

PAPER II

Ann-Elise O. Jordal, Ivar Hordvik, Maurice Pelsers, David Bernlohr, Bente E. Torstensen FABP3 and FABP10 in Atlantic salmon (*Salmo salar* L.), – general effects of dietary fatty acid composition, and life cycle variations.

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FABP3 and FABP10 in Atlantic salmon (Salmo salar L.), – general effects of dietary fatty acid composition, and life cycle variations.

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Fatty acid binding proteins in Atlantic salmon

Abstract

The increased use of dietary plant oil supplementation combined with high dietary lipid loads challenges the lipid transport systems of cultivated fish species. Fatty acid binding proteins (FABPs) have been thoroughly studied as intracellular fatty acid transporters in vertebrates, but no data have been reported in Atlantic salmon. In the present study, comparative characterisations were performed, and dietary influence of plant oil supplementation on FABP3 and FABP10 expression was studied for several tissues in two separate dietary trials. In trial I, groups (6 fish each) were fed diets for 42 weeks (body mass 142 ± 1 - 1463 ± 83 g) (mean \pm SD), containing graded levels of rapeseed oil substituting for fish oil using a linear regression design. In trial II, groups (3 fish each) were fed 100% fish oil or 100% plant oil for 22 months (0.160 ± 0.052 to 2523 ± 590 g) (mean \pm SD) and sampled at regular intervals. Liver and muscle tissues appeared to express several FABPs possibly linked to different metabolic functions. FABPs mRNA expression did not change with dietary inclusion of 75% rapeseed oil, whereas FABP3 protein expression seemed to be affected by dietary rapeseed oil inclusion. Significant changes in red muscle FABP3 mRNA expression correlate to significant changes in total β -oxidation capacity during the energy consuming process of smoltification.

Keywords: Atlantic salmon, FABP, fatty acids, fatty acid oxidation, FABP1, FABP3, FABP4, FABP10, Quantitative-PCR

1. Introduction

The substitution of plant oils in diets is used increasingly in aquaculture, due to limited supplies of marine fish oils (FO) (Barlow, 2000). Plant oils have a different fatty acid composition than marine oils, and these differences are believed to challenge lipid metabolism and transport.

Functionally, fatty acid-binding proteins (FABPs) are involved in lipid transport and metabolism (Ockner et al., 1972; Spener et al., 1989; Veerkamp et al., 1991; Veerkamp and Maatman, 1995; Massolini and Calleri, 2003), and exhibit differences in ligand specificity (Glatz and van der Vusse, 1990; Veerkamp et al., 1991) as well as affinity for specific fatty acids (Veerkamp et al., 1990). FABPs belong to the conserved multigene family of intracellular lipid binding proteins (iLBPs) that are individual genes arising from an ancestral iLBP gene through gene duplication and diversification (Schaap et al., 2002). The nomenclature for iLBPs suggested by Hertzler and Bernlohr (2000) will be used in this report (**Table 1**). Phylogenetic analysis suggests that FABP3 (Ando et al., 1998) and FABP10 (DiPietro et al., 1997) diverged from a FABP progenitor before the divergence of fish and mammals. FABP3 has been characterized in heart ventricle of four Antarctic teleosts (Vayda et al., 1998), in rainbow trout (*Oncorhynchus mykiss*) (Ando et al., 1998) (**Table 2**) and in barnacle geese (*Branta leucopsis*) (Pelters et al., 1999). FABP10 has been characterized in catfish (*Rhamdia sapo*) (Di Pietro et al., 1996; DiPietro et al., 1997), zebrafish (*Danio rerio*) (Denovan-Wright et al., 2000), axolotl (*Ambystoma mexicanum*) (Di Pietro et al., 1999), argentine toad (*Bufo arenarum*) (Di Pietro et al., 2001; Di Pietro et al., 2003), lungfish (*Lepidosiren paradoxus*) (Di Pietro and Santome, 2001) and chicken (*Gallus gallus*) (Nichesola et al., 2004; Nolan et al., 2005) (**Table 3**).

Atlantic salmon both oxidize (Froyland et al., 2000; Torstensen et al., 2000; Stubhaug et al., 2005a) and store lipids in red- and white muscles (Zhou et al., 1995). Functionally, FABP3 is thought to function as a transport protein for mitochondrial β -oxidation in muscle tissues (Haunerland and Spener, 2004). However, FABP have also been suggested as a transport protein for lipid storage, for genetic variants of FABP3 in pigs (Gerbens et al., 1999; Zeng et al., 2005), and through FABP in vitro over expression and antisense studies (Makowski and Hotamisligil, 2004 and references therein).

Liver docosahexaenoic acid (DHA) content, but not FABP10 expression, changed with dietary DHA enrichment in Tsaiya duck (Anas platyrhynchos) (Ko et al., 2004). Furthermore, Nolan and co-workers recently (2005) renamed FABP10 as bile acid binding protein (BABP) on its sequence identity to FABP6 (formerly known as BABP) (Nichesola et al., 2004). FABP6 belonging to the same iLBP subfamily as FABP10 (Haunerland and Spener, 2004), has not been characterised in fish.

Our focus was to characterise FABP3 and FABP10, and to evaluate whether dietary inclusion of rapeseed oil (RO) changed mRNA expression in the metabolically active organs liver, red and white muscle. Furthermore, FABP3 protein expression in heart, red- and white- muscle was examined. Since Atlantic salmon muscle is used for lipid storage and β -oxidation, FABP3 mRNA expression and its relation to changes in β -oxidation and total lipids between life cycle stages during production life cycle was examined. The possible involvement of liver FABPs as transport proteins to β -oxidation was also examined.

2. Materials and Methods

2.1 Dietary trials

This study was based on two dietary experiments termed trial I and II. Trial I was performed at Gildeskaal Research Station, Inndyr, Norway, 67° North from May 2001 to March 2002 (total 42 weeks). Approximately 600 post-smolt Atlantic salmon, mean body mass 142 g, were distributed to 7 net pens of 125 m³. Five experimental diets where fish oil was replaced by respectively 25%, 50%, 75% and 100% RO and 50% olive oil (OO) were fed to each group of Atlantic salmon (**Table 4**). The control diet containing 100 % fish oil was fed using two parallels. All diets, produced by Nutreco ARC, were formulated to contain 300 g kg⁻¹ lipid, 450 g kg⁻¹ protein, 60 g kg⁻¹ moisture, 70 g kg⁻¹ ash and 120 g kg⁻¹ Nitrogen free extract. Mean monthly temperature through the experiment varied from 4 °C during winter to 15 °C during summer. Due to low expected differences in mRNA expression between groups at low RO inclusion and decreased growth in the 100% RO group after 22 weeks 100% FO and 75% RO were selected as candidates for microarray (Jordal et al., 2005) and RT-Q-PCR analysis.

