The problem of meeting dietary protein requirements in intensive aquaculture of marine fish larvae, with emphasis on Atlantic halibut (*Hippoglossus hippoglossus* L.)

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Abstract

Atlantic halibut (*Hippoglossus hippoglossus*) achieve a mature gastrointestinal tract approximately 2 months after first feeding (12 °C). The immature digestion may be the reason that compound diets fail to sustain growth and survival in first feeding halibut larvae and in larvae of other marine fish species. On the other hand, larvae fed with live feeds are capable of extraction of sufficient quantities of nutrients to sustain high growth rates. A lower availability of the protein in formulated diets compared with live prey is considered to be an important reason for the low performance of formulated diets. One approach to increase dietary protein availability is supplementation of pre-digested proteins. Experiments using tube fed individual larvae show that halibut larvae are able to utilize hydrolysed protein more efficiently than intact protein. However, Atlantic halibut in culture did not respond well to dietary supplementation of hydrolysed protein, in contrast to some other species. One reason may be extensive leaching of pre-hydrolysed proteins from the microparticulate feed. Atlantic halibut are slow feeders and may thus suffer more from nutrient leaching than species eating more rapidly. Feed formulation techniques affect dietary protein leaching, and in this paper, different techniques and their impact on feed properties are described. Microbound diets are most widely used in larval rearing, but show high rates of nutrient leaching. Lipid-based capsules seem to have the best potential to prevent leaching, however, they are not able to deliver a complete diet. The high need for improvements in larval feed formulation techniques are clearly stated, and some suggestions are given. Among these are production of complex particles, where small lipid-based capsules or liposomes containing the low molecular weight water-soluble nutrients are embedded. In such feed particles the water-soluble molecules are protected from leaching. Techniques for delivery of water-soluble nutrients that are needed in large quantities, i.e. free amino acids or hydrolysed and water-soluble protein, remain to be developed.

KEY WORDS: Atlantic halibut, compound diets, digestion, larvae, leaching, protein

Received 3 February 2006, accepted 23 October 2006

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Introduction

The Atlantic halibut (*Hippoglossus hippoglossus* L.) is an interesting species for aquaculture because of its high filet outcome, high market value and low annual wild catches. Success in rearing of this species relies on stable, year-round production of high-quality juveniles, which involves the use of artificial diets. The drawbacks with use of live feed regarding workload, costs (Person Le Ruyet *et al.* 1993) and nutritional implications (Næss *et al.* 1995; Næss & Lie 1998; Hamre *et al.* 2002, 2005), suggests early transition to formulated diet. Early weaning is, however, only obtained for larvae of a few marine fish species (Cahu *et al.* 1998, 1999, 2003, 2004; Baskerville-Bridges & Kling 2000a,b; Hoehne-Reitan *et al.* 2001). This review aims to discuss some recent findings and future perspectives in protein nutrition of early stages of the Atlantic halibut. Many of the topics discussed apply for other marine fish species too, and whenever literature is scarce on halibut, findings in other fish species are included.

Fish larvae in general have a great potential of growth in terms of specific growth rate (SGR; Houde 1989; Kamler 1992; Conceição *et al*. 1997). Even in cold water species such as the Atlantic cod, growth rates close to 30% per day in early larvae have been reported (Otterlei *et al*. 1999). The highest SGR values reported on the Atlantic halibut larvae is 17.7% (Næss & Lie 1998); however, this is not necessarily the biological maximum for this species but merely the result of the best feeding and culture conditions provided in experiments so far. Maximum growth potential for the Atlantic halibut larvae can first be assessed when optimized feed and culture conditions are established.

Larval growth is primarily deposition of proteins (Houlihan *et al*. 1995). Ten of the twenty amino acids (AA) that serve as substrates in the protein synthesis are indispensable to fish (Wilson 2002) and hence have to be supplied through the diet. The suitability of dietary proteins as AA sources is thus an important issue in relation to larval nutrient requirements. In addition, AA seems to be a preferred energy substrate in larvae of the Atlantic halibut (Rønnestad *et al*. 1993; Rønnestad & Naas 1993) as it is for Atlantic cod larvae (Finn *et al*. 2002). This point further increases the quantitative importance of the dietary supply of AA to larvae.

The assumedly high dietary protein requirement in Atlantic halibut larvae is a paradox in light of some morphological and physiological features that indicate low digestive capacity for proteins in larvae relative to older stages. Alternative mechanisms for protein digestion in larvae have been suggested. Such special larval features and their possible implications for the Atlantic halibut larvae nutritional requirements are to be addressed in this paper.

Development of the gastrointestinal tract in halibut larvae

The development of the gastrointestinal tract of Atlantic halibut resembles the general development in other marine fish species that do not have a functional stomach at first feeding. A special feature for Atlantic halibut compared with (most) other species is their long yolk sac stage before they are ready for first feeding (35–45 days at 6–7 °C; Næss *et al*. 1995; Harboe & Mangor-Jensen 1998; Næss & Lie 1998). Along with most other marine fish larvae, they have so far been dependent of live feed for a comparative long period before successful weaning is performed (12 °C; Hamre *et al*. 2001; Kvåle *et al*. 2002). During the period from hatching until after metamorphosis the halibut gastrointestinal tract goes through large changes. At hatching the gastrointestinal tract is straight (Pittman *et al*. 1990) and separated in three regions, foregut, midgut and hindgut, by constrictions (Kjørsvik & Reiersen 1992). The lumen is open 2 days after hatching (dah; 6 °C; Kjørsvik & Reiersen 1992), and between approximately 14 and 28 dah the gut forms a loop from the posterior end of the foregut (6 °C; Pittman *et al*. 1990; Kjørsvik & Reiersen 1992). During the yolk sac stage the epithelium in the gut becomes thicker and more mature, especially in the mid- and hindgut (Kjørsvik & Reiersen 1992). At approximately 3 dah, the liver, gall bladder, pancreatic tissue, kidney and urinary bladder seem to be functional (6 °C; Pittman *et al*. 1990; Kjørsvik & Reiersen 1992). Histomorphologically the gastrointestinal tract appears ready for first feeding from approximately 24 to 26 dah (150 day-degrees) at about 50% yolk absorption (Kjørsvik & Reiersen 1992). However, studies of enzymatic activity revealed a peak in activity of a number of digestive enzymes at a later time, indicating that proper time for first exogenous feeding is between 37 and 44 dah (230–276 day-degrees; Gawlicka *et al*. 2000), which is in agreement with observed feeding behaviour of halibut larvae (260–290 day-degrees; Harboe & Mangor-Jensen 1998).

