

**LIPID HOMEOSTASIS IN FARMED FISH:
ROLE OF PEROXISOME-PROLIFERATOR
ACTIVATED RECEPTOR-GAMMA (PPAR γ)**



THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

By

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DECLARATION

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree.

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ABSTRACT

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily. Three PPARs (PPAR α , PPAR β and PPAR γ) exist in mammals, and all are activated by binding lipid molecules, including fatty acids and their derivatives, and also by synthetic drug ligands. Together, these three receptors are critical regulators of lipid and energy homeostasis in mammals. PPAR γ is a central factor in fat uptake and storage and is required for adipocyte differentiation. Fish are now known to have homologues of the three PPAR isotypes, although in many species there is more than one representative of each. Piscine PPAR γ is of particular interest in finfish aquaculture, since under aquaculture conditions fish often accumulate excess visceral and hepatic fat. This can affect the health and welfare of the fish, and also represents an economic waste of valuable resources that might otherwise be channelled into growth. However, piscine PPAR γ has some important structural differences to the mammalian counterpart, and is not activated by fatty acids or synthetic ligands. Although presumed to have an important role in fat accumulation, further research on piscine PPAR γ has been hampered by this failure to identify activating compounds.

The aim of this project is to identify activators for piscine PPAR γ , and then to discover the effects of PPAR γ activation on fish lipid and energy metabolism. In addition, given the variability in numbers of PPAR genes in fish species, the PPAR complement of the salmon genome was investigated. Atlantic salmon is

an important aquaculture species and unlike most other vertebrates, was found to contain two PPAR γ genes with distinct tissue expression profiles. To discover activating compounds for fish PPAR γ , total lipid was extracted from salmon liver tissue and fractionated into different lipid classes. Lipid fractions obtained were then tested in a high-throughput cell-based transactivation screen for fish PPAR activity in a Chinook salmon embryo (CHSE-214) cell line. Two polar lipid fractions believed to contain ceramides significantly increased PPAR γ -dependent transactivation of a luciferase reporter gene. The molecular species in two of these fractions were analysed by LC-MS, confirming the presence of various ceramide and sphingolipid species. Application of pure glucosylceramide (GlcCer) in the cell transfection assay resulted in PPAR γ activation. The identification of activating lipids for piscine PPAR γ will enable further study on the physiological functions of this receptor in fish and under aquaculture conditions. Ultimately this knowledge could lead to improvements in finfish feed formulation to better optimise the relationship between lipid input, fat accumulation and growth.

TABLE OF CONTENTS

DECLARATION.....	2
ACKNOWLEDGEMENTS	3
ABSTRACT	4
LIST OF TABLES	13
LIST OF FIGURES	15
LIST OF ABBREVIATIONS	23
1 General Introduction.....	26
1.1 Current status of aquaculture.....	26
1.1.1 Use of lipids and fatty acids in aquaculture feeds.....	26
1.2 Lipids.....	27
1.2.1 Polar lipids or phospholipids.....	28
1.2.2 Neutral lipids.....	30
1.3 Peroxisome proliferator-activated receptors (PPARs).....	31
1.3.1 Origin of PPARs and their structure	31
1.3.2 Discovery of piscine PPARs and their structure.....	34
1.3.3 Tissue-specific distribution of piscine PPARs	38

1.3.4	PPAR activation.....	45
1.4	Cell culture	49
1.5	Thesis aims and objectives	51
2	General materials and methods.....	53
2.1	Preparation of media and buffers	53
2.1.1	Preparation of 50x TRIS/acetate/EDTA (TAE) buffer.....	53
2.1.2	Preparation of Luria-Bertani (LB) broth	53
2.1.3	Preparation of antibiotic ampicillin solution.....	53
2.1.4	Preparation of Luria-Bertani (LB) agar with ampicillin.....	54
2.2	Preparation of pure plasmid DNA	54
2.2.1	Transformation of plasmid DNA into <i>Escherichia coli</i> (<i>E. coli</i>)	54
2.2.2	Inoculation of bacterial cells containing plasmid DNA.....	55
2.2.3	Purification of plasmid DNA.....	55
2.2.4	Determination of plasmid integrity by Agarose Gel Electrophoresis.....	57
3	Development of a luciferase reporter assay and the optimisation of transfection methods using branched polyethylenimine (bPEI) transfection reagent	59
3.1	Introduction.....	59

