Molecular cloning, tissue expression and regulation of Liver X Receptor (LXR) transcription factors of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Fish are important sources of high quality protein, essential minerals such as iodine and selenium, vitamins including A, D and E, and omega-3 fatty acids in the human diet. With declining fisheries worldwide, farmed fish constitute an ever-increasing proportion of fish in the food basket. Sustainable development of aquaculture dictates that diets will have to contain increasing levels of plant products that are devoid of cholesterol, but contain phytosterols that are known to have physiological effects in mammals. Liver X receptors (LXR) are transcription factors whose activity is modulated by sterols, with activation inducing cholesterol catabolism and de novo fatty acid biosynthesis in liver. Transcriptomic analysis has shown that substitution of fish meal and oil with plant products induces genes of cholesterol and fatty acid metabolism in salmonids. Here we report the cloning of LXR cDNAs from two species of salmonid fish that are important in aquaculture. The full-length cDNA (mRNA) of LXR obtained from salmon was shown to be 3766 bp, which included a 5'-untranslated region (UTR) of 412 bp and a 3'-UTR of 1960 bp and an open reading frame (ORF) of 1394 bp, which specified a protein of 462 amino acids. The trout LXR full-length cDNA was 2056 bp, including 5'- and 3'-UTRs of 219 and 547 bp, respectively, and an ORF of 1290 bp, which specified a protein of 427 amino acids. The protein sequences included characteristic features of mammalian LXRs, including the DNA binding (DBD), containing P-box, ligand binding (LBD) and activation function-2 (AF-2) domains, D-box, D (hinge) region, and eight cysteines that belong to the two zinc fingers. Phylogenetic analysis clustered

the salmonid LXRs together, more closely with zebrafish and more distantly from medaka and stickleback. A pair-wise comparison among vertebrate LXR sequences showed the amino acid sequence predicted by the salmon LXR ORF showed greatest identity to that of trout 97%, and 97%, 87% and 81% identity to LXRs of zebrafish, frog and human (LXRα). The trout LXR ORF showed 96%, 92% and 82% identity to LXRs of zebrafish, frog and human (LXRα). Surprisingly, the expression of LXR was lowest in liver of all tissues examined and in salmon the greatest expression was observed in pyloric caeca with liver showing intermediate expression. It is likely that tissue expression was affected by the physiological status of the sampled animals. Certainly, nutritional, environmental and/or developmental regulation was evident in salmon, where the expression of LXR in liver was higher in fish in seawater than in freshwater, and higher in fish fed fish oil compared to fish fed vegetable oil in adult salmon.

Keywords: Atlantic salmon; Rainbow trout; Liver X receptor; cDNA; Nutritional regulation; Fish oil; Vegetable oil; Cholesterol

1. Introduction

Around one third of fish in the human food basket are now farmed and, with global fisheries generally in decline, this proportion is showing an ever-increasing trend (FAO, 2006; Worms et al., 2006). In Europe, aquaculture is largely focussed on carnivorous species and so diets have traditionally been based on fish meal and fish oil (FO), paradoxically themselves derived from feed-grade fisheries that have reached their sustainable limit (Pike, 2005). Therefore, for continued expansion and development of aquaculture, sustainable alternatives to fish meal and fish oil are urgently required (Tacon, 2004). Plant products including a variety of grain/cereal/legume meals and vegetable oils (VO) are currently the most obvious choice and are being intensively studied as replacements for the marine products (Torstensen et al., 2005; de Francesco et al., 2004; Kaushik et al., 2004; Albalat et al., 2005; Izquierdo et al., 2005; Espe et al., 2006). Much work has focussed on effects of these changes in terms of essential amino acids and n-3 polyunsaturated fatty acids (PUFA) (Gómez-Requeni et al., 2004; Torstensen et al., 2005). However, the plant meals and oils are also deficient in terms of phospholipid and do not contain cholesterol, although they do contain phytosterols (Padley et al., 1994; Tocher et al., 2008). Consequently, we have recently shown that replacement of dietary FO with VOs in Atlantic salmon (*Salmo salar*) resulted in up-regulation of genes of cholesterol biosynthesis and sterol regulatory element binding protein 2 (SREBP-2), a

member of a family of transcription factors that regulate lipid homeostasis including cholesterol metabolism (Taggart et al., 2008; Leaver et al., 2008).

Other transcription factors involved in cholesterol homeostasis are liver X receptors (LXRs), which regulate cholesterol catabolism, storage, absorption and transport through the transcriptional regulation of key target genes involved in these processes (Aranda and Pascual, 2001). They belong to the class I subfamily of nuclear hormone receptors and their activity is modulated by the binding of oxysterols, products of cholesterol metabolism (Aranda and Pascual, 2001). In the liver, activation of LXR induces the catabolism of cholesterol through the induction of cholesterol 7α-hydroxylase (CYP7A1) expression, and *de novo* fatty acid biosynthesis (through SREBP1c), which has led to the suggestion that LXRs are sensors of the balance between cholesterol and fatty acid metabolism (Peet et al., 1998; Repa et al., 2000). The fact that unsaturated fatty acids can function as LXR antagonists, and thereby create a feedback mechanism, supports this suggestion further (Ou et al., 2001). Additionally, LXRs have recently been implicated in negative regulation of inflammatory gene expression (Marathe et al., 2006), and as key regulators of genes governing carbohydrate metabolism (Mitro et al., 2007).

LXRs are zinc finger proteins which confers the ability to bind DNA, enabling the trans-activation of LXR target genes. The LXR molecule consists of 4 principal domains (Fig.1) including an N-terminal ligand-independent activation function domain (AF-1), a DNA-binding domain (DBD) containing two zinc finger regions, a hydrophobic ligand-binding domain (LBD) required for ligand binding and receptor dimerization, and a C-terminal ligand-dependent transactivation sequence, also referred to as activation function-2 (AF-2), which stimulates transcription in response to ligand binding and is required for binding to co-activators or corepressors and trans-activation (Aranda and Pascual, 2001). In mammals, the LXR subfamily consists of LXRα and LXRβ, encoded by two genes, with the β isoform having 77% amino acid identity to the α isoform (Vaya and Schipper, 2007). Little is known about LXRs in fish. The complete sequencing of the Fugu (*Fugu rubripes*) genome showed it contained a single LXR gene (Maglich et al., 2003), and analysis of genomic sequences and evolutionary genomics of nuclear receptors suggested that an LXRα ortholog may be present in zebrafish (*Danio rerio*) (Bertrand et al., 2004). Subsequently, a cDNA coding for a protein with high similarity to mammalian LXRα was identified in zebrafish (Archer et al., 2008). There are no reports of LXR in salmonids.

Our overarching hypothesis is that understanding the molecular basis of lipid metabolism and regulation in fish will enable the efficient and effective use of sustainable plant-derived alternatives to marine products in aquaculture. The specific aims of the present study were to

clone and characterise cDNAs for LXRs from the major salmonid species being cultured, Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*). The tissue distribution of LXR was determined in both species, and the nutritional, developmental and environmental regulation of LXR gene expression in salmon liver was investigated at various points in the two-year growth cycle in fish fed diets in which FO was replaced by VO.