The digestive capacity is not well described in halibut larvae, but some studies exist (Table 1). Higher specific activities of the pancreatic enzymes trypsin, amylase and non-specific lipase and the intestinal enzyme alkaline phosphatase are indicated in metamorphic larvae (34 days post first-feeding (dpff); 11–12 °C) compared with first feeding larvae (Gawlicka *et al*. 2000). However, these analyses were conducted on different larval fractions, whole larvae at first feeding and the dissected digestive system at 34 dpff. Rojas-García & Rønnestad (2002) found that tryptic activity increased from 13 to 26 dpff [ambient temperature, 6.2 °C (0 dpff)–11.8 °C (33 dpff)]. An abrupt increase in the specific activities of the brush border membrane-bound leucine aminopeptidase N and alkaline phosphatase was found from about 30 to 50 dpff (20–114 mg wet weight; 15–22 mm SL; Kvåle, *et al*., accepted), just before climax of metamorphosis (Sæle *et al*. 2004). The specific activity of leucine-alanine peptidase increased from first feeding to 28 dpff (11 mg wet weight, 14 mm SL), and thereafter remained unchanged until past metamorphosis (Kvåle, *et al*., accepted). The maturational increase in activities of brush border membrane enzymes is in accordance with findings in other marine fish species, while the activity of leucine-alanine peptidase was expected to decrease as pinocytosis diminishes during metamorphosis (Ribeiro *et al*. 1999; Zambonino Infante & Cahu 2001).

Cholecystokinin (CCK) is a central stimulator for release of bile, secretion of pancreatic enzymes, intestinal peristalsis

Table 1 Ontogenetic development of the specific activities of some digestive enzymes in Atlantic halibut larvae and the number of cholecystokinin producing cells in their intestines

Age[1]	Day-degrees	Weight[2] (mg)	Trypsin	Amylase	NS lipase	AP	LAP	Leu-Ala	CCK-IR cells	References and units[3]
40/7		0.94 ± 0.10	0.17 ± 0.07							1
46/13			0.18 ± 0.05							
52/19			0.28 ± 0.05							
59/26		2.41 ± 0.72	1.22 ± 0.39							
37/0	230	5.8 ± 0.8	16.7 ± 0.7	27.0 ± 6.2	13.4 ± 0.5	38.5 ± 1.1				2
44/0	276	5.3 ± 0.5	11.6 ± 0.5	32.9 ± 6.1	22.6 ± 0.7	61.2 ± 3.4				
78/34	660	56.0 ± 9.0	98.6 ± 18.6	1634 ± 401	37.3 ± 2.8	111.9 ± 7.2				
43/1	253	5.5 ± 0.6				192 ± 112	190 ± 93	55 ± 15		3
71/29	~ 574	11.2 ± 3.5				217 ± 71	204 ± 31	342 ± 23		
94/52	~ 850	114 ± 24				1277 ± 225	984 ± 123	399 ± 28		
120/78 [4]	~1162	237 ± 68				925 ± 235	404 ± 102	420 ± 61		
120/78[4]	~1162	442 ± 75				2699 ± 344	931 ± 93	428 ± 46		
33/0	238								0	4
39/6	282								0	
45/12	332								–[5]	
52/19	399								23	
66/33	559								146	

NS lipase, non-specific lipase; AP, alkaline phosphatase; LAP, leucine aminopeptidase N; Leu-Ala, leucine-alanine peptidase; CCK-IR cells, cholecystokinin immunoreactive cells.

[1] Age is noted as days post hatch/days post first feeding.

[2] Weight is dry body weight in reference 1 and wet body weight in references 2 and 3.

[3] 1: Rojas-García & Rønnestad (2002); ambient temperature, 6.2 °C (0 dpff)–11.8 (33 dpff); unit used and tissue analysed are U (mg dry weight)$^{-1}$ and whole larvae, respectively. 2: Gawlicka *et al.* (2000); 11–12 °C; unit used is mU (mg protein)$^{-1}$, and tissues analysed are whole larvae at first feeding and digestive tissues including liver, pancreas and partly digested *Artemia* at 34 dpff (enzymes from *Artemia* were estimated to represent 2.6% of the lipase activity, 8.4–9.6 of trypsin and AP activities and 52.3% of amylase activity). 3: Kvåle, *et al.*, accepted; 12 °C; unit used is mU (mg protein)$^{-1}$, and tissues analysed are intestinal segments (Leu-Ala) and brush border membrane extracts of intestinal segments (AP and LAP), respectively. 4: Kamisaka *et al.* (2001); ambient temperature, 6.2 °C (0 dpff)–11.8 (33 dpff); unit is total number of CCK-IR cells in the midgut of each larva.

[4] The two samples at 78 dpff are separated on basis of larval size.

[5] 3–5 CCK-IR cells were present in two of six larvae.

and satiety (Louie 1994), and thus an important modulator of digestion and feed ingestion. This gut hormone is not detected in first feeding halibut (33–39 dah; 238–282 day-degrees; Table 1). CCK-immunoreactive (CCK-IR) cells appear around 45–52 dah, or 10–20 dpff (332–399 day-degrees; Kamisaka *et al.* 2001). This indicates that digestion and feed ingestion is not well regulated in first feeding larvae, or that regulation occurs by other mediators than CCK. Studies on other gastrointestinal hormones that take part in the regulation of the digestion are scarce for marine fish larvae (Krogdahl 2001).

The metamorphic climax in Atlantic halibut is considered to take place when the larvae standard body length (SL) is 22–24 mm (12 °C; Sæle *et al.* 2004). This corresponds to approximately 120–170 mg wet weight (Kvåle, *et al.*, accepted). During the metamorphic climax the foregut starts to expand and differentiate into a stomach with gastric glands (35–40 to 50–55 dpff at 12 °C; Luizi *et al.* 1999). Fully developed gastric digestion is apparently reached at approximately 80 dpff (Stoss *et al.* 2004). However, although gastric secretion is evident, high individual variation in the ability to acidify a buffered proteinic solution was found in juveniles after metamorphosis, using a tube feeding technique (S.K. Tonheim, unpublished data).

In the anterior end of the midgut, approximate to the pyloric sphincter, pylorus caeca are formed during the course of metamorphosis. Different numbers of caeca (2–5) are reported (Luizi *et al.* 1999; Rønnestad *et al.* 2000). During metamorphosis, the intestinal epithelium also becomes heavily folded and the wave-like structure of the mucosa at earlier stages changes into conical folds (12 °C; Luizi *et al.* 1999). All these changes tend to increase the absorptive surface of the gut.

Increased ability of absorption is also evidenced by observation of large supranuclear vacuoles (SNV) in the epithelium in both mid- and hindgut. Of particular interest with regard to protein digestion is protein-containing vacuoles in the hindgut (see below). Such vacuoles were present in the hindgut epithelium of larvae, but tended to disappear during metamorphosis (Luizi *et al.* 1999).

At the end of metamorphosis, Atlantic halibut appears to have a mature gastrointestinal tract with high digestive and absorptive capabilities (Luizi *et al*. 1999).