3.1.1	Development of a reporter gene construct.....	59
3.1.2	Transfection	65
3.1.3	Transfection of fish cell lines	70
3.2	Materials and methods.....	72
3.2.1	Construction of Gal4-PPAR γ expression plasmid.....	72
3.2.2	Cell culture	77
3.2.3	Optimisation 1: Transfection of cell lines.....	80
3.2.4	Optimisation 2: Transfection using CHSE-214 cell line.....	88
3.2.5	Treatment of transfected CHSE-214 cells with PPAR ligands and fatty acids	89
3.3	Results.....	91
3.3.1	Construction of Gal4-PPAR γ expression plasmid.....	91
3.3.2	Transfection efficiency of bPEI reagent	94
3.3.3	Re-optimisation of AS and CHSE-214 cell lines.....	97
3.3.4	Transactivation of Gal4-PPAR with PPAR ligands and fatty acids.....	103
3.4	Discussion.....	105
3.4.1	Determination of a suitable cell line	105
3.4.2	Ratio of bPEI:DNA and transfection efficiency	107

3.4.3	Response of Gal4-PPARs to PPAR agonists.....	109
3.4.4	Response of Gal4-PPARs to fatty acids	111
3.4.5	Use of non-commercial dual luciferase assay system	112
3.5	Conclusions	114
4	Lipid extraction and response of piscine PPARγ to lipid fractions	115
4.1	Introduction.....	115
4.2	Materials and methods.....	118
4.2.1	Lipid and fatty acid analyses.....	118
4.2.2	Treatment of transfected CHSE-214 cells with lipids	123
4.2.3	Identification of molecular components in lipid samples	126
4.3	Results.....	128
4.3.1	Lipid content and lipid class composition of liver tissues.....	128
4.3.2	Response of Gal4-PPAR γ to polar and neutral lipids.....	133
4.3.3	Determination of molecular components in lipids.....	134
4.4	Discussion.....	137
4.4.1	Phospholipids (polar lipids) and neutral lipids composition	137
4.4.2	Activation of Gal4-PPAR γ by polar lipids.....	140

4.4.3	Identification of polar lipid fractions.....	140
4.5	Conclusions	142
5	Response of fish PPARγ to sphingolipids.....	143
5.1	Introduction.....	143
5.2	Materials and methods.....	149
5.2.1	Seeding and transfection of CHSE-214 cells.....	149
5.2.2	Treatment of transfected cells with sphingolipids and their metabolites 150	
5.2.3	Luciferase activity, data normalisation and statistical analysis	150
5.3	Results.....	152
5.4	Discussion.....	154
5.4.1	Ceramide and its precursors.....	155
5.4.2	Glucosylceramide as a potential activating compound of PPAR γ	157
5.5	Conclusions	160
6	Phylogeny and tissue distribution of peroxisome proliferator- activated receptors (PPARs) in Atlantic salmon (<i>Salmon salar</i>)	161
6.1	Introduction.....	161
6.2	Materials and methods.....	164

6.2.1	Sampling.....	164
6.2.2	RNA extraction	164
6.2.3	Cloning of Atlantic Salmon PPAR γ L cDNA.....	166
6.2.4	Complementary DNA (cDNA) synthesis.....	166
6.2.5	Quantitative polymerase chain reaction (qPCR) primers	168
6.2.6	Tissue expression analysis.....	170
6.2.7	Data normalisation and statistical analysis	171
6.2.8	Phylogenetic analysis	172
6.3	Results.....	173
6.3.1	PPAR γ L mRNA sequence.....	173
6.3.2	Phylogenetic analysis	173
6.3.3	Distribution patterns of Atlantic salmon PPARs	180
6.4	Discussion.....	186
6.5	Conclusion	191
7	General discussion and conclusions.....	192
7.1	Introduction.....	192
7.2	Suitable cell line for cellular transactivation assay.....	193

