Available online at www.sciencedirect.com
ScienceDirect
Aquaculture 275 (2008) 217–224
Aquaculture
www.elsevier.com/locate/aqua-online

Pre-hydrolysis improves absorption of neutral lipids in Atlantic halibut (*Hippoglossus hippoglossus*, L.) larvae

T.A. Mollan [a,*,1], S.K. Tonheim [b], K. Hamre [a]

[a] *National Institute of Nutrition and Seafood Research (NIFES) PO Box 2029, Nordnes, N-5817 Bergen, Norway*
[b] *Department of Biology, University of Bergen, P.O. Box 7800, NO-5020 Bergen, Norway*

Received 29 June 2007; received in revised form 7 December 2007; accepted 10 December 2007

Abstract

To investigate if digestion is limiting for absorption of dietary neutral lipids in Atlantic halibut (*Hippoglossus hippoglossus*, L.) larvae, absorption of triacylglycerol (TAG), diacylglycerol, (DAG), and monoacylglycerol, (MAG) as well as phosphatidylcholine (PC) was studied by tube-feeding larvae with radio-labelled lipids and tracing the radioactivity. PC was included to evaluate any difference in absorption of neutral versus polar lipids. The labelled lipids where deposited by tube-feeding before larvae were incubated individually for 18 h with collection of expired CO_2. At sampling, the gut was separated from the body carcass and radioactivity traced in four different compartments, namely body carcass, gut, incubation water and CO_2 expired from larvae. The relative (%) isotope distribution of each compartment was calculated and the dose size effect analysed by regression analysis.

The results show that there is an increasing amount of evacuated unabsorbed neutral lipids as the complexity of the lipid increases (TAG>DAG>MAG). The larval faecal evacuation ranged from 66±20% of TAG to 9±6% of MAG. DAG was intermediate with 52±21% evacuated. Of the labelled PC, 37±16% was absorbed, but this can not be directly compared to the neutral lipids due to the different digestive enzymes that specifically hydrolyse neutral and polar lipids. Increasing the administered amount of lipids only slightly increased the total amount of labelled TAG and DAG that were actually absorbed, while there was a linear correlation between fed and absorbed MAG. The absorption of PC was also linearly related to the administered amount. The difference in net absorption of labelled TAG, DAG and MAG diets indicates that digestion is a limiting factor for absorption of neutral lipids in Atlantic halibut larvae.

Keywords: Atlantic Halibut; Digestion; Absorption; Lipids; Tube-feeding

1. Introduction

The Atlantic halibut is a potential species for commercial production in aquaculture, however, annual production of juveniles is low (850,000 in 2004, Harboe and Adoff, 2005) and juvenile production is regarded as a major bottleneck. There have been several problems in the development of reliable and efficient production of juveniles in intensive production systems, such as high mortality, and high incidences of deformities and malpigmentation, factors that are often closely related to each other. The average survival reported in 2004 was 5% from hatched larvae to juveniles weaned on a formulated diet (Harboe and Adoff, 2005).

Lipids have multiple roles in nutrition, being a main source of metabolic energy, and of essential fatty acids which are precursors of important signalling molecules (eicosanoids), and components of biological membranes (Sargent et al., 1993). There is evidence of lipid digestion in fish larvae as early as two hours after first ingestion. This is confirmed by the appearance of lipid droplets in the epithelial cells of the gut mucosa (Luizi et al., 1999), and the presence of lipid-digesting enzymes, lipases (Izquierdo et al., 2000). Lipases cut the ester-bonds in lipids by hydrolysis, liberating free fatty acids. Bile salts secreted from the liver, aid lipid digestion by emulsifying the lipids and thereby increasing the interface between the lipid and the lipases. Several types of lipases have been detected in the

* Corresponding author. Tel.: +47 91610235; fax: +47 55697036.
E-mail address: ted.andreas.mollan@ewos.com (T.A. Mollan).
[1] Current address: EWOS AS, C. Sundts gate 17/19 N-5802 Bergen, Norway.

0044-8486/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquaculture.2007.12.009

digestive tract of larvae and juvenile fish. Among them is the bile-salt activated lipase (BAL) (Izquierdo et al., 2000; Murray et al., 2006). Its activity is thought to play a major role in the digestion of neutral lipids in many fish species (Iijima et al., 1998; Olsen et al., 1998; Murray et al., 2003; Perez-Casanova et al., 2004). Gene expression of BAL has been detected by RT-PCR in Atlantic halibut larvae at least from the time of first feeding (Murray et al., 2006). The specificity of BAL towards the three fatty acid positions of TAG may be dependent of the fatty acid chain length and their degree of saturation (Olsen and Ringø, 1997; Tocher, 2003; Gottsche et al., 2005; Oxley et al., 2007). Phospholipid (PL) are mainly digested by pancreatic phospholipase A2 (PLA2), which catalyses the hydrolysis of the fatty acid ester bond at the sn-2 position of PL, and produces a free fatty acid and a lysophospholipid (lysoPL) (Izquierdo et al., 2000). Its activity is dependent on the presence of bile salts (Nordskog et al., 2001). PLA_2 specific activity has been detected in pooled ($n=3$) 6 day old turbot larvae, and from day 9 in individual larvae, (Hoehne-Reitan et al., 2003). Gene expression of PLA_2 has been detected and quantified in European sea bass (Zambonino Infante and Cahu, 1999).

