Available online at www.sciencedirect.com
ScienceDirect
Aquaculture 262 (2007) 426–435
Aquaculture
www.elsevier.com/locate/aqua-online

In vitro digestibility of water-soluble and water-insoluble protein fractions of some common fish larval feeds and feed ingredients

S.K. Tonheim [a,b], A. Nordgreen [a], I. Høgøy [c], K. Hamre [a], I. Rønnestad [b,*]

[a] *National Institute of Nutrition and Seafood Research, Bergen, Norway*
[b] *Department of Biology, University of Bergen, Bergen, Norway*
[c] *Maripro AS, Austevoll, Norway*

Received 24 April 2006; received in revised form 27 October 2006; accepted 27 October 2006

Abstract

In vitro methods have previously been utilised for the rapid and reliable evaluation of protein digestibility in fish. In this study we used *in vitro* methods to compare the digestibility of various live and artificial larval feeds and feed ingredients. Given previous suggestions that water-soluble dietary proteins are efficiently digested and utilised by stomachless fish larvae, we also analysed the content of water-soluble nitrogen in the feeds and feed ingredients and then measured the specific *in vitro* digestibility (simulated midgut conditions) of the water-soluble and insoluble fractions. The soluble nitrogen fractions were generally more digestible than the insoluble nitrogen fractions ($P<0.05$). A soluble reference protein (Na^+-caseinate) was digested faster than the similar but insoluble reference protein (casein) although their final digestibility was the same (94%). Frozen live feeds (*Artemia fransiscana* and *Calanus finmarchicus*) contained high fractions of soluble nitrogen (54–67%) and also had high digestibility *in vitro* (84 and 87%, respectively). The *in vitro* digestibility of two formulated larval feeds tested was lower (53 and 70%) than the frozen live feeds. The digestibility of the ingredients of the protein-encapsulated feeds particles was reduced as a result of the production process (from 71 to 53%, respectively). Three meals of marine origin (fish meal, squid meal and fish roe meal) all had low contents of water-soluble nitrogen (11–17%) but showed different degrees of digestibility (77, 77 and 49%, respectively). The results also demonstrated that while pre-hydrolysis of a feed ingredient (fresh frozen cod fillet) almost doubled the water-soluble nitrogen fraction (from 34 to 65%) the positive effect of pre-hydrolysis on *in vitro* digestibility was much lower (from 80 to 86%). This demonstrates the complexity of assessment of bioavailability of dietary protein sources in larval fish; a number of factors such as leaching rates from feed particles, digestibility, digestion rates and absorption rates all need to be taken into account.

Keywords: Protein; Digestibility; Fish larvae; Soluble protein; Compound diets

1. Introduction

Most marine fish species that are either established or candidates for aquaculture start exogenous feeding at an early stage in ontogeny, and long before the digestive system has fully matured (Vu, 1983; Blaxter, 1988; Luizi et al., 1999). The lack of a functional acid-secreting stomach in particular may negatively affect

* Corresponding author. Department of Biology, University of Bergen. Allégaten 41, N-5007 Bergen, Norway. Tel.: +47 55 58 25 75; fax: +47 55 58 96 67.
E-mail address: ivar.ronnestad@bio.uib.no (I. Rønnestad).

0044-8486/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquaculture.2006.10.030

protein digestion (e.g. Jany, 1974; Rønnestad et al., 2003; Tonheim et al., 2005). In a functional gastric stomach, proteins are exposed to proteolytic active pepsin under denaturing acid conditions (pH 2 to 5), which may accelerate the proteolysis of ingested dietary proteins. In stomachless larvae dietary protein digestion has to rely solely on the intestinal digestion which is performed by pancreatic proteases and mucosa-associated proteases. It has also been shown that dietary protein can be taken up by pinocytosis in larvae and be digested intracellularly in the enterocyte by lysosomal proteolysis (Watanabe, 1981; Govoni et al., 1986), although the quantitative significance of this route of absorption remains to be determined (Rønnestad and Conceição, 2005).

Fish larvae have high dietary amino acid (AA) requirements due to their high growth potential (e.g. Houde, 1989; Conceição et al., 1997; Otterlei et al., 1999) and extensive combustion of AA in their energy metabolism (Rønnestad and Naas, 1993; Finn et al., 1995, 2002). The digestibility of dietary proteins is therefore crucial to their utilisation efficiency and an understanding of the specific digestibility of feed ingredients is important for the formulation of optimal diets for larvae in aquaculture. However, little is known about the digestibility of various protein sources used in compound diets for first-feeding marine larval fish. The digestibility of different sources of protein in adult fish and other animal models is of only limited relevance to larvae, because of differences in the digestive physiology of larval and juvenile/adult stages.

Current best practice for first-feeding fish larvae involves the extensive use of live feeds such as rotifers, *Artemia* and copepods (e.g. Kolkovski, 2001). Experimental studies have shown that it is possible to first-feed some stomachless marine fish larvae exclusively on compound diets (sea bass, *Dicentrarchus labrax*, Cahu et al., 1998; Japanese eel, *Anguilla japonica*, Pedersen et al., 2003; gilthead sea bream, *Sparus aurata*, Robin and Vincent, 2003. However, both sea bass and gilthead sea bream larvae grew more poorly than larvae fed live feeds (Cahu et al., 1998; Robin and Vincent, 2003). Growth on compound diets was also poor in the Japanese eels (Pedersen et al., 2003), but this species had not previously been successfully first-fed in an artificial system. A sufficient supply of dietary amino acids is a prerequisite for high growth rates. Compound feeds usually have high nutrient density, high relative protein content and are based on ingredients that have good amino acid balance as far as sustaining growth in juvenile fish is concerned. It is therefore unlikely that inferior growth in fish larvae fed compound diets is the result of low quantitative protein content or a poor amino acid profile, but is more likely to be a result of other qualitative differences and specific larval dietary requirements.