Trial II was a complete production life cycle study, performed at Lerang Research Station, Nutreco ARC, Stavanger, Norway from April 2002 to January 2004. Samplings were performed regularly through 2002 to January 2004 related to changes in life stage, pellet size and dietary lipid content. Dietary total lipid increased and total protein decreased with increasing pellet size through Atlantic salmon lifecycle as previously reported by (Torstensen et al., 2005). Two diets were produced by Nutreco ARC, Stavanger, and fed to triplicate tanks of Atlantic salmon.

Capelin oil was the fish oil used in the 100% FO diet. The 100% plant oil diet was made up of a mixture of 55 % RO, 30 % palm oil and 15 % linseed oil to obtain a lipid profile of saturated, monounsaturated and PUFA as similar as possible to capelin oil. Mean monthly temperature in fresh water (FW) varied between 7.8 and 12.6 °C and in seawater (SW) between 4 °C during winter to 17 °C during summer. Samplings were performed regularly after 3 (July, 2003), 6 (October 2002), 9 (January 2003), 14 (June 2003), 16 (August 2003), and 22 months (January 2004).

For both dietary trials, diets were fed to satiation by hand and the exact amounts consumed were recorded. Mortalities were recorded and dead fish removed daily. Biomass and average individual body masses were determined by bulk weighing and counting of all fish at each sampling or individual weighing of all the sampled fish (Trial II, SW phase).

2.2 Sampling procedure

The fish were deprived of food for 24 hours before sampling. Fish were randomly selected and anaesthetised with methiomidate ($7\text{g}\cdot\text{L}^{-1}$; Norsk medisinal depot, Oslo, Norway) before being killed with a blow to the head. For trial I, five and six fish from each diet were sampled for sandwich ELISA and PCR analysis, respectively. For trial II three fish were sampled from each net pen. Approximately 1 g of liver, heart, red- and white- muscle were dissected out, immediately transferred to liquid nitrogen, and stored at $-80\text{ }^{\circ}\text{C}$. White muscle samples were taken from within the Norwegian Quality Cut (NQC).

2.3 Proximate composition of the diets

Proximate analyses were done on feed samples of each of the diets in both dietary trials (see (Torstensen et al., 2005) for data from trial II). Dry matter was determined gravimetrically after freeze-drying the samples, and total lipid was determined gravimetrically following extraction with ethyl acetate. Crude protein ($N \times 6.25$) was analysed with a nitrogen analyser (PE 2410 series II, USA). Total lipid in diets was measured gravimetrically after acid hydrolysis with boiling HCl followed by ethyl acetate extraction.

2.4 Dietary fatty acid composition

Fatty acid composition was analysed in the diets from trial I (Table 4) and II. Details from trial II were reported previously (Torstensen et al., 2005). Lipids from the samples were extracted by adding chloroform/methanol (2:1, v/v). Samples were filtered before being saponified and methylated using 12% BF_3 in methanol. Fatty acid composition of total lipids was analysed using methods previously described (Lie and Lambertsen, 1991) where the methyl esters were separated using a Trace gas chromatograph 2000 (Fison) ("cold on column" injection, 60°C for 1 min 25°C/min 160°C for 28 min 25°C/min 190 °C for 17 min 25°C/min 220°C for 10 min), equipped with a 50m CP-sil 88 (Chromopack) fused silica capillary column (id: 0.32 mm). Fatty acids were identified by retention time using standard mixtures of methylesters (Nu-Chek, Elyian, USA), and the fatty acid composition (weight %) was calculated using an integrator (Chromkvest ver. 2.52, Thermoquest CE Instruments, Milan, Italy), connected to the GLC.

2.5 Cloning of FABP3 and assembly of FABP10 sequences

Total RNA was purified from flash frozen white muscle (Chomczynski and Sacchi, 1987). First strand cDNA synthesis was performed using 2 µg total RNA and oligo (dT)₁₈ incubated at 70 °C for 10 min before cooling and addition of DTT and dNTP mix. After pre-incubation at 42 °C for 2 min Superscript™ II was added for cDNA synthesis and a fragment encoding FABP3 was amplified by PCR on basis of primers derived from rainbow trout (GenBank accession no. **U95296**) (Ando et al., 1998): 5` primer:

AGTCATGGATCCCCTCCACACTGCTAACAACATG

3` primer: AGTCATAAGCTTTCCTCGGCCTTGACGTAGGA. An initial cycle of 94 °C for 5 min was followed by 30 cycles of 48 °C for 30 s, and 72 °C for 45 s. A PCR product of approximately 0.5 kb was purified using a Qiagen gel extraction kit (QIAGEN, prod. No. 28704), digested with BamHI and HindIII, and ligated into pRSETB vector. Sequencing was performed at the Obesity Centre, University of Minnesota. Atlantic salmon FABP10 was represented by the EST sequences **BG935663**, **BG935111**, **BG935057** and **BG934924**. These sequences had a protein identity index to zebrafish FABP10 (GenBank accession no. **AF254642**) on 83 %.

2.6 Software

Percent protein residue identities to other species (Tables 1-3) were determined through translation and BLAST submission at ExPASy/SIB of FABP3 (Gen Bank Acc. no **AY509548**) and FABP10 (Gen Bank accession no **BG935057**) virtual_Swiss Prot entry. Translation and

alignment of amino acid sequences was done using Baylor College of Medicine Search launcher (<http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html>) and verified using Clustal W (<http://www.ebi.ac.uk/clustalw/>).

