food in aquaculture. *Hydrobiologia* **186**: 387–400.

Lubzens E, Zmora O, Barr Y (2001) Biotechnology and aquaculture of rotifers. *Hydrobiologia* **446**: 337–353.

Mæland A, Rønnestad I, Fyhn HJ, Berg L, Waagbo R (2000) Water-soluble vitamins in natural plankton (copepods) during two consecutive spring blooms compared to vitamins in *Artemia franciscana* nauplii and metanauplii. *Marine Biology* **136**: 765–772.

Mæland A, Rønnestad I, Waagbo R (2003) Folate in eggs and developing larvae of Atlantic halibut, *Hippoglossus hippoglossus* L. *Aquaculture Nutrition* **9**: 185–188.

Mai MG, Engrola S, Morais S, Portella MC, Verani JR, Dinis MT *et al.* (2009) Co-feeding of live feed and inert diet from first-feeding affects *Artemia* lipid digestibility and retention in Senegalese sole (*Solea senegalensis*) larvae. *Aquaculture* **296**: 284–291.

Martins DA, Estévez A, Stickland NC, Simbi BH, Yúfera M (2010) Dietary lecithin source affects growth potential and gene expression in *Sparus aurata* larvae. *Lipids* **45**: 1011–1023.

Mazurais D, Glynatsi N, Darias MJ, Christodoulopoulou S, Cahu CL, Zambonino-Infante JL *et al.* (2009) Optimal levels of dietary vitamin A for reduced deformity incidence during development of European sea bass larvae (*Dicentrarchus labrax*) depend on malformation type. *Aquaculture* **294**: 262–270.

McEvoy LA, Estévez A, Bell JG, Shields RJ, Gara B, Sargent JR (1998) Influence of dietary levels of eicosapentaenoic and arachidonic acid on the pigmentation success of turbot (*Scophtalmus maximus*) and halibut (*Hippoglossus hippoglossus*). *Bulletin of the Aquaculture Association of Canada* **98** (4): 17–20.

van der Meeren T, Olsen RE, Hamre K, Fyhn HJ (2008) Biochemical composition of copepods for evaluation of feed quality in production of juvenile marine fish. *Aquaculture* **274**: 375–397.

Merchie G, Lavens P, Sorgeloos P (1997) Optimization of dietary vitamin C in fish and crustacean larvae: a review. *Aquaculture* **155**: 165–181.

Mollan TA, Tonheim SK, Hamre K (2008) Pre-hydrolysis improves absorption of neutral lipids in Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae. *Aquaculture* **275**: 217–224.

Monroig O, Navarro JC, Amat F, González P, Bermejo A, Hontoria F (2006) Enrichment of *Artemia* nauplii in essential fatty acids with different types of liposomes and their use in the rearing of gilthead sea bream (*Sparus aurata*) larvae. *Aquaculture* **251**: 491–508.

Montero D, Tort L, Izquierdo MS, Robaina L, Vergara JM (1998) Depletion of serum alternative complement pathway activity in gilthead seabream caused by alpha-tocopherol and n-3 HUFA dietary deficiencies. *Fish Physiology and Biochemistry* **18**: 399–407.

Morais S, Conceição LEC (2009) A new method for the study of essential fatty acid requirements in fish larvae. *British Journal of Nutrition* **101**: 1564–1568.

Morais S, Conceição LEC, Dinis MT, Rønnestad I (2004a) A method for radiolabeling *Artemia* with applications in studies of food intake, digestibility, protein and amino acid metabolism in larval fish. *Aquaculture* **231**: 469–487.

Morais S, Lacuisse M, Conceição LEC, Dinis MT, Rønnestad I (2004b) Ontogeny of the digestive capacity of (*Solea senegalensis*), with respect to and metabolism of amino acids from Senegalese sole digestion, absorption *Artemia*. *Marine Biology* **145**: 243–250.

Morais S, Koven W, Rønnestad I, Dinis MT, Conceição LEC (2005a) Dietary protein/lipid ratio affects growth and amino acid and fatty acid absorption and metabolism in Senegalese sole (*Solea senegalensis* Kaup 1858) larvae. *Aquaculture* **246**: 347–357.