Carvalho et al. (2004), who studied freshwater common carp, *Cyprinus carpio*, larvae, found that replacing insoluble casein with soluble Na^+-caseinate in a starter diet improved both growth and survival. The protein sources used in their study are very similar except for their water solubility. For this reason, and because common live feeds, unlike formulated feeds, contain a high proportion of water-soluble protein (Fyhn et al., 1993; Hamre et al., 2002; Helland et al., 2003; Carvalho et al., 2003), Carvalho et al. (2004) suggested that protein solubility is important as a determinant of digestibility in larvae.

FAA and small peptides are usually water soluble and are efficiently absorbed from the intestine without previous digestion, and can thus be regarded as pre-digested dietary protein. Although pre-hydrolysis will enhance digestibility, substantial supplementation of compound diets with pre-hydrolysed protein has been reported as having a negative impact on the growth and survival of some marine fish larvae (*S. aurata*, Kolkovski and Tandler, 2000; *D. labrax*, Cahu et al., 1999). On the other hand, Day et al. (1997) found that substituting 100% of fish meal with pre-hydrolysed fish protein in a compound weaning diet for Dover sole (*Solea solea*) resulted in improved survival.

The specific digestibility of soluble versus insoluble proteins has not been studied in fish larvae. The positive effects of Na^+-caseinate as reported by Carvalho et al. (2004) may be due to soluble proteins being more exposed to intestinal proteases or being more efficiently taken up by pinocytosis. High feed intake and gut evacuation rates have been reported in fish larvae, (Govoni et al., 1986), and these may be important factors in poor protein digestion and utilisation in larvae (Govoni et al., 1986, Tonheim et al., 2005). An important prerequisite of successful compound starter diets for fish larvae may thus be sufficient quantities of highly digestible dietary proteins.

The true digestibility of different protein sources in larvae can only be measured *in vivo*. However, methods for *in vitro* digestion assessment using commercial available proteases have been used for rapid, easy and reproducible evaluation of the digestibility of feeds and feedstuffs (Hsu et al., 1977; Saterlee et al., 1979; Lazo et al., 1998). Chong et al. (2002) compared dry matter and protein digestibility in discus fish (*Symphysodon aequifasciata*) assessed by three different methods: the *in vitro* protocols of Hsu et al. (1977), Saterlee et al.

(1979), Lazo et al. (1998); *in vitro* digestion using gut extract from the discus fish; and *in vivo* digestibility assessed in feeding trials with fish itself. They found that relative digestibility, as measured by simple methods involving only a few proteases in a single reaction step, correlated well with digestibility measured *in vivo*. *In vitro* digestibility can thus be a useful first approach to selecting candidate protein sources for follow-up studies in larvae.

In order to improve our understanding of bioavailability of larval feeds, we investigated the solubility and *in vitro* digestibility of the water-soluble and water-insoluble fractions of live feeds and selected protein sources that are widely used or have a potential for use in larval compound diets.

2. Materials and methods

2.1. Feed and feed ingredients

A total of 13 feeds and feed ingredients was evaluated (Table 1). The live feeds, squid meal, fish roe meal, fish meal, fresh frozen cod fillet and pepsin-hydrolysed cod fillet were homogenised in phosphate buffer (pH 8.0) at 4 °C. The insoluble fraction of each homogenate was separated from the soluble fraction by centrifugation. Pellets were resuspended in phosphate buffer and centrifuged twice in order to wash the pellet.

Two compound larval diets were included in the study: one commercial microbound diet (Minipro™) and one protein-encapsulated micro-diet. Minipro™ was based on fish meal, fish protein hydrolysate, squid meal and herring stick-water as the main ingredients and the feed particles were prepared by using a carbohydrate binder and a spray technique. The protein-encapsulated diet was based on Na^+-caseinate, 39%; squid meal, 12%; cod roe, 12%; pepsin hydrolysed cod roe, 12%; dextrin, 10%; soya lecithin, 5%; cod liver oil, 10%. The microcapsules were prepared according to Yufera et al. (1999), with some modifications. The dissolved feed ingredients (water phase) were immersed in a non-polar solvent before the addition of an agent (1,3,5-benzene-tricarboxylic acid chloride), which induced cross-linking between the dietary protein molecules at the interface between the two phases.

2.2. In vitro measurements

Several different methods and procedures have previously been used to measure protein digestibility *in vitro*. In this study, digestibility was assessed as a percentage of nitrogen in the soluble fraction after treatment of the protein source with proteases and subsequent precipitation of undigested protein by trichloroacetic acid (TCA).

