\(^{15}\)N: an Indicator of the Assimilation of Dietary Proteins for *Oreochromis niloticus*

Hanno Slawski\(^1\)*, Klaus-Peter Götz\(^2\), Carsten Schulz\(^1,3\)

\(^1\)Gesellschaft für Marine Aquakultur mbH, Hafentörn 3, 25761 Büsum, Germany

\(^2\)Department of Crop Science Tropics and Subtropics, Humboldt-Universität zu Berlin, Albrecht-Thaer-Weg 5, 14195 Berlin, Germany

\(^3\)Department of Marine Aquaculture, Christian-Albrechts-Universität zu Kiel, Olshausenstr. 40, 24098 Kiel, Germany

(Received 21.10.09, Accepted 24.11.09)

Key words: dietary markers, stable isotope, \(^{15}\)N ammonia, nitrogen assimilation

**Abstract**

The potential of enriched stable isotopes (\(^{15}\)N) for protein digestion and assimilation studies in juvenile fish was evaluated. Fishmeal and maggot meal were enriched with \(^{15}\)N, incorporated into two diets (crude protein 30.2\%). Four groups of ten *Oreochromis niloticus* (47.5±8.3 g) were fed an experimental diet at the same starting time. After 15 min, 2 h, 4 h, and 6 h, five fish were selected, the stomach and gut were extracted, and the liver, kidney, gills, and *musculus lateralis magnus* were sampled. Concentrations of \(^{15}\)N in the stomach 2-6 h after feeding indicate that fishmeal passed through the stomach faster than maggot meal. Differences in digestive tract passage between protein sources were probably due to the hard digestible chitin present in the maggot meal. In the liver, kidney, gills, and *musculus lateralis magnus*, \(^{15}\)N in fishmeal and maggot meal nitrogen was assimilated at similar speeds. Results indicate that \(^{15}\)N can provide comprehensive information about protein digestion and assimilation in fish nutrition studies.

* Corresponding author. E-mail: hanno.slawski@vti.bund.de
Use of $^{15}$N as a dietary marker

Introduction
Fishmeal production has remained stable from the late 1980s at approximately 6-7.2 million tons per annum (FAO, 2007). Limitation is a cause of serious concern for the long-term availability of fishmeal as a protein source in fish diets and the growth of aquaculture production (Benedito-Palos et al., 2007). Substitutes for fishmeal must be evaluated for their nutritional value, as well as digestibility and assimilation of the main nutrients to clarify their potential as fish feed ingredients (Glencross et al., 2007).

Digestibility of individual dietary ingredients is commonly determined directly or indirectly by the amount of nutrients ingested and later excreted. Direct methods measure the amounts of feed consumed by fish and the resulting excreta in an aquatic metabolism chamber (Smith, 1971). Indirect methods are based on nondigestible markers, such as chromic oxide, yttrium oxide, crude fiber, or other (Weatherup and McCracken, 1998; Middleton et al., 2001; Vandenbergh and De La Noüe, 2001; Opstvedt et al., 2003; Toppe et al., 2006). It is assumed that the ratio of the marker in the feed and feces remains constant throughout gut passage and that it will be found in the collected feces. The difference between the feed and fecal concentrations of the marker and the nutrient makes calculation of nutrient digestibility possible (Cho et al., 1982).

Common direct and indirect methods provide acceptable approximation of real nutrient digestibility. But both methods gain no direct information about temporary nutrient absorption and assimilation in fish. Enriched stable isotopes as dietary markers could provide insight into the patterns of passage of dietary proteins in the digestive tract and their assimilation in tissues of fish. Enriched stable isotopes have been used in nutrition studies on fish larvae (see review by Conceição et al., 2007) and shrimps (Preston et al., 1996). We used $^{15}$N labeled fishmeal and maggot meal in a feeding experiment on juvenile Oreochromis niloticus, paying special interest on the potential of $^{15}$N to show temporary nitrogen assimilation in selected fish tissues. By this, we wish to provide an insight into the assimilation of nitrogen from dietary proteins by tilapia.

Materials and Methods
$^{15}$N enrichment of protein sources. Labeled dietary protein sources were not obtainable, therefore, fishmeal and maggot meal were enriched with $^{15}$N. In the case of fishmeal, 75 carps (Cyprinus carpio L.; 22.3 ± 1.5 g) were kept in a 140-l fish tank containing $^{15}$N labeled water (100 mg $^{15}$N excess/l water) from ammonium chloride ($^{15}$NH$_4$Cl, 95 atom%). The fish were given protein-free feed for 12 days and then were killed. Complete fish bodies were freeze-dried and ground to pass through a 1-mm screen (Bergner et al., 1993).