7.3	Lipid extraction from liver tissues	194
7.4	Piscine PPARγ and its potential ligand	195
7.5	Phylogeny and tissue expression patterns of Atlantic salmon PPARs	
	198	
7.6	Conclusions	200
REFERENCES	201

LIST OF TABLES

Table 3-1 Primer (sequences) used in the sequencing of pBIND vector containing ligand-binding domain of PPAR γ	76
Table 3-2 The four transfection mixes prepared using two-part mixing in salt-free buffer method with bPEI transfection reagent, each containing the Gal-PPAR α , pGL4.31[<i>luc2P/Gal4UAS/hygro</i>] and pGL4.75[<i>hRluc</i> /CMV] at a ratio of 1:1:1 and different amounts of bPEI reagent. bPEI reagent was added before each of the four transfection mixes was split between 3 wells containing AS cells.....	82
Table 3-3 The three transfection mixes prepared using two-part mixing in salt-free buffer method with bPEI transfection reagent, each containing DNA mix of Gal4-PPAR α , pGL4.31[<i>luc2P/Gal4UAS/hygro</i>] and pGL4.75[<i>hRluc</i> /CMV] at different ratio 1:1:1, 1:1:0.5 and 1:1:0.2, respectively. bPEI reagent was added before each of the three transfection mixes was split between 3 wells containing AS cells.....	86
Table 3-4 The six transfection mixes prepared using two-part mixing in salt-free buffer method with bPEI transfection reagent, each containing the Gal4-PPAR α , pGL4.31[<i>luc2P/Gal4UAS/hygro</i>] and pGL4.75[<i>hRluc</i> /CMV] at ratio of 1:1:0.5, respectively. The bPEI:DNA ratio set ups were 1:1, 2.5:1, 4:1, and doubling the amount of bPEI and DNA to the three ratios. bPEI reagent was added before each of the six transfection mixes was split between 3 wells containing CHSE-214 cells.....	88

Table 4-1 Lipid content and lipid class composition of liver tissues.	129
Table 4-2 Fatty acid composition (%) of two pure lipid fractions UNK1 (unknown polar lipid) and SF (solvent front). Fatty acid is given as a percentage of the total fatty acid content. Abbreviation: polyunsaturated fatty acid (PUFA).....	135
Table 5-1 Sphingolipids classes and subclasses. Adopted from Fahy <i>et al.</i> (2005).....	144
Table 6-1 Nucleotide sequences of primers used for PCR amplification of cDNA fragments encoding ssPPARs.....	169

LIST OF FIGURES

- Figure 1-1 Schematic structure of phosphoglyceride..... 29
- Figure 1-2 Functional domains of peroxisome proliferator-activated receptors (PPARs) (adopted from Schoonjans *et al.*, 1996) 33
- Figure 3-1 Schematic diagram of the two constructs in the Gal4-UAS system.
A: The Gal4-PPAR construct consisted of PPAR-LBD cloned downstream of the Gal4-DBD, whose expression was driven by the CMV promoter. B: The Gal4UAS-luc construct consisted of 5 Gal4UAS sequences upstream of a TATA box, which was upstream of a firefly luciferase enzyme..... 62
- Figure 3-2 Schematic illustration of the Gal4-UAS assay system. A: The interaction between the two plasmid constructs involves the binding of the Gal4UAS sequences with the Gal4-DBD, forming a complex, which remains transcriptionally inactive due to the association of PPAR-LBD with co-repressor proteins. B: Binding of ligand to the ligand-binding domain of PPAR leading to a conformational change in the Gal4-PPAR construct, releasing the co-repressor proteins and subsequently, recruitment of co-activator proteins. AF-2 becomes activated and this ultimately results in an increase in the transcription of the firefly luciferase reporter enzyme. 63
- Figure 3-3 Nucleotide and deduced amino acid sequences of Gal4-PPAR γ contigs assembled using SeqManPRO software. The first and stop codons of PPAR ligand-binding domains are boxed in blue. The BamH1

