Weaning of Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*)

Studying effects of dietary hydrolysed protein and intestinal maturation as a marker for readiness for weaning

Audil Kvåle

Dissertation for the degree of doctor scientiarum

at the University of Bergen

December 2006

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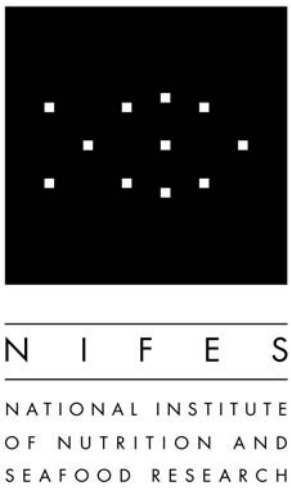

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Paper II Kvåle A, Yúfera M, Nygård E, Aursland K, Harboe T, Hamre K, 2006. Leaching properties of three different microparticulate diets and preference of the diets in cod (*Gadus morhua* L.) larvae. Aquaculture 251, 402-415.

Paper III Kvåle A, Harboe T, Espe M, Næss T, Hamre K, 2002. Effect of predigested protein on growth and survival of Atlantic halibut larvae (*Hippoglossus hippoglossus* L.). Aquacult. Res. 33, 311-321.

Paper IV Kvåle A, Harboe T, Mangor-Jensen A, Hamre K. Effects of hydrolysed protein in weaning diets for Atlantic cod (*Gadus morhua* L.) and Atlantic halibut (*Hippoglossus hippoglossus* L.). (Aquacult. Nutr., submitted).

In the following, these four papers are referred to in the text by their roman numerals.

Abbreviations

AA(s)	amino acid(s)
AP	alkaline phosphatase
bbm	brush border membrane
^{14}C-HCO_3	radiolabelled hydrogen carbonate
dd	day-degrees
dpff	days post first-feeding
dph	days post hatch
FAA(s)	free amino acid(s)
HCl	hydrochloric acid
HRP	horseradish peroxidase
LAP	leucine aminopeptidase N
LSC	liquid scintillation counting
Leu-ala	leucylalanine peptidase
LP	live prey
LWM	lipid-walled microencapsulated diet
PWM	protein-walled microencapsulated diet
SGR	specific growth rate
SL	standard length
TL	total length
wt.	weight

Abstract

The gastrointestinal tract of marine fish larvae undergoes extensive changes during the larval stage. The success in weaning increases as the juvenile stage approaches and the gastrointestinal tract attains a more adult-like form and functionality. In the present study, intestinal maturation in Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippolgossus*) larvae was compared with weaning studies to give an indication whether intestinal maturation and weaning success were correlated. Activities of the brush border membrane (bbm) enzymes alkaline phosphatase (AP) and leucine aminopeptidase N (LAP) were used as markers for for intestinal maturation. In addition, the activity of intestinal leucylalanine peptidase (Leu-ala) was used as a marker for larval mode of digestion, but this marker gave no information in decline in larval digestive features in cod and halibut. The specific activities of AP and LAP increased abruptly from 30 to 40-50 dpff in both species, corresponding to 50-120 mg wet weight in halibut and 7-9 mm standard length (SL) in cod. These increases mark the time when the intestine achieves a more adult-like functionality, and thus, the fish larvae were considered to be better prepared to digest microparticulate diets. Comparison of intestinal maturation with early weaning studies with cod and halibut indicated that intestinal maturation was important for succeeding in weaning of these two species. However, several factors are supposed to influence the weaning results, and in particular the suitability of the diet.

Marine fish larvae absorb hydrolysed protein more efficiently than intact protein. Thus, including part of the dietary protein in a hydrolysed form is thought to enhance growth and development during the larval stage when the gastrointestinal tract is not fully mature. Graded levels of pepsin hydrolysed protein (from 0 to 40-45 %) were included in the weaning diets

for cod (11 mg wet weight) and halibut (120 and 220 mg wet weight) in order to define the optimal level of hydrolysed protein. Cod obtained the highest survival rate when 40 % of the dietary protein was exchanged with pepsin hydrolysed protein. Higher inclusion levels were not tested. In contrast, the diets added none or 10 % pepsin hydrolysed protein supported the highest survival rates in halibut. Specific activities of AP and LAP showed positive correlation with the survival results, supporting that cod and halibut took benefit of high and low levels of dietary hydrolysed protein, respectively. Dietary hydrolysed protein did not affect growth significantly. The main reason for the different results obtained with cod and halibut is considered to be a combination of different feeding practise in cod and halibut, and high leaching rates of hydrolysed protein from microparticulate diets. Determination of leaching rates from three microparticulate diets showed that nutrient losses were extensive, but to some extent dependent on type of feed, feed particle size and molecular size of the dietary peptides (*i.e.* amino acids>hydrolysed protein>intact protein). Halibut ingests diet particles slowly, allowing extensive losses of nutrients from the diet prior to ingestion. Cod ingests diet particles rapidly, and thus when the particles still contain some hydrolysed protein. The protein content in the diets offered to halibut was probably reduced comparatively with hydrolysed protein inclusion level, and since juvenile halibut is considered to have a high dietary protein requirement, a sub-optimal level of protein may have been reached when the diets contained high levels of hydrolysed protein. This may explain the low optimal level of dietary hydrolysed protein to halibut.

1 Introduction

1.1 Early weaning of marine fish larvae

To establish a sustainable rearing industry of Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*), as well as other marine fish larvae that hatch at a similar early stage of development, a stable, year-round supply of high quality juveniles is of outmost importance. Today, rearing of larvae heavily relies on feeding live feed for several weeks until weaning can be conducted. The use of live feed has several drawbacks. The most widely used live feeds are brine shrimp (*Artemia* ssp) and rotifers (*Brachionus plicatilis*) which are expensive and labour-intensive (Person Le Ruyet *et al*, 1993; Lavens and Sorgeloos, 2000; Olsen, 2004; Stoss *et al*, 2004). Moreover, the nutrient composition of these prey organisms is inadequate for the marine fish larvae, and the enrichment techniques only partly compensate for this (Næss *et al*, 1995; Shields *et al*, 1999; Hamre *et al*, 2002; 2005a). Wild copepods are the natural prey for cod and halibut larvae and thus an excellent feed organism providing the nutrients necessary for growth and development. Unfortunately, as they only are harvested from coastal lagoons seasonally, the industrial potential as a year-round larval feed is limited. In addition, the quality and availability of copepods vary and they may introduce parasites and disease agents (Olsen, 2004).

To avoid or minimise these problems with the live feed organisms, early weaning is demanded. Formulated diets have long shelf life, their nutritional composition and particle size are easy to manipulate, and they need minimal preparations before feeding. With a few exceptions, formulated diets have unfortunately not been able to support growth and survival of marine fish larvae to the same extent as live feeds. Table 1 gives an overview of some early weaning results of different marine fish species. In European seabass (*Dicentrarchus labrax*),

Table 1. Early weaning* of marine fish larvae. Growth and survival of marine fish larvae fed mictoparticulate diets from different developmental stages.

Fish species	Microdiet feeding period	Larval performances		References	Approximate time for onset of stomach development
		Survival (%)	SGR % day^{-1} **		
European seabass *Dicentrarchus labrax*	6 (0 dpff)-34 dph	14-35	4-7 [w]	Cahu *et al*, 1998a	55 dph, 21 mm TL (18-19ºC, García Hernández *et al*, 2001)
	LP control, 6-20 dph	—	17 [w]		
	6 (0 dpff)-27 dph	28-57	8-11 [w]	Fontagné *et al*, 2000	
	9 (0 dpff)-40 dph	22-73	3-13 [w]	Cahu *et al*, 2003	
	6 (0 dpff)-42 dph	29-45	5-9 [w]	Cahu *et al*, 2004	
Gilthead seabream *Sparus aurata*	3 (0 dpff)-21 dph	7-25	—	Robin and Vincent, 2003	50-60 dph (18-19ºC, Elbal *et al*, 2004)
	8-25 dph	38-48	5-7 [d]	Yúfera *et al*, 2003	
	8-25 dph, co-fed all days	63-70	8-10 [d]		
	LP control	99	10 [d]		
	8-15 dph	62-70	9-13 [d]	Yúfera *et al*, 1999	
	LP control	73	14 [d]		
Senegalese sole *Solea senegalensis*	3 (0 dpff)-23 dph	1	8	Cañavate and Fernández-Díaz, 1999	27 dph (16.5-19ºC, Ribeiro *et al*, 1999a)
	3 (0 dpff)-70 dph, co-fed 40days	16-17	11		
	43-70 dph	0	0		
	LP control, 3-70 dph	52	12		
	5-37 dph	5	7	Fernández-Díaz *et al*, 2001	
	LP control, 5-28 dph	> 70	21-23		
	36-82 dph	58	2 [d]	Ribeiro *et al*, 2002	
	LP control	93	4 [d]		
	13-29 dph	65	5-6 [d]	Yúfera *et al*, 2003	
	LP control	100	13 [d]		
	8-30 dph	75-76	3-5	Fernández-Díaz *et al*, 2006	
	LP control	81	13		

Table 1 continued.

Fish species	Microdiet feeding period	Larval performances		References	Approximate time for onset of stomach development
		Survival (%)	SGR % day^{-1} **		
Barramundi	2 (0 dpff)-28 dph	0.1	1.3 mg wet wt.	Curnow *et al*, 2006	13 dph (unspecified temp., Walford and Lam, 1993)
Lates calcarifer	2 (0 dpff)-28 dph, co-fed 3 days	2.0	23 mg		
	4-28 dph, co-fed 5 days	2.5	95 mg		
	9-28 dph, co-fed 4 days	5.9	253 mg		
	6-28 dph, co-fed 14 days	8.6-13.5	209-218 mg		
Atlantic cod	17-39 dph, co-fed 16 days	5-23	2.2-6.6 ^{d}	Baskerville-Bridges and Kling, 2000a	15 mm SL (Pedersen and Falk-Petersen 1992).
Gadus morhua	LP control	36	8.2 ^{d}		
	8-71 dph, co-fed 14 days	34	8.6 ^{d}	Baskerville-Bridges and Kling, 2000b	
	15-71 dph, co-fed 7 days	35	8.2 ^{d}		
	29-70 dph, co-fed 7 days	35	9.0 ^{d}		
	8-64 dph, co-fed 14 days	7	5.4 ^{d}	Callan *et al*, 2003	
	36-64 dph, co-fed 14 days	25	7.7 ^{d}		
Atlantic halibut	20-73 dpff, co-fed 7 days	64	4.6 ^{w}	Næss *et al*, 2001 / Hamre *et al*, 2001	35-40 dpff (10-12ºC, Luizi *et al*, 1999)
Hippoglossus hippoglossus	26-73 dpff, co-fed 7 days	64	4.8 ^{w}		
	34-73 dpff, co-fed 7 days	80	4.4 ^{w}		
	46-73 dpff, co-fed 7 days	96	5.0 ^{w}		

Abbreviations: dpff = days post first-feeding, dph = days post hatch,, LP = live prey, SGR = specific growth rate, SL = standard length, TL = total length, wt. = weight. * Weaning is regarded as being early when conducted before or at onset of stomach development. ** Daily SGR is calculated as: (e^{g} -1)*100 %, where g = (ln(final wt.)-ln(initial wt.))/experimental days (Houde and Schekter, 1981). Values marked with ^{w} or ^{d} mean that calculations are based on wet or dry larval weights, respectively.

survival and specific growth rates (SGR, % day^{-1}) up to 35-73 % and 7-11 %, respectively, have been achieved when fed solely on formulated diets (Cahu *et al*, 1998a; 2003; 2004; Fontagné *et al*, 2000). Early weaning (*i.e.* before or at the onset of gastric development) has also been proved possible with other fish species, but as the live feed period is shortened, larval growth and survival are impaired (Table 1; Hoehne-Reitan *et al*, 2001; Robin and Vincent, 2003; Yúfera *et al*, 2003; Curnow *et al*, 2006). Gilthead seabream (*Sparus aurata*) larvae perform poorly on compound diets at first-feeding (Table 1; Yúfera *et al*, 2000; Robin and Vincent, 2003), but when fed rotifers for 2-4 days before weaning, improved growth and survival have been observed (Yúfera *et al*, 1999; 2000). The highest rate of weaning success is, however, reached when weaning is conducted at 50 days post hatch (dph), which is about the time when the stomach starts to turn acidic (Yúfera *et al*, 2004). Similarly as gilthead seabream, turbot (*Scophthalmus maximus*) larvae are also possible to wean (24 dph) just before the stomach starts to turn acidic (28 dph), while earlier weaning results in growth retardation (Hoehne-Reitan *et al*, 2001). Co-feeding live and compound diets for a certain period of time may improve the success of the diet change, as clearly can be seen for gilthead seabream, Senegalese sole (*Solea senegansis*) and barramundi (*Lates calcarifer*) in Table 1 (Cañavate and Fernández-Díaz, 1999; Yúfera *et al*, 2003; Curnow *et al*, 2006).

As shown in Table 1, introducing compound diets to Atlantic cod between 8 and 17 dph succeeded when 1-2 weeks co-feeding was included (Baskerville-Bridges and Kling, 2000a; 2000b; Callan *et al*, 2003). In these experiments, the larvae were fed exclusively compound diets from a size of 8-9 mm standard length (SL). According to Stoss *et al* (2004), the rate of weaning success in cod increases as the fish reaches 100 mg wet weight (wt.; corresponding to approximately 24 mm SL, Finn *et al*, 2002) and that size is likely reached between 50 and 75 dph when assuming 8-12 % daily SGR and 16 % dry matter content.

Halibut larvae introduced to compound diets from approximately 20, 26 or 34 days post first-feeding (dpff; 70-160 mg wet wt.) and subsequently co-fed *Artemia* for 1 week, obtained almost similar growth as the *Artemia* control group, but survival rates were reduced (Table 1; Hamre *et al*, 2001). The early weaning groups grew poorer in the beginning of the weaning period as compared to the control, but this was partly compensated after weaning of all the experimental groups (Hamre *et al*, 2001; Næss *et al*, 2001). According to Stoss *et al* (2004), safe weaning of halibut can be conducted at 250 mg wet weight which is likely to be reached between 40 and 80 dpff when assuming 5-10 % daily SGR.