2.7 Relative quantification of FABP3 in Atlantic salmon tissues

Muscle homogenates (15-20% wt/vol) were prepared by first adding nine volumes wet weight of ice-cold homogenization buffer (250 mM sucrose, 2 mM EDTA, and 10 mM Tris-HCl pH 7.4), then by homogenizing with an Ultra-Turrax homogenizer (3 × 15 s at 24,000 rpm, with 15-s intervals of cooling on ice), and finally by sonicating (4 × 15 s, MSE ultrasonic disintegrator). Aliquots of 100 and 500 µL homogenate were stored at 80°C. Before FABP measurement, samples were thawed and centrifuged for 10 min at 15,000 X g and 4°C. The supernatant was then used for FABP measurements. FABP3 was determined in white- and red muscle and heart using an enzyme linked immunosorbent assay of the antigen capture type (sandwich ELISA) as described previously (Wodzig et al., 1997). Specific monoclonal antibodies (Roos et al., 1995) directed to human heart-type FABP, but cross-reactive with Atlantic salmon FABP3 (western blot, data not shown) was coated on 96-well microtitre plates in 0.1 mol/L carbonate buffer, pH 9.4, at 4°C overnight. After coating and five washes with phosphate buffer (pH 7.2; 0.1% BSA and 0.05% Tween 20; PBT), 50 µL of a secondary antibody directly conjugated with horseradish peroxidase and 50 µL of sample or standard (recombinant human FABP3, 0-12 µg/L) were incubated at room temperature for 60 min in a humid environment with gentle shaking. After five washes with PBT, 100 µL tetramethyl benzidine substrate mixture was added to each well. The reaction was stopped after seven min with 50 µL 2 mol/L H₂SO₄, and the absorbance at 450

nm was measured with a microplate reader. The detection limit of the assay was 0.3 µg/L (mean + 2SD of the zero standard; n = 35). Data were relatively expressed as human mg FABP3/g protein measured, because no purified Atlantic salmon FABP3 was available.

2.8 Q-PCR assays

The primer and probe sequences (5'-3') were for FABP3 (GenBank accession no. AY509548) CACCGCTGACGACAGGAAA (F), TGCACGTGAACCATCTTACCA(R) and **FAM-TCAAGTCCCTAATAACG-MBGNFQ** (probe) and for FABP10 (GenBank accession no **BG935057**) CCGACATCACCACCATGGA (F), GCTTCCCTCCC TCCAGTTTG (R), and **FAM-CAGTGCACTTGAGCTT- MGBNFQ** (reverse probe) (Fig.1). The primer and probe sequences for the reference genes 18S rRNA (Hevrøy et al., 2006; Jordal et al., 2005) and elongation factor 1- α (EF1A_A) (Moore et al., 2005) have been published earlier. For more details on the use of normalisation controls (Olsvik et al., 2005).

2.9 RT-PCR

Before cDNA synthesis, total RNA was purified using TRIZOL (Invitrogen, California, USA), before subjected to DNase treatment (DNA-free™, Ambion, Austin, USA). RNA quality and quantity were determined by denaturing gel electrophoresis and spectrophotometry (A_{260/280}).

Six purified white muscle, red muscle and liver samples per diet (100% FO and 75% RO) were analysed from trial I. From trial II, liver, red and white muscle samples from three fish per replicate (Two replicates of 100% FO and one replicate of 100% plant oil) were analysed.

cDNA (125 ng) was synthesized following a modified protocol from the Taq Man Reverse Transcription Reagents kit (Applied Biosystems, New Jersey, USA). Modifications included increasing the reaction volume to 30 μ L, using 1X TaqMan RT buffer, 5.5 mmol/L MgCl₂, 500 μ mol/L dNTP each, 0.4 U/ μ L RNase Inhibitor, 1.67 U/ μ L Multiscribe™ Reverse Transcriptase and 2.5 μ mol/L oligo d(T)₁₆/random hexamers (18S rRNA). The reactions (triplicates) were incubated at 25 °C for 10 min and 48 °C for 60 min, and the reverse transcriptase was inactivated at 95 °C for 5 min followed by a decrease to 4 °C. RT-PCR efficiency was monitored using a four-step two-fold dilution curve of RNA (from 250 ng total RNA) for FABP3, FABP10, and EF1A_A and from 0.25 ng total RNA for 18S rRNA. White muscle FABP3 curves started at 500 ng, and 500 ng (\pm 5%) were used for sample analysis, for trial-I only.

2.10 RT- Quantitative PCR (RT-Q-PCR)

Quantitative PCR was performed using FAM fluorescent chemistry on an ABI prism 7000 (Applied Biosystems). The reaction mixture (25 μ L) contained primers (900 nmol/L each), FAM probe (200 nmol/L), 1X TaqMan universal PCR master mix (Applied Biosystems art. nr. 430 4437) and 5 μ L cDNA. All samples were run in triplicate with non-template controls (NTC) on the same plate. Reactions were incubated at 50 °C for 2 min and followed by 95 °C for 10 min and 50 cycles of 95 °C for 10 s and 60 °C for 15 s.

2.11 Normalisation analysis

18S rRNA had the lowest expression stability value through lifecycle using geNorm software (Vandesompele et al., 2002) for red muscle. However the expression stability value for EF1A_A was comparable, as seen earlier (Olsvik et al., 2005). For liver and white muscle, EF1A_A was evaluated as the best reference gene. Hence, EF1A_A was used for all tissues to calculate relative expression levels for FABP3 and FABP 10 using the Q gene method (Simon, 2003). However, as there seem to be some variability in reference gene stability (results not shown) through lifecycle, relative expression levels using both reference genes were presented graphically (Fig. 4).

2.12 Statistical analysis

Relative expression data from Q-gene were subjected to statistical analysis. Mann Whitney U test (MWU) and Kolmogorov Smirnov test (K-S) (Statistica 6.1, Statsoft Inc., USA) were used to analyse normalised RT-Q-PCR (n=6) and sandwich ELISA data from trial I (n=5), due to heterogeneity in variance. Relative mRNA expression values from Atlantic salmon fed 100% plant oil and 100% FO diet in the lifecycle study (trial II) were combined, before statistical analysis. This was done as no significant differences in mRNA expression were seen between dietary groups in dietary trial I. One way ANOVA using Tukey's HSD test (n=3) was used to analyse differences between samplings. However, since white muscle FABP3 and liver FABP10 mRNA expression data showed heterogeneity in variance, these data were subjected to a nonparametric Kruskal-Wallis test.

