Evaluation of feed protein quality by measuring plasma free amino acids in Atlantic salmon (*Salmo salar* L.) after dorsal aorta cannulation

J. SUNDE[1], A. KIESSLING[1], D. HIGGS[2], J. OPSTVEDT[3], G. VENTURINI[4] & K. RUNGRUANGSAK-TORRISSEN[1]

[1] *Department of Aquaculture, Matre Aquaculture Research Station, Institute of Marine Research, Matredal, Norway;* [2] *Department of Fisheries and Oceans, West Vancouver Laboratory, West Vancouver, BC, Canada;* [3] *Dept SSF, Norwegian Institute of Fisheries and Aquaculture Research, Fyllingsdalen, Norway;* [4] *ASA srl-Agridea, Servizio Nutrizione, San Martino Buon Albergo, Verona, Italy*

Abstract

Two successive experiments were conducted in order to assess plasma free amino acid (FAA) profiles as a method for evaluating protein quality of fish feeds for Atlantic salmon, *Salmo salar* (L.). In experiment 1, the importance of meal size and inter-fish variation was assessed by using dorsal aorta cannulated fish and diets that contained different sources of fishmeal (menhaden versus herring) which in the case of herring, had been dried at either 70 or 100 °C. In experiment 2, an attempt was made to mimic a production situation by comparing the FAA profiles in salmon fed diets containing two commercially available fishmeals that had been produced in accordance with industrial standards (Norse-LT94® and NorSeaMink®; Norsildmel AL, Fyllingsdalen, Norway). FAA profiles in plasma 6 h after feeding were compared with feed true protein digestibility as determined in mink. Cannulated fish, held in individual tanks, were hand-fed twice daily to pellet rejection (satiety) and daily records of the actual rations consumed were maintained. A total of 24 different amino acids and other amino-containing compounds were detected using high-pressure liquid chromatography. Morning and evening meal size showed significant correlations. Meal size had a significant effect on blood levels of the majority of essential free amino acids (EAA) as well as the total sum of FAA (TFAA). In experiment 1, a marked inter-individual effect was found, possibly because of incipient sexual maturation. FAA profiles were therefore corrected for meal size by linear regression while repeated sampling via the permanently implanted cannula allowed paired comparisons of the different test diets, minimizing inter-individual variation. Significant differences in plasma FAA profile, EAA and TFAA were detected between fish fed all diets in both experiments. The preceding parameters for fish ingesting each feed were directly related to their respective mink protein digestibility in experiment 1, but not in experiment 2. Our results show that dietary protein quality can be differentiated by the aforementioned protocol, and by using fish with a low metabolic rate feed qualities could be ranked correctly as in experiment 1.

KEY WORDS: Atlantic salmon, cannulation, feed intake, feed quality, plasma free amino acids

Received 20 September 2002; accepted 1 April 2003

Correspondence: Jan Sunde, Department of Aquaculture, Matre Aquaculture Research Station, Institute of Marine Research, N-5984 Matredal, Norway. E-mail: jan.sunde@imr.no

Introduction

Global production of farmed fish and shellfish has more than doubled in the past 15 years (Naylor *et al.* 2000). This increase in fish farming has created an accelerating demand for quality fishmeals for fish feed production. Fishmeals comprised of different raw materials vary in their biological and nutritional value and several commercially implemented methods currently exist for evaluating the quality of their protein. These include *in situ* digestibility, and chemical and *in vitro* enzyme assays (reviewed by Anderson *et al.* 1993; Rungruangsak-Torrissen *et al.* 2002). At present, costly and time-consuming large-scale growth experiments are still the norm, but new alternatives are being

© 2003 Blackwell Publishing Ltd

sought. Optimal growth and feed utilization in fish and other animals depend on a balanced supply of free amino acids (FAAs) to the plasma and muscle tissue as well as an optimum and appropriate source of nonprotein energy. FAA pools in blood and organs influence a range of physiological and biochemical parameters in the organism (Millward & Rivers 1988). Comparatively large amounts of amino acids from the diet are, however, deaminated and used as a source of energy in fish, giving a net retention of dietary nitrogen in the range of only 30–40% (Cowey 1994). Further, deficiencies or low biological availability of certain amino acids in the diet lead to poor protein utilization and consequently lower growth and feed utilization.

Studies on pellet-fed rainbow trout (Nose 1972; Walton & Wilson 1986), and Atlantic salmon fed pelleted feed (Torrissen *et al.* 1994) and intact and prehydrolysed cod protein (Espe *et al.* 1993; Torrissen *et al.* 1995), have shown peak values of total and essential amino acids (EAA) in the plasma 6 h after feeding. EAA profiles of plasma follow those of the feed (Nose 1972; Espe *et al.* 1993; Shuhmacher *et al.* 1995), but individual fish and day-to-day variation in appetite cause marked short-term fluctuations in plasma levels of FAA and probably makes pooled and group values unsuitable for diet evaluation in fish (Carter *et al.* 2001). However, by using individual dorsal aorta cannulated fish, these factors can be circumvented. Dorsal aorta cannulation, as described by Soivio *et al.* (1975), and as used by Kiessling *et al.* (1995) in nutritional studies, offers several unique advantages that are useful for metabolic studies by allowing repeated blood sampling of normally feeding individuals over long periods of time with minimal stress.

The first aim of this study was to test the precision of the preceding method for predicting the quality of protein in feeds with different sources of fishmeal processed in dissimilar ways. The second aim was to evaluate the dependence of the method to internal and external factors proving its usefulness also in studies of protein metabolism. This was carried out by comparing differences between fish in plasma FAA profiles and, first, relating these to the ranking of the quality of protein in these feeds by other means, e.g. mink true protein digestibility estimates and, secondly, to individual and environmental differences within and between the two studies. To our knowledge, this is the first study that has investigated the potential for using plasma FAA profiles in fish fitted with permanent dorsal aorta cannulas as a method for evaluating protein quality in fish feeds.