Morais S, Koven W, Rønnestad I, Dinis MT, Conceição LEC (2005b) Dietary protein: lipid ratio and lipid nature affects fatty acid absorption and metabolism in a teleost larva. *British Journal of Nutrition* **93**: 813–820.

Morais S, Rojas-Garcia CR, Conceição LEC, Rønnestad I (2005c) Digestion and absorption of a pure triacylglycerol and a free fatty acid by *Clupea harengus* L. larvae. *Journal of Fish Biology* **67**: 223–238.

Morais S, Torten M, Nixon O, Lutzky S, Conceição LEC, Dinis MT *et al.* (2006) Food intake and absorption are affected by dietary lipid level and lipid source in seabream (*Sparus aurata* L.) larvae. *Journal of Experimental Marine Biology and Ecology* **331**: 51–63.

Moren M, Naess T, Hamre K (2002) Conversion of beta-carotene, canthaxanthin and astaxanthin to vitamin A in Atlantic halibut (*Hippoglossus hippoglossus* L.) juveniles. *Fish Physiology and Biochemistry* **27**: 71–80.

Moren M, Opstad I, Berntssen MHG, Infante JLZ, Hamre K (2004) An optimum level of vitamin A supplements for Atlantic halibut (*Hippoglossus hippoglossus* L.) juveniles. *Aquaculture* **235**: 587–599.

Moren M, Gundersen TE, Hamre K (2005) Quantitative and qualitative analysis of retinoids in *Artemia* and copepods by HPLC and diode array detection. *Aquaculture* **246**: 359–365.

Morris AL, Hamlin HJ, Francis-Floyd R, Sheppard BJ, Guillette LJ (2011) Nitrate-induced goiter in captive whitespotted bamboo sharks *Chiloscyllium plagiosum*. *Journal of Aquatic Animal Health* **23**: 92–99.

Mourente G, Rodríguez A, Grau A, Pastor E (1999a) Utilization of lipids by *Dentex dentex* L-(*Osteichthyes, Sparidae*) larvae during lecitotrophia and subsequent starvation. *Fish Physiology and Biochemistry* **21**: 45–58.

Mourente G, Tocher DR, Diaz-Salvago E, Grau A, Pastor E (1999b) Study of the n-3 highly unsaturated fatty acids requirement and antioxidant status of *Dentex dentex* larvae at the *Artemia* feeding stage. *Aquaculture* **179**: 291–307.

Næss T, Lie Ø (1998) A sensitive period during first feeding for the determination of pigmentation pattern in Atlantic halibut, *Hippoglossus hippoglossus* L., juveniles: the role of diet. *Aquaculture Research* **29**: 925–934.

Nankervis L, Southgate PC (2006) An integrated assessment of gross marine protein sources used in formulated microbound diets for barramundi (*Lates calcarifer*) larvae. *Aquaculture* **257**: 453–464.

Nankervis L, Southgate P (2009) Enzyme and acid treatment of fish meal for incorporation into formulated microbound diets for barramundi (*Lates calcarifer*) larvae. *Aquaculture Nutrition* **15**: 135–143.

Navarro JC, Henderson RJ, McEvoy LA, Bell MV, Amat F (1999) Lipid conversion during enrichment of *Artemia*. *Aquaculture* **174**: 155–166.

Nguyen VT, Satoh S, Haga Y, Fushimi H, Kotani T (2008) Effect of zinc and manganese supplementation in *Artemia* on growth and vertebral deformity in red sea bream (*Pagrus major*) larvae. *Aquaculture* **285**: 184–192.

Nicklason PM, Johnson RB (2008) Real-time measurement of protein leaching from micro-particulate larval fish feeds. *Aquaculture Research* **39**: 1793–1798.

Nordgreen A, Hamre K, Langdon C (2007) Development of lipid microbeads for delivery of lipid and water-soluble materials to *Artemia*. *Aquaculture* **273**: 614–623.

Nordgreen A, Yúfera M, Hamre K (2008) Evaluation of changes in nutrient composition during production of cross-linked protein microencapsulated diets for marine fish larvae and suspension feeders. *Aquaculture* **285**: 159–166.

Nordgreen A, Tonheim S, Hamre K (2009) Protein quality of larval feed with increased concentration of hydrolysed protein: effects of heat treatment and leaching. *Aquaculture Nutrition* **15**: 525–536.