Crude protein content ($N \times 6.25$) was determined in all separated fractions and in the compound feeds by an FP-528 nitrogen analyser (Leco, MI, USA). The previously separated insoluble fractions, dry compound feeds and insoluble casein were resuspended in 1 ml of phosphate buffer (pH 8.0) in Eppendorf tubes in amounts corresponding to 20 mg of crude protein. The previously separated soluble fractions, soluble Na^+-caseinate, whey protein and stick-water were added to Eppendorf tubes in amounts corresponding to 20 mg of crude protein and diluted with phosphate buffer (pH 8.0)

Table 1
Feeds and feed ingredients evaluated

Feeds and feed ingredients	Origin
[1]*Artemia franciscana* (nauplii)	Decapsulated and hatched at IMR*, Austevoll, Norway
Calanus finmarchicus (copepod)	Captured and frozen at sea, Calanus AS, Tromsø, Norway
**Minipro™	Produced by Maripro AS, Austevoll, Norway
**Protein-encapsulated feed	Produced by NIFES according to Yufera et al., 1999
Micronised squid meal	Obtained from Rieber, Bergen, Norway
Micronised fish meal	Obtained from Rieber, Bergen, Norway
Micronised fish roe meal	Obtained from Rieber, Bergen, Norway
[2]Pepsin-digested cod fillet	Wild captured, hydrolysed at NIFES, Bergen, Norway
[3]Fresh frozen cod fillet	Wild captured
Stick-water	Water-soluble fraction from boiled herring, produced by Maripro AS, Austevoll, Norway
Casein	Tine AS, Oslo, Norway
Na^+-caseinate	Tine AS, Oslo, Norway
Whey protein	Tine AS, Oslo, Norway

*Institute of Marine Research;** Formulated diets; [1]Decapsulated, hatched and enriched for 20 h according to standard protocols at IMR before being rinsed with water, frozen and stored at −20 °C; [2]Ground fillets (136 g) were acidified by addition of 6 M of HCl (46 ml, pH 4.2). Hydrolysis was initiated by adding a mixture of water (18 ml), glycerol (9.5 ml) and pepsin (10.2 g) and hydrolysis allowed to continue at room temperature over night. The hydrolysate was thereafter neutralised to pH 7.6 by adding 6 M NaOH (55 ml). [3]A fresh fillet, frozen and stored at −20 °C.

to a final volume of 1 ml. A mixture (final volume 100 µl) of trypsin (type IX, *bovine* pancreas), chymotrypsin (type II, *bovine* pancreas) and bacterial protease (type XIV, *Streptomyces griseus*), all obtained from Sigma-Aldrich, MO, USA, was added to each tube to final concentrations of 73, 145 and 64 mg ml^{-1}, respectively. Digestion was performed at room temperature (22 °C). Proteins were precipitated and the *in vitro* digestion terminated by adding 250 µl of 40% TCA. For each component analysed, four parallel tubes were terminated and sampled 0, 1 and 12 h after addition of the proteases. The tubes were centrifuged and the crude protein content in the supernatant (350 µl), which contained proteolysis products, FAA and small peptides, was analysed and regarded as representing digested protein. Crude protein in the precipitate, containing intact protein and large peptides, was regarded as representing undigested protein. Casein was introduced as a reference protein every time *in vitro* digestion was performed, as a control of reproducibility.

Minor errors (<3%) in the calculation of supernatant volume due to changes in suspended material according to the degree of proteolysis were corrected for.

3. Results and discussion

Digestibility was assessed by elementary nitrogen analysis of the TCA solubles after treatment with crystalline proteases in an alkaline buffer. Compared to the results of Garcia-Ortega et al. (2000), who separated digested nitrous compounds from undigested nitrous compounds by filtration, the present analyses of the soluble fraction represent an improvement as most of the samples contained more nitrogen in the soluble than the insoluble fraction. In future studies, further improvements could be made by determining the increase in free amino acids by amino acid analysis, or by detecting free amino groups, as demonstrated by Lindner et al. (1995).

3.1. Contents of water-soluble nitrogen

The distribution of crude protein into water-soluble and insoluble fractions varied among the feeds and feedstuffs (Table 2). The live feeds, *Artemia* and *Calanus*, contained high fractions of soluble nitrogen; 67 and 54% of total N respectively. This was in accordance with high levels of water-soluble nitrogen in *Artemia* and rotifers (54 and 61%, respectively), as previously reported by Carvalho et al. (2003). Fresh frozen cod fillet contained 35% of water-soluble nitrogen. Pepsin hydrolysis almost doubled the water-soluble nitrogen fraction in cod fillet to 65%. The squid, fish and roe meals contained lower levels of 11, 17 and 11% respectively.

Table 2
Distribution of crude protein ($N \times 6.25$) into water-soluble and water-insoluble fractions

Sample	Water-soluble fraction (%)	Insoluble fraction (%)	S.D.	*n* (analytical)
Artemia (nauplii)	67	33	2.8	4
Calanus (copepod stage)	54	46	2.2	4
Squid meal	11	89	2.6	4
Fish meal (Rieber, Norway)	17	83	1.6	4
Roe meal	11	89	1.1	4
Trypsin-digested cod filet	65	35	3.0	3
Fresh frozen cod fillet	34	66		1
Stick-water	100			–
Casein		100		–
Na^{+}-caseinate	100			–
Whey protein	100			–

3.2. In vitro digestibility

The *in vitro* digestibility after 12 h, assessed as percentage of analysed nitrogen in the TCA-soluble fraction, showed that the water-soluble fractions from the live feeds, the hydrolysed cod fillet and the meals were digested to 90% or above (Table 3). The water-soluble fraction of the fresh frozen cod fillet, together with whey protein and stick-water, both of which consists entirely of water-soluble proteins, were digested to some lesser extent, 75±6.4%, 68±1.9 and 71±0.7, respectively.