3. Results

3.1 Characterisation of FABP3 and FABP10

A muscle cDNA encoding FABP3 was cloned and characterized (**Fig. 1**). The predicted FABP3 protein was 133 amino acids long, had a deduced molecular weight of 14630.5 g/mol and a theoretical pI of 5.52. Atlantic salmon FABP3 was clearly more similar to FABP3 than other FABP isoforms (Table 1) and the protein identity index ranged from 68 to 98 % to FABP3 compared to other fish species (Table 2). Protein residue alignments to the four species of Antarctic teleost (Table 2) clearly suggested that Atlantic salmon FABP3 was more similar to the FABP3 than the FABP4 isoform (described as the H_{ad} type, also known as the H6-isoform (Vayda et al., 1998)). The characteristic β -barrel formed by 10 β -sheets (Q6R758) closed off by a helix turn helix is highly conserved and there are no differences between salmonids within described positions in the binding cavity (positions: 34, 50, 85, 91 and 125) (results not shown) (Ando et al., 1998).

The Atlantic salmon FABP10 sequence was identified on the basis of similarity to zebrafish FABP10. The cDNA encodes a 126 amino acid protein with a deduced molecular weight of 14023.2 g/mol and a theoretical pI of 8.52. Its characteristic pI, protein identity index (64-84%) to other FABP10s (Table 3) clearly suggest the existence of the Atlantic salmon FABP10 isoform. Atlantic salmon FABP10 protein identity index to human (Table 1) and axolotl FABP1 (Table 3) were only 43% and 42%, respectively. Atlantic salmon FABP10 predicted the

characteristic three dimensional structure, which was resolved for chicken FABP10 (Nichesola et al., 2004) and 13 of 17 amino acids involved in ligand binding and hydrophobic interactions were conserved between Atlantic salmon and chicken (results not shown).

Atlantic salmon FABP3 and FABP10 exhibits 28.6 %protein residue identity (Fig. 1) typical for different FABP family members (Hertzel and Bernlohr, 2000). Independent of reference gene used, FABP3 transcripts were higher than FABP10 transcripts levels in both muscle tissues (Fig. 2 A-D), as would be expected. Furthermore FABP10 gene transcripts levels were on average 65 fold greater than the level of FABP3 gene transcript in liver (Fig. 2 E and F). Although, normalised toward a reference gene, mRNA expression of FABP3 and FABP10 were higher in red muscle than white muscle.

3.2 Diets and fish growth

The body mass of Atlantic salmon fed the experimental diets in trial I, increased from initially 142 ± 1 g (mean \pm SD) to 1463 ± 82 g (mean \pm SD) after 42 weeks. No significant differences were observed in growth between the dietary groups. Data on growth and feed efficiency and fatty acid composition in the analysed tissues are reported elsewhere (Torstensen et al., 2004a and b). For the experimental diets the sum of monounsaturated FA, n-6 and n-9 fatty acids increased, while the sum of saturated FA and n-3 fatty acids decreased with an increased inclusion of dietary RO (Table 4). Specifically, the levels of 18:3n-3, 18:2n-6 and 18:1n-9 increased, while the long

chained n-3 fatty acids, 22:6n-3 and 20:5n-3 decreased with increased inclusion of RO in the diet.

In trial II, the body mass of Atlantic salmon fed the experimental diets increased from 0.160 ± 0.052 g (mean ± SD) to 2523 ± 590 g (mean ± SD) after 22 months. The growth rate was high in both experimental groups and no differences were observed, except after 22 months when the 100 % plant oil group showed significantly increased body mass compared to the 100 % FO group. Mortalities were negligible throughout the experimental period for both dietary trials. Further details on growth, feeding efficiency and fatty acid composition in mixed muscle tissue were reported (Stubhaug, 2005; Torstensen et al., 2005). The proximate composition of diets changed, with an increase in total lipid and a decrease in total protein with increasing pellet size through the production lifecycle of Atlantic salmon. These results have been reported previously (Torstensen et al., 2005). There was a small decrease in average dietary lipid content between diets fed within the FW period, followed by an increase of ca. 10 % lipid (wet weight) between pellets fed at SW transfer and the 9 mm pellets.

3.3 Atlantic salmon FABP3 and FABP10 mRNA expression

FABP3 and FABP10 mRNA expression in muscle tissues (**Fig.2 A-D**) and liver (Fig. 2 E-F) was not significantly changed between fish fed the 75% RO or 100% FO diet.

3.4 Atlantic salmon FABP3 protein expression

FABP3 protein levels in red and white muscle using a sandwich ELISA directed to human FABP3 (**Fig. 3**), partially reflected the dietary RO inclusion levels. FABP3 protein was significantly higher in red muscle of Atlantic salmon fed 100% RO than 50 % RO, and in white muscle of Atlantic salmon fed 100% RO than 25 % RO. For heart muscle, no correlation between dietary RO inclusion and protein expression was observed. No significant differences between FABP3 protein levels in Atlantic salmon fed 75% RO than 100% FO were observed for all tissues tested. Independent of diet, FABP3 protein levels were greater in red muscle than in white muscle. The heart contained the lowest levels of FABP3.

3.5 Atlantic salmon FABP3 and FABP10 mRNA expression during the production life cycle study

Mean red muscle FABP3 mRNA expression did increase 60% between samplings in the FW and first sampling in SW followed by a 50% reduction in relative FABP3 expression between first and second sampling in SW (**Fig. 4 A**, normalized to EF1A_A). Only modest changes in white muscle FABP3 mRNA expression levels between the different life stages were observed (Fig. 4 C normalised to EF1A_A). However, mean FABP3 mRNA expression levels were reduced by 65% from samplings at FW compared to SW stages. Furthermore, FABP3 gene transcript levels were higher in red than white muscle, although not correlated against total mRNA expression in the individual tissues.

Mean liver FABP3 gene transcript levels decreased 50% from the first to the subsequent samplings during the FW stage (Fig. 4 E, normalised to EF-1A_A). Between the first sampling in

FW to the final sampling in SW the relative FABP3 gene transcript levels decreased with 50%. Mean liver FABP10 expression changes between life stages in a similar matter as FABP3, and FABP10 expression decreases through production lifecycle (Fig. 4 G normalised to EF-1A_A). Between 3 and 9 months of feeding there was an 80% reduction in mean FABP10 gene transcript levels.

4. Discussion

In the present study, neither FABP3 nor FABP10 mRNA expression in Atlantic salmon tissues was significantly changed as a consequence of replacing the 100% diet with a 75% RO replacement diet (Fig. 2A-E). Furthermore, no significant differences were observed between protein levels of red and white muscle FABP3 protein from Atlantic salmon fed 75% RO and 100% FO (Fig. 3). This may indicate a correlation between gene transcript and protein levels of FABP3. Possibly suggesting control of FABP expression at the level of transcriptional initiation as suggested for FABP in desert locust (Schistocerca gregaria) flight muscle (Zhang and Haunerland, 1998).