After the digestion, products like MAG, FFA, glycerol and lysoPL are absorbed across the brush border membrane and into the intestinal epithelial cells (enterocytes). Inside the enterocytes, re-esterification of the digestion products may take place following one of two pathways: 1) the MAG pathway in the smooth endoplasmatic reticulum which produces TAG from MAG and FFA, and, 2) the glycerol-3-phosphate pathway in the rough and smooth endoplasmatic reticulum which produces both TAG and PL from glycerol liberated by complete hydrolysis of TAG (Johnson, 1977). When the lipids are re-esterified they appear as lipid droplets in the epithelial cells. These vacuoles are probably a temporary lipid store. The lipid molecules will form chylomicrons or very low density lipoprotein (VLDL) together with apo-lipoproteins, PL and cholesterol. This will generate a hydrophilic surface of the lipid complex so that it can be transported by the circulatory system to the liver or other targets in the body (Iijima et al., 1990). Accumulation of lipid droplets in the gut mucosa, probably TAG, has been observed in carp fed diets deficient of PL, and this may be due to a reduced ability to produce lipoproteins (Fontagne et al., 1998). It is proposed that fish larvae have a limited ability to biosynthesise PL *de novo* and therefore have a partial dietary requirement of PL (Teshima et al., 1987). European sea bass larvae fed compound diets containing high levels of dietary PL from first feeding showed better maturation of the digestive tract, better survival, better growth and less malformed larvae than larvae fed low levels of phospholipids (Cahu et al., 2003). The authors also concluded that sea bass utilise dietary PL more efficiently than neutral lipids.

In larval protein nutrition, pre-digested protein has been used to study the underlying mechanisms of the limitations of protein digestion. Pre-digested protein has been found to be more efficiently absorbed and utilised than intact protein when fed directly to Atlantic halibut larvae by tube feeding (Tonheim et al., 2005). By using a similar approach, and pre-digested lipids, our aim was to elucidate if digestion is limiting for absorption of neutral lipids into the body of Atlantic halibut (*Hippoglossus hippoglossus*, L.) larvae, and to investigate possible differences in the assimilation of neutral and polar lipids. Oleic acid (OA) was tube-fed to the larvae as undigested, partly digested and digested radio-labelled lipid (TAG, DAG, MAG or as intact PL) to study if there was any effect of the chemical form of lipid fed to the larvae. The labelled lipids where also fed in different amounts to study the effect of meal size on digestion, absorption and distribution of radio labelled lipids.

2. Materials and methods

2.1. Preparation of diets

To compare absorption efficiencies and metabolic distributions of TAG, DAG, MAG and the phospholipid phosphatidylcholine (PC), four different test solutions containing radio-labelled representatives of these lipid types were produced. The four test solutions were basically identical and the labelled lipids added constituted only an insignificant part of the total dietary lipid. All lipids in the test solutions, both labelled and unlabelled, contained only OA. Labelled PC [dioleoyl-1-^{14}C] and TAG (triolein [carboxyl-^{14}C]) were obtained from ARC (St. Louis, MO, USA). Labelled DAG and MAG were produced by enzymatic hydrolysis of the labelled TAG.

Triolein [carboxyl-^{14}C] was treated with lipase from porcine pancreas type II (Sigma-Aldrich CO, St. Louis, MO, USA) to hydrolyse ester bonds and thereby produce both mono-and dioleoylglycerol. The digestion was performed by mixing 50 mg ^{14}C-labelled triolein, 50 mg lipase and 2 mL buffer (1 M Tris-pH 8, 10% (w/v) gum arabic and 2.5% (w/v) $CaCl_2$).

The mixture was vortexed and incubated at 37 °C for 5 min before the lipids where extracted by 4×4 mL diethyl ether. The extract was passed through a filter containing anhydrous Na_2SO_4 to remove residual water, and was then dried under nitrogen and resolved in 200 μL chloroform. The triolein hydrolysis products were separated by thin layer chromatography (TLC) The hydrolysate was applied to a 20×20 cm TLC silica gel plate (Merck, 1.0 mm), 1.0 cm from both sides and 1.5 cm from the lower edge. The TLC plate was then developed in chloroform: acetone (88:12 v/v) until the front reached the top end. The plate was dried and sprayed with 0.1% 2.7-dichlorofluorescein. Bands were visualised by UV-light and the gel bands containing TAG, DAG and MAG was scraped off the plate with a scalpel and placed into separate tubes. The lipids were then extracted from the silica gel by 4×3 mL chloroform: acetone (90:10 v/v). To rinse lipids from fluorescein, 3.5 mL of water and 200 μL 1 M NaOH was added to the pooled extract. After mixing and centrifugation the upper water layer was removed and the lipids dried down and resolved in 100 μL chloroform.

The basic diet emulsion vector consisted of 60% filtrated seawater, 35% oil (triolein) and 5% Tween 20 (Polyoxyethylene-Sorbitan Monolaurate) Both from Sigma-Aldrich CO St. Louis, MO, USA. One day prior to the experiment the labelled lipids where dried under nitrogen and dissolved by sonication into the emulsion. Due to some instability, the test solutions were re-dispersed into an emulsion each day of the experiment. The mean specific activities for the different test solutions were 8, 10, 190 and 52 DPM nL^{-1} for TAG, DAG, MAG and PC, respectively.

2.2. Larvae

Atlantic halibut (*H. hippoglossus*) larvae, 38 to 42 days post first feeding (dpff), where obtained from a commercial hatchery, Nordic Seafarms (Askøy, Norway). The larval weight (wet weight) ranged from 270 to 1050 mg, with an average weight of 654 mg.

Larvae originated from eggs from seven different females. Eggs where stripped and fertilised before incubation at 6 °C. Eggs hatched 14 days post fertilisation and they were then transferred into an incubation silo. At day 42 the larvae where transferred into a feeding tank and offered *Artemia* enriched with Multigain® (Danafeed, Horsens, Denmark). Larvae were kept at 11.5 °C under continuously artificial illumination. The seawater treatment consisted of protein skimmer, mechanical filtration (20 μm), and UV-filtration. The water was heated and de-gassed before entering the feeding tank.