2. Materials and Methods

2.1. Fish and diets

Samples of Atlantic salmon were obtained from fish fed either FO or VO in a trial conducted over an entire two-year production cycle (Zheng et al., 2005a). Briefly, the two diets were fed to triplicate tanks/cages at Marine Harvest Ltd. facilities at Invergarry (freshwater) and Loch Duich, Lochalsh (seawater), Scotland. Fry were distributed randomly into 6 tanks (3 m x 3 m, depth 0.5 m) at a stocking level of 3000 per tank, and weaned onto extruded feeds containing 20 % added oil which was either FO (capelin oil) or a VO blend, containing rapeseed, palm and linseed oils in a 3.7 : 2 : 1 ratio, replacing 75 % of the FO. Fish were fed the diets for 53 weeks until seawater transfer, at which point fish (mean weight ~ 50 g) were transferred into 5 m × 5 m net pens at 700 fish per pen. The fish were fed the same diet in seawater as in freshwater although the dietary oil levels were increased to 25 % (3mm pellet) and 32 % (9mm pellets) through the year-long seawater phase. The diets were formulated to satisfy the nutritional requirements of salmonid fish (NRC, 1993), and manufactured by Skretting ARC, Stavanger, Norway. Livers were collected from six fish at all time points, with two time-points during the freshwater phase (36 and 52 weeks post-hatch) and a further two time-points in seawater (55 and 86 weeks post-hatch) (Taggart et al., 2008). A range of tissues was also sampled from a further set of four fish fed FO for 86 weeks. All liver and tissue samples were immediately frozen in liquid nitrogen and stored at – 70 ^{o}C prior to extraction.

Rainbow trout between 200 and 250 g were obtained from the “Truchas del Segre” fish farm (Lleida, Spain). Fish were acclimatized to environmental conditions at 18 ºC and natural photoperiod in facilities at the Faculty of Biology, University of Barcelona in closed circuit flow systems. They were fed daily ad libitum with a commercial diet based on fishmeal and fish oil (DibaqAquatex 22, Segovia, Spain). Samples of tissues (liver, intestine, kidney, spleen, gills, adipose tissue, heart, red muscle, white muscle and brain) were collected from five fish and placed immediately in RNAlater solution (Ambion, Madrid, Spain) at a ratio or around 200 mg tissue to 2 ml RNAlater and subsequently stored according to manufacturers instructions.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted by homogenising tissue samples in TriReagent (Ambion, UK). The quantity and quality of isolated RNA was determined by spectrophotometry with an ND-1000 Nanodrop (Labtech Int., East Sussex, UK) and electrophoresis using 1 µg of total RNA in a 1 % denaturising agarose gel. Prior to cDNA synthesis, total RNA (3µg) was incubated at 70 °C for 5 min followed by 2 min on ice. For cDNA synthesis, 1 µl of a 3:1 blend of random hexamers (400 ng/µl) / oligo dT (500 ng/µl), 2 µl dNTP (5 mM), 1 µl of reverse transcriptase, and 1 µl of RNase inhibitor were mixed with kit buffer in a final volume of 20 µl (Verso™ cDNA kit, ABgene, UK), and incubated at 42 °C for 60 min, followed by 95 °C for 2 min to inactivate the enzymes.

2.3. Isolation of salmon and trout LXR cDNAs

The 5' and 3' ends of LXR were amplified by Rapid Amplification of cDNA Ends (RACE) PCR using the FirstChoice RLM-RACE kit (Ambion, UK). 5' RACE amplicons were generated using the primer 5'LXR-R (for both species, 60 ºC annealing temperature) which was designed on conserved areas from alignments of DNA sequences of *Homo sapiens* (NM_005693), *Gallus gallus* (NM_204542) and *Danio rerio* (NM_001017545) LXRs. 3'RACE amplicons were obtained using 3'ssLXR-F (63 ºC annealing temperature) and 3'omLXR-F (58 ºC annealing temperature), designed on the expressed sequence tags (ESTs) for salmon (BG935168) and trout (BX303228), respectively. ESTs were obtained from the database "Computational Biology and Functional Genomes Laboratory" (http://compbio.dfci.harvard.edu/tgi/tgipage.html). The final full-length PCR products were synthesised using ssLXR Full-F and ssLXR Full-R for salmon, and omLXR Full-F and omLXR Full-R for trout, designed on corresponding 5` and 3' fragments. All primer sequences are shown in Table 1. PCR reactions were performed with a PCR Master Mix (ABgene, UK), 2 µl of cDNA reaction and the primers at a concentration of 0.5 µM in final volume of 20 µl. PCR products obtained were cloned in a pCR2.1 vector (Topo TA, Invitrogen, UK) and sequenced.

2.4. Sequence analysis

Sequencing was performed using a CEQ-8800 autosequencer (Beckman Coulter Inc., Fullerton, USA) and Lasergene SEQman software (DNASTAR, www.dnastar.com) was used to edit and assemble DNA sequences. ClustalW was used to generate multiple alignments of deduced protein sequences (Thompson et al., 2000). To deduce and bootstrap phylogenetic

trees using the neighbour joining method (Saitou and Nei, 1987) MEGA version 4 was used (Tamura et al., 2007).

2.5. Quantitative reverse transcription PCR (qRT-PCR)

QPCR measurements were performed applying the primers at 0.5 µM with one fortieth of the cDNA synthesis reaction and SYBR-green qPCR mix (ABgene, UK) in a total volume of 20 µl. QPCR primer sequences for target genes, salmon and trout LXRs, and reference genes β-actin, glyceraldehyde phosphate dehydrogenase (Gapdh) and elongation factor 1α (ElonF1α) are shown in Table 1. Reactions were performed in a Techne Quantica thermocycler at an annealing temperature of 58 °C for salmon LXR, 62 ºC for trout LXR and 61 °C for β-actin, Gapdh and ElonF1α to give PCR products of 210, 171, 120, 204 and 175 bp respectively. Each QPCR product was sequenced to confirm identity and all were found to be 100 % identical to its respective sequence. Quantification was achieved running the samples with a parallel set of reactions containing standards consisting of serial dilution of spectrophotometrically determined, linearised plasmid containing the cloned gene of interest. Reference genes were processed by geNorm software that used all three reference genes copy number values to elaborate a normalization factor (NF), which is then used to normalize the target genes (TGs) (copy number of TGx divided by NFx) (Vandesompele et al., 2002).

As the reference genes used above for the nutritional regulation study showed variations between tissues, 18S ribosomal RNA (AJ427629) was chosen as a reference gene for the normalization of the expression of the salmon and trout LXR genes across a wide range of tissues. Normalization was achieved by dividing the copy numbers of the target gene by the copy numbers of 18S rRNA. The real-time PCR reactions were performed in triplicates for the nutritional regulation study in salmon LXR and in duplicates for the LXR tissue distribution for both species.

2.6. Statistical analyses

Statistical analysis was performed using the InStat Statistical package (V 3.01; GraphPad Software Inc. USA). Data was first assessed for normality with the Kolmogorov-Smirnov test and for homogeneity of variances by Bartlett's test. Data were compared by one-way analysis of variance (ANOVA) except for regulation of salmon liver LXR data that were analysed by two-way ANOVA (time and diet). Post hoc multiple comparisons were applied using Tukey's test. A significance of $p < 0.05$ was applied to all statistical tests performed. All data are presented as mean ± S.E.M. (n as indicated).

