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Pre-hydrolysis improves utilisation of dietary protein in the larval teleost Atlantic halibut (*Hippoglossus hippoglossus* L.)

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Abstract

A protein preparation labelled by incorporation of [U]^{14}C-AA was hydrolysed to various degrees and administered to a teleost fish larva (Atlantic halibut, *Hippoglossus hippoglossus* L.) by tube-feeding, and its post-administration utilisation was studied. Three treatments were prepared: IntP—intact protein, PHP—pepsin-hydrolysed protein, and HHP—highly hydrolysed protein (using pepsin, trypsin, endoproteinase Glu-C, Asp-N, and Pro-C). At small doses (11.4±1.5 μg larvae^{-1}), the intact protein (IntP) was digested and absorbed to 36±5.5%. However, the relative absorption efficiency of the intact protein was reduced as the dose increased. Absorption efficiency was higher when the protein was hydrolysed prior to feeding the larvae and was constant at 63% (R^2=98) independent of degree of proteolysis and dose (ranging from 3.5 to 35 μg larvae^{-1}). The initial absorption rate increased with the degree of hydrolysis. Calculations based on data collected during the first 30–120 min show that the absorption of PHP and HHP into extra-intestinal body tissues was 2.2 and 3 times as fast, respectively, as that of intact protein. However, the rates of absorption did not influence the distribution of absorbed AA into either catabolism or anabolism, as all larvae, independent of which protein solution they were given, catabolised 42±7% of the absorbed AA, and accumulated 49±6% into the body tissue, during the 20 h post-feeding incubation period.

Larval age and size did not influence the absorption and utilisation of the hydrolysed protein preparations. This was different from the intact protein, as significantly higher fractions of the intact protein were absorbed by the larvae at 31 days past first feeding (dpff) than by larvae at 25 dpff. Analysis of the faecal evacuation suggested that the poor protein utilisation in the younger larvae was due to enhanced faecal evacuation, which in some larvae was more than 50% only 4 h post diet administration, at a time when the process of digestion and absorption was far from complete. This indicated that faecal evacuation is a critical factor in the utilisation of slowly digested and absorbed feed components, such as intact proteins, by fish larvae.

Keywords: Teleost fish larvae; Protein; Hydrolysate; Amino acids; Tube-feeding; Digestion; Absorption; Assimilation; Catabolism; Faecal evacuations

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1. Introduction

Despite several decades of research, the nutrient processing and transfer efficiency of the digestive system in larval teleosts remain a topic of debate. Like the larvae of other animals, early stages of fish have very high potential rates of growth (Houde, 1989; Kamler, 1992; Conceição et al., 1997; Otterlei et al., 1999). However, in contrast to many freshwater species like salmonides, it has been impossible to achieve these growth rates under laboratory and culture conditions for marine teleosts unless live feed is used. Despite recent progress in the development of live feed substitutes (Yúfera et al., 1999; Cahu and Zambonino-Infante, 2001; Koven et al., 2001), live feed is still necessary for cultured marine fish larvae. A few authors have reported that marine fish larvae have been reared solely using artificial diets, although at low survival and growth rates (e.g., Cahu et al., 1998; Pedersen et al., 2003; Robin and Vincent, 2003).

High growth rates require an abundant supply of dietary amino acids for anabolic as well as energetic purposes (Rønnestad and Naas, 1993; Finn et al., 1995, 2002). Most marine fish larvae start exogenous feeding at an early stage of development, and before the digestive system is completely developed (Govoni et al., 1986; Blaxter, 1988; Luizi et al., 1999). Although proteolytic pancreatic enzymes are present at the onset of exogenous feeding (Zambonino-Infante and Cahu, 1994, Gawlicka et al., 2000; Srivastava et al., 2002), a functional stomach is absent until metamorphosis (Vu, 1983; Blaxter, 1988; Luizi et al., 1999). In general, the stomach plays an important role in protein digestion due to the concomitant action of proteolytically active pepsin and the denaturing conditions of the HCl, besides serving as a reservoir of nutrients between meals. It is still not known whether—and, if so, to what extent—the functional status of the gut imposes limitations on the digestion and absorption efficiency of formulated diets and dietary prey animals not encountered in the wild. Low digestive efficiency has been implicated in AA deficiency due to low availability of the dietary proteins (Fyhn, 1989).

Pre-hydrolysed protein has been used in artificial diets in order to improve protein availability and thus enhance the growth of marine larval fish. However, even though moderate supplementation of pre-hydrolysed proteins in artificial diets has been shown to improve larval growth (Zambonino-Infante et al., 1997), a depression of growth is usually experienced when pre-hydrolysed proteins are included at levels above 25–30% (Carvalho et al., 1997; Cahu et al., 1999; Cahu and Zambonino-Infante, 2001).

Free AA (FAA), dipeptides, and tripeptides are readily absorbed by enterocytes and may thus enter the organism at higher rates than proteins that need to be split up by the proteolytic enzymes before they become available for absorption (Ganapathy et al., 1994). Enhanced absorption is therefore expected from diets supplemented with FAA, peptides, or pre-hydrolysed proteins. However, such supplementations may lead to saturation of intestinal transporters mechanisms because of resulting high luminal peptide and FAA concentrations, and was suggested by Cahu et al. (1999) as a possible reason for inferior growth in sea bass (*Dicentrarchus labrax*) larvae fed diets with high inclusions of pre-hydrolysed protein compared to larvae fed with fish meal-based diets. Maturation of transporters mechanisms may result in absorptive antagonism (Berge et al., 1999) and thus to imbalanced AA absorption. Imbalanced AA absorption leads to increased AA oxidation and reduced dietary protein retention (Kim et al., 1983; Tacon and Cowey, 1985; Aragão et al., 2004). This made it pertinent to study the absorption and postprandial utilisation of protein hydrolysates vs. intact proteins in marine fish larvae.

In contrast to feeding trials with terrestrial vertebrates, nutrient leakage is a notorious problem in water and particularly in studies with fish larvae when artificial feeds are used. A typical size of the marine fish studied at the onset of exogenous feeding is 3–5 mm length. Thus, there is large surface-to-volume ratio and a short diffusion distance involved for the tiny feed particles used, 50–200 μm (López-Alvarado et al., 1994). Leakage of the nutrients in artificial feeds is related to their solubility in water (Yúfera et al., 2002) and molecular size, and can be very difficult to assess under realistic conditions (O. Garatun-Tjeldstø, University of Bergen, personal communication). The results of feeding trials designed to test the nutritional value of protein hydrolysate vs. intact protein in diets for marine fish larvae may therefore be influenced by differences in the profile and total amounts of AA actually ingested by larvae and/or be due to different rates of leakage of the feed protein and hydrolysate fractions. Indeed, the latter may offer

an alternative interpretation of the reported slower growth of larvae fed diets containing large fractions of hydrolysed proteins.