The insoluble fractions of the live feeds, hydrolysed (pepsin-digested) cod fillet, fish and squid meals were all digested to above 70% (71–76%), while the insoluble fraction of the fish roe meal was digested to a lesser extent (43±1%). The insoluble fraction of the fresh frozen cod fillet had the highest digestibility of the insoluble fractions (82±2.5%). These results suggest that, in a pancreatic digestive system, the water-soluble protein fractions of live feeds and feed ingredients are more digestible than the insoluble protein fractions ($P<0.05$), when all insoluble fractions are compared with all soluble fractions, All water-soluble fractions contained a high initial proportion of TCA-soluble nitrogen (36–93%). This fraction was, according to the definition used in this study, already digested before the proteases were added.

The initial levels of TCA-soluble nitrogen in the *Calanus* soluble fraction consisted of 93% of total

Table 3
In vitro digestibility of some feeds and feed ingredients used in starter diets for fish larvae in Aquaculture

	Digestibility of water-soluble fractions (%)				Digestibility of insoluble fractions (%)				[a]Total digestibility (%)			
	0 h	1 h	12 h	*n* (analytic)	0 h	1 h	12 h	*n* (analytic)	0 h	1 h	12 h	*n* (analytic)
Artemia	37±1.9	66±6.7	91±7.0	4	17±0.2	46±0.4	71±3.6	2	30	59	84	–
Calanus	93±3.8	88±8.9	96±6.1	4	9±0.2	45±1.0	72±0.8	2	54	68	87	–
Cod fillet	36±2.5	55±7.4	75±6.4	4	8±0.1	55±1.3	82±2.5	2	18	55	80	–
Hydrolysed cod fillet	55±2.7	80±4.8	92±2.7	4	10±1.4	42±1.9	76 (*n*=1)	2	39	67	86	–
Fish meal	56±7.0	99±4.8	103±2.4	4	6±0.9	46±0.8	71±0.2	2	15	55	77	–
Squid meal	79±7.5	85±4.3	90±7.1	4	7±1.4	42±0.5	75±2.3	2	15	47	77	–
Fish roe meal	57±5.4	71±5.6	91±0.9	4	6±0.4	21±0.8	43±1.0	2	12	27	49	–
Casein									5±0.0	61±4.0	94±3.0	2
Na^+-caseinate									6±0,6	82±2.4	94±1.7	6
Whey protein									14±1.2	24±3.9	68±1.9	4
Stick-water									63±0.6	73±0.0	71±0.7	4
Minipro™									32±1.9	47±2.0	70±3.0	2
Protein-encapsulated diet									1±0.4	29±1.2	53±2.3	4
Mixed ingredients for protein-encapsulated diet									8±0.6	54±2.1	71±1.4	4

[a] Total digestibility was calculated as the weighed digestibility based on the digestibility of the water-soluble and water-insoluble fractions. Hence, errors and analytical parallels are not presented.

nitrogen. This strongly suggests that autolysis had taken place in this sample, a possibility that cannot be rejected, as the *Calanus* sample was obtained frozen from a commercial supplier and its history of treatment from capture to freezing was not known in detail. The *Artemia* sample, on the other hand, was rapidly frozen and kept frozen at −20 °C until analysis, and during extraction of water-soluble nitrogen the temperature was kept at 4 °C in order to avoid autolysis. In spite of this *Artemia* also contained a high initial level of TCA solubles (37.8%), far above the levels of free amino acids (FAA) that have been reported for *Artemia* in the literature (Hamre et al., 2002; Helland et al., 2003). Carvalho et al. (2003) found that 89% of the *Artemia* nitrogen was in macromolecules larger than 500 Da. As peptides larger than this may be soluble in TCA, these values may represent the true level of FAA and small peptides in *Artemia*. Alternatively, they may include some hydrolysis products of autolysis in spite of the rapid freezing and low temperature during extraction. However, if autolysis during the extraction contributed to the high initial content of water-soluble nitrogen in the *Artemia*, the same contribution from autolysis to the total proteolysis in the gut after ingestion by fish larvae would be expected. The high initial levels of TCA-soluble components in the live feed samples thus suggest high protein availability in larvae. On the other hand, there is a possibility that the freezing and thawing of the *Artemia* sample in the present study contributed to some extent to protein degradation and the increased solubility and digestibility of the *Artemia*, beyond that of live *Artemia* ingested by fish larvae.

The measured digestibility of the soluble Na^+-caseinate after 12 h did not differ ($P>0.05$) from that of insoluble casein. After 1 h, however, the digestibility of soluble Na^+-caseinate was significantly higher ($P<0.05$), suggesting a lower initial digestion rate of insoluble casein. One possible reason for the difference observed after 1 h is the poor hydroscopic properties of the casein, which formed large hydrophobic aggregations in the reaction tubes, which would have reduced the effective contact surface between the casein molecules and the proteases. Carvalho et al. (2004) reported significantly improved survival and some improved growth in first-feeding carp larvae on compound diets when insoluble casein was replaced by Na^+-caseinate as the only protein source. Since feed ingestion rate was not monitored in their experiment, the possible positive effect of the solubility of Na^+-caseinate cannot be separated from possible differences in palatability between diets. However, no evidence for such a different effect on palatability has been presented and due to the high molecular weight of casein, differences in leaching between the two diets were unlikely to occur. The measured difference in initial digestibility between insoluble casein and Na^+-caseinate in the present study supports