In the case of maggot meal, a substrate with a high $^{15}$N concentration was provided to mature egg-laying flies. For this, 4000 mg $^{15}$N excess (ammonium chloride, 95 atom%), 3.0 kg pig liver, and 2.0 kg semolina were homogenized in a cutter. A PVC box (0.8 x 0.6 x 0.3 m) was filled with the substrate and the box was exposed to the sun. The box was covered with a black lid to
support warmth. Slits in the sides of the box provided access for bluebottle fly species (Calliphoridae) to the egg-laying substrate. When enough developing eggs were observed, the entrance slits were closed. Larvae started feeding on the substrate immediately after hatching. Outgrown maggots were collected by hand 14-20 d after hatching and killed with hot water. They were washed thrice with cold water, cleaned from substrate remains, dried for 48 h at 60°C in an oven, crushed with a mortar and pestle, and stored in a refrigerator at 4°C before use in feed production. The protein sources were analyzed for nutrient composition and $^{15}$N concentrations were determined (Table 1).

**Preparation of experimental diets.** Two diets (30.2% protein) were enriched with $^{15}$N based on fishmeal and maggot meal (Table 2). Further, two unlabeled diets identical to the experimental diets were formulated for enzymatic habituation in fish digestive tract before feeding with the labeled diets. Dry diet components were mixed with fish oil and water (50°C) was added to obtain a liquid mixture. The mixture was poured onto flat plates in thin layers and put in an oven at 30°C for drying. After 48 h, the dry mixture was scratched off the plates with a knife, producing feed flakes. Experimental diets were stored in a refrigerator at 2°C until use.

Table 1. Nutritional composition of fishmeal and maggot meal.

<table>
<thead>
<tr>
<th></th>
<th>Fishmeal</th>
<th>Maggot meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter (DM, %)</td>
<td>96.8</td>
<td>95.6</td>
</tr>
<tr>
<td>Crude protein (% DM)</td>
<td>58.6</td>
<td>66.0</td>
</tr>
<tr>
<td>Crude fat (% DM)</td>
<td>18.4</td>
<td>20.8</td>
</tr>
<tr>
<td>Ash (% DM)</td>
<td>14.4</td>
<td>4.7</td>
</tr>
<tr>
<td>Atom% $^{15}$N excess</td>
<td>1.91</td>
<td>0.90</td>
</tr>
<tr>
<td>mg $^{15}$N excess/g DM</td>
<td>1.428</td>
<td>0.983</td>
</tr>
</tbody>
</table>

Table 2. Ingredients and nutritional composition of experimental diets.

<table>
<thead>
<tr>
<th>Ingredient (g/kg organic matter)</th>
<th>Fishmeal</th>
<th>Maggot meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fishmeal</td>
<td>511.5</td>
<td>0</td>
</tr>
<tr>
<td>Maggot meal</td>
<td>0</td>
<td>454.5</td>
</tr>
<tr>
<td>Pea seed meal</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fish oil</td>
<td>35.9</td>
<td>35.3</td>
</tr>
<tr>
<td>Vitamin/mineral mix*</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Potato starch</td>
<td>392.6</td>
<td>450.2</td>
</tr>
<tr>
<td>Wheat gluten</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nutritional composition</th>
<th>Fishmeal</th>
<th>Maggot meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter (DM, %)</td>
<td>93.6</td>
<td>93.6</td>
</tr>
<tr>
<td>Crude protein (%DM)</td>
<td>33.4</td>
<td>33.3</td>
</tr>
<tr>
<td>Crude fat (%DM)</td>
<td>13.8</td>
<td>13.3</td>
</tr>
<tr>
<td>Ash (%DM)</td>
<td>10.1</td>
<td>4.9</td>
</tr>
<tr>
<td>mg $^{15}$N excess/g DM</td>
<td>0.795</td>
<td>0.466</td>
</tr>
</tbody>
</table>

* Spezialfutter Neuruppin -VM BM 55/13 Nr. 7318 (per 1 kg dry feed): vitamin A 12000 IE, vitamin D3 1600 IE, vitamin E 160 mg, vitamin K3 6.4 mg, vitamin B1 12 mg, vitamin B2 16 mg, vitamin B6 12 mg, vitamin B12 26.4 μg, nicotinic acid 120 mg, biotin 800 μg, folic acid 4.8 mg, pantothenic acid 40 mg, inositol 240 mg, vitamin C 160 mg, antioxidants (BHT) 120 mg, iron 100 mg, zinc 24 mg, manganese 16 mg, cobalt 0.8 mg, iodine 1.6 mg, selenium 0.08 mg
Nutritional analysis of experimental diets. Experimental diets were analyzed for nutritional composition and ash content. Each analysis was carried out in duplicate. Dry matter was determined by freeze-drying for constant weight. Crude protein (N x 6.25) was determined according to the Kjeldahl method (Kjeltec System; Tecator, Hoganas, Sweden). Crude fat was analysed by petroleum ether extraction in a Soxhlet apparatus (Soxtec System HT; Tecator). Ash was determined by burning in a muffle furnace at 550°C for 10 h.