One possible way of alleviating the problem of leakage and controlling the amount ingested in studies of digestive and absorptive efficiency is to use in vivo tube-feeding (Rust, 1995; Rønnestad et al., 2001a) where the nutrient is deposited directly into the digestive tract. In addition, by using ^{14}C-labelled AA and a CO_2 trap as described by Rønnestad et al. (2001a), the experimental set-up permits ^{14}C-labelled evacuated nutrients to be distinguished from $^{14}CO_2$ originating from catabolism of the absorbed nutrients. In combination with scintillation counting of dissected organs or body compartments, the system provides a useful framework for investigating features of gut absorption, oxidation, and retention (assimilation) of nutrients.

This study aims to elucidate the kinetics of absorption and postprandial utilisation of dietary intact and hydrolysed proteins in marine teleost fish larvae in stages before the stomach is developed, in order to increase our knowledge of possible restrictions in protein digestion and possible effects of different absorption rates on AA oxidation and utilisation for growth.

2. Materials and methods

2.1. Preparation of protein test solutions

Various test protein solutions were prepared from a salmon serum protein (SSP) preparation labelled by in vivo incorporation of (U) ^{14}C-labelled AA in Atlantic salmon, identical (same batch) to the ^{14}C-SSP described by Tonheim et al. (2004). The protein concentration of the ^{14}C-SSP was 81 mg ml^{-1}, the specific activity was 3.6 kBq mg^{-1} protein, and the initial pH was 6.7. The amino acid composition and amino acid specific radioactivity are listed in Table 1.

Three treatments were prepared: intact protein (IntP), pepsin-hydrolysed protein (PHP), and highly hydrolysed protein (HHP). All solutions were adjusted to a final pH of 8.5 to mimic conditions in the stomachless larval digestive tract (Rønnestad et al., 2000a). For IntP, a sub-sample (50 μl) of the ^{14}C-SSP was transferred to a 600-μl Eppendorf tube and the pH was then adjusted using 1.0 M NaOH. For PHP, a sub-sample (150 μl) of the ^{14}C-SSP was transferred to a 600-μl Eppendorf tube. The pH was adjusted to 4.0 by the addition of 10 μl of 1.0 M HCl and thereafter the sample was hydrolysed by adding 150 μg of pepsin (porcine; Sigma, St. Louis, MO, USA) and incubated at 37 °C for 4 h. Hydrolysis was stopped by elevating the pH to 8.5 by adding 1.0 M NaOH. A sample of 5 μl was collected for electrophoresis. An aliquot of 60 μl (PHP) was transferred to a 600-μl Eppendorf tube and heated to 80° C for 10 min in order to denature the added pepsin. The HHP solution was prepared from the remaining pepsin hydrolysate, to which was added 60 μg of trypsin (bovine pancreas; Sigma, St. Louis, MO, USA) before being hydrolysed for 2 h at 23 °C, with the process being stopped by heating to 80 °C for 5 min. The pH was then adjusted to 7.0 by addition of 1.0 M HCl and further hydrolysed at 23 °C after the addition of 60 μg of endoproteinase Glu-C (*Staphylococcus aureus* V8; Fluka, Bucks, Switzerland), 90 μg of endoproteinase Asp-N (*Pseudomonas fragi*; Fluka), and 2 μg of endoproteinase Pro-C (*Escherichia coli*; Fluka). After 2 h, hydrolysis was terminated by heating to 80 °C for 5 min. The final highly hydrolysed protein (HHP) was adjusted to pH 8.5 using 1.0 M NaOH. A sample of 2 μl was collected for electrophoresis. All test solutions were stored at −80 °C until use.

Table 1
Amino acid composition and amino acid specific activity of the (U)-^{14}C-labelled salmon serum protein (^{14}C-SSP)

Amino acid	Molar composition (%)	Specific activity (Bq μmol^{-1})
Asp+Asn	9.7	113
Ser	1.9	122
Gly	9.3	124
Pro	6.6	238
Glu+Gln	7.9	92
Ala	13.9	78
Tyr	3.9	2895
Thr	5.0	217
Val	7.7	211
Met	2.9	164
Ile	3.9	268
Leu	8.9	202
Phe	3.3	2250
Lys	8.5	293
His	2.5	150
Arg	4.1	500

Values are percent of analysed AA. Trp was not analysed (from Tonheim et al., 2004).

2.2. Tube-feeding experiment

2.2.1. Fish larvae

Pre-metamorphosed Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae were obtained from a commercial hatchery, Nordic Seafarms (Askøy, Norway), and sampled from one tank on 24–32 days post first feeding (dpff). The eggs originated from three different females, stripped, and artificially fertilised on two adjacent days; incubated at 6±0.2 °C; and hatched after 13–14 days post fertilisation. At 55 and 56 days post fertilisation, the larvae were transferred to the start feeding tank and offered *Artemia* enriched with Selco™ (INVE, Ghent, Belgium) and Aglo Norse™ (EWOS, Bergen, Norway). From the onset of feeding, larvae were on constant artificial light. The water supply was UV-filtered seawater (33 g l^{-1}) kept at 6± 0.2 °C until start of feeding, when the temperature was gradually increased. The tank water temperature varied from 10.8 °C to 12.0 °C during the experimental period.

Larvae were carefully sampled using a cup to avoid injuring the delicate epidermis, and transferred to a 20-l beaker. The beaker was completely filled with water from the tank and then closed before transport to the laboratory. This procedure prevented the rough wave motion that occurs during transport in tanks with an air compartment. In the laboratory, the larvae were acclimatised to 9±1 °C and kept at this temperature throughout the tube-feeding experiment. Water was exchanged with acclimatised seawater when the larvae had emptied their gut. The laboratory water supply was filtered (0.2 μm) seawater (34 g l^{-1}).

2.2.2. Tube-feeding, incubation, and sampling procedure

Tube-feeding, incubation with a ^{14}C-CO_2 trap, sampling of larvae, dissection, larval tissue extraction, and sample preparation were all performed as previously described by Rønnestad et al. (2001a). However, a modified incubator and CO_2-trapping system based on 20-ml scintillation vials was used (Tonheim et al., 2004). Expired CO_2 was trapped at an efficiency of 97±2%.

Larvae were randomly collected from the laboratory tank one by one and slightly anaesthesised by brief immersion (2–3 min) in MS-222 (0.1 g l^{-1}; Sigma, St. Louis, MO, USA) before the protein solutions were tube-fed into the mid-gut via 0.34-mm OD plastic capillary tubes (Sigma). After tube-feeding, each larva and the immersion water used during tube-feeding were gently flushed into a tray filled with 8 ml of seawater and the larvae were allowed to recover (assessed by recovery of equilibrium and swimming movements) from the anaesthesia for 2.5±1 min before they were transferred with a minimum of water to incubators filled with 8 ml of seawater, in which they were incubated singly. The air pocket in the incubator was continuously aerated during incubation. The larvae were kept immersed in water during all operations in order to avoid epidermal injuries. Total handling time from when the larvae were collected from the laboratory tank until they were placed into the incubators was 7±2.0 min.