The poorer ability of compound diets than live feeds to sustain larval growth and development is probably caused by a combination of several factors as palatability of the diet, particle locomotion (sinking velocity, movement pattern), nutritional composition, nutrient availability, digestibility and absorption. Digestibility is of particular concern because marine fish larvae hatch at a very early developmental stage, and the gastrointestinal tract, along with other organs, is not fully matured at first-feeding (Kjørsvik *et al*, 2004).

1.2 Development of the gastrointestinal tract in marine fish larvae

Marine fish larvae that hatch from pelagic eggs undergo fairly similar ontogenetic changes of the gastrointestinal tract and of other organs as well (Kjørsvik *et al*, 2004). At hatching, the gastrointestinal tract is a closed, straight and undifferentiated tube, and the larvae obtain nutrients from the yolk-sac (Pittman *et al*, 1990; Kjørsvik *et al*, 1991; 2004; Ribeiro *et al*, 1999a; García Hernández, 2001; Elbal *et al*, 2004). Shortly after hatching, the gastrointestinal tract becomes separated into four distinct regions noted as the oesophagus, foregut, midgut and hindgut. Furthermore, the liver, pancreas and the enterocytes with the brush border (*i.e*.

microvilli) start to differentiate and become functional. At this developmental stage, the pancreatic duct also opens into the anterior end of the midgut and digestive enzymes are synthesised (Beccaria *et al*, 1991; Kjørsvik and Reiersen, 1992; Segner *et al*, 1994; Ribeiro *et al*, 1999a; Kjørsvik *et al*, 2004). Before the yolk is totally resorbed, the mouth and anus open and the larvae are ready to commence exogenous feeding (Blaxter *et al*, 1983; Pittman *et al*, 1990; Kjørsvik and Reiersen, 1992; Segner *et al*, 1994; Ribeiro *et al*, 1999a; García Hernández *et al*, 2001; Elbal *et al*, 2004; Kjørsvik *et al*, 2004). Later during the larval stage, the digestive and absorptive functions improve and nutrient utilisation becomes more efficient. The gut grows faster relative to the body length, and hence forms one or more loops (Kjørsvik *et al*, 1991; Luizi *et al*, 1999; Ribeiro *et al*, 1999a; García Hernández *et al*, 2001; Elbal *et al*, 2004). The intestinal epithelium thickens and becomes increasingly folded (Kjørsvik *et al*, 1991; Segner *et al*, 1994; Luizi *et al*, 1999; García Hernández *et al*, 2001; Elbal *et al*, 2004). Along with formation of a few or more pyloric caecae in the anterior end of the midgut at about the time of stomach development, the increased mucosal folding and gut elongation increase the intestinal surface at which digestion and absorption occur (Segner *et al*, 1994; Luizi *et al*, 1999; Kjørsvik *et al*, 1991; García Hernández *et al*, 2001; Elbal *et al*, 2004).

At the time of metamorphosis, when the larvae transform into its juvenile form, the foregut differentiates into a stomach. During this process, the constriction separating the fore- and midgut differentiates into a muscular sphincter, the pyloric sphincter, and the foregut area expands to allow feed storage (Pedersen and Falk-Petersen, 1992; Segner *et al*, 1994; Luizi *et al*, 1999; García Hernández *et al*, 2001). Furthermore, gastric glands, in which pepsin and hydrochloric acid are synthesised, start to develop in the mucosa (Pedersen and Falk-Petersen, 1992; Luizi *et al*, 1999, Kjørsvik *et al*, 2004). The stomach seems to achieve full functionality

by the end of the larval stage (Luizi *et al*, 1999; Ribeiro *et al*, 1999a; García Hernández *et al*, 2001; Elbal *et al*, 2004). In cod, however, a delayed development of the stomach is observed (Pedersen and Falk-Petersen, 1992), but also in other species, the stomach appears to be further differentiated after metamorphosis (Luizi *et al*, 1999; Ribeiro *et al*, 1999b; Kjørsvik *et al*, 2004; Yúfera *et al*, 2004).

Digestive enzymes are synthesised in gastric glands in the stomach wall, in acinar cells in the exocrine pancreas and in enterocytes (Table 2; Krogdahl, 2001). Pepsin from the gastric glands is not synthesised until the end of the larval period when the stomach develops. The other main digestive enzymes are present from the onset of first-feeding or shortly afterwards (Table 2; Zambonino Infante and Cahu, 2001; Kjørsvik *et al*, 2004), but the amount of digestive enzymes seem to be low at first-feeding (Munilla-Moran *et al*, 1989; Gawlicka *et al*, 2000; Kjørsvik *et al*, 2004). The levels of enzymes increase as the digestive organs become more elaborated and feeding activity increases (Munilla-Moran and Stark, 1989; Zambonino Infante and Cahu, 2001; Kjørsvik *et al*, 2004). The digestive enzymes produced by the enterocytes and anchored in the brush border membrane (bbm) display a special developmental pattern. A sudden increase in specific activities of bbm-bound alkaline phosphatase (AP) and leucine aminopeptidase N (LAP) is observed at a species-specific developmental stage and marks the moment when the enterocytes mature (Figure 1; Ribeiro *et al*, 1999b; Zambonino Infante and Cahu, 2001). A similar pattern also has been described in postnatal mammals (Henning *et al*, 1994; Reisenauer *et al*, 1992). The increases in AP and LAP specific activities appear to coincide with the period of time when the intestinal mucosa becomes more extensively folded (Zambonino Infante and Cahu, 2001), and a coordinated occurrence of these events seems logical. In a mature intestine, enterocytes proliferate in the bases of the mucosal folds and differentiate and attain mature functions as they approach the

fold tips (Ugolev and De Laey, 1973; Krogdahl, 2001). An efficient differentiation process likely develops as the "true" folds appear. The digestive functions will be treated in more details later (Chapter 1.4).

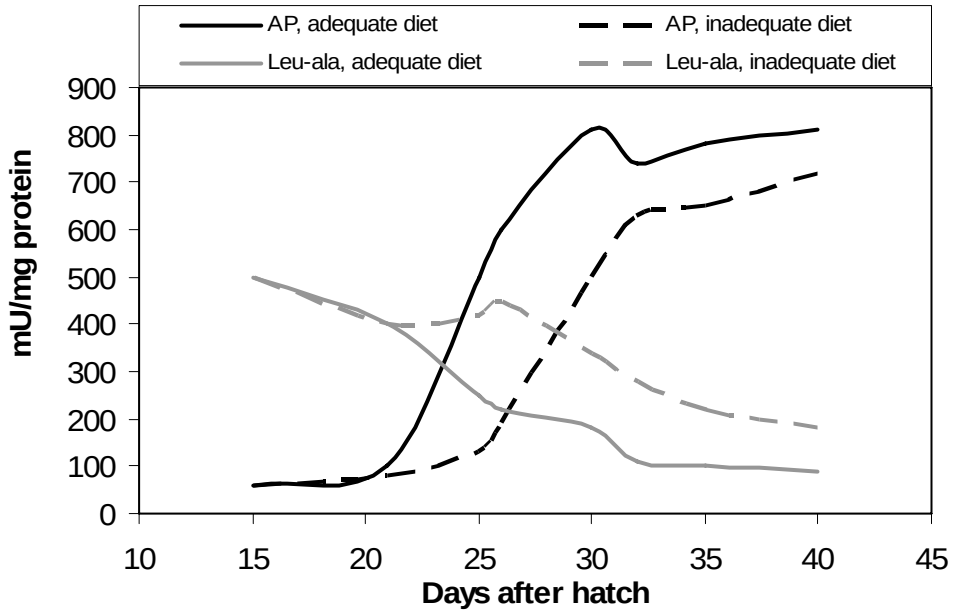


Figure 1. Ontogenetic changes in activities of brush border membrane bound alkaline phosphatase (AP) and cytosolic leucylalanine peptidase (Leu-ala) in European seabass larvae fed adequate and inadequate diets. AP is a marker of enterocyte maturation and thus increased activity indicates when improved luminal digestion is obtained. Reduced activity of Leu-ala is thought to indicate declined intracellular digestion due its proposed role in intracellular digestion. Reproduced from Zambonino Infante and Cahu (2001).

Nutrient absorption is evidenced by the presence of supranuclear vacuoles in the enterocytes. Shortly after first-feeding, lipid vacuoles are seen in the midgut mucosa and protein containing vacuoles in the hindgut mucosa (Kjørsvik *et al*, 1991; Segner *et al*, 1994; Luizi *et al*, 1999; García Hernández *et al*, 2001; Elbal *et al*, 2004). Both of them increase in size and number as the young larvae develop. The presence of the lipid vacuoles is thought to indicate

immature ability to transport lipids to the liver, and when the vacuoles decline after development of the stomach, lipid mobilisation has likely improved (Elbal *et al*, 2004; Kjørsvik *et al*, 2004). The protein containing vacuoles in the hindgut indicate that protein is digested intracellularly in larvae (Watanabe, 1982; Govoni *et al*, 1986). When luminal digestion markedly improves after development of the stomach, these vacuoles cease (Luizi *et al*, 1999; Elbal *et al*, 2004). The intracellular digestion of protein will be discussed in more detail later (Chapter 1.4).

1.3 Larval growth and protein demands

Fish larvae have a high capacity to direct energy into growth (Finn *et al*, 1995a; Pedersen, 1997), and thus, they grow exceptionally fast (Kjørsvik *et al*, 2004). In warm-water species (18-20 ºC) such as turbot, white seabream (*Diplodus sagrus*), gilthead seabream and European seabass, SGR of 14-20 % day^{-1} is commonly obtained when using *Artemia* and/or rotifers as feed organisms (Cousin *et al*, 1987; Cahu *et al*, 1998a; b; Sarasquete *et al*, 1995; Cara *et al*, 2003). Despite lower rearing temperatures, similar and even faster growth has been obtained in Atlantic cod (SGR 25-30% day^{-1}, 10-17ºC; Otterlei *et al*, 1999; Finn *et al*, 2002) and Atlantic halibut larvae (SGR 14-18 % day^{-1}, 12±1ºC; Næss and Lie, 1998) when fed natural copepods**.** More frequently, a daily SGR of 8-13 % is obtained in cod fed rotifers (7.5-12.6ºC; Baskerville-Bridges and Kling, 2000b, Callan *et al*, 2003; van der Meeren and Ivannikov, 2006), and 5-12 % in halibut fed *Artemia*, copepods or both (10-12ºC; Harboe *et al*, 1998; Næss and Lie, 1998; Shields *et al*, 1999; Hamre *et al*, 2002).

Growth in fish larvae mainly constitutes an increase in muscle tissue (*i.e*. protein deposition; Houlihan *et al*, 1995; Rønnestad *et al*, 2003). The fast larval growth therefore demands high levels of dietary amino acids (AAs) the building blocks of protein. Moreover, AAs derived

both as free and protein-bound also is the main energy substrate for cod larvae and juveniles (Finn *et al*, 2002), and likely also for halibut larvae, as the energy budgets are fairly similar for cod and halibut in the yolk-sac phase (Finn *et al*, 1995b; c). In first-feeding cod (4-7 mm SL) AA oxidation accounts for 70-95% of the energy dissipation, while the ratio decreases to approximately 50-70% in the juveniles (12-40 mm SL; Finn *et al*, 2002).

1.4 Protein digestion in marine fish larvae and adults

The high growth rate and high need for protein in fish larvae contrasts to their immature gastrointestinal tract at first-feeding which alter their digestion of protein.

Adult digestion

In adults, protein digestion is initiated in the stomach by the actions of hydrochloric acid (HCl) and the gastric enzyme pepsin which both are produced in mucosal glands in the stomach. Pepsin is synthesised and stored in an inactive form, as pepsinogen, and has to be activated by HCl. HCl also denatures the dietary protein and this leaves more peptide bonds available for enzymatic cleavage by pepsin (Krogdahl, 2001). Pepsin acts as an endopeptidase and thus hydrolyses the dietary protein into smaller fragments. This hydrolysation increases the solubility of the protein and hence eases the subsequent intestinal digestion. Besides the actions of HCl and pepsin, muscular contractions in the stomach wall also contribute in dissolving the ingested diet by thoroughly mixing the chyme before it is released in controlled portions through the pyloric sphincter into the midgut (Krogdahl, 2001). Due to the strong pyloric sphincter and elastic stomach mucosa, the stomach also has an important role in storing ingested feed. This allows intake of large meals, whereas only suitable small portions enter the intestine for efficient digestion and absorption (Sire and Vernier, 1992).

Once entering the midgut, the chyme is neutralised by bicarbonates secreted from the intestinal mucosa and pancreas and further digested by pancreatic and mucosal enzymes. The most important pancreatic peptidases are trypsin, chymotrypsin, elastase and carboxypeptidases, while aminopeptidases and di- and tripeptidases dominate among the mucosal ones (Table 2). The pancreatic juice is secreted though one or more ducts which enter the anterior end of the midgut. The secreted enzymes are released in an inactive form, but are activated by enzymatic cleavage. Among the proteolytic enzymes released, trypsin is activated by enterokinase, an enzyme produced in the intestinal mucosa, whereas the others are activated by trypsin (Alpers, 1987; Krogdahl, 2001). The digestive enzymes in the gut act either as endo- or exopeptidases which respectively hydrolyse the peptides in the middle or terminal ends (Table 2). As digestion continues, the free amino acids (FAAs), di- and tripeptides liberated are absorbed by the enterocytes through different transport pathways (Krogdahl, 2001). In this context, the bbm-bound aminopeptidases, di- and tripeptidases play a central role, as the close presence of these to the absorptive site facilitates efficient absorption once an AA, di- or tripeptide is liberated from a larger peptide. The fast removal of proteolytic end products is important since accumulation of intermediary products inhibits the luminal digestive enzymes (Ugolev and De Laey, 1973). In fish, digestion and absorption take place throughout the whole gut, but with decreasing contribution of the gut segments in the following order pyloric caecae > midgut > hindgut (Sire and Vernier, 1992; Krogdahl, 2001).

Larval digestion

Due to the absence of gastric digestion, marine fish larvae heavily depend on intestinal protein digestion. Pancreatic and intestinal enzymes are synthesised from the time of exogenous feeding (Table 2; Gawlicka *et al*, 2000; Zambonino Infante and Cahu, 2001;

Table 2. Main digestive enzymes, their sites of synthesis and action, role in digestion and approximate time when they are detected in marine fish larvae.