Materials and methods

Feed production

Experiment 1. Two fishmeals produced from fresh spring spawning Norwegian herring (*Clupea harengus*) were dried at low temperature (air dryer, duration 20–30 s, outlet meal temperature 70 °C) and at high temperature (steam dryer, duration >1 h, outlet meal temperature 100 °C) to provide two meals that differed in protein quality based upon potential differences in the bioavailability of their respective amino acids. These meals were subsequently ranked as FM1 > FM2, based on their true protein digestibility in mink. Thereafter, they were used for the preparation of two feeds (3.5 mm ∅ cylindrical pellets) in pilot scale facilities (Norwegian Institute of Fisheries and Aquaculture Research, Dept SSF, Fyllingsdalen, Norway). A detailed description of the production and characterization of these meals and feeds were given in Opstvedt *et al.* (2003). The feed designated as FM0 was made from menhaden fishmeal and it was extruded as 8.5 mm ∅ cylindrical pellets at the facilities of Skretting (formerly Moore-Clark), Vancouver, BC, Canada.

The concentrations of proximate constituents in the feeds, FM0, FM1 and FM2 were similar (on average 510 g kg^{-1} crude protein, 256 g kg^{-1} fat and 84 g kg^{-1} carbohydrates as N-free extract). True protein digestibility percentages were determined in mink to be 90.8 and 89.5 for feeds FM1 and FM2, respectively, while not measured for FM0. All pellets were coated with 2% krill extract in fish oil at the Department of Fisheries and Oceans, West Vancouver Laboratory, Vancouver, BC, Canada, to minimize potential differences in diet acceptance (palatability) caused by differences in ingredient composition.

Experiment 2. Two commercial fishmeals (Norsildmel AL, Fyllingsdalen, Norway) designated as FM3 and FM4, were certified as low temperature (LT) fishmeal Norse LT94® and NorSeaMink® fishmeal, respectively. Two feeds were prepared from these fishmeals and the protein quality in these was subsequently ranked as FM3 > FM4, based on their true protein digestibility in mink. The extrusion processing of the feeds to produce 3.5 mm ∅ cylindrical pellets was performed in a commercial plant (ASA srl-Agridea, Cologna Veneta, VR, Italy). A detailed description of the production and characterization of these meals and feeds is given in Opstvedt *et al.* (2003). The levels of proximate constituents in feeds FM3 and FM4 were similar (on average 391 g kg^{-1} crude protein, 275 g kg^{-1} fat and 184 g kg^{-1} carbohydrates

as N-free extract) with true protein digestibility percentages in mink of 91 and 87.4, respectively.

Fish rearing conditions and handling

Experiment 1. Atlantic salmon (*Salmo salar* L.) were held in outdoor 8 m^3 covered round fibreglass tanks supplied with bio-ring aerated and filtered pumped seawater, with a temperature of 11–14 °C and a salinity of 24–29 g L^{-1} at the facilities of the Department of Fisheries and Oceans (49°N).

After a 2-month acclimation of the salmon to the FM0 feed, fish with an average initial weight of 747 ± 49 g (mean ± SEM) were transferred to individual 200 L semi-round fibreglass tanks (80 × 50 cm) that were located indoors. A 14 h light (L) : 10 h dark (D) photo cycle with dawn and dusk periods, was provided using simulated natural light fluorescent tubes. All handling of the fish (see below for a description of fish cannulation procedures) was preceded by sedation with 0.25 mg L^{-1} Marinil® (metomidate hydrochloride; Wildlife Laboratories, Inc., Fort Collins, CO, USA) for 15 min.

Water was supplied to the experimental tanks through a column with bio-rings and the incoming water jet was directed parallel to, but 1 cm above, the water surface in order to create a circular current in the tank and to avoid the risk of tangling of the cannula in the water pipes. For the same reason, all tanks were fitted with external standpipes. A clamp-on frame with fine mesh net covered the tanks between feedings. During the experiment, the fish were hand-fed twice daily at 09.00 and 16.00 hours, until pellet rejection (satiety). The number of pellets ingested was recorded and back calculated based on the mean pellet air-dry weight to provide grams of feed ingested per fish. Uneaten pellets were rapidly removed through the bottom outlet in each tank by the current.

At the start of the experiment, the fish were divided into two groups comprising seven and six individuals (total of 13 tanks), respectively. The menhaden meal-based feed, FM0, was used as the holding feed until baseline blood samples were obtained on day 3. After the evening meal on day 3, the groups of fish were presented to their prescribed herring-based feeds FM1 and FM2 and blood was subsequently sampled on days 5, 8 and 10. After the blood sampling of the fish on day 10, the groups were switched to the other feed and new blood samples were collected on days 13 and 15. At the end of the experiment on day 15, all fish were sedated and killed after the blood sampling of each individual. All plasma samples were analysed for FAA content using high-pressure liquid chromatography (HPLC) (see methodology below).

Experiment 2. The experiment was conducted at Matre Aquaculture Research Station, Institute of Marine Research, Matredal, Norway (61°N). Atlantic salmon were held in indoor 1 m^3 covered fibreglass tanks which were each supplied with bio ring aerated and filtered pumped seawater, with a temperature of 10–11 °C and a salinity of 26–28 g L^{-1}. The fish in all tanks were subjected to a 24 h L : 0 h D photoperiod (beginning in January) using simulated natural light mounted in the tank lids.

Fish with an average initial weight of 749 ± 98 g (mean ± SEM) were transferred to individual 200 L square fibreglass tanks (70 × 80 cm) using the same sedation protocol as previously described and keeping the 24 h L : 0 h D photoperiod. Water was supplied under pressure through a pipe mounted in the tank wall so that a water jet and, in turn, a circular current were provided in each tank. This protocol was followed to avoid tangling of the cannula, reduce fish stress due to the holding of individual large fish in small tanks, and to allow resumption of normal feeding behaviour (Kiessling *et al.* 1995). All tanks were fitted with external standpipes and covered with nontransparent lids between feedings to provide an undisturbed environment. The feeding protocol was identical to that described for experiment 1, except in this case the feeds FM3 and FM4 were employed. Also, during weekends, the fish were fed using automatic feeders between 08.00 and 16.00 hours each day for a total amount of pellets approximating four meals over the course of 2 days. Estimates of the actual rations ingested by the fish were thus not available for Saturdays and Sundays. Fish blood sampling was performed as described in experiment 1, except instead of sedation of the fish with metomidate, 15 min prior to sampling, the room was maintained in darkness with only the tank light switched on. This situation created a mirror effect on the water surface, and minimized external disturbance of the fish during sampling. Blood samples were taken on Tuesdays and Thursdays so that there were accurate estimates of the actual rations consumed by the fish.