Higher levels of FABP3 proteins in red muscle than white muscle, irrespective of diet (Fig. 3) may agree with the observation that Atlantic salmon red muscle has a higher capacity for oxidizing fatty acids (Froyland et al., 2000; Torstensen et al., 2000; Stubhaug et al., 2005a). Earlier studies in red muscle of rainbow trout have shown that saturated FA and 18:1n-9 may be preferentially utilized for energy production (Kiessling and Kiessling, 1993). The sum of saturated FA and 18:1n-9 decreased with reduced RO inclusion in the diets (Table 4), as did

FABP3 protein levels in red muscle (100 %-50% RO) and white muscle (100%-25% RO).

However, FABP3 protein expression only partly correlated with decreased β - oxidation capacity in red muscle (75% -25% RO) (Stubhaug et al., 2005a). Additionally, 18:1n-9 has been shown to induce FABP3 gene transcript expression in vitro in cultured rat muscle cells (Chang et al., 2001).

Ando and co-workers (1998) argued that it was very unlikely that amino acid substitutions in the binding site of rainbow trout FABP3 had affected the binding behaviour of rainbow trout FABP3 compared to that of human FABP3. The FABP3s of salmonids exhibit identical amino acid composition within described positions in the binding cavity. Thus, one may assume that Atlantic salmon FABP3 exhibit similar binding specificity and behaviour as human FABP3. In vitro binding studies for FABP3 have shown high binding affinity for 18:1n-9 and n-6 fatty acids in mammals (Zimmerman et al., 2001; Hanhoff et al., 2002). 18:1n-9 was a prominent fatty acid in the rapeseed oil diet (Table 4). 18:2n-6 was also present at high levels in the rapeseed oil diets. Thus, these fatty acids may mediate differential protein expression of Atlantic salmon muscle FABP3 as a response to selective uptake mechanisms.

One could expect that changes in muscle FABP3 mRNA expression levels between life-stages would coincide with changes in total β -oxidation capacity through Atlantic salmon production lifecycle. Since FABP3 is believed to be a transport protein for β -oxidation (Hauerland and Spener, 2004). Three months after seawater transfer total β -oxidation capacity in Atlantic salmon red muscle was significantly higher than in samplings in FW (Stubhaug, 2005) which coincide with the observed significant increase in FABP3 mRNA expression (Fig. 4. A, normalised to EF-

1A_A). Moreover, no significant change in total β -oxidation capacity between samplings in FW and a decreased β -oxidation capacity during late seawater stages coincided with changes in FABP3 mRNA expression. Changes in white muscle FABP3 mRNA expression only partly reflected changes in total β -oxidation through production life cycle (Fig. 4.C, normalised to EF-1A_A). Higher FABP3 mRNA expression before SW transfer (9 months) than during late SW phase coincide with changes in white muscle total β -oxidation capacity (Stubhaug, 2005). Furthermore, increased total β -oxidation capacity and its positive correlation to dietary RO inclusion (25%-75% RO) was observed for white muscle tissue (Stubhaug et al., 2005a). A positive correlation between FABP3 expression and dietary RO inclusion was indicated by significant differences in FABP3 protein expression levels between Atlantic salmon fed 100% and 25% RO (Fig. 3). Together, these observations may suggest that FABP3 may act as a transport protein for muscle β -oxidation. However further studies must be conducted to determine the specific *in vivo* function of FABP3 in Atlantic salmon muscle tissues, especially in white muscle.

Atlantic salmon red muscle stores more lipids than white muscle (Zhou et al., 1995). Atlantic salmon parr store lipid as triacylglycerol in muscle, and this stock is depleted during parr-smolt transformation (Sheridan, 1989 and 1994). No decrease was observed for red or white muscle FABP3 mRNA expression (Fig. 4, A-D), which coincide with fatty acid transport mediating a decrease in total lipid content in mixed muscle tissue, as observed during parr-smolt transformation in the present study (Stubhaug, 2005). Total lipid depletion in this period was also indicated by analyses indicating low growth and low fatty acid retention in whole fish. Preliminary studies (results not shown) indicate the presence of mRNA of the FABP4 isoform

(Vayda et al., 1998) in Atlantic salmon. This FABP4 form may be associated with the need for muscle fatty acid transporters for lipid storage and hence depletion.

Changes in FABPs mRNA expression in Atlantic salmon liver between different life stages (Fig. 4 E and G, normalised to EF-1A_A) do not seem to coincide with life stage dependent changes in total β -oxidation capacity (Stubhaug, 2005). Atlantic salmon liver FABP3 may also mediate fatty acid transport to the process of lipogenesis, as suggested for FABP3 present in zebrafish liver (Liu et al., 2003a); however, further studies are needed to evaluate the function of Atlantic salmon liver FABP3.

Liver FABP10 gene expression was not influenced by partial dietary replacement with rapeseed oil (Fig. 2) confirming results from microarray analyses (Jordal et al., 2005). Consequently, the two fold higher level of dietary DHA in the 100% FO than the 75% RO diet (Table 4), did not induce liver FABP10 gene expression. In the present study a 2 percent point higher level of DHA was seen in liver of Atlantic salmon fed 100% FO than the 75% RO diet (Torstensen et al., 2004a). Thus, consistent with results reported in Tsaiya duck were dietary DHA affected the liver DHA content but not liver FABP10 mRNA expression (Ko et al., 2004). It has been shown that FABP10s isolated from lungfish and catfish binds 18:1n-9 with high affinity in vitro (Di Pietro et al., 1996; Di Pietro and Santome, 2001). 18:1n-9 was present in high levels in RO diets (Table 4), and may have induced FABP10 protein expression in liver of Atlantic salmon. However, as no assay for FABP10 was available at the time of the study, several studies are needed to investigate dietary influence on in vivo liver FABP10 protein expression.

