

PAPER IV

**Effects of hydrolysed protein in weaning diets for Atlantic cod
(*Gadus morhua* L.) and Atlantic halibut (*Hippoglossus
hippoglossus* L.)**

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Abstract

Predigested dietary protein may enhance the utilisation of formulated diets at weaning, and also stimulate intestinal maturation. In this study, Atlantic cod (*Gadus morhua* L.; 41 days post hatch (dph)) and Atlantic halibut (*Hippoglossus hippoglossus* L.; 63 days post first feeding (dpff)) were weaned onto diets with graded levels of where varying parts of the protein was substituted with pepsin hydrolysed protein, exchanged with dietary protein. By increasing dietary content of hydrolysed protein from none to 40 %, cod increased the rate of survival from 7 ± 1 % to 18 ± 2 % (82 dph; Regression, $P=4*10^{-7}$). In halibut, the survival rate decreased from 57 ± 9 % to 22 ± 7 % as the content of hydrolysed protein increased from none to 45 % (119 dpff; Regression, $P=8*10^{-5}$). Growth was not affected in any of the species. A 3-fold higher specific activity of leucine aminopeptidase (LAP) in cod was found in the groups fed 20 and 30 % hydrolysed protein than in the group fed non-hydrolysed protein (82 dph; ANOVA, $P<0.05$), indicating a more mature intestine in the former groups. In halibut, specific activity of alkaline phosphatase (AP) was 3-6-fold higher in the groups receiving non- and 15 % hydrolysed protein than in the groups receiving 30 and 45 % hydrolysed protein (91+119 dpff; ANOVA, $P<0.05$). The lower preference optimal level of hydrolysed protein in halibut than in cod is suggested to mainly be caused by due to a slower feeding habit practice in halibut which allow more extensive nutrient leaching before ingestion.

Introduction

The high growth rate at the larval and early juvenile stages of fishes, as compared to later life developmental stages (Otterlei *et al.* 1999; Kjørsvik *et al.* 2004), witnesses their very efficient utilisation of the nutrients in (natural) live prey. Still, the gastrointestinal tract at this early life stage in the fish larvae is not completely differentiated and all digestive functions are not developed (Pedersen & Falk-Petersen 1992; Luizi *et al.* 1999; Zambonino Infante & Cahu 2001). Moreover, digestion seems to be a limiting step for efficient utilisation of dietary protein (Tonheim *et al.* 2005), even though amino acids (AAs), the building blocks in protein, are considered to be the quantitatively major nutrient component for fish larvae (Rønnestad *et al.* 1999; Finn *et al.* 2002). High levels of AAs are required for muscular protein deposition during this period of fast growth (Houlihan *et al.* 1995; Rønnestad *et al.* 2003). Furthermore, AAs are also used as the main energy source (Rønnestad *et al.* 1999; Finn *et al.* 2002).

Marine fish larvae and early juveniles utilise formulated diets less efficiently than live prey, indicated by lower growth and survival (Baskerville-Bridges and Kling 2000; Hamre *et al.* 2001; Callan *et al.* 2003; Leifson 2003). Likely, this can partly be explained by the combination of an immature gastrointestinal tract in larvae and more complex protein incorporated into formulated diets. A major digestive organ that develops at the end of the larval stage is the stomach in which protein digestion is initiated by the actions of hydrochloric acid and pepsin. Furthermore, the stomach has high capacity to store ingested feed and thus allow efficient regulation of the passage of ingesta through the gastrointestinal tract (Krogdahl 2001). In larvae, digestion seems to be less adjustable and relies on activities of pancreatic and intestinal enzymes,

contribution of live feed enzymes and possibly on intracellular digestion of pinocytosed protein absorbed by pinocytosis (Watanabe 1982; Zambonino Infante & Cahu 2001; Kolkovski 2001).

In order to ease digestion of dietary protein, pre-digested protein has been added to compound diets, but with varying results. High levels of hydrolysed protein have been found detrimental in seabass (*Dicentrarchus labrax*, Cahu *et al.* 1999), seabream (*Sparus aurata*, Kolkovski & Tandler 2000), halibut (*Hippoglossus hippoglossus*, Kvåle *et al.* 2002) and carp (*Cyprinus carpio*, Carvalho *et al.* 2004), but beneficial in Dover sole (*Solea solea*, Day *et al.* 1997). In carp and seabass, low to medium levels of hydrolysed protein supported the highest growth and survival rates (Zambonino Infante *et al.* 1997; Cahu *et al.* 1999; Carvalho *et al.* 2004).

An appropriate diet is considered to be important for normal maturation of the gastrointestinal tract of fish larvae (Zambonino Infante & Cahu 2001). In this respect, an optimal level of hydrolysed protein is found to stimulate intestinal maturation in seabass larvae when maturation was estimated by indicators using markers for typical larval and adult modes of digestion characters, respectively (Cahu *et al.* 1999).

Activity of a cytosolic enzyme, leucylalanine aminopeptidase (Leu-Ala), that is thought to be involved in the terminal steps of intracellular digestion of protein (Henning, 1987), was used as a marker indicator of larval mode of digestion, and the activities of the brush border membrane (bbm) enzymes alkaline phosphatase (AP) and leucine aminopeptidase N (LAP) was used as indicators markers for achievement of a more adult-like digestion.