Dietary protein digestion in adult and larval fish

Protein digestion in adult fish is similar to vertebrates in general (Krogdahl 2001). Ingested feed enters the stomach and is mixed with acidic secretion form the stomach wall. The lowered pH contributes to protein denaturation and tissue decomposition. This increases the effect of pepsin, which is also secreted from the stomach wall and performs maximum proteolytic activity in the low pH range (Gildberg *et al*. 1990). The partly digested stomach contents enter the intestine through the pyloric sphincter at controlled rates, regulated by neural and hormonal feedback mechanisms. In the intestine, the digesta are neutralized by alkaline secretion from the pancreas and the gall bladder and the protein is being further digested by the action of the various proteases and peptidases that are secreted from the pancreas or associated with the intestinal mucosa (Krogdahl 2001). In fish larvae, on the other hand, no stomach is developed and ingested feed enters the intestine at rates controlled by ingestion only, with no previous digestion. The importance of the gastric digestion for efficient protein digestion was illustrated by *in vitro* experiments by Jany (1976). The absence of gastric digestion (Luizi *et al*. 1999) and the relative low specific protease activity in early feeding larvae (Gawlicka *et al*. 2000; Rojas-García & Rønnestad 2002; Kvåle, *et al*., accepted) may lead to the suggestion that other proteolytic mechanisms, such as pinocytosis followed by intracellular digestion, is quantitatively important for first feeding Atlantic halibut, as also suggested for other stomach-less fish larvae (Govoni *et al*. 1986). Based on knowledge on larvae from other species, protein-containing SNV found in Atlantic halibut larvae hindgut enterocytes can be associated with pinocytosis and intracellular protein digestion (Luizi *et al*. 1999). Watanabe (1981, 1982) and Nakamura *et al*. (2004) proved by the use of marker proteins (horseradish peroxidase and rabbit IgG, respectively) that orally administered proteins were absorbed by pinocytosis and present in SNV in intact form in all out of six studied teleost species. Kishida *et al*. (1998) presented evidence that also nutritionally significant proteins (*Artemia* lipovitellin) were engulfed in antigenically intact form by the teleost larvae hindgut enterocytes. SNV usually disappear around the time of metamorphosis, when the gastric secretion appears in larvae that develop a stomach (Govoni *et al*. 1986; Luizi *et al*. 1999), but continue to exist in stomach-less juvenile fish (Gauthier & Landis 1972). This further suggests that pinocytosis and intracellular protein digestion is a special adaptation to compensate for the absence of a gastric stomach in larvae, while extracellular digestion becomes more important as the intestine, pancreatic secretion and gastric stomach mature towards metamorphosis (Govoni *et al*. 1986; Rust 1995, 2002). In this respect, Atlantic halibut larvae seem to share the typical larval morphology and physiology (Luizi *et al*. 1999).

Pinocytosis and intracellular digestion in fish larvae are well described (Watanabe 1984; reviewed by Govoni *et al*. 1986 and Sire & Vernier 1992) and resemble the processes in mammals (Henning 1987; Sire & Vernier 1992). In pinocytosis, small vesicles which include intact proteins are formed by invaginations of the cell membrane. These vesicles move towards the nucleus region and fuse with each other to form SNV. The SNV associate with primary lysosomes which lower the pH in the SNV and supply them with proteolytic enzymes that efficiently digest the engulfed proteins under approximately similar denaturing condition (low pH) as in the stomach (Watanabe 1984; Sire & Vernier 1992). The intracellular digestion thus has the potential to digest a broad range of different proteins, including proteins that are not efficiently digested under the proteolytic conditions that take place in the intestinal lumen (neutral pH). However, one limitation that probably is important to the efficiency of protein uptake by pinocytosis is particle size. Different dietary proteins will exist in the gut at different structurally organized levels. Free amino acid (FAA) and small peptides will exist as free molecules which are being efficiently absorbed by specific transport mechanisms in the midgut (Sire & Vernier 1992; Bakke McKellep *et al*. 2000). Soluble intact proteins and polypeptides, which also exist as single molecules, seem to be taken up by the unspecific mechanisms of pinocytosis. Insoluble proteins, on the other hand, will more likely exist as larger hydrophobic aggregates or complex particles and uptake efficiency of this fraction by pinocytosis remains unknown. Despite the significant amount of SNV in hindgut mucosa of marine fish larvae, the quantitative contribution of intracellular digestion to protein nutrition is yet to be determined.

The dependence of most marine fish larvae on live prey at first feeding suggests that live prey may assist in larval digestion. Contribution of proteolytic enzymes from live feed is estimated to amount to only a minor part (1–8%; Munilla-Moran & Stark 1989; Kurokawa *et al*. 1998; Gawlicka *et al*. 2000), but live prey may enhance larval digestion and diet utilization in other ways, possibly by stimulating larval

enzyme secretion (Munilla-Moran & Stark 1989; Beccaria *et al*. 1991) and increasing larval diet ingestion (Kolkovski *et al*. 1997).

Dietary protein supply to first feeding larvae

Copepods are dominating wild populations of marine zooplankton and are the natural first feed for the pelagic larvae of Atlantic halibut and other marine fish species. Planktonic copepods can thus serve as reference for Atlantic halibut larvae nutritional requirements (Fyhn *et al*. 1993; Holmefjord *et al*. 1993; Næss & Lie 1998; Evjemo *et al*. 2003; Helland *et al*. 2003).

Marine copepods are osmoconformers and typically contain large fractions of FAA (Fyhn *et al*. 1993; Table 2) which are effective osmolytes (Yancey *et al*. 1982). Based on this, it has been suggested that efficient protein digestion is of less importance in larvae on marine plankton diets because the dietary protein requirement can be sustained by extraction and absorption of FAA (Fyhn 1989; Rønnestad *et al*. 2003). FAA absorption is rapid and efficient in first feeding Atlantic halibut larvae (Applebaum & Rønnestad 2004), as in other fish larvae studied (Rønnestad *et al*. 2000, 2001; Conceição *et al*. 2002). Variable, but generally lower levels of FAA when compared with natural zooplankton, are reported in *Artemia* and rotifers, which are commonly used as live feed in intensive culture of fish larvae (Table 2). Differences in FAA have been discussed as a potential problem with regard to meeting larval dietary requirement for AA in intensive production (Helland *et al*. 2003; Rønnestad *et al*. 2003). Although some comparative feeding experiments have revealed poorer growth and survival in Atlantic halibut larvae fed with *Artemia* when compared with larvae fed with copepods (Holmefjord *et al*. 1993; Harboe *et al*. 1998; Næss & Lie 1998), no such differences was found by others (Næss *et al*. 1995; Shields *et al*. 1999).

Instead of focusing on live feed contents of FAA alone, Carvalho *et al*. (2003) focused on live feed contents of total soluble nitrogen, which in addition to FAA also includes water-soluble intact proteins and peptides. Live larval feeds are generally high in water-soluble nitrogen compounds and quantitative differences between live feed species are less pronounced than differences in FAA (Table 2). The large variation in results between the different studies referred in the table is likely caused by the different analytical methods used. Carvalho *et al*. (2004) showed that increasing the ratio of soluble protein in formulated feed to first feeding carp larvae improved larval growth and survival, indicating the potential impact of soluble protein on larval nutrition. Increased protein utilization by increasing the soluble protein fraction may have contributed positively by either soluble proteins being more efficiently digested by pancreatic enzymes in the intestinal lumen or by soluble protein being more efficiently taken up by pinocytosis, or more likely, by a combination of both these factors.