The high expression of Atlantic salmon liver FABP10 compared to liver FABP3 may indicate an important function in metabolism of one or several ligands. Lungfish (Di Pietro and Santome, 2001) and catfish (Di Pietro et al., 1997) FABP10s exhibit a broad binding specificity. However, both FABP10s have higher affinity for bile salts than fatty acids. Thus, this may indicate a potential role for fish FABP10 in the metabolism of bile salts, as recently suggested for chicken FABP10 (Nichesola et al., 2004; Nolan et al., 2005) and FABP1 in mice through *in vivo* knock-out studies (Martin et al., 2005).

In conclusion, assuming control of salmon FABP3 expression at the level of transcriptional initiation, one may link FABP3 to the process of fatty acid oxidation in red muscle related to coordinate changes in energy demanding parts of the Atlantic salmon production lifecycle. For both muscle tissues, changes in total fatty acid oxidation capacity and muscle FABP3 protein expression patterns respond similarly to dietary inclusion of RO. The established sandwich ELISA assay may be used to monitor changes in muscle FABP3 protein levels, and consequently its seemingly selective transport of dietary fatty acids. No conclusions can be made on the function of liver FABPs in the present study. Overall, FABP3 expression have been characterised in Atlantic salmon liver, heart and red and white muscle tissue. Liver and muscle tissues appeared to express several FABPs possibly linked to different metabolic functions. Since other presently unidentified fatty acid transporters in Atlantic salmon also may contribute in mediating selective transport of fatty acids; further studies are clearly needed to fully elucidate mechanisms for fatty acid transport in Atlantic salmon.

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Figure Legends

Fig. 1: Atlantic Salmon FABP3 and FABP10.

cDNA sequences encoding FABP3 (GenBank accession no. **AY509548**) and FABP10 (GenBank accession no. **BG935057**) are listed in the upper and lower lines respectively. Letters are positioned by numbers at the left. The translated amino acid sequences are shown in mid lines, indicated by their gene name and positioned through numbers at the right. Start codons are underlined, and primer and probe (italics) sequences are written in bolded letters.

Fig. 2: Relative FABP3 (A, C, E) and FABP10 (B,D,F) mRNA expression in red- (A, B) and white muscle (C, D) and liver (E, F) tissues from Atlantic salmon fed 100% FO and 75% RO for 42 weeks.

RT-Q-PCR results using FABP3 and FABP10 specific primers and probes on individual liver, white and red muscle samples from 100% FO and 75% RO after 42 weeks of feeding (trial I). Data are presented as mean \pm SD (n=5 and n=6). FABP expression normalised to 18 S rRNA was multiplied by 100, except for red muscle FABP3 (A) and white muscle FABP 10 (C) where values were multiplied by 1000. Note that the scaling on the y-axis differs between tissues and between FABPs. No significant differences in mRNA expression between Atlantic salmon fed the two diets were seen using nonparametric statistical analysis.

Fig. 3: FABP3 protein expression in heart, red- and white muscle in Atlantic salmon fed 100% FO and RO replacements diets for 42 weeks.

Results from sandwich ELISA analysis (mg FABP3 g protein⁻¹) on individual heart, red and white muscle samples from 100 % FO (data from 2 net pens combined), 100,75, 50 and 25% RO fed Atlantic salmon after 42 weeks of feeding (trial I). Data are presented as mean \pm SD (n=5). Different letters denote significant differences (P<0.05) when using nonparametric statistical analysis.

Fig. 4: Relative FABP mRNA expression in red- (A and B) and white muscle (C and D) and liver tissue (E-H) during Atlantic salmon production life cycle.

Mean normalised FABP3 (A-F) and FABP10 (G-H) expression to EF1A_A (A, C, E and G) and 18S rRNA (B, D, F and H) based on RT-Q-PCR results using specific primers and probes on individual muscle and liver samples. Data are presented as mean \pm SD. SW transfer was performed after 10 months of feeding, indicated by the vertical line. Samples were taken after 3, 6, 9, 14, 16 and 22 months of feeding. Note that the scaling on the y-axes differs. Different letters denotes significant differences (P<0.05) seen using One way ANOVA.

Table 1.

Atlantic salmon FABP3 and FABP10 compared to FABPs from Danio rerio and Homo sapiens

General characterisation				Sequence identity to Atlantic salmon isoforms	
Gene	Common name	Tissues expressed	GenBank Accession. no	Swiss Prot entry	FABP3 FABP10
<u>Danio rerio</u> ^a					
FABP2	Intestinal	Intestine, brain, muscle, liver, heart, skin, ovary, testis (Q-PCR)	<u>AF541953</u>	Q8AX65	29 25
FABP3	Heart	Ovary, liver (ISH) heart, muscle, brain (RT-PCR)	<u>AF448057</u>	Q8UUVG7	80 31
FABP7	Brain	Fabp7a :Brain, liver, testis, intestine (RT-PCR), heart, muscle (Q-PCR)	<u>AF237712</u>	Q918N9	63 29
			<u>AY380814</u>	Q6UIJ7	62 31
FABP10	Liver basic	Fabp7b: liver, intestine, brain and testis (RT-PCR) FABP10 liver (ISH ^b , EA)	<u>AF25462</u>	Q918L5	28 84
<u>Homo sapiens</u>					
FABP1	Liver	Liver, intestine, kidney, lung	<u>M10617</u>	P07148	29 43
FABP2	Intestinal	Intestine	<u>BC069466</u>	P12104	30 29
FABP3	Heart muscle	Heart, mammary, skeletal muscle	<u>U17081</u>	P05413	74 31
FABP4	Adipocyte	Adipose tissue, macrophages	<u>BT006809</u>	PI5090	62 32
FABP5	Epidermal	Heart, skeletal muscle ^c	<u>BT007449</u>	Q01469	48 21
FABP6	Ileal	Ileum	<u>U19869</u>	P51161	27 40
FABP7	Brain	Brain, olfactory bulb	<u>CR457057</u>	O15540	67 31
FABP9	Testis	Testis	-	-	-
MP2	Myelin P2	Schwann cells	<u>BC034997</u>	P02689	56 31

^a Data presented from adult zebra fish only, and with reference to work done by Wright and co-workers (Denovan-Wright et al., 2000);

Liu et al., 2003, a and b; Liu et al., 2004; Sharma et al., 2004). ^b Abbreviations used: ISH: in situ hybridization, EA: emulsion

autoradiography, Q-PCR: quantitative RT-PCR, RT-PCR: reverse transcriptase PCR ^c See Hertz and Bernlohr (2000) for a complete listing.