Larvae were sampled from the incubators with a forceps, dried on a filter paper, and weighed before being killed by severing the spinal chord. The gut was immediately separated from the body carcass by dissection under a dissecting microscope at 11° C. The carcass and gut were then extracted twice for 24 h at 4 °C in 2 and 1 ml of 6% trichloro-acetic acid (TCA), respectively, in order to ensure sufficient separation of free AA (FAA) and small peptides from proteins. The TCA precipitates of the larval carcass and gut were dissolved in 2 and 1 ml, respectively, of Solvable™ (Packard Bioscience, Groningen, The Netherlands), and all four fractions were analysed for radioactivity.

Incubators were resealed immediately after sampling, and the pH was brought to below 2.0 in order to remove all CO_2/HCO_3 from the incubation water. The radioactivity in compounds evacuated from the larvae, apart from the CO_2, remained in the incubation water and was analysed. The radioactivity in CO_2 traps was analysed and was presumed to have been derived from AA catabolism. Solubilised and liquid samples were analysed for radioactivity by 10 min counting in a Tri-Carb 2300 TR™ (Packard Instrument, Meriden, USA) liquid scintillation counter (LSC) after thorough mixing with Ultima Gold XR™ (Packard Bioscience) LSC cocktail.

The total contents of protein and FAA were analysed in 15 larvae sampled from the laboratory tank after tube feeding at 31 and 32 dpff. These larvae were sampled, weighed, killed, and dissected as described above. Carcasses were immediately frozen and stored at −20 °C for protein and FAA analyses.

2.3. Experimental design

The tube-feeding experiment was performed over a period of several days due to the large number of larvae used. In order to avoid the results being influenced by circadian variation in larval metabolism, all larvae were sampled, tube-fed, and incubated in accordance with a standard schedule. Larvae were collected at 08:00 h and tube-fed the following day between 14:00 and 18:30 h. For practical reasons, only one test solution was tube-fed on any 1 day of the experiment. IntP was tube-fed at 25 and 31 dpff, PHP was tube-fed at 24 and 32 dpff, and HHP was tube-fed at 26 and 30 dpff.

For the study of the kinetics of absorption and postprandial utilisation of AA, 150 nl of the test solutions was tube-fed to larvae. After feeding, the larvae were incubated before being sampled at 0.5, 1, 2, 4, 10, and 20 h. Six larvae were sampled after 20 h, while five larvae were sampled at the other sampling times on each day of tube feeding.

In order to evaluate the effects of the amount of protein administered on absorption and postprandial AA utilisation, groups of 20–25 larvae at each day were tube-fed various doses of the protein solutions (50 nl, 150 nl, 300 nl, and 450 nl) and incubated for 20 h before sampling and compartmental analysis.

2.4. Chemical analysis

Protein concentrations in the three test solutions were calculated from the specific activity of the ^{14}C-SSP (3.6 Bq μg^{-1}) and designated in terms of microgram of protein without considering whether the radioactivity was present in proteins, peptides, or FAA.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 10–20% linear gradient Tris–Tricine/Peptide Ready Gel™. Proteins were visualised by Bio-Safe Coomassie™. Ready Gel™, Comassie stain, premixed–tricine sample buffer, and premixed Tris–tricine–SDS running buffer were obtained from Bio-Rad Laboratories (Hercules, CA, USA).

Frozen larvae sampled for analysis of proteins and FAA were lyophilised and extracted into 6% TCA for 24 h. The extract was analysed for AA by reversed-phase chromatography with an HPLC connected to an ASTED™ sample robot, both from Gilson Medical Electronics (Middelton, WI, USA). AA were detected fluorometrically after derivatisation with OPA (*o*-phthaldialdehyde) and FMOC (9-fluoromethyl-chloroformate) reagents, and quantified by comparisons to an external AA standard (24 AA; Sigma-Aldrich, St. Louis, MO, USA). The TCA-washed larval carcass was solubilised in 0.5 M NaOH and the protein quantified by the method of Lowry et al. (1951), using bovine serum albumin as standard.

2.5. Statistical analysis

Regression models for total absorbed radioactivity and radioactivity in the incubation water as functions of doses and for initial absorption rates were performed using Statistica 6.0 (StatSoft, Tulsa, OK, USA). Multiple comparisons among slopes were performed using the Tukey test according to Zar (1996). Outliers were eliminated.

Correlation between administered amounts of protein and radioactivity in CO_2 traps and in body TCA precipitate as fraction of total absorbed radioactivity was tested by simple regression performed using Statistica 6.0. Differences between larvae fed different protein solutions in distribution of radioactivity into CO_2 or body TCA precipitate, as fractions of absorption, were tested by one-way ANOVA, and homogeneity of variance was tested by Levenes test, both performed using Statistica 6.0.

Data are presented as mean±S.E., if no other is noted. A confidence interval of 95% was adopted.

3. Results

3.1. Test solutions and administration

SDS-PAGE confirmed that the experimental protein solutions differed in their distribution of molecular sizes (Fig. 1). IntP consisted of proteins varying in size from approximately 15 kDa to above 250 kDa. Most of the proteins, however, were between 65 kDa and 120 kDa, as described in Tonheim et al. (2004). PHP contained proteins within more or less the same range of molecular sizes as IntP, except for the presence of protein in the lower region (low molecular range) of the gel. However, unlike from IntP, defined protein

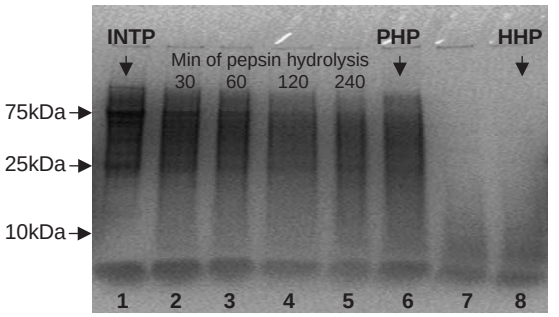


Fig. 1. SDS-PAGE of the experimental protein preparations made from ^{14}C salmon serum protein (^{14}C-SSP) used for tube feeding Atlantic halibut larvae. Lane 1: Intact protein preparation (IntP). Lanes 2–5: Degradation of IntP with pepsin sampled at different times. Lane 6: ^{14}C-SSP hydrolysed with pepsin at pH 4.0 (PHP). Lane 8: ^{14}C-SSP hydrolysed with pepsin at pH 4.0, followed by hydrolysis with trypsin at pH 8.5 and neutral hydrolysis with endoproteinase Glu-C, *Staphylococcus aureus* V8; endoproteinase Asp-N, *Pseudomonas fragi*; and endoproteinase Pro-C, *Escherichia coli* (HHP).

bands were not present in the gel and less protein material seems to appear in the upper region of the gel. In HHP, neither protein bands were seen and all the stained protein materials present in the gel were in the lower region corresponding to peptides less than 25 kDa, and only little protein material is left in the gel as the major part of the originally present proteins was hydrolysed into peptides and FAA that were too small to be retained by the gel used (<1.4 kDa).