(GGATCC) and Kpn1 (GGTACC) enzyme recognition sites are boxed in red. 94

Figure 3-4 The mean transfection efficiencies (+ SD) at four different bPEI reagent to DNA ratios (μg to μg), containing equal amounts of plasmid DNA i.e. Gal4-PPAR α , pGL4.31[*luc2P/Gal4UAS/hygro*] and pGL4.75[*hRluc*/CMV]. Different ratios of bPEI:DNA were used to transfect AS and CHSE-214 before they were assayed for *Renilla* luciferase signal, used as a measurement of transfection efficiency..... 95

Figure 3-5 The mean firefly luciferase signals (+ SD) measured from transfected AS cells at 2.5:1, bPEI to DNA ratio, with different amounts of plasmid DNAs, Gal4-PPAR α , pGL4.31[*luc2P/Gal4UAS/hygro*] and pGL4.75[*hRluc*/CMV], respectively and treated with 95 % ethanol vehicle and PPAR α agonist, WY-14,643 compound. 96

Figure 3-6 The mean firefly luciferase signals (+ SD) from the previous transfection experiment (labelled "old", from Figure 3-2), plotted against the latest firefly luciferase signals measured from the same experiment (labelled "new"). AS cells were transfected with different amounts of plasmid DNAs, Gal4-PPAR α , pGL4.31[*luc2P/Gal4UAS/hygro*] and pGL4.75[*hRluc*/CMV], at 2.5:1, bPEI to DNA ratio, and treated with 95 % ethanol vehicle and PPAR α agonist, WY-14,643 compound. 98

Figure 3-7 Morphology of “new” AS cells (at passage number 121) transfected with pEGFP overnight. Cells were transfected with the following bPEI:DNA ratios: (A) 1:1 (B) 2 x 1:1 (C) 2.5:1 (D) 2 x 2.5:1 (E) 4:1 (F) 2 x 4:1. Bar is approximately 100 μm 100

Figure 3-8 Morphology of CHSE-214 cells transfected with pEGFP overnight. Cells were transfected with the following bPEI:DNA ratio: (A) 1:1 (B) 2 x 1:1 (C) 2.5:1 (D) 2 x 2.5:1 (E) 4:1 (F) 2 x 4:1. Bar is approximately 100 μm 101

Figure 3-9 The mean transfection efficiencies (+ SD) at three different bPEI reagent to DNA ratios (μg to μg). Different ratios of bPEI:DNA were used to transfect CHSE-214 before they were assayed for *Renilla* luciferase signal, used as measurement of transfection efficiency..... 102

Figure 3-10 Transactivation of Gal4-PPAR α and Gal4-PPAR γ in response to PPAR activators and fatty acids. CHSE-214 cells were transiently transfected with Gal4-PPAR α firefly luciferase reporter plasmid pGL4.31 and an internal *Renilla* luciferase reporter used to correct for transfection efficiencies pGL4.75. Post-transfection cells were treated with 25 μM Wy-14,643, 50 μM rosiglitazone and 100 μM fatty acids. Data are the mean (+ SD) in which each treatment was applied in triplicates, expressed as the fold increase over ethanol control of normalised firefly luciferase activity. Asterisks (*) indicate activities significantly different from those in ethanol-treated cells (EtOH=1)... 104

Figure 4-1 HPTLC chromatogram of lipid class compositions in single dimension, double development system of the total lipid extracted from liver tissues, as described in text. Total lipid was run with two lipid standards: cod roe total lipid (TL) and neutral lipids, to identify the location of each lipid class. The plate was stained with 3 % cupric acetate in 8 % phosphoric acid. Abbreviations refer to Table 4-1..... 131