Experimental design. The feeding trial was conducted in the experimental facilities of the Institute of Animal Science, Humboldt-Universität zu Berlin, Germany. Ten tilapia (47.5±8.3 g) were stocked in each of eight 240-l experimental tanks, organized in two recirculation systems. Each system comprised four tanks and a filtration unit with a sedimentation chamber and a trickling filter for nitrification. Water quality was similar in both systems: mean temperature was 24.2±0.29°C in system A and 24.6±0.25°C in system B, mean pH was 8.00±0.08 in system A and 7.82±0.15 in system B, O₂ content was 5.6±0.23 mg/l in system A and 5.4±0.28 mg/l in system B, and conductivity was 816±5.7 μs/cm in system A and 828±5.2 μs/cm in system B.

To ensure enzymatic habituation prior to the actual experiment, fish were fed similar unlabeled diets seven days before the feeding trial. The daily feed amount was 3% of fish body weight given in two portions. On the morning of day 8, fish were fed ¹⁵N labeled diets at 1.5% of their body weight. They received the diets eagerly and came to rest a few minutes after feeding, swimming calmly in the tanks. One fish group of ten individuals was taken out of tanks fifteen minutes, 2, 4, and 6 hours after initial feeding. Fish were sacrificed and laid in icy water. The abdominal cavity was opened and the gut and stomach, with its contents, were extracted. Both organs were manually cleaned from fat remains, placed in plastic bags, and immediately cooled in liquid nitrogen. The same was done with samples of liver, kidney, gills, and musculus lateralis magnus. Tissue samples were stored in a refrigerator at -18°C until analysis.

Determination of ¹⁵N in tissues. The abdominal cavity was opened and, as far as detectable, sex was determined. Only male fish with low developed gonads were further investigated. About five of the ten fish were male. Samples of gut and stomach (n = 5) were analyzed for ¹⁵N enrichment by emission spectrometry (Isonitromat 5200, Fa. Statron, Fürstenwalde, Germany). The enrichment of liver, kidney, gills, and musculus lateralis magnus (n = 5) was measured by mass spectrometry using Finnigan Delta C MS (ThermoFinnigan, Bremen, Germany) coupled with Conflo II Interface on Carlo Erba 1108 (Carlo Erba, Milano, Italy). Analyses were carried out in triplicate.

Statistics. The data were analyzed using statistical software from SPSS, version 14.0 (ANOVA) coupled with Tukey’s test (p<0.05).
Results

$^{15}$N concentrations in fishmeal, maggot meal, and diets. The protein sources were successfully enriched with $^{15}$N at but at different concentrations depending on protein source, protein content, labeling procedure, and label uptake. As a result, the diets contained different $^{15}$N concentrations.

$^{15}$N concentrations in the digestive tract. Due to different organ weights between individuals and the small size of the fish, it was unfeasible to compare accurate quantitative stomach and gut concentrations. Thus, investigation of stomach and gut $^{15}$N contents were based on relative $^{15}$N concentrations. In fish fed the fishmeal diet, concentrations in the fish stomach plus its content significantly differed 2-6 h after ingestion from the initial concentration at 15 min after ingestion (Fig. 1). Concentrations in the fish gut plus its content 15 min after feeding stayed stable. There were no significant differences between samples in fish fed the maggot meal diet.

$^{15}$N excess in liver, kidney, gills, and musculus lateralis magnus. There were differences between protein sources in protein nitrogen assimilation as seen by temporary $^{15}$N enrichment (atom% $^{15}$N excess) in sampled tissues. In the fishmeal diet, the $^{15}$N excess in liver, kidney, and gills of fish significantly differed 4 h after feed intake from the initial status; in the musculus lateralis magnus, the $^{15}$N enrichment significantly differed 2-6 h after feed intake from the initial $^{15}$N enrichment at 15 min after feed ingestion (Fig. 2). In fish fed the maggot meal diet, the $^{15}$N enrichment significantly differed in the liver, kidney, gills, and musculus lateralis magnus of fish 2 h after diet intake. Except for the musculus lateralis magnus, the $^{15}$N enrichment further increased significantly 4 h after diet intake. The $^{15}$N excess continued to increase in the gills and musculus lateralis magnus 6 h after ingestion.

