Aquaculture Nutrition

Aquaculture Nutrition **2009 15;** 525–536 doi: 10.1111/j.1365-2095.2008.00619.x

Protein quality of larval feed with increased concentration of hydrolysed protein: effects of heat treatment and leaching

A. NORDGREEN[1], S. TONHEIM[1,2] & K. HAMRE[1]

[1] *National Institute of Nutrition and Seafood Research (NIFES), Nordnes, Bergen, Norway;* [2] *Ewos AS, Bergen, Norway*

Abstract

Four heat coagulated early weaning diets with increasing concentrations of pepsin hydrolysed protein, were investigated with regard to the change in protein quality during feed production and exposure to leaching. Water-soluble N, trichloroacetic acid-soluble N and amino acid (AA) profiles were determined in finished diets and in diets leached for 6 min. *In vitro* diet digestibility was measured and related to increasing inclusion of hydrolysed protein and N leakage. Seventeen to 47% of soluble N in the feed ingredients was made insoluble by heat denaturation during feed production, but the concentration of peptides and free amino acids (FAA) were not influenced. All peptides/FAA and 70–80% of water-soluble protein were lost after exposure to leaching. Increased inclusion of hydrolysed protein increased the loss of crude protein (15–30%). All taurine and 30% of histidine was lost during leaching, no other major changes in AA profile were found. There was no difference in digestibility between diets exposed to leaching. However, leached diets showed reduced digestibility as compared to diets that had not been exposed to leaching. In conclusion micro-bound type diets as used in this study have a low efficiency in delivering soluble N to fish larvae and should be carefully considered for this purpose.

KEY WORDS: fish larvae, formulated diet, *in vitro* digestibility, protein hydrolysate, protein quality

Received 27 February 2008, accepted 2 June 2008

Correspondence: A. Nordgreen, National Institute of Nutrition and Seafood Research (NIFES), Post-box 2029, Nordnes, 5817 Bergen, Norway. E-mail: andreas.nordgreen@nifes.no

Introduction

Marine fish larvae have great growth potential (Houde 1989; Kamler *et al.* 1992; Conceicao *et al.* 1997) and even in cold water species such as the Atlantic cod (*Gadus morhua*) specific growth rates exceeding 25% day^{-1} have been reported (Otterlei *et al.* 1999). The larval body is lean and growth is therefore mainly the result of protein deposition (Houlihan *et al.* 1995). Accordingly, both high quality and quantity of dietary protein are required. At an early stage larvae lack a functional stomach (Govoni *et al.* 1986; Pittman *et al.* 1990; Segner *et al.* 1994) and may have low digestive enzyme activity (Cousin *et al.* 1987; Kvåle *et al.* 2007). Larval ability to digest complex protein has thus been questioned (Tonheim *et al.* 2004). Tube feeding showed that larval Atlantic Halibut (*Hippoglossus hippoglossus*) had a higher absorption efficiency of hydrolysed protein as compared to intact soluble protein (Tonheim *et al.* 2005). Stomachless fish larvae fed formulated diets have reduced growth and survival compared to larvae fed live feed (Howell *et al.* 1998; Cahu & Zambonino-Infant 2001; Kolkovski *et al.* 2001). The high concentration of water-soluble nitrogen (N) (copepods 54 ± 2%, rotifers 56.9 ± 0.8% and *Artemia* 54 ± 0.4% of total N, respectively, Tonheim *et al.* 2007; Srivastava *et al.* 2006; Carvalho *et al.* 2003) and high concentration of low molecular weight N (Carvalho *et al.* 2003; Tonheim *et al.* 2007) in live feed compared to formulated diets, is a suggested explanation for the reduced growth and survival reported from trials with stomachless fish larvae fed on formulated feeds (Carvalho *et al.* 2004). Carvalho *et al.* (2003) showed that nearly 90% of the soluble N in rotifers and *Artemia* was less than 500 D in size.

To mimic the free N pool of live feed and get improved growth and survival, free amino acids (FAA) or hydrolysed protein have been added to formulated feeds for early stage larvae (Carvalho *et al.* 1997, 2004; Cahu *et al.* 1999; Hamre *et al.* 2001; Kvåle *et al.* 2002). Although results are not always consistent, low inclusion levels of hydrolysed protein in diets have been shown to improve survival and growth (Zambonino-Infante *et al.* 1997; Cahu *et al.* 1999; Carvalho *et al.* 2004) while higher inclusion levels have shown negative effects on larval performance (*Sparus aurata* L., Kolkovski &

Tandler 2000; *Cyprinus carpio*, Carvalho *et al*. 1997, 2004; *Dicentrarchus labrax*, Cahu *et al*. 1999; *H. hippoglossus* Kvåle 2007). Supplementation of 40% pepsin hydrolysed protein to a heat coagulated diet (Hamre *et al*. 2001) improved survival rates in cod (*G. morhua*) larvae, compared to lower levels of supplementation (Kvåle 2007). On the other hand, Atlantic Halibut (*H. hippoglossus*) juveniles fed identical experimental diets showed a declining survival rate as the inclusion level of hydrolysed protein increased (Kvåle 2007). It is suggested that the reduced survival in halibut fed increased levels of hydrolysed protein is caused by their typical slow feeding behaviour (Stoss *et al*. 2004; Kvåle 2007). Feed will therefore stay in the water for a longer time before being eaten compared to species with a more aggressive feeding behaviour. The loss due to leaching of water-soluble nitrogenous compounds will be correspondingly higher and proportional to the inclusion level of hydrolysed protein. This will consequently result in lowered levels of both total protein and water-soluble N in consumed feed particles, which will in turn affect the amount of digestible protein and amino acids available for the larvae.

Loss of low molecular weight nutrients such as FAA and peptides are severe in almost all tested formulated larval diets supplemented with such hydrolysed protein (Lopez Alvarado *et al*. 1994; Kvåle *et al*. 2006). The loss of FAA was as high as 90% within less than 2 min in diets tested by Carvalho *et al*. (2004). Experimental diets have been developed which to some extent can reduce the loss of low molecular weight nutrients during feeding (Villamar & Langdon 1993; Lopez Alvarado *et al*. 1994; Yufera *et al*. 2002; Onal & Langdon 2005). However, nearly all studies conducted to investigate the nutritional effect of hydrolysed protein on larvae of different fish species have been carried out with micro-bound or ground diets that all have in common rapid leaching of soluble compounds (see review by Langdon 2003).

In addition to high loss during feeding, water-soluble protein is susceptible to loss or modification during production, such as denaturation (de Wet 1983; Boye *et al*. 1997; Mohammed *et al*. 2000), crosslinking (Jones *et al*. 1974; Langdon 1989; Yufera *et al*. 1996), Maillard reactions (Plakas *et al*. 1985; Deng *et al*. 2005) and leaching (Nordgreen *et al*. 2007). The feed production process can therefore significantly change protein quality and reduce the diet digestibility and thereby mask any biological effects of increased supplementations of hydrolysed protein. When investigating the effect of such supplementations to formulated diets, it is thus of great importance to investigate the effect of production process and leaching on the remaining protein quality.

The present study was designed to investigate changes in protein quality due to production process and leaching in four diets with increasing levels of hydrolysed protein. A heat coagulated diet was used (Hamre *et al*. 2001), that had previously been used for weaning of cod and Atlantic halibut larvae in several studies (Hamre *et al*. 2001, 2002, 2003, 2005; Kvåle *et al*. 2002; Moren *et al*. 2004). The diet had also been used in several studies to investigate possible effects of hydrolysed protein on performance of Atlantic halibut larvae and Atlantic cod larvae (Kvåle *et al*. 2002; Kvåle 2007). To understand the changes in protein quality and bioavailability during production and leaching of diets, the present study investigated changes in crude protein, water-soluble N, trichloroacetic acid (TCA)-soluble N (FAA and peptides), amino acid profile and *in vitro* digestibility.