The exact amount of administered protein in micrograms was calculated per single larva by dividing the sum of radioactivity from all larval compartments, including incubation water and CO_2, by the specific activity of the ^{14}C-SSP (3.6 Bq μg^{-1}). The doses varied among the different experimental protein solutions due to differences in the hydrolysis procedures, which involved pH adjustments and heating during the preparation of the experimental solutions. The doses also varied between larvae fed identical protein solutions due to the inaccuracy of the micro-injector system and regurgitation of administered protein from the larvae during tube feeding and the subsequent stay in the wash tray. The amounts of protein administered and retained in the larvae by tube feeding IntP, PHP, and HHP in the kinetic study were (mean±S.D.) 11.4±1.5 μg, 12.9±0.9 μg, and 10.5±0.7 μg, respectively.

3.2. Larval performance and weight

A transient high incidence of mortality during the acclimatisation period of 53%, 40%, 58%, 17%, 15%, and 21% on the respective days was registered and is believed to be related to stress caused by sampling, transport, and transfer of the larvae to the holding tank at the laboratory. Mortality during the post tube-feeding incubation period, however, was low, with 1, 4, 0, 5, 3, and 2 larvae dying on the respective days of tube feeding, out of a total of 281 tube-fed larvae, representing a total mortality of 5.3% during the tube-feeding experiment. Dead larvae were not included in the analysis. The numbers of larvae sampled and analysed for each sampling time in the kinetic study are shown in Table 2.

Larval body weights (mean wet weight±S.D.) tube-fed on the respective days were: 27.0±5.5, 28.3±5.2, 29.5±6.0, 37.4±7.1, 42.4±8.0, and 40.4±6.1 mg, and thus increased significantly during the experimental period. Larval carcass dry mass content was (mean±S.D., n=15) 11.7±0.6%. Proteins and FAA accounted for (mean±S.D., n=15) 500±40 and 8.8±1 μg mg carcass weight (dry weight)$^{-1}$, respectively. Free AA consisted of: aspartic acid 4.4±1.7%, glutamic acid 19±2.3%, cysteine 0.42±0.29%, asparagine 1.2±0.49%, serine 5.1±1.0%, glutamine 9.3±1.95, histidine 13±1.35, glycine 11±2.9%, threonine 8.0±0.86%, arginine 2.1±0.41%, alanine 5.8±2.7%, tyrosine 1.6±0.39%, valine 6.5±6.3%, methionine 0.80±0.17%, tryptophan 0.60±0.12%, phenylalanine 0.29±0.22%, isoleucine 1.1±0.17%, leucine 2.0±0.37%, and lysine 7.5±1.6%. Proline was not analysed.

3.3. Regression analysis of protein utilisation at 20 h post tube-feeding

Linear regression models for larval absorption (body TCA precipitate, body TCA soluble, and CO_2

Table 2
Number of larvae included in the analysis of the kinetic study

Sample (h past tube feeding)	0.5	1	2	4	10	20
IntP	10	10	9	10	8	7
PHP	3	10	10	9	10	12
HHP	9	10	10	10	10	12

fractions) and faecal evacuation of the protein solutions (incubation water) demonstrate higher absorption efficiency of the hydrolysed protein solutions (PHP and HHP) than of intact protein (Fig. 2A). Absorption and evacuation in larvae fed PHP and HHP were not significantly different, and these data were therefore pooled into a common regression model (Fig. 2A and B). For both relationships, the predicted interceptions were close to zero, and both models could be forced through zero, resulting in a slight change in slope, but no noticeable changes in the regression coefficients (R^2). This suggests that the utilisation of the hydrolysed protein preparations (PHP and HHP) was constant throughout the entire range of doses tested. Thus, independent of administered load of hydrolysed protein, absorption was 63% and faecal evacuation was 25% 20 h after tube feeding. The remaining 12% were in the dissected gut.

Utilisation of the intact protein solution was more complex. In spite of wide variation in the results, the analysis demonstrated that larval age (25 vs. 31 dpff) significantly ($P<0.05$) affected utilisation. The data could therefore not be pooled into a common regression but were described by two separate models (Fig. 2). The regression slope of the absorption at 25 dpff was gentle and the predicted intercept was positive, suggesting a discontinuous or non-linear relationship between absorbed and administered amounts of protein. At small doses (<5 μg protein), the intact protein was absorbed in amounts comparable to the absorption of the hydrolysed protein solutions. However, as the doses increased, the percentage of absorbed protein fell, and at the highest amounts (30 μg), the regression predicted absorption efficiency as low as 12%. The absorption of the intact protein in the older larvae (31 dpff) lies between the absorption efficiency of larvae tube-fed the hydrolysed protein solutions and younger larvae (25 dpff) tube-fed the intact protein, both as regards slope and interception of the regression. At small doses, the absorption efficiency of IntP at 31 dpff was comparable to that of the hydrolysed protein preparations (64%), but the predicted absorption efficiency fell to 43% when the doses of the intact protein rose by a factor of 10. Faecal evacuation in larvae fed the intact protein increased in proportion to the decrease in absorption efficiency (Fig. 2).

Assimilated protein (body TCA precipitate, body TCA soluble) and oxidised protein, as fraction of total absorbed protein, were not correlated with adminis-