Dietary protein utilization efficiency in larvae and juveniles

Rust (1995) studied AA assimilation in fish larvae from different species by tube feeding small amounts of radiolabelled FAA, peptides or intact proteins. For species of stomach-less

Table 2 Some values for dry matter (DM), protein, free amino acids (FAA) and soluble protein in copepods, *Artemia* and rotifers as found in recent literature

	Copepods	*Artemia*	Rotifers	Reference
Dry matter (g kg^{-1})	110 ± 20	110 ± 10	110–160	Lie *et al.* (1997), Hamre *et al.* (2002)
Protein (g kg^{-1} DM)	720 ± 80[1]	620 ± 40[1]	570[1]	Lie *et al.* (1997), Øie *et al.* (1997),
	310–540[2]	310 ± 80[2]	340–360[3]	Hamre *et al.* (2002), Helland *et al.* (2003),
	380–570[2]	280–320[2]	370–410[4]	van der Meeren (2003), Srivastava *et al.* (2006)
FAA (g kg^{-1} protein)	230 ± 60 –	36–63[5]	21[5]	Øie *et al.* (1997), Hamre *et al.* (2002),
	260 ± 60[6]	140 ± 20	33–42	Carvalho *et al.* (2003), Helland *et al.* (2003),
	250 ± 24	180 ± 33	53–66[5]	Srivastava *et al.* (2006)
		65 ± 8		
Soluble protein (g kg^{-1} protein)	540 ± 22	540	470–610	Carvalho *et al.* (2003), S.K. Tonheim (unpublished data),
		670 ± 28	440–520	Srivastava *et al.* (2006)

[1] Protein analysed as N × 6.25.
[2] Analysed by the method of Lowry.
[3] Protein determined as average of N × 4.1 and total water free amino acids.
[4] Analysed as total amino acids.
[5] Free amino acids as per cent of total amino acids.
[6] Calculated from molar to weight basis by using weighted average amino acid molecular weight of 119.4 g mol^{-1} (Creighton 1993).

larvae, he found markedly increasing assimilation efficiency as the complexity of the AA source decreased (FAA > peptide > protein). Tonheim *et al.* (2004, 2005) performed series of tube feeding experiments with Atlantic halibut using an improved tube feeding setup (Rønnestad *et al.* 2001), measuring total absorption of a model protein in larvae and juveniles and not only AA assimilation into body tissues. The results showed that while juveniles after metamorphosis and development of a functional gastric stomach absorbed the administered model protein to 59% in average, larvae absorbed only 25% of the model protein when administered at identical amounts relative to body size. Further, it was shown that pre-digestion of the model protein with proteases prior to tube feeding, improved the mean absorption efficiency in larvae to a level similar to the absorption efficiency in juveniles (64% versus 59%, Tonheim *et al.* 2004, 2005). This is indicative of that the larval ability for efficient utilization of dietary protein is limited by low proteolytic activity and not by the means of absorptive capacity. Further, the pinocytotic activity and intracellular protein digestion occurring in the larval Atlantic halibut hindgut appears unable to fully compensate for the low extracellular proteolytic capacity. The model protein in the experiments of Tonheim *et al.* (2004, 2005; concentrated salmon serum proteins) was constituted solely of highly soluble proteins which can be expected to be efficiently taken up by pinocytosis.

Tonheim *et al.* (2005) also showed that pre-digested protein was absorbed with the same efficiency in larvae (64%) irrespective of administered amount within the tested range, while intact protein was absorbed with decreasing efficiency as the administered amount increased. However, although mean absorption efficiency for intact protein was low in larvae administered the higher doses, some individuals performed well. Early defecation was accompanied by low absorption efficiency in individuals with low digestive performance.

Increasing the dose of protein fed may have increased the intestinal flow and is a possible reason for the decreasing absorption efficiency in larvae tube fed with intact model protein. Increased intestinal flow will potentially reduce the protein to protease ratio and the time for proteolysis to occur in the midgut. It will also potentially reduce the efficiency of protein taken up by pinocytosis. Individual larvae which managed to keep the model protein in the gut for a longer time would thus increase the absorption efficiency relative to those with shorter passage time. High intestinal flow rate should, however, be considered to be less deleterious on the absorption efficiency in larvae fed with pre-hydrolysed model protein, as hydrolysed model protein was absorbed 2.2–3 times faster than the intact model protein (Tonheim *et al.* 2005).

Øie *et al.* (1997) performed a study on nitrogen and carbon retention in larvae from another flat-fish species, the turbot (*Scophthalmus maximus*). Larvae were fed differently enriched rotifers in tanks with or without supply of algae (green water). Supplementation of algae induced increased ingestion rate leading to higher growth rates. Substantial poorer protein retention was found in the more rapidly growing larvae. Similar relation between the rates of feed ingestion, growth and protein retention has also been reported in other larval species (reviewed by Govoni *et al.* 1986). Gut passage time in larvae is generally short and is positively correlated with rate of ingestion (Govoni *et al.* 1986). Regulation mechanisms for the intestinal flow in the Atlantic halibut larvae is uncertain as they lack both a stomach with feed storing capacity and hormones such as the CCK (Kamisaka *et al.* 2001) which play key roles in feed ingestion, gastric emptying and intestinal flow in later stages. Generally, poor digestion is reported in Atlantic halibut larvae (Luizi *et al.* 1999; Rust 2002).

Lowered nitrogen retention at high feed ingestion rates in larvae may not only be an effect of metabolic factors, but also an effect of reduced protein absorption efficiency due to increased intestinal flow rate, as indicated by the tube feeding experiments (Tonheim *et al.* 2005). Intracellular protein digestion occurs in the hindgut which constitute only about one-fourth to one-fifth of the total intestinal length in Atlantic halibut larvae (Kamisaka *et al.* 2001). High intestinal flow rates may thus exert a stronger negative impact on the efficiency of protein taken up by pinocytosis and even stronger negative impact on insoluble proteins that have to be digested to some extent before they can be efficiently taken up by pinocytosis. Based on this reasoning, there will be a hierarchy with regard to absorption efficiency and absorptive rates in larvae. FAA, di- and tripeptides are fast and efficiently absorbed in the midgut and will be on top, followed by soluble proteins, with insoluble proteins coming last. Increased intestinal flow will probably strengthen this hierarchy. At high feed availability and ingestion rates, higher total AA absorption can be achieved despite increased intestinal flow rates and reduced protein absorption efficiency. At lower feed availability, dietary protein utilization efficiency can be increased by reduced intestinal flow rate and hence prolonged time for both extracellular proteolysis and pinocytosis to occur.

From an ecological point of view, the marine fish larvae seem to be well adapted to the complex composition and graded availability of dietary protein in natural diets with

regard to optimizing growth. This indicates that in order to maximize growth in larvae in intensive aquaculture, the fraction of rapidly absorbed and metabolized protein such as FAA, pre-hydrolysed protein and soluble intact protein sources should be increased in the diets. Insoluble protein, on the other hand, seems less efficiently utilized (Carvalho *et al.* 2004).