3. Results

3.1. Salmon and trout LXR cDNAs

The full-length cDNA (mRNA) of LXR obtained from salmon (GenBank accession no. FJ470290) was shown to be 3764 bp, which included a 5'-untranslated region (UTR) of 411 bp and a 3'-UTR of 1967 bp. Sequencing revealed an open reading frame (ORF) of 1386 bp, which specified a protein of 462 amino acids. The trout LXR full-length cDNA (GenBank accession no. FJ470291) was 2021 bp, including 5'- and 3'-UTRs of 218 and 537 bp, respectively, and an ORF of 1266 bp, which specified a protein of 422 amino acids (Fig.2). Alignment of the deduced amino acid sequence of salmon, trout and zebrafish LXRs and those deduced from genomic database information for medaka (*Oryzias latipes*) and stickleback (*Gasterosteus aculeatus*), frog (*Xenopus tropicalis*) and chick (*Gallus gallus*), and human LXRα and β, showed the salmonid sequences included characteristic features of mammalian LXRs, including the DNA binding (DBD), containing P-box, ligand binding (LBD) and activation function-2 (AF-2) domains, D-box, D (hinge) region, and eight cysteines that belong to the two zinc fingers (Fig.2). The only major difference between the salmonif LXR cDNA sequences was at the N-terminal region containing the ligand-independent activation function domain (AF-1), which was considerably shorter in the trout. A pair-wise comparison among vertebrate LXR sequences showed the amino acid sequence predicted by the salmon LXR ORF showed greatest identity to that of trout 97%, and 97%, 87% and 81% identity to LXRs of zebrafish, frog and human (LXRα). The trout LXR ORF showed 96%, 92% and 82% identity to LXRs of zebrafish, frog and human (LXRα). Phylogenetic analysis of the salmonid LXRs and the LXRs deduced from the genomic sequences of zebrafish, medaka, stickleback and reptile (frog), bird (chicken) and mammal (human) is shown in Fig.3. The fish sequences clustered together and were more similar to mammalian LXRα than LXRβ. The salmonid LXRs were more closely related to zebrafish LXR, with medaka and stickleback more distant, which agrees with the general phylogeny of these species.

3.2. Tissue distribution of salmon and trout LXR

There were major quantitative and qualitative differences in the tissue distribution of LXR genes in trout and salmon (Fig.4). In trout, the expression of LXR was surprisingly low in liver with the rank order being spleen > heart > gill and distal intestine > brain and kidney > proximal intestine > white muscle > adipose tissue and red muscle > liver (Fig. 4A). In salmon the greatest expression was observed in intestinal tissue (pyloric caeca) followed by gill and brain, then liver and heart, spleen, white muscle, red muscle and kidney (Fig.4B).

3.3. Regulation of salmon LXR gene expression

The effects of diet and environment and/or development in the regulation of LXR gene expression in liver of Atlantic salmon are shown in Fig. 5. The expression of LXR in liver was significantly higher in fish in seawater than fish in freshwater. However, there may be an effect of development as there was a trend of decreasing expression of LXR from young parr through to two year-old adult salmon. There was no effect of diet on LXR expression in liver of salmon parr, but there was a trend of decreasing expression in fish fed VO compared to fish fed FO in salmon from pre-smolt onwards although the difference was only significant in the adult fish (Fig.5).

4. Discussion

Understanding the molecular basis of lipid metabolism and regulation in fish will facilitate the efficient and effective use of sustainable plant-derived alternatives to marine products in aquaculture. Previously, we have focussed on fatty acid metabolism, investigating key candidate genes including fatty acyl desaturases and elongases (Hastings et al., 2005; Zheng et al., 2005a,b) and transcription factors such as peroxisome proliferators activated receptors (PPARS) (Leaver et al., 2005, 2007) known to be intimately involved in the regulation of fatty acid metabolism (Tocher 2003). However, genomic analysis of transcriptomes from salmon fed diets with high levels of replacement of marine products with plant products showed that cholesterol metabolism was also greatly affected (Taggart et al., 2008; Leaver et al., 2008). This was perhaps not surprising as plant products are devoid of cholesterol, but contain phytosterols that, in addition to reducing intestinal cholesterol absorption (Calpe-Berdiel et al., 2006; Moghadasian 2006), are known to have physiological effects on cholesterol homeostasis (Yang et al., 2004). Some of these effects of phytosterols may be mediated through effects on LXR activity (Plat et al., 2005; Yang et al., 2004, 2006). Therefore, we broadened our studies to also include cholesterol metabolism. As a first step, we have cloned and characterised full-length cDNAs of Atlantic salmon and rainbow trout that code for LXRs, transcription factors known to be important in cholesterol metabolism in mammals and, most likely, fish (Archer et al., 2008).

The salmon and trout cDNAs cloned in the present study show that orthologs of mammalian LXR are expressed in salmonid fish. Previously, the complete sequencing of the Fugu (*Fugu rubripes*) genome showed it contained a single LXR gene with 75% sequence identity to the human LXRα LBD and 65% to the human LXRβ LBD (Maglich et al., 2003). Similarly, a cDNA coding for a protein with high similarity to mammalian LXRα was

identified in zebrafish (Archer et al., 2008). The phylogenetic analysis in the present study shows that the salmonid and all other fish LXRs are clearly more similar to the mammalian LXRα than to the LXRβ isoforms. Previously, it was suggested that LXRβ was absent in fish as a result of gene loss during evolution (Archer et al., 2008). Studies of co-transfection in mammalian HEK293 cells and zebrafish ZFL cells with zebrafish LXR expression plasmid showed that synthetic ligands GW3965 and T091317, and natural LXR ligand 22-R-hydroxycholesterol increased reporter gene activity (Archer et al., 2008). Furthermore the protein domains DBD and LBD show a high degree of sequence conservation across fish, mammals and amphibians, as previous studies have shown comparing the LBD of human, mouse, zebrafish and frog sequences (Reschly et al., 2008). The salmon and trout LXR cDNAs were very similar but showed a significant difference at the N-terminal region with the trout containing just 54 amino acids of AF-1 domain. However, this is not particularly unusual as this region is considerably less conserved than the other domains over all species, suggesting the function may also be less conserved and, by inference, less important. It has been speculated that varying lengths of the A/B domain may be related to differential use of start codons (Archer et al., 2008). Two alternative transcripts of human LXRα, arising from alternative splicing, were identified with one of them lacking the first 45 aa through the use of an alternative promoter and first exon (Chen et al., 2005).