NRC (2011) *Nutrient Requirements of Fish and Shrimp*. National Academic Press, Washington D.C.

Ohkubo N, Sawaguchi S, Nomura K, Tanaka H, Matsubara T (2008) Utilization of free amino acids, yolk protein and lipids in developing eggs and yolk-sac larvae of Japanese eel *Anguilla japonica*. *Aquaculture* **282**: 130–137.

Øie G, Makridis P, Reitan KI, Olsen Y (1997) Protein and carbon utilization of rotifers (*Branchionus plicatilis*) in first feeding of turbot larvae (*Scophthalmus maximus* L.). *Aquaculture* **153**: 103–122.

Oikawa S, Itazawa Y (1984) Allometric relationship between tissue respiration and body-mass in the carp. *Comparative Biochemistry and Physiology A: Physiology* **77**: 415–418.

Oliva-Teles A, Cerqueira AL, Gonçalves P (1999) The utilization of diets containing high levels of fish protein hydrolysate by turbot (*Scophthalmus maximus*) juveniles. *Aquaculture* **179**: 195–201.

Olsen Y (2004) Live food technology of cold-water marine fish larvae. In: Kjørsvik EM, Olsen Y (eds) *Culture of Cold-water Marine Fish*, pp. 73–193. Blackwell Publishing, Oxford, UK.

Onal U, Langdon C (2004) Lipid spray beads for delivery of riboflavin to first-feeding fish larvae. *Aquaculture* **233**: 477–493.

Onal U, Langdon C (2005) Development and characterization of complex particles for delivery of amino acids to early marine fish larvae. *Marine Biology* **146**: 1031–1038.

Osse J, Boogaart J (1995) Fish larvae, development, allometric growth, and the aquatic environment. *ICES Marine Science Symposium* **201**: 21–34.

Otterlei E, Nyhammer G, Folkvord A, Stefansson SO (1999) Temperature- and size-dependent growth of larval and early juvenile Atlantic cod (*Gadus morhua*): a comparative study of Norwegian coastal cod and northeast Arctic cod. *Canadian Journal of Fisheries and Aquatic Sciences* **56**: 2099–2111.

Ozkizilcik S, Chu FLE (1996) Preparation and characterization of a complex microencapsulated diet for striped bass *Morone saxatilis* larvae. *Journal of Microencapsulation* **13**: 331–343.

Parra G, Yúfera M (2001) Comparative energetics during early development of two marine fish species, *Solea senegaensis* (Kaup) and *Sparus aurata* (L.). *Journal of Experimental Biology* **204**: 2175–2183.

Penglase S, Nordgreen A, van der Meeren T, Olsvik PA, Sæle O, Sweetman JW *et al.* (2010) Increasing the level of selenium in rotifers (*Brachionus plicatilis* 'Cayman') enhances the mRNA expression and activity of glutathione peroxidase in cod (*Gadus morhua* L.) larvae. *Aquaculture* **306**: 259–269.

Penglase S, Hamre K, Sweetman JW, Nordgreen A (2011) A new method to increase and maintain the concentration of selenium in rotifers (*Brachionus* spp.). *Aquaculture* **315**: 144–153.

Perumal P, Rajkumar M, Santhanam P (2009) Biochemical composition of wild copepods, *Acartia spinicauda* and *Oithona similis*, from Parangipettai coastal waters in relation to environmental parameters. *Journal of Environmental Biology* **30**: 995–1005.

Pinto W, Figueira L, Dinis MT, Aragão C (2009) How does fish metamorphosis affect aromatic amino acid metabolism? *Amino Acids* **36**: 177–183.

Pinto W, Figueira L, Ribeiro L, Yúfera M, Dinis MT, Aragão C (2010) Dietary taurine supplementation enhances metamorphosis and growth potential of *Solea senegalensis* larvae. *Aquaculture* **309**: 159–164.

Quantz G (1985) Use of endogenous energy-sources by larval turbot *Scophthalmus maximus*. *Transactions of the American Fisheries Society* **114**: 558–563.

Rainuzzo JR, Reitan KI, Jorgensen L (1992) Comparative-study on the fatty-acid and lipid-composition of 4 marine fish larvae. *Comparative Biochemistry and Physiology B* **103**: 21–26.

