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## Multiple peroxisome proliferator-activated receptor $\beta$ subtypes from Atlantic salmon (*Salmo salar*)

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### Abstract

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily that function as critical regulators of lipid and energy homeostasis. Although intensively studied in mammals, their basic biological functions are still poorly understood. The objective of this work was to characterise PPAR $\beta$  subtypes in a fish the Atlantic salmon (*Salmo salar*) in order to address PPAR function and the regulation of lipid homeostasis in lower vertebrates. The screening of an Atlantic salmon genomic library revealed the presence of four genes for PPAR $\beta$  subtypes. Based on comparisons of exons and exon-flanking regions, these genes were assigned into two families, ssPPAR $\beta$ 1 and ssPPAR $\beta$ 2, each family containing two isoforms; ssPPAR $\beta$ 1A and  $\beta$ 1B and ssPPAR $\beta$ 2A and  $\beta$ 2B. Two full-length cDNAs for ssPPAR $\beta$ 1A and ssPPAR $\beta$ 2A were isolated. Transcripts for ssPPAR $\beta$ 1A and ssPPAR $\beta$ 2A have distinct tissue expression profiles, with ssPPAR $\beta$ 1A predominating in liver and ssPPAR $\beta$ 2A predominating in gill. Expression levels of mRNA of either isoform were up to ten fold lower in kidney, heart, spleen, muscle, and brain. In cellular transfection assays ssPPAR $\beta$ 1A is activated by monounsaturated fatty acids, 2-bromopalmitate and by the mammalian PPAR $\beta$ -specific ligand GW501516. In contrast, PPAR $\beta$ 2A was not activated by any of the compounds tested. Furthermore, ssPPAR $\beta$ 2A repressed both basal reporter gene activity and the GW501516-induced activity of ssPPAR $\beta$ 1A. The results indicate unexpected levels of variety and complexity in PPAR subtype and mechanism of action in lower vertebrates.

Footnote: The sequences referred to have been deposited in the EMBL/NCBI databases under accession numbers AM229297 to AM229306.

Abbreviations: CAT, chloramphenicolacetyltransferase; FBS, fetal bovine serum; PBS, phosphate buffered saline; ONPG, 0-nitrophenolgalactoside; LBD, ligand binding domain; NHR, nuclear hormone receptor; nt, nucleotide; PPAR, peroxisome proliferator-activated receptor; PPARE, PPAR response elements; QPCR, quantitative PCR; RACE,

rapid amplification of cDNA ends; RNase, ribonuclease; AS, Atlantic salmon; SMART, switching mechanism at 5' end of RNA transcript.

## Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily. Originally isolated from humans, rodents and amphibians, they are now widely accepted to be critical regulators of lipid and energy homeostasis (Isseman and Green, 1990; Krey et al, 1993; Desvergne and Wahli, 1999; Hihi et al., 2002). In mammals, one gene for each of three PPAR subtypes, PPAR $\alpha$ , PPAR $\beta$  (also known as PPAR $\delta$ ) and PPAR $\gamma$  is present (Michalik and Wahli, 1999). The role of PPAR $\alpha$  is hypothesised to be primarily in controlling the reversible induction of  $\beta$ -oxidation in specific tissues, especially liver, as a response to changing energy requirements and nutritional status. The evidence for this comes most directly from rodents where PPAR $\alpha$  is expressed in cells with high catabolic rates of fatty acid oxidation such as hepatocytes, cardiomyocytes, kidney proximal tubules and intestinal mucosa (Escher et al., 2001). Indeed, PPAR $\alpha$ -null mice are incapable of up-regulating fatty acid oxidation during fasting (Kersten et al., 1999; Leone et al, 1999). In contrast, mammalian PPAR $\gamma$  is considered to play a critical role in fat accumulation particularly in adipocytes, and in lipid-accumulating macrophages (Rosen and Spiegelman, 2001). The role of PPAR $\beta$  is less well understood. Various studies suggest that PPAR $\beta$  has a role in global control of lipid homeostasis in mammals. It is moderately activated by a range of unsaturated fatty acids (Forman et al., 1997) and has a broad tissue expression profile (Escher et al, 2001). PPAR $\beta$ -null mice show reduced adipose stores (Peters et al., 2000) which cannot be explained by adipose-specific PPAR $\beta$  deficiency (Barak et al., 2002; Wang et al., 2003). Furthermore, in the absence of ligand, PPAR $\beta$  can act as a repressor of PPAR $\alpha$  and PPAR $\gamma$  action (Shi et al., 2002). Similarly in cell overexpression systems and in the absence of activating ligand, PPAR $\beta$  can down-regulate genes involved in lipid and energy metabolism (Tachibana et al., 2005). Recently highly specific and potent synthetic ligands for mammalian PPAR $\beta$  have been developed. Treatment of animals with these compounds has demonstrated that ligand-activated PPAR $\beta$  directly controls lipid utilisation through up-regulation of genes involved in  $\beta$ -oxidation and energy uncoupling in various tissues (Dressel et al., 2003, Tanaka et al., 2003; Wang et al., 2003, Tachibana et al., 2005). In consequence PPAR $\beta$  ligands can have beneficial effects in correcting dyslipidemic states in various animal disease models. Thus, PPAR $\beta$  ligands are now receiving considerable attention as potential pharmaceuticals for the treatment of a variety of human diseases associated with dyslipidemia (Desvergne et al., 2004).