A total of 10 fish was divided into two groups and individual fish in each of the two groups of five tanks were presented feeds FM3 and FM4, respectively. After blood sampling on days 5 and 7, the groups were switched from one feed to the other and subsequently blood samples were taken from each fish on days 12 and 14. Thereafter, the groups were again switched back to their original diet in the evening of day 14 and then samples of blood were collected on days 15

and 17. At the end of the experiment, all fish were sedated and killed. Then all plasma samples were analysed for FAA content using HPLC (see below).

Cannulation and blood sampling procedures

Dorsal aorta cannulation was performed using a slightly modified protocol of Soivio *et al*. (1975) as described in Kiessling *et al*. (1995). After each fish resumed normal feeding behaviour following their transfer to individual tanks, the fish were sedated with metomidate hydrochloride (0.25 mg L^{-1}), according to Kreiberg & Powell (1991), and transferred to an oxygenated bath where they were anaesthetized with tricaine methanesulphonate, MS-222, at a concentration of 60 mg L^{-1}. The fish were left in the MS-222 bath until their swallowing reflex was suppressed before surgery was initiated. The insertion of the cannula was completed within 4–5 min, and during this time the fish were covered with a damp cloth to avoid afflicting damage to the skin. A PE-50 polyethylene tube (Intramedic®, Clay Adams, Parsipan, NJ, USA) was heated and extended to make a sharpened end that was inserted into the dorsal aorta through an incision in the roof of the mouth. At a point 6–7 cm from the end, the tube was heated and bent to a 90° angle to allow its exit through a hole drilled in the roof of the snout that was sheathed by a PE-90 polyethylene tube. Each cannula was filled with an isotonic (0.9% NaCl) solution of 1000 IU of heparin (Sigma, St Louis, MO, USA) and heat-sealed. All fish resumed normal feeding shortly after surgery, indicating that an insignificant level of stress was induced by this procedure.

During blood sampling, the fish in experiment 1 were sedated for 15 min in advance by using metomidate at a concentration of 0.25 mg L^{-1}, whereas the fish in experiment 2 were sampled without prior sedation. All samples were collected 6 h after the morning feeding through the cannula using a syringe. The end of the cannula was cut off and then the heparin lock and 0.1 mL of blood were discarded. A blood sample of 0.3–0.5 mL was then withdrawn with a 1-mL heparinized syringe. The cannula was then refilled with the isotonic heparin solution and re-sealed. Care was taken not to introduce any air bubbles into the cannula or the blood stream during this procedure. Each blood sample was transferred to a 0.5-mL Eppendorf tube, spun down at $1000 \times \boldsymbol{g}$ for 1 min, and then the plasma fraction was subsequently frozen at −80 °C pending analysis.

Plasma FAAs analysis

The FAA concentrations in the plasma were determined according to the method developed by Rungruangsak-Torrissen & Sundby (2000), using α-amino butyric acid as an internal standard in a final concentration of 100 nmol mL^{-1}. The plasma supernatant was deproteinized with a Millipore Ultrafree-MC 10 000 NMWL Filter Unit (Millipore, Bedford, MA, USA) at $5000 \times \boldsymbol{g}$ for 30 min at 4 °C. The filtrate of the plasma supernatant was derivatized with Waters AccQ·Tag reagent, according to Waters (1993) and analysed with a buffer gradient system according to Rungruangsak-Torrissen & Sundby (2000) using the Alliance HPLC system (Waters 2690 Separations module), Waters 996 Photodiode Array Detector ($\lambda = 254$ nm) and Waters 474 Scanning Fluorescence detector ($\lambda_{ex} = 250$ nm, $\lambda_{em} = 395$ nm). Separation of the FAA was performed using a Waters Nova-Pak C_{18} column (60 Å, 4 μm, 3.9 × 300 mm). The tryptophan derivative was not reliably detected in this analysis.

Statistical analysis

Multivariate tests were conducted using the statistical software SAS System 6.12 for Windows (SAS Institute, Inc., Cary, NC, USA). All data exhibited normal distribution and a main factorial model (the *glm* procedure) was employed, incorporating the effects of meal size prior to sampling as a covariate by linear regression. Free amino acid profiles in fish samples were compared between diets using pair-wise comparison (*t*-test). On all occasions when the fish refused to feed at the meal preceding blood sampling, the measurements were omitted from the subsequent statistical analysis.

Conclusions regarding feed intake variation were confirmed using R 1.2.1 software (http://www.r-project.org), for constructing and testing feeding models.

Results

Feed intake and influence of sampling

The size of the morning and evening meals showed a strong positive correlation in both experiments. This was especially apparent for fish held under the 14 h L : 10 h D photo cycle in experiment 1 ($r = 0.628$, $P < 0.0001$), compared with fish held under continuous light in experiment 2 ($r = 0.218$, $P < 0.02$). Individual meal size ranged from 0.05 to 3.85 g in experiment 1 (up to 0.5% of body weight) and between 0.08 and 6.94 g (up to 0.9% of body weight) in experiment 2. In experiment 1, appetite was suppressed in the morning following sampling (18-h postblood sampling). By contrast, appetite was restored by the afternoon (24-h postblood sampling). No marked appetite depression was seen in experiment 2 following sampling.