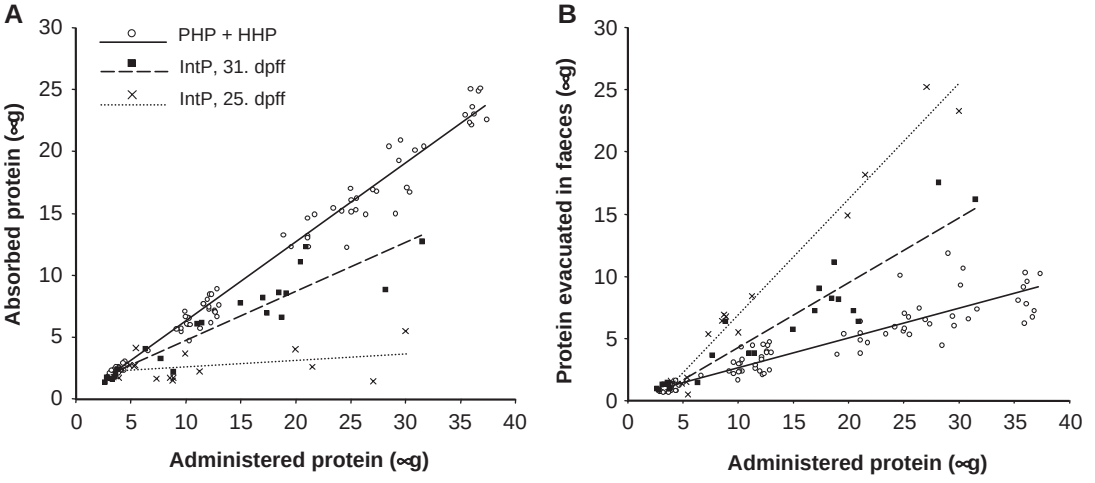


Fig. 2. Total absorption (A) and faecal evacuation (B) of the experimental protein preparations 20 h after being tube-fed to Atlantic halibut larvae. Larvae fed the hydrolysed protein preparations (PHP and HHP) did not differ significantly between days or diets and were therefore pooled into common regression models: Absorbed protein $_{\text{PHP and HHP}}$=0.64 administered protein−0.24 (R^2=0.98); Evacuated faecal protein $_{\text{PHP and HHP}}$=0.24 administered protein+0.23 (R^2=84). Absorption and faecal evacuation in larvae fed the intact protein preparation (IntP) differed between the days of tube feeding and were therefore described by separate models: Absorbed protein $_{\text{IntP, 25 dpff}}$=0.05 administered protein+2.0 (R^2=0.16); Absorbed protein $_{\text{IntP, 31 dpff}}$=0.40 administered protein+0.72 (R^2=0.87); Evacuated faecal protein $_{\text{IntP, 25 dpff}}$=0.94 administered protein−2.6 (R^2=0.97); Evacuated faecal protein $_{\text{IntP, 31 dpff}}$=0.52 administered protein−0.9 (R^2=0.89).

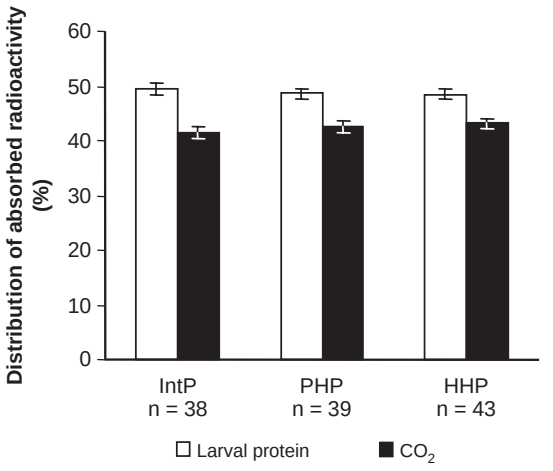


Fig. 3. Distribution of radioactivity in body TCA precipitate and CO_2 as fractions of absorbed radioactivity at 20 h post tube feeding. Values are mean±S.E.M. and include all larvae sampled at 20 h post tube feeding. Differences between treatments were analysed by ANOVA. No differences were found.

tered amounts (P>0.05). Fractional oxidation and assimilation were 41.4±1.2%, 42.6±1.0%, 43.2±0.9%, and 49.4±1.0%, 48.7±0.9%, 48.4±0.9% in larvae fed with IntP, PHP, and HHP, respectively. The differences were not statistical significant either for fractional assimilation (P=0.77) nor fractional oxidation (P=0.47), even though a weak tendency of increased oxidation and decreased assimilation for increased degree of hydrolysis of the administered protein was found (Fig. 3). Homogeneity in variance was found.

3.4. Kinetics of digestion, absorption, and post-absorptive AA utilisation

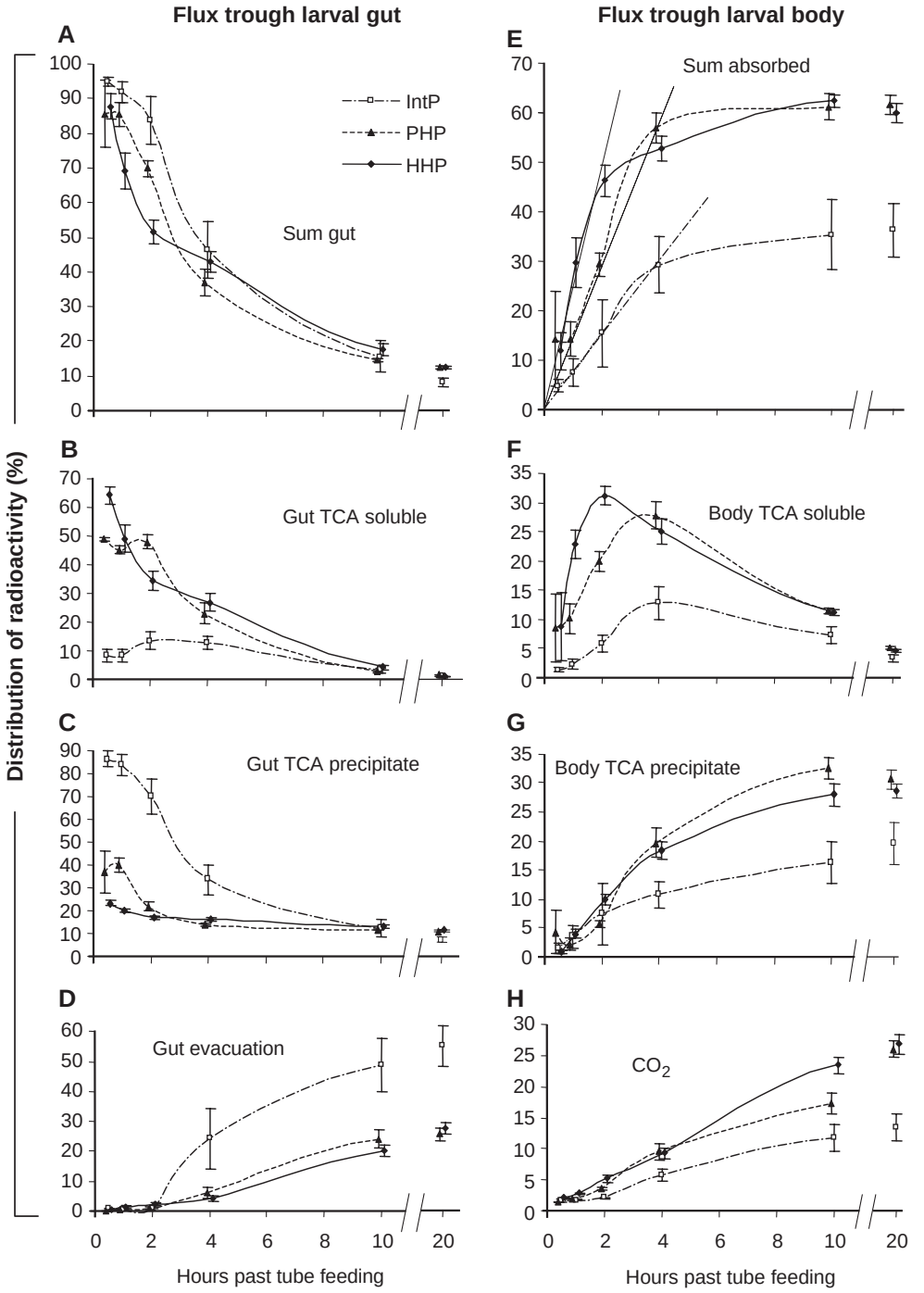
The rate of absorption into the larval body increased with the degree of hydrolysis of the protein, as can be seen from the higher initial accumulation rate of radioactivity in the absorbed fractions (Fig. 4E). Calculations based on data collected during the first 30–120 min show that the absorption rates of PHP and HHP into extra-intestinal body tissues were 2.2 and 3 times as fast as that of intact protein (P<0.05). However, the higher absorption rate in larvae tube-fed the HHP than those given PHP was only significant at a 10% level (P=0.1). A faster larval absorption of HHP relative to PHP was further indicated by higher accumulation of radioactivity in the body TCA-soluble fraction, containing AA in the larval free pool, at 2 h post tube feeding, when accumulation of AA in larvae tube-fed HHP reached the maximum level of 31±2.4% of the dose (n=10; Fig. 4F). The accumulation of radioactivity in the body TCA-soluble fractions in larvae, which had been fed IntP and PHP, reached their corresponding maximum levels (13±2.7% (n=10) and 28±2.2% (n=9), respectively) later, at about 4 h post administration.