Enzymes	Site of synthesis	Site of action	Role in digestion	Time of detection
Pepsin	Stomach	Stomach	Endopetidase	Around metamorphosis [1,2]
Trypsin	Pancreas	Intestine	Endopetidase	Before or at first-feeding [1,2]
Chymotrypsin	Pancreas	Intestine	Endopetidase	Before or at first-feeding [2]
Elastase	Pancreas	Intestine	Endopetidase	Before or at first-feeding [1,2]
Carboxypeptidases A, B	Pancreas	Intestine	Exopeptidases. Cleave off C-terminal AAs	Before or at first-feeding [1]
Aminopeptidases (including LAP)	Intestine	Intestine, bbm and cytosol	Exopeptidases. Cleave off N-terminal AAs	Before or at first-feeding [1,2]
Di- and tripeptidases (including Leu-ala) [2]	Intestine	Intestine, bbm and cytosol	Hydrolyse di- or tripeptides.	Before or at first-feeding [2]
Amylase	Pancreas	Intestine	Hydrolyses starch (α-1,4-glucosidic bonds)	Before or at first-feeding [1,2]
Maltase	Intestine	Intestine, bbm-bound	Hydrolyses maltose	Before or at first-feeding [1]
Lipases	Pancreas	Intestine	Hydrolyses triacylglyserols	Before or at first-feeding [1,2]
Co-lipase	Pancreas	Intestine	Auxiliary enzyme for lipase	—
Phospholipases	Pancreas	Intestine	Hydrolyse phospholipids	Few days after first-feeding [1,2]
Alkaline phosphatase (AP)	Intestine	Intestine	Hydrolyses monophosphoesters.	Before or at first-feeding [1,2]

Abbreviations: AAs = amino acids, AP = alkaline phosphatase, bbm = brush border membrane, LAP = leucine aminopeptidase, Leu-ala = leucylalanine peptidase. References are Krogdahl (2001), except [1]Kjørsvik *et al* (2004) and [2]Zambonino Infante and Cahu (2001).

Kjørsvik *et al*, 2004; Perez-Casanova *et al*, 2006) and the pancreatic enzymes also seem to have been transported through the ducts to the gut at this time (Beccaria *et al*, 1991; Kurokawa and Suzuki, 1998). The digestive capacity, however, seems to be low in young larvae, indicated by the observations of undigested feed in the gut lumen (Walford *et al*, 1991; López-Alvarado *et al*, 1994; Luizi *et al*, 1999; Næss *et al*, 1995), defecations of large amount of undigested protein (Tonheim *et al*, 2005) and low activities of digestive enzymes (Munilla-Moran and Stark, 1989; Kjørsvik *et al*, 2004). A progressive improvement in digestive capacity seems to occur as the gastrointestinal tract attains its mature form (Munilla-Moran and Stark, 1989; Zambonino Infante and Cahu, 2001; Kjørsvik *et al*, 2004).

In adults, the digestion is regulated through several gastrointestinal hormones along with the nervous system. In young fish larvae, hormonal regulation of the digestive processes appears to be weak since the presence of all of these hormones is more uncertain at first-feeding, at least in halibut (Kamisaka *et al*, 2001). Late development of the pyloric sphincter and feed storage capacity in the stomach also suggest that passage of the diet through the gastrointestinal tract is less adjustable in fish larvae as compared to in the older stages. In a well-regulated digestion system, gastrointestinal transit time is regulated to optimise the utilisation efficiency of protein (Zhao *et al*, 1997), whereas this seems not to be the case in marine fish larvae (Tonheim *et al*, 2005). However, it may appear as the sphincter between the mid- and hindgut can to some extent regulate feed passage, and thus facilitate for feed storage and mastication by peristaltic contractions in the midgut (Rønnestad *et al*, 2003).

Alternative digestive processes may compensate for the seemingly low digestive capacity in fish larvae. Live feeds may assist in digestion of first-feeding larvae by being a source for proteolytic enzymes or enhancing diet utilisation in other ways. Low contributions of live

feed proteases are estimated in metamorphic halibut (34 dpff; 8 %, Gawlicka *et al*, 2000), larval Japanese sardine (*Sardinops melanotictus*, 10 dph, 0.6 %, Kurokawa *et al*, 1998) and larval turbot (6 dph, 2-10 %; Munilla-Moran and Stark, 1989). In the latter study with turbot, besides the protease contribution estimated from live feed, protease activity in unfed larvae constituted 33-34 % of the activity present in the fed larvae (pH 7-8). The remaining 57-64 % of the activity appeared to be a result of stimulatory effects of ingested rotifers (Munilla-Moran and Stark, 1989), indicating that the digestive capacity heavily depend on stimulation from the diet offered. Microscopic studies of pancreas development indicated that live feed stimulated release of pancreatic enzymes in seabass larvae, while this stimulation was not achieved by a compound diet (Beccaria *et al*, 1991). Furthermore, studies using ^{14}C-labelled compound diets have also indicated that live feed positively affects ingestion rates as well as the assimilation of compound diets (Kolkovski *et al*, 1997a; 1997b). Thus, live feed organisms appear not only to supply the larvae with nutrients, but may also be important in stimulating the digestive processes in fish larvae.

An alternative pathway of protein digestion in marine fish larvae, as well as in postnatal mammals, has been proposed (Watanabe, 1981; 1982; 1984; Govoni *et al*, 1986; Henning, 1987). Absorption of intact protein in larvae of several fish species is indicated by the presence of large acidophilic, supranuclear vacuoles in the hindgut enterocytes before gastric digestion commences (Watanabe, 1981; 1982; Kjørsvik *et al*, 1991; Segner *et al*, 1994; Luizi *et al*, 1999; Ribeiro *et al*, 1999a; Elbal *et al*, 2004). By studying the uptake of native horseradish peroxidase (HRP) or other tracers, the transport and intracellular processing of the absorbed protein has been investigated (Watanabe, 1982; 1984; reviewed by Govoni *et al*, 1986 and Sire and Vernier, 1992; Kishida *et al*, 1998). The tracer protein is absorbed by invaginations in the bases of the microvilli, forming small vesicles inside the enterocytes.

These vesicles tend to coalesce and accumulate to large vacuoles that move toward the supranuclear region of the cell (Watanabe 1982; 1984; Govoni *et al*, 1986). Primary lysosomes associate with the vacuoles and acidify the interior of the vacuoles and provide the vacuoles with proteases that hydrolyse the protein (Watanabe, 1984; Govoni *et al*, 1986; Sire and Vernier, 1992). Evidenced by their loss of activity, the tracer proteins are digested within the vacuoles (Watanabe, 1982; 1984; Kishida *et al*, 1998). Important lysosomal proteases are cathepsins B and D (Sire and Vernier, 1992). The properties of cathepsin D are quite similar to those of pepsin, being an endopeptidase that acts at acidic pH values. Whereas cathepsin D initiates protein degradation, cathepsin B acts secondarily by removing dipeptides from the peptide chains (Sire and Vernier, 1992). Lysosomes have low dipeptidase activity, and thus, dipeptides are transported into the cytosol for final hydrolysis (Thamotharan *et al*, 1997). Cathepsins B and D activities follow a similar ontogenetic pattern as pinocytotic absorption in the distal intestine of suckling rats, with increasing activities the first days after birth followed by decreasing activities when approaching the weaning stage (Davies and Messer, 1984). Some cytosolic dipeptidases in the distal intestine also show this developmental profile, suggesting that these enzymes are important in the final hydrolysis of pinocytosed protein (Vaeth and Henning, 1982; Henning, 1987). A similar development of the protein degradation pattern as present in mammals is also thought to apply to fish larvae (Watanabe, 1982; 1984; Govoni *et al*, 1986; Sire and Vernier, 1992; Zambonino Infante and Cahu, 2001). In seabass and Senegalese sole (*Sole senegalensis*), a gradual decline in the activity of intestinal leucylalanine dipeptidase (Leu-ala) occurs as the larvae develop, and this has been proposed to indicate the gradual decline in larval mode of digestion (Zambonino Infante and Cahu, 2001; Ribeiro *et al*, 1999b). The decreasing specific activity of Leu-ala and concomitantly increasing AP specific activity during the larval stage of seabass is showed in Figure 1, and this pattern is considered to illustrate the course of intestinal maturation in fish larvae.

Protein-containing vacuoles are also observed in fish that possess gastric digestion (Georgopoulou *et al*, 1986), but the number of vacuoles are reported to decline as gastric digestion commences (Luizi *et al*, 1999; Elbal *et al*, 2004) and with an apparent longer digestion time (Watanabe, 1982). The marked decline in their presence at onset of gastric digestion and their absence in starving larvae (Yúfera *et al*, 1993) suggest that they have significant importance for protein nutrition in larvae when the gastrointestinal tract still is not fully mature However, there is argument against this. Since the vacuoles are observed to not disappear until 4-10 hours after tracer administration in different larval fish (Watanabe, 1982; Kishida *et al*, 1998), the digestion time appears to be too long to provide significant amount of AAs (Rønnestad *et al*, 2003). Furthermore, appreciable amounts of tracer proteins are also absorbed in older fish that possess efficient luminal digestion (Sire and Vernier, 1992). Rather than in nutrition, another possible role of pinocytosis and intracellular digestion is in immunisation, since a small fraction of protein absorbed in an intact form by the enterocytes appears to escape from degradation inside the enterocytes and enters the blood circulation (Sire and Vernier, 1992; McLean *et al*, 1999).

1.5 Inclusion of hydrolysed protein in weaning diets for marine fish larvae

One possible way to enhance the utilisation of dietary protein in fish larvae is to pre-digest the protein prior to addition to a compound diet. By enzymatic pre-hydrolysis, the gastric digestion is imitated by cutting the protein into smaller fragments and thus increasing its solubility. The smallest protein fragments (*i.e*. di- and tripeptides) thus will be available for direct uptake and the longer ones are more available for larval digestive enzymes and possibly for uptake by pinocytosis. Indeed, tube-feeding experiments with halibut larvae have shown that pre-hydrolysis induces faster absorption of the protein and that the absorption is similar over a large range of doses. Intact protein is both more slowly absorbed and absorbed at a

decreasing rate as doses of protein increase (Tonheim *et al*, 2005). This indicates that increased complexity of the dietary protein reduces its utilisation.

Despite the findings by Tonheim *et al* (2005), high inclusions of hydrolysed protein (above 50 %) in weaning diets have been found to be detrimental to several fish species (*Sparus aurata*, Kolkovski and Tandler, 2000; *Cyprinus carpio*, Carvalho *et al.*, 1997; 2004; *Dicentrarchus labrax*, Cahu *et al.*, 1999), although with a few exceptions (*Solea solea*, Day *et al*., 1997; *Scophthalmus maximus*, Oliva-Teles *et al*., 1999). On the other hand, diets containing low to medium fractions of dietary hydrolysed protein, has been reported to improve larval performance (Zambonino Infante *et al*, 1997; Cahu *et al.*, 1999; Carvalho *et al.*, 2004). Collectively, performances in growth, survival and rate of malformation was best in seabass larvae fed 25 % hydrolysed protein, compared to none or higher levels (Table 3). The larvae of common carp (*Cyprinus carpio*) produced the highest biomass, as estimated by combining survival and growth, when fed 6 % hydrolysed protein (Carvalho *et al*, 2004). Although common carp is a fresh-water species with different nutritional requirements than marine fish species, the differences might be smaller at the larval stage when their gastrointestinal tract is not fully mature.

An optimal level of hydrolysed protein may also stimulate the maturation of the intestine as is seen by feeding live feed as compared to compound feed (Cahu and Zambonino Infante, 1995). This is illustrated in Figure 1 which indicates a delay in achievement of adult mode of digestion and a prolonged larval mode of digestion when larvae are fed nutritionally inadequate diets (Zambonino Infante and Cahu, 2001). The activity of intestinal Leu-ala was used as a marker for the larval mode of digestion, and activity of bbm-bound AP was used as a marker for the adult mode of digestion. Improved intestinal maturation in seabass larvae fed

an optimal level of hydrolysed protein was shown by Cahu *et al* (1999; Table 3), using the principles outlined in Figure 1. By comparing activities of AP and LAP vs Leu-ala, offering a diet in which 25 % of the protein was substituted with hydrolysed protein resulted in highest stimulation of intestinal maturation. This is in accordance with the performances in growth, survival and rate of malformation described in the previous section (Table 3; Cahu *et al*, 1999).

Table 3. Effects of graded levels of hydrolysed protein, exchanged with intact protein, in diets for European seabass (*Dicentrarchus labrax*) larvae fed from 10 to 41 dph. Survival rate, growth and rate of malformation at 41 dph and ratio of activities of alkaline phosphatase (AP) and leucine aminopeptidase N (LAP) vs leucylalanine peptidase (Leu-ala) at 20 dph are given. From Cahu *et al*, 1999.

Hydrolysed protein (%)	0	25	50	75
Survival (%)	39±2 [b]	47±4 [a]	34±1 [bc]	33±3 [c]
Final wet weight (mg)	5.5±2.6 [a]	5.7±2.8 [a]	3.3±1.0 [b]	2.6±0.7 [b]
Rate of malformation (%)	13±3.5 [a]	6±1 [b]	5±1 [b]	2±0.5 [c]
AP – Leu-ala ratio [1]	30±11 [b]	52±16 [a]	21±4 [b]	15±5 [b]
LAP – Leu-ala ratio [1]	37±8 [b]	81±14 [a]	25±8 [bc]	19±6 [c]

[1] At 41 dph, these enzyme activity ratios were higher, but showed a similar pattern in response to dietary hydrolysed protein.

1.6 Microparticulate diets

A wide range of microparticulate diets for marine fish larvae have been developed and tested in feeding studies (Reviewed by Langdon, 2003). Mainly, they can be grouped according to their particle structure as microbound or microencapsulated. Microbound diets have in common that they consist of particles that have a uniform matrix throughout each particle. In

production, the ability of a polymer to form a gel in which the other ingredients are embedded is utilised. The gel-forming polymer can either be an added binder, such as alginate, carageenan, zein or gelatine (López-Alvarado *et al*, 1994; Baskerville-Bridges and Kling, 2000a; Guthrie *et al*, 2000; Önal and Langdon, 2005) or a natural diet ingredient such as starch or protein (Hoestmark and Nygaard, 1992; Fontagné *et al*, 2000; Hamre *et al*, 2001). Particles can be formed by crushing the dried gel (López-Alvarado *et al*, 1994, Baskerville-Bridges and Kling, 2000a; Hamre *et al*, 2001) or by forming beads by spraying the diet mixture into a gelling bath or drying chamber (Önal and Langdon, 2000; 2005). Microbound diets can also be produced by means of different agglomeration, pelleting or extruding techniques (Hoestmark and Nygaard, 1992; Fontagné *et al*, 2000; Guthrie *et al*, 2000).