The tissue expression profiles obtained in the present study showed that LXR is expressed in a wide range of tissues in salmonids. In mammals, although the LXRα and LXRβ genes are both expressed in the enterohepatic system, each has a distinct tissue expression profile. In humans, LXRα is expressed at the highest level in tissues involved in lipid metabolism, such as liver and kidney, while LXRβ is expressed more widely in brain (2-5 fold higher levels than in the liver) (Vaya and Schipper, 2007). Studies in mice revealed that LXRα is expressed in a number of metabolically active tissues, such as liver, adipose, intestine and macrophages, whereas LXRβ is ubiquitously expressed (Zhang and Magelsdorf, 2002; Tontonoz and Magelsdorf, 2003), and more highly expressed in tissues of neuronal and endocrine origin (Annicotte et al., 2004). Therefore, despite a higher sequence similarity to vertebrate LXRα, the tissue expression pattern of salmonid LXR was more reminiscent of vertebrate LXRβ. The expression of LXR in zebrafish was highest in liver followed by brain, tissues that in mice expressed high levels of LXRα and LXRβ, respectively (Annicotte et al., 2004). However, the tissue expression pattern of the Fugu LXR was more similar to mammalian LXRβ, being expressed in brain, gill, gut, heart, liver and ovary (Maglich et al., 2003). In chicken, as well as in fish, there is only LXRα isoform that show 80-81% of homology with mamalian LXRα and it was widely expressed in liver, brain, heart, kidney, spleen, skeletal muscle, abdominal

fat, pancreas and hypothalamus (Proszkowiec-Weglarz et al., 2008). These data may indicate that the progenitor of the mammalian LXRs may have carried out a physiological role more analogous to mammalian LXRβ than LXRα. The LXRα gene, with a more restricted tissue expression pattern, may have arisen to function in specific tissues with key roles in cholesterol/lipid metabolism, such a liver, macrophages and adipose tissue (Maglich et al., 2003).

Although both showed ubiquitous expression, the contrasting tissue profiles obtained in salmon and trout was surprising. As tissue expression was related to 18S RNA in both species, it is possible to compare the levels to some extent. Expression in trout was generally much greater than in salmon. The trout were in freshwater, and younger and smaller fish than the two year-old salmon whose tissues were sampled. In salmon, our data showed that the expression of LXR in liver was 2-3 fold greater in parr than in adult fish and so part of the difference between trout and salmon could be due to developmental stage. The decreasing trend in LXR expression with development and growth in salmon may be partly related to the variations in growth hormone and insulin production during the smoltification process. High LXR expression in parr could be expected as this stage is characterised by accumulation of energy stores to enhance/perform smoltification, and LXR enhances expression of lipogenic genes such as acetyl CoA carboxylase and fatty acid synthase through activation of SREBP-1c (Al-Hansani and Joost, 2005; Zhang et al., 2001). During this initial phase of parr to smolt transformation insulin levels are increased (Gutiérrez and Plisetskaya, 1991; Mommsen and Plisetskaya, 1991). Insulin stimulates fatty acid synthesis through activation of LXR, mediated by PPARα in mammals (Shalev et al., 1996; Juge-Aubry et al., 1999; reviewed Tobin et al., 2002), and insulin action is considered one of the important factors responsible of the anabolic processes at this stage of salmon growth (Mommsen and Plisetskaya, 1991). As smoltification proceeds further, energy demand is high and growth hormone increases (Björnsson, 1997; Nordgarden et al., 2007) and insulin level decreases (Mommsen and Plisetskaya, 1991). Growth hormone has been shown to decrease the expression of LXR and SREBP-1c associated with decreased insulin sensitivity in rat liver (Améen et al., 2004). Thus growth hormone down regulation of LXR expression, together with the decrease in insulin levels and possibly its tissue sensitivity, would promote release of energy necessary in smoltification.

Further, the apparent differences in relative expression between tissues in the salmonid species may reflect differences in nutritional/physiological status between the sampled fish. In salmon, expression in pyloric caeca far exceeded that in any other tissue and this may be related to the fact that fish were being fed a high fat diet (32%) based on fish meal and oil,

and so would be relatively rich in cholesterol, which may have induced increased LXR expression in intestine. In contrast, LXR expression in trout was highest in spleen. Spleen will contain macrophages and, in mammals, macrophage-specific expression of LXRα is suggested to prevent the transformation of macrophages into foam cells by amplifying the process of reverse cholesterol transport (Joseph et al., 2003; Sakamoto et al., 2007). Reverse cholesterol transport is mediated by specific target genes of LXR, ABCA1 transporters, which promote the efflux of intracellular and plasma membrane cholesterol to the nascent high density lipoprotein (HDL) particles thus promoting cholesterol efflux from tissues to the liver for excretion (Repa et al., 2000).

The nutritional regulation of LXR expression observed in salmon liver in the present study could be related to either dietary cholesterol/phytosterol contents or to fatty acid compositions. Liver LXR expression was lower in adult salmon fed VO compared to fish fed FO. This could be argued to be consistent with lower dietary cholesterol in VO-fed fish, but phytosterols have been reported to increase LXR activity in mammalian systems although not the LXR expression (Kaneko et al., 2003; Plat et al., 2005). Furthermore it has lately been suggested that phytosterols are not involved in the cholesterol homeostasis through LXR pathways (Calpel-Berdiel et al., 2006; Plösch et al., 2006; Calpel-Berdiel et al., 2008). Therefore, the decrease in liver LXR expression in salmon fed with VO could be an effect of the low dietary cholesterol and not for the presence of phytosterols.

Unsaturated fatty acids also affect activation of LXR, as they were shown to act as antagonists to the oxysterol activation of LXRα, but their effect on expression is not clear (Ou et al., 2001; Pawar et al., 2002). Highly unsaturated and polyunsaturated fatty acids (HUFA and PUFA) are competitive antagonists with the hierarchy being HUFA > PUFA > monounsaturated fatty acids, with saturated fatty acids having no effect (Pawar et al., 2002). The FO and VO diets have the same proportions of saturated and monounsaturated fatty acids, but the n-3 HUFA (20:5n-3 and 22:6n-3) in the FO diet are replaced by C18 PUFA (18:2n-6 and 18:3n-3) in the VO diet. This difference in fatty acid composition may affect the regulation of LXR expression, perhaps through PPARα. LXR is a target gene of PPARα (Tobin et al., 2002), and thus ligands of PPARα may consequently regulate LXR expression. The HUFA present in FO, 20:5n-3 and 22:6n-3, are known to up-regulate PPARα (Desvergne and Wahli, 1999), therefore PPARα could be relatively down-regulated in the animals fed VO, with consequent down regulation of LXR.

In conclusion, the present study reports the first cloning of LXR cDNAs from salmonid fish. In comparison to mammals it appears fish express a single LXR gene that, based on sequence, is most similar to mammalian LXRα, but has a more ubiquitous tissue expression pattern

more similar to mammalian LXRβ. The salmon and trout LXRs were similar in sequence, but there were major quantitative and qualitative differences in tissue expression that may be related to environmental, developmental or nutritional state of the sampled animals. Certainly, liver LXR in salmon was shown to be regulated by both nutritional and developmental/environmental factors. Further functional studies focussing on target genes for LXR are required, as greater understanding of the role of LXR in the regulation of lipid metabolism in fish will facilitate the development of sustainable diets based on plant-derived alternatives to dwindling marine resources.

Acknowledgements

LC was funded by a predoctoral fellowship (BES-2005-9566) from the Ministerio de Educación y Ciencia (project AGL-2004-06319-C02-02). MM and the work described in this paper were partly funded by a research grant from the Norwegian Research Council (NFR project nr. 172641/S40).