Rainuzzo JR, Reitan KI, Olsen Y (1997) The significance of lipids at early stages of marine fish: a review. *Aquaculture* **155**: 103–115.

Ribeiro ARA, Ribeiro L, Sæle O, Hamre K, Dinis MT, Moren M (2011) Iodine-enriched rotifers and *Artemia* prevent goitre in Senegalese sole (*Solea senegalensis*) larvae reared in a recirculation system. *Aquaculture Nutrition* **17**: 248–257.

Rodríguez C, Perez JA, Lorenzo A, Izquierdo MS, Cejas JR (1994) N-3 HUFA requirement of larval gilthead seabream *Sparus aurata* when using high-levels of eicosapentaenoic acid. *Comparative Biochemistry and Physiology A: Physiology* **107**: 693–698.

Rodríguez C, Perez JA, Diaz M, Izquierdo MS, Fernández-Palacios H, Lorenzo A (1997) Influence of the EPA/DHA ratio in rotifers on gilthead seabream (*Sparus aurata*) larval development. *Aquaculture* **150**: 77–89.

Rodríguez C, Perez JA, Badia P, Izquierdo MS, Fernández-Palacios H, Hernández AL (1998) The n-3 highly unsaturated fatty acids requirements of gilthead seabream (*Sparus aurata* L.) larvae when using an appropriate DHA/EPA ratio in the diet. *Aquaculture* **169**: 9–23.

Rodríguez C, Perez JA, Izquierdo MS, Mora J, Lorenzo A, Fernández-Palacios H (1993) Essential fatty acid requirements for larval gilthead sea bream (*S. aurata*). *Aquaculture and Fisheries Management* **24**: 295–304.

Rojas-García CR, Rønnestad I (2003) Assimilation of dietary free amino acids, peptides and protein in post-larval Atlantic halibut (*Hippoglossus hippoglossus*). *Marine Biology* **142**: 801–808.

Romero-Romero S, Yúfera M (2012) Contribution of gut content to the nutritional value of Brachionus plicatilis used as prey in larviculture. *Aquaculture* (in press).

Rønnestad I, Conceição LEC (2012) Artemia protein is processed very fast in Solea senegalensis larvae: a dynamic simulation model. *Aquaculture* **350**: 154–161.

Rønnestad I, Conceição LEC, Aragão C, Dinis MT (2000) Free amino acids are absorbed faster and assimilated more efficiently than protein in postlarval Senegal sole (*Solea senegalensis*). *Journal of Nutrition* **130**: 2809–2812.

Rønnestad I, Finn RN, Groot EP, Fyhn HJ (1992a) Utilization of free amino-acids related to energy-metabolism of developing eggs and larvae of lemon sole *Microstomus kitt* reared in the laboratory. *Marine Ecology Progress Series* **88**: 195–205.

Rønnestad I, Fyhn HJ, Gravningen K (1992b) The importance of free amino-acids to the energy-metabolism of eggs and larvae of turbot (*Scophthalmus maximus*). *Marine Biology* **114**: 517–525.

Rønnestad I, Groot EP, Fyhn HJ (1993) Compartmental distribution of free amino-acids and protein in developing yolk-sac larvae of Atlantic halibut (*Hippoglossus hippoglossus*). *Marine Biology* **116**: 349–354.

Rønnestad I, Finn RN, Lein I, Lie Ø (1995) Compartmental changes in the contents of total lipid, lipid classes and their associated fatty acids in developing yolk-sac larvae of Atlantic halibut, *Hippoglossus hippoglossus* (L.). *Aquaculture Nutrition* **1**: 119–130.

Rønnestad I, Lie Ø, Waagbø R (1997) Vitamin B-6 in Atlantic halibut, *Hippoglossus hippoglossus* – endogenous utilization and retention in larvae fed natural zooplankton. *Aquaculture* **157**: 337–345.

Rønnestad I, Hemre G-I, Finn RN, Lie Ø (1998) Alternate sources and dynamics of vitamin A and its incorporation into the eyes during the early endotrophic and exotrophic larval stages of Atlantic halibut (*Hippoglossus hippoglossus* L.). *Comparative Biochemistry and Physiology Part A*, **119**: 787–793.