the notion of improved digestibility as an explanation for the improved performance of the soluble Na^+-caseinate in the early feeding larvae in the study of Carvalho et al. Visual observations made during this study showed that all suspended insoluble fractions were more hydrophilic than the insoluble casein and were therefore better dispersed in the buffer in the reaction tubes. Even so, the average initial digestibility (at 0 h) of insoluble casein exceeded that of all the other insoluble fractions (Table 3). This suggests that the insoluble fractions of the fish-, squid- and fish roe-derived ingredients are even less available than the insoluble casein that produced poor growth and survival in carp larvae (Carvalho et al., 2004). This further supports the idea that low dietary protein availability could be a limiting factor for growth and survival in first-feeding larvae fed compound diets based on such protein sources. Feeding experiments with compound diets for marine larvae (sea bass, *D. labrax*) have shown that casein may support growth and survival in the early feeding stage (sea bass, Cahu and Zambonino Infante, 1995; sea bream, *S. aurata*, Robin and Vincent, 2003) when intestinal proteolysis is considered to be poor. On the other hand, diets with casein as the only protein source did not to support growth in sea bass larvae over time just as well as fish-based or mixed protein sources (Cahu and Zambonino Infante, 1995), suggesting that casein as the only protein source did not fully meet the amino acid requirements of the larvae of this species. However, in these experiments insoluble casein or a mix of insoluble casein and soluble casein and pre-hydrolysed casein were used. To the best of our knowledge, marine larvae have yet to be fed with compound diets based on Na^+-caseinate as the only protein source, as in the study of Carvalho et al., 2004 on freshwater carp larvae.

The total digestibility of the live feeds and the complex ingredients was not analysed *per se*, but was calculated from the specific digestibility of the water-soluble and insoluble fractions (Table 3). Theoretically, this value can differ from the digestibility of the unseparated feed ingredients due to protease inhibition from other ingredient constituents (Alarcón et al., 1999). The *in vitro* digestibility of the live feeds (84 and 87%) was greater than that of the compound diets (Minipro™, 70±3%, protein encapsulated diet, 53±2.3%). Of the feed ingredients, cod fillet was more digestible *in vitro* than the squid, fish and fish roe meals. Fish roe meal distinguishes itself as the least digestible protein source in this study, at 48%. All the other feeds, feedstuffs and reference proteins were digested to between 68 and 95%.

3.3. Pre-hydrolysis of dietary protein

The two live feeds tested contained high levels of TCA-soluble nitrogen which, as pointed out above, seems to be readily available for utilisation by the fish larvae. The feed ingredients tested contained various levels of TCA-soluble nitrogen, although these levels may be increased by hydrolysis before incorporation into feed particles, as shown for cod fillet (Table 3). Pre-hydrolysed protein sources are frequently added to compound larval diets (Cahu et al., 1999; Hamre et al., 2001; Kvåle et al., 2002) in order to increase the availability of dietary AA. However, this strategy may fail if nutrient leaching is not prevented due to the increased solubility and lowered molecular weight of the hydrolysis products and thus the potential leaching from feed particles before ingested by the larvae. Rapid rates of loss (>90% in 2 min) have been reported for free amino acids supplementing compound diets (López-Alvarado et al., 1994; Kvåle et al., 2006). Free amino acids should be regarded as extremes with regards to leaching rates; however, extensive leaching of nitrous compounds from microdiets based on intact fish protein sources (Opstad and Hamre, 2003) demonstrates that leaching will occur also for water-soluble macromolecules. Leaching rates of peptides should be regarded as intermediate and a function of molecular weight and to a certain extent, of the amino acid composition too. Our cod fillet results show that pre-hydrolysis with pepsin almost doubles the content of water-soluble nitrogen, while total digestibility increased only from 55 to 67% at 1 h and from 79 to 86% at 12 h. In a feeding trial, pre-hydrolysis of a protein source such as cod fillet might therefore reduce rather than improve the total availability of the protein if a significant proportion of the water-soluble cod fillet or pre-hydrolysed cod fillet is lost by leaching to the water before being ingested. Nutrient leaching is time-dependent and nutrient loss may therefore also depend on tank design, feeding protocols and the feeding activity of the species concerned. Supplementing pre-hydrolysed protein sources in compound diets may thus be advantageous in species that ingest formulated feeds rapidly and that can be kept in small systems, while this strategy may fail and even have negative effects in species whose feeding behaviour is more sedate and thus eat more slowly, and that also require larger tank systems, such as larvae of Atlantic halibut (Kvåle et al., 2006). Negative absorptive and metabolic effects of pre-hydrolysed protein sources in compound larval diets have been observed and discussed by several authors (e.g. Cahu et al., 1999; Kolkovski and Tandler, 2000; Dabrowski et al., 2003). Unfortunately, the leaching

rates of the micro-particulate diets in these experiments were not reported, making it difficult to distinguish between the possible negative effect of ingested pre-hydrolysed protein on absorption and metabolism (flooding) and the negative effects of reduced total dietary and digestible protein ingested due to substantial leaching from the feed. In our opinion this makes it very difficult to evaluate the effects of such inclusions in feeding trials. At the same time, some leaching of nutrients are positive due to their roles as attractants (Kolkovski et al., 1997; Koven et al., 2001).