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Legends to Figures

Fig 1. Schematic drawing of Liver X Receptor (LXR) mechanism. In the absence of ligand, the LXR and the retinoic X receptor (RXR) are associated with corepressors (NCoR/SMRT). On ligand-binding, the corepressors are disassociated and the coactivators are recruited, the RXR and LXR receptors are translocated from the cytoplasm to the nucleus and they act by binding to the LXR response elements (LXRE) in the promoter of the target genes. The RXR-LXR dimerize through the ligand binding domain (LBD) and the DNA binding domain (DBD). The DBD contain two zinc fingers that are required for the DNA binding. The ligands bind in the core of the LBD, a conformational change involving AF-2 region takes place, introducing a binding site for coactivators that belong to p160 family (SRC-1, SRC-2, SRC-3). These coactivators interact with the transcriptional cointegrators CBP and p300 that possess histone acetyltransferase activity that allows chromatin decompactation and the gene transcription. LXR regulate the expression of ABC transporters family (ABCA1/G5/G8), apolipoprotein E (ApoE), cholesteryl ester transfer protein (CETP) and cholesterol 7α-hydroxylase (CYP7A1) involved in the cholesterol transport and catabolism. The LXR also regulates the fatty acid metabolism through the up-regulation of genes like the sterol regulatory element-binding protein 1c (SREBP-1c), fatty acid synthase (FAS) and lipoprotein lipase (LPL).

Fig. 2. Alignment of the deduced amino acid sequence of salmon and trout LXRs and those of other fish species, medaka, stickleback and zebrafish, and frog, human and chick LXRα. Similarities are indicated by (*) indentical amino acids; (:) conserved substitutions; (.) semi-conserved substitutions; and (-) represent gaps. Protein structural domains are indicated; AF-1 (N-terminal ligand-independent activation function domain); DBD (DNA binding domain) containing P-box, D-box (black shading), and eight cysteines (grey shading) that belong to the two zinc fingers; D region, Linker between LBD and DBD; LBD (ligand binding domain) with the AF-2 (activation function-2) region.

Fig. 3. Phylogenetic plot of LXR polypeptide sequences of salmon (FJ470290), trout (FJ470291), medaka (ENSORLT00000001582), stickleback (ENSGACT00000022713), zebrafish (NM_001017545), frog (NM_001079385), chick LXR-alpha (NM_204542), human (alpha NM_005693 and Beta NM_007121), Mouse (alpha NM_013839 and beta

NM_009473). Deduced protein LXR sequences were used to generate alignments using ClustalW and phylogenetic tree using MEGA4. Numbers (bootstrap values) represent the percentage of times the associated branch topology was returned after 100 iterations of tree generation.

Fig.4. Tissue expression profile of mRNA of LXR in salmon and trout as determined by quantitative PCR. Results are expressed as copy number relative to 18S RNA as described in the Methods section and are means ± S.E.M. (n = 5 and 4 for trout and salmon, respectively). Different letters indicate significant differences between tissues. DistI, distal intestine; ProxI, proximal intestine; PC, pyloric caeca; RM, red muscle; WM, white muscle.

Fig.5. Effect of dietary vegetable oil (VO) and fish oil (FO) on expression of LXR mRNA of Atlantic salmon liver as determined by quanititative PCR. Liver samples were collected from fish sampled at 36 (parr), 52 (pre-smolt), 55 (post-smolt) and 86 (adult) weeks post-hatch. Results are expressed as copy number normalised to a set of reference genes as described in the Methods section and are means ± S.E.M. (n = 6). Different letters indicate significant differences between time-points and an asterisk indicates a significant effect of diet.

Table 1. Sequences of primers used for cloning salmon and trout LXRs and for quantitative PCR

Primer name	Sequence 5' →3'
Salmon	
5'LXR-R	cacttgcggcgcatgtacatgtccat
3'ssLXR-F	gatgccctcacggaactcttg
ssLXRFull-F	cacgtgaccgacgacagagggattt
ssLXRFull-R	tcgtgtttgggtagcctgggagac
ssLXRqPCR-F	gccgccgctatctgaaatctg
ssLXRqPCR-R	caatccggcaaccaatctgtagg
Gapdh-F	tctggaaagctgtggagggatgga
Gapdh-R	aaccttcttgatggcgtcgtagc
EF1α-F	tctggagacgctgctattgttg
EF1α-R	gactttgtgaccttgccgcttgag
β-actin-F	atcctgacagagcgcggttacagt
β-actin-R	tgcccatctcctgctcaaagtcca
18s-F	ggcgccccctcgatgctctta
18s-R	cccccggccgtccctcttaat
Trout	
5'LXR-R	cacttgcggcgcatgtacatgtccat
3'omLXR-F	ctccaaggctacccaaacacgacgaa
OmLXR-Full-F	cacagatgttggtcctgggtgag
OmLXR-Full-R	cttggaggcatcgcttcattct
omLXRqPCR-F	tgcagcagccgtatgtgga
omLXRqPCR-R	gcggcgggagcttcttgtc
18s-F	ggcgccccctcgatgctctta
18s-R	cccccggccgtccctcttaat