Figure 4-2 HPTLC chromatogram of pure polar and neutral lipids in 10 mg/ml C:M (2:1) prepared from TLC plates developed in single dimension, double development system, as described in text, before each lipid fraction was reconstituted in 5 mg/ml absolute ethanol. Abbreviations refer to Table 4-1..... 132

Figure 4-3 Response of PPAR γ to polar lipids and neutral lipids. Transfected cells were treated with two lipid concentrations: 25 μ g/ml and 50 μ g/ml. Data are the means \pm SD of two independent experiments (n=4 for each experiment). Results are expressed as the fold increase over ethanol control of normalised firefly luciferase activity. Asterisk (*) represents statistically significant difference (P<0.05) to activities in ethanol controls..... 133

Figure 4-4 LC-MS analyses of the (A) unknown polar lipid (UNK1) and (B) solvent front (SF) previously selected as transfected CHSE-214 cells treated with these two lipids fractions showed significantly high PPAR γ activity..... 136

Figure 5-1 Some representative structures for sphingolipids. Adopted from Fahy <i>et al.</i> (2005).....	145
Figure 5-2 Sphingolipid metabolic pathways. Adopted from Halt and Maiti (2017).....	147
Figure 5-3 Response of PPAR γ to sphingolipids at different concentrations. Data are the means \pm SD of three independent experiments. Results are expressed as the fold increase over ethanol control of normalised firefly luciferase activity. Two asterisks (**) represent statistically significant difference (P<0.01) and one asterisk (*) represents statistically significant difference (P<0.05) to activities in ethanol controls.	153
Figure 5-4 Response of empty pBIND to sphingolipids at 5 μ M. Data are the means \pm SD and results are expressed as the fold increase over ethanol control of normalised firefly luciferase activity.	154
Figure 6-1 Alignment of deduced amino acid sequences of salmon and human PPARs. Ligand-binding domains are shown.	174

Figure 6-2 Phylogenetic tree for the full-length PPARs from diverse species.

The numbers represent the frequencies (%) with which the tree was generated using maximum likelihood method with MEGA 7 software. Receptor sequences used were: (1) *Gallus gallus* (chicken) PPARa (NP_001001464), PPARb (NP_990059), PPARg (NP_001001460); (2) *Homo sapiens* (human) PPARa (NP_005027), PPARb (NP_006229), PPARg (NP_005028); (3) *Xenopus laevis* (African clawed frog) PPARa (P37232), PPARb (NP_001081310), PPARg (XP_018095920) (4) *Esox Lucius* (Northern pike) PPARa1 (XP_010882200), PPARa2 (XP_010885449), PPARba (XP_010891645), PPARbb (XP_012993730), PPARga (XP_010872599), PPARgb (XP_010900626); (5) *Sparus aurata* (gilthead seabream) PPARa1 (not submitted to database), PPARa2 (AAT85613), PPARb (AAT85615), PPARg (AAT85618); (6) *Pleuronectes platessa* (European plaice) PPARa2 (CAD62447), PPARb (CAD62448), PPARg (CAB51618); (7) *Danio rerio* (zebrafish) PPARaa (NP_001154805), PPARab (NP_001096037), PPARba (XP_699900), PPARbb (NP_571543), PPARg (NP_571542); (8) *Salmon salar* (Atlantic Salmon) PPARa1a (PPARa, NP_001117032), PPARa1b (PPARaL, XP_014025332), PPARb1a (Q1XE69_SALSA), PPARb1b (Q1XE68_SALSA), PPARb2a (NP_001117031), PPARb2b (XP_014002724), PPARg (XP_014000887), PPARgL (not submitted to database)..... 177