Together with the protein-walled microencapsulated diet described below, two microbound diets are of particular interest in the current study. The first one has been used successfully for weaning of halibut (Table 1; Hamre *et al*, 2001; Næss *et al*, 2001). In this diet, proteins from raw fish fillets are used as the main binder, while additional binding is achieved upon addition of a small amount of starch. By comminution, all the diet ingredients are mixed and the native fish muscle proteins are restructured and later converted to a gel upon heat exposure. The diet is then dried, crushed and sieved into appropriate particle sizes, the latter production step being necessary in all microdiets.

Another microbound diet that utilises the properties of native fish protein as a binder is produced by agglomeration (Hoestmark and Nygaard, 1992). In this diet, micronised fishmeal and stick water, the latter derived from fishmeal production, are the main ingredients. The collagen in these ingredients is protected from denaturation during meal production. When all the ingredients are mixed, the diet is heated and a gel is formed by the collagen during

cooling. By using the agglomerating technique, compact, spherical particles are formed. This diet is well accepted by cod and halibut at weaning, and also by other species (*Solea senegalensis*, Engrola *et al*, 2001; *Solea solea*, Rueda-Jasso *et al*, 2001)

In order to produce diets with better ability to retain water-soluble nutrients, different microencapsulated diets have been developed. In a microcapsule, a distinct membrane surrounds the nutrients, aiming to reduce nutrient leaching (Langdon, 2003). Microencapsulated diets using protein as the encapsulating medium have been studied thoroughly (Jones, 1980; Langdon, 1989; Ozkizilcik and Chu, 1996; Yúfera *et al*, 1999; 2000). This diet can be made by emulsifying the diet ingredients in a cyclohexane – lecithin solution and further adding a highly reactive cross-linker under continuous high-speed stirring (Yúfera *et al*, 1999). The ingredient list has to include a soluble protein that will act as an emulsifier, together with lecithin, and hence to be positioned at the droplet surface. The cross-linker polymerises the surface protein to form a membrane that stabilises the particles. The produced particles are then dispersed in a gelatine solution, and thereafter washed to remove any debris. The final process stages are freeze-drying and sieving (Yúfera *et al*, 1999). This diet has been found to support good growth and survival in short term feeding experiment with seabream larvae from 4 dph onwards (Table 1; Yúfera *et al*, 1999; 2000). A drawback with this diet is the excessive use of organic solvents that limits its up-scaled production (*i.e*. above lab scale).

Lipid-based diets include lipid-walled microencapsulated diets and lipid spray beads. Their production techniques and particle structure differ, but both of them consist of hardened lipid droplets containing water-soluble nutrients either within small vesicles or as particles (Langdon, 2003). These diets can not be used as complete diets for fish larvae as their lipid to

core material ratio is too high, and because lipids with high melting points are needed to stabilize the particles (López-Alvarado *et al*, 1994). This opposes with both digestibility (López-Alvarado *et al*, 1994) and the larval demands for long-chain, ω-3 fatty acids (Olsen *et al*, 2004). Despite these drawbacks, the diets are still interesting due to the fact that they retain water-soluble nutrients better as compared to other microparticulated diets (see later). Furthermore, they can be embedded into microbound or microencapsulated diets and thus reduce leaching rates of water-soluble nutrients from these (Ozkizilcik and Chu, 1996; Baskerville-Bridges and Kling, 2000a; Önal and Langdon, 2005). The production and use of such complex diets need to be further studied to prove their suitability for fish larvae (Langdon, 2003).

The diets described above differ in several properties such as water stability, sinking velocity, nutrient leaching, palatability and digestibility. This affects their suitability as a feed for different larval fish species. Diet particles that float on the water surface or sink too fast will be less available for the fish larvae. Some of the binders and encapsulation media used are less digestible because fish larvae lack or have low levels of enzymes that are capable to digest these compounds (reviewed by Langdon, 2003). On the other hand, loosely bound diet particles, which may be readily digested, will have lower water stability, facilitating for rapid nutrient leaching. Also different production processes may impair the nutritional quality of the diets by reducing digestibility and displacing or inactivating nutrients. Protein is basically highly digestible, but this can be altered during feed formulation due to heating, protein cross-linking and other production steps (Cheftel *et al*, 1985; Walford *et al*, 1991; García-Ortega *et al*, 2001). Moreover, micronutrients and water-soluble nutrients might be susceptible to inactivation and displacement if exposed to degrading conditions (heating, extreme pH, chemical agent) or excessive water (particle washing, gelling bath), respectively

(Tannenbaum *et al*, 1985; Yúfera *et al*, 2002; 2003). Thus, several diet properties have to be considered carefully for designing suitable diets for marine fish larvae.

1.7 Nutrient leaching from microparticulate diets

Nutrient retention is of great concern in larval diets. The small particle sizes imply that the diffusion distance from the core of the particle to the surface is very short, and this facilitates leaching of water-soluble nutrients when the particles are exposed to water. Besides several micronutrients, the leaching problem concerns the delivery of FAAs, peptides and soluble protein. A full picture of leaching of hydrolysed and soluble protein is difficult to obtain since variation in peptide length, AA composition, along with the binding properties of the diet, affect the rate of diffusion from the diet particles. FAAs are identical with completely hydrolysed protein, and leaching of FAAs from several fish larval diets have been well examined of which some studies are cited in Table 4. López-Alvarado *et al* (1994) found that 3 different microbound diets lost more than 80 % of the initial FAAs within 2 minutes immersion in water, whereas a protein-walled microencapsulated diet lost about 60 %. Although coating of the microencapsules with lipids improved the FAA retention, the diet still showed considerable loss (39 %). In the study by López-Alvarado *et al* (1994), a lipid-walled diet showed the lowest FAA leaching. A high content of saturated fatty acids was, however, necessary to keep the leaching rate low and, as discussed in section 1.6, this discords with larval needs. A similar conclusion can be drawn from the complex diets in which lipid based particles are embedded. Such diets have shown higher capacity to retain FAAs as compared to the basal diets (Table 4; Ozkizilcik and Chu, 1996; Önal and Langdon, 2005), but not when high proportions of lipids with low melting points are used as wall material (Table 4; Baskerville-Bridges and Kling, 2000a).

Table 4. Leaching rates of free amino acids (FAAs) and protein from different compound diets for marine fish larvae after 1 to 60 minutes immersion in water.

Diet	Leaching compound	Leaching rates (%) after:				Reference
		1-2 min	5 min	30 min	60 min	
Microbound (3 diets)	FAAs	81 – 91	—	—	97	López-Alvarado
PWM	FAAs	59	—	—	—	*et al*, 1994
Lipid coated PWM	FAAs	39	—	—	—	
LWM (tripalmitin)	FAAs	4	—	—	10	
LWM (tripalmitin/ triolein)	FAAs	47	—	—	—	
Microbound	FAAs	7	22	72	85	Yúfera *et al*,
Protein encapsulated	FAAs	1	8	16	17	2002
Protein encapsulated	FAAs	1	2	9	15	
Microbound	FAAs	87	—	97	99	Önal and
Complex diet	FAAs	56	—	79	86	Langdon, 2005
Microbound	FAAs	58-60	—	80	80-85	Baskerville-
Complex diet	FAAs	58-62	—	78-80	78-82	Bridges and Kling, 2000a
PWM	Lysine	—	45	71	83	Ozkizilcik and
Complex diet	Lysine	—	19	21	33	Chu, 1996
Microbound (4 diets)	Protein	18 – 42	—	26 - 50	—	Hamre, 2006

Abbreviations: FAAs = free amino acids, LWM = lipid-walled microencapsulated diet, PWM = protein-walled microencapsulated diet.

Hydrolysed and soluble protein might be better retained within the feed particles than FAAs due to the higher molecular weight of these compounds. Leaching of protein from 4 different microbound diets reached 18 to 42 % after 2 minutes in water when leaching was evaluated against dietary crude protein (Table 4; Hamre, 2006). These leaching rates could appear lower

as compared to leaching rates of FAAs (Table 4; López-Alvarado *et al*, 1994; Baskerville-Bridges and Kling, 2000a; Yúfera *et al*, 2002; Önal and Langdon, 2005), but may have corresponded to most of the soluble protein originally present in the diets.

Larval diets not only loose a high ratio of the water-soluble nutrients through leaching, but this leaching also occurs very rapidly. Maximum leaching levels are almost reached within 2 to 5 minutes immersion in water (Table 4; López-Alvarado *et al*, 1994; Ozkizilcik and Chu, 1996; Önal and Langdon, 2005; Hamre, 2006). A fast ingestion of the diet particles by the fish larvae therefore is crucial for catching particles still containing a high nutrient content. Although a certain level of leaching is desirable for release of attractants, the high levels actually measured will impact the delivery of a nutritionally balanced diet to the marine fish larvae.

2 Aims of the study

The present study aimed:

1. To describe the process of the intestinal maturation in cod and halibut larvae to indicate their digestive capacities and predict the time at which these species are ready for weaning.

2. To define the level of hydrolysed protein in weaning diets for Atlantic cod and Atlantic halibut that maximise growth, survival and intestinal maturation.

To achieve these aims, the following study objectives were addressed:

1. Describe the ontogeny of alkaline phosphatase, leucine aminopeptidase N and leucylalanine peptidase in cod and halibut from first-feeding until after metamorphosis (**Paper I**)
2. Study the delivery efficiency of hydrolysed protein in three different larval compound diets, based on leaching properties and ingestion rates (**Paper II**)
3. Investigate effects on growth, survival and intestinal maturation when graded levels of hydrolysed protein substituted the intact protein in weaning diets for cod and halibut (**Papers III and IV**)

3 Methodological considerations

3.1 Enzyme activities and intestinal maturation

Enzyme activity assays

To obtain valid data from enzyme activity assays, the rate of product formation or substrate utilisation needs to be linear during the assay time-course (Tipton, 2002). In the analyses of AP and LAP, automatic recording of the absorbance every 20 second during the 2 minutes assay duration ensured this. Furthermore, the AP and LAP assays were optimised toward pH, temperature and substrate concentration, both to maximise enzyme activity potential and to ensure a linear product formation. Because of the complexity of the Leu-ala assay, pH and temperature optimisation was more difficult, but performing the assay in two steps would probably have solved the optimisation problems. The assay was checked for linear time-course of product formation during the 20 minutes assay duration and therefore considered to produce valid data.

Sample collection in relation to feeding and circadian rhythm

Samples for the enzyme activity determinations were supposed to be collected before the morning feeding and at approximately the same hour at every sampling days since the presence of feed in the gut in general have an impact on digestive enzyme activities (Cousin *et al*, 1987; Munilla-Moran *et al*, 1989) and the activities may also vary during a day and night. The morning basal enzyme activities were analysed in the experiment with halibut in paper I and in both the experiments in paper IV, whereas cod in paper I was by a mistake sampled after the morning feed distribution. This can be assumed as the main cause for the large variation in Leu-ala specific activity (Figure 4c, paper I). Since the AP and LAP specific

activities varied to a lesser extent and in addition followed the expected developmental profile with a distinct elevation during the larval stage, they appear to have been less affected by feeding than Leu-ala. This agrees with observation of LAP in a semi-quantitative study where LAP activity, in contrast to the activities of amylase and general proteases, appeared to be unaffected by feeding (Cousin *et al*, 1987). Thus, the AP and LAP activity profiles were assumed to represent the basal level of these enzymes in cod. The Leu-ala specific activity profile, on the other hand, appears to add little information about developmental progress in cod as well as the digestive capacity.

Ontogenetic changes in enzyme activities

Although enzyme activity profiles generally reflect ontogenetic changes in enzyme activities, other factors may also impact on the profiles. This applies in particular to changes in soluble protein content in the enzyme extract which is considered as a possible explanation for apparent decline in enzyme specific activities (Zambonino Infante and Cahu, 2001). A purification procedure of the bbm was carried out to up-concentrate bbm enzymes at the expense of other proteins (Papers I and IV). Furthermore, to exclude any impact from soluble protein changes, activities of AP and LAP were additionally given as bbm-bound activities in percent of total activity of the respective enzyme in the tissue homogenate (Figures 5a-b, paper I). Similar profiles of the specific activity and the percentage of bbm-bound activity would thus support that the activity profiles actually refer to changes in activity of the enzyme studied. Conversely, dissimilar profiles would indicate influence of other factors and the real profile of the enzyme would be more uncertain.