In addition to functioning as a regulator of energy metabolism PPAR $\beta$  has also been shown to have significant roles in the control of cellular proliferation and differentiation. Studies on PPAR $\beta$ -null mice have indicated the PPAR $\beta$  functions in skin wound healing (Michalik et al, 2001), keratinocyte differentiation (Schmuth et al, 2004; Kim et al., 2006) in apoptosis (Di Poi et al., 2002) and in skin and colon carcinogenesis (Harman et al., 2004; Kim et al., 2004)

Despite these developments *bona fide* endogenous ligands for PPAR $\beta$  have still not been conclusively defined and the precise role of PPAR $\beta$  in lipid and energy metabolism, and how this relates to PPAR $\beta$  function in cellular differentiation and proliferation, is not yet understood. The study of PPARs in lower vertebrates such as fish offers an opportunity to compare expression, to define common ligands and infer common functions across vertebrates, thus informing studies on PPARs in humans. Recently a study on two members of a major fish clade, the Perciformes, reported the identification and characterisation of homologues of PPAR $\alpha$ ,  $\beta$  and  $\gamma$  (Leaver et al., 2005). Comparison of these gene sequences with information from the pufferfish (*Takafugu rubripides* and *Tetraodon nigriviridis*, also Perciformes) genome sequencing projects suggested that these fish possessed a single PPAR $\beta$  gene and that it shared many of the features of its mammalian counterpart. However, examination of the zebrafish genome database indicated that there are two PPAR $\beta$  genes in this Cypriniform species. Consequently, the exact number of genes and/or the presence of distinct PPAR isoforms in fish have yet to be determined. Here we show that multiple PPAR $\beta$  genes are present in Atlantic salmon, a commercially important member of a third major fish clade, the Salmoniformes. Two of these gene products are demonstrated to differ in tissue expression profile and transactivation properties.

## Materials and Methods

### Isolation of Atlantic salmon PPAR genes

An Atlantic salmon genomic DNA library was constructed in  $\lambda$ FIXII (Stratagene, La Jolla, CA) from DNA isolated from blood from a single individual. This library was screened with a  $^{32}$ P labelled DNA probe corresponding to the ligand-binding domain (LBD) of plaice (*Pleuronectes platessa*) PPAR $\beta$  (Leaver et al., 2005). Hybridising clones were isolated, phage DNA purified, restriction digested and subjected to Southern blot analysis using the plaice PPAR $\beta$  probe. Hybridising restriction fragments were then subcloned to plasmid vectors and sequenced using an ABI 477 autosequencer and BigDye sequencing reagents. Sequences were analysed using Autoassembler software supplied by ABI.

### Isolation of Atlantic salmon PPAR $\beta$ cDNAs

Full length cDNAs were isolated from total salmon liver RNA using reverse transcription, PCR and RACE (SMART RACE system, BD Clontech, Oxford, UK). Gene specific primers (P1, P2, P3; Fig 1., Table 1) were directed to the regions immediately adjacent to PPAR $\beta$  termination codons predicted from genomic sequencing. These primers were combined in 5'RACE PCR reactions with SMART liver cDNA and the SMART system universal primer. Further RACE PCR was performed with primers synthesised to specific regions within products arising from the first round of reactions. These primers (P4, P5; Fig 1., Table 1) were used in nested PCR procedures. Finally full length open reading frames for salmon PPAR $\beta$  cDNAs were obtained using both forward and reverse gene specific primers (P1, P6, P7, P8; Fig 1., Table1.), designed from

consideration of the previously isolated RACE products. All products were ligated to *E. coli* plasmid vectors and sequenced.

**Table 1. Oligonucleotide primers used for Atlantic salmon PPAR $\beta$  cDNA isolation**

Primer	Oligonucleotide 5' to 3'
P1	TAAACCAGTCCAGTCAGTACATGTCC
P2	CAGTCAGTACATGTCCTTATAGATC
P3	TTCCAGTCAGTACAGATTTAGTCCGGT
P4	CTTGGGGAAGACATAGAGGGAGTCCTG
P5	TCGTTAAGGAAGAGATCCACAAACCCT
P6	CAGGATCCATGGGTAGCCAACAGAATGGAGAGATTG
P7	AAGGTACCAGTCAGTACATGTCCTTATAGATCTCCT
P8	CTTGGATCCTGGGACATGAAG

### Tissue Expression

Total RNA was isolated from tissues of three Atlantic salmon (average mass = 200g) using TriReagent (Sigma, Poole, UK). For QPCR (quantitative PCR) 1 $\mu$ g of total RNA were converted to cDNA using a blend of poly-dT oligonucleotide primer (25nM) and random hexamers (75nM), and Reverse-iT reverse transcriptase (AMV/MMuLV blend; ABgene, Epsom UK). QPCR reactions of 20 $\mu$ l contained 100nM each primer, cDNA from the equivalent of 25ng total RNA for PPAR $\beta$  reactions and from the equivalent of 0.25ng RNA for 18S rRNA reactions. Amplicons were quantified using SYBR Green PCR mastermix (Abgene, Epsom, UK) and a Techne Quantica QPCR instrument. Cycling parameters consisted of a 15 min 95°C soak to activate the polymerase followed by 40 cycles of 95°C for 15 secs and 60°C for 1 minute. Primers were directed to areas where the two cDNAs exhibited low nucleotide identity, either side of the junction corresponding to the first exon of the LBD of each PPAR. For PPAR $\beta$ 1A these were 5'-GACCACCAACCCCAATGGCTCGGAT and 5'-CAGCCCATTCTCAGCCTGGCACAAG, and for PPAR $\beta$ 2A, 5'-CCCCACCATCTTGGTGGCTCAGA and 5'-TAGACCACTCTCTGCTTGCCACAGG. The PCR products produced from these primers under QPCR conditions were checked by gel electrophoresis and sequencing before undertaking QPCR. In each case the predicted products of 176 base pairs and 190 base pairs for PPAR $\beta$ 1A and PPAR $\beta$ 2A respectively were amplified. Values for PPAR $\beta$  were normalised to 18SrRNA levels measured with primers: 5'-CTGCCCTATCAACTTTCGATGGTACT and 5'-AAAGTGTACTCATTCCAATTACGGGG.