In a previous study (Kvåle *et al.* 2002), the weaning diet to halibut (*Hippoglossus hippoglossus* L.) containing 10 % pepsin hydrolysed protein supported the highest survival rate, although not significantly higher than the non-hydrolysed diet and a diet where 10 % of the protein was hydrolysed with pepsin and trypsin. The aims of the present study were to confirm this result and to investigate the influence effect of dietary hydrolysed protein on growth and survival of cod (*Gadus morhua* L.) at weaning. Additionally, the study aimed to investigate dietary effects on intestinal maturation in both cod and halibut. In the present study, the dietary protein source was the same in all diets fed to each species, securing ensuring that all diets fed to cod and halibut, respectively, had similar AA composition. To mimic the gastric function, part of the dietary protein was hydrolysed with pepsin, an endopeptidase thought to produce relatively long peptides. Hydrolysates containing long peptides have been considered superior compared to those containing short peptides (Kvåle *et al.* 2002; Carvalho *et al.* 2004). To investigate whether supplementation of hydrolysed protein had an effect on maturation of the intestinedigestive tract, activities of the intestinal enzymes AP, LAP and Leu-Ala ala were analysed.

Materials and Methods

The diets

Four and five isoenergetic and isonitrogenous diets with varying graded amounts of pepsin hydrolysed protein were produced for halibut and cod, respectively, at NIFES (Bergen, Norway) as described previously (by Hamre *et al.* (2001; Kvåle *et al.* 2002).

Fresh fish fillets (saithe, *Pollachius virens* L., in the diets to cod and cod in the diets to halibut), pepsin hydrolysed fish mince (see below) and squid (*Loligo vulgaris*, Lamarck 1798) mantles were the main ingredient in the diets, being thoroughly mixed with the other ingredients in a high speed cutter (Tables 1 and 2). The produced paste was formed into diets strings that were heat coagulated in a continuous microwave oven and then dried over night in a warm air tunnel dryer (with immobile conveyor; 60 °C). The dried diets were crushed and sieved into appropriate particle sizes.

Pepsin hydrolysed protein was made of a mince of saithe fillets and squid mantles (9:1) in the diets to cod, experiment and of a mince of cod fillets in the diets to halibut experiment. The mince was, after adjusting the pH to 4.2 by addition of 5M HCl, incubated at ambient temperature with pepsin ($7.5 \text{ mg (kg mince)}^{-1}$; Sigma-Aldrich Corp., St.Louis, Mo, USA) dissolved in glycerol ($0.93 \text{ ml (g pepsin)}^{-1}$; Merck, Darmstadt, Germany) and distilled water ($1.73 \text{ ml (g pepsin)}^{-1}$). After 24 hours incubation at ambient temperature, the hydrolysate was neutralized by 5 M NaOH, and then stored frozen (-30 °C) until diet preparation. The hydrolysate constituted 0, 10, 20, 30 and 40 % of the dietary protein in the diets for cod (Table 1), and 0, 15, 30 and 45 % of the dietary protein in the diets for halibut (Table 2).

Rearing of cod

The rearing of cod was conducted at the Institute of Marine Research (Storebø, Norway). Fertilised eggs were collected from natural spawns of a captive broodstock and hatched in 100-L conical cylinders (6-8 °C). From day four to 38 post hatch (dph), the larvae were offered rotifers (*Brachionus plicatilis*) grown on baker's yeast (Idun

Industri AS, Oslo, Norway) and Rotimac (Biomarine Aquafauna Inc., Hawthorne, CA, USA) and thereafter *Artemia* (Artemia Systems Inc., Gent, Belgium) enriched with DC DHA Selco (INVE Aquaculture NV, Dendermonde, Belgium) from 33 to 41 dph. Green water was used from four to 38 dph by adding Instant Algae® (*Nannochloropsis*; Reed Mariculture, Campbell, CA, USA) to the rearing water. Further details in the treatment of eggs and first feeding larvae are described by van der Meeren & Ivannikov (2006). At 41 dph the larvae were transferred to circular 50-L experimental tanks, 400-600 larvae per tank, and weaned onto the experimental diets in randomly distributed triplicate tanks. The tanks received a constant supply (0.5 L min^{-1}) of sand filtered seawater (34.5 ‰ salinity) maintaining 11 °C (range 10-12 °C) and were supplied with continuous light. On a daily basis, wastes and dead larvae were removed. The larvae were continuously fed the experimental diets by belt-feeders (Hølland Tech. Inc., Sandnes, Norway). Additionally, *Artemia* was given the first five days of the experiment. Formulated feed rations and particle size were approximately 7 ml and 0.3-0.6 mm, respectively, at start of the experiment and later increased to 10 ml and 0.3-0.6 mm, respectively. The trial was terminated after 41 days of experimental feeding (82dph).