In the first half of the cod experiment (0-29 dpff) in paper I, enzyme extracts were made of whole larvae, whereas in the second half of the experiment (37-72 dpff), the enzyme extract

was prepared of the segment of the larvae between the end of the periculum and the anus, which included the abdominal cavity. This simple and quick dissection, previously used by others (Cahu and Zambonino Infante, 1994; Ribeiro *et al*, 1999b; Ma *et al*, 2005), is in the present study noted to represent the intestinal segment of the larvae and is an alternative to using the whole larvae in the enzyme extract when the small size of the larvae complicates dissection of the gut. The shift in dissection practise coincided with detection of a marked increase in AP and LAP specific activities (Figures 4a-b, paper I), and therefore introduced an uncertainty as to whether these activity changes were due to real changes in enzyme activities or a result of the change in method. To address this problem, the three samples following the dissection shift (37-51 dph) were reanalysed with regard to AP and LAP activities in homogenates of both whole larvae and intestinal segments. The number of sampled larvae did not allow extraction of bbm in these whole larvae samples. As shown in Figure 2, activities in the intestinal segments and whole larvae were similar, except for marked differences in

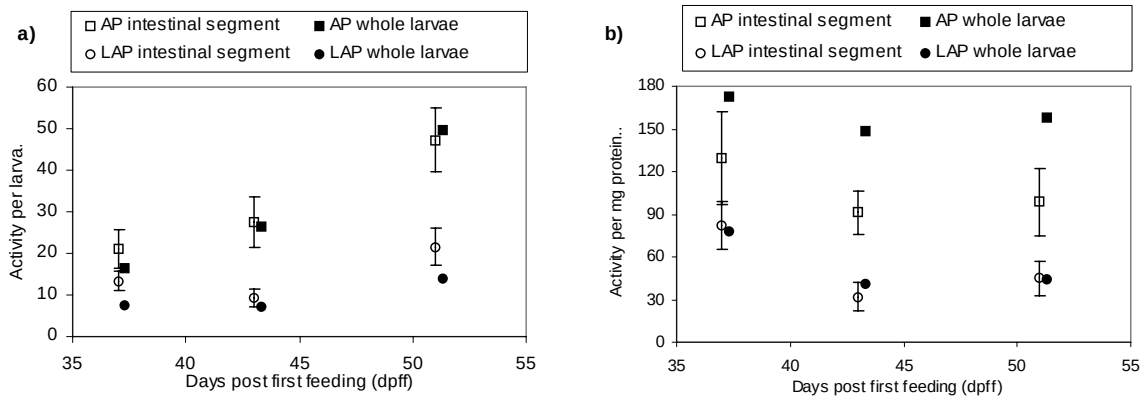


Figure 2. Comparisons of AP and LAP a) individual and b) specific activities in homogenates of either whole larvae (n=1) or intestinal segments (n=3) of cod to verify that measured enzyme activity profiles were due to real changes in enzyme activities and not due to shift in sampling methodolgy.

specific activity of LAP. This difference was, however, in favour of further increase in LAP activity from 29 to 37 dpff compared to that reported in paper I. Further support for elevated bbm enzyme activities from 29 dpff is the prolonged increase in bbm-bound activity of AP in percent of total AP activity until 51 dpff (Figure 5b, paper I). Based on these results, one may therefore assume that AP and LAP specific activities increased between 29 and 37 dpff.

Estimation of intestinal maturation

Opposite profiles of bbm-bound enzyme and Leu-ala specific activities as shown in Figure 1 was expected and thought to indicate intestinal maturation. Despite the failure of Leu-ala to follow the expected profile, which will be further discussed in section 4.1, intestinal maturation was estimated by the distinct increases in AP and LAP activities. LAP in cod did, however, make a less clear picture due to the different activity profiles when given on basis of soluble protein and as percentage of total LAP activity (Figures 4b and 5b, paper I), and due to the less marked increase in specific activity as compared to AP (Figures 4a-b in Paper I). However, AP exhibited marked and concomitant increase in activity when expressed both as specific activity and as activity in percent of total intestinal activity in both cod and halibut, and thus appeared to be a robust marker for intestinal maturation.

3.2 Choice of feed for delivery of hydrolysed protein

Before conducting weaning studies with various dietary levels of hydrolysed protein, a suitable feed for such studies had to be defined. Microparticulate diets loose a significant portion of water-soluble nutrients such as FAAs by leaching (Table 4; López-Alvarado *et al*, 1994; Ozkizilcik and Chu, 1996; Baskerville-Bridges and Kling, 2000a; Yúfera *et al*, 2002; Önal and Langdon, 2005; Hamre, 2006), whereas leaching of hydrolysed protein is less

studied. A better understanding of the leaching of hydrolysed protein was considered important before the planned weaning experiments were carried out. Moreover, the diet had to be well accepted by the fish larvae. The following three microdiets were considered as potential diets for use in weaning experiments with cod and halibut: 1) A heat-coagulated, protein-bound diet which previously has given good results in weaning of halibut (Table 1; Hamre *et al*, 2001; Næss *et al*, 2001; Paper III). 2) An agglomerated, proteinbound diet which also is considered successful in weaning of marine fish larvae. 3) A protein-walled microencapsulated diet which has given good results in short term weaning studies with gilthead seabream (Yúfera *et al*, 2002) and which also has shown better nutrient retention properties than microbound diets (Table 4; López-Alvarado *et al*, 1994; Yúfera *et al*, 2002). Therefore, these three diets were compared with regard to leaching rates and diet preference for selection of the most suitable diet in the weaning studies with cod and halibut.

3.3 Nutrient leaching

Determination of leaching rates

Leaching rates obtained in experiments may not completely correspond to the actual leaching rates from diet particles distributed in fish tanks. In order to obtain reliable and reproducible leaching data, leaching rates are determined in small water volumes and with far higher feed concentration than used in fish tanks. This may lead to saturated levels of water-soluble nutrients in the water and hampered diet particle hydration due to accumulation of feed at the bottom of the vial, and thus probably underestimate leaching. In the present study (Paper II), gentle agitation was used to disperse the particles throughout the water volume to prevent such particle accumulation, and this may have accelerated leaching from the diet particles. However, diet particles released in a fish tank are also subjected to movements relative to the water. Despite all these factors that may modify the determined leaching figures, the leaching

model used in the study in paper I gives a good indication of the leaching potential of different compounds in specific diets and is useful for comparing the ability of the diets to retain water-soluble nutrients.

Leaching rates were determined by liquid scintillation counting (LSC) of the water in which a diet containing a ^{14}C-labelled compound had been submerged. This method was considered to be well suitable since the study involved comparisons of different diets and different leaching compounds (i.e. serine (an FAA), pepsin hydrolysed algae extract, intact algae extract). LSC allows precise quantification of the ^{14}C-labelled compounds regardless of their chemical structure. Moreover, the ^{14}C-label is not affected by the sample matrix or by biochemical processes. A drawback with the use radiolabelling is the limited availability of certain labelled compounds, such as proteins. Protein that is labelled by ^{14}C-methylation is commercially available at high prices. Biochemically, this protein has modified properties, and the protein is not uniformly labelled. Possibly, ^{14}C-met-protein could have been used in the leaching study without influencing the results, but because leaching was planned measured from both intact and enzymatically hydrolysed protein, a laboratory produced ^{14}C-algae protein was prepared for the leaching study (Paper II). The alga *Chlamydomonas reinhardtii* was used to label protein. By supplying the algae with ^{14}C-HCO_3 as the sole carbon source, ^{14}C is built into all their biochemical compounds. Efficient extraction of the protein would thus result in an extract containing uniformly ^{14}C-labelled proteins.

Algae protein extraction

The protein content of the algae extract was very low, only 8.5 % (Paper II). However, the proportion of protein in the labelled compounds was probably higher since cutoff centrifugation of intact and hydrolysed algae extract revealed that 30 % of the label changed

position on a molecular size scale due to hydrolysis (Table 4, paper II). In the intact extract, this portion of the label was found in the unrecovered cutoff fraction, and thus probably was associated with large, complex molecules, such as glycoproteins and cell membrane remnants. Upon hydrolysis of the extract with pepsin, smaller molecules were released from the complex molecules and appeared in a lower molecular weight fraction (*i.e*. below 18 kD, <3k cutoff). These molecules were probably peptides since they were released with pepsin. Hence, this indicates that protein was associated with at least 30 % of the labelled compounds in the extracts. The intact extract also contained a small portion (14 %) of compounds ranging in molecular size from 9-18 to 300-600 kDa (from 3 to 100 k cutoff; Table 4, paper II) and a large portion (42 %) of compounds ranging in molecular size from 1.2 (dialysing tubing specification) to 9-18 kDa (<3 k cutoff). These were likely of protein or carbohydrate origin. Low molecular weight compounds (<1.2 kDa) were likely efficiently removed by dialysis, RNA and DNA by digestion and dialysis and cell wall was not present since a wall-less mutant of *C. reinhardtii* was used. The high level of compounds in the algae extract that was not of protein origin creates a problem in interpretation of the leaching data as leaching of intact and hydrolysed protein. A new estimate for leaching rates were made based on molecular weight characterisation of the algae extract and this is discussed in Paper II and in section 4.4.

3.4 Feed preference

Feed preference was determined by including a unique tracer, yttrium (Y), lanthanum (La) or ytterbium (Yb), in each of the three larval compound diets studied, and simultaneous feeding of the three diets to cod (Paper II). These tracers have previously been used in preference studies with juvenile cod and shown to be equally ingested when given as a mixture of mono-labelled diets (Otterå *et al*, 2003; Garatun-Tjeldstø *et al*, 2006) and also to be well suited for

apparent digestibility studies in salmonids (Austreng *et al*, 2000). Differently from the preference studies with juvenile cod (Otterå *et al*, 2003; Garatun-Tjeldstø *et al*, 2006), the diets used in the present study differed a lot in physical properties (colour, sinking velocity, shape, particle size distribution, leaching) and in nutritional and ingredient composition. The diets may therefore mutually have affected the ingestion rates of each other. Possibly, one diet may have triggered feeding, and thus increased the ingestion rates of the other diets. In order to test whether the analysed ingestion rates were influenced by the presence of the other diets in the tank, additional feeding of the diets in separate tanks might have been conducted, but in that case, the experiment would have been appreciably extended.

3.5 Statistical methods

Results have been analysed by regression and analyses of variance (ANOVA) and covariance (ANCOVA). Where significant treatment effects were detected by ANOVA or ANCOVA, the significant differences between means were analysed using Tukey's HSD. Normal probability plots and plots of fitted values versus residuals were used to examine the data for normal distribution of residuals and homogeneous variance, respectively. These plots are considered to be well suited for testing the assumption for the validity of the statistical models (Festing *et al*, 2002; Crawley, 2004). When deviations from these assumptions were present, appropriate data transformation were used as specified (Papers I, II and IV), or a non-parametric analysis was conducted (Kruskal-Wallis; Paper IV).

In the ontogenetic studies (Paper I), the samples could not be said to be independent of each other since they were collected from the same tanks and because randomisation due to time is impossible. Thus, repeated measures ANOVA was used to compare all observations of each

response variable in cod and halibut, respectively, and regression lines were fitted to parts of the data to confirm the significance of increases observed in enzyme activity or growth.

The leaching experiment in paper II also included measurements of samples collected at different time points. In this experiment, ANCOVA with the time variable (immersion time) specified as the covariate was used to solve the problem with dependent data. Thus, a summary measurement of immersion time, the slope of the regression line, was used in the comparison of experimental groups (Festing *et al*, 2002).

Normal error distribution and constant variance may not be achieved in proportion of binary data, such as survival rate. In paper IV, the survival data was analysed using logistic (and not linear) models. Such models take into account both the number of surviving and the number of dead larvae, and not only their relative proportion. By logit transformation, the odds for surviving and for dying are compared and a linear predictor is made. This allows prediction of a model using the maximum-likelihood method where binomial error distribution is specified. The models for survivals of cod and halibut in paper IV were a little over-dispersed, meaning that the models did not completely fit the data. Significance of the models was therefore tested by F-tests and not Chi-square which is usual with binary data (Crawley, 2004; Sokal and Rohlf, 1995).

4 General discussion

4.1 Intestinal maturation in cod and halibut

Decline in larval mode of digestion

Leu-ala specific activity was used as a marker for larval mode of digestion and therefore expected to decrease at the end of the experiments with both cod and halibut (Paper I). Our data fail to show this since no significant decreases in Leu-ala specific activity was found in halibut and the data on cod showed large variation throughout the sampling period, which makes it difficult to see any trend with respect to developmental progress (Figures 3c and 4c in Paper I). In seabass and Senegalese sole, Leu-ala specific activity decreased significantly from approximately 20 to 30 dph (Figure 1; Ribeiro *et al*, 1999b; Zambonino Infante and Cahu, 2001). The present study thus opposes with findings in other species and gives no indication of decline in larval mode of digestion in cod and halibut although the sampling period can be considered to cover the entire larval period of both species.

The unexpected Leu-ala specific activity profiles in cod and halibut may have several explanations. The lack of trends in Leu-ala specific activity in cod may have been caused by external influences on the ontogenetic profile and the sampling of larvae after the morning feed distribution is the most likely influential factor as discussed previously (Chapter 3.1), but also the shifts in diets during the study from rotifers to *Artemia* at 35-40 dpff and then to a compound diet at 50 dpff may have influenced the Leu-ala specific activity profile in cod (Paper I). However, Leu-ala specific activity in halibut also deviated from the expected outcome, although the data appeared valid. Possibly, the use of a homogenate of the the intestinal segment of the larvae as the enzyme extract obscured detection of ontogenetic

changes of intestinal Leu-ala specific activity since different types of tissues thus were included in the enzyme extract. Leu-ala is present in several tissues (Kim *et al*, 1972). Furthermore, dipeptidases are in general responsible for the final step of protein digestion both when the preceding steps in digestion are carried out intracellularly and in the intestinal lumen (Alpers, 1987: Henning, 1987; Krogdahl, 2001; Zambonino Infante and Cahu, 2001). In mature digestion, dipeptides present in the intestinal lumen are either hydrolysed by bbm-bound dipeptidases immediately before absorption or by cytosolic dipeptidases just afterwards (Alpers, 1987: Krogdahl, 2001; Bakke-McKellep *et al*, 2000). These two transport pathways are difficult to distinguish and their relative importance in digestion is still uncertain (Sire and Vernier, 1992). In the intestine of rat, 70-90 % of the Leu-ala activity is determined in the cytosolic cell fraction (Kim *et al*, 1972; Nicholson and Kim, 1975), indicating that Leu-ala mainly represents cytosolic dipeptidase activity. However, the bbm-bound fraction can be underestimated due to incomplete separation of cell fractions and disintegration of enzymes from the bbm during sample preparation (Kim *et al*, 1972; Ugolev and De Laey, 1973). Another issue that may mask the detection of a relationship between Leu-ala activity and intracellular digestion is that intracellular digestion takes place in the hindgut, whereas Leu-ala is present also in other parts of the intestine. In the rat, certain dipeptidases present in the distal intestine are shown to have temporary higher activity during the suckling period, suggesting involvement in the final step of intracellular digestion (Vaeth and Henning, 1982; Henning, 1987). However, the proximal section of the intestine in the suckling rat is shown to have a different ontogentic profile of the same dipeptidases (Vaeth and Henning, 1982; Henning, 1987). Thus, although other studies using the similar dissection method as in paper I have shown significant decrease in Leu-ala specific activity during the larval stage of other species (Cahu *et al*, 1998b; Ribeiro *et al*, 1999b; Zambonino Infante and Cahu, 2001), the

presence of more tissues in the enzyme extract may have obscured detection of the developmental pattern expected for Leu-ala specific activity in halibut in paper I.