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621

Fig.1. Schematic drawing of Liver X Receptor (LXR) mechanism

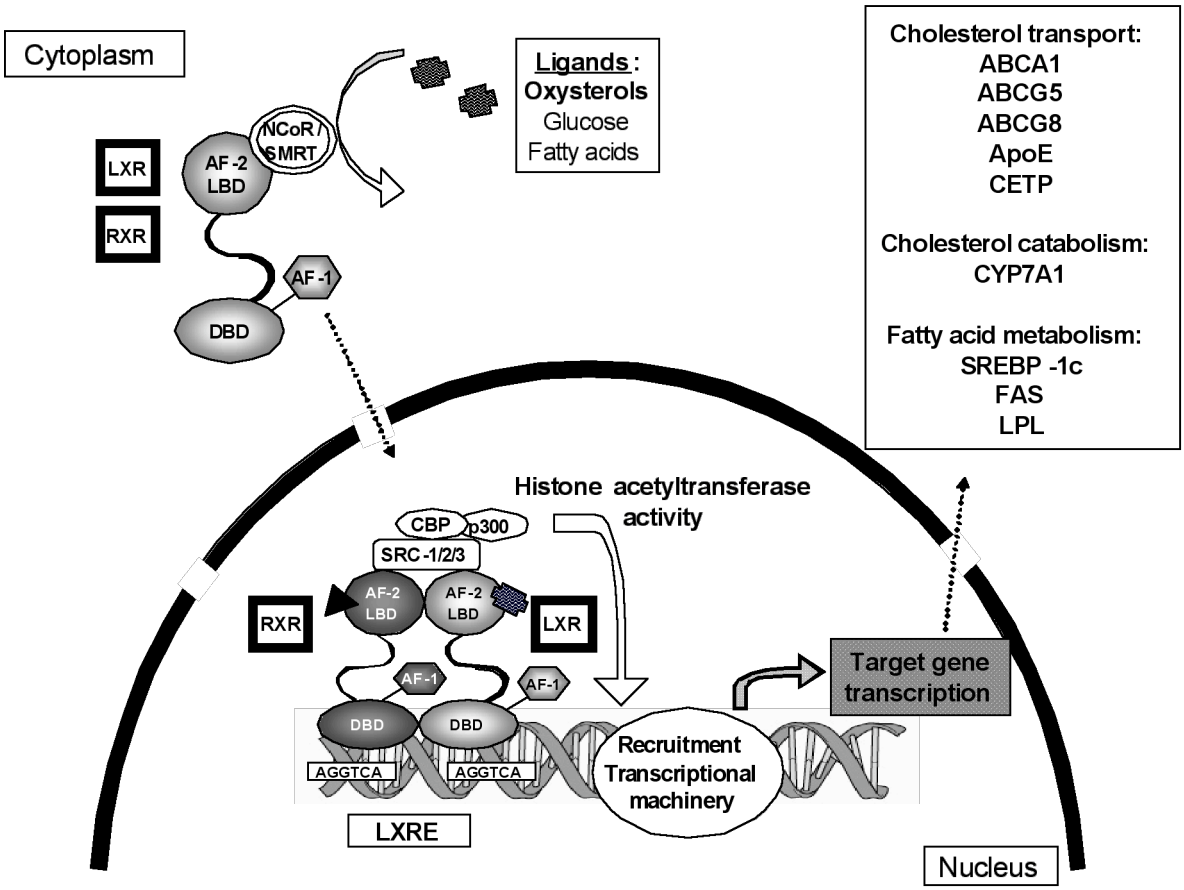
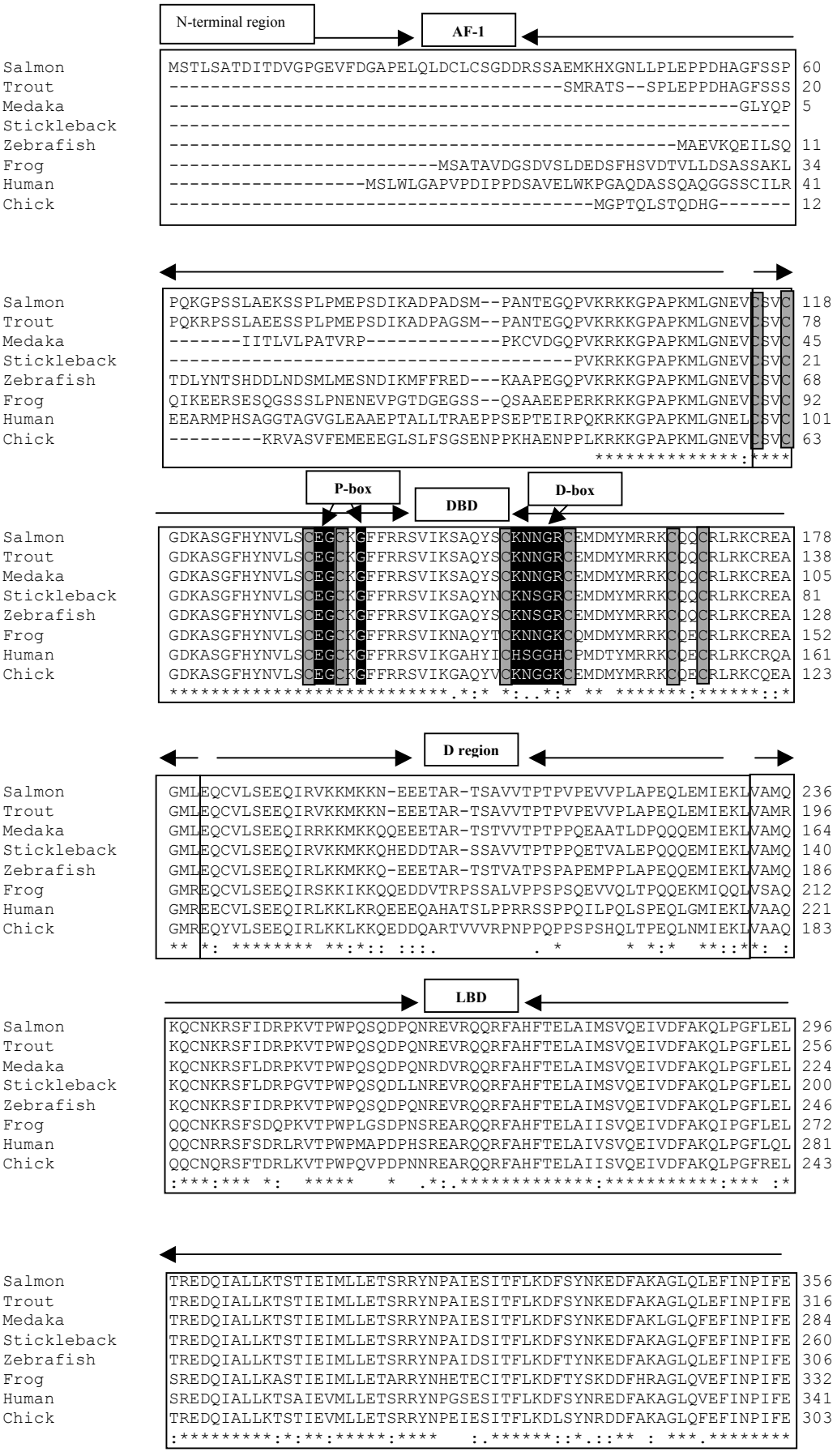


Fig.2.