Effects of hydrolysed protein in weaning diets

Based on the above, one should think that pre-hydrolysed protein would be an advantageous supplement in compound diets for halibut larvae. By enzymatic hydrolysis the proteins are cut into smaller fragments and such treatment will both reduce the average molecular size and increase the solubility of dietary protein sources. Thus, the smallest protein fragments (di- and tripeptides) are available for direct uptake and the longer ones are more available for digestive enzymes and possibly for uptake by pinocytosis. 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 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), while in an experiment with cod (*Gadus morhua*), supplementation of 400 g kg^{-1} protein, pepsin hydrolysed protein improved survival rates, compared with lower levels of supplementation (A. Kvåle, unpublished data). Inclusion levels above 500 g kg^{-1} of the protein seem to be detrimental to several fish species (*Sparus aurata*, Kolkovski & Tandler 2000; *Cyprinus carpio*, Carvalho *et al.* 1997, 2004; *Dicentrarchus labrax*, Cahu *et al.* 1999), although not to all (*Solea solea*, Day *et al.* 1997; *Scophthalmus maximus*, Oliva-Teles *et al.* 1999).

Two weaning experiments have been conducted to investigate effects of protein hydrolysate inclusion level and degree of hydrolysation in Atlantic halibut larvae (Kvåle *et al.* 2002; A. Kvåle, unpublished data). Enzymatic treatment of the dietary protein with only pepsin led to better survival compared with further treatment with both trypsin and chymotrypsin (Kvåle *et al.* 2002). A pepsin and trypsin-treated hydrolysate gave an intermediate result. Furthermore, 100 g kg^{-1} inclusion of pepsin hydrolysed protein supported the highest rate of survival, although not significantly higher than the non- or 100 g kg^{-1} pepsin and trypsin hydrolysed protein. The fact that halibut fed the least solubilized hydrolysate and the lowest hydrolysate inclusion level showed the best performance, leads to the conclusion that early weaned Atlantic halibut (120 ± 40 mg wet weight) should not be supplemented with high levels of protein hydrolysate (Kvåle *et al.* 2002). In a recent experiment where graded levels of pepsin hydrolysed protein were fed to slightly larger halibut (222 ± 69 mg wet weight), survival was highest in the group fed the non-hydrolysed protein and decreased gradually with higher inclusion levels up to 450 g kg^{-1} protein (A. Kvåle, unpublished data). The different results in these two experiments may be related to differences in fish size, as the fish in the latter experiment had obtained a more juvenile character and thus probably a more mature gastrointestinal tract, which was able to digest the dietary intact protein more efficiently.

An optimal level of dietary hydrolysed protein has been found to promote maturation of the gastrointestinal tract in seabass larvae when intestinal maturation was estimated by comparing indicators for adult versus larval mode of digestion (Cahu *et al.* 1999). Specific activities of the brush border membrane enzymes leucine aminopeptidase N and alkaline phosphatase can be used as indicators for maturation of extracellular digestive mechanisms (adult mode), while the specific activity of the cytosolic enzyme leucine-alanine peptidase may serve as an indicator for intracellular digestive mechanisms (larval mode; Zambonino Infante & Cahu 2001). In Atlantic halibut, the specific activity of leucine-alanine peptidase did not respond to different levels of dietary hydrolysed protein, while the specific activities of leucine aminopeptidase N and alkaline phosphatase were significantly higher when weaning was conducted by a non-hydrolysed diet compared with diets containing 300–450 g protein hydrolysate kg^{-1} protein (A. Kvåle, unpublished data). This is in accordance with the results on survival (see above). Altogether, the two experiments indicate that the supplementation of hydrolysed protein in weaning diets to Atlantic halibut should be kept low and at maximum 100 g kg^{-1} of the dietary protein.

The apparent disadvantage of including hydrolysed protein in weaning diets to halibut, compared with other fish species, may be explained by their slow feeding behaviour. The microparticulate diets used at weaning, rapidly leach water-soluble nutrients, including soluble protein (Kvåle *et al.* 2006). Thus, the level of dietary hydrolysed protein at the time of ingestion may have been reduced comparatively to the inclusion level. As dietary protein requirement for juvenile Atlantic halibut is reported to be very high (580 g kg^{-1} dry diet, Hamre *et al.* 2003), this could lead to sub-optimal protein levels at the time of ingestion when hydrolysates are supplemented at high levels.

Formulated diets for fish larvae

Few studies are dealing with formulated diets for halibut larvae specifically. This and the following chapters will therefore review information on fish larvae in general. There is a demand for a formulated diet that can be fed to fish larvae from first feeding, which does not lead to depressed growth and survival in comparison with live feeds. Commercially, the use of a formulated diet is a matter of cost and benefit considerations. Formulated diets are, however, also demanded for scientific purposes, to increase the possibilities to manipulate and control the dietary nutritional composition in feeding trials with fish larvae. Much effort has been put into development of suitable feed formulation concepts, and this has resulted in increased success during the last years in early weaning of fish larvae (Baskerville-Bridges & Kling 2000a,b; Hoehne-Reitan *et al*. 2001; Cahu *et al*. 1998, 2003, 2004). Especially seabass was fed solely on a formulated diet with SGR and survival rates of 7–11% and 35–70, respectively (Cahu *et al*. 1998, 2003, 2004). However, studies focusing on the properties of existing formulated diets, put doubt on their ability to deliver controllable amounts of both macro- and micronutrients (see later; Blair *et al*. 2003; Langdon 2003; Hamre 2006).

Several different formulation concepts have been investigated as potential for fish larval diets. These can mainly be put into two categories: microbound particles and microencapsulated particles. Different types of microbound diets are widely used for marine fish larvae (see review by Langdon 2003). These have in common that the particles consist of a uniform matrix throughout each particle without a distinct surrounding wall (Langdon 2003; Önal & Langdon 2005). The matrix is bound by either carbohydrate or protein binders (gelatine, zein, alginate, carboxymethyl-cellulose, soluble fish protein, chitin, carrageenan, etc.; Hamre *et al*. 2001, López-Alvarado *et al*. 1994, Person Le Ruyet *et al*. 1993; Baskerville-Bridges & Kling 2000a; Guthrie *et al*. 2000; Önal & Langdon 2000, 2005; García-Ortega *et al*. 2001; Yúfera *et al*. 2002; Hogoy 2005). These binders create a non- or low-soluble matrix in the feed particle which holds the nutrients within the particle. The different binders need different treatments (heating, cooling, drying or chemical cross-linking) to produce the matrix. The particles can be produced by using a spray nozzle (Önal & Langdon 2005), agglomerating technology (Guthrie *et al*. 2000) or by crushing and sieving particles to wanted size (Guthrie *et al*. 2000; Hamre *et al*. 2001). The binder used affects the properties of the particles (Person Le Ruyet *et al*. 1993; López-Alvarado *et al*. 1994; Guthrie *et al*. 2000). Weak binders might give particles that are highly digestible, but that easily disintegrate in the water, and thus become less available for ingestion by the fish larvae. Binders that are favourable with regard to minimizing particle disintegration and nutrient leaching may be unfavourable with regard to digestibility in the fish larvae (Person Le Ruyet *et al*. 1993; Guthrie *et al*. 2000). Different fish species may have different success with different binders (Partridge & Southgate 1999). Halibut larvae have a low tolerance for carbohydrates in the diet (Hjertnes *et al*. 1993; Hamre *et al*. 2003), indicating that formulated diets that need a high amount of digestible carbohydrates as a binding material should be considered carefully. Generally, microbound feeds can be produced on a large scale at relatively low production costs.