Achievement of adult mode of digestion

Distinct elevation in bbm enzyme specific activities is considered to indicate a significant step of intestinal maturation in both fish and mammals (Reisenauer *et al*, 1992; Henning *et al*, 1994; Zambonino Infante and Cahu, 2001). AP and LAP specific activities in cod and halibut increased significantly from 30 to 40-50 dpff, corresponding to larval sizes 7-9 mm SL in cod and 18-22 mm SL and 50-120 mg wet weight in halibut (Figures 3a-b, 4a-b and 5a-b, paper I). A concomitant increase in bbm-bound AP activity in percent of of total AP activity in the intestinal segment of the larvae from 2-5 % to 15-20 % , which also applied to LAP in halibut (Figures 5a-b, paper I), suggests an increase in the importance of bbm-bound enzyme in digestion from the time the increases occurred. Similar abrupt increases in bbm-bound AP and LAP specific activities have previously been found in senegalese sole, seabass and large yellow craoker at around 25 dph (Ribeiro *et al*, 1999b; Zambonino Infante and Cahu, 2001; Ma *et al*, 2005). Hence, the increases in bbm-enzyme activities seem to reflect a general developmental pattern in marine fish larvae and therefore paper I appears to specify the time when the intestine in cod and halibut attains its mature function.

As compared to AP, LAP showed a less marked increase in specific activity in cod, and also a delayed increase in activity ratio of bbm-bound LAP versus total LAP in the intestinal segment of the larvae (Figures 3b and 5b, paper I). This complicates interpretation of the data. It is argued in section 3.1 that the methodological shift between 29 and 37 dpff, which corresponded to the time when the bbm-enzyme specific activities markedly increased, did not cause the elevation in bbm-enzyme specific activities. The inconsistent LAP activity

profiles may relate to the feeding state of the larvae as is also described in section 3.1. However, Cousin *et al* (1987) noted in a semi-quantitative study with turbot larvae that LAP was not affected by feeding. Another possible explanation is that LAP reaches a maturational activity level at a later stage than AP in cod. A less steep increase in the activity of LAP than of AP is also described in other species (*Pseudoscriaena crocea*; Ma *et al*., 2005). The activity of LAP may depend on surplus availability of oligopeptides as substrate in the gut lumen for being induced, and thus on the diet composition and on the activities of gastric and pancreatic enzymes which act on the dietary protein prior to LAP. A possible relationship between maturational increase in LAP activity and the development of gastric digestion has been suggested in the rat (Reisenauer *et al*., 1992; Reisenauer and Castillo, 1994). In cod, the stomach is considered to develop lately as compared to development of other organs that characterise the larvae to juvenile transition (Pedersen and Falk-Petersen, 1992). Pedersen and Falk-Petersen (1992) found that the stomach started to develop at 15 mm SL in cod, which in the present study corresponds to the size of the larvae at 60-65 dpff and to the time when the activity ratio of bbm-bound LAP to total LAP in the intestinal segment of the larvae increased. Thus, the less clear profile of LAP activity may relate to the developmental process of the stomach.

The elevated activities of bbm enzymes logically occur when the intestinal mucosa becomes extensively folded. At the early larval stages, the intestinal mucosa goes from having a smooth surface at hatching to having wave-like folds in the early first-feeding stage before conical folds become abundant (Kjørsvik and Reiersen, 1992; Kurokawa and Suzuki, 1998, Luizi *et al*, 1999; García Hernández *et al*, 2001; Elbal *et al*, 2004). In these folds, mature enterocytes are found in the fold apices (Ugolev and De Laey, 1973; Cousin *et al*, 1987; Krogdahl, 2001). Concomitant appearance of intestinal folds and increased bbm-enzyme

activities are found in other species (Zambonino Infante and Cahu, 2001). When comparing the current results of elevated bbm-enzyme activities with histological studies (Kjørsvik and Reiersen, 1992; Luizi *et al*, 1999), parallel development of these characteristic events were present in cod, but to a lesser degree in halibut (Paper I). The latter might be due to difficulties in comparing the studies, especially since larval growth was not noted by Luizi *et al* (1999), as well as due to different rearing conditions used.

4.2 Protein digestive capacity in cod and halibut larvae

To obtain a comprehensive overview of the capacity of protein digestion, the study conducted in paper I together with published data on other proteases will give some indications. In both cod and halibut, only small changes in AP and LAP activities were present during the first 30 days after first-feeding (Figures 2-5, paper I), suggesting low digestive capacity during this period. However, activities of other digestive enzymes, especially those secreted from the pancreas, will also contribute to the digestive capacity. In cod, trypsin-like and general protease specific activities were constant until 6 dph (60 daydegrees; dd) and then increased two- to threefold at 8 dph (78 dd, Perez-Casanova *et al*, 2006). Later, decreases in trypsin-like and general protease specific activity occurred, and these decreases can probably be attributed to a dilution effect related to increase in soluble protein as the larvae develop (Zambonino Infante and Cahu, 2001), or increasing levels of protease inhibitors in the enzyme extract (Perez-Casanova *et al*, 2006). In halibut, unchanged trypsin weight-specific activity from 7 to 13 dpff was found when assayed in whole larvae, and then a twofold increase from 13 to 26 dpff occurred when assayed in dissected guts (0.9-2.0 mg dry wt., corresponding to 5.5-12.5 mg wet wt. when assuming 16 % dry matter content, Rojas-García and Rønnestad, 2002). Thereafter, a fourfold increase until 40 dpff was found. A similar increase in trypsin specific activity from first-feeding to 34 dpff (56±9 mg

wet wt.) in halibut is confirmed by Gawlicka *et al* (2000). These results indicate that pancreatic enzymes also stay low in activity in cod and halibut during the 1-2 first weeks after first-feeding, but thereafter contribute to improve digestive capacity at an earlier developmental stage than bbm enzymes.

The increase in Leu-ala specific activity in halibut from 1 to 34 dpff may indicates increasing protein digestive activity during this developmental period (from 5.4 to 23.7 mg wet wt.; Figure 3c, paper I). Regardless of the presence in the cytosol or bbm, and regardless of larval or adult mode of digestion, dipeptidases are thought to carry out the final step in protein digestion (Vaeth and Henning, 1982; Alpers, 1987; Krogdahl, 2001; Zambonino Infante and Cahu, 2001). The increases in Leu-ala and tryptic activity (Gawlicka *et al*, 2000; Rojas-García and Rønnestad, 2002) appear to be positively correlated and thus they may be assumed to reflect the theory that trypsin and dipeptidases initiates and finish protein digestion, respectively (Sire and Vernier, 1992; Krogdahl, 2001; Zambonino Infante and Cahu, 2001). 2-3.5-fold increase in Leu-ala specific activity also occurred in seabass larvae from 8 to 12 dph, and thereafter the activity was fairly similar for 4 days (Cahu *et al*, 1998b). The opposite has, on the other hand, been observed in Senegalese sole, where Leu-ala specific activity was 3-4 times higher from 2 to 6 dph than from 9 to 18 dph (Ribeiro *et al*, 1999b). However, this early decline in Leu-ala activity might not be related to digestion as it occurred only few days after the start of exogenous feeding. It is therefore possible that the increased Leu-ala specific activity indicates improved digestive capacity in halibut during the first 5 weeks after first-feeding (Paper I).

The elevated bbm-enzyme activities present at 40-50 dpff in both cod and halibut indicate improved digestive capacity from this developmental stage. Also, the elevations indicate that

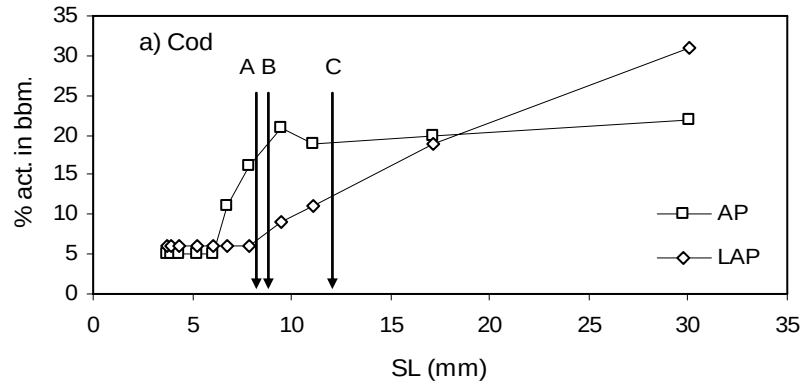
absorption might be more efficient from this stage. The position of the bbm enzymes close to the absorptive site facilitates efficient removal of liberated AAs and small peptides from the lumen (Alpers, 1987; Krogdahl, 2001). Conversely, accumulation of digestive products in the lumen reduces the efficiency of digestive enzymes due to end-product inhibition (Ugolev and De Laey, 1973). Hence, the digestion is probably markedly improved from the time when the bbm-bound enzymes show the maturational increase in activity.

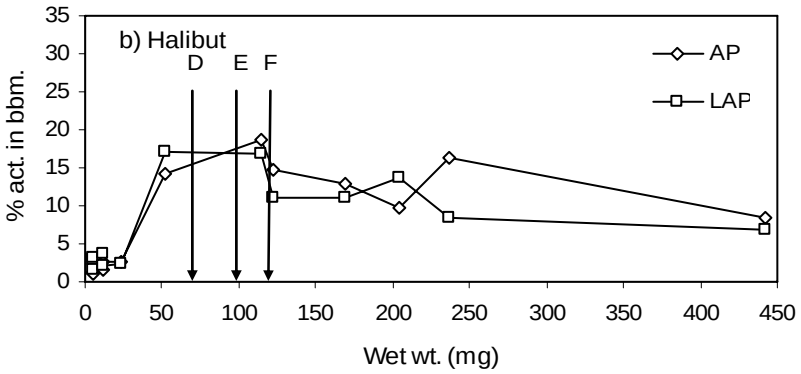
The more than twofold higher AP and LAP specific activities in the group of halibut sampled at 78 dpff that had settled to the botton as compared to the pelagic group sampled at the same day and also as compared to the two previous samples, suggest improved digestive capacity in the settled group (Figure 3a, paper I). The group that had settled consisted of individuals that had achieved a juvenile appearance, and the pelagic group consisted of individuals that were smaller and had a more larval pigmentation pattern, but still had achieved asymmetric body (Paper I). Luizi *et al* (1999) describe development of the stomach during the metamorphic stage in halibut and Stoss *et al* (2004) reports that gastric digestion becomes fully developed at approximately 80 dpff (11°C). Thus, the stomach had probably started to develop in both groups sampled at 78 dpff, but the higher AP and LAP specific activities in the most developed group might be a response of a more developed stomach.

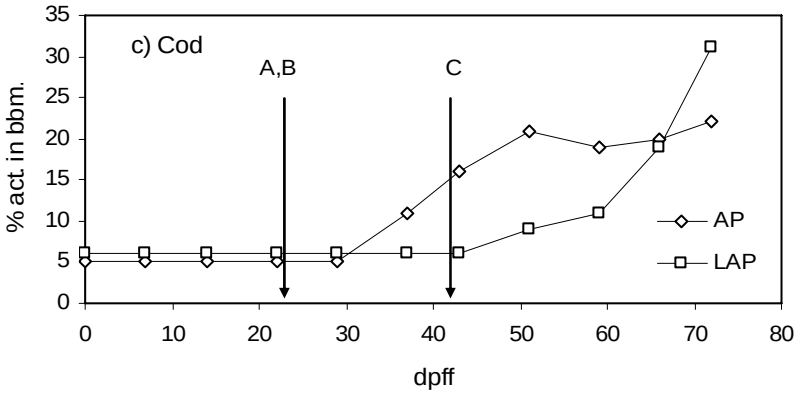
In cod, the stomach is found to develop in individuals larger than 15 mm SL and gastric glands are noted in larvae with a SL of 18-20 mm (Pedersen and Falk-Petersen, 1992). Furthermore, pepsin transcript and activity is detected from 45 dph (476 dd, 9-12°C, Perez-Casanova *et al*, 2006). Thus, the stomach had probably started to develop at the end of the experiments with cod also (72 dpff, 30 mm SL, Paper I).

4.3 Prediction of a time for early weaning of cod and halibut

The problem with reduced growth and survival in early weaning of marine fish larvae is thought to be related to the immature gastrointestinal tract, causing the digestive capacity to be low. Thus, intestinal maturation may increase the probability for successful weaning Comparing the time-course of intestinal maturation described in paper I with the early weaning studies with cod and halibut presented in Table 1 (Baskerville-Bridges and Kling, 2000b; Hamre *et al*, 2001; Næss *et al*, 2001; Callan *et al*, 2003) and papers III and IV may indicate whether weaning success and intestinal maturation is correlated. These weaning studies can be considered to fairly well indicate the earliest time when cod and halibut can be weaned onto current available compound diets since: 1) Co-feeding with live feed was necessary. Baskerville-bridges and Kling (2000b) assumed that the cod larvae were not prepared for receiving only compound diet after co-feeding for one week and therefore continued with rotifer supplementation for one more week. 2) High mortality of cod larvae just after termination of rotifer supplementation indicated difficulties in digesting the compound diet (Paper IV). 3) The lipid content in the weaned halibut was reduced at the end of the study, but not in the *Artemia* control group (Paper III), and 4) growth in the early weaned groups was reduced as compared to live feed controls or larvae weaned at a later developmental stage (Baskerville-Bridges and Kling, 2000b; Hamre *et al*, 2001; Paper III; Callan *et al*, 2003). According to these studies, cod and halibut can be completely weaned from the ages of 22-42 dpff (*i.e*. 20-38 dph) and 29-47 dpff, respectively, corresponding to 8-12 mm SL and 70-120 mg wet weight in cod and halibut, respectively. When comparing on basis of the larval sizes, the time of weaning and intestinal maturation seem to be correlated (Figures 3a-b), but when comparing on basis of larval ages, the weaning studies seem to have started when the intestine still was immature (Figures 3c-d). Larval development, however, usually shows higher correlation to larval size than to larval age since fish are ectothermic and







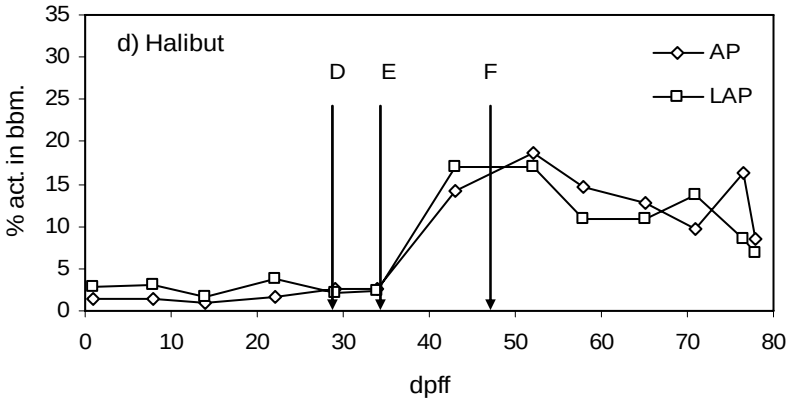


Figure 3. Comparisons of intestinal maturation and time of weaning in Atlantic cod (a and c) and Atlantic halibut (b and d) when comparisons are based on larval size (a and b) and on larval age (c and d). References for the weaning studies are: A, Callan *et al*, 2003; B, Baskerville-Bridges and Kling, 2000b; C, Paper IV; D and E, Hamre *et al*, 2001 / Næss *et al*, 2001; F, Paper III. Weaning is registered when supplementation of live feed was finished, except for that larval size was only determined before the co-feeding period (approximately 1 week) in references C-F.

rearing condition will affect (almost) all developmental processes including growth. This favours the comparisons based on larval size, and thus supports that there might be a connection between intestinal maturation and success of weaning**.** Further support for this is given by the weaning practise of cod by a commercial hatchery where compound diets are introduced from 7.5-8 mm SL, corresponding to 22-27 dph, followed by a gradual reduction in rotifer supplementation during 8 days (Børre Erstad, CCN, pers.com.). However, the weaning period can also start from 6 mm SL, although with a small reduction in biomass outcome. Based on this, there are some indications that intestinal maturation can increase the success of weaning, but a clear conclusion is impossible to make, partly due to the practice of co-feeding which complicates the interpretation of when the larvae actually start digesting the compound diet without any influence of the live feed offered.