Figure 6-3 Phylogenetic tree for the ligand-binding domain (LBD) of PPARs from diverse species. The numbers represent the frequencies (%) with which the tree was generated using maximum likelihood method with MEGA 7 software. the tree topology presented was replicated after 1,000 replications. Receptor sequences used were: (1) *Gallus gallus* (chicken) PPARa (NP_001001464), PPARb (NP_990059), PPARg (NP_001001460); (2) *Homo sapiens* (human) PPARa (NP_005027), PPARb (NP_006229), PPARg (NP_005028); (3) *Xenopus laevis* (African clawed frog) PPARa (P37232), PPARb (NP_001081310), PPARg (XP_018095920) (4) *Esox Lucius* (Northern pike) PPARa1 (XP_010882200), PPARa2 (XP_010885449), PPARba (XP_010891645), PPARbb (XP_012993730), PPARga (XP_010872599), PPARgb (XP_010900626); (5) *Sparus aurata* (gilthead seabream) PPARa1 (not submitted to database), PPARa2 (AAT85613), PPARb (AAT85615), PPARg (AAT85618); (6) *Pleuronectes platessa* (European plaice) PPARa2 (CAD62447), PPARb (CAD62448), PPARg (CAB51618); (7) *Danio rerio* (zebrafish) PPARaa (NP_0011154805), PPARab (NP_001096037), PPARba (XP_699900), PPARbb (NP_571543), PPARg (NP_571542); (8) *Salmon salar* (Atlantic Salmon) PPARa1a (PPARa, NP_001117032), PPARa1b (PPARaL, XP_014025332), PPARb1a (Q1XE69_SALSA), PPARb1b (Q1XE68_SALSA), PPARb2a (NP_001117031), PPARb2b (XP_014002724), PPARg (XP_014000887), PPARgL (not submitted to database)..... 179

Figure 6-4 Tissue expression profile of female Atlantic salmon PPAR α isotypes. Relative expression of each PPAR was normalised to PPAR expression in muscle. Data are the means \pm SD of results from four individual salmon fish..... 181

Figure 6-5 Tissue expression profile of female Atlantic salmon PPAR γ isotypes. Relative expression of each PPAR was normalised to PPAR expression in muscle. Data are the means \pm SD of results from four individual salmon fish..... 182

Figure 6-6 Tissue expression profile of female Atlantic salmon PPAR β 1 isotypes. Relative expression of each PPAR was normalised to PPAR expression in muscle. Data are the means \pm SD of results from four individual salmon fish. Asterisk (*) represents PPAR expression value, which differs significantly from muscle (P<0.05). 183

Figure 6-7 Tissue expression profile of female Atlantic salmon PPAR β 2 isotypes. Relative expression of each PPAR was normalised to PPAR expression in muscle. Data are the means \pm SD of results from four individual salmon fish. Asterisk (*) represents PPAR expression value, which differs significantly from muscle (P<0.05). 184

Figure 6-8 Gene expression of PPARs in each tissue where the data is normalised to muscle. Data are the means \pm SD of results from four individual salmon fish. Asterisk (*) represents PPAR expression value, which differs significantly from muscle (P<0.05). 185

LIST OF ABBREVIATIONS

AF	Activation Function
AS	Atlantic salmon
BHT	Butylated hydroxytoluene
BLAST	Basic Local Alignment Search Tool
bPEI	Branched polyethylenimine
C1P	Ceramide-1-phosphate
cDNA	Complementary DNA
CHOL	Cholesterol
CHSE-214	Chinook salmon Embryo 214
C:M	Chloroform : Methanol
DBD	DNA-binding domain
DHA	Docosaheptaenoic acid
DPBS	Dulbecco's phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
EMEM	Eagle's Minimum Essential Medium
EPA	Eicosapentaenoic

FAMES	Fatty acid methyl esters
FBS	Fetal bovine serum
FFA	Free fatty acids
GC	Gas chromatography-flame ionisation detection
GlcCer	Glucosylceramide
GlcT-1	Glucosylceramide synthase
HPTLC	High-performance thin-layer chromatography
LB	Luria-Bertani
LBD	Ligand-binding domain
LC-MS	Liquid chromatography-mass spectrometry
LPC	Lysophosphatidylcholine
OEA	Oleoylethanolamide
OFN	Oxygen-free nitrogen
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
pEGFP	Enhanced green fluorescent protein plasmid
PPAR	Peroxisome Proliferator-Activated Receptor