N-terminal region
AF-1
Salmon MSTLSATDITDVGPGEVFDGAPELQLDCLCSGDDRSSAEMKHXGNLLPLEPPDHAGFSSP 60
Trout ----------SMRATS--SPLEPPDHAGFSSS 20
Medaka ----------GLYQP 5
Stickleback
Zebrafish ----------MAEVKQEILSQ 11
Frog ----------MSATAVDGSDVSLDEDSFHSVDTVLLDSASSAKL 34
Human ----------MSLWLGAPVPDIPPDSAVELWKPGAQDASSQAQGGSSCILR 41
Chick ----------MGPTQLSTQDHG------- 12
Salmon PQKGPSSLAEKSSPLPMEPSDIKADPADSM--PANTEGQPVKRKKGPAPKMLGNEVDSVC 118
Trout PQKRPSSLAEESSPLPMEPSDIKADPAGSM--PANTEGQPVKRKKGPAPKMLGNEVDSVC 78
Medaka -------IITLVLPATVRP----------PKCVDGQPVKRKKGPAPKMLGNEVDSVC 45
Stickleback ----------PVKRKKGPAPKMLGNEVDSVC 21
Zebrafish TDLYNTSHDDLNDSMLMESNDIKMFFRED---KAAPEGQPVKRKKGPAPKMLGNEVDSVC 68
Frog QIKEERSESQGSSSLPNENEVPGTDGEGSS--QSAAEEPERKRKKGPAPKMLGNEVDSVC 92
Human EEARMPHSAGGTAGVGLEAAEPTALLTRAEPPSEPTEIRPQKRKKGPAPKMLGNELDSVC 101
Chick ---------KRVASVFEMEEEGLSLFSGSENPPKHAENPPLKRKKGPAPKMLGNEVDSVC 63
P-box
DBD
D-box
Salmon GDKASGFHYNVLSCEGCKGFFRRSVIKSAQYSCKNNGRCEMDMYMRRKCQQCRLRKCREA 178
Trout GDKASGFHYNVLSCEGCKGFFRRSVIKSAQYSCKNNGRCEMDMYMRRKCQQCRLRKCREA 138
Medaka GDKASGFHYNVLSCEGCKGFFRRSVIKSAQYSCKNNGRCEMDMYMRRKCQQCRLRKCREA 105
Stickleback GDKASGFHYNVLSCEGCKGFFRRSVIKSAQYNCKNSGRCEMDMYMRRKCQQCRLRKCREA 81
Zebrafish GDKASGFHYNVLSCEGCKGFFRRSVIKGAQYSCKNSGRCEMDMYMRRKCQQCRLRKCREA 128
Frog GDKASGFHYNVLSCEGCKGFFRRSVIKNAQYTCKNNGKCQMDMYMRRKCQECRLRKCREA 152
Human GDKASGFHYNVLSCEGCKGFFRRSVIKGAHYICHSGGHCPMDTYMRRKCQECRLRKCRQA 161
Chick GDKASGFHYNVLSCEGCKGFFRRSVIKGAQYVCKNGGKCEMDMYMRRKCQECRLRKCQEA 123
D region
Salmon GMLEQCVLSEEQIRVKKMKKN-EEETAR-TSAVVTPTPVPEVVPLAPEQLEMIEKLVAMQ 236
Trout GMLEQCVLSEEQIRVKKMKKN-EEETAR-TSAVVTPTPVPEVVPLAPEQLEMIEKLVAMR 196
Medaka GMLEQCVLSEEQIRRKKMKKQQEEETAR-TSTVVTPTPPQEAATLDPQQQEMIEKLVAMQ 164
Stickleback GMLEQCVLSEEQIRVKKMKKQHEDDTAR-SSAVVTPTPPQETVALEPQQQEMIEKLVAMQ 140
Zebrafish GMLEQCVLSEEQIRLKKMKKQ-EEETAR-TSTVATPSPAPEMPPLAPEQQEMIEKLVAMQ 186
Frog GMREQCVLSEEQIRSKKIKKQQEDDVTRPSSALVPPSPSQEVVQLTPQQEKMIQQLVSAQ 212
Human GMREECVLSEEQIRLKKLKRQEEEQAHATSLPPRRSSPPQILPQLSPEQLGMIEKLVAAQ 221
Chick GMREQYVLSEEQIRLKKLKKQEDDQARTVVVRPNPPQPPSPSHQLTPEQLNMIEKLVAAQ 183
LBD
Salmon KQCNKRSFIDRPKVTPWPQSQDPQNREVRQQRFAHFTELAIMSVQEIVDFAKQLPGFLEL 296
Trout KQCNKRSFIDRPKVTPWPQSQDPQNREVRQQRFAHFTELAIMSVQEIVDFAKQLPGFLEL 256
Medaka KQCNKRSFLDRPKVTPWPQSQDPQNRDVRQQRFAHFTELAIMSVQEIVDFAKQLPGFLEL 224
Stickleback KQCNKRSFLDRPGVTPWPQSQDLLNREVRQQRFAHFTELAIMSVQEIVDFAKQLPGFLEL 200
Zebrafish KQCNKRSFIDRPKVTPWPQSQDPQNREVRQQRFAHFTELAIMSVQEIVDFAKQLPGFLEL 246
Frog QQCNKRSFSDQPKVTPWPLGSDPNSREARQQRFAHFTELAIISVQEIVDFAKQIPGFLEL 272
Human QQCNRRSFSDRLRVTPWPMAPDPHSREARQQRFAHFTELAIVSVQEIVDFAKQLPGFLQL 281
Chick QQCNQRSFTDRLKVTPWPQVPDPNNREARQQRFAHFTELAIISVQEIVDFAKQLPGFREL 243
Salmon TREDQIALLKTSTIEIMLLETSRRYNPAIESITFLKDFSYNKEDFAKAGLQLEFINPIFE 356
Trout TREDQIALLKTSTIEIMLLETSRRYNPAIESITFLKDFSYNKEDFAKAGLQLEFINPIFE 316
Medaka TREDQIALLKTSTIEIMLLETSRRYNPAIESITFLKDFSYNKEDFAKLGLQFEFINPIFE 284
Stickleback TREDQIALLKTSTIEIMLLETSRRYNPAIDSITFLKDFSYNKEDFAKAGLQFEFINPIFE 260
Zebrafish TREDQIALLKTSTIEIMLLETSRRYNPAIDSITFLKDFTYNKEDFAKAGLQLEFINPIFE 306
Frog SREDQIALLKASTIEIMLLETARRYNHETECITFLKDFTYSKDDFHRAGLQVEFINPIFE 332
Human SREDQIALLKTSAIEVMLLETSRRYNPGSESITFLKDFSYNREDFAKAGLQVEFINPIFE 341
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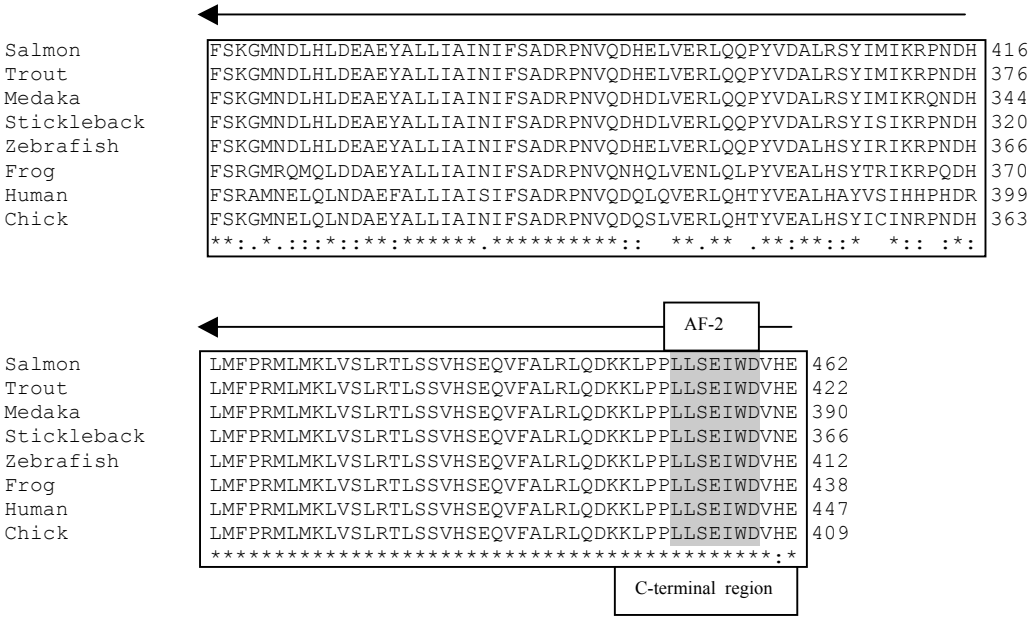
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Medaka FSKGMNDLHLDEAEYALLIAINIFSADRPNVQDHDLVERLQQPYVDALRSYIMIKRQNDH 344
Stickleback FSKGMNDLHLDEAEYALLIAINIFSADRPNVQDHDLVERLQQPYVDALRSYISIKRPNDH 320
Zebrafish FSKGMNDLHLDEAEYALLIAINIFSADRPNVQDHELVERLQQPYVDALHSYIRIKRPNDH 366
Frog FSRGMRQMQLDDAEYALLIAINIFSADRPNVQNHQLVENLQLPYVEALHSYTRIKRPQDH 370
Human FSRAMNELQLNDAEFALLIAISIFSADRPNVQDQLQVERLQHTYVEALHAYVSIHHPHDR 399
Chick FSKGMNELQLNDAEYALLIAINIFSADRPNVQDQSLVERLQHTYVEALHSYICINRPNDH 363