While microbound particles have a uniform matrix through the whole particles, microencapsulated particles have an insoluble membrane surrounding a core matrix. The concept of microencapsulation is often used with the aim to reduce or control leaching of soluble components from the core matrix, a strategy that has been proven effective, dependent on the encapsulation technique that is used (López-Alvarado *et al*. 1994). Chitin/alginate, lipid and protein encapsulated particles can be produced by spraying particles into a bath or through a vapour that contains a cross-linking agent or by using emulsifying technology (López-Alvarado *et al*. 1994; Ozkizilcik & Chu 1996; Yúfera *et al*. 2000; Önal & Langdon 2000). A well studied diet is a protein encapsulated diet that is made with an emulsifying technology (Jones *et al*. 1974; Hayworth 1983, Yúfera *et al*. 2000; Jones 1980; Langdon 1989; Önal & Langdon 2000). An aqueous dietary solution is emulsified with an organic solvent. The small emulsified aqueous droplets containing the dietary nutrients and the wall forming protein are exposed to a chemical cross-linking agent. The chemical cross-linker polymerizes the water-soluble proteins at the interface between water and the organic solvent phase, resulting in a stable capsule of cross-linked protein that envelopes each particle. Although there have been promising results with the use of the protein encapsulated diet feeding marine fish larvae, most of the feeding trials are preliminary and few feeding studies have investigated growth and survival over a longer time period. Seabass (*Lates calcarifer*) larvae fed the protein encapsulated diet from first feeding were all dead at day 10 and larvae fed microcapsules together with rotifers for 5 days and then microcapsules alone for 1 week had a mean survival rate of 2.4% (Walford *et al*. 1991). Fernández-Díaz & Yúfera (1995) discovered that by dispersing the protein cross-linked particles in a gelatine solution, the digestibility of the particles increased. The use of this diet gave good

growth and survival for seabream (*Sparus aurata* L.) larvae after only feeding rotifers for the first 4 days (Yúfera *et al.* 2000). The technique is well suited for small-scale laboratory preparation and no expensive equipment is necessary, but there are high production costs due to the use of organic solvents and the cross-linking agent.

Production of capsules with chitin, alginate or both by procedures that are relatively easy and cheap, is well documented in the medical literature. A well studied particle consists of a microbound alginate bead coated with a thin membrane of chitin. The particles are produced by spraying an aqueous dietary solution containing alginate into a solution of chitosan and calcium chloride (Gaserod *et al.* 1998, 1999; Vandenberg & De La Noue 2001; Vandenberg *et al.* 2001). By varying the exact procedures, technical properties of the particles, including leaching, can to some degree be manipulated (Gaserod *et al.* 1998, 1999; Vandenberg & De La Noue 2001; Vandenberg *et al.* 2001). There are few published studies that address the use of chitin capsules as a formulated diet for marine fish larvae (Hogoy 2005), and it is not established to what degree these larvae can digest the chitin membrane. Production processes and leaching properties of chitin/alginate capsules are well described for medical purposes (Chen *et al.* 1996, 2002; Huguet & Dellacherie 1996; Huguet *et al.* 1996; Bartkowiak & Hunkeler 1999; Shu *et al.* 2001; Vandenberg & De La Noue 2001; Vandenberg *et al.* 2001; Shu & Zhu 2002), and should be further investigated for use in larval rearing.

Lipid-based particles cannot be used as a complete diet due to a high lipid to core material ratio and clumping during feeding (Önal & Langdon 2005). Nevertheless, they have interesting properties for larval rearing purposes. In lipid-based particles, water-soluble nutrients are encapsulated within a lipid matrix, and the technologies used are based on the lipid's ability to solidify at low temperature, either in a cooled water bath for lipid walled capsules (LWC; López-Alvarado *et al.* 1994) or in cooled air for lipid spray beads (LSB; Buchal & Langdon 1998). LSB can deliver a higher payload than LWC (Buchal & Langdon 1998). Studies have shown that up to 210 g kg^{-1} glycine can be incorporated within an LSB (Önal & Langdon 2004a), while only a 60 g kg^{-1} FAA payload has been achieved with LWC (López-Alvarado *et al.* 1994). To get a satisfactory low leaching, the amount of saturated FA with a high melting point is important (López-Alvarado *et al.* 1994; Buchal & Langdon 1998). The problem that has to be solved is to make stable capsules with lipids that are digestible and have a satisfactory nutritional value. Japanese flounder younger than 20 days did not manage to digest tripalmitin LWC (López-Alvarado *et al.* 1994). A similar type of capsule was broken down at an earlier stage when oils with a lower melting point were used, however, at the expense of significantly increased leaching of the entrapped water-soluble component (Table 3; López-Alvarado *et al.* 1994; Buchal & Langdon 1998). LSB made with menhaden stearine was broken down by 3-day-old clown fish (Önal & Langdon 2004a). Menhaden stearine has an acceptable technical quality and a higher content of n-3 fatty acids than lipids that have been used previously for production of lipid-based particles (Önal & Langdon 2004a), and could thus be an interesting candidate for further investigations.

Table 3 Leaching of free amino acids from different particle types after 2 min of hydration (López-Alvarado *et al.* 1994)

Particle type	Leaching (%)
Microbound carrageenan	85 ± 7
Microbound alginate	81 ± 2
Microbound zein	91 ± 2
Protein encapsulated	59 ± 1
Protein encapsulated and lipid coated	39 ± 2
Lipid walled (tripalmitin + triolein)	47 ± 9
Lipid walled (tripalmitin)	4 ± 2

Qualitative changes during manufacturing of larval diets

During production of formulated diets for fish larvae there can be a severe loss of nutrients and change in the nutritional quality due to heating, chemical interactions or leaching during bead formation and washing stages (Gabaudan *et al.* 1980; García-Ortega *et al.* 2000; Yúfera *et al.* 2002; Yúfera *et al.* 2003; Önal & Langdon 2005). Diets that are produced using a spray nozzle to form beads in a chemical solution, such as the chitosan/alginate diets, can have significant loss of nutrients during the stay in the chemical solution, due to leaching (Huguet & Dellacherie 1996; Vandenberg & De La Noue 2001; Vandenberg *et al.* 2001). This will also apply for diets that go through washing stages. A cross-linked protein walled diet (Yúfera *et al.* 1999) had more than 90% loss of low molecular weight water-soluble nutrients, such as FAA, due to the washing stages during production (Yúfera *et al.* 2002, 2003).