Materials and methods

Feed and feed ingredients

Four heat coagulated diets based on fresh cod fillet and increasing concentrations of pepsin hydrolysed cod fillet as the major protein sources were produced at the National Institute of Nutrition and Seafood Research (NIFES), Bergen, Norway, as described by Hamre *et al*. (2001). Different inclusion levels of pepsin hydrolysed cod fillet represented 0, 15, 30 and 45% of diet total protein content (Table 1). Feed ingredients were blended in a high speed cutter to a thick dough and finger thick strings of the feed blend were fed onto a conveyor belt. The feed was first heated

Table 1 Composition of the formulated diets investigated in this study (g kg^{-1} dry weight)

Diets	0	15	30	45
Ingredients (dw)	g kg^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}
Suprex wheat[1]	35	35	35	35
Squid mantle[2]	81	81	81	81
Cod fillet[3]	725	604	483	362
Pepsin hydrolysed cod fillet[4]	0	121	242	362
Fish oil[5]	90	90	90	90
Lecithin[6]	30	30	30	30
Vitamin mixture[7]	10	10	10	10
Minerals[8]	30	30	30	30

[1] Suprex wheat.
[2] Fresh frozen squid mantle.
[3] Fresh cod.
[4] See description in Materials and methods.
[5] Epax A/S, Lysaker, Norway.
[6] Soy lecithin, Norsk Medisinaldepot, Bergen, Norway.
[7] As recommended by NRC (1993) except for alfa-tocopheryl acetate supplemented at 200 mg kg^{-1} and vitamin C at 400 mg kg^{-1}.
[8] As recommended by NRC (1993).

using electromagnetic energy and then dried in a warm air tunnel dryer over night (with immobile conveyor; 60 °C). Thereafter pellets were crushed and sieved. The size fraction sieved through a 600 μm sieve and collected on a 300 μm sieve was studied.

Pepsin hydrolysis was carried out according to the method described by Kvåle *et al*. (2002). Fresh cod fillet was minced and the pH was lowered to 4.2 by addition of 6 M HCl. A solution consisting of pepsin (7.5 g kg^{-1} mince; Sigma-Aldrich Corp., St Louis, MO, USA), glycerol (93 mL kg^{-1} mince; Merck, Darmstadt, Germany) and distilled water (173 mL kg^{-1} mince) was mixed into the minced fish solution and incubated at room temperature for 24 h under continuous shaking. Thereafter the pH was raised to 7.2 by addition of 5 M NaOH. The hydrolysed solution was frozen (−30 °C) until use. The enzyme was inactivated by heat during feed production.

Particle size was evaluated by taking pictures under the microscope (Olympus BX 51, Tokyo, Japan) at 40× magnification. The width and length of a minimum of 90 feed particles of each diet produced were measured with Olympus DP-soft.

Change in protein quality due to the production process

Crude protein (N × 6.25), water-soluble N and trichloroacetic acid (TCA) soluble N were measured in all raw materials and the concentrations of the different N fractions in the raw material blends were calculated based on results from Tonheim *et al*. (2007). Crude protein, water-soluble N and TCA soluble N were also analysed in the four diets. For analysis of water-soluble N, diets were crushed and 44–49 mg was weighed into 1.8 mL Eppendorf tubes. A volume of 1 mL of 80 mM phosphate buffer (pH 8) was added and tubes where shaken vigorously on a shaking table for 5 h. Tubes were then centrifuged at 8000 rpm (Eppendorf Centrifuge 5415 C, Hamburg, Germany) for 9 min. The supernatant was removed and the pellet was washed and centrifuged twice in phosphate buffer. The pellet was transferred to evaporating tinfoil cups (5 mL, Elemental Microanalysis Limited, Cambridge, UK) and residues in the tube were rinsed into the tinfoil cups. The tinfoil cups were dried at 65 °C overnight before analysing for crude protein (N × 6.25) by total combustion. TCA soluble N was analysed by weighing 30 mg of each of the four diets ($n = 4$) into 1.8 mL Eppendorf tubes. A volume of 1.1 mL of phosphate buffer was added and tubes were shaken vigorously before 250 μL of TCA (40%) was added to each sample. Samples were incubated at 4 °C until the next day before centrifugation at 8000 rpm (Eppendorf Centrifuge 5415 C) for 9 min. Finally 350 μL of supernatant was added to tin capsules (Smooth wall, Round base; Elemental Microanalysis Limited) and analysed for total N by total combustion.

Leaching study

To investigate the difference in leaching between the four diets, five parallel samples with 40–50 mg of each diet were suspended in tubes containing 1.5 mL of phosphate buffer for 6 min. Tubes were rotated slowly for 6 min to keep feed in suspension. Tube contents were then filtered onto a 0.65 μm N free Durapore membrane filter (25 mm). Filters were wrapped in tin foil cones (Elemental Microanalysis Limited) and analysed for crude protein (N × 6.25) by total combustion. The loss of N was calculated by dividing the N content in the leached samples with the analysed N content in the preleached samples. To calculate the remaining concentration of TCA soluble N after leaching the diet was lyophilized before 30 mg of the each diet ($N = 4$) were weighed into 1.8 mL Eppendorf tubes, precipitated with TCA and analysed as described above.

In vitro *digestibility*

The protocol for *in vitro* digestion used in the present study was based on the methods described by Hsu *et al*. (1977) and Saterlee *et al*. (1979) and was performed according to the modified method described by Tonheim *et al*. (2007). Diet samples equivalent to 20 mg of crude protein were suspended in 1 mL of phosphate buffer in 1.8 mL Eppendorf tubes. A mixture of trypsin (type IX, bovine pancreas) chymotrypsin (type II, bovine pancreas) and bacterial protease (type XIV, *strepomyces griseus*), all obtained from Sigma–Aldrich, MO, USA were added to each tube to final concentrations of 73, 145 and 64 μg mL^{-1} phosphate buffer, respectively. Digestion was performed at room temperature (22 °C). Samples were placed on a shaking table at low speed during the digestion period. Undigested protein was precipitated and the *in vitro* digestion terminated by adding 250 μL of TCA (40%). For each analysed diet and treatment four parallel tubes were analysed at 0 h, ½ h, 1 h and 12 h, respectively. All tubes were centrifuged and the crude protein content in the supernatant (350 μL), containing proteolysis products, i.e. FAA and small peptides, was analysed and regarded as representing digested protein. Each sample of supernatant (350 μL) was added to a tin capsule (Smooth wall, Round base; Elemental Microanalysis Limited) and analysed for total N by total combustion. Crude protein (N × 6.25) in the

precipitate, containing intact protein and large peptides, was regarded as representing undigested protein.

To remove water-soluble N from the four diets prior to *in vitro* digestibility, 1 g of each diet was suspended in 10 mL phosphate buffer and shaken for 2 min. Tubes were centrifuged at 3220 ***g*** for 30 s and the supernatant was removed. This was repeated once; the feed was in solution for approximately 6 min to equal the leaching study. The leached diets were frozen and freeze dried and a sub sample was analysed for N content before the *in vitro* digestibility studies.