3.4. The effect of feed preparation on dietary protein digestibility and availability

The *in vitro* digestibility of the mixed ingredients of the protein-encapsulated particles was equal to the sum of the analysed digestibility of the individual protein sources, suggesting that there were no significant protease inhibitor effects of any of the ingredients of this diet formulation (Alarcón et al., 1999). The *in vitro* digestibility of the finished protein-encapsulated particles, however, was significantly ($P<0.05$) reduced in comparison with that of the mixed ingredients. After 1 h, the digestibilities of the particulated feed and the mixed ingredients were 29% and 54% respectively, but the relative difference decreased thereafter, eventually ending at 53% and 71% after 12 h (Table 3). A lowering of the digestibility as a result of the diet formulation process could be expected, due to the cross-binding of proteins which probably yields fewer degradable reaction products.

The final digestibility at 12 h was higher for the other formulated feed tested (Minipro™, 70±3%) than for the protein-encapsulated particles (53±2.3%). In comparison with the protein-encapsulated particles, Minipro™ contained substantially higher initial levels of TCA solubles (31.8±2.0% vs 1±0.4%) and was thus more similar to the composition of live feeds. However, in contrast to live feeds, in which the nutrient content is protected by biomembranes and homeostatic regulation, small soluble components supplementing compound feeds may largely be lost by leaching in water (López-Alvarado et al., 1994, Kvåle et al., 2006). The availability of the Minipro™ TCA soluble protein fraction to larvae will thus depend on the rate and extent to which it leaks from the feed particles after it has been added to the water. The finished protein-encapsulated feed leaches its water-soluble added ingredients at a low rate (Yufera et al., 2002). However, a recent study (A. Nordgreen, NIFES, Bergen, Norway, pers. comm.) indicates that repeated washing of microcapsulate feeds in water during the production process seems to leave little of leachable components. Any leakage during production may alter the composition of the microcapsules in favour of the water-insoluble fractions. This may thus also have direct implications for the total protein digestibility of this feed. The other micro-diet was presumedly more similar to the list of ingredients with regards to biochemical composition when the *in vitro* digestibility was performed. Thus, it is difficult to compare and evaluate these specific micro-diet preparation methods in terms of their impact on protein digestibility, on the basis of the present study.

A potential strategy for improving dietary protein availability in compound feeds would be to prevent leaching by efficient encapsulation of feed particles that are supplemented with high levels of FAA, peptides and soluble protein, in order to mimic live feeds. Another potential strategy would be to design feeds that contain intact protein sources which would resist leaching due to their large molecular size, but that are still highly digestible. Na+-caseinate appears to be a candidate in the latter category, due to its high digestibility, although it might be necessary to supplement it with other amino acid sources in order to satisfy the larval demands for amino acids. However, our results confirm that the digestibility of intact proteins that are highly digestible *per se* may be significantly reduced during the feed preparation process. Thus, some effort still needs to be put into developing micro-feed preparation techniques that combine particle stability and nutrient leaching on one side with adequate digestibility on the other.

3.5. Correlation between in vitro and in vivo digestibility

Various approaches have been tried in order to develop reliable and cost-efficient methods for the evaluation of protein digestibility. Compared to *in vivo* measurements, *in vitro* assays are easier to perform and are much more cost efficient; however, their relevance can only be confirmed by *in vivo* experiments. *In vitro* studies may be based on intestinal extract from a species of particular interest (e.g. Jany, 1974; Nankervis and Southgate, 2006) or on commercial available crystalline proteases (e.g. Hsu et al., 1977; Saterlee et al., 1979). In this study, our aim was to evaluate the digestibility of some feeds and potential protein sources in stomachless fish larvae in general. Commercial enzymes were thus used as this was expected to improve the reproducibility of data as compared to using larval intestinal extracts from one species of fish. This approach was also supported by the findings of Chong et al. (2002), who

showed that *in vitro* protein digestibility rates, as evaluated by three different protocols, correlated well among themselves and also with *in vivo* digestibility in juvenile discus fish. The protocol used in the present experiment was a combination of two of the protocols (Hsu et al., 1977; Saterlee et al., 1979) tested by Chong et al. (2002), with the exception that Trizma-base (pH 8.0) was replaced by the nitrogen-free phosphate buffer (pH 8.0) since our protocol was based on nitrogen analysis. Although Chong et al. found some differences in absolute digestibility between protocols, the feed ingredient digestibility ranking made by all protocols agreed well with the *in vivo* digestibility ranking. They used juvenile discus fish that had developed functional gastric digestion and thus differed from the typical marine fish larvae. However, the *in vitro* digestion protocol consists of a single chamber reaction at alkaline pH, which is more similar to the type of digestion that takes place in marine larvae. The results of Chong et al. suggest that *in vitro* protocols based on commercial crystalline enzymes can be used with success to predict the relative digestibility of different feedstuffs *in vivo*. However, in the end, the true digestion of a diet has to be measured in the target larvae for the studies. Such *in vivo* digestibility studies are not easily performed in small larvae, due to technical difficulties concerning the quantification of ingestion and faeces collection. An assessment of the digestibility of dietary protein can be performed in fish larvae by controlled tube-feeding of a radioisotope-labelled protein followed by compartmental analysis of the distribution of the tracer in a hot-chase approach (Rønnestad et al., 2001). Using this method, Tonheim et al. (2004, 2005) measured absorption rates and the utilisation efficiency of a salmon serum protein in Atlantic halibut larvae. However, this method requires labelling techniques that do not alter the properties of the protein (Rojas-García and Rønnestad, 2003; Tonheim et al., 2004) and so far only model proteins have been tested. If this problem can be adequately dealt with, tube-feeding experiments may be included in future studies to assess the correlation between the *in vitro* digestibility of a particular dietary protein and the digestibility of the identical but labelled protein in larvae. A range of other tracer methodologies for the assessment of digestibility is currently available (Conceição et al., 2006 but due to their individual constraints it is advisable that long-term effects should also be assessed in subsequent validation growth trial-type experiments. However, *in vitro* digestibility experiments can be a very useful tool for screening larval feeds and feed ingredients and reducing the number of dietary treatments to be tested in growth-trial studies.