Diets produced with either heating or cross-linking of proteins will have a significant decrease in water-soluble protein due to denaturation and/or polymerization (Boye *et al.* 1997; García-Ortega *et al.* 2000), and this may oppose with larval preferences (Carvalho *et al.* 2004). A cross-linked protein walled diet (Yúfera *et al.* 1999) and a heat coagulated

diet (Hamre *et al*. 2001) had decreases in water-soluble protein from 62% to 2.4% and from 34% to 18% during production, respectively (Nordgreen *et al*. 2006). FAA and small peptides in these diets were only to a small extent affected by chemical cross-linking or heat denaturation (Nordgreen *et al*. 2006). When using fish meal in the diet, the heat sensitive proteins are already denatured and only the heat stable proteins are left soluble. Another protein source used in microdiets, sodium caseinate, is less influenced by heat treatment and can thus be used as a water-soluble protein source in diets that are exposed to heat during production. The use of chitosan and/or alginate binding is a 'mild' procedure that allows the protein within the feed particle to maintain native properties (Leonard *et al*. 2004). On the other hand, there is often high protein diffusion both during production (Wheatley *et al*. 1991; Rilling *et al*. 1997; Vandenberg & De La Noue 2001; Vandenberg *et al*. 2001) and feeding (López-Alvarado *et al*. 1994) from such feeds.

Digestibility of the raw materials and inactivation of nutrients can also be affected by the production processes of feeds (Teshima & Kanazawa 1983; García-Ortega *et al*. 2000). *In vitro* studies of the cross-linked protein walled diet, where the protein was exposed to pancreatic proteases to simulate the conditions in the larval intestine, showed that there was a decrease in the protein digestibility during production from 70.6 ± 1.4 to 54.3 ± 1.0 (A. Nordgreen, unpublished data). Loss of vitamin C due to unknown inactivation processes is found to occur in protein cross-linked particles (Yúfera *et al*. 2003).

As the nutritional value of the feed ingredients may change during production, the production method and its impact on the ingredients that are being used must be considered in the feed formulation in order to obtain the wanted nutritional values in the final diet.

Nutrient leaching from formulated larval diets

Nutrient leaching during feeding is a large problem with regard to being able to supply fish larvae with water-soluble nutrients such as FAA, peptides, water-soluble proteins, vitamins and minerals (López-Alvarado *et al*. 1994; Kvåle *et al*. 2002; Yúfera *et al*. 2002; Önal & Langdon 2004a). Furthermore, leaching appears to be a larger problem for halibut than for other marine fish larvae (Stoss *et al*. 2004). Losses of water-soluble compounds are severe in almost all described formulated diets for marine fish larvae (López-Alvarado *et al*. 1994; Hamre 2006; Kvåle *et al*. 2006) and above 90% loss of FAA in less than 2 min has been measured (Table 3; López-Alvarado *et al*. 1994). The high leaching rate from microfeeds is a result of rapid hydration and diffusion because of short diffusion distance within the small particles. In the protein encapsulated diets, there is a significant higher leaching rate of FAA from the diets produced in accordance with the method of Langdon (1989) and Ozkizilcik & Chu (1996) (50–60% in 2 min; López-Alvarado *et al*. 1994; Ozkizilcik & Chu 1996) compared with the method of Fernández-Díaz & Yúfera (1995) (8% in 5 min; Yúfera *et al*. 2002). The significant difference in leaching rate is due to large losses of FAA (90%) during production in the latter method (Yúfera *et al*. 2002) as discussed above. LWC and LSB can deliver significant amounts of FAA, small peptides and micronutrients in a controlled manner (Table 3; Önal & Langdon 2004a,b, 2005), but these diets are not capable of delivering a complete diet and the lipid with high melting points needed to efficiently prevent leaching oppose with the nutritional requirements of fish larvae (López-Alvarado *et al*. 1994; Buchal & Langdon 1998). Therefore, at the present time, there is no available formulated diet that can deliver significant amounts of low molecular weight proteinic compounds in a single digestible diet with the appropriate proportions of macro- and micronutrients.

Leaching increases with decreasing molecular size of the AA source (protein < hydrolysed protein < serine; Kvåle *et al*. 2006). However, in some diets the leaching of hydrolysed protein appears to be as high as leaching of FAA. In two microbound diets, a heat coagulated and an agglomerated diet (Hoestmark & Nygaard 1992; Hamre *et al*. 2001), up to 54% and 98% of the hydrolysed protein, respectively, was lost from the diet particles within 5 min immersion in water (Kvåle *et al*. 2006). Additionally, 80% of the water-soluble crude protein (N × 6.25) had leached within 6 min from the heat coagulated diet (A. Nordgreen, unpublished data). For a protein encapsulated diet (Yúfera *et al*. 1999) the leaching was lower, less than 6% (Kvåle *et al*. 2006), but in this diet most of the hydrolysed protein was probably lost during manufacturing due to cross-linking and/or washing of the particles (Yúfera *et al*. 2002), as discussed above. The leaching potential from the small feed particles (<1 mm) seems to be reached within the first 1–5 min after immersion in water (Fig. 1; López-Alvarado *et al*. 1994; Hamre 2006; Kvåle *et al*. 2006), but increasing the particle size to the upper range of that acceptable for the larvae, will reduce the nutrient loss (Kvåle *et al*. 2006).

The loss of crude protein was severe from all diets containing significant amounts of water-soluble protein (Fig. 1; Hamre 2006; Kvåle *et al*. 2006). Figure 1 shows that 18–42% of intact protein had diffused out of the diet particles of two experimental and two commercial microbound diets within

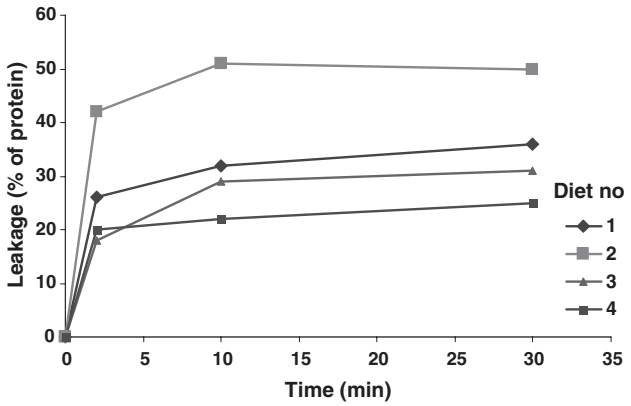

Figure 1 Leaching of crude protein (%) from formulated larval feeds. Diets 1 and 4 are experimental feeds, diet 2 and 3 are commercial feeds. All diets were micro-bound. Leaching was measured by incubating 1 g of diet in 100 mL seawater for variable time intervals. Protein leached to the water was measured as N × 6.25 after filtration and partly evaporation of the water phase (Hamre 2006).