Fig. 1. Concentration of $^{15}$N (mg $^{15}$N/g) in the stomach and gut and their content of fish fed the fishmeal and maggot meal diets 15 min, 2, 4, and 6 h after feeding (means±standard error, n = 5). Different superscripts indicate significant differences of $^{15}$N excess between sampling times of stomach or gut (Tukey’s test, p<0.05).
Use of $^{15}$N as a dietary marker

**Discussion**

Enriched stable nitrogen isotopes have been used as tracers in nutrition studies on shrimps (Preston et al., 1996) and fish larvae (see review by Conceição et al., 2007). The $^{15}$N tracer is a diet element that can give insight on digestive tract passage patterns of dietary nutrients and their assimilation in fish tissues. This is an advantage over non-nutritive markers such as chromic oxide and others that can influence the digestion process itself or show different passage patterns along the gastro intestinal tract with respect to the digesta (Hanley, 1987; Vandenberg and De La Noüe, 2001; Glencross et al., 2005) without providing data on nutrient assimilation.

Our experiment provides an insight into temporary passage patterns of protein from fishmeal and maggot meal along the gastro intestinal tract of juvenile Nile tilapia fed a compound diet. We observed faster stomach passage of $^{15}$N from fishmeal as compared to maggot meal. The differences between fishmeal and maggot meal in stomach evacuation time could be due
to anti nutritional factors present in maggot meal. Aminopolysaccharide chitin (1,4β-N-acetyl glucosamine) is the most prominent anti nutritional factor in maggot meal. Anti nutritional factors can delay transit time of ingested food from the stomach to the intestine of aquatic organisms (Storebakken, 1985; Shiau et al., 1988; González-Peña et al., 2002). Chitin decreases the stomach evacuation rate in rainbow trout as chitin particles can be larger than the pylorus in the fish stomach, even after whole organic substances are digested (Kionka and Windell, 1972). Small chitin particles passed the fish stomach at a similar speed as the organic substances. It can be assumed that the chitin present in maggot meal caused the longer stomach evacuation time of maggot meal in contrast to fishmeal.

Differences in $^{15}$N enrichment in sampled tissues make temporary protein nitrogen assimilation in fish body visible. In general, most absorbed amino acids proceed to the portal venous blood and are transported to the liver. After deamination of amino acids in the liver the resulting ammonia is transported to the gills and kidney for excretion while amino acids enter fish muscles (Smutná et al., 2002). The $^{15}$N assimilation was similar in tissues of fish fed fishmeal and maggot meal diets, similar to hydrolyses, digestion, and absorption of fishmeal and maggot meal proteins. This was seen in the similar assimilation patterns of $^{15}$N from fishmeal and maggot meal throughout the period. However, when comparing the protein sources (fishmeal and maggot meal) for $^{15}$N enrichment in tissues, the different $^{15}$N enrichment of the diets must be considered (1.51 atom% $^{15}$N excess for fishmeal and 0.86 for maggot meal), i.e., the maggot meal diet contained relatively less $^{15}$N than the fishmeal diet. Therefore, the efficiency of nitrogen absorption and assimilation in fish tissues seems relatively higher for maggot meal than for fishmeal.

The total $^{15}$N concentrations in the fish gastro intestinal tract indicate that digestion and absorption of fishmeal and maggot meal was not complete until 6 h after diet ingestion. Studies indicate that juvenile Nile tilapia can effectively use maggot meal as a source of protein (Ajani et al., 2004; Ogunji et al., 2007). However, when low quality fishmeal was substituted with maggot meal, decreased digestibility of dietary protein for tilapia was observed (Fashina-Bombata and Balogun, 1997). This finding may be related to maggot meal chitin. Beside our assumption that chitin slowed stomach passage of $^{15}$N from maggot meal, chitin could also depress protein digestibility in fish gut. Chitin is not digested by three tilapia species (Buddington, 1980). It seems possible that, after whole gut passage, relative nitrogen absorption from maggot meal might be lower than observed. It seems likely that an observation period including nutrient passage along the whole gastro intestinal tract will provide different results for nitrogen absorption and assimilation from fishmeal and maggot meal.

In conclusion, the use of $^{15}$N as a dietary marker of a compound diet provided insight into the temporary passage, absorption, and assimilation patterns of different dietary proteins in the gastro intestinal tract and organ tissues of juvenile Nile tilapia. Enrichment of proteins with $^{15}$N can help maximize protein and amino acid assimilation from aquaculture diets. Our limited observation period did not permit complete assessment of protein
Use of $^{15}$N as a dietary marker

digestion plus nitrogen absorption and assimilation from fishmeal and maggot meal. This will be the aim of further investigations.

Acknowledgements
We thank Mrs. Susanne Moryson (HU zu Berlin, Germany) for her skilled analytical assistance, and Dr. K. Dittert (CAU zu Kiel, Germany) for providing data of mass spectrometric analysis.

References
González-Peña M.C., Gomes S.Z. and G.S. Moreira, 2002. Effects of dietary fiber on growth and gastric emptying time of the freshwater prawn