### Cell Transfection Experiments

Salmon PPAR $\beta$  cDNAs were ligated to pcDNA3 and used to transfect AS (derived from Atlantic salmon, epitheloid: Nicholson and Byrne, 1973) cells with pCMV $\beta$ gal and a reporter construct containing a PPRE from the mouse cyp4A6 promoter (Ijpenberg *et al.*, 1997) linked to a chloramphenicol acetyltransferase (CAT) gene. AS cells were routinely grown and passaged in DMEM, 10% FBS. Prior to transfection cells were passaged into DMEM, plus charcoal/dextran-stripped 10% FBS (Pierce, Rockford, IL ). 1.5 $\mu$ g of total

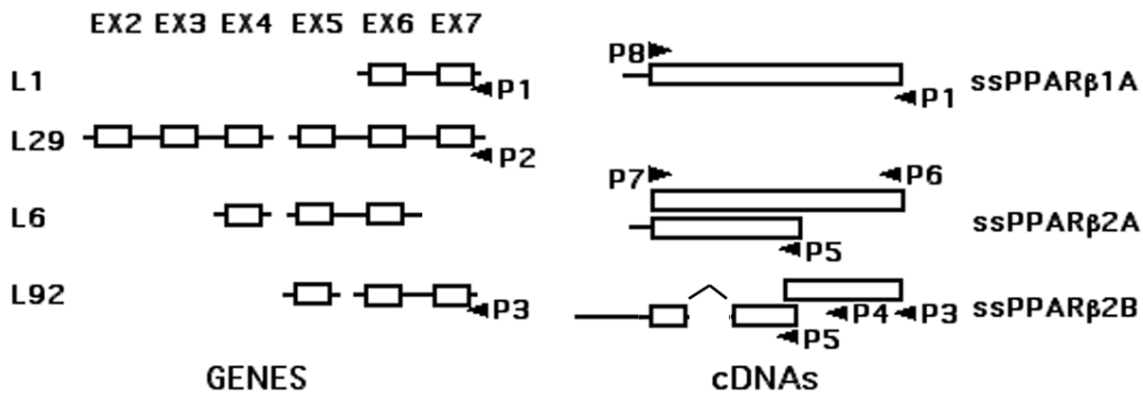
plasmid DNA were transfected to each well of 12 well tissue culture plates using 7.5 $\mu$ l of Superfect reagent according to the manufacturers instructions (Qiagen, Crawley, UK). Twenty four hours after transfection cells were treated with fatty acids (100 $\mu$ M, Sigma, Poole, UK), Wy16463 (50 $\mu$ M), rosiglitazone, GW501516 (10 $\mu$ M, Alexis Corporation, UK), and L165041 (10 $\mu$ M, Calbiochem) in 5 $\mu$ l ethanol. Cells were harvested 24 hours after treatment, lysed in 300  $\mu$ l of detergent-based buffer and CAT protein was measured using an ELISA method (Roche, Lewes, UK).  $\beta$ -galactosidase activity was measured in a microtitre plate based assay. Briefly 20 $\mu$ l of cell lysate was incubated with 130 $\mu$ l of PBS containing 5mM MgCl<sub>2</sub>, 10mM  $\beta$ -mercaptoethanol, 1.5mM ONPG. After 30mins the reaction was stopped by the addition of 75 $\mu$ l of 1M Na<sub>2</sub>CO<sub>3</sub> and the A<sub>420</sub> measured. CAT quantity was normalised to  $\beta$ -galactosidase activity and, after subtracting mock-transfected blank values, values for each treatment were expressed relative to the ethanol control for pcDNA3 (empty vector). All treatments were performed in triplicate.

## Results

### Atlantic salmon possess four distinct PPAR $\beta$ genes

The salmon genomic library was screened with a probe corresponding to the LBD of a plaice PPAR $\beta$  (Leaver et al., 2005) and a total of nine distinct phage inserts were found to contain PPAR $\beta$ -related sequences. Assembly of the sequences from these nine inserts produced four distinct clone sets, each comprised of overlapping genomic fragments. Comparisons of exon sequences corresponding to the LBD of PPAR $\beta$  suggested that they contained distinct sequences. Based on the genomic sequence information, oligonucleotide primers were designed to be specific for each PPAR $\beta$  sequence. Initially primers were directed to the regions adjacent to the termination codons of each of three gene sequences (Fig 1; L1, L29 and L92 clone sets). For the L1 clone set a single cDNA was isolated (designated ssPPAR $\beta$ 1A) which was found to contain a full-length open reading frame for PPAR $\beta$ . For L92 a partial cDNA was obtained containing only the coding region corresponding to the LBD of PPAR $\beta$ . This cDNA was distinct from ssPPAR $\beta$ 1A. No PPAR $\beta$  cDNAs were obtained for the L29 clone set (designated as ssPPAR $\beta$ 1B). Using the partial cDNA sequence derived from the L92 clone set, two more oligonucleotides were designed to areas within the region corresponding to the LBD. These were used in a nested 5'-RACE procedure and two distinct cDNAs were obtained. One of these cDNAs contained an open reading frame for a PPAR $\beta$  isoform distinct from both ssPPAR $\beta$ 1A and the partial L92 cDNA, and was designated ssPPAR $\beta$ 2A (corresponding to the L6 clone set). The other cDNA corresponded, with 100% nucleotide identity in the overlapping region, to the previously isolated partial L92 cDNA. This cDNA contained an open reading frame which lacked a DNA-binding domain but possessed an intact A/B domain and LBD and together with the gene in the L92 clone set was designated ssPPAR $\beta$ 2B. The gene and cDNA sequences corresponding to the exons encoding the LBD of each of the presumed PPAR $\beta$  genes were aligned, together with exon flanking regions (Fig 2.). The percentage identity matrices for these alignments showed that one of the cDNA sequences (sPPAR $\beta$ 1A) was identical to the gene present in the L1 clone set. The other full length cDNA (sPPAR $\beta$ 2A) showed

between 98% and 100% identity to clone set L6, whilst the atypical ssPPAR $\beta$ 2B cDNA showed between 99 and 100% identity to the L92 clone set. Further attempts were made to isolate a cDNA (ssPPAR $\beta$ 1B) corresponding to the L29 genomic sequence from gill, kidney and muscle cDNA but were unsuccessful. It should be noted that the materials for the construction of genomic and cDNA libraries were derived from different individuals, thus the small differences observed between these cDNAs and genes are most likely the result of allelic variation within salmon populations. Also apparent from the identity matrices is that the clone sets fall into two groups of two genes each, with about 80% identity between members from different groups. Exon flanking sequences from the genes were compared to determine whether the four genes were from distinct loci, or were allelic variants from two loci. From Fig 2., it is apparent that, although there is some similarity in the exon flanking regions between L29 and L1, and between L6 and L92 clone sets, there is a lower level of identity than might be expected of allelic variants. This indicates that there are at least four distinct PPAR $\beta$  genes in salmon.