PPRE	Peroxisome Proliferator-Response Element
PUFA	Poly unsaturated fatty acids
qPCR	Quantitative polymerase chain reaction
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RXR	Retinoid X receptor
S1P	Sphingosine-1-phosphate
SE	Sterol esters
SF	Solvent front
SM	Sphingomyelin
TAE	TRIS/acetate/EDTA
TAG	Triacylglycerols
TL	Total lipid
TLC	Thin-layer chromatography
UAS	Upstream Activation Sequence
UNK1	Unknown polar lipid
UNK2	Unknown neutral lipid
WY-14,643	4-Chloro-6-(2,3-xylodino)-2-pyrimidinylthioacetic acid

1 General Introduction

1.1 Current status of aquaculture

While fish production continues to outpace the global growth population, finfish aquaculture remains as one of the fastest-growing food producing sectors (FAO, 2017). In general, the larger aquaculture sector now provides almost 50 % of all fish and seafood for human consumption. The growth in finfish aquaculture is to a large extent dependent on the use of artificial diets and feeds which are developed and formulated to meet the essential requirements that include amino acids, fatty acids, vitamins and minerals, and provide macronutrients, namely, protein, lipid, carbohydrate, in order to provide energy to optimize growth. Hence, the understanding of fish nutrition, including lipid and fatty acid metabolism and its regulation, is critical in order to satisfy the requirements of finfish aquaculture (Leaver *et al.*, 2008).

1.1.1 Use of lipids and fatty acids in aquaculture feeds

Lipids and fatty acids, besides, proteins, are major macronutrients for fish (Sargent *et al.*, 2002). Fish do not utilize carbohydrates efficiently, probably because they are not large components of their diets and glycogen used to meet short-term energy demands is a product of gluconeogenesis. Dietary lipids and fatty acids in fish are used to synthesize new cell membranes/flesh, oxidized to provide energy or are stored in adipose or other tissues (Tocher, 2003). Thus, it is important that the lipid content and fatty acid composition of feeds are optimized to achieve high growth rates

whilst maintaining health and nutritional benefits of fish for human consumption (Sargent *et al.*, 2002; Tocher, 2003).

Because lipids are high-energy nutrients, a recent trend in finfish aquaculture feeds is the utilization of higher levels of dietary lipid to partially spare (or substitute for) protein (Hemre and Sandnes, 1999). Although increasing dietary lipid is widely accepted as it can help reduce the high costs of diets by partially sparing protein in feed, its effectiveness still remains unclear for any fish (Company *et al.*, 1999). The use of lipid has now been maximized in dietary formulations due to commercial pressure to increase growth rates, and, at the same time, to reduce production times and as a consequence can lead to excess adiposity (or fat) in the carcass (Hemre and Sandnes, 1999). This does not only affect the fish health and welfare, but also represents an economic waste of valuable feed that might otherwise be channelled into overall growth.

1.2 Lipids

Lipids are described as biological substances that are hydrophobic in nature and are often soluble in organic solvents, such as chloroform, hydrocarbons or alcohols, represent a large group of chemically heterogeneous compounds with long-chain fatty acids (Smith, 2000). Lipids can be divided into “simple” and “complex” groups based on their molecular structure; simple lipids are lipids that yield at most two products on hydrolysis, for example, fatty acids, sterols and acylglycerols, and complex lipids are those that yield one or more products on hydrolysis, such as, glycerophospholipids and glycosphingolipids (Fahy *et al.*, 2005). Lipids can also be categorised by their

composition, for example, lipids that contain glycerol are called glycerolipids, lipids containing phosphate group are called phospholipids, lipids that consist of carbohydrates are glycolipids, lipids with sulphur-containing group are named sulpholipids, lipids containing sphingosine or other long-chain bases are called sphingolipids and ether lipids are lipids with long-chain alkyl moieties in addition to having ether bonds to fatty acids. Lipids can also be classified according to their chemical functional backbone as polyketides, acylglycerols, sphingolipids, prenols and saccharolipids (Fahy *et al.*, 2005). In animals, including fish, lipids can be classified into two groups, according to their solubility. Neutral lipids include triacylglycerols (TAG), wax esters, sterols, steryl esters and free fatty acids and polar lipids include phosphoglycerides, sphingolipids, sulpholipids and glycolipids.