Rønnestad I, Hamre K, Lie Ø, Waagbø R (1999) Ascorbic acid and alpha-tocopherol levels in larvae of Atlantic halibut before and after exogenous feeding. *Journal of Fish Biology* **55**: 720–731.

Rønnestad I, Rojas-García CR, Tonheim SK, Conceição LEC (2001) *In vivo* studies of digestion and nutrient assimilation in marine fish larvae. *Aquaculture* **201**: 161–175.

Rønnestad I, Yúfera M, Ueberschär B, Ribeiro L, Sæle Ø. Izquierdo M, Boglione C (2012) Feeding behaviour and digestion physiology in larval fish – current knowledge and gaps and bottlenecks in research. *Reviews in Aquaculture* (in press).

Roo FJ, Hernández-Cruz CM, Socorro JA, Fernández-Palacios H, Montero D, Izquierdo MS (2009) Effect of DHA content in rotifers on the occurrence of skeletal deformities in red porgy *Pagrus pagrus* (Linnaeus, 1758). *Aquaculture* **287**: 84–93.

Rubio VC, Sánchez-Vázquez FJ, Madrid JA (2003) Macronutrient selection through postingestive signals in sea bass fed on gelatine capsules. *Physiology and Behavior* **78**: 795–803.

Saavedra M, Conceição LEC, Pousão-Ferreira P, Dinis MT (2006) Amino acid profiles of *Diplodus sargus* (L., 1758) larvae: implications for feed formulation. *Aquaculture* **261**: 587–593.

Saavedra M, Beltran M, Pousão-Ferreira P, Dinis MT, Blasco J, Conceição LEC (2007) Evaluation of bioavailability of individual amino acids in *Diplodus puntazzo* larvae: towards the ideal dietary amino acid profile. *Aquaculture* **263**: 192–198.

Saavedra M, Conceição LEC, Helland S, Pousão-Ferreira R, Dinis MT (2008a) Effect of lysine and tyrosine supplementation in the amino acid metabolism of *Diplodus sargus* larvae fed rotifers. *Aquaculture* **284**: 180–184.

Saavedra M, Conceição LEC, Pousão-Ferreira P, Dinis MT (2008b) Metabolism of tryptophan, methionine and arginine in *Diplodus sargus* larvae fed rotifers: effect of amino acid supplementation. *Amino Acids* **35**: 59–64.

Saavedra M, Pousão-Ferreira P, Yúfera M, Dinis MT, Conceição LEC (2009) A balanced amino acid diet improves *Diplodus sargus* larval quality and reduces nitrogen excretion. *Aquaculture Nutrition* **15**: 517–524.

Salhi M, Izquierdo MS, Hernández-Cruz CM, González M, Fernández-Palacios H (1994) Effect of lipid and n-3 HUFA levels in microdiets on growth, survival and fatty-acid composition of larval gilthead seabream (*Sparus aurata*). *Aquaculture* **124**: 275–282.

Salhi M, Izquierdo MS, Hernández-Cruz CM, Socorro J, Fernández-Palacios H (1997) The improved incorporation of polyunsaturated fatty acids and changes in liver structure in larval gilthead seabream fed on microdiets. *Journal of Fish Biology* **51**: 869–879.

Salhi M, Hernández-Cruz CM, Bessonart M, Izquierdo MS, Fernández-Palacios H (1999) Effect of different dietary polar lipid levels and different n-3 HUFA content in polar lipids on gut and liver histological structure of gilthead seabream (*Sparus aurata*) larvae. *Aquaculture* **179**: 253–263.

Samaee SM, Estévez A, Giménez G, Lahnsteiner F (2009) Evaluation of quantitative importance of egg lipids and fatty acids during embryos and larvae development in marine pelagophil teleosts: with an emphasis on *Dentex dentex*. *Journal of Experimental Zoology Part A* **311A**: 735–751.

Sánchez-Vázquez FJ, Yamamoto T, Akiyama T, Madrid JA, Tabata M (1999) Macronutrient self-selection through demand-feeders in rainbow trout. *Physiology and Behavior* **66**: 45–51.