**Figure 1. Schematic representation of Atlantic Salmon PPAR $\beta$  genes and cDNAs.**

L1, L29, L6 and L92 are overlapping phage insert sets and the exons present are indicated as open boxes. ssPPAR $\beta$ 1A,  $\beta$ 2A and  $\beta$ 2B are the cDNAs isolated using the primers P1 to P8 (see text), with the open reading frames indicated by open boxes. The ssPPAR $\beta$ 1B cDNA, corresponding to insert set L29 was not isolated. The diagram is not to scale.

### Atlantic salmon PPAR $\beta$ phylogeny

Comparison of the deduced amino acid sequences of the full-length ssPPAR $\beta$ 1A and ssPPAR $\beta$ 2A cDNAs clearly demonstrated that both salmon PPARs phylogenetically clustered with PPAR $\beta$  subtypes from diverse vertebrates (Fig 3). The salmon PPARs were most closely related to PPARs from other fish species. However, it is notable that, within the PPAR $\beta$  phylogeny, the two zebrafish PPAR $\beta$  isoforms do not resolve on the same branches as the salmon isoforms, and in any case their positions in the phylogeny are not well supported by bootstrap values. Most amino acid identity between the salmon isoforms and PPAR $\beta$  from other vertebrates was evident in the DNA-binding and ligand-binding regions, although there were also areas of cross-species identity in both the AB-domain and D-domain (Fig 4). In both ssPPAR $\beta$ 1A and ssPPAR $\beta$ 2A the amino acid residues which interact with fatty acid ligand and with co-activator proteins are conserved. (Nolte et al., 1998; Xu et al., 1999; Fyffe et al., 2006, Fig 4).

EXON 5								
	$\beta$ 1A	$\beta$ 1A	L29	L6	L92	$\beta$ 2A	$\beta$ 2B	
	$\beta$ 1A	—	93.2	80.7	79.2	79.7	78.7	
TAACTAATACCCCTCCCCATAG	L29	93.2	—	80.2	78.7	79.7	78.3	GATATACAGAACACACCAATT
TTTCTCTCTCTCTCCCCGGCAG	L6	80.7	80.2	—	90.8	98.1	90.3	GATGGTATGGACCACGGTCTCC
TTGCTTTCTCTCTGTCCCCTCAG	L92	79.2	78.7	90.8	—	91.3	99.5	GTACGGCACGGAGATAGAGATAT
	$\beta$ 2A	79.7	79.7	98.1	91.3	—	90.8	
	$\beta$ 2B	78.7	78.3	90.3	99.5	90.8	—	
EXON 6								
	$\beta$ 1A	$\beta$ 1A	L1	L29	L6	$\beta$ 2A	$\beta$ 2B	
	$\beta$ 1A	—	100	93.9	86.9	87.3	87.3	
TGGTTCTCCCTCCCC-CCACCAG	L1	100	—	93.9	86.9	87.3	87.3	GATGTGCAGATTCTATACTGGT
CTGTTCTCCCTCCCCGCCACCAG	L29	93.9	93.9	—	86.1	85.7	85.7	GATGTGCTGGTTCTGTACTGGT
ATTTACCTGCCTGCTTCCATCAG	L6	86.9	86.9	86.1	—	94.3	94.3	GATGGTATACTTTTGACTAGT
AATGTTTTGCTAGTCTGCCTCAG	L92	88.1	88.1	86.5	95.1	99.2	99.2	GATGGTTTACTTCTGTAGGAGT
	$\beta$ 2A	87.3	87.3	85.7	94.3	—	99.2	
	$\beta$ 2B	87.3	87.3	85.7	94.3	99.2	—	
EXON 7								
	$\beta$ 1A	$\beta$ 1A	L1	L29	L92	$\beta$ 2A	$\beta$ 2B	
	$\beta$ 1A	—	100	94.8	82.3	82.3	82.3	
TTCCCTCTCTCTCCTCCTGTCTAG	L1	100	—	94.8	82.3	82.3	82.3	TGACTGGACTGGTTTAGCTTCCT
CCTCAGCTCTCTCCTCCTCCACAG	L29	94.8	94.8	—	82.7	82.7	82.7	TGACTGGACTGACTGGACCAGA
AACTATTCTCTATCCATCTGCAG	L92	82.3	82.3	82.7	—	100	100	TGACTGGACCGGACTAATCTGT
	$\beta$ 2A	82.3	82.3	82.7	100	—	100	
	$\beta$ 2B	82.3	82.3	82.7	100	100	—	

**Figure 2. Identity matrices and exon flanking regions of Atlantic salmon PPAR $\beta$  genes and cDNAs comprising the region corresponding to the ligand-binding domain.**

Numbers indicate the percentage identity between pairs of sequences. L1, L6, L29 and L92 are the gene sequences (boxed). The flanking exon sequences are shown with the AG/GT splice junctions and TGA termination codons underlined.  $\beta$ 1A,  $\beta$ 2A and  $\beta$ 2B are the ssPPAR $\beta$  cDNA sequences.