Change in amino acid profile due to leaching and in vitro *digestibility*

Change in amino acid (AA) profile due to leaching and *in vitro* digestibility was analysed with the diet containing 30% hydrolysed protein (Table 2). The diet was leached according to the method described above. The preleached and leached diets were analysed in triplicate. To investigate the AA profile of the digested diet, the preleached and leached diets were *in vitro* digested in triplicate for 12 h according to method described. Samples of the supernatants were analysed for AA content.

Analytical methods

N was determined by total combustion using a nitrogen analyser (Leco FP-528, St Joseph, MI, USA). Crude protein was calculated as N × 6.25. Total AA profile was analysed according to the method described by Cohen *et al.* (1989). Samples were hydrolysed in 6 M HCl for 22 h at 110 °C. The hydrolysed solution containing free AA was then analysed using the Waters HPLC analyser system (Pico Tag) after prederivatization with phenyl isothiocyanate (PITC) using norleucine as internal standard. AA were detected by UV at 254 nm and identified by retention time. Tryptophan is degraded during acidic hydrolysis and is therefore not analysed.

Statistical methods

Data are expressed as means ± standard deviation. To investigate the effect of pepsin hydrolysis on change in crude protein content (N × 6.25), water-soluble N and TCA soluble N data were analysed by a *T*-test. The effects of the production process on water-soluble protein content and TCA soluble content in different diets were analysed by regression analysis along with one-way analysis of variance (ANOVA; Sokal & Rohlf 1969) followed by Tukey's HSD multiple comparison test. Change in AA profile due to production process, leaching and *in vitro* digestibility was analysed by one-way analysis of variance (ANOVA; Sokal & Rohlf 1969) followed by Tukey's multiple comparison test. The effect of increased inclusion of hydrolysed protein on particle size was analysed by regression analysis along with one-way ANOVA. Means with P values less than 0.05 were considered significantly different. All statistical analyses were performed using STATISTICA 7.1 (Statsoft Inc., Tulsa, OK, USA).

Table 2 Analysis of the different N fractions of minced cod fillet before and after pepsin hydrolysis (means ± SD, $n = 3$)

	Water-soluble N % of tot N	TCA soluble N % of tot N
Cod fillet	34 ($n = 1$)	18 ± 2.4
Pepsin hydrolysed cod fillet	65 ± 3	40 ± 4.2

Results

Pepsin hydrolysis of the cod fillet led to a 91% increase in water-soluble N (Table 2) and a 122% increase in TCA soluble N (Table 2).

Due to the crushing and sieving process most of the particles were rod shaped (Fig. 1) and it was therefore necessary to measure both width and length of the particles. The particles were nearly twice as long as they were wide and had a mean length 593 ± 184 μm and a mean width of 320 ± 81 μm. There was no significant correlation ($R^2 = 0.006$, $P = 0.35$) between increased hydrolysed protein and particle size and no significant difference (ANOVA, $P > 0.05$) in particle size between any of the diets.

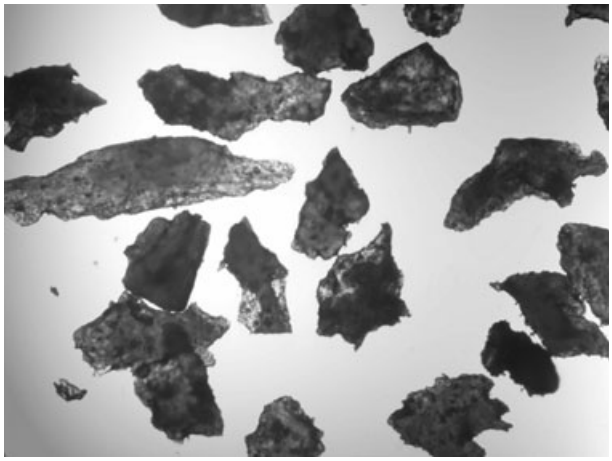

Figure 1 Sample of a diet containing 15% hydrolysed protein. The picture is taken with differential interference contrast (DIC) (Olympus BX 51; magnification 40×).

Change in total N

There was a negative correlation ($R^2 = 0.91$, $P < 0.00001$, $\beta = -0.96$) between concentration of hydrolysed protein and crude protein (N × 6.25) in the four diets (Fig. 2) with a 5% reduction in crude protein from the diet containing 0% hydrolysed protein to the diet with 45% hydrolysed protein (Fig. 2).

Leaching was positively correlated ($R^2 = 0.96$, $P = 0.000$, $\beta = 0.982$) with inclusion of hydrolysed protein. Loss of crude protein ranged from 14.9 ± 0.5% to 30.3 ± 0.4% of total crude protein from the diet containing 0% hydrolysed protein to the diet containing 45% hydrolysed protein (Fig. 2).

Change in water-soluble N

The calculated increase in water-soluble N in the raw ingredients, with an increase of hydrolysed protein from 0 to 45% hydrolysed protein, was 38% (Fig. 3). Increased inclusion of hydrolysed protein was positively correlated ($R^2 = 0.89$, $P < 0.00001$, $\beta = 0.943$) with concentration of water-soluble N in the diet (Fig. 3), but there was a significant decrease ($R^2 = 0.73$, $P < 0.00001$, $\beta = -0.86$) in relative loss (%) of water-soluble N with inclusion of hydrolysed protein during feed production (Fig. 3). The loss of water-soluble N due to heat denaturation ranged from 46.8 ± 2.7% to 17.4 ± 2.4%

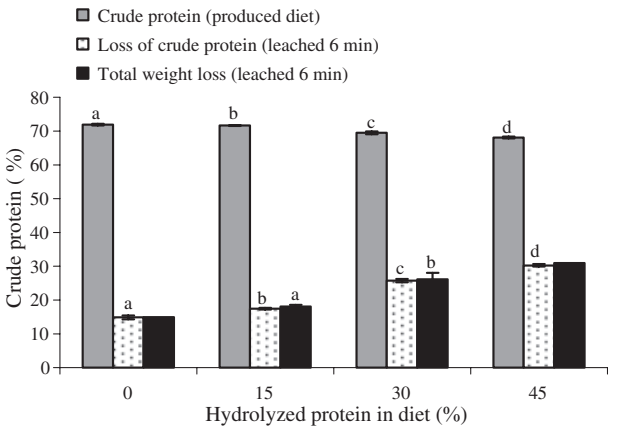

Figure 2 Grey bar is concentration of crude protein (N × 6.25) in for four diets with increasing inclusion of pepsin hydrolysed protein ranging from 0 to 45% of the total protein ($n = 3$). The white spotted bars are loss of crude protein (N × 6.25) in the same diets after 6 min leaching ($n = 5$). The black spotted bars are loss (%) of total weight after 6 min leaching ($n = 4$). The weight loss from the diet containing 0 and 45% hydrolysed protein has $n = 1$ and statistic analysis is therefore not performed. Otherwise error bars represent standard deviations. Significant differences between the diets are shown by different letters (ANOVA, followed by Tukey HSD, $P < 0.05$).