4. Conclusions

1. The live feeds and the cod fillet contained high fractions of water-soluble protein.
2. The digestibility of the water-soluble protein was generally higher than that of the insoluble protein fraction.
3. The digestion rates of soluble Na^{+}-caseinate were higher than those of insoluble casein, although they reached the same final value for digestibility after 12 h.
4. Pepsin hydrolysis almost doubled the water-soluble nitrogen fraction in cod fillet, but produced only a small increase in total digestibility.
5. The final assessment of protein sources for diets for marine fish larvae should include evaluation of digestibility and bioavailability from prepared particles that have been immersed in water for a realistic period of time.

Acknowledgements

This study was supported by the Research Council of Norway, grant no. 163520/S40. Edel Erdal was responsible for nitrogen analysis at NIFES.

References

Alarcón, F.J., Moyano, F.J., Díaz, M., Fernández-Díaz, C., Yufera, M., 1999. Optimization of the protein fraction of microcapsules used in feeding of marine fish larvae using in vitro digestibility techniques. Aquac. Nutr. 5, 107–113.

Blaxter, J.H.S., 1988. Pattern and variety in development. In: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology, vol. XI. Academic Press, Inc., San Diego, pp. 1–58.

Cahu, C.L., Zambonino Infante, J.L., 1995. Maturation of the pancreatic and intestinal digestive function in sea bass (*Dicentrarchus labrax*): effect of weaning with different protein sources. Fish Physiol. Biochem. 14, 431–437.

Cahu, C.L., Zambonino Infante, J.L., Estaffre, A.M., Bergot, P., Kaushik, 1998. Preliminary results on sea bass (*Dicentrarchus labrax*) larvae rearing with compound diet from first feeding. Comparison with carp (*Cyprinus carpio*) larvae. Aquaculture 169, 1–7.

Cahu, C.L., Zambonino Infante, J.L., 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 (1–2), 109–119.

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

Carvalho, A.P., Sá, 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.

Chong, A.S.C., Hashim, R., Ali, A.B., 2002. Assessment of dry matter and protein digestibility of selected raw ingredients by discus fish

(*Symphysodon aequifasciata*) using *in vivo* and *in vitro* methods. Aquac. Nutr. 8, 229–238.

Conceição, L.E.C., van der Meeren, T., Verreth, J.A.J., Evjen, M.S., Houlihan, D.F., Fyhn, H.J., 1997. Amino acid metabolism and protein turnover in larval turbot (*Scophthalmus maximus*) fed natural zooplankton or *Artemia*. Mar. Biol. 129, 255–265.

Conceição, L.E.C., Morais, S.J., Rønnestad, I., 2006. Tracer studies in fish larvae. 12th Int. Symp. Nutr. Feed. Fish. Biarritz, France, May 28–June 1.

Dabrowski, K., Lee, K.J., Rinchard, J., 2003. The smallest vertebrates, teleost fish, can utilize synthetic dipeptide-based diets. J. Nutr. 133, 4225–4229.

Day, O.J., Howell, B.R., Jones, D.A., 1997. The effect of dietary hydrolysed fish protein concentrate on the survival and growth of juvenile Dover sole, *Solea solea* (L.), during and after weaning. Aquac. Res. 28, 911–921.

Finn, R.N., Rønnestad, I., Fyhn, H.J., 1995. Respiration, nitrogen and energy metabolism of developing yolk-sac larvae of Atlantic halibut (*Hippoglossus hippoglossus* L.). Comp. Biochem. Physiol. 111A, 647–671.

Finn, R.N., Rønnestad, I., van der Meeren, T., Fyhn, H.J., 2002. Fuel and metabolic scaling during the early life stages of Atlantic cod *Gadus morhua*. Mar. Ecol., Prog. Ser. 243, 217–234.

Fyhn, H.J., Finn, R.N., Helland, S., Rønnestad, I., Lømsland, E., 1993. Nutritional value of phyto- and zooplankton as live food for marine fish larvae. In: Reinertsen, H., Dahle, L.A., Jørgensen, L., Tvinnerheim, K. (Eds.), Fish Farming Technology. Balkema, Rotterdam, pp. 121–126.

Garcia-Ortega, A., Koussoulaki, A., Boer, H., Verreth, J., 2000. In vitro protein digestibility of *Artemia* decapsulated cysts and nauplii, and of microbound diets for larval fish. Aquac. Res. 31, 475–477.

Govoni, J.J., Boehlert, G.W., Watanabe, Y., 1986. The physiology of digestion in fish larvae. Env. Biol. Fisches 16, 59–77.

Hamre, K., Næss, T., Espe, M., Holm, J.C., Lie, Ø., 2001. A formulated diet for Atlantic halibut (*Hippoglossus hippoglossus*, L.) larvae. Aquac. 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*. Aquac. Nutr. 8, 139–148.

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.