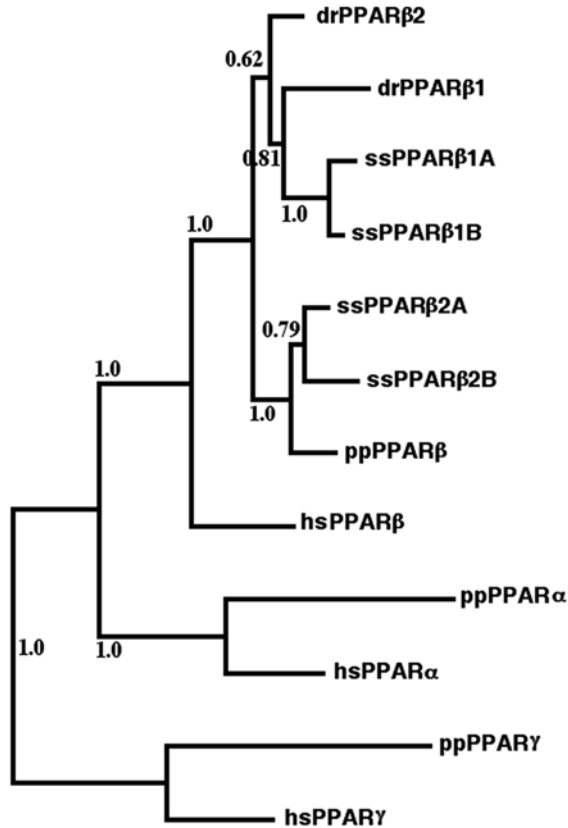
### Differential tissue expression of salmon PPAR $\beta$ genes

QPCR analysis of salmon PPAR $\beta$  expression across a range of tissues indicated that there were differences in relative expression levels (Fig 5). SsPPAR $\beta$ 1A was most highly expressed in liver and adipose, whilst ssPPAR $\beta$ 2A was most highly expressed in gill and adipose. The levels of ssPPAR $\beta$ 1A exceeded those of ssPPAR $\beta$ 2A in liver. In gill PPAR $\beta$ 2A was the predominant isoform and both were expressed equally in adipose. The expression levels in other tissues were 3 to 5 fold lower than in liver, gill and adipose and there were no differences between the expression level of PPAR $\beta$ 1A and ssPPAR $\beta$ 2A.

### Differential transactivation of salmon PPAR $\beta$ subtypes

In cellular transfection assays (Fig. 6) there was a relatively small but significant activation of ssPPAR $\beta$ 1A by palmitoleic acid. In contrast there was no apparent effect of oleic, linolenic, arachidonic, eicosapentenoic or docosahexaenoic acids. There was a much greater response to the mammalian PPAR $\beta$ -specific ligand GW501516 and to bromo-palmitate. However L165041, another mammalian PPAR $\beta$ -selective ligand, was not an effective activator of salmon ssPPAR $\beta$ 1A. Note that all compounds that resulted in