Comparison of amino acid profiles between experiments

No significant differences existed in the amino acid profiles between the FM1 and FM2 feeds and between the FM3 and FM4 feeds. A total of 21 peaks that corresponded to 24 different amino acids and other amino-containing compounds were detected in the plasma samples (Table 1). In addition, inconsistent appearances of 1-methyl-histidine and 3-methyl-histidine, led to the exclusion of these from the analysis. Aspartic acid and citrulline were detected in the plasma samples taken in experiment 2 only, where commercially available fishmeals were used to produce the feeds. Asparagine, cysteine and tryptophan were found to be below detectable concentrations in all samples. Concentrations of glycine and the sulphur-containing compound taurine were markedly higher in plasma samples collected from fish in experiment 2, compared with those noted for fish in experiment 1. Hydroxyproline concentrations also differed between the experiments and were markedly lower in the plasma from fish in experiment 2. The total EAA in the plasma of salmon were significantly affected by diet treatment in both experiments.

Changes in plasma FAA profiles

Table 2 presents each amino acid and the significance of the main factors contributing to their variations in the plasma, viz. individual fish, feed type and meal size preceding sampling. All statistical evaluations were carried out separately for each experiment. In both experiments, sums for essential and total FAAs were affected by feed type as well as meal size. Therefore, FAA profiles were corrected for meal size in order to compare the plasma FAA profiles between fish given the

Table 1 Plasma free amino acid profiles (nmol mL^{-1}) in experiments 1 and 2, corrected for feed intake and individual fish variation (*lsmeans* procedure). Significant differences ($P \leq 0.05$) are marked with different superscripts. All numbers are given as least square mean values ± standard error (n.d. = not detected). Pair-wise comparisons of free amino acid profiles showed significant differences between all feeds within respective experiments

	Experiment 1			Experiment 2	
Free amino acid	FM0	FM1	FM2	FM3	FM4
Ala	272.7 ± 19.2^{a}	345.0 ± 15.0^{b}	335.3 ± 15.0^{b}	264.7 ± 36.7	292.7 ± 30.9
Arg + GABA*	166.2 ± 14.9^{a}	259.5 ± 11.6^{b}	234.0 ± 11.6^{b}	144.0 ± 24.7^{c}	221.3 ± 18.9^{d}
Asn	n.d.	n.d.	n.d.	n.d.	n.d.
Asp	n.d.	n.d.	n.d.	6.22 ± 0.80	7.20 ± 0.80
β-Ala + Sar*	113.1 ± 10.3^{a}	61.2 ± 8.0^{b}	56.1 ± 8.0^{b}	84.9 ± 12.6	92.4 ± 10.2
Cit	n.d.	n.d.	n.d.	62.6 ± 14.0	55.8 ± 11.4
Cys	n.d.	n.d.	n.d.	n.d.	n.d.
Gln	149.0 ± 14.5^{a}	238.6 ± 11.3^{b}	242.8 ± 11.3^{b}	109.0 ± 17.3	151.1 ± 14.1
Glu	28.2 ± 4.3	26.6 ± 3.6	33.4 ± 3.7	35.6 ± 3.25	35.9 ± 2.66
Gly	396.0 ± 31.4^{a}	279.3 ± 24.5^{b}	294.1 ± 24.5^{b}	754.6 ± 124.7	741.2 ± 101.8
His	55.6 ± 10.3^{a}	83.9 ± 8.0^{b}	60.1 ± 8.0^{a}	24.1 ± 3.9^{c}	35.7 ± 3.2^{d}
Hyp	104.6 ± 10.5^{a}	117.7 ± 8.2^{b}	106.3 ± 8.2^{b}	36.1 ± 5.2	47.8 ± 3.9
Ile	135.6 ± 9.4^{a}	216.3 ± 7.4^{b}	205.9 ± 7.4^{b}	160.9 ± 16.8	200.8 ± 13.7
Leu + Orn*	185.2 ± 10.1^{a}	236.2 ± 7.9^{b}	225.5 ± 7.9^{b}	360.2 ± 43.5^{c}	476.5 ± 35.5^{d}
Lys	274.0 ± 25.0^{a}	364.1 ± 19.5^{b}	301.7 ± 19.5^{a}	132.6 ± 30.6	165.3 ± 25.0
Met	107.5 ± 6.0	113.8 ± 4.8	115.0 ± 4.8	80.0 ± 11.1^{a}	115.2 ± 9.1^{b}
Phe	96.1 ± 5.7^{a}	118.7 ± 4.5^{b}	120.3 ± 4.5^{b}	80.8 ± 8.9^{c}	108.4 ± 7.3^{d}
Pro	30.0 ± 5.7	31.4 ± 5.3	35.6 ± 5.2	30.7 ± 5.3	19.8 ± 4.5
Ser	111.8 ± 8.0	127.9 ± 6.5	107.5 ± 6.2	135.5 ± 15.0	163.4 ± 12.2
Tau	165.5 ± 33.1	182.8 ± 26.8	171.4 ± 25.9	712.7 ± 136.7	703.3 ± 111.5
Thr	242.4 ± 14.0	264.5 ± 11.6	247.4 ± 10.9	178.2 ± 21.3^{a}	246.3 ± 17.3^{b}
Trp	n.d.	n.d.	n.d.	n.d.	n.d.
Tyr	40.8 ± 3.3^{a}	52.1 ± 2.5^{b}	53.1 ± 2.5^{b}	44.5 ± 5.2^{d}	64.5 ± 4.5^{e}
Val	333.7 ± 17.3^{a}	436.5 ± 13.7^{b}	414.2 ± 13.7^{b}	325.3 ± 38.2	373.4 ± 31.2
ΣEAA	1435 ± 75^{a}	1785 ± 58^{b}	1666 ± 58^{b}	1195 ± 132^{c}	1620 ± 108^{d}
Σ NEAA	1563 ± 94	1695 ± 73	1660 ± 73	2503 ± 221	2596 ± 181
ΣTFAA	2998 ± 142^{a}	3479 ± 111^{b}	3326 ± 111^{b}	3697 ± 195^{c}	4216 ± 159^{d}

*Unable to achieve peak separation.

Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; β-Ala, β-alanine; Cit, citrulline; Cys, cysteine; GABA, γ-aminobutyric acid; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Hyp, hydroxyproline; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Orn, ornithine; Phe, phenylalanine; Pro, proline; Sar, sarcosine; Ser, serine; Tau, taurine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine; ΣEAA, sum of essential free amino acids; ΣNEAA, sum of nonessential free amino acids; ΣTFAA, sum of total free amino acids.