Studies with other species may help to elucidate the importance of intestinal maturation for achieving good weaning results. Successful weaning of seabass larvae has been obtained from first-feeding or after 5 days on live feed (Cahu *et al*, 1998a; 1999; 2003; 2004; Fontagné *et al*, 2000; Zambonino Infante and Cahu, 2001), indicating that the protein in the compound diets used was available to these larvae before maturation of the intestine at approximately 30 dph (Zambonino Infante and Cahu, 2001). In Senegalese sole, a mature intestine is obtained at similar age as in seabass (Ribeiro *et al*, 1999b). Weaning studies starting earlier than this, *i.e*. at 3-8 dph, have reported mortalities above 95 % (Cañavate and Fernández-Díaz, 1999; Fernández-Díaz *et al*, 2001) or, when obtaining high survival (> 70 %), the larvae showed clear signs of impaired nutrient assimilation in the liver and intestine (Fernández-Díaz *et al*, 2006). This indicates that the sole larvae were not well prepared for weaning at this early stage. Weaning at later stages (beyond 30 dph) has, with some exeptions, given good results (Table 1; Cañavate and Fernández-Díaz, 1999; Engrola *et al*,

2001; Ribeiro *et al*, 2002). These studies may not be fully representative for comparing intestinal maturation and success of weaning, but they indicate that a clear conclusion on this topic is difficult to make. Several factors will affect the weaning results, and in particular the composition of the diet and how well the diet fulfils the requirement of the fish larvae. Furthermore, there might be species differences in development and behaviour that alter their readiness for digesting compound diets. Clearly, more research is needed for increasing the knowledge of what factors that impact the weaning outcome and also how to improve compound diets to meet larval demands.

4.4 Selection of a feed for delivery of hydrolysed protein

To increase the success in weaning of fish larvae, the diets need to fulfil the larval nutrient requirements. One method that may increase the availiablity of protein is to pre-digest it before inclusion into the diet. However, one has to be sure that the diet used is able to deliver this ingredients to the fish larvae.

Nutrient leaching

High leaching rates of FAAs from microparticulate diets (Table 4; López-Alvarado *et al*, 1994; Ozkizilcik and Chu, 1996; Baskerville-Bridges and Kling, 2000a; Yúfera *et al*, 2002; Önal and Langdon, 2005) suggest that losses of hydrolysed protein can be significant. Paper II confirms this hypothesis, although the leaching rates of hydrolysed protein were not exactly determined due to the low protein content of the algae extract, as is discussed in section 3.3. The almost similar leaching rates of hydrolysed protein as compared to leaching of FAAs from the microbound diets are illustrated in Figure 4 which shows the determined leaching rates of serine and estimated leaching rates of hydrolysed protein and intact protein from the three diets tested. The figure also clearly shows that leaching rates differed among the diets,

with the lowest leaching rates obtained for the protein-walled microencapsulated diet. This agrees with other studies where leaching rates of FAAs from protein-walled microencapsulated and microbound diets are compared (Table 4; López-Alvarado *et al*, 1994; Yúfera *et al*, 2002). Thus, the leaching study in paper II reflects previous findings and in addition shows that leaching of hydrolysed protein may approach FAA leaching in some diets.

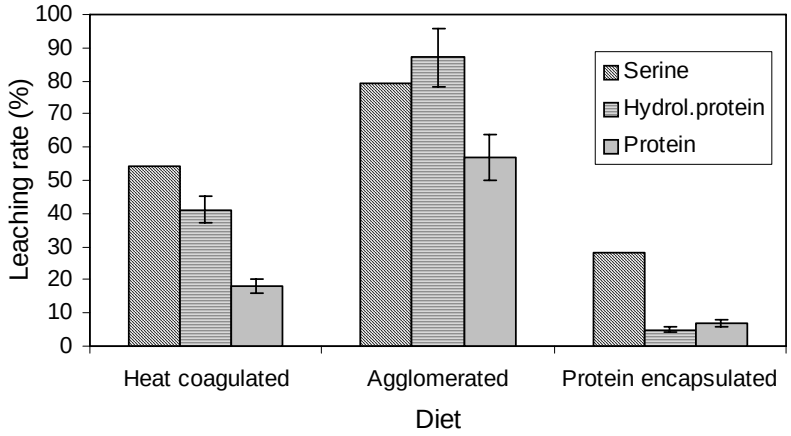


Figure 4. Leaching rates of serine, hydrolysed protein and intact protein from the heat-coagulated, the agglomerated and the protein-walled microencapsulated diets after 5 min submersion in water. The data represent estimated leaching rates of hydrolysed protein and intact protein and measured leaching rates of serine. The bars represent upper and lower leaching estimates as described in paper II. Particle sizes are 0.3-0.6 mm for the heat-coagulated and agglomerated diets and 0.12-0.32 mm for the protein-walled microencapsulated diet.

The lower leaching rates from the protein-walled microencapsulated suggests this as a suitable diet for delivery of hydrolysed protein to marine fish larvae. However, the most readily leached labelled compounds were probably removed from this diet during processing due to several washing steps. This has recently been found to occur with low-molecular

weight water-soluble compounds (A. Nordgreen, pers. com.), and is also indicated by the low encapsulation efficiency of FAAs (7.5 %, Yúfera *et al*, 2002) and the higher leaching from protein-walled microencapsulated diets which were not washed during production (FAAs, 45-60 % after 2-5 min. submersion in water; Table 4; López-Alvarado *et al*, 1994; Ozkizilcik and Chu, 1996) as compared to diets that were washed (FAAs, 2-28 % after 5 min. submersion in water; Table 4; Figure 4; Yúfera *et al*, 2002; Paper II). In addition, it was uncertain whether the labelled compounds were covalently bound in the particles due to the cross-linking process, and this is supported by a recent finding that almost all of the soluble protein disappears from the diet after the polymerisation process (A. Nordgreen, pers. com.). Thus, although this diet appeared to have very low losses of water-soluble nutrients, the quantity of hydrolysed protein present in this diet in free form, was uncertain.

Extensive release of the labelled compounds was observed from the two microbound diets, and especially from the agglomerated diet, where leaching of hydrolysed protein was estimated to be 40-50 % after 1 minute and twice as high after 5 minutes in water when the diet particle size was 0.3-0.6 mm (Figure 4**;** Paper II). Compared to the two other diets, the agglomerated diet disintegrated more easily in water, which may explain the higher leaching observed. The heat-coagulated diet leached to a lesser extend, although it lost approximately 40 % of the hydrolysed protein within 5 minutes in water (Particle size 0.3-0.6 mm, Figure 4). The rapid and high leaching from these diets agrees with other leaching studies with microbound diets where losses of FAAs are found to be above 60 % within 2 minutes immersion in water (López-Alvarado *et al*, 1994; Baskerville-Bridges and Kling, 2000a; Önal and Langdon, 2005; Hamre, 2006). Altogether, this significant reduction in the content of FAA, hydrolysed protein and intact protein from the microbound diets suggests leaching as a

major problem in feeding of fish larvae and other small organisms living in water with microparticulate diets.

The high and rapid loss of water-soluble nutrients from microbound diets makes it necessary to improve these diets. The study in paper II showed that leaching rates were significantly affected by the size of the diet particles. Thus, as long as the particles can be ingested by the larvae, small adjustment of particle size may reduce the nutrient leaching. Furthermore, the data in paper II also showed a general decline in leaching rates as the molecular size of the leaching compound increased (protein<hydrolysed protein<serine; Table 5, paper II), and thus suggests addition of soluble protein instead of hydrolysed protein in weaning diets, to reduce losses from the diet particles. Although intact, soluble protein is less efficiently utilised by halibut larvae than is hydrolysed protein (Tonheim *et al*, 2005), Carvalho *et al* (2004) showed that soluble protein was a potent diet ingredient for improving growth and survival of carp larvae. Another possibility for improving nutrient retention capacity of microbound diets is embedding lipid-based particles into microbound diets. This has a potential to reduce leaching rates depending on the lipid composition of the lipid-based particles (Table 4; Baskerville-Bridges and Kling, 2000a; Önal and Langdon, 2005). Hence, there are possibilities for modulating the losses of water-soluble nutrients, although a considerable amount of work seems to remain for development of a microparticulate diet with high capacity to retain water-soluble nutrients.

Feed preference in cod

Determination of the ingestion rates of the three diets gives an indication of the potentials of the diets to support larval growth and development. Based on previous feeding studies with different marine fish larval species, all three diets could be considered to be suitable weaning

diets for cod and halibut (Yúfera *et al*, 2000; Hamre *et al*, 2001; Næss *et al*, 2001; Engrola *et al*, 2001; Rueda-Jasso *et al*, 2001). In the preference study in paper II, the cod larvae showed the highest ingestion rates of the heat-coagulated and agglomerated diets when the experiments were conducted from 39 dph (5.6±2.5 mg wet wt.) and 49 dph (15. 8 ±7.2 mg wet wt.), respectively. The protein-walled microencapsulated diet was ingested at the lowest rate in both experiments. Therefore, the heat-coagulated, protein-bound and the agglomerated diets were considered more suitable in early weaning of cod than the protein-walled microencapsulated diet.

Comparison of the feeds

The low leaching rate from the protein-walled microencapsulated diet compared to the heat-coagulated and agglomerated diets, suggests this diet as a suitable tool for delivery of hydrolysed protein to cod and halibut larvae. However, the low preference in cod, production disadvantages (*i.e*. lab-scale production facilities, excess use of organic solvent) along with uncertainty of binding of peptides during cross-linking and leaching during production, excluded this diet from further studies. Although cod had high preference for the agglomerated diet, the high leaching rates of hydrolysed protein from this diet made it unsuitable for our purposes. The heat-coagulated, protein-bound diet therefore was chosen for further studies, even though leaching rates were also considerable from this diet.

4.5 Effects of hydrolysed protein in weaning diets for cod and halibut

The significant leaching of hydrolysed protein from the heat-coagulated, protein-bound diet and the high nutrient leaching from formulated microdiets in general (López-Alvarado *et al*, 1994; Ozkizilcik and Chu, 1996; Baskerville-Bridges and Kling, 2000a; Önal and Langdon, 2005; Hamre, 2006; Paper II) question the use of FAAs and hydrolysed protein in larval

compound diets. However, significant biological effects of dietary supplementation with hydrolysed protein and other water-soluble nutrients, as measured as increase in larval performance, have been found (Table 3; Pérez *et al*, 1997; Zambonino Infante *et al*, 1997; Cahu *et al*, 1999; Dabrowski *et al*, 2003; Carvalho *et al*, 2004), indicating that microdiets are able to deliver such compounds.

Effects on growth, survival and intestinal maturation in cod

Survival rates of cod weaned from 41 dph increased with increasing content of hydrolysed protein when 0 to 40 % exchange of dietary protein with hydrolysed protein were tested (Paper IV). Better survival when cod are weaned onto diets added 40 % hydrolysed protein as compared to weaning onto diets not added hydrolysed protein has later been confirmed in a study with juveniles (Åsnes, 2006). The level of dietary hydrolysed protein did not affect growth in any of these studies (Åsnes, 2006; Paper IV). In some other species, 50 % or higher inclusions of hydrolysed protein seem disadvantageous (Cahu *et al*, 1999; Carvalho *et al*, 2004; Kolkovski and Tandler, 2000), but with some exceptions (Day *et al*, 1997; Oliva-Teles *et al*, 1999). In paper IV, specific activities of bbm-bound LAP supported that hydrolysed protein supplementation was beneficial for intestinal maturation, but the activity was highest in cod receiving 20 and 30 % hydrolysed protein. This indicates that far higher levels of hydrolysed protein could be disadvantageous. Thus, 40 % exchange of dietary protein with hydrolysed protein appears optimal in weaning diets for cod.

Effects on growth, survival and intestinal maturation in halibut

In paper III, three different methods for dietary protein hydrolysis were tested because peptide composition was thought to affect larval performance. Survival rates of halibut was higher when the larvae received pepsin hydrolysed protein as compared to larvae receiving more

extensively hydrolysed protein, indicating that the protein hydrolysate preferably should consist of longer peptides. This agrees with a study revealing beneficial effects when increasing the molecular size of the peptides included in the protein hydrolysate of a diet for carp larvae (Carvalho *et al*, 2004). Thus, supplementation of a gently hydrolysed/solubilised protein appears to improve larval performances as compared to more extensively hydrolysed protein.