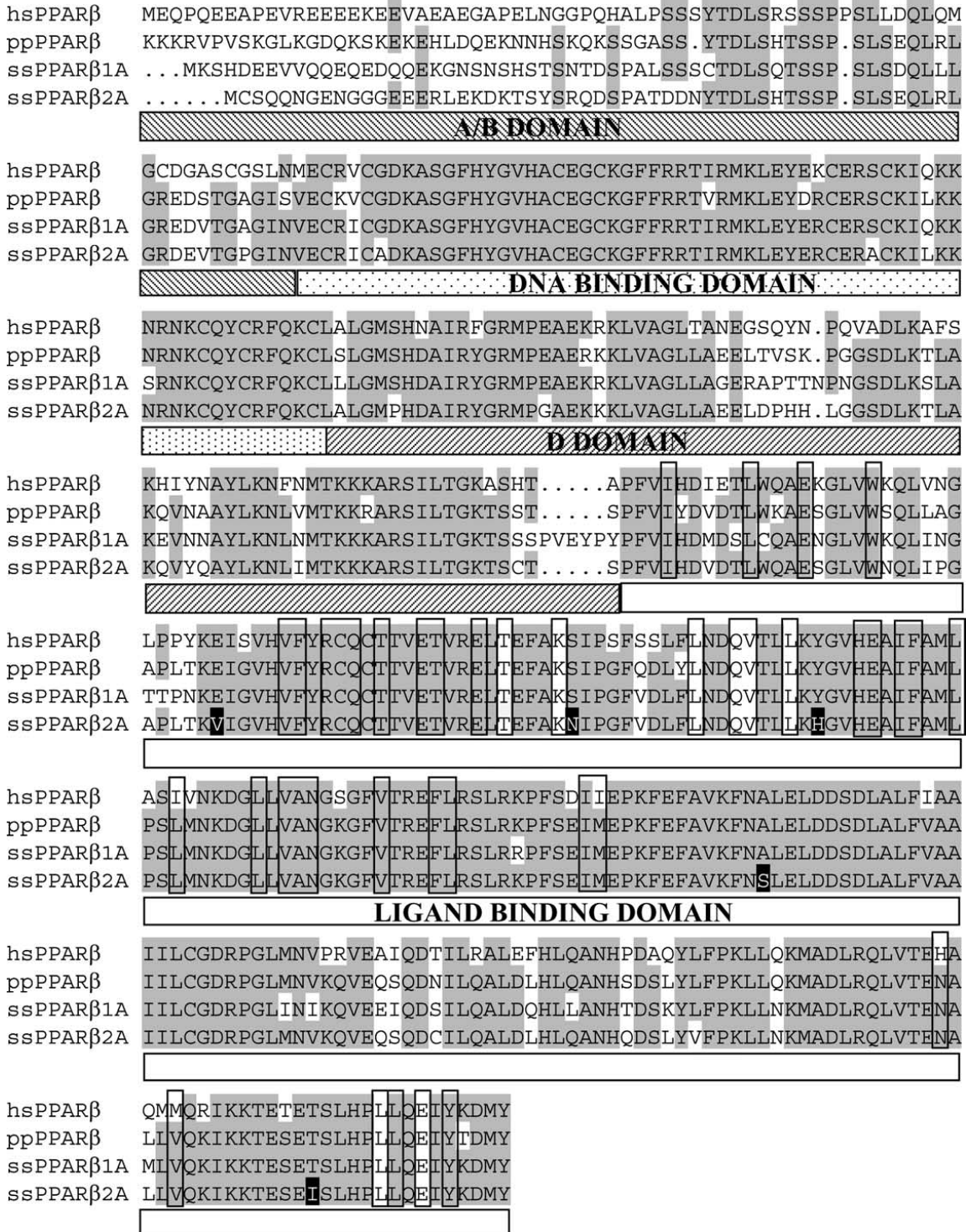


**Figure 3. Phylogenetic tree for the ligand binding domain (LBD) of PPARs from diverse species.**

The deduced amino acid sequences corresponding to the LBD of the PPAR isotypes from plaice (pp), Atlantic salmon (ss), human (hs), and zebrafish (dr) were aligned using ClustalW and the resulting data used to construct an unrooted tree using Bayesian inference (MrBayes 3; Ronquist & Huelsenbeck, 2005). The tree was plotted with NJplot. Confidence values of branch topologies are indicated.

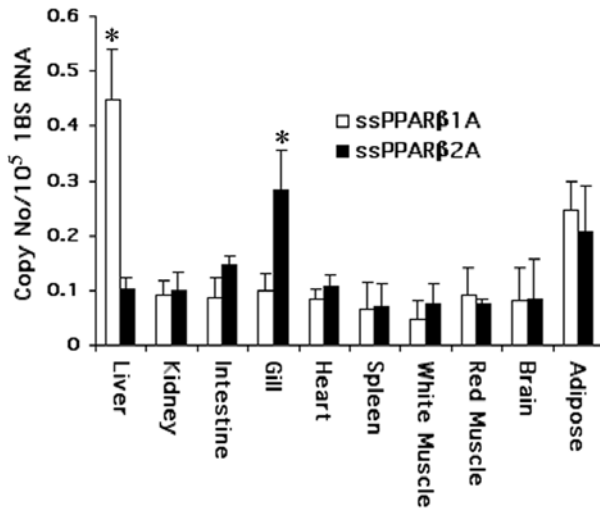
the activation of ssPPAR $\beta$ 1A also induced the basal activity of the reporter construct suggesting the presence of endogenous ssPPAR $\beta$ 1A. Indeed, QPCR indicated that ssPPAR $\beta$ 1A was present (not shown) in the AS cell line although at lower levels per unit input RNA than any of the salmon tissues tested. This may indicate that fatty acids are effective activators of ssPPAR $\beta$ 1A, since they all induced reporter gene activity significantly above that induced by the vehicle ethanol. Transfection of the ssPPAR $\beta$ 2A significantly reduced the basal level of transcription from the reporter construct with all tested compounds (Fig 6), suggesting repression of endogenous ssPPAR $\beta$ 1A activity in the AS cell line. To test this AS cells were transfected with both ssPPAR $\beta$ 1A and ssPPAR $\beta$ 2A and reporter gene activation measured after GW501516 treatment. The results confirmed (Fig 7) that ssPPAR $\beta$ 2A repressed both the basal and GW501516-induced activity of ssPPAR $\beta$ 1A as well as the endogenous basal and induced activity.

## Atlantic salmon PPAR $\beta$



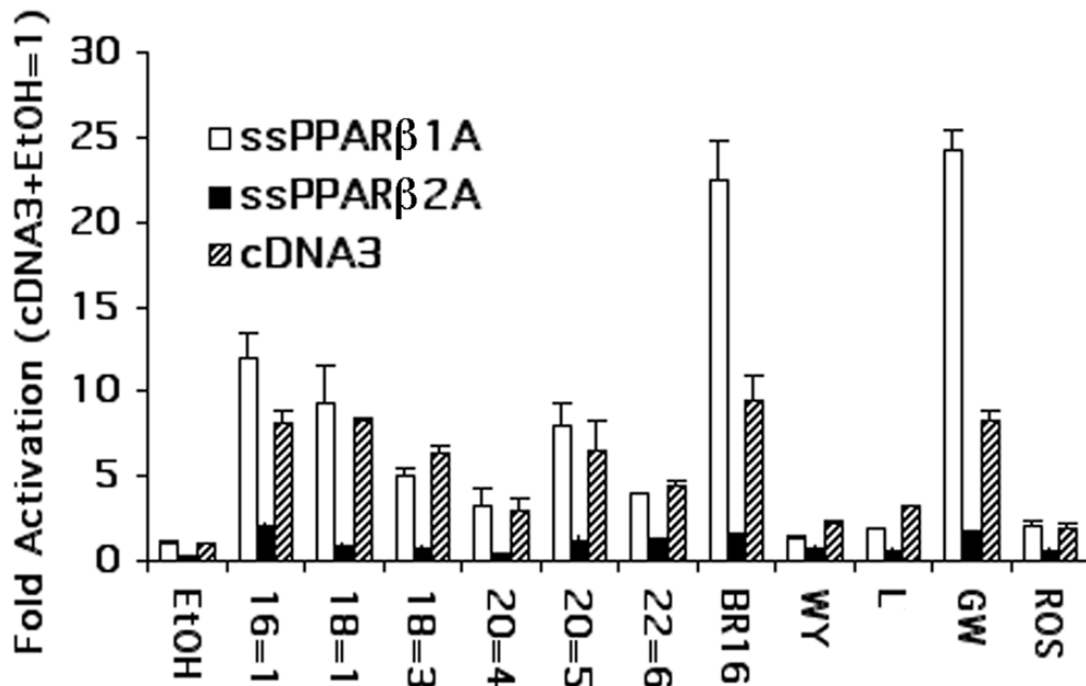
**Figure 4. Comparison of PPAR $\beta$  from Atlantic salmon, plaice and human**

Alignment was generated with ClustalW and identical residues present in 3 or more of the four sequences are shaded. The functional domains of the proteins are indicated by shaded or unshaded, labeled boxes below the sequence line. The positions of residues which are not conserved in ssPPAR $\beta$ 2A are highlighted. Residues involved in human PPAR $\beta$  ligand binding are boxed and shaded. Residues involved in human PPAR co-activator binding are boxed and unshaded.