Table 2 Values of *F* and associated probabilities of detecting significant differences between the means for plasma free amino acids, considering the effect of individual fish, feed type and meal size before sampling as source factors in a main factorial model (*glm* procedure) in both experiments. Significant variations ($P \leq 0.05$) are marked with asterisks (n.d. = not detected)

	Experiment 1						Experiment 2					
	Fish		Feed type		Meal size		Fish		Feed type		Meal size	
Free amino acid	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Ala	5.81	<0.001*	4.68	0.01*	0.27	0.60	0.68	0.72	0.34	0.57	0.23	0.63
Arg + GABA**	2.24	0.02*	11.98	<0.001*	40.12	<0.001*	1.23	0.33	6.15	0.02*	0.81	0.38
Asn	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Asp	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.58	0.04*	0.79	0.39	16.34	<0.001*
β-Ala + Sar**	1.17	0.33	10.46	<0.001*	0.46	0.50	1.29	0.30	0.21	0.65	2.33	0.14
Cit	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.44	0.90	0.14	0.71	0.41	0.71
Cys	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gln	2.23	0.02*	15.04	<0.001*	0.38	0.54	0.54	0.83	3.53	0.07	0.04	0.84
Glu	3.10	<0.003*	0.89	0.42	5.29	<0.03*	2.41	0.04*	0.01	0.93	2.20	0.15
Gly	0.91	0.55	4.58	0.02*	0.25	0.62	0.43	0.90	0.01	0.93	0.90	0.35
His	1.32	0.24	2.94	0.06	0.53	0.47	0.40	0.92	5.31	0.03*	1.33	0.26
Hyp	25.95	<0.001*	0.63	0.54	0.56	0.46	1.36	0.27	3.22	0.09	1.04	0.32
Ile	2.76	0.006*	24.20	<0.001*	59.78	<0.001*	0.23	0.98	3.36	0.08	1.45	0.24
Leu + Orn**	2.22	0.02*	7.93	0.001*	82.33	<0.001*	0.71	0.70	4.27	0.05*	1.85	0.19
Lys	0.78	0.68	4.39	0.02*	8.23	0.006*	0.81	0.61	0.68	0.42	1.70	0.21
Met	2.64	0.008*	0.49	0.61	68.53	<0.001*	0.62	0.77	6.01	0.02*	3.26	0.08
Phe	3.13	0.002*	6.25	0.004*	42.64	<0.001*	0.58	0.80	5.70	0.03*	0.95	0.34
Pro	1.72	0.15	0.34	0.71	0.87	0.36	3.13	0.01*	2.41	0.14	4.33	0.05*
Ser	2.86	0.004*	2.51	0.09	13.84	<0.001*	0.41	0.92	2.08	0.16	1.01	0.32
Tau	1.26	0.27	0.08	0.92	3.31	0.075	0.84	0.59	<0.01	0.96	0.41	0.53
Thr	4.80	<0.001*	0.81	0.45	7.41	<0.001*	0.39	0.93	6.13	0.02*	0.17	0.68
Trp	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tyr	2.43	0.01*	5.02	0.01*	47.38	<0.001*	1.56	0.19	8.48	0.01*	4.14	0.05*
Val	2.42	0.02*	10.83	<0.001*	67.9	<0.001*	0.34	0.95	0.95	0.34	2.22	0.15
ΣEAA	1.51	0.15	6.54	0.003*	45.26	<0.001*	0.89	0.55	6.25	0.02*	4.09	0.05*
ΣNEAA	1.63	0.11	0.61	0.55	5.79	0.02*	1.29	0.30	0.11	0.75	1.60	0.22
ΣTFAA	1.16	0.34	3.44	0.04*	26.16	<0.001*	1.78	0.13	4.24	0.05*	7.85	0.01*

**Unable to achieve peak separation.

See Table 1 for abbreviations.

different diets. In this regard, the profile of the FM0 group was noted to be distinguished from those of the FM1 and FM2 groups because of significant differences in the concentrations of 13 FAA (Table 1). By comparison, only two FAA, namely, histidine and lysine, showed significantly different concentrations in the plasma between fish consuming the FM1 and FM2 diets, with the high quality herring meal-based feed, FM1, yielding higher concentrations than the low quality herring meal-based feed, FM2 (Table 1). Pair-wise comparison of FAA profiles (*t*-test) showed that the FM0 group (menhaden feed) differed significantly from that of the FM1 group (high quality herring feed, $P < 0.02$), and the FM2 group (low quality herring feed, $P < 0.04$). In addition, the herring meal-based feed groups FM1 and FM2 were different when compared by a paired test ($P < 0.02$), despite fewer differences in individual FAA concentrations. The method was thus able to distinguish between the plasma amino acid profiles of fish fed all three of the different feeds.

In experiment 2, feeds FM3 and FM4 were separated by significant differences in concentrations of seven plasma FAAs, as well as the sums for EAA and total FAA. In contrast to the observations in experiment 1, in cases where FAA concentrations differed between the two feed groups, fish consuming the high quality feed FM3 exhibited lower plasma amino acid concentrations than those ingesting the low quality FM4 feed. Pair-wise comparison of FAA profiles, corrected for variation in meal size, revealed in agreement with the results obtained for FM1 and FM2 in experiment 1, a significant difference ($P < 0.002$) between the two feeds.

Regulation of meal size following blood sampling

Of all amino acid concentrations, only methionine showed significant positive correlation ($P < 0.002$) with meal size immediately after sampling (0 h), whereas glutamine showed positive correlations with the size of the following morning

meal (18 h, $P = 0.054$), as well as the following evening meal (24 h, $P < 0.006$). In addition, fish appetite on the morning following sampling (meal size at 18 h) showed significant variation between individuals.