Houde, E.D., 1989. Comparative growth, mortality, and energetics of marine fish larvae: temperature and implied latitudinal effects. Fish. Bull. U.S. 87, 471–495.

Hsu, H.W., Vavak, D.L., Saterlee, L.D., Miller, G.A., 1977. A multi-enzyme technique for estimating protein digestibility. J. Food Sci. 42, 1269–1273.

Jany, K.D., 1974. Studies of the digestive enzymes of the stomachless bonefish *Carassius auratus gibelio* (Bloch): endopeptidases. Comp. Biochem. Physiol. 53B, 31–38.

Kolkovski, S., 2001. Digestive enzymes in fish larvae and juveniles–implications and applications to formulated diets. Aquaculture 200, 181–201.

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

Kolkovski, S., Arieli, A., Tandler, A., 1997. Visual and chemical cues stimulate microdiet ingestion in sea bream larvae. Aquac. Int. 5, 527–536.

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

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

Kvåle, A., Yúfera, M., Nygård, E., Aursland, K., Harboe, T., Hamre, K., 2006. Leaching properties of three different microparticulate diets and preference of the diets in cod (*Gadus morhua* L.) larvae. Aquaculture 28, 402–415.

Lazo, J.P., Romaire, R.P., Reigh, R.C., 1998. Evaluation of three in vitro enzyme assays for estimating protein digestibility in Pacific white shrimp *Penaeus vannamei*. J. World Aquac. Soc. 29, 441–449.

Lindner, P., Eshel, A., Kolkovski, S., Tandler, A., Harpaz, S., 1995. Proteolysis by juvenile sea bass (*Dicentrarchus labrax*) gastrointestinal enzymes as a method for the evaluation of feed proteins. Fish Physiol. Biochem. 14, 399–407.

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

Luizi, F.S., Gara, B., Shields, R.J., Bromage, N.R., 1999. Further description of the digestive organs in Atlantic halibut (*Hippoglossus hippoglossus*) larvae, with notes on differential absorption of copepod and *Artemia*. Aquaculture 176, 101–116.

Nankervis, L., Southgate, P.C., 2004. An integrated assessment of gross marine protein sources used in formulated microbound diets for barramundi (*Lates calcarifer*) larvae. Aquaculture 257, 453–464.

Opstad, I., Hamre, K., 2003. Evaluering av formulerte fôr med hensyn på ernæringsmessig sammensetning, tekniske egenskaper og egnethet for weaning av torskelarver. Research Council of Norway. Report from grant no. 141768/120.

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. Fish. Aquat. Sci. 56, 2099–2111.

Pedersen, B.H., Ueberschär, B., Kurokawa, T., 2003. Digestive response and rates of growth in pre-leptocephalus larvae of the Japanese eel *Anguilla japonica* reared on artificial diets. Aquaculture 215, 321–338.

Robin, J.H., Vincent, B., 2003. Microparticulate diets as first food for gilthead sea bream larva (*Sparus aurata*): study of fatty acid incorporation. Aquaculture 225, 463–474.

Rojas-García, C.R., Rønnestad, I., 2003. Assimilation of dietary free amino acids, peptides and protein in post-larval Atlantic halibut (*Hippoglossus hippoglossus*). Mar. Biol. 142, 801–808.

Rønnestad, I., Conceição, L.E.C., 2005. Aspects of protein and amino acid digestion and utilization by marine fish larvae. In: Wang, J.M. (Ed.), Physiological and Ecological Adaptations to Feeding in Vertebrates. Science Publishers, Inc, Enfield, NH, pp. 389–416.

Rønnestad, I., Naas, K.E., 1993. Oxygen consumption and ammonia excretion in larval Atlantic halibut (*Hippoglossus hippoglossus* L.) at first feeding: a first step towards an energetic model. In: Walther, B.T., Fyhn, H.J. (Eds.), Fish Larval Physiology and Biochemistry. University of Bergen, Bergen, pp. 279–284.

Rønnestad, I., Rojas-García, C.R., Tonheim, S.K., Conceição, L.E.C., 2001. In vivo studies of digestion and nutrient assimilation in marine fish larvae. Aquaculture 201, 161–175.

Rønnestad, I., Tonheim, S.K., Fyhn, H.J., Rojas-Garcia, C.R., Kamisaka, Y., Koven, W., Finn, R.N., Terjesen, B.F., Barr, Y.,

Conceição, L.E.C., 2003. The supply of amino acids during early feeding stages of marine fish larvae: a review of recent findings. Aquaculture 227, 147–164.

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

Tonheim, S.K., Espe, M., Raae, A.J., Darias, M.J., Rønnestad, I., 2004. In vivo incorporation of [U]-^{14}C-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., Rønnestad, I., 2005. Pre-hydrolysis of protein improves utilisation of dietary protein in the larval teleost Atlantic halibut (*Hippoglossus hippoglossus*, L.). J. Exp. Mar. Biol. Ecol. 321, 19–34.

Vu, T.T., 1983. Etude histoenzymologique des activités protéasiques dans le tube digestif des larves et des adultes de bar, *Dicentrarchus labrax*. Aquaculture 109, 57–69.

Watanabe, Y., 1981. Intracellular digestion of horseradish peroxidase by the intestinal cells of teleost larvae and juveniles. Bull. Jpn. Soc. Sci. Fish. 48, 37–42.

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

Yufera, M., Pascual, E., Fernandez-Diaz, C., 1999. A highly efficient microencapsulated food for rearing early larvae of marine fish. Aquaculture 177, 249–256.