**Figure 5. Tissue expression profile of Atlantic salmon PPAR $\beta$  isotypes**

Results of QPCR assays as described in text. Data represents mean  $\pm$  SD from three individual salmon. PPAR $\beta$  was normalised to 18S RNA. Both genes were quantified by comparison with a standard curve of linearised plasmid containing the corresponding insert cDNA. Asterisks indicate tissues in which one of the PPAR subtypes significantly ( $P < 0.05$ ; t-test) differs from the other.



**Figure 6. Transactivation of Atlantic salmon PPAR $\beta$**

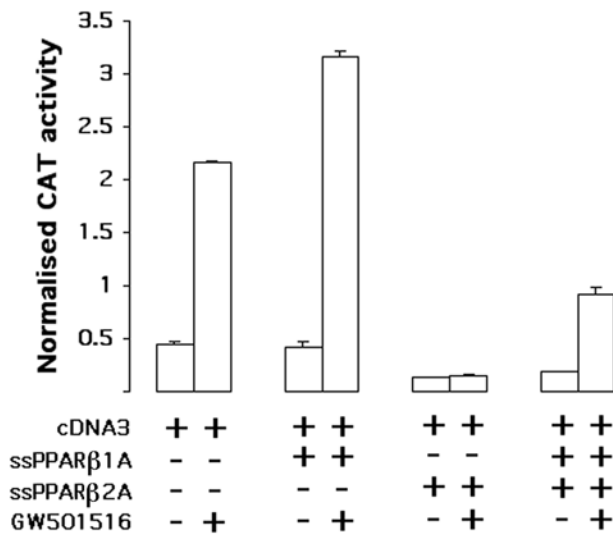
AS cells were transfected with the expression vector pcDNA3, alone (cDNA3) or containing the coding sequences for the two Atlantic salmon PPARs, a CAT reporter construct containing the Cyp4A6-Z PPRE and a  $\beta$ -galactosidase expression plasmid. Cells were treated with 100 $\mu$ M palmitoleic acid (16=1), oleic acid (18=1), linolenic acid (18=3), arachidonic acid (20=4), eicosapentenoic acid (20=5), docosahexenoic acid (22=6), 50 $\mu$ M 2-bromopalmitate (BR16) 50 $\mu$ M Wy-14,643 (WY) 10 $\mu$ M L165041 (L), 10 $\mu$ M GW501516 (GW), 10 $\mu$ M rosiglitazone (ROS) or the vehicle ethanol (EtOH). Results are expressed as the fold increase in CAT (after subtraction of mock transfected background and normalisation to  $\beta$ -galactosidase) with respect to the ethanol control values for empty vector (pcDNA3). Figures represent a single experiment out of two repetitions and all treatments were in triplicate. Error bars correspond to standard deviations of the mean.

## Discussion

Atlantic salmon are both a declining wild species with a complex and characteristic life cycle, and a commercially valuable farmed species. The management of both wild and farmed stocks is as rich in social, economic and political issues as its resource base is rich biologically. However a particular biological problem which concerns both the survival of wild fish and the quality of farmed fish is related to the control of energy and particularly lipid homeostasis. PPARs are now known to be critical regulators of these processes. The study presented here is the first report of the functional characterisation of PPAR subtypes from a salmonid species, the Atlantic salmon. Phylogenetic analysis shows that the four genes described here all belong to the PPAR $\beta$  subfamily. In mammals, where PPARs have been studied most intensively, a single gene encodes for PPAR $\beta$  and studies in other fish species have identified varying numbers of PPAR $\beta$  subtypes. BLAST searches of near complete genome sequences for the pufferfishes, *Takafugu rubripes* and *Tetraodon nigroviridis*, reveal the presence of only one PPAR $\beta$  gene. Similarly in plaice and sea bream it has previously been reported that only one PPAR $\beta$  gene is detectable (Leaver et al., 2005). This compares to the situation in zebrafish, where, from genomic sequence and published information, there is clear evidence of two PPAR $\beta$  isoforms (Robinson-Rechavi et al, 2001; Leaver et al., 2005). Of the four Atlantic salmon sequences identified here at least two appear to be functional genes for PPAR $\beta$  (termed here ssPPAR $\beta$ 1A and ssPPAR $\beta$ 2A) as shown by the identification of corresponding cDNAs containing complete coding sequence. It is not clear whether our failure to isolate fully resolved cDNAs for the other two gene sequences is because that these are not functional genes or because they are not expressed. It is nevertheless important to note that for one of the other genes (ssPPAR $\beta$ 2B) we were able to isolate a cDNA with an open reading frame containing an AB-domain, a D-domain and a ligand-binding domain but lacking a DNA-binding domain. It is not yet clear whether this represents an aberrantly or partially processed mRNA or codes for an atypical but functional PPAR $\beta$  subtype. For the other gene (ssPPAR $\beta$ 1B), through sequence homology, we could identify six of seven coding exons on a single lambda genomic clone (not shown: Acc nos AM229298 and AM229299). The first coding exon of ssPPAR $\beta$ 1B could not be definitively established because of the uncertainty in predicting this less conserved region of the gene by homology. Nevertheless, it is likely that this gene also encodes a functional PPAR. Attempts to isolate a cDNA for this gene from a variety of tissue were not successful suggesting that either it is not expressed or it is expressed in a tissue or developmentally restricted manner.