Total absorption (body TCA precipitate, body TCA soluble, and CO_2 fractions) in larvae fed 11.4±1.5 μg of the intact protein at the final sampling (20 h post administration) was 36±5.5% (n=7; Fig. 4E).

Total radioactivity in the gut showed the opposite picture to that of initial absorption since no evacuation occurred during the first 2 h. Larvae tube-fed with HHP absorbed the protein dose from the gut faster than larvae fed PHP, as shown by the lower level of radioactivity in the gut compared to larvae fed the other protein solutions, at 1 and 2 h post administration (Fig. 4A). Larvae given PHP were intermediate and showed lower levels of radioactivity in the gut at 2 h post administration than larvae fed the intact protein. From 4 h post tube feeding onwards, the levels of radioactivity in the gut were almost identical in all groups. Studies of the individual components of total gut radioactivity showed that the three protein solutions were initially differently distributed into the TCA-soluble and TCA-precipitate fractions (Fig. 4B and C). At the first sampling, 0.5 h

Fig. 4. Distribution of radioactivity in Atlantic halibut larvae during the 20 h incubation period after tube feeding with an intact protein (^{14}C-SSP, IntP), pepsin hydrolysed ^{14}C-SSP (PHP), and further hydrolysation of PHP with trypsin and endoproteinase Glu-C, endoproteinase Asp-N, and endoproteinase Pro-C (HHP). Values are mean±S.E.M. Numbers of larvae sampled at each sampling time are shown in Table 2. Sum absorbed (E) is the summed distribution of radioactivity into body TCA soluble fraction, which includes FAA (F), body TCA precipitate which includes proteins (G), and CO_2 containing the radioactivity from catabolised AA (H). Initial absorption rates in larvae tube fed the respective diets were: $IntP_{0.5–4\ h}$=0.87 μg h^{-1} (R^2=0.36); $PHP_{0.5–4\ h}$=1.92 μg h^{-1} (R^2=0.77); $HHP_{0.5–2\ h}$=2.61 μg h^{-1} (R^2=0.54). Sum gut (A) is the summed distribution of radioactivity into gut TCA soluble fraction, which includes FAA and small peptides (B) and gut TCA precipitating compounds, including proteins (G). It is not possible to separate label in the gut lumen from label in the gut tissues. (D) shows the radioactivity in evacuated materials.

post administration, 8.2±2.1% (n=10), 49±0.5% (n=3), and 64±2.9% (n=9) of the radioactivity was present in the TCA-soluble fractions of larvae tube-fed IntP, PHP, and HHP, respectively (Fig. 4B), while 86±3.2%, 37±9.2,% and 23±1.0% were found in the gut TCA precipitates (Fig. 4C). The remainder had



already been absorbed. In larvae fed HHP, the level of radioactivity in the gut TCA-soluble fraction constantly fell during the first 2 h after tube feeding, while larvae given PHP displayed constant levels during this period. In larvae tube-fed intact protein, there was a tendency in the direction of an increase in the TCA-soluble fraction at 2 and 4 h after tube feeding, suggesting that active proteolysis of TCA-precipitating proteins was taking place in these larvae at that time. In all groups of larvae, the TCA-soluble fraction approached zero at the final sampling at 20 h post tube feeding. Radioactivity in the TCA-precipitate fractions, on the other hand, stabilised at 7.2±1.2%, in larvae tube-fed the intact protein, and at 11.2±0.6 for larvae fed the hydrolysed protein solutions (Fig. 4G).

Virtually no radioactivity could be measured in the incubation water after removal of CO_2 during the first 2 h post administration, from larvae tube-fed any of the protein solutions (Fig. 4D). From this point on, however, evacuation of the gut seemed to start. Faecal evacuation in larvae tube-fed either of the hydrolysed protein solutions was similar. Four hours after the diet was administered, less than 5% was evacuated into the incubation water, while by 10 h, evacuation was almost complete, with 24±3.0% (n=10) and 20±1.9% (n=10), in larvae fed with PHP and HHP, respectively. Evacuation at 20 h post tube feeding was 26±2.0% (n=12) and 28±2.0% (n=12), respectively. Mean evacuation in larvae tube-fed the intact protein was substantial higher than in larvae fed hydrolysed protein solutions, at 4, 10, and 20 h post tube feeding. However, the individual variation was substantial, and differences between larvae tube-fed intact protein at 25 dpff and 31 dpff were indicated by two-way ANOVA (P<0.05), although homogeneity in variance was not found (P>0.05). At 4 h post administration, the mean evacuated fractions in the younger larvae were 35±18% (n=5) vs. 13±10% (n=5) in the older larvae (Fig. 5). By 10 h, evacuation in the younger larvae was almost completed at 64±7.8% (n=5), while the older larvae had evacuated less (24±6.8%, n=3). The older larvae continued evacuation between 10 and 20 h post tube feeding and ended at 46±8.9% (n=4), which was between the final level evacuated by the younger larvae (67±6.2%, n=3) and larvae administered the hydrolysed diets.