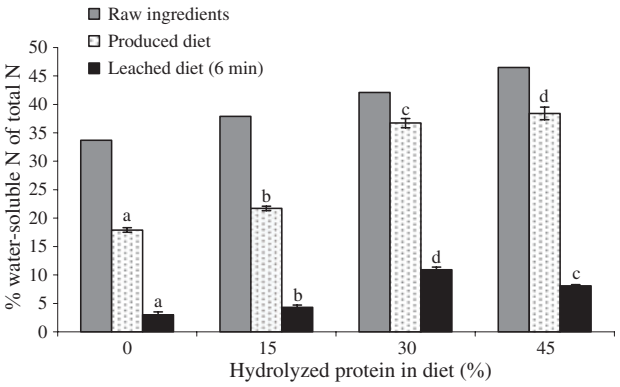

Figure 3 Concentration of water-soluble N in raw ingredients (calculated), produced diet ($n = 4$) and diets leached for 6 min ($n = 5$) for four diets with inclusion of pepsin hydrolysed protein ranging from 0 to 45% of the total protein. Error bars represent standard deviations. Significant differences between the diets are shown by different letters (ANOVA, followed by Tukey HSD, $P < 0.05$).

of water-soluble N from the diet containing 0% hydrolysed protein to the diet containing 45% hydrolysed protein (Fig. 3).

Total loss of water-soluble N due to leaching was positively correlated with concentration of hydrolysed protein (Fig. 3), and the remaining concentration of water-soluble N ranged from 3.0 ± 0.5% water-soluble N for the diet containing 0% hydrolysed protein to 11.0 ± 0.5% water-soluble N for the diet containing 30% hydrolysed protein (Fig. 3). There was no correlation between concentration of hydrolysed protein and relative loss of water-soluble N by leaching and the relative loss of water-soluble N ranged from a minimum of 70% to a maximum of 83% of total soluble N in the respective diets.

Change in TCA-soluble N

The raw ingredients had a 77% increase in TCA soluble protein from the diet containing 0% hydrolysed protein to the diet containing 45% hydrolysed protein (Fig. 4). The concentration of TCA soluble N was not affected by feed production (Fig. 4) and the produced diets had a significant increase ($R^2 = 0.9$ $P < 0.0001$, $\beta = 0.949$) in TCA soluble N from 10.6 ± 0.6% of total N to 21.6 ± 0.9% of total N (Fig. 4).

There was an almost complete loss of TCA soluble N due to 6 min leaching and no significant difference ($P>0.05$) in concentration of retained TCA soluble N between diets (Fig. 4).

In vitro *digestibility*

There was a significant increase (ANOVA, $P < 0.05$) in digestibility with time for both the leached and unleached

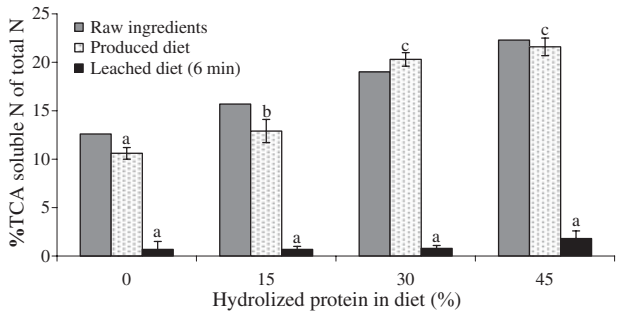

Figure 4 Concentration of TCA soluble N in diets with increasing inclusion of pepsin hydrolysed protein ranging from 0 to 45% of the total protein. The concentration of TCA soluble N is investigated in the raw ingredients (calculated) the produced diets ($n = 4$) and in the diets after 6 min leaching ($n = 4$). Error bars represent standard deviations. Significant differences between the diets are shown by different letters (ANOVA, followed by Tukey HSD, $P < 0.05$).

diets (Fig. 5). The unleached diets all had a significantly higher (ANOVA, $P < 0.05$) digestibility at all investigated time intervals than the leached diets (Fig. 5). Increased digestibility correlated with increased concentration of hydrolysed protein for time 0 h, ½ h and 1 h for the unleached diets and there was no significant difference in digestibility between the four diets at 12 h *in vitro* digestibility (Fig. 5). For the leached diets, no significant differences (ANOVA, $P \geq 0.05$) in digestibility between the diets at any of the investigated time intervals were found (Fig. 5).

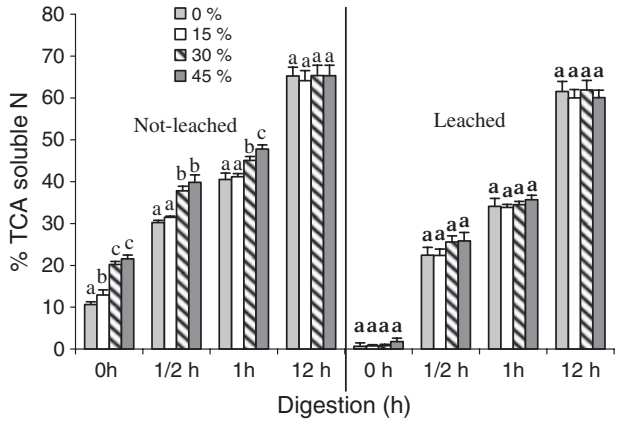

Figure 5 *In vitro* digestibility of protein in four diets with increasing concentration of hydrolysed protein ranging from 0 to 45% of the total protein. *In vitro* digestibility was investigated for both not leached and leached (6 min) diets. The digestibility was investigated at four time intervals from 0 h to 12 h ($n = 4$). Error bars represent standard deviations. Significant differences between the diets at each time interval are shown by different letters (ANOVA, followed by Tukey HSD, $P < 0.05$).

Change in AA profile

There were significant changes in AA profile due to leaching for 6 min, but the only severe change was a 100% loss of taurine and a 30% loss of histidine (Fig. 6). No significant ($P \geq 0.05$) differences in AA profile were found comparing the preleached diet with the digested preleached diet and the leached diet with the digested leached diet (Fig. 6). The significant difference in the AA profile between the *in vitro* digested preleached and leached diet was a consequence of the changes occurring during the leaching process (Fig. 6).

Discussion

These experiments were performed to evaluate changes in protein quality as a consequence of production procedures and leaching in a heat coagulated diet (Hamre *et al.* 2001) with increasing inclusion levels of prehydrolysed protein. The results clearly indicated that both the production process and leaching can dramatically change the protein quality and could possibly mask the biological effects of including a high concentration of hydrolysed protein. Different larval diets have shown to have different leaching rates (Lopez Alvarado *et al.* 1994; Kvåle *et al.* 2006), the findings of this study are valid for micro-bound type diets, but may differ with other diet production technologies such as microencapsulated diets. Considering the concentration of soluble N in the raw ingredients, there were relatively small differences in soluble N between the four diets after exposure to water for 6 min. For TCA soluble N there were no differences between the four diets after exposure to water. This shows the difficulties in accomplishing feeding studies to find the optimal concentration of hydrolysed protein for different species of marine fish larvae and could explain the varying results in feeding trials with formulated diets containing different concentrations of hydrolysed protein, as suggested by Kvåle *et al.* (2006).