Discussion

Based on our data, we conclude that plasma profiles of FAAs in individual dorsal aorta cannulated Atlantic salmon can function as an assessment tool of dietary protein quality if variations in FAA because of different meal sizes and inter individual differences are controlled. Hence, the technique of dorsal aorta cannulation of salmon provides an important and relatively rapid complement for handling these factors to more traditional approaches, such as apparent digestibility and growth studies. For instance, in contrast to the current experiment, growth studies utilizing the same feeds did not find quality differences between the FM1 and FM2 feeds to be large enough to result in significant differences in either specific growth rate (SGR) or feed conversion efficiency (FCE) after 3 months of feeding salmon with a start weight of 150 g. However, in salmon with a start weight of 2 kg, the FM3 diet resulted in significantly higher total FAA in the plasma, SGR and FCE compared with the FM4 diet, after 3 months of experiment (Sunde *et al*. 2003). This may be due to the fact that the difference in mink protein digestibility between the FM3 and FM4 feeds (3.6%) was higher than between the FM1 and FM2 feeds (1.3%). Compared with the traditional apparent digestibility measurements, measuring plasma levels of FAA offers an advantage in its use of individual whole animal allowing different metabolic factors and states to be included in the model. It also involves effects on enterocytes (gut lining) metabolism and/or metabolic effects of anti-nutritional factors to be included in the efficiency calculation. Given the same feed, apparent digestibility was not found to differ between fish with different trypsin phenotypes, exhibiting different digestive efficiencies (Torrissen & Shearer 1992), while plasma FAA levels were higher in fish that showed higher digestive efficiency (Torrissen *et al*. 1994), suggesting that monitoring of plasma FAA levels may have a potentially higher resolution when measuring amino acid digestibility.

Feed intake and influence of sampling

In both experiments, a lower than expected feed intake [<0.7% (body weight) per day] was observed. It is possible that this was a consequence of too small a pellet size, which may have led to cessation of feeding behaviour before actual satiation was reached. In this regard, the time needed to dispense feed through hand feeding increases dramatically when the pellet size is below the optimum in relation to fish size. The reduction in fish appetite each morning after blood sampling can most likely be ascribed to an effect of stress caused either by the loss of blood during sampling, the sampling routines, or by the administration of anaesthetic. A maximum of 0.3 mL of blood was sampled nine times per fish over a 15-day period and represents roughly 7% of the total blood volume in the size of fish used in this study. However, if the removal of this amount of blood actually presented a serious problem to the fish, one would expect that the effect would escalate with time and also that there would be attendant decreases in percentage of haematocrit. Neither situation was observed. If sedation of the fish at the time of sampling was the problem, the effect should have differed between the two experiments. In fact, this was the case, because no marked appetite depression was seen 18 h postsampling of the fish in experiment 2. We therefore conclude that either the fish were stressed before sedation in experiment 1 or that the anaesthetic or the procedure of sedation *per se* induced a stress response. However, metomidate is known to inhibit cortisol synthesis and is thereby effective in removing the cortisol response to handling stress (Olsen *et al*. 1995). Therefore it is probable that the stress resulted from increased human activity around the tanks before administration of the anaesthetic, as the transparent tank lids used in experiment 1 offered no protection against visual disturbance of the fish during daylight hours.

Comparison of amino acid profiles between feed groups

The aim of this study was to determine whether feeds with different protein qualities led to differences in plasma FAA profiles in dorsal aorta cannulated salmon once sources of FAA variation as a result of meal size and individual fish had been removed through statistical means. In relation to this goal, we were successful in distinguishing between all feeds used in both experiments. When Anderson *et al*. (1995) compared amino acid and protein digestibility of several fishmeals in Atlantic salmon, menhaden meal had a lower apparent crude protein digestibility coefficient than three of the four herring meals assessed in our study and these meals differed in the bioavailable levels and composition of amino acids. Similarly, *in vitro* enzyme digestibility for the menhaden fishmeal was found to be lower than that of the herring-based meals (Anderson *et al*. 1993). Theoretically, FM0 used in the present study should therefore have a lower digestibility than the feeds containing herring fish meals (FM1 and

FM2). Our plasma FAA results also confirm this, although mink protein digestibility of the menhaden meal-based feed was not measured. Plasma amino acid concentrations, in particular, sums of EAA and TFAA, were generally lower in fish fed the diet with FM0 when compared with those fed the diets FM1 and FM2, and values for histidine and lysine in fish fed FM2 were lower than those found in fish fed FM1 (Table 1). Based on these findings, a ranking of the feeds could be made as FM1 > FM2 > FM0, which supports the assumption that FM0 had lower digestibility than FM1, with FM2 displaying an intermediate digestibility. In experiment 2, the opposite situation was seen, with lower plasma FAA concentrations noted in the (high quality) FM3 group, compared with the (low quality) FM4 group. Based on the arguments given above, one would have expected to see similar differences as in experiment 1. The reasons for the differences seen in absolute FAA concentrations in plasma between the two experiments (Table 1) are at present not clear, but may relate to several factors such as differences in metabolic state caused by a higher stress level (see discussion above regarding tank lids) or incipient maturation (see discussion below) reducing feed motivation in experiment 1. Genetic differences manifested by selective breeding of fish in experiment 2 for higher growth rate (increased metabolic rate) and reduced occurrence of early maturation could also be the underlying cause for the difference in feed motivation observed between the two experiments. Different photoperiods used in the two experiments may also effect metabolic status of fish and thus tissue and plasma FAA concentrations, as was seen by Sunde *et al.* (2001).