AF-2
Salmon LMFPRMLMKLVSLRTLSSVHSEQVFALRLQDKKLPPLLSEIWDVHE 462
Trout LMFPRMLMKLVSLRTLSSVHSEQVFALRLQDKKLPPLLSEIWDVHE 422
Medaka LMFPRMLMKLVSLRTLSSVHSEQVFALRLQDKKLPPLLSEIWDVNE 390
Stickleback LMFPRMLMKLVSLRTLSSVHSEQVFALRLQDKKLPPLLSEIWDVNE 366
Zebrafish LMFPRMLMKLVSLRTLSSVHSEQVFALRLQDKKLPPLLSEIWDVHE 412
Frog LMFPRMLMKLVSLRTLSSVHSEQVFALRLQDKKLPPLLSEIWDVHE 438
Human LMFPRMLMKLVSLRTLSSVHSEQVFALRLQDKKLPPLLSEIWDVHE 447
Chick LMFPRMLMKLVSLRTLSSVHSEQVFALRLQDKKLPPLLSEIWDVHE 409
C-terminal region

Fig.3.

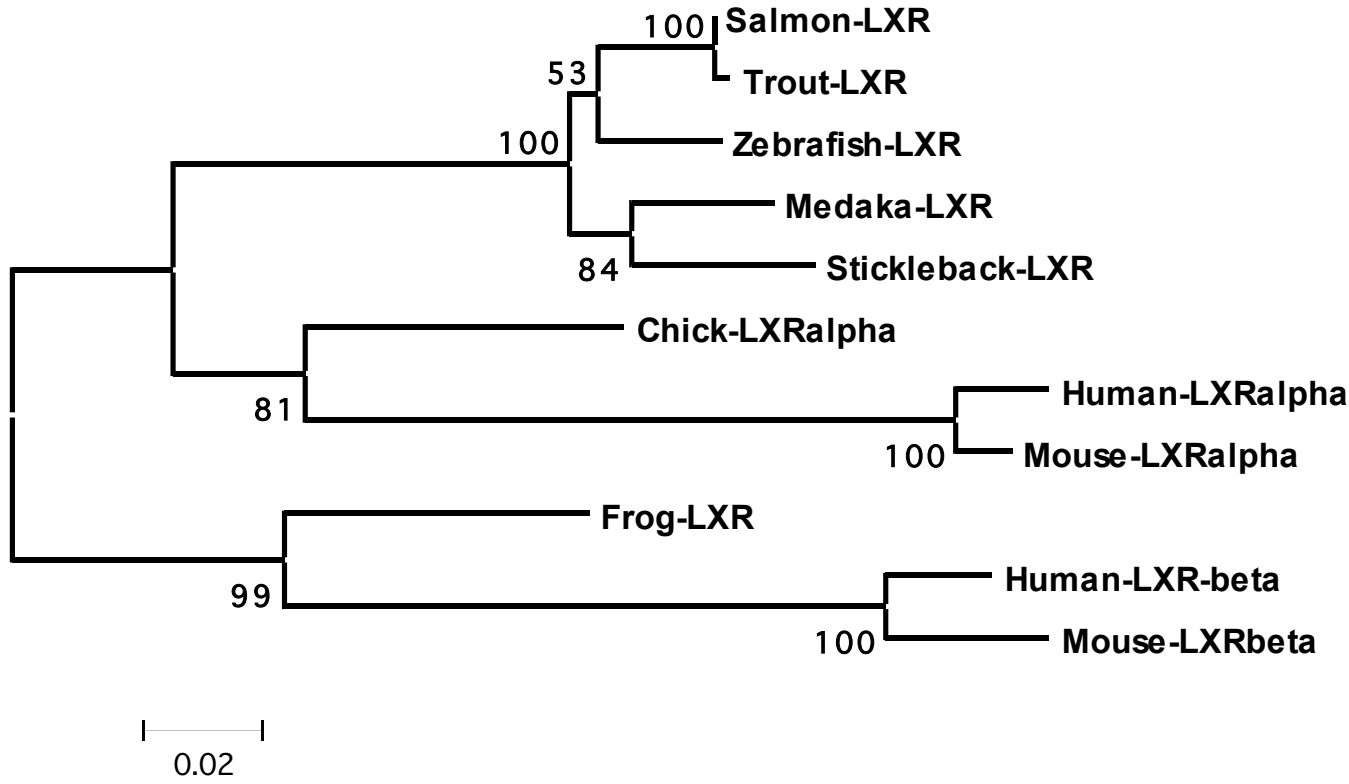

100
Salmon-LXR
53
Trout-LXR
100
Zebrafish-LXR
Medaka-LXR
84
Stickleback-LXR
Chick-LXRalpha
Human-LXRalpha
81
100
Mouse-LXRalpha
Frog-LXR
Human-LXR-beta
99
100
Mouse-LXRbeta
0.02

Fig.4.

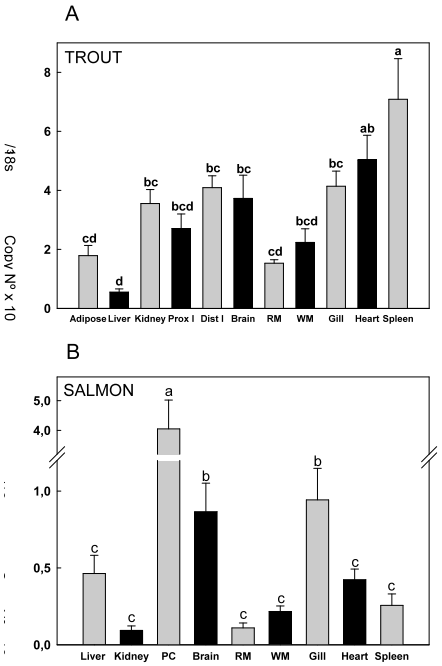
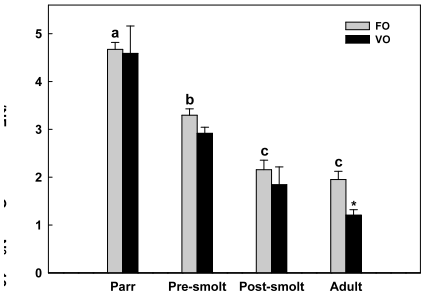

A
TROUT
/18s
Copy N° x 10
8
6
4
2
0
cd
d
bc
bcd
bc
bc
cd
bcd
bc
ab
a
Adipose Liver Kidney Prox I Dist I Brain RM WM Gill Heart Spleen
B
SALMON
5,0
4,0
1,0
0,5
0,0
c
c
a
b
c
c
b
c
c
Liver Kidney PC Brain RM WM Gill Heart Spleen

Fig.5.



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