Quality of the hydrolysed protein

Fresh cod fillet has a relatively high initial concentration of soluble N compared to commonly used feed ingredients such as heat processed fish meal (Tonheim *et al.* 2007). Pepsin hydrolysis of cod fillet leads to a doubling in concentration of both water-soluble N, from 34 ($n = 1$) to 65 ± 3%, and TCA soluble N, from 18 ± 2.4 to 40 ± 4.2% of total N. Tonheim *et al.* (2007) showed that 12 h pancreatic hydrolysis of cod fillet gave a product with 79 ± 9.1% TCA soluble N while a pepsin + pancreatic (24 h + 12 h) gave a product

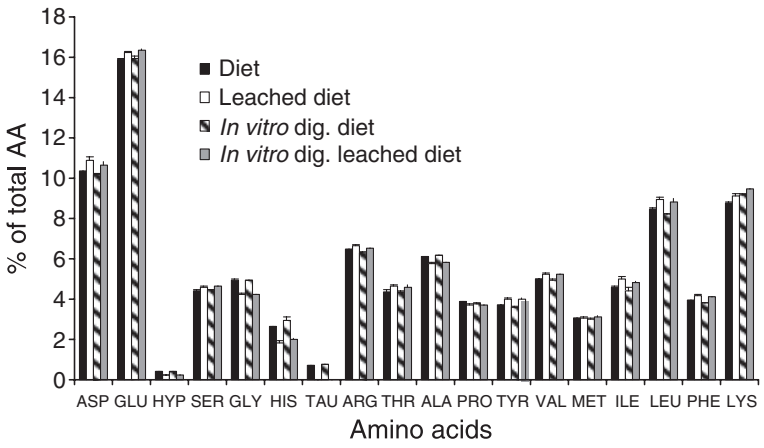

Figure 6 AA profile of the heat coagulated diet containing 30% hydrolysed protein before and after exposure to leaching for 6 min and the AA profile of the *in vitro* digested TCA precipitated supernatant of the not leached and leached diet. Error bars represent standard deviations ($n = 3$).

with 86 ± 2.7% TCA soluble N. This shows that different degrees of hydrolysis leads to large changes in product quality which might affect fish larvae during feeding trials. Due to possibly large differences in protein quality between hydrolysed protein sources, it is of importance to describe the quality of hydrolysed protein fractions and the total protein of diets used in feeding trials. Due to the high initial concentration of soluble N in cod fillet, inclusion of 45% hydrolysed protein led only to a 37% increase in water-soluble N in the raw ingredient mixture in this diet, compared to the diet containing 0% hydrolysed protein. However, there was a 104% increase in TCA soluble N.

Inclusion of pure peptides and FAA mixtures could be used instead of hydrolysed marine ingredients (Dabrowski *et al*. 2003; Zhang *et al*. 2006). Although there might be better control of the concentration and quality of the soluble N fraction by using pure peptides and FAA mixtures, use of hydrolysed marine products (random peptide mixtures), such as fish fillet used in this study, is a cheap and effective way of producing a product with a good AA profile. When hydrolysing the main protein source in the diet, instead of adding increasing amounts of a different hydrolysed protein source, the diet will keep the same AA profile only differing in solubility of the N fraction. This is in accordance with Dabrowski *et al*. (2003), who argued that the same source of protein had to be fed in both intact and hydrolysed forms to investigate if inclusion of protein hydrolysates leads to enhanced growth in larval fish.

Binding and particle quality

A protein bound heat coagulated diet is bound together by thermal denaturation of the protein. A diet with increased hydrolysed protein will contain less protein which is able to bind and contribute to matrix formation and might therefore result in decreased stability of the feed particles. The binding properties of the four diets were not investigated, however heat coagulated diets containing similar concentrations of hydrolysed protein have been used previously without detecting problems with the particle stability (Kvåle, pers. comm, NIFES, Bergen, Norway). Crushing and sieving led to rod shaped particles with shorter distance to the centre of the particles and larger surface area compared to round particles with the same volume. Increase of surface to volume ratio can lead to an increased leaching rate (Lee & Rosenberg 2000, 2001; Kvåle *et al*. 2006). However, rod shaped particles can make it possible for marine fish larvae to swallow larger particles that might compensate for the increased leaching due to the increased surface area.

Effect of production process on protein quality

As expected there was a reduction in soluble N due to thermal denaturation, but the concentration of TCA soluble N did not seem to be affected by heat treatment. However, the concentration of TCA soluble N in the raw ingredients was calculated from the pooled ingredients (Tonheim *et al*. 2007) so no statistics could be run. Increasing inclusion levels of hydrolysed protein increased the total content of water-soluble N from the ingredients and a relatively higher percentage of this water-soluble N remained water soluble after feed production as compared to feeds with lower inclusion levels of hydrolysed protein. The most likely reason for this is that hydrolysed protein participated to a lesser extent in matrix binding due to reduced molecular size, and thus remains unbound and water soluble after particle formation. Loss of soluble N due to heat denaturation was reduced from 45 ± 4% in the diet containing 0% hydrolysate to 17 ± 2% in the diet containing 45% hydrolysate. The increased loss of

soluble N due to heat denaturation from the diets containing zero or low levels of pepsin hydrolysed cod is due to the higher concentration of heat unstable intact soluble protein. Use of protein as the binder can be desirable in order to reduce the concentration of binders with low or non-nutritional qualities or to reduce the concentration of carbohydrates for species with a low tolerance such as halibut (Hjertnes *et al*. 1991; Hamre *et al*. 2003). However, different processes that cause protein matrix formation such as heat or crossbinding (Jones 1980) might also negatively affect the digestibility. For diets exposed to heat during feed production it is necessary to include soluble N in the form of FAA, peptides and thermally stable proteins such as casein, to avoid reductions in the concentration of soluble N due to denaturation.

Effect of leaching on protein composition of submerged feed particles

This study confirms the high loss of water-soluble N fractions when formulated diets with a small particle size are submerged in water (Lopez Alvarado *et al*. 1994; Kvåle *et al*. 2006). A 6 min leaching trial is relatively long and except for slow feeding species such as halibut, most feed particles would most likely be ingested within minutes. According to leaching trials on heat coagulated diets produced using the same protocol (Kvåle *et al*. 2006), the burst release was over after 5 min and there was still 50% retention. Based on these results, a 6-min leaching trial seemed to be an appropriate time interval to investigate the relative difference in leaching rate between the diets. However, the present study showed a higher rate of leaching compared to studies on heat coagulated diets produced using the same protocol (Kvåle *et al*. 2006). Kvåle *et al*. (2006) investigated leaching with the use of radioactively marked algae extract. The ^{14}C-labelled algae extract contained only 8% protein and presumably a large content of ^{14}C-labelled carbohydrates. The reduction of soluble ^{14}C-labelled algae extract due to heat treatment during production was not investigated; denaturing of the protein as shown in this study or a severe Maillard reaction of the carbohydrates (Deng *et al*. 2005) during production could explain the reduced leaching rate.

In the leaching studies by Kvåle *et al*. (2006) diets were submerged in 3% NaCl in comparison to this study where diets were leached in phosphate buffer. The leaching may therefore be overestimated due to a stronger osmotic gradient than with saltwater as leaching media. However, to better compare changes in concentration of soluble N in raw ingredients (Tonheim *et al*. 2007), produced diets, leached diets and finally *in vitro* digested diets, it was of importance to use the same buffer to better compare results. The choice of leaching media will presumably not affect the relative difference in leaching rate between diets.

Loss of soluble N from diets with high concentrations of hydrolysed protein will not only cause a shift in quality of the remaining protein, but also to a large extent affect protein quantity. Soluble N lost from the diet containing 45% hydrolysed protein accounted for 30% of the total protein fraction. As earlier hypothesized by Kvåle *et al*. (2006) amongst others, it might be the reduction in dietary crude protein levels that led to the reduced survival of Atlantic halibut juvenile fed increased concentrations of hydrolysed protein (Kvåle 2007).

Depending on hydrolysis method there may be a significant change in AA profile between soluble and insoluble fraction. For diets containing a large concentration of soluble N this may lead to a change in the AA profile of ingested diet, compared the diet as formulated, due to leaching. For the investigated diet containing 30% hydrolysed protein there were no major changes in AA profile due to leaching except a 32% loss of histidine and a 100% loss of taurine. Histidine is an essential AA and a high loss may lead to malnutrition; studies by Aragao *et al*. (2004) and Saavedra *et al*. (2007) suggests that histidine may be the first limiting AA when fish larvae are fed on rotifers. Taurine is not really an amino acid as it lacks the carboxylic group, and is not incorporated in protein. Taurine can thus only be ingested in the free form and not as part of dietary protein.