1.2.1 Polar lipids or phospholipids

Phospholipids are abundant in all biological membranes. In general, a structure of a phospholipid molecule consists of two hydrophobic fatty acid “tails” and a hydrophilic “head” that consists of a phosphate group modified by an alcohol and a platform on which phospholipids are built, for example, a glycerol (a 3-carbon alcohol) or a sphingosine (a more complex alcohol). Phospholipids that derived from glycerol are called phosphoglycerides (Berg *et al.*, 2002).

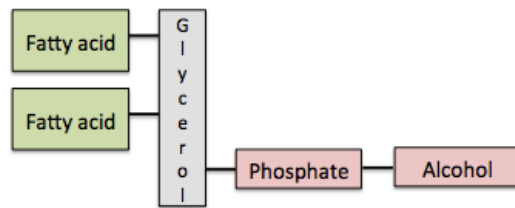


Figure 1-1 Schematic structure of phosphoglyceride

The simplest phosphoglyceride, phosphatidate (diacylglycerol 3-phosphate), is formed through esterification between the C-1 and C-2 hydroxyl groups of glycerol and the carboxyl groups of the two fatty acid chains and esterification between the C-3 hydroxyl group of glycerol and phosphoric acid (Berg *et al.*, 2002). Although only small amounts of phosphatidate are present in membranes, it is an intermediate key molecule in the formation of the other phosphoglycerides formed through esterification between the phosphate group of phosphatidate and the hydroxyl group of the alcohols, such as, choline, serine, inositol and ethanolamine, to form phosphatidylcholine, phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine, respectively.

Sphingomyelin is a phospholipid that is also found in membranes, derived from the more complex alcohol, sphingosine that consists of an amino alcohol with a long, unsaturated hydrocarbon chain. In sphingomyelin, a fatty acid is linked through an amide bond to the amino group of the sphingosine backbone (Berg *et al.*, 2002).

1.2.2 Neutral lipids

Neutral lipids are compounds that are hydrophobic in nature and are found in all cells with critical roles from energy storage to signal transduction. Neutral lipids mainly include triacylglycerols, diacylglycerols, monoacylglycerols, cholesterol esters and cholesterol. TAGs consist of a glycerol moiety with each hydroxyl group esterified to a fatty acid (Christie and Han, 2010), whereas diacylglycerols and monoacylglycerols, which are rarely present in animal tissues, contain two moles and one mole of fatty acids per mole of glycerol, respectively (Christie and Han, 2010). Cholesterol, on the other hand, is the main sterol present in animal tissues with a vital role in maintaining membrane fluidity and cholesterol esters are a storage form of cholesterol found in lipid bodies of all cells.

1.3 Peroxisome proliferator-activated receptors (PPARs)

1.3.1 Origin of PPARs and their structure

Peroxisomes are small, membrane-enclosed organelles found in all eukaryotic cells. They contain a variety of enzymes that are mostly involved in lipid homeostasis including the degradation of fatty acids and their derivatives through β -oxidation (Reddy and Mannaerts, 1994). Peroxisomes in mice respond to treatment with certain compounds called peroxisome proliferators by increasing in size and number and it was discovered in 1990 that these peroxisome proliferators activate a nuclear receptor known as peroxisome proliferator-activated receptor (PPAR, later PPAR α) (Issemann and Green, 1990).

Peroxisome proliferator-activated receptors (PPARs) are ligand-inducible transcription factors that belong to the nuclear hormone receptor superfamily (Michalik and Wahli, 1999). The isolation of PPARs was originally performed in humans, rodents and amphibians, and they have now been identified as receptors that are critical in the regulation of lipid and energy homeostasis. PPARs occur in three different isotypes, namely, PPAR α (the original receptor responsible for peroxisome proliferation in mice), PPAR