Sandel E, Nixon O, Lutzky S, Ginsbourg B, Tandler A, Uni Z *et al.* (2010) The effect of dietary phosphatidylcholine/phosphatidylinositol ratio on malformation in larvae and juvenile gilthead sea bream (*Sparus aurata*). *Aquaculture* **304**: 42–48.

Sargent JR, McEvoy LA, Bell JG (1997) Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. *Aquaculture* **155**: 117–127.

Sato M, Yoshinaka R, Kuroshima R, Morimoto H, Ikeda S (1987) Changes in water-soluble vitamin contents and transaminase-activity of rainbow-trout egg during development. *Nippon Suisan Gakkaishi* **53**: 795–799.

Seiliez I, Bruant JS, Zambonino-Infante JL, Kaushik S, Bergot P (2006) Effect of dietary phospholipid level on the development of gilthead sea bream (*Sparus aurata*) larvae fed a compound diet. *Aquaculture Nutrition* **12**: 372–378.

Shields RJ, Bell JG, Luizi FS, Gara B, Bromage NR, Sargent JR (1999) Natural copepods are superior to enriched *Artemia* nauplii as feed for halibut larvae (*Hippoglossus hippoglossus*) in terms of survival, pigmentation and retinal morphology: relation to dietary essential fatty acids. *Journal of Nutrition* **129**: 1186–1194.

Sire MF, Lutton C, Vernier JM (1981) New views on intestinal absorption of lipids in teleostean fishes – an ultrastructural and biochemical study in the rainbow trout. *Journal of Lipid Research* **22**: 81–94.

Solbakken JS, Berntssen MHG, Norberg B, Pittman K, Hamre K (2003) Differential iodine and thyroid hormone levels between Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae fed wild zooplankton or *Artemia* from first exogenous feeding until post metamorphosis. *Journal of Fish Biology* **61**: 1345–1362.

Srivastava A, Hamre K, Stoss J, Chakrabarti R, Tonheim SK (2006) Protein content and amino acid composition of the live feed rotifer (*Brachionus plicatilis*): with emphasis on the water soluble fraction. *Aquaculture* **254**: 534–543.

Srivastava A, Stoss J, Hamre K (2011) A study on enrichment of the rotifer *Brachionus* 'Cayman' with iodine and selected vitamins. *Aquaculture* **319**: 430–438.

Srivastava A, Hamre K, Stoss J, Nordgreen A (2012) A study on enrichment of the rotifer *Brachionus* 'Cayman' with iodine from different sources. *Aquaculture* **334**: 82–88.

Sturman JA (1993) Taurine in development. *Physiological Reviews* **73**: 119–147.

Tocher DR, Sargent JR (1984) Analyses of lipids and fatty-acids in ripe roes of some northwest European marine fish. *Lipids* **19**: 492–499.

Tocher DR, Fraser AJ, Sargent JR, Gamble JC (1985a) Fatty-acid composition of phospholipids and neutral lipids during embryonic and early larval development in Atlantic herring (*Clupea harengus*, L). *Lipids* **20**: 69–74.

Tocher DR, Fraser AJ, Sargent JR, Gamble JC (1985b) Lipid class composition during embryonic and early larval development in Atlantic herring (*Clupea harengus*, L). *Lipids* **20**: 84–89.

Tocher DR, Bendiksen EA, Campbell PJ, Bell JG (2008) The role of phospholipids in nutrition and metabolism of teleost fish. *Aquaculture* **280**: 21–34.

Tonheim SK, Koven W, Rønnestad I (2000) Enrichment of *Artemia* with free methionine. *Aquaculture* **190**: 223–235.

Tonheim SK, Espe M, Hamre K, Rønnestad I (2005) Pre-hydrolysis improves utilisation of dietary protein in the larval teleost Atlantic halibut (*Hippoglossus hippoglossus* L.). *Journal of Experimental Marine Biology and Ecology* **321**: 19–34.

Tonheim SK, Nordgreen A, Hogoy I, Hamre K, Rønnestad I (2007) *In vitro* digestibility of water-soluble and water-insoluble protein fractions of some common fish larval feeds and feed ingredients. *Aquaculture* **262**: 426–435.

Tulli F, Tibaldi E (1997) Changes in amino acids and essential fatty acids during early larval rearing of dentex. *Aquaculture International* **5**: 229–236.