Rearing of halibut

The rearing of halibut was conducted at the Institute of Marine Research (Storebø, Norway). Eggs were stripped from one female and fertilized with sperm from two males. Details in the treatment of eggs, yolk sac and first feeding larvae are given by Mangor-Jensen *et al.* (1998) and Harboe *et al.* (1994; 1998). The larvae were first fed with *Artemia* enriched with Multigain (Danafeed, Horsens, Denmark) from 43 dph (265

daydegrees) in 1500 L tanks. At 63 days post first feeding (dpff), the larvae were transferred from two first feeding tanks and distributed into circular 50-L experimental tanks where they were weaned onto the experimental diets. The fishes were stocked in randomly distributed triplicate tanks, each tank receiving 57-99 larvae. At start, the tanks were supplied with 0.7-1.0 L min⁻¹ of sand filtered seawater (34.5 ‰ salinity) maintaining 12 °C (range 11-13 °C), and later the water flow was increased in two steps to 1.5 L min⁻¹. Oxygen saturation was maintained above 80 % saturation, and dead larvae and wastes were daily removed. The tanks were supplied with continuous light. During the first three experimental days, the feeds were given manually, approximately once per hour, 12 hours per day. Thereafter, the feeds were given continuously, administered by belt feeders (Hølland Tech. Inc., Sandnes, Norway). The daily ration of feed was 7 ml and feed particle size was 0.3-0.6 mm the first 20 days of the experiment and thereafter 0.3-1.0 mm. The experiment was terminated after 56 days (119 dpff).

Sampling procedures

Larvae or post-larvae were collected at start (day 0) and end (days 41 and 56 for cod and halibut, respectively) of the experiments for analysis of enzyme activities and growth. In the experiment with halibut, additional samples were collected at days 13 and 35. At day 13, only the smallest larvae in each tank were sampled since dietary hydrolysed protein may change effect when juvenile digestive characteristics features are attained. Cod and halibut were anaesthetised with benzocain and metacain, and then immediately frozen on dry ice and stored at -80 °C until analysis. The individual dry weights of 30 cod larvae were measured at day 0 of the experiment and the individual wet weights of 19 to 105 post-larvae at day 41 prior to freezing. The initial wet weights

were estimated by assuming that the dry matter comprised 16 % of the larvae (Finn *et al.* 2002). The individual wet weights of halibut was measured on semi-thawed larvae/post-larvae at all samplings, 27 individuals at day 0, 10 at days 13 and 35, and 12 to 53 at day 56. Specific growth rates (SGR; % day⁻¹), estimated from wet weights, were calculated as:

$SGR = (e^g - 1) * 100$, where $g = (\ln(\text{final weight}) - \ln(\text{initial weight})) / \text{experimental days}$ (Houde & Schekter, 1981).

Survival rates of cod are based on approximate number of fishes distributed into the tanks and exact number of surviving fishes, whereas survival rates of halibut are based on exact numbers of dead and surviving fishes.

Enzyme preparations and assays

Dissected guts were used in all enzyme preparations, except that the liver (only days 13 and 35) and stomach were included in the preparations of halibut. All preparations and assays were in accordance with Cahu *et al.* (1999), and are previously described by Kvåle *et al.* (submitted/accepted). The preparations were made of pooled samples of seven to 30 larvae or post-larvae and kept on ice during all preparation steps. The bbm were extracted from the samples of halibut as described by Crane *et al.* (1979). The assays for AP, LAP and Leu-ala are originally given by Bessey *et al.* (1946), Maroux *et al.* (1973) and Nicholson & Kim (1975), respectively, and the substrates used were p-nitrophenyl phosphate, leucine-p-nitroaniline and Leucylalanine leucylalanine (all from Sigma-Aldrich Corp., St.Louis, Mo, USA), respectively. Protein contents of homogenates and bbm extracts were determined by Bio-Rad protein micro assay (Bio-

Rad Laboratories, Hercules, CA, USA) using bovine serum albumin (BSA) as standard. The enzyme activity unit U was defined as the amount of enzyme that hydrolysed 1 μ mole of substrate per minute at the specified conditions and is given as specific activity ($\text{U (mg protein)}^{-1}$), *i.e.* on the basis of soluble protein content in the enzyme extract. The calculations are described by Kvåle *et al.* (submitted).

Chemical analysis

Content of dry matter in the diets was measured gravimetrically after 24 h at 104 °C, and total lipid was extracted with ethyl acetate:isopropanol (7:3) followed by gravimetric determination (Norsk Standard, 1996). Crude protein ($\text{N} \times 6.25$) in the cod and halibut diets were determined by Kjeldahl digests (Crooke & Simpson 1971) and by total combustion using a nitrogen analyzer (PE 2410 SERIES II, Perkin Elmer, Oslo, Norway), respectively. Dietary protein solubility was estimated by determining the amount of free α -amino groups reacting with trinitrobenzenesulphonic acid (TNBS) on basis of crude protein content in the diet. The binding of TNBS to free α -amino groups was determined colorimetrically using L-leucine as standard (Adler-Nissen 1979).

Statistics and calculations

Statistical analyses were conducted by S-plus (version 6.1; Insightful Corp., Seattle, WA, USA), by following procedures given by Crawley (2004). Possible effects of the dietary levels of protein hydrolysate was analysed by regression and analysis of variance (ANOVA). Upon detection of significant effects, ANOVA was followed by Tukey's honest significant difference (HSD) test. In analyses of effect on survival,

generalised linear models (glm) were used, assuming binomial error distribution and by using a logit link functions ($\ln(\text{probability to survive} : \text{probability to die})$). F-tests were used to specify the simplest model that best described the data. Kruskal-Wallis test was additionally used in analysis of effects on survival of cod and final weight of halibut due to heterogeneous variance. Effects on growth and enzyme activities were analysed by linear models (lm), using adequate transformations of the data when this was necessary to produce linearity between the explanatory and response variables, normal distribution of errors and constant variance. The models were inspected by diagnostic plots (normal probability, fitted values (fv) vs residuals, fv vs square root of the absolute values of the residuals, response variable vs fv and Cook's distance) and outliers detected as described by Crawley (2004).

The analyses were based on triplicates, except for duplicates in the H30 group of halibut, in survival and enzyme analyses of the H10 and H20 groups in cod and in the analyses of diet composition. The H30 halibut group was accidentally lost during trial run, and the H10 and H20 cod groups were excluded from the enzyme analyses due to too few surviving larvae and from the survival analysis due to outlying tanks (See results). Data is given as mean \pm SD if not elsewhere stated, and considered significantly different when $P < 0.05$.

Estimates of specific growth rate ($\% \text{ day}^{-1}$) were based on wet weights and calculated as:

$\text{SGR} = (e^g - 1) * 100$, where $g = (\ln(\text{final weight}) - \ln(\text{initial weight})) / \text{experimental days}$ (Houde & Schekter, 1981).

Results

The diets

The five and four diets given to cod and halibut, respectively, were fairly similar in content of dry matter, crude protein and crude lipid (Tables 1 and 2). Dietary protein solubility increased significantly with increasing content of protein hydrolysate (Cod, protein solubility = $261+6*H$, $R^2=96.5$, $P=4*10^{-7}$; Halibut, protein solubility = $308+13*H$, $R^2=93.8$, $P=8*10^{-5}$; H = % hydrolysed protein). The protein solubility values were two- and threefold higher in the most hydrolysed diets than in the non-hydrolysed diets to cod and halibut, respectively (Tukey HSD, $P<0.05$; Tables 1 and 2). Further, there was larger variation in protein solubility between the diets to halibut than between those to cod, evidenced by the twice as steep slope of the regression line.

Growth and survival

The final wet weight of cod was 303 ± 61 mg and was not affected by the dietary content of protein hydrolysate (Table 3). The dry weight of the larvae at start of the experiment was 1.72 ± 0.34 mg, and, when assuming 16 % dry matter in the larvae, the estimated SGR was on average 8.2 ± 0.5 % day^{-1} . The survival of cod varied from 7 ± 1 % in the group receiving the non-hydrolysed diet to 18 ± 2 % in the group receiving the diets with 40 % hydrolysate (Fig. 1). The increase in survival due to dietary hydrolysate level was significant when two outlying tanks receiving 10 or 20 % protein hydrolysate were excluded (Regression, $P=8*10^{-5}$, Fig. 1). By Tukey HSD, significant differences between groups were detected, except that the 20 % hydrolysate group was not

significantly different from the 30 and 40 % hydrolysate groups, and that the 30 % hydrolysate group was not significantly different from the 10 % hydrolysate group. Including the outlying tanks and performing a non-parametric statistical test, survival increased with a significance of 6 % (Kruskal-Wallis, $P=0.054$).

The wet weight of halibut was 221 ± 69 mg at start of the experiment, and on average 448 ± 65 mg and 974 ± 558 mg at days 35 and 56, respectively (Table 4). The weights did not differ significantly due to diet, but there was a tendency for lower weight in the two groups receiving 30 and 45 % hydrolysed protein, compared to the groups receiving non- or 15 % hydrolysed protein (Kruskal-Wallis, $P=0.10$). The SGR was on average 2.4 ± 0.9 % day^{-1} from start to end of the experiment. The survival rates declined with increasing addition of protein hydrolysate in the diets, being 56.6 ± 8.6 % in the group fed the non-hydrolysed diet and gradually decreasing to 22.4 ± 6.5 % in the group fed the diet added 45 % protein hydrolysate (Regression, $P=0.0035$; Fig. 2). Of the levels of dietary protein hydrolysate tested, only survival of fish fed the non- and most hydrolysed diets were significantly different (Tukey HSD, $P<0.05$).

Activity of intestinal enzymes

Activity of the cytosolic enzyme Leu-ala was unaffected by the diets used for both cod and halibut. The activity of this enzyme was on average 1688 ± 482 mU (mg protein) $^{-1}$ in cod at day 41, and 686 ± 38 , 1001 ± 209 , 627 ± 81 and 1032 ± 217 mU (mg protein) $^{-1}$ in halibut at days 0, 13, 35 and 56, respectively.

The specific activity of LAP in cod at day 41 was affected by the diets and exhibited the highest activity in the groups receiving the medium levels of protein hydrolysate (Table 3). The specific activity of LAP in the groups fed the 20 and 30 % protein hydrolysate was threefold higher than in the group fed non-hydrolysed protein (Tukey HSD, $P < 0.05$; $LAP = 0.22 + 0.04 * H - 0.0007 * H^2$, $R^2 = 73.4$, $P = 0.0014$;). The specific activity of AP was not affected by the diets (Table 3).

After 13 days of feeding the experimental diets, the specific activities of AP and LAP in halibut did not differ between the experimental groups (Table 4). The average wet weight at this sampling was 184.4 ± 16.9 mg, which was lower than at the experimental start. At day 35, both LAP and AP activities significantly decreased with increasing content of protein hydrolysate in the diet ($AP = 6.05 * e^{-0.035 * H}$, $R^2 = 74.6$, $P = 0.0006$; $LAP = 1.36 * e^{-0.019 * H}$, $R^2 = 60.3$, $P = 0.005$; Table 4). The specific activity of AP in the group receiving the non-hydrolysed diet was approximately fourfold higher than in the two groups receiving 30 and 45 % protein hydrolysate (Tukey HSD, $P < 0.05$; Table 4). Likewise, the specific activity of LAP in the group receiving the non-hydrolysed diet was twofold higher than in the two groups fed 30 and 45 % protein hydrolysate (Tukey HSD, $P < 0.05$; Table 4). The activities of both enzymes in the group receiving 15 % protein hydrolysate were intermediate, only the AP activity being significantly higher than in the groups receiving more hydrolysed diets (Tukey HSD, $P < 0.05$).

The specific activity of AP in halibut at day 56 resembled the pattern at day 35 (Table 4). In the groups receiving the non- and 10 % protein hydrolysate diets, the specific AP activity was sixfold higher than in the groups delivered 30 and 45 % protein hydrolysate

(Tukey HSD, $P < 0.05$). The pattern in activity of LAP at day 56 was less clear. The activity in the group fed the non-hydrolysed diet was threefold higher than in the group fed 30 % protein hydrolysate, while the LAP activity in the groups fed 15 and 45 % protein hydrolysate were similar and intermediate.

Discussion

In this study, a very different optimal level of hydrolysed protein was found for cod and halibut at weaning. The highest level of dietary hydrolysed protein tested, 40 %, promoted the highest survival in cod. Oppositely, the other end of the range of dietary hydrolysed protein tested, the non-hydrolysed diet, supported the highest rate of survival in halibut. The results with both species are confirmed in related studies (Kvåle *et al.* 2002; Åsnes 2006). The difference between the species is remarkable since both cod and halibut are considered to have high and fairly similar needs for easily available dietary protein during the larval stage. They both have a high growth rate (Harboe *et al.* 1998; van der Meeren & Ivannikov 2002; Otterlei *et al.* 1999) which implies that they have high demands for AAs for muscle accretion (Houlihan *et al.* 1995; Rønnestad *et al.* 2003). Furthermore, they derive a major part of the metabolic energy from AAs (Rønnestad *et al.* 1999; Finn *et al.* 2002). In combination with the immature gastrointestinal tract during the larval stage (Pedersen & Falk-Petersen 1992; Luizi *et al.* 1999; Kjørsvik *et al.* 2004), these aspects suggest that hydrolysed dietary protein is advantageous in weaning diets to both cod and halibut, since less digestion is needed on such a protein before it can be absorbed. This consideration is supported by a recent study demonstrating that pre-hydrolysed protein, given by controlled tube-feeding to

halibut larvae, was faster absorbed and resulted in a higher portion of absorbed protein, compared to the same protein given in intact form (Tonheim *et al.* 2005).

A more juvenile character of halibut at onset of the experiments may partly explain why hydrolysed protein was less beneficial for halibut than for cod. In halibut, dissections revealed that the stomach was in progress, although the status of functionality was not determined. The stomach in cod presumably began to develop at the time of or later than the start of the present study (Pedersen & Falk-Petersen 1992; Perez-Casanova *et al.* 2006). However, cod is found to survive better when given 40 % hydrolysed protein, as compared to none, also when they are weaned at the juvenile stage (330 mg, Åsnes 2006)

A more likely explanation for the different optimal level of hydrolysed protein found for cod and halibut is different feeding behaviour. Unfortunately, their feeding practises were not studied during the experiments, but halibut is considered to feed slowly (Stoss *et al.* 2004). Since halibut partly had settled on the bottom of the tank when the experiment started, old diet particles were highly available. Cod, on the other hand, probably ingested the feed particles as they sank through the water column, and thus quicker than halibut. Feed particles ingested by halibut, may therefore have lost a higher portion of their water soluble nutrients. Leaching rates of free amino acids (FAAs), hydrolysed protein and other highly water soluble nutrients are high from the small feed particles used at weaning (Lopez-Alvarado *et al.* 1994; Kvåle *et al.* 2006; Hamre 2006). The protein content of the diet at the time of ingestion was probably reduced in proportion with the inclusion level of hydrolysed protein due to leaching and might

have reached a suboptimal level. The protein requirement for juvenile halibut is estimated to be very high, 58 %, and might be reached when high level of hydrolysed protein is included in the diet (Hamre *et al.* 2003; 2005; Kvåle *et al.* 2006). Juvenile cod, on the other hand, seem to tolerate lower protein levels and is found to grow well on dietary protein content above 40 % (Hamre & Mangor-Jensen 2006; Åsnes 2006).

If the diets to halibut had suboptimal protein content at time of ingestion, one would expect this to influence the growth, as seen in protein requirement studies (Hamre *et al.* 2003; Åsnes 2006; Kim *et al.* 2001; Lupatsch *et al.* 2001). Although there was a tendency for reduced growth of halibut in the groups receiving 30 and 45 % hydrolysed protein, the variance in growth was very heterogenousheterogeneous and thus interfering any conclusion with regard to dietary effect. In other studies, high levels of hydrolysed protein or free amino acids in weaning diets have often resulted in reduced growth, as well as survival (Carvalho *et al.* 2004; 2004; Cahu *et al.* 1999; Kolkovski & Tandler 2000; Dabrowski *et al.* 2003). This may have beenight be caused by the high leaching of these nutrients, as discussed above.

In a previous weaning study with halibut, 10 % supplementation of hydrolysed protein is indicated to improve survival, although not significantly (Kvåle *et al.* 2002). The smaller size of the larvae in this previous study than in the present study (120 ± 40 vs 221 ± 69 mg, respectively) suggests that hydrolysed protein might be beneficial at early weaning of this species when the gastrointestinal tract is less mature. Alternatively, the use of shallow raceways in Kvåle *et al.* (2002) may have facilitated faster removal of

old diet particles from the fish tank, and thus ingestion of more newly fed diet particles which still contained some hydrolysed protein.

Higher specific activities of AP and LAP at days 35 and 56 in halibut receiving the non- and 10 % hydrolysed diets, confirmed the results on survival. These enzymes are regarded as markers for a well differentiated intestinal bbm and have previously been found to exhibit high specific activities in fish larvae fed optimal diets (Zambonino Infante *et al.* 1997; Bakke-McKellep *et al.* 2000; Moren *et al.* 2004). In cod, the highest specific activities of AP and LAP were reached in the groups fed 20 and 30 % hydrolysed protein. Further, the activities tended to decrease in the group fed the highest inclusion level, in which the highest survival rate was obtained. A possible explanation for the slight discordance in enzymatic and survival results might be a temporary higher optimal level of hydrolysed protein at onset of weaning. During the cod experiment, there was a high incidence of mortality when co-feeding with *Artemia* ceased five days after the experiment started. Throughout the rest of the experiment, the mortality was low. The survival result might thus be regarded as an effect obtained early in the experiment, between the 5th and 10th experimental days, while enzyme activities were analysed in fishes sampled at day 41 when they had become juveniles (wet wt 311 ± 38 mg). A temporary beneficial effect of hydrolysed protein at the onset of weaning has previously been suggested by Carvalho *et al.* (2004), who found that carp larvae enhanced growth and survival when fed hydrolysed protein during the first week after weaning. Thereafter, only minor improvements in performances were achieved by further administration of hydrolysed protein. A related result was achieved in juvenile Dover sole (Day *et al.* 1997).

A temporary higher optimal level of hydrolysed protein at onset of weaning could explain why the AP and LAP specific activities in halibut at day 13 did not correspond by the activity levels at days 35 and 56. At this first sampling, only the smallest larvae were sampled in order to detect possible dietary effect when larval digestive characteristics still were dominating. In fact, there was a tendency for lower AP activity at day 13 in the group receiving non-hydrolysed protein compared to the other groups, but interpretation was difficult because the AP activity in the fishes in one of the tanks fed non-hydrolysed protein was much higher than in the other replicates (AP activity 0.55 vs 0.10-0.14 mU (mg protein)⁻¹). Another possible explanation for the absence of effects at day 13 is that dietary effects were not induced at this early point in the experiment.

The specific activity of intestinal Leu-ala is thought to decline as the gastrointestinal functions mature and the fish larvae approach the juvenile stage follow a similar ontogenetic profile as pinocytosis, *i.e.* a decrease in activity as the gastrointestinal functions mature (Henning, 1987; Zambonino Infante & Cahu 2001). Furthermore, feeding a more adequate diet is thought to promote an earlier decline in Leu-ala activity (Zambonino Infante & Cahu 2001). The absence of dietary effects on the activity of this enzyme is in accordance with its unexpected ontogenetic profile previously found in cod and halibut (Kvåle *et al.* accepted, submitted), but opposes with results in seabass and Senegalese sole (Ribeiro *et al.* 1999; Zambonino Infante & Cahu 2001). Thus, and indicate the less clear role of this enzyme in larval mode digestion appears unclear.

In conclusion, assessed on based onis of survival rates and markersestimation of intestinal maturation, a weaning diet without hydrolysed protein is optimal for halibut (221 mg wet wt.). However, a small supplementation of hydrolysed protein might be advantageous at early weaning (120 mg wet wt., Kvåle *et al.* 2002). Cod (11 mg wet wt.), on the other hand, achieved the highest survival rate when weaned onto a diet with 40 % hydrolysed protein as compared to lower inclusion level of hydrolysed protein. This result has been confirmed in weaning of juvenile cod (Åsnes 2006).

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Figure legends

Figure 1. Survival rates of Atlantic cod fed diets where 0-40 % of the protein is exchanged with pepsin hydrolysed protein from weaning (41 dph) to 82 dph. The regression model is: $\text{Survival} = 0.085 * e^{0.023 * H} * (1 + 0.085 * e^{0.023 * H})^{-1}$, $p = 8 * 10^5$ (binomial distribution, logit link function). The circles denote individual tanks and the line the regression model. The two tanks with low survival are outliers and not included in the regression model.

Figure 2. Survival rates of Atlantic halibut fed diets where 0-45 % of the protein is exchanged with pepsin hydrolysed protein from weaning (63 dpff) to 119 dpff. The regression model is: $\text{Survival} = 0.994 * e^{-0.029x} * (1 + 0.994 * e^{-0.029x})^{-1}$, $p = 0.0035$ (binomial distribution, logit link function). The circles denote individual tanks and the line the regression model.

Tables

Table 1. Formulation and proximate composition of the experimental diets added 0 to 40 % protein hydrolysate (H0-H40) and fed to Atlantic cod from 41 to 82 dph.

Raw materials (mg (g) ⁻¹ , dry wt)	H0	H10	H20	H30	H40	Pooled SD
Saithe fillet	724.5	652.1	579.6	507.1	434.7	
Squid mantles	80.5	72.4	64.4	56.4	48.3	
Protein hydrolysate ¹	0.0	80.5	161.0	241.5	322.0	
Constant components ^{2,3,4}	195.0	195.0	195.0	195.0	195.0	
Dry matter (DM; mg (g) ⁻¹)	955.8	941.5	932.5	927.4	935.5	1.3
Crude lipid (mg (g DM) ⁻¹)	148.8	144.5	137.9	138.6	140.7	0.8
Crude protein (mg (g DM) ⁻¹)	796.0	744.9	768.5	783.4	782.05	0.9
Protein solubility (Leu-equiv. ⁵)	260 ^c	326 ^{bc}	375 ^b	467 ^a	500 ^a	18

Values marked with different letters in the same row are significantly different. ¹

Pepsin hydrolysed fish mince as explained in materials and methods. ² Extruded wheat (Condrico BV, Amsterdam, The Netherlands), 35 mg (g)⁻¹; Fish oil (Epax 3000 TG; Pronova Biocare AS, Sandefjord, Norway), 90 mg (g)⁻¹; Soy lecithin (Norsk Medisinaldepot, Bergen, Norway), 30 mg (g)⁻¹; Vitamins³, 30 mg (g)⁻¹; Minerals³, 30 mg (g)⁻¹. ³ As recommended by NRC (1993) except for vitamins C and E supplemented at 400 (as Stay-C) and 200 mg kg⁻¹ (as α -tocopheryl acetate), respectively. Vitamins supplied from Hoffmann–La Roche Ltd (Basel, Switzerland) and minerals from Merck (Darmstadt, Germany). ⁴ Additional ingredients: Tocopherol-mix (Nutrilo GmbH, Cuxhaven, Germany), 0.75 g kg⁻¹; Ascorbic acid (Norsk Medisinaldepot, Bergen, Norway), 1.2 g kg⁻¹; Astaxanthin (8 %; Roche, Basel, Switzerland), 20 mg kg⁻¹; β -caroten (8 %; Roche, Basel, Switzerland), 20 mg kg⁻¹. ⁵ Leu-equiv. = Equiv Leu- α -NH₂ (g protein)⁻¹.

Table 2. Formulation and proximate composition of the experimental diets added 0 to 45 % protein hydrolysate (H0-H45) and fed to Atlantic halibut from 63 to 119 dpff.

Raw materials (mg (g) ⁻¹ , dry wt)	H0	H15	H30	H45	Pooled SD
Cod fillet	724.5	603.7	483.0	362.2	
Squid mantles	80.5	80.5	80.5	80.5	
Protein hydrolysate ¹	0.0	120.8	241.5	362.3	
Constant components ^{2,3,4}	195.0	195.0	195.0	195.0	
Dry matter (DM; mg (g) ⁻¹)	922.0	909.5	900.2	908.6	0.1
Crude lipid (mg (g DM) ⁻¹)	144.8	139.4	123.38	129.0	1.6
Crude protein (mg (g DM) ⁻¹)	788.1	796.7	782.5	755.9	1.3
Protein solubility (Leu-equiv. ⁵)	333 ^b	434 ^b	757 ^a	874 ^a	40

Footnotes as in table 1.

Table 3. Atlantic cod final wet weights, specific growth rates (SGR) and specific activities of alkaline phosphatase (AP) and leucine aminopeptidase N (LAP) when fed 0-40 % protein hydrolysate (H0-H40) from 41 to 82 dph.

	H0	H10	H20	H30	H40
Final wet wt. (mg)	303±55	292±101	282±27	377±54	301±21
SGR (% day ⁻¹) ²	8.5±0.5	8.3±1.1	8.3±0.3	9.1±0.4	8.5±0.2
AP ¹	1.04±0.39	1.39±0.24 ^{*3}	1.23±0.15 [*]	1.55±0.13	1.40±0.14
LAP ¹	0.22±0.11 ^b	0.52±0.07 ^{ab,*}	0.65±0.25 ^{a,*}	0.68±0.14 ^a	0.53±0.07 ^{ab}

Values marked with different letters in the same row are significantly different.¹

Enzyme activities are expressed as U (mg protein)⁻¹.² SGR are based on wet weights and assuming a start weight of 10.7 mg (dry wt. 1.7 mg, 16 % dry matter).³ Values marked with * are based on n=2, elsewhere n=3.

Table 4. Atlantic halibut final wet weight, specific growth rates (SGR) and specific activities of alkaline phosphatase (AP) and leucine aminopeptidase N (LAP) when fed 0-45 % protein hydrolysate (H0-H45) from 63 to 119 dpff.

	H0	H15	H30	H45
Final wet wt. (mg)	1217±302	1230±861	437±45 ^{*2}	741±301
SGR (% day ⁻¹) ³	3.0±0.4	2.9±1.1	1.2±0.2 [*]	2.1±0.7
AP day 13 ¹	0.26±0.24	0.39±0.10	0.33±0.06 [*]	0.22±0.09
LAP day 13 ¹	0.29±0.11	0.42±0.08	0.36±0.08 [*]	0.33±0.07
AP day 35 ¹	6.27±1.27 ^a	4.36±1.52 ^a	1.26±0.45 ^{b,*}	1.57±0.33 ^b
LAP day 35 ¹	1.34±0.28 ^a	1.20±0.20 ^{ab}	0.61±0.31 ^{b,*}	0.65±0.09 ^b
AP day 56 ¹	14.82±3.09 ^a	15.63±6.75 ^a	2.47±0.65 ^{b,*}	2.67±1.34 ^b
LAP day 56 ¹	2.43±0.78 ^a	1.68±0.43 ^{ab}	0.81±0.28 ^{b,*}	1.64±0.38 ^{ab}

Values marked with different letters in the same row are significantly different.¹

Enzyme activities are measured after 13, 35 and 56 of experimental feeding are expressed as U (mg protein)⁻¹.² Values marked with * are based on n=2, elsewhere n=3. ³ SGR are based on wet weights and a start weight of 221 mg.

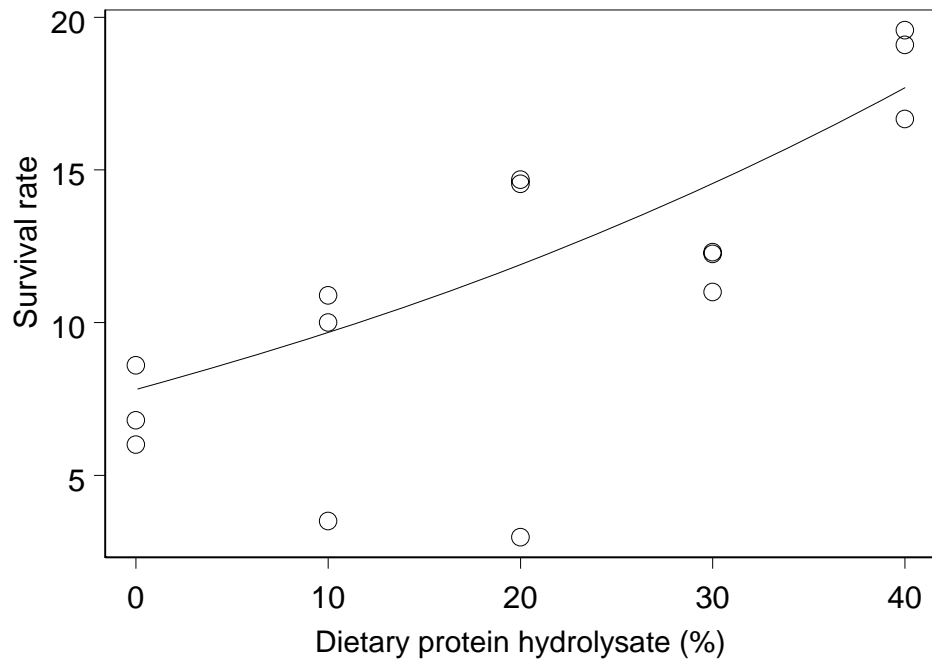


Figure 1.

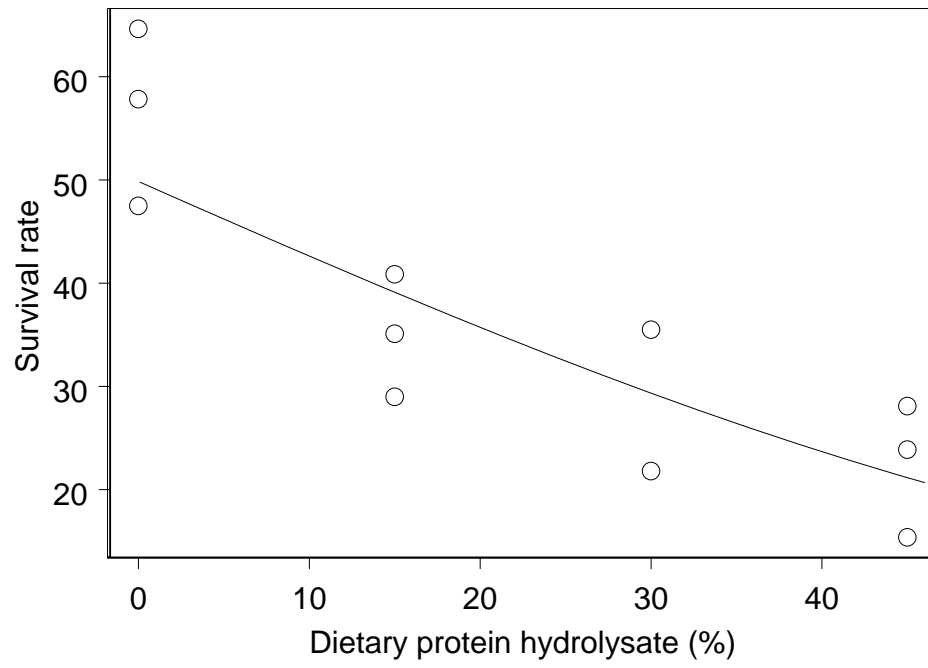


Figure 2.