The accumulation of radioactivity into the body TCA precipitate and CO_2 fractions in larvae tube-fed

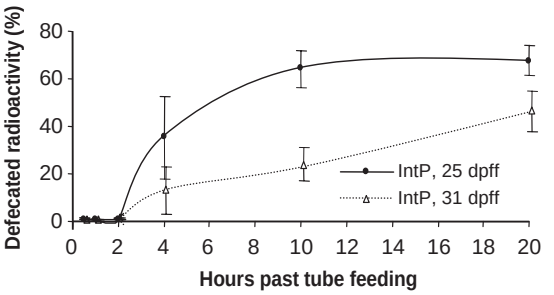


Fig. 5. Faecal evacuation of the intact protein (IntP) tube fed to larvae at 25 dpff (n=5) and 31 dpff (n=3), respectively. Values are mean±S.E.M. Mean evacuation was substantial higher in the youngest larvae at 4, 10, and 20 h post tube-feeding.

the hydrolysed protein solutions (Fig. 4G and H) was similar except for a faster accumulation of radioactivity in CO_2 in larvae given HHP, shown by higher mean level of radioactivity in the CO_2 at 10 h post tube feeding. The accumulated levels in larvae tube-fed any of the hydrolysed protein solutions at 20 h after feeding were 30±1.0% (n=24) and 26±1.0% (n=24) in the body TCA precipitate and CO_2 fractions, respectively. Accumulation of radioactivity in the body TCA precipitate and CO_2 fractions in larvae given intact protein, during the 20 h incubation period, was lower at 20±3.6% and 13±2.9%, respectively. The faecal evacuation was inversely related to absorption and post-absorptive utilisation of AA. Substantial variation and differences between larvae tube-fed with intact protein at 25 dpff and at 31 dpff were thus registered in total absorption and accumulation of radioactivity in the TCA precipitate and CO_2 fractions, as was also true for faecal evacuation (data not shown).

4. Discussion

4.1. Low capacity to digest and absorb protein

The gut of marine teleost fish larvae has been suggested to efficiently digest and absorb AA from ingested feeds, despite the absence of a functional stomach. This is based on the presence of pancreatic and mucosa associated proteases (Hjelmeland et al., 1988; Cahu and Zambonino-Infante, 1995; Gawlicka et al., 2000; Srivastava et al., 2002) and by the observed pinocytotic uptake of intact proteins over the

hind-gut epithelium (Watanabe, 1981a; Govoni et al., 1986; Kishida et al., 1998). The proteins taken up by pinocytosis are subsequently proteolysed by lysosomal enzymes, and thus made available to the larvae, although the available data suggest that the flux rates are low (Watanabe, 1981b). However, the present study demonstrates that even though small administered loads of intact protein (3.5 μg) were proteolytically degraded and absorbed by the Atlantic halibut larvae, with efficiency comparable to hydrolysed proteins, the larvae were not able to utilise the intact protein as efficient as the hydrolysed protein when the administered load increased (Fig. 2). This indicates that although proteases are present, and even though pinocytosis of intact proteins may occur in the gut of stomachless fish larvae, they seem to have a limited capacity for handling proteins, particularly when the substrate load is high. Indeed, this suggests that the proteolytic activity in marine fish larvae is one of the factors that limit growth. The average absorption efficiency (36%) in larvae administered 11.4 μg of the intact protein, as reported in the present study, was even higher than reported in a previous study (25±13%), where comparable amounts of the model protein (same batch) were tube-fed to slightly larger Atlantic halibut larvae (36 dpff; 60±12 mg wet weight; Tonheim et al., 2004). In the same study, intact protein was tube-fed to Atlantic halibut post-larvae (78 dpff, 250±52 mg body wet weight) in equal amount relative to body size, and the intact protein was absorbed almost identically efficient in post-larvae (59±13%) as was pre-hydrolysed protein in larvae of the present study (63%; Fig. 2). Preparative pepsin hydrolysis of diet proteins prior to feeding fish larvae, thus, seems to compensate for the absence of a complete gastric digestive system in fish larvae. These data support earlier studies of Rust (1995), Rønnestad et al. (2000b), and Rojas-Garcia and Rønnestad (2003), who suggested that larvae that develop gastric digestion during metamorphosis initially assimilate simple forms of amino acids more efficiently than more complex forms. The incorporation of tracer into the body TCA precipitate (Fig. 4G) further supports that FAA and hydrolysate are incorporated into body proteins and indicate that it may serve as an anabolic substrate given that it is supplied in a balanced diet. However, although a previous study (Tonheim et al., 2004) showed that intact protein was absorbed and utilised more efficient in juvenile Atlantic halibut as compared to larvae, absorption and utilisation of hydrolysed protein compared to intact protein should be tested in juveniles also to be able to state fundamental differences between larvae and juveniles in their ability to digest and utilise dietary protein.

4.2. Higher absorption rates of hydrolysate

Hydrolysis of the protein before administration to the Atlantic halibut larvae did not only improve the absorption efficiency, but also increased the initial rates of absorption (IntP<PHP<HHP; Fig. 4E). The SDS-PAGE data (Fig. 1) suggested that PHP and HHP in increasing order to a great extent consisted of protein fragments, small peptides, or FAA that are more readily absorbed or that only involve peptidases present at the apical membrane or in the cytosol of the enterocytes (Ganapathy et al., 1994). Faster absorption from HHP was thus expected, and faster absorption of peptides and FAA compared to intact proteins has previously been reported in fish larvae (Rønnestad et al., 2000b; Rojas-Garcia and Rønnestad, 2003) as well as in adult fish (Espe et al., 1993; Berge et al., 1994; Sveier et al., 2001). Absorption of larger peptides and proteins from IntP and, to some extent, also PHP into tissues outside the mucosa will include a time delay for the digestion.

The anabolic utilisation of rapidly digested and absorbed protein (whey protein) vs. slowly digested and absorbed protein (casein) has been studied in humans using single dose administration of ^{14}C[leu]-labelled proteins (Boirie et al., 1997; Dangin et al., 2001). Their data show that both “fast” and “slow” proteins stimulated AA oxidation and protein synthesis. However, the “slow” protein inhibited protein degradation, a feature that was attributed to prolonged elevated plasma FAA. This was not observed in subjects administered the “fast” protein. In sum, the “slow” protein was more efficiently retained than the “fast” protein after 7 h. No prolonged period with elevated plasma or whole body FAA was indicated in the fish larvae administered the intact protein (Fig. 4F) vs. the pre-hydrolysed protein preparations, despite many fold differences in absorptive rates (Fig. 4E). This was probably because early defecation from larvae prevented prolongation of the

absorptive period (Fig. 4D). Accordingly, no differences in retention of slowly absorbed dietary protein vs. rapidly absorbed dietary protein due to different inhibition of protein degradation should be expected in the larvae administered on single dose of the respective protein preparations. Whey protein administered in repeated small portions instead of one single meal inhibited protein degradation equally efficient as casein (Dangin et al., 2001). Thus, if protein synthesis and degradation are regulated by the same mechanisms in fish larvae and humans, greater anabolic stimulation from slowly vs. rapidly digested proteins should not be expected in continuously fed stomachless fish larvae as well. Hence, the concept of fast and slow proteins and their different anabolic stimulation is probably of low relevance in larvae nutrition.