Taurine will therefore be lost rapidly from most formulated diets as reported for a protein encapsulated diet that also had a 100% loss after exposure to water (Nordgreen *et al*. 2007). Taurine is described as a non-essential nutrient, however, there are several indications that taurine is essential during early development of fish. Enrichment of live feed with taurine leads to increased growth of Japanese flounder (*Paralichtys olivaceus*) (Takeuchi *et al*. 2001; Chen *et al*. 2002, 2004, 2005; Kim *et al*. 2003, 2005; Matsunari *et al*. 2003) and red sea bream (*Pagrus major*) (Chen *et al*. 2004). In addition to a complete loss by exposure to leaching, the initial concentration of taurine (4.7 mg g^{-1} dw) was one quarter of that found in copepods (18 mg g^{-1} dw, van der Meeren *et al*. 2008) and 0.28 times that in *Artemia* (13 mg g^{-1} dw, van der Meeren *et al*. 2008). However, the concentration was 7.5–22 times higher than in rotifers (0.21–0.62 mg g^{-1} dw, van der Meeren *et al*. 2008; Srivastava *et al*. 2006). Kim *et al*. (2005) found that juvenile Japanese flounder required at least 15 mg g^{-1} taurine in the diet, confirming that formulated diets based on meals need

extra addition of taurine (Kim *et al*. 2005; Nordgreen *et al*. 2007). However, the taurine requirement for Japanese flounder established by Kim *et al*. (2005) might be overestimated due to leaching.

Effect of heat treatment on digestibility

Heat processing during feed production can decrease both the *in vivo* and *in vitro* protein digestibility of marine protein sources significantly (Yanes *et al*. 1970; Opstvedt *et al*. 1984; Lan & Pan 1993; Garcia-Ortega *et al*. 2000). The *in vitro* digestibility of the raw ingredients used in this study was unfortunately not measured. On the other hand, the individual digestibility of the protein sources *in vitro* was reported by Tonheim *et al*. (2007), using an identical batch of enzymes and protocol with this study. The results indicate that there is a decrease in digestibility from approximately 80% for the pooled ingredients (Tonheim *et al*. 2007) to 65% for the finished diets, suggesting a reduction in protein digestibility due to the production process.

Effect of leaching on N availability

As shown in this study, pepsin hydrolysis of fish fillet led to a doubling in both TCA and water-soluble N. However 35% of the hydrolysed product was still insoluble protein. The complete hydrolysed product with its remaining insoluble N was included in the formulated diet as in previous studies (Kvåle *et al*. 2002; Kvåle 2007). It is likely that the N fraction of the raw material that will be solubilized by enzymatic hydrolysis is the same fraction that would first be digested by marine fish larvae. A high inclusion of hydrolysed protein, with subsequent leaching during feeding, may therefore lead to an ingested diet with not only a reduced concentration of total protein, but also having lower quality of the remaining protein compared to a formulated diet using the same protein source unhydrolysed. This could explain the lower digestibility in the leached diets compared to the diets that had not been exposed to leaching. When high inclusion of hydrolysed protein is required, separation of the insoluble fraction before inclusion into a formulated diet should be considered. The FAA pool in live feed (Frolov *et al*. 1991; Hamre *et al*. 2002; Carvalho *et al*. 2003; Helland *et al*. 2003; van der Meeren *et al*. 2008; Srivastava *et al*. 2006) has been suggested to be one of the reasons for increased growth of larvae fed these feeds, compared to larvae fed formulated diets. However, later studies have also suggested that larger soluble nitrogenous compounds in live feed organisms could be as beneficial as FAA for increasing growth. More than 85% of the soluble N in *Artemia* and rotifers was larger than 500 D (Carvalho *et al*. 2003) and studies have indicated improved digestibility (Tonheim *et al*. 2007) and growth with the use of water-soluble proteins compared to insoluble proteins (Carvalho *et al*. 2004). There was a lower loss of water-soluble N than TCA soluble N in the present study. This is in accordance with Kvåle *et al*. (2006) who showed that increased size of the soluble N fraction led to a significant reduction in leaching rate. The use of higher molecular weight soluble N, rather than FAA and small peptides, should be considered in order to reduce the leaching rate from formulated diets.

For diets that had not been exposed to leaching there was a decreasing difference in digestibility between diets with increase in time of digestibility. The difference in digestibility at ½ h and 1 h is most likely a logical consequence of initial difference in TCA soluble N between diets. At 12 h there were no significant differences between diets, indicating that increased concentration of hydrolysed protein did not lead to increased digestibility of the non-TCA soluble N fraction. The benefit of using pepsin hydrolysates in larval diets may therefore be the increased fraction of highly available peptides and FAA and not an increased fraction of partly digested non-TCA soluble N. However, this hypothesis needs to be confirmed by further studies. The diets tested in the present study performed similarly with regards to digestibility *in vitro*, to commercially available Minipro™ after both 1 h and 12 h but better than a protein encapsulated diet that was digested to only 50% after 12 h (Tonheim *et al*. 2007).

Diets exposed to leaching prior to digestibility had a significantly lower digestibility at all time intervals compared to diets that were not leached. At ½ h and 1 h digestion the lower digestibility is most likely a consequence of loss of TCA soluble N. After 12 h, on the other hand, difference in digestibility might be due to difference in protein quality. Similar digestion of diets exposed to leaching at all time intervals, confirms suggestions by Kvåle *et al*. (2006) that leaching can change the quality of the ingested diets. Reduction in crude protein due to leaching was suggested by Kvåle *et al*. (2006) to be a possible reason for the lower survival of halibut juvenile fed diets with increased inclusion levels of hydrolysed protein. This is not possible to evaluate when using *in vitro* digestibility methods. The protocol for *in vitro* digestion used in the present study was based on methods described by Hsu *et al*. (1977) and Saterlee *et al*. (1979) and has been thoroughly discussed by Chong *et al*. (2002) and Tonheim *et al*. (2007). Chong *et al*. (2002) found a good correlation in relative digestibility between *in vitro* and *in vivo* measurements using juvenile discus fish

(*Symphysodon aequifasciata*) although there were some differences in absolute digestibility. Thus, if aiming to investigate true digestion of a feed or feed ingredients, *in vivo* measurements in the target species should be performed. As discussed by several authors it is difficult to estimate digestibility of different protein sources in small fish larvae. Tube feeding of radio-labelled protein to marine fish larvae (Rønnestad *et al*. 2001; Tonheim *et al*. 2004, 2005) has proved to be a good method to evaluate protein digestibility in such small animals. The method can, however, only be used when tube feeding soluble model proteins (Tonheim *et al*. 2004) and insoluble dietary proteins can therefore not be investigated by this method.

Conclusion

Micro-bound type diets as the one used in this study have a low efficiency in delivering soluble N to fish larvae and should be carefully considered for this purpose. However, it should be emphasized that the results in this study are valid for micro-bound type diets, but may differ with other production technologies. This study clearly indicates that feed production and leaching can have significant effects on protein quality and that optimal level of inclusion of hydrolysed protein in formulated diets for marine fish larvae may be difficult to determine. The optimum levels may have to be set for every individual diet, protein source and adapted to species feeding behaviour. The severe changes in protein quality during feed production and leaching show the importance of considering both the protein sources used and the type of diet produced when effects of hydrolysed proteins are investigated. Production process, leaching rate, and type and quality of the hydrolysed product used, have to be considered.