Udagawa M (2001) The effect of dietary vitamin K (phylloquinone and menadione) levels on the vertebral formation in mummichog *Fundulus heteroclitus*. *Fisheries Science* **67**: 104–109.

Vetter RD, Hodson RE, Arnold C (1983) Energy-metabolism in a rapidly developing marine fish egg, the red drum (*Sciaenops ocellata*). *Canadian Journal of Fisheries and Aquatic Science* **40**: 627–634.

Villalta M, Estévez A, Bransden MP (2005a) Arachidonic acid enriched live prey induces albinism in Senegal sole (*Solea senegalensis*) larvae. *Aquaculture* **245**: 193–209.

Villalta M, Estévez A, Bransden MP, Bell JG (2005b) The effect of graded concentrations of dietary DHA on growth, survival and tissue fatty acid profile of Senegal sole (*Solea senegalensis*) larvae during the *Artemia* feeding period. *Aquaculture* **249**: 353–365.

Villeneuve L, Gisbert E, Delliou HL, Cahu CL, Zambonino-Infante JL (2005a) Dietary levels of all-*trans* retinol affect retinoid nuclear receptor expression and skeletal development in European sea bass larvae. *British Journal of Nutrition* **93**: 791–801.

Villeneuve L, Gisbert E, Zambonino-Infante JL, Quazuguel P, Cahu CL (2005b) Effect of nature of dietary lipids on European sea bass morphogenesis: implication of retinoid receptors. *British Journal of Nutrition* **94**: 877–884.

Walford J, Lam TJ (1987) Effect of feeding with microcapsules on the content of essential fatty-acids in live foods for the larvae of marine fishes. *Aquaculture* **61**: 219–229.

Watanabe T (1993) Importance of docosahexaenoic acid in marine larval fish. *Journal of the World Aquaculture Society* **24**: 151–161.

Watanabe T, Izquierdo MS, Takeuchi T, Satoh S, Kitajima C (1989) Comparison between eicosapentaenoic and docosahexaenoic acids in terms of essential fatty-acid efficacy in larval red seabream. *Nippon Suisan Gakkaishi* **55**: 1635–1640.

Weirich CR, Reigh RC (2001) Dietary lipids and stress tolerance of larval fish. In: Lim C, Webster CD (eds) *Nutrition and Fish Health*, pp. 301–312. Food Products Press, New York, NY.

Woodward B (1994) Dietary vitamin requirements of cultured young fish, with emphasis on quantitative estimates for salmonids. *Aquaculture* **124**: 133–168.

Yúfera M (2007) Swimming behaviour of *Brachionus plicatilis* in relation to food concentration and feeding rates. *Hydrobiologia* **593**: 13–18.

Yúfera M, Parra G, Santiago R, Carrascosa M (1999a) Growth, carbon, nitrogen and caloric content of *Solea senegalensis*

(Pisces: *Soleidae*) from egg fertilization to metamorphosis. *Marine Biology* **134**: 43–49.

Yúfera M, Pascual E, Fernández-Díaz C (1999b) A highly efficient microencapsulated food for rearing early larvae of marine fish. *Aquaculture* **177**: 249–256.

Yúfera M, Fernández-Díaz C, Pascual E, Sarasquete MC, Moyano FJ, Díaz M *et al.* (2000) Towards an inert diet for first-feeding gilthead seabream *Sparus aurata* L. larvae. *Aquaculture Nutrition* **6**: 143–152.

Yúfera M, Kolkovski S, Fernández-Díaz C, Dabrowski K (2002) Free amino acid leaching from a protein-walled microencapsulated diet for fish larvae. *Aquaculture* **214**: 273–287.

Yúfera M, Kolkovski S, Fernández-Díaz C, Rinchard J, Lee KJ, Dabrowski K (2003) Delivering bioactive compounds to fish larvae using microencapsulated diets. *Aquaculture* **227**: 277–291.

Yúfera M, Fernández-Díaz C, Pascual E (2005) Food microparticles for larval fish prepared by internal gelation. *Aquaculture* **248**: 253–262.

Zambonino-Infante JL, Cahu CL (2007) Dietary modulation of some digestive enzymes and metabolic processes in developing marine fish: applications to diet formulation. *Aquaculture* **268**: 98–105.