The individual variation of free hydroxyproline that was observed in experiment 1 is intriguing as it was independent of both feed type and meal size (Table 2). Hydroxyproline does not originate from the feed, but is a result of collagen breakdown in the connective tissues, and may be a possible substrate in anaerobic energy production (Van Waarde 1988), suggesting that changes in free hydroxyproline concentration may indicate changes in protein metabolism. For instance, free hydroxyproline in white muscle increased with increasing growth rates in salmon reared under natural and continuous light regimes (Sunde *et al.* 2001), whereas lower concentrations were seen in plasma in salmon exhibiting higher digestive efficiency (Torrissen *et al.* 1994). The change in protein metabolism associated with vitellogenesis and sexual maturation in female spawning ayu, *Plecoglossus altivelis*, was related to increased plasma levels of hydroxyproline (Toyohara *et al.* 1997). Continuous light is known to lower the incidence of sexual maturation in Atlantic salmon (Hansen *et al.* 1992; Taranger *et al.* 1999) and one should therefore expect a lower risk of incipient maturation in fish of experiment 2. However, the inclusion of individual growth rates in our statistical model did not yield a significant effect. One of course has to bear in mind the uncertainty in estimation of fish growth rates because of the short experimental period. Sunde *et al.* (2003), who studied FAA in plasma using the traditional single observations of one sample per fish and without individual feed intake measurements, found no differences in plasma FAA when no differences in growth and feed conversion efficiency were apparent between diet groups. On the other hand, the same author reported higher plasma TFAA levels in fish fed on higher digestibility feed displaying increased feed conversion efficiency, but without a growth increase. Torrissen *et al.* (1994) also showed that during a short-term experiment with no differences in growth, Atlantic salmon with higher digestive efficiency had higher levels of postprandial plasma FAA than those with lower digestive efficiency. Based on these earlier experiments and the present one it is tempting to formulate a working hypothesis to be tested in future work. In fish with a high metabolic rate, a limiting factor for growth could be the uptake from the gut of essential FAA necessary for protein synthesis, thereby resulting in a low plasma level of these FAA compared with fish ingesting a protein with low digestibility that does not stimulate protein synthesis to the same degree, consequently resulting in differences in growth. Such an effect would be even more accentuated in fish given a reduced ration. In the situation of a low metabolic rate/slow growth, one would expect the opposite pattern in plasma FAA levels. Thus as with a highly digestible protein, FAA would still be transported across the gut-blood barrier more effectively but would not be removed as rapidly from the plasma, i.e. the limiting factor to growth in this case would not be the uptake of FAA from the gut but instead protein synthesis itself, resulting in an accumulation of FAA in plasma; an effect more pronounced in fish consuming a feed with higher digestibility.

Regulation of future feed intake

It has been postulated that absorbed FAA, in particular essential amino acids, serve an important transient role as regulators of metabolism and tissue protein synthesis (Millward & Rivers 1988). Further, the concentration differences of FAA in plasma between individual fish may indicate metabolic differences between these fish that result in varying levels of appetite stimulation (Carter *et al.* 2001). Our experimental design facilitates the study of such questions. As a result of the limited number of blood samplings of the fish in

the present experiment, no final conclusions can be made based on the present data set. Even so, when the trends for changes in meal size of fish are examined in relation to their variations in plasma profiles of EAA in experiment 1, there were indications of long-term effects of plasma FAA concentrations on subsequent feed intake. For instance, plasma methionine concentrations positively correlated with meal sizes immediately after blood sampling. This trend conceivably could have been an artefact as the fish at this time probably were still under the influence of the anaesthetic. On the other hand, methionine is important for initiation of protein synthesis (Stryer 1988a), and an increase in the blood may indicate a preparation for protein synthesis that induces a higher appetite. In addition, it is interesting to note that cotton pellets soaked in an aqueous solution of methionine facilitated a positive feeding response in rainbow trout, *Oncorhynchus mykiss* (Jones 1989). One might therefore speculate that plasma methionine levels may provide a positive feedback regulation of either feed intake or feeding behaviour through its concentration in the plasma. Similarly, glutamic acid, which is also a pivotal molecule in nitrogen metabolism (Stryer 1988b) showed a strong positive correlation with meal sizes on the evening following blood sampling. At this point normal fish feeding behaviour had resumed. Consequently, the size of this meal should have reflected an undisturbed situation in the fish. Both glutamic acid concentrations in the plasma and the size of this meal showed concomitant variations between individuals. This suggests that differences in glutamic acid metabolism between individual fish may play a role in the regulation of feed intake. This intriguing possibility certainly warrants further research.

Acknowledgements

This study was carried out with financial support from the Commission of the European Communities, Agriculture and Fisheries, specific RTD programme FAIR-CT96-1329, 'Effect of processing technology on the quality of aquaculture feeds'. The paper does not necessarily reflect its views and in no way anticipates the Commission's future policy in this area. The authors wish to thank the Department of Fisheries and Oceans, Aquaculture Division, Pacific Region of Canada. We are also in debt to Dr Kari Ruohonen, Finnish Game and Fisheries Research Institute, Evo Fisheries Research Station, Evo, Finland for invaluable discussion and assistance in operating the R 1.2.1 software during the evaluation of this data set for regulation of voluntary feed intake. This part of the project was supported by travel grants from COST action 827.

References

Anderson, J.S., Lall, S.P., Anderson, D.M. & McNiven, M.A. (1993) Evaluation of protein quality in fishmeals by chemical and biological assays. *Aquaculture*, **115,** 305–325.

Anderson, J.S., Lall, S.P., Anderson, D.M. & McNiven, M.A. (1995) Availability of amino acids from various fish meals fed to Atlantic salmon (*Salmo salar*). *Aquaculture*, **138,** 291–301.

Carter, C.G, Houlihan, D., Kiessling, A., Médale, F. & Jobling, M. (2001) Physiological effects of feeding. In: *Food Intake in Fish* (Houlihan, D., Boujard, T. & Jobling, M. eds), pp. 297–331. Blackwell Science, Oxford, UK.

Cowey, C.B. (1994) Amino acid requirements of fish: a critical appraisal of present values. *Aquaculture*, **124,** 1–11.

Espe, M., Lied, E. & Torrissen, K.R. (1993) Changes in plasma and muscle free amino acids in Atlantic salmon (*Salmo salar*) during absorption of diets containing different amounts of hydrolysed cod muscle protein. *Comp. Biochem. Physiol.*, **105A,** 555–562.

Hansen, T., Stefansson, S. and Taranger, G.L. (1992) Growth and sexual maturation in Atlantic salmon, *Salmo salar* L., reared in sea cages at two different light regimes. *Aquacult. Fish. Manage.*, **23,** 275–280.

Jones, K. A. (1989) The palatability of amino acids and related compounds to rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Biol.*, **34,** 149–160.