Table 2.
Comparison of Atlantic salmon FABP3 with FABP3 and FABP4s in other fish species.

	Species							
	<i>Oncorhynchus mykiss</i>	<i>Fundulus heteroclitus</i>	<i>Gobionotothen gibberifrons</i>	<i>Cryodraco antarcticus</i>	<i>Notothenia coriiceps</i>	<i>Chaenocephalus aceratus</i>	<i>Anguilla japonica</i>	
Atlantic salmon FABP3^a	98	78	73 ^b /54 ^c	75 ^b /55 ^c	75 ^b /54 ^c	75 ^b /55 ^c	68	
Identities								
UniprotKB/TrEMBLentry	O13008	Q90W92	O57670/O57665	O57668/O57691	O57669/O57663	O57667/Q788S9	Q9J896	
GenBank accession.nos	U95296	AY034789	U92451/U92446	U92449/U92443	U92450/U92444	U92448/U92442	AB039665/6	

UniprotKB/TrEMBLentries[©] and GeneBank accession numbers (where known) were listed for all species. For several sequence

comparisons of FABP3 please view work by Ando and co-workers (Ando et al., 1998).^a Atlantic salmon FABP3 have been characterized through UniprotKB/TrEMBLentry **Q6R758**.^b Indicates the FABP3 isoform ^c Indicate the FABP4 isoform (renamed after h6/h_{ad} isoform (Vayda et al., 1998))

Table 3.
Comparison of Atlantic salmon FABP10 with sequenced FABP10 and FABP1 in other species.

	Species						
	<i>Rhamdia sapo</i>	<i>Ambystoma mexicanum</i>	<i>Halaetus bivius</i>	<i>Gallus gallus</i>	<i>Bufo arenarum</i>	<i>Lepidosiren paradoxus</i>	<i>Fundulus heteroclitus</i>
Atlantic salmon FABP10							
Identities	83	72 ^a /42 ^b	64	72	66	65	77
UniprotKB/TrEMBLentry	P80856	P81400/P81399	P81653	P80226	P83409	P82289	Q645P9
GenBank accession. Nos.							AY725224

UniprotKB/TrEMBLentries[©] and GeneBank accession numbers (were known) were listed for all species. No FABP10 protein

was identified in Salmonides through queries in salmon genome database (<http://www.salmongenome.no/cgi->

[bin/sqp.cgi](http://www.salmongenome.no/cgi-bin/sqp.cgi)).^aIndicate the FABP10 isoform ^b Indicate the FABP1 isoform present in axolotl liver (Di Pietro et al., 1999)

Table 4.

Dietary fatty acid composition (wet weight % of total fatty acids) of the six experimental diets.

	100% FO	25 % RO	50 % RO	75 % RO	100 % RO	50 % OO
Fatty acid composition (% w.w of total fatty acids)						
16:0	11.6	10.0	8.9	7.5	5.7	11.7
18:1n-9	11.2	22.5	32.4	42.4	53.6	37.3
20:1n-9	17.1	13.3	9.8	6.1	2.1	9.0
22:1n-11	13.3	10.1	7.3	4.3	1.0	5.4
18:2n-6	3.5	7.6	11.5	15.4	19.5	7.7
18:3n-3	1.1	3.0	4.7	6.6	8.6	2.6
20:5n-3	5.9	4.5	3.4	2.1	0.7	3.2
22:6n-3	4.6	3.7	3.0	2.1	1.0	2.9
∑ saturated	20.4	17.4	14.9	12.5	9.1	18.0
∑ monoenes	57.1	58.4	59.6	60.4	61.1	61.2
∑ n-6 PUFA	4.1	8.1	11.5	15.4	19.5	7.9
∑ n-3 PUFA	15.8	14.2	13.3	11.9	10.6	10.9
n-3/n-6 ratio	3.9	1.8	1.2	0.8	0.5	1.4

Data are presented as mean (n=2). (When data is below 0.1, it is denoted by -). The remaining constituent in replacement diets were FO.

Fig. 1.

	1	<u>atggctgaggcatttcgaggcacatggaacctgaaggacagcaagaacttt</u>	
FABP3:	>	M A E A F A G T W N L K D S K N F	17
FABP10:	>	M A F S G T W Q V Y A Q E N Y	15
	1	<u>atggccttcagtggaacatggcaggtgtatgctcaggagaactac</u>	
	52	gatgaatacatgaaggctctgggtgtgggctttgcgacacgccaggttggc	
FABP3:		D E Y M K A L G V G F A T R Q V G	34
FABP10:		E E F L R A I S L P E D V I K L A	32
	46	gaggagttcctcagggccatctcactcccagaagatgttatcaagctggcc	
	103	ggtatgaccaagcccaccaccatcatcgaggtagctggagacacagtcact	
FABP3:		G M T K P T T I I E V A G D T V T	51
FABP10:		K D I K P V T E I Q Q N G N D F V	49
	97	aaagacatcaagcccgtgactgagatccagcagaacggcaatgacttcgtc	
	154	ctgaagacacagagcactttcaagaacaccgagatctccttcaactagga	
FABP3:		L K T Q S T F K N T E I S F K L G	68
FABP10:		I T S K T P G K S I T N S F T I G	66
	148	atcacctccaaaactcctggcaagtccatcaccaactccttcacatcggc	
	205	gaagagttcgcagagac caccgctgacgacaggaaagtcaagtc ccctaata	
FABP3:		E E F D E T T A D D R K V K S L I	85
FABP10:		K E A D I T T M D G K K L K C T V	83
	198	aaagagg ccgacatcaccaccatgga tggcaag aagctcaagtgactgtc	
	256	acg atagacgg tggtaagatggttcacgtgca aaagtgggacggcaaggag	
FABP3:		T I D G G K M V H V Q K W D G K E	102
FABP10:		K L E G G K L M C N - - - - T D K	96
	250	aaactggagggaggggaagc tgatgtgcaac acagataaa	
	307	accactctggttcgcgaagtgcgccaacgccctcgaacgtactctgact	
FABP3:		T T L V R E V S G N A L E R T L T	119
FABP10:		F T H I Q E L K G G E M V E T L T	113
	289	ttcacacacatccaggagctcaaaggaggagagatggttgagacgctgaca	
	358	ctgggtgacgtcgtctccacacgctcctacgtcaaggccgag	
FABP3:		L G D V V S T R S Y V K A E	133
FABP10:		V G S T S L I R R S K K M	126
	340	gtgggctctacgtcactcatcaggaggagcaaaaagatg	

Fig. 2.