Survival rates of halibut larvae increased with reduced inclusion of hydrolysed protein (Papers III and IV). Despite this, paper III does not exclude that a 10 % supplementation of hydrolysed protein could be beneficial since the survival rate was higher, although not significantly higher, in the larvae fed 10 % hydrolysed protein as compared to the larvae fed the non-supplemented diet (67±4 % vs 57±5 % survival rates, respectively). In paper IV, the relationship between inclusion level of hydrolysed protein and survival was more linear through all experimental groups. Moreover, specific activities of AP and LAP supported the conclusions based on survival, being in general highest in the larvae fed none or 15 % hydrolysed protein. Cod fillet was used as the main protein source in the diet for halibut, and fish fillet is known to contain FAAs and soluble proteins (Huss, 1988). Thus, the studies suggest that the protein source used in the weaning diet contained fairly adequate level of soluble protein compounds and that no or only a small dietary supplement of hydrolysed protein was needed in the weaning diet to halibut.

The slight disagreement in optimal level of hydrolysed protein in weaning diets for halibut may relate to the shallow raceways with low water level used in paper III. The raceways may have facilitated for a quicker ingestion rate and thus a lower leaching rate prior to ingestion by the larvae than did the tanks used in paper IV. Alternatively, the possible benefit of 10 %

supplementation of hydrolysed protein indicated in paper III, but not in paper IV, might also be due to a smaller size of the larvae at onset of the experiment in paper III (120±40 mg wet wt.) as compared to in paper IV (221±69 mg wet wt.). However, a combination of both explanations may also be possible.

In the present study, growth appeared as a less sensitive response parameter than survival. In papers III and IV, no growth differences were present in any of the experiments with halibut or cod (Papers III and IV), although halibut in paper IV tended to grow faster when receiving none or 15 % hydrolysed protein as compared to the higher inclusion levels. In weaning studies with seabass and carp larvae, optimal level of hydrolysed protein was indicated by performances in both growth and survival (Zambonino Infante *et al*, 1997; Cahu *et al*, 1999; Carvalho *et al*, 2004), whereas in other studies either growth (Kolkovski and Tandler, 2000) or survival (Day *et al*, 1997) have been altered by the dietary level of hydrolysed protein. In cases when growth effects are not detected, this may be due to that retardation of growth early in the experiment can be compensated later. The discrepancies in response of growth and survival in these weaning studies is difficult to explain and may relate to variation in the experiments, rearing conditions and in the health and developmental state of the larvae. In any case, both growth and survival are important parameters for evaluation of experiments with fish larvae, although more treatment-specific parameters are needed to increase the knowledge of the biological mechanisms that cause treatment effects.

Temporary beneficial effects of hydrolysed protein at weaning

Hydrolysed protein may have a temporary beneficial effect in a short period after weaning when the larvae or juveniles adapt to the compound diet. This is suggested by growth and survival rates obtained in weaning studies with carp larvae and juvenile Dover sole where

positive effects of hydrolysed protein supplementation was found at onset of weaning. After one or four weeks, respectively, there were no effects (*Solea solea*, Day *et al*, 1997; Carvalho *et al*, 2004). The present study supports this suggestion, but the effects are not statistically significant. In cod, LAP specific activity tended to be highest in the groups supplemented with 20 and 30 % hydrolysed protein, suggesting this as the optimal hydrolysed protein level at the end of the study. Survival rates, which showed that 40 % supplementation was optimal, likely was determined at the start of the experiment when supplementation with *Artemia* finished at day 5 of the experiment. Just after this, there was a high rate of mortality, whereas later during the experiment, survival rates remained low (Paper IV). According to AP specific activity in halibut, 15 to 30 % hydrolysed protein appeared optimal on day 13 of the experiment, whereas survival rates and AP and LAP specific activities analysed later showed that hydrolysed protein supplementation was disadvantageous (Paper IV). Thus, paper IV supports the hypothesis that hydrolysed protein supplementation is most important for a short period at onset of weaning. However, more studies are needed to confirm this for cod and halibut.

Comparison of cod and halibut

The clear differences in optimal hydrolysed protein in weaning diets for cod and halibut is surprising since both species are expected to have high requirement of highly digestible protein due to the combination of a long period of development of the digestive tract and high demands for protein (Kjørsvik *et al*, 1991; Pedersen and Falk-Petersen, 1992; Luizi *et al*, 1999; Rønnestad *et al*, 2003). Moreover, halibut larvae utilise hydrolysed protein more efficiently than intact protein when given by tube-feeding (Tonheim *et al*, 2005). This contrasts to the negative effects of hydrolysed protein supplementation in weaning diets found in papers III and IV. Comparing with other species, 20 to 25 % substitution of the dietary

protein with hydrolysed protein was optimal for seabass larvae (Zambonino Infante *et al*, 1997; Cahu *et al*, 1999), whereas 6 % appears optimal for carp larvae, given that 25 % of the dietary protein was soluble (Carvalho *et al*, 2004). Thus, the optimal level of hydrolysed protein seems to vary considerably between species, although they can be expected to have fairly similar benefits of readily digested protein.

Dietary hydrolysed protein is likely more beneficial before the gastrointestinal tract has matured. In the experiments described in paper IV, the cod larvae were probably weaned at an earlier developmental stage than halibut. This may explain some of the different results obtained for the two species. However, cod weaned at 85 dph and 330 mg wet weight still took advantage of dietary hydrolysed protein (Åsnes, 2006), indicating that developmental stage at onset of weaning was not the major cause for the differences found between cod and halibut.

The different optimal levels of hydrolysed protein for cod and halibut may relate to the fact that halibut are slow feeders whereas cod consume the feed offered much faster. The slow feeding rate of halibut, combined with the rapid nutrient loss from microparticulate diet particles (Paper II), may result in almost equal content of hydrolysed protein in the diet particles at ingestion regardless of inclusion level. Moreover, the total protein content of the diet is also likely reduced in correlation with the hydrolysed protein inclusion level. Juvenile halibut appears to have a high dietary protein requirement (58 %, Hamre *et al*, 2003; 2005b), and thus the reduced survival rates observed when the larvae were fed a high level of hydrolysed protein in paper III and IV might reflect protein deficiency. Cod, on the other hand, has both a lower protein requirement (40 %, Hamre and Mangor-Jensen, 2006; Åsnes,

2006) and ingest the feed rapidly. Therefore, high leaching rates will probably have much less impact on the performance of cod than on the performance of halibut.

5 Conclusions

Intestinal maturation

- Intestinal maturation in Atlantic cod and Atlantic halibut was estimated by abrupt increases in specific activities of brush border membrane (bbm) alkaline phosphatase (AP) and leucine aminopeptidase N (LAP).
- In cod, intestinal maturation was estimated at 40-50 dpff (43-53 dph), which corresponded to the time when the larval size was 7-9 mm standard length (SL).
- In halibut, intestinal maturation was estimated at 40-50 dpff, which corresponded to the time when the larvae were 18-22 mm SL and 50-120 mg wet weight.
- Decline in larval mode of digestion was expected indicated by decrease in leucylalanine peptidase (Leu-ala) specific activity, but this decrease did not occur in our experiments with cod and halibut**.**
- Increasing Leu-ala specific activity from first-feeding to 34 dpff in halibut indicates gradual improvement in digestive capacity during this period. Further improved digestive capacity is suggested in both species at the time of maturational increase in AP and LAP specific activities.
- Intestinal maturation is suggested to be a significant factor for successful early weaning, but a clear conclusion was not possible to make.

Effects of hydrolysed protein in weaning diets for cod and halibut

- Hydrolysed protein was found to leach rapidly from microparticulate diets, depending on type of feed, feed particle size and the molecular weight of the dietary peptides (*i.e.* protein<hydrolysed protein<FAA). This will influence the optimal level of hydrolysed protein determined for cod and halibut.

- When cod larvae were weaned onto a diet with graded levels of pepsin hydrolysed protein up to 40 % exchange of the protein, survival rates increased with increasing levels of hydrolysed protein.
- Similar studies with halibut showed that survival rates decreased with increasing levels of hydrolysed protein, although in one of the studies, 10 % supplementation of pepsin hydrolysed protein appeared to be advantageous, however not significantly.
- Specific activities of AP and LAP were correlated with survival rates, supporting the conclusions of optimal level of hydrolysed protein.
- Dietary hydrolysed protein had minimal effects on growth rates.
- The different optimal levels of hydrolysed protein in cod and halibut were considered mainly due to different feeding behaviour in these two species, combined with the high leaching rates of hydrolysed protein. A slow feeding rate in halibut allows extensive losses of water-soluble nutrients from the diet particles prior to ingestion. Cod ingest the diet particles quicker and thus when the particles still contain some hydrolysed protein. The diet particles ingested by halibut might have had sub-optimal level of protein when inclusion level of hydrolysed protein was high, which may explain the reduced performance of the larvae with increasing level of dietary hydrolysed protein.

6 Future perspectives

Larval mode of digestion

A lot of questions about the larval mode of digestion remain to be answered, both concerning the digestive processes and the regulatory mechanisms of digestion. The importance of intracellular protein digestion for nutritional purposes need to be better understood. If a significant portion of the dietary protein ingested by the larvae is assimilated via intracellular digestion, this will likely put other demands for the dietary protein quality than will the digestive processes in the lumen. Thus, elucidation of the importance of intracellular digestion in dietary protein assimilation will give valuable information for diet formulation.

The lower ability of compound diets as compared to live feed to sustain larval growth and development suggests that live feed assist in larval digestion. The supply of digestive enzymes from live feeds appears to be low (Munilla-Moran and Stark, 1989; Kurokawa *et al*, 1998; Gawlicka *et al*, 2000), while stimulatory effects of live feed appears more predominant (Munilla-Moran and Stark, 1989; Beccaria *et al*, 1991; Kolkovski *et al*, 1997a; b). The mechanisms for this stimulation of larval nutrient assimilation may perhaps relate to alternative regulation of digestion in larvae than in adults since digestive hormones may be present at lower levels in larvae (Kamisaka *et al*, 2001). More knowledge about this would be useful for improvement of compound diets and for design of feeding regimes, especially concerning co-feeding live and inert diets.

The Leu-ala results in the present study, along with the uncertainty of the role of intracellular digestion in nutrition and the diverse role of dipetidases in cellular metabolism, suggest that

Leu-ala activity may not be a suitable marker for the larval mode of digestion. In the rat, the hypothesis that some dipetidases are involved in the final step in intracellular protein digestion is based on enzyme activity determinations in the cytosolic fraction of the distal section of the intestine (Vaeth and Henning, 1982). However, dipetidases are present as cytosolic enzymes also in the proximal intestine and in other tissues, and they are present in the bbm as well (Kim *et al*, 1972; Vaeth and Henning, 1982; Krogdahl, 2001). Thus, Leu-ala activity measured in the intestinal segment of the larvae or in whole larvae may not reflect a typical larval digestive feature properly and this suggests that more markers for the larval mode of digestion should be defined.

Digestive capacity and achievement of adult mode of digestion

For improvement of digestive capasity, three maturational processes in the gastrointestinal tract are of particular importance. These are the development of an efficient pancreatic enzyme secretion, maturational elevation of bbm-enzyme activities, and development of the stomach with gastric digestion and feed storage capacity (Zambonino Infante and Cahu, 2001). Although a comprehensive study on digestive capacity with cod exists (Perez-Casanova *et al*, 2006) and more studies give indications of the digestive capacities in cod and halibut (Gawlicka *et al*, 2000; Hjelmeland *et al*, 1984; Rojas-García and Rønnestad, 2002; Paper I), the maturational processes of the gastrointestinal tract need to be better characterised in these two species. Furthermore, the regulation of these processes in fish needs to be better understood. The ability to secrete pancreatic enzymes are especially interesting to know more about since these enzymes are very important at the larval stage before gastric digestion develops. Next, the less clear time of intestinal maturation in cod observed in paper I, indicates that intestinal maturation should be further studied in cod and that such a study also should focus on stomach development for examining whether achievement of a mature level

of LAP activity is coordinated with development of the stomach. Furthermore, comprehensive studies on the onset of gastric digestion, including peptic activity analyses, stomach histology and stomach acidification, in both species would give valuable information.

Nutrient leaching

The high leaching rate from microparticulate diets suggests this as a major problem since many essential nutrients are water-soluble. Therefore, techniques for improving the ability of microdiets to retain water-soluble nutrients need to be developed. One possible solution for this problem is the use of complex diet particles (Langdon, 2003; Önal and Langdon, 2005), and another possibility is to offer the larvae several types of diets which in combination serve the larvae with the required nutrients. Improvements in nutrient retention capacities in compound diets are evidently demanded and suitable techniques for achieving this are highly requested.

Protein quality in weaning diets for marine fish larvae

Marine fish larvae utilise protein given in hydrolysed form more efficiently than intact protein (Tonheim *et al*, 2005). However, the high leaching rates of water-soluble nutrients suggest this as a cause for unbeneficial effects of high supplementation of hydrolysed protein in weaning diets. The improvements in growth and survival of carp larvae when fed diets containing soluble protein (Carvalho *et al*, 2004) combined with the results showing reduced leaching of intact protein versus hydrolysed protein (Paper II), suggest further work in optimisation of the protein composition in weaning diets. As observed with the effect of hydrolysed protein to cod and halibut (Paper IV), one has to be aware that slow-feeders and quick-feeders may have quite different specification for dietary protein composition.

Early weaning time

The discussion of the importance of intestinal maturation for successful weaning in section 4.3 did not give any conclusion. A clear conclusion is also difficult to make due to differences between species and because the diet used may be differently suited for the fish larvae. However, evaluation of the importance of different developmental changes for the possibility to succeed in weaning is useful. Regarding digestion, the importance of pancreatic secretion capacity and intestinal maturation for weaning success would be interesting to know more about.

Furthermore, to succeed with earlier weaning, development of compound diets that meet the larval requirements with regard to nutrient composition, physical properties, digestibility etc is crucial. Of the topics treated in this study, improved leaching properties of the weaning diets and an optimal dietary protein composition would ensure delivery of a more adequately composed diet that can function as the sole feed for the fish larvae at an early stage. Continuation of ongoing work in improvement of weaning diets regarding both nutrient composition and physical properties of the diet is important for reducing the dependence of using live feed in rearing of marine fish larvae.

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