In humans (Boirie et al., 1997; Dangin et al., 2001), both protein synthesis and AA oxidation occurred at higher rates in subjects fed with "fast" vs. "slow" protein. This was probably a direct effect of the higher plasma FAA levels of the former group, as there was no difference in plasma insulin between the two treatments. The hydrolysed protein preparations were oxidised and assimilated into larval protein faster and to a greater extent than intact protein, and a greater fraction of the HHP was oxidised by the larvae at 10 h past administration compared to the PHP (Fig. 4H). This could at first be taken as evidences for increased protein synthesis and AA oxidation in larvae due to increasing FAA levels. However, the rates of protein synthesis and AA oxidation could not be evaluated in the present experiment because the specific activity of the substrates was not measured in the larvae. Differences in rates of tracer accumulation into CO_2 and body TCA precipitate due to differences in metabolic rates could thus not be distinguished from the effect of changed specific activity of AA in the larval free pool.

As an alternative method for evaluation of the anabolic efficiency of intact protein vs. pre-hydrolysed protein, the accumulation of radioactivity in CO_2 and in the body TCA precipitate as fractions of absorbed radioactivity was measured. A weak tendency of increased oxidation and reduced assimilation relative to absorption was found for increasing degree of hydrolysis of the administered protein. This weak indication of a negative effect from pre-hydrolysed dietary protein in the larvae may be explained by saturation of the intestinal transport mechanisms, leading to imbalanced AA absorption. This is in accordance with earlier studies that have shown that an imbalanced dietary AA composition leads to increased AA oxidation (Kim et al., 1983; Tacon and Cowey, 1985; Aragão et al., 2004). However, the differences were only marginal and insignificant ($P>0.05$), and the potential reduction in anabolic efficiency was compensated for many times by the more efficient absorption of pre-hydrolysed protein by the stomachless larvae. The present results can thus not explain why inferior growth has been the result in previously reported studies where intact dietary proteins have been substituted with dipeptides and tripeptides and hydrolysed proteins in feeding experiments with stomachless marine fish larvae (Carvalho et al., 1997; Cahu et al., 1999; Cahu and Zambonino-Infante, 2001).

The leakage of water-soluble nutrients from compound feeds can be great (López-Alvarado et al., 1994; Rust, 2002) and variable, according to molecular size and water solubility (Yúfera et al., 2002). Hence, AA imbalances in larvae fed diets with high inclusions of low molecular dietary protein might have been introduced by leakage, and cannot be excluded as a reason to the negative effect of such supplementations in these experiments. In terrestrial animals (the broiler chick), it has previously been showed that adequate growth (more than 20% day^{-1}) can be achieved by diets based on balanced FAA as the only AA source (Roth et al., 2001).

4.3. The model protein

The same ^{14}C-labelled salmon serum protein was precursor for all three protein preparations tested in larvae, and thus, equal AA composition and AA specific activities were assured. The AA composition (Table 1) was naturally well balanced according to general fish requirements for indispensable AA (Wilson, 2002). The protein was, however, not uniformly labelled, as the constituting AA residues had different specific activities (Table 1). It is known that fish larvae discriminate between the different AA in their metabolism and that dispensable AA are catabolised to a higher degree (Rønnestad et al., 2001b; Conceição et al., 2002). The relative distribu-

tion of radioactivity into the different larval compartments in the present results is, thus, not necessarily identical to the distribution of AA in total. For instance, the AA alanine, aspartic acid, glutamine, and glutamic acid, which are shown to be preferentially oxidised in the energy metabolism of rats (Tanaka et al., 1995), together constituted a specific activity of approximately one fifth of the average specific activity in the other AA. Thus, oxidation of AA may have been underestimated in the present study. The specific activities of dispensable vs. indispensable AA in the protein were, however, almost identical, and as the AA profile and AA-specific activities were identical between the experimental protein solutions, inter-dietary comparisons was assumed to be safe.

The administered nutrient load is of importance for protein utilisation efficiency, as shown in the present study (Fig. 2) and previously reported (Boehlert and Yoklavich, 1984; Øie et al., 1997). Hence, studies of protein digestion and AA metabolism in rapidly growing larvae should be performed with high protein loads. The protein load administered by tube feeding to fish larvae is restricted by gut volume and the solubility of the administered protein/hydrolysate. However, by tube feeding fed larvae instead of larvae with empty guts, somewhat similar to the experiment of Aragão et al. (2004), protein assimilation and retention can be studied in larvae with a high protein load, which is an interesting future perspective.

4.4. Larval age and evacuation rate

The larval age influenced the ability to utilise dietary intact protein and suggested the older larvae to have enhanced proteolytic capacity. However, in all larvae, the test solutions were tube-fed directly into the larval mid-gut and the older larvae could therefore not possibly have any advantages from establishment of gastric functions. Increased levels of mid-gut proteases during the Atlantic halibut larvae ontogeny have been reported (Gawlicka et al., 2000) and are a possible explanation. However, differences in the kinetics of faecal evacuation between larvae tube-fed the intact protein at different days (Fig. 5) and between larvae tube-fed IntP and PHP and HHP (Fig. 4D) suggested an alternative explanation. Between 2 and 4 h post tube feeding, the larvae tube-fed the intact protein evacuated on average 35% and 12% of the administered protein at 25 and 31 dpff, respectively. In the same period of time, 15% of the intact protein was absorbed at both days. Thus in larvae fed intact protein, faecal evacuation started at a time when the absorption was high and still in progress. This suggests that the protein utilisation could be improved in these larvae by delaying the evacuation process. The halibut larvae tube-fed the hydrolysed protein preparations, on the other hand, had at 4 h post tube feeding already absorbed 50–60% of the administered protein (approximately 90% of the final absorption measured at 20 h post tube feeding) and evacuated less than 5%. The larvae fed the PHP and HHP, thus, experienced reduced risk of losing digestible proteins and peptides due to a faster absorption rate. It could be suspected that fast evacuation rates were induced by stress related to the tube feeding method. However, similar fast gut evacuation in stomachless fish larvae is previously reported for other species (*Leiostomus xanthurus*, Govoni et al., 1982; *Clupea herengus*, Pedersen, 1984; *Anguilla japonica*, Pedersen et al., 2003), and will have a negative effect on nutrient assimilation (Govoni et al., 1986).

A gut evacuation rate that exceeds the level at which maximum feed conversion is obtained may seems odd. However, it may be a good strategy in marine fish larvae due to their poorly developed digestive system, and a suitable adaptation to their natural habitat. Fish larvae are carnivores, feeding on live planktonic prey, which is reported to contain high amounts of soluble proteins, small peptides, and FAA (Fyhn, 1989; Carvalho et al., 2003; Helland et al., 2003). FAA are readily absorbed and utilised by fish larvae (Rønnestad et al., 2001b; Conceição et al., 2002; Applebaum and Rønnestad, 2004; Aragão et al., 2004), and soluble proteins are probably more available for digestion and absorption in the stomachless fish larvae than insoluble tissue proteins. Thus, due to restrictions in storage and proteolytic capacity, provided feed availability is sufficient, a high feed intake rate and extensive utilisation of the most available AA sources may be a better strategy for stomachless fish larvae with the aim of maximising growth, than are the maximisation of feed conversion by a prolonged passage time through the intestine.

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