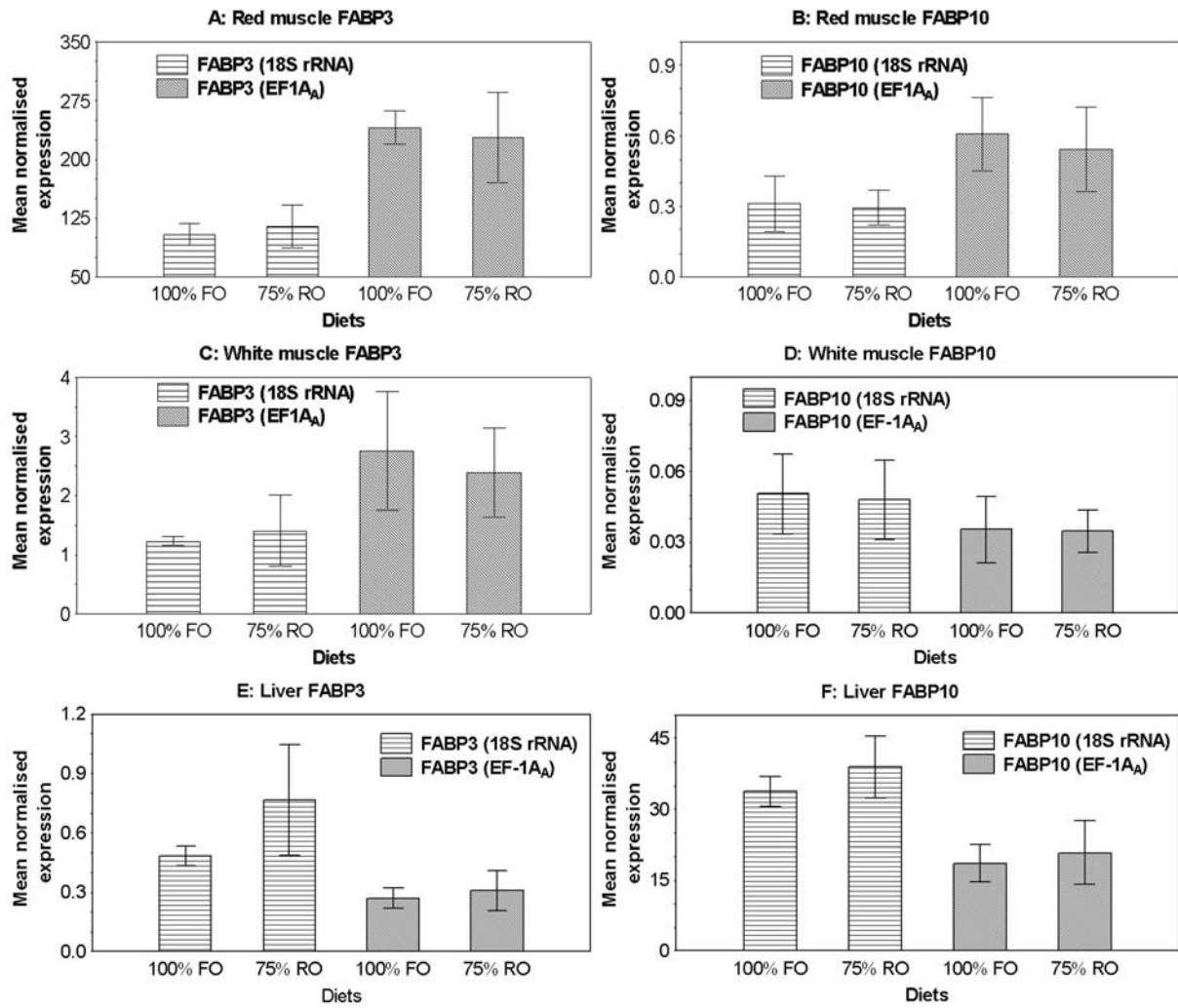


Fig. 3

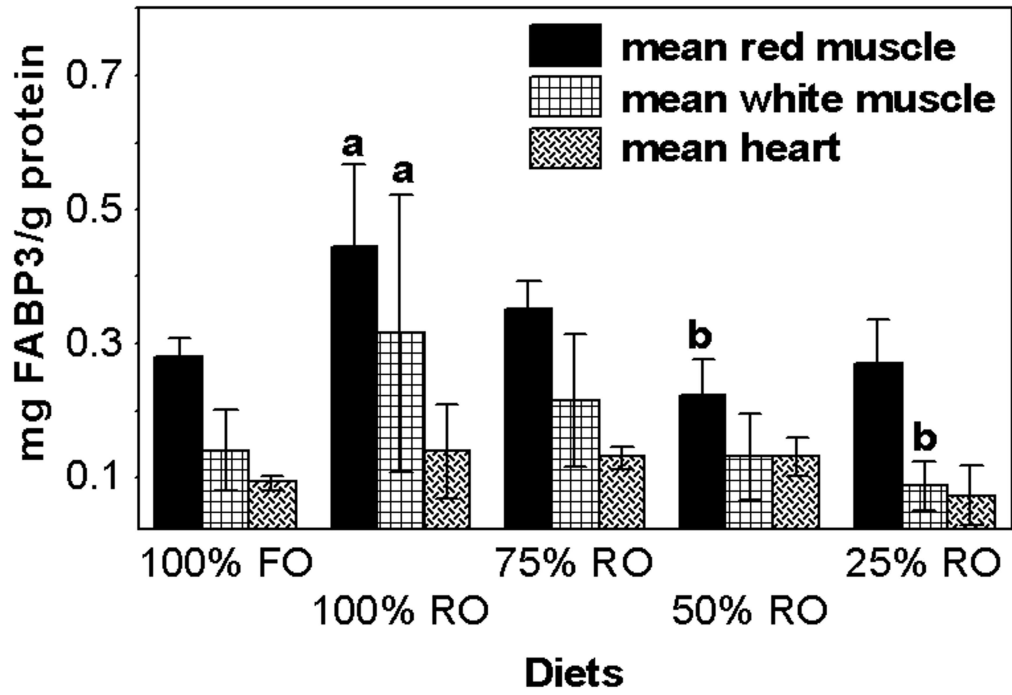


Fig. 4.