Kiessling, A., Dosanjh, B., Higgs, D., Deacon, G. & Rowshandeli, N. (1995) Dorsal aorta cannulation: a method to monitor changes in blood levels of astaxanthin in voluntarily feeding Atlantic salmon, *Salmo salar* L. *Aquacult. Nutr.*, **1,** 43–50.

Kreiberg, H. & Powell, J. (1991) Metomidate sedation reduces holding stress in chinook salmon. *World Aquacult.*, **22,** 58–59.

Millward, D.J. & Rivers, J.P.W. (1988) The nutritional role of indispensable amino acids and the metabolic basis for their requirements. *Eur. J. Clin. Nutr.*, **42,** 367–393.

Naylor, R.L., Goldburg, R., Primavera, J.H., Kautsky, N., Beveridge, M.C.M., Clay, J., Folke, C., Lubchenko, J., Mooney, H. & Troell, M. (2000) Effect of aquaculture on world fish supplies. *Nature*, **405,** 1017–1024.

Nose, T. (1972) Changes in the pattern of free plasma amino acids in rainbow trout after feeding. *Bull. Freshw. Fish. Res. Lab.* (*Tokyo*), **22,** 137–144.

Olsen, Y.A., Einarsdottir, I.E. & Nilssen, K.J. (1995) Metomidate anaesthesia in Atlantic salmon, *Salmo salar*, prevents cortisol increase during stress. *Aquaculture*, **134,** 155–168.

Opstvedt, J., Nygård, E., Samuelsen, T.A., Venturini, G., Luzzana, U. & Mundheim, H. (2003) Effect on protein digestibility of different processing conditions in the production of fish meal and fish feed. *J. Sci. Food. Agric.*, **83,** 775–782.

Rungruangsak-Torrissen, K. & Sundby, A. (2000) Protease activities, plasma free amino acids and insulin at different ages of Atlantic salmon (*Salmo salar* L.) with genetically different trypsin isozymes. *Fish Physiol. Biochem.*, **22,** 337–347.

Rungruangsak-Torrissen, K., Rustad, A., Sunde, J., Eiane, S.A., Jensen, H.B., Opstvedt, J., Nygård, E., Samuelsen, T.A., Mundheim, H., Luzzana, U. & Venturini, G. (2002) *In vitro* digestibility based on fish crude enzyme extract for prediction of feed quality in growth trials. *J. Sci. Food Agric.*, **82,** 644–654.

Shuhmacher, A., Schön, J., Goldberg, M. & Gropp, J.M. (1995) Plasma amino acid levels in rainbow trout (*Oncorhynchus mykiss*). *J. Appl. Ichthyol.*, **11,** 309–316.

Soivio, A., Nyholm, K. & Westman, K. (1975) A technique for repeated sampling of the blood of individual resting fish. *J. Exp. Biol.*, **62,** 207–217.

Stryer, L. (1988a) Protein synthesis. In: *Biochemistry*, 3rd edn (Stryer, L. ed.), pp. 733–766. W.H. Freeman, New York.

Stryer, L. (1988b) Biosynthesis of amino acids and heme. In: *Biochemistry*, 3rd edn (Stryer, L. ed.), pp. 575–600. W.H. Freeman, New York.

Sunde, J., Taranger, G.L. & Rungruangsak-Torrissen, K. (2001) Digestive protease activities and free amino acids in white muscle as indicators for feed conversion efficiency and growth rate in Atlantic salmon (*Salmo salar* L.). *Fish Physiol. Biochem.*, **25,** 335–345.

Sunde, J., Eiane, S.A., Rustad, A., Jensen, H.B., Opstvedt, J., Nygård, E., Venturini, G. & Rungruangsak-Torrissen, K. (2003) Effect of fish feed processing conditions on digestive protease activities, free amino acid pools, feed conversion efficiency and growth in Atlantic salmon (*Salmo salar* L.). *Aquacult. Nutr.* (in press).

Taranger, G.L., Haux, C., Hansen, T., Stefansson, S.O., Björnsson, B.T., Walther, B.T. & Kryvi, H. (1999) Mechanisms underlying photoperiodic effects on age at sexual maturity in Atlantic salmon *Salmo salar*. *Aquaculture*, **177,** 47–60.

Torrissen, K.R. & Shearer, K.D. (1992) Protein digestion, growth and food conversion in Atlantic salmon and Arctic charr with different trypsin-like isozyme patterns. *J. Fish Biol.*, **41,** 409–415.

Torrissen, K.R., Lied, E. & Espe, M. (1994) Differences in digestion and absorption of dietary protein in Atlantic salmon (*Salmo salar*) with genetically different trypsin isozymes. *J. Fish Biol.*, **45,** 1087–1104.

Torrissen, K.R., Lied, E. & Espe, M. (1995) Differences in utilization of dietary proteins with varying degrees of partial pre–hydrolysis in Atlantic salmon (*Salmo salar* L.) with genetically different trypsin isozymes. In: *Biopolymers and Bioproducts: Structure, Function and Applications* (Svasti, J., Rimphanitchayakit, V., Soontaros, S., Tassanakajorn, A., Pongsawasdi, P., Sonthayanon, B., Packdibamrung, K., Limpaseni, T., Wilairat, P., Boonjawat, J. & Kamolsiripichaiporn, S. eds), pp. 432–442. Proceedings of the 11th FAOBMB Symposium, Samakkhisan (dokya) Public Company Ltd, Bangkok, Thailand.

Toyohara, H., Ito, K., Touhata, K., Kinoshita, M., Kubota, S., Sato, K., Ohtsuki, K. & Sakaguchi, M. (1997) Effect of maturation on the contents of free and bound forms of hydroxyproline in Ayu muscle. *Fish. Sci.*, **63,** 843–844.

Van Waarde, A. 1988. Biochemistry of non-protein nitrogenous compounds in fish including the use of amino acids for anaerobic energy production. *Comp. Biochem. Physiol.*, **91B,** 207–228.

Walton, M.J. & Wilson, R.P. (1986) Postprandial changes in plasma and liver free amino acids of rainbow trout fed complete diets containing casein. *Aquaculture*, **51,** 105–115.

Waters (1993) *Waters AccQTag Chemistry Package Instruction Manual.* Waters Corporation, Milford, MA.