The presence of four PPAR $\beta$  genes in salmonids is most probably a result of a relatively recent polyploidisation event (60-90 million years) which is clearly evident in the evolution of salmonids (Allendorf and Thorgaard, 1984). Moreover more ancient polyploidisation events have been proposed as a driving force for the evolution of the vertebrate lineage (Ohno, 1970). One of these has been proposed to have occurred in the early evolution of ray-finned fishes (Taylor et al., 2003) and recent comparative syntenic analysis of zebrafish and pufferfish genomes (Woods et al., 2005) have supported this event and inferred subsequent lineage-specific duplications or losses to account for the differences in gene numbers between these species. The four PPAR $\beta$  genes in Atlantic

salmon clearly group into two subfamilies most likely representing the polyploidization-dependent duplication of two PPAR $\beta$  subtypes from an ancestral diploid salmonid, these two genes arising from the more ancient ray-finned fish event. The phylogenetic analysis suggests that these two salmon subfamilies are not orthologous to the two PPAR $\beta$  subtypes in zebrafish, although it should be noted that the zebrafish sequences are not placed in the tree with high confidence and a definitive conclusion must await functional characterisation of the zebrafish PPAR $\beta$  subtypes.



**Figure 7. ssPPAR $\beta$ 2A represses the activity of ssPPAR $\beta$ 1A**

AS cells were transfected with cDNA3 (no PPAR) alone, or in combination with ssPPAR $\beta$ 1A and/or ssPPAR $\beta$ 2A. Cells were treated with GW501516 or with the vehicle, ethanol, as indicated. Results are expressed as arbitrary units of normalised CAT activity. Figures represent a single experiment out of two repetitions and all treatments were in triplicate. Error bars correspond to standard deviations of the mean.

From an evolutionary perspective, a key question surrounding the process of polyploidization is how an individual with a polyploid genome would gain a selective advantage in a population and thence give rise to a new polyploid species. For such polyploidized individuals to reproduce successfully they are required to undergo a process of diploidization concurrent with functional divergence of duplicated loci to obtain a selective advantage over their diploid relatives (Wolfe, 2001). The genetic divergence of PPAR $\beta$  subtypes in salmon may be an example of the outcome of this diploidization process, and as such these genes would be expected to exhibit functional divergence. Indeed, our results indicate that ssPPAR $\beta$ 1A or ssPPAR $\beta$ 2A are differentially expressed and exhibit distinct activation characteristics. Levels of ssPPAR $\beta$ 1A predominate in liver and ssPPAR $\beta$ 2A predominate in gill and the differential expression of salmon PPAR $\beta$  isoforms has functional significance when the cellular transfection results are considered. It would appear that ssPPAR $\beta$ 1A is similar in ligand-activation profile to PPAR $\beta$  from other species (Forman et al 1997; Oliver et al., 2001; Leaver et al., 2005) being responsive to palmitoleic and oleic acids and to 2-bromopalmitate. Importantly it is highly activated by the mammalian PPAR $\beta$ -selective ligand GW501516, but not activated by the PPAR $\alpha$ -specific ligand WY146463. The response to GW501516 suggests that, as with PPAR $\beta$  in mammals, this compound is a ligand for ssPPAR $\beta$ 1A. The identification of highly selective and potent ligands for fish PPARs is a necessary step in advancing understanding in this area and further studies are required to test whether GW501516 will be as selective and potent in salmon as it is in

mammals. On the other hand, the lack of response of ssPPAR $\beta$ 2A to fatty acids or PPAR $\beta$ -selective ligands is intriguing. Consideration of the LBD of ssPPAR $\beta$ 2A suggests that this may be because there are critical differences between the sequence of this isoform and the sequences of mammalian or other characterized fish PPAR $\beta$  homologues. A number of amino acids residues are not conserved in the ssPPAR $\beta$ 2A subtype (Fig 4) which may change the characteristics of the LBD. However, none of these substitutions are at positions shown to be involved in either fatty acid ligand binding (Xu et al, 1999; Fyffe et al., 2006) or in co-activator binding to human PPAR (Nolte et al, 1998), suggesting that ssPPAR $\beta$ 2A may be capable of binding and being activated by as yet undiscovered ligands. It is possible that, if the two salmon subtypes have conserved functions and binding specificity, the true endogenous ligand for vertebrate PPAR $\beta$  may not be fatty acids but might in future be identified as a compound which activates both ssPPAR $\beta$ 1A and ssPPAR $\beta$ 2A. Alternatively, the non-conserved substitutions or other characteristics of ssPPAR $\beta$ 2A may prohibit ligand-activation under any circumstances and its sole function may be to repress the activity of other PPARs. Mammalian PPAR $\beta$  has been shown to repress the activity of the other PPAR subtypes by competing for binding to PPRES (Shi et al., 2002). In this respect, the repressive ability of ssPPAR $\beta$ 2A in both basal and GW501516-induced transcriptional activity is significant. The high levels of expression of ssPPAR $\beta$ 2A in gill, in combination with its repressive activity, may have specific implications for the action of other PPAR subtypes in this tissue. As the fish gill is in close contact with the external environment, a single layer of epithelial cells separating the blood from the water, it is possible that in this tissue ssPPAR $\beta$ 2A functions to repress the activity of other PPARs in order to prevent their un-programmed activation by exogenous compounds or contaminants. Thus Atlantic salmon may have evolved novel PPAR $\beta$ -dependent processes. In this regard it is important to note that other salmonids, such as rainbow trout and brown trout (*Onchorhynchus mykiss*; *Salmo trutta*) are also polyploid and would thus contain homologues of the four salmon PPAR $\beta$  genes. Previous studies (Liu et al, 2005; Batista-Pinto et al., 2005) of tissue expression profiles and PPAR-agonist responses in these species have only considered a single PPAR $\beta$  subtype identified from partial cDNA sequences. In future, and in the light of these results from salmon, such studies should be conducted with the expectation of the presence of multiple PPAR subtypes with divergent function in trout and other salmonids.

In conclusion, these studies demonstrate the possibility for unexpected levels of variety in PPAR $\beta$  subtype and mechanism of action in Atlantic salmon, showing that further complexity in physiological control may result from functional divergence of duplicated nuclear receptor genes. Further studies are required to understand the role of these important regulators of lipid homeostasis in life cycle and evolutionary adaptation in salmonids and to determine common PPAR-dependent processes across all vertebrates.

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