2 min of immersion in water (Hamre 2006). To get a better understanding of leaching properties and to make it easier to compare different diets and diet recipes, leaching of protein should be measured and evaluated against the diets' content of water-soluble nitrogen and not only towards the crude protein content (N × 6.25), as a diet with a low concentration of water-soluble nitrogen may seem to have a low leaching rate, even if there is a 100% loss of the water-soluble nitrogen.

Studies on chitosan/alginate capsules show that the leaching of soluble protein is dependent on the isoelectric point of the protein, pH, interaction with the particle matrix, ion concentration of the matrix, ion concentration of the leaching medium, as well as the molecular size (Huguet *et al.* 1996; Gaserod *et al.* 1999). The leaching of neutral molecules, such as the carbohydrate dextran, is mostly correlated to the molecular size and to some extent the flexibility of the molecule (Huguet & Dellacherie 1996).

Buoyancy and floating characteristics of a particle will also influence amount of available nutrients at time of ingestion. An optimal diet for fish larvae should have a low sinking rate and a good distribution throughout the tank. Because of the high surface tension of water, some particles will not penetrate the water surface, but stay on top of the water column and thus be unavailable for the larvae and cause environmental problems in the tank (Rønnestad & Hamre 2001; Leifson *et al.* 2003). To avoid this problem, some hatcheries are pre-hydrating the feed in water before feeding to the larvae. Although this efficiently prevents floating, the negative effect of pre-hydration might be increased leaching. A particle's sinking rate is dependent on size, structure and density (Vilstrup 2001), and also chemical interactions between the water and the surface of the particle can influence particles of small size. A particle that is sinking too fast will be less catchable for the larvae, and thus excessive feeding is needed to increase feed availability. This has both cost and environmental implications.

Strategies for reducing leaching from formulated diets

Several of the mentioned formulated diets have been developed with the intention to decrease leaching, but without the wanted results. There is no diet at the present time that seems to be significantly superior to the others with regard to the compromise between digestibility and appropriate nutrient composition at one side, and leaching at the other (López-Alvarado *et al.* 1994). Microbound diets have, however, been used in weaning of larval fish species with good results (Cahu *et al.* 1999; Baskerville-Bridges & Kling 2000a,b; Hoehne-Reitan *et al.* 2001), despite the extensive leaching from such diets (López-Alvarado *et al.* 1994; Hamre 2006; Kvåle *et al.* 2006). To reduce leaching from microbound or protein encapsulated diets, coating with lipid has been tried (Table 3; López-Alvarado *et al.* 1994). Unfortunately, the lipids necessary to reduce the leaching have an unacceptable nutritional value, and the total lipid level can become too high, as with the LWC.

Even though lipid-based particles cannot deliver a complete larval diet, these particles are still interesting candidates for incorporation into microbound particles to form a complex feed. There are, however, technical problems with incorporating LSB into complex particles due to leaching from the LSB into the surrounding feed solution during the production process, or the LSB can melt due to heating during production (Önal & Langdon 2005). A complex diet with promising properties concerning above-mentioned problems has been developed (Önal & Langdon 2005). The high lipid to core material ratio necessary to obtain stable LSB clearly limits the potential of LSB to enrich formulated diets with large amounts of water-soluble feed ingredients such as FAA and hydrolysed proteins, but can be a useful tool in delivery of water-soluble micronutrient that are needed in small amounts.

Liposomes are lipid vesicles mainly made of phospolipids and have been proven successful carriers for water-soluble nutrients (New 1990). Unfortunately, the documented payload of water-soluble nutrients in liposomes (Touraki *et al.*

1995) is low compared with the payload obtainable with LSB (Önal & Langdon 2004a). Although liposomes have been fed directly to small fish larvae prior to first feeding (Koven *et al*. 1999), liposomes probably have a larger potential for incorporation into complex particles. The problems with liposomes are high cost of production and low particle stability.

Another approach to solve the problem of nutrient leaching is to substitute water-soluble micronutrients with fat-soluble derivatives. For a successful use of a fat-soluble derivative it is necessary that the derivative can be converted into its bioavailable and bioactive form by the larvae. Ascorbyl palmitate (AP) (Merchie *et al*. 1997) and Lipiodol^{TM} (Moren *et al*. 2006) enriched *Artemia* have successfully been used to increase the supply of their respective water-soluble derivative (ascorbic acid and iodine) to fish larvae, although it has been shown that seabass fed AP enriched dry diets had a lower assimilation and deposition compared with seabass fed a diet with supplemented ascorbyl polyphosphate (Merchie *et al*. 1996). Benfothiamine (Geyer *et al*. 2000) and riboflavin tetrabutyrate (Yagi *et al*. 1970) are derivatives that appear promising for chicken and rats and their potential for supplementing thiamine and riboflavin to fish larval diets should be investigated.

Conclusions and future perspectives

The background for this review is the lower ability of compound diets, when compared with live prey, to sustain growth and survival in Atlantic halibut larvae and other stomach-less fish larvae. A lower availability of AA sources in formulated diets is discussed as one of the main reasons of this problem. The larvae have large demands for suitable AA sources for muscle deposition due to fast growth, and for catabolic energy, whereas their capability to digest complex proteins is still not fully developed. By the absence of a stomach, larval protein digestion relies on intestinal digestion and a possibly significant contribution of intracellular digestion after pinocytoses. Thus, larvae may have different demands for protein digestibility than juvenile and adult fish. As a whole, the larval mode of processing protein is less understood than adult digestion and absorption and more knowledge is needed in the fields of larval digestion, nutrient absorption and the regulation of these processes. This knowledge is important for designing suitable formulated larval diets.

Larval digestion seems to be very well adapted to digest their natural live prey, which contains high proportions of highly available AA sources such as FAA and soluble proteins. Moreover, controlled feeding studies (using tube-feeding techniques), have shown that hydrolysed forms of the protein is more efficiently utilized than the intact form. Despite the need for highly available protein, halibut larvae seem not to benefit from dietary hydrolysed protein. This is thought to be largely due to high leaching rates of water-soluble nutrients from the small feed particles used in weaning of fish larvae. Nutrient leaching is a general problem in larval rearing, but is particularly severe for Atlantic halibut which feed slowly. Besides leaching, digestibility is another challenge in weaning diets, as feed processing, such as heating and protein cross-linking, reduce digestibility of the feed ingredients. The different types of larval diets used seem either to have high leaching during production (microencapsulated diets) or during feeding (microbound diets), or to contain high amounts of lipid of inadequate quality (LWC, LSB and coated microbound diets). Development of formulated microdiets with improved properties is highly demanded. Suggestion for reducing leaching of water-soluble micronutrients are development of complex particles where either liposomes or other lipid-based particles are embedded in microbound particles, or the use of fat-soluble derivatives of water-soluble nutrients. These suggestions need, however, to be further investigated before they can be implemented in rearing of marine fish larvae. As these suggestions likely will apply only for nutrients needed in small quantities, techniques for enhancing the retention within the feed particles of FAA, peptides and water-soluble protein are still highly requested.

Acknowledgements

This study has been supported by the Research Council of Norway, grant numbers 130195/140, 141758/120, 152931/120.

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