Acknowledgements

Audil Kvåle PhD at National Institute of Nutrition and Seafood Research (NIFES) is greatly acknowledges for discussions and suggestions on methodological issues. A special thank to Joseph Malaiamaan (NIFES) for AA analysis and feed manufacturing. This study was part of a project financed by the Norwegian Research Council (Project No. 14768/120).

References

Aragao, C., Conceicao, L.E.C., Fyhn, H.J. & Dinis, M.T. (2004) Estimated amino acid requirements during early ontogeny in fish with different life styles: gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*). *Aquaculture*, **242,** 589–605.

Boye, J.I., Ma, C.-Y. & Hardwalker, V.R. (1997) Thermal denaturizing and coagulation of proteins. In: *Food Proteins and Their Applications* (Damodaran, S. & Paraf, A. eds), pp. 25–56. Marcel Dekker, New York.

Cahu, C.L. & Zambonino-Infant, J.L. (2001) Substitution of live food by formulated diets in marine fish larvae. *Aquaculture*, **200,** 161–180.

Cahu, C.L., Infante, J.L.Z., Quazuguel, P. & Le Gall, M.M. (1999) Protein hydrolysate vs. fish meal in compound diets for 10-day old sea bass *Dicentrarchus labrax* larvae. *Aquaculture*, **171,** 109–119.

Carvalho, A.P., Escaffre, A.M., Teles, A.O. & Bergot, P. (1997) First feeding of common carp larvae on diets with high levels of protein hydrolysates. *Aquacult. Int*., **5,** 361–367.

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

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

Chen, X.G., Zheng, L., Wang, Z., Lee, C.Y. & Park, H.J. (2002) Molecular affinity and permeability of different molecular weight chitosan membranes. *J. Agricult. Food Chem*., **50,** 5915–5918.

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

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

Chong, A.S.C., Hashim, R. & Ali, A.B. (2002) Assessment of dry matter and protein digestibilities of selected raw ingredients by discus fish (*Symphysodon aequifasciata*) using in vivo and in vitro methods. *Aquacult. Nutr*., **8,** 229–238.

Conceicao, L.E.C., Houlihan, D.F. & Verreth, J.A.J. (1997) Fast growth, protein turnover and costs of protein metabolism in yolk-sac larvae of the African catfish (*Clarias gariepinus*). *Fish Physiol. Biochem*., **16,** 291–302.

Cousin, J.C.B., Baudinlaurencin, F. & Gabaudan, J. (1987) Ontogeny of enzymatic-activities in fed and fasting turbot, *Scophthalmus Maximus* L. *J. Fish Biol*., **30,** 15–33.

Dabrowski, K., Lee, K.J. & Rinchard, J. (2003) The smallest vertebrate, Teleost fish, c can utilize synthetic dipeptide-based diets. *J. Nutr*., **133,** 4225–4229.

Deng, D.F., Hemre, G.I., Storebakken, T., Shiau, S.Y. & Hung, S.S.O. (2005) Utilization of diets with hydrolyzed potato starch, or glucose by juvenile white sturgeon (*Acipenser transmontanus*), as affected by Maillard reaction during feed processing. *Aquaculture*, **248,** 103–109.

Frolov, A.V., Pankov, S.L., Geradze, K.N. & Pankova, S.A. (1991) Influence of salinity on the biochemical-composition of the rotifer *Brachionus Plicatilis* (Muller) – aspects of adaptation. *Comp. Biochem. Physiol. A Physiol*., **99,** 541–550.

Garcia-Ortega, A., Verreth, J., Van Hoornyck, A. & Segner, H. (2000) Heat treatment affects protein quality and protease activity in decapsulated cysts of *Artemia* when used as starter food for larvae of African catfish Clarias gariepinus (Burchell). *Aquacult. Nutr*., **6,** 25–31.

Govoni, J.J., Boehlert, G.W. & Watanabe, Y. (1986) The physiology of digestion in fish larvae. *Environ. Biol. Fishes*, **16,** 59–77.

Hamre, K., Naess, T., Espe, M., Holm, J.C. & Lie, O. (2001) A formulated diet for Atlantic halibut (*Hippoglossus hippoglossus*, L.) larvae. *Aquacult. Nutr*., **7,** 123–132.

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

Hamre, K., Ofsti, A., Naess, T., Nortvedt, R. & Holm, J.C. (2003) Macronutrient composition of formulated diets for Atlantic halibut (*Hippoglossus hippoglossus*, L.) juveniles. *Aquaculture*, **227,** 233–244.

Hamre, K., Moren, M., Solbakken, J., Opstad, I. & Pittman, K. (2005) The impact of nutrition on metamorphosis in Atlantic halibut (*Hippoglossus hippoglossus* L.). *Aquaculture*, **250,** 555–565.

Helland, S., Terjesen, B.F. & Berg, L. (2003) Free amino acid and protein content in the planktonic copepod Temora longicornis compared to *Artemia franciscana*. *Aquaculture*, **215,** 213–228.

Hjertnes, T., Gulbrandsen, K.E., Johnsen, F. & Opstvedt, J. (1991) Effect of dietary protein, carbohydrate and fat levels in dry feed for juvenile halibut (*Hippoglossuss hippoglossus* L.). In: *Fish Nutrition in Practice* (Kaushik, S.J. & Luquet, P. ed.), pp. 493–496, Biarritz, France, 493–496.

Houde, E.D. (1989) Comparative growth, mortality, and energetics of marine fish larvae – temperature and implied latitudinal effects. *Fishery Bull.*, **87,** 471–495.

Houlihan, D.F., Pedersen, B.H., Steffensen, J.F. & Brechin, J. (1995) Protein-synthesis, growth and energetics in larval herring (*Clupea Harengus*) at different feeding regimes. *Fish Physiol. Biochem.*, **14,** 195–208.

Howell, B.R., Day, O.J., Ellis, T. & Baynes., S.M. (1998) Early life stages of farmed fish. In: *Biology of Farmed Fish* (Black, K.D. & Pickering, A.D. eds), pp. 27–66. Sheffield Academic Press, Sheffield.

Hsu, H.W., Vavak, D.L., Satterlee, L.D. & Miller, G.A. (1977) Multienzyme technique for estimating protein digestibility. *J. Food Sci.*, **42,** 1269–1273.

Jones, D.A. (1980) Microencapsulation process. In: *Europena Patent Office*, Vol. Patent No. GB 2040863, Munich, Germany.

Jones, D.A., Munford, J.G. & Gabbott, P.A. (1974) Microcapsules as artificial food particles for aquatic filter feeders. *Nature*, **247,** 233–235.

Kamler, E., Szlaminska, M., Raciborski, K., Barska, B., Jakubas, M., Kuczynski, M. & Przybyl, A. (1992) Bioenergetic evaluation of 4 formulated diets for carp (*Cyprinus-Carpio* L) larvae as compared with zooplankton fed and starved larvae. *J. Anim. Physiol. Anim. Nutr.*, **67,** 1–15.

Kim, S.K., Takeuchi, T., Yokoyama, M. & Murata, Y. (2003) Effect of dietary supplementation with taurine, beta-alanine and GABA on the growth of juvenile and fingerling Japanese flounder *Paralichthys olivaceus*. *Fisheries Sci.*, **69,** 242–248.

Kim, S.K., Takeuchi, T., Yokoyama, M., Murata, Y., Kaneniwa, M. & Sakakura, Y. (2005) Effect of dietary taurine levels on growth and feeding behavior of juvenile Japanese flounder *Paralichthys olivaceus*. *Aquaculture*, **250,** 765–774.

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

Kolkovski, S., Koven, W. & Tandler, A. (2001) Digestive enzymes in fish larvae and juveniles-implications and applications to formulated diets. *Aquaculture*, **200,** 181–201.

Kvåle, A. (2007). *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*. PhD thesis, pp. 82. University of Bergen, Bergen, Norway.

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

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

Kvåle, A., Mangor-Jensen, A., Moren, M., Espe, M. & Hamre, K. (2007) Development and characterisation of some intestinal enzymes in Atlantic cod (*Gadus morhua* L.) and Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae. *Aquaculture*, **264,** 457–468.

Lan, C.C. & Pan, B.S. (1993) In-vitro digestibility simulating the proteolysis of feed protein in the midgut of grass shrimp (*Penaues monodon*). *Aquaculture*, **109,** 59–70.

Langdon, C.J. (1989) Preparation and evaluation of protein microcapsules for a marine suspension-feeder, the Pacific oyster *Crassostrea Gigas*. *Mar. Biol.*, **102,** 217–224.

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

Lee, S.J. & Rosenberg, M. (2000) Microencapsulation of theophylline in whey proteins: effects of core to wall ratio. *Int. J. Pharm.*, **205,** 147–158.

Lee, S.J. & Rosenberg, M. (2001) Microencapsulation of thephylline in composite wall system consisting of whey proteins and lipids. *J. Microencapsul.*, **18,** 309–321.

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

Matsunari, H., Takeuchi, T., Murata, Y., Takahashi, M., Ishibashi, N., Chuda, H. & Arakawa, T. (2003) Changes in the taurine content during the early growth stages of artificially produced yellowtail compared with wild fish. *Nippon Suisan Gakkaishi*, **69,** 757–762.

van der Meeran, T., Olsen, R.E., Hamre, K. & Fyhn, H.J. (2008) Biochemical composition of copepods for evaluation of feed quality in production of juvenile marine fish. *Aquaculture*, **274,** 375–397.

Mohammed, Z.H., Hill, S.E. & Mitchell, J.R. (2000) Covalent crosslinking in heated protein systems. *J. Food Sci.*, **65,** 221–226.

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

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

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

Opstvedt, J., Miller, R., Hardy, R.W. & Spinelli, J. (1984) Heat-induced changes in sulfhydryl-groups and disulfide bonds in fish-protein and their effect on protein and amino- acid digestiblity in rainbow-trout (*Salmo Gairdneri*). *J. Agricult. Food Chem.*, **32,** 929–935.

Otterlei, E., Nyhammer, G., Folkvord, A. & Stefansson, S.O. (1999) Temperature- and size-dependent growth of larval and early juvenile Atlantic cod (*Gadus morhua*): a comparative study of Norwegian coastal cod and northeast Arctic cod. *Can. J. Fisheries Aquat. Sci.*, **56,** 2099–2111.

Pittman, K., Skiftesvik, A.B. & Berg, L. (1990) Morphological and behavioral-development of halibut, *Hippoglossus Hippoglossus* (L) larvae. *J. Fish Biol.*, **37,** 455–472.

Plakas, S.M., Lee, T.C., Wolke, R.E. & Meade, T.L. (1985) Effect of maillard browning reaction on protein-utilization and plasma amino-acid response by rainbow-trout (*Salmo Gairdneri*). *J. Nutr.*, **115,** 1589–1599.

Rønnestad, I., Rojas-Garcia, C.R., Tonheim, S.K. & Conceicao, L.E.C. (2001) *In vivo* studies of digestion and nutrient assimilation in marine fish larvae. *Aquaculture*, **201,** 161–175.

Saavedra, M., Beltran, M., Pousao-Ferreira, P., Dinis, M.T., Blasco, J. & Conceicao, L.E.C. (2007) Evaluation of bioavailability of individual amino acids in *Diplodus puntazzo* larvae: towards the ideal dietary amino acid profile. *Aquaculture*, **263,** 192–198.

Saterlee, L.D., Marshall, H.F. & Tennyson, J.M. (1979) Measuring protein quality. *J. Am. Oil Chem. Soc.*, **56,** 103–109.

Segner, H., Storch, V., Reinecke, M., Kloas, W. & Hanke, W. (1994) The development of functional digestive and metabolic organs in turbot, *Scophthalmus Maximus*. *Mar. Biol.*, **119,** 471–486.

Sokal, R.R. & Rohlf, F.J. (1969) *Biometry*. pp. 776. W.H. Freeman, San Francisco.

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

Stoss, J., Hamre, K. & Otterå, H. (2004) Weaning and nursery. In: *Culture of Cold-Water Marine Fish* (Moksness, E., Kjørsvik, E. & Olsen, Y. eds), pp. 337–362. Blackwell Publishing, Oxford.

Takeuchi, T., Park, G.S., Seikai, T. & Yokoyama, M. (2001) Taurine content in Japanese flounder *Paralichthys olivaceus* T. & S. and red sea bream Pagrus major T. & S. during the period of seed production. *Aquacult. Res.*, **32,** 244–248.

Tonheim, S.K., Espe, M., Raae, A.J., Darias, M.J. & Ronnestad, I. (2004) In vivo incorporation of [U]C-14-amino acids: an alternative protein labelling procedure for use in examining larval digestive physiology. *Aquaculture*, **235,** 553–567.

Tonheim, S.K., Espe, M., Hamre, K. & Ronnestad, I. (2005) Prehydrolysis improves utilisation of dietary protein in the larval teleost Atlantic halibut (*Hippoglossus hippoglossus* L.). *J. Exp. Mar. Biol. Ecol.*, **321,** 19–34.

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

Villamar, D.F. & Langdon, C.J. (1993) Delivery of dietary-components to larval shrimp (*Penaeus Vannamei*) by means of complex microcapsules. *Mar. Biol.*, **115,** 635–642.

de Wet, P.J. (1983) Effect of processing on nutritive value of feeds: protein. In: *Handbook of Nutritive Value of Processed Food* (Hudson, B.J.F. ed.), pp. 321–341. CRC Press Inc, Boca Raton, FL.

Yanes, E., Ballester, D. & Donoso, G. (1970) Effect of drying temperature on quality of fish protein. *J. Sci. Food Agricult.*, **21,** 426.

Yufera, M., Sarasquete, M.C. & FernandezDiaz, C. (1996) Testing protein-walled microcapsules for the rearing of first-feeding gilthead sea bream (*Sparus aurata* L) larvae. *Mar. Freshwater Res.*, **47,** 211–216.

Yufera, M., Kolkovski, S., Fernandez-Diaz, C. & Dabrowski, K. (2002) Free amino acid leaching from a protein-walled micro encapsulated diet for fish larvae. *Aquaculture*, **214,** 273–287.

Zambonino-Infante, J.L.Z., Cahu, C.L. & Peres, A. (1997) Partial substitution of di- and tripeptides for native proteins in sea bass diet improves *Dicentrarchus labrax* larval development. *J. Nutr.*, **127,** 608–614.

Zhang, Y., Dabrowski, K., Hliwa, P. & Gomulka, P. (2006) Indispensable amino acid concentration decrease in tissues of stomachless fish, common carp in response to free amino acid- or peptide-based diets. *Amino Acids*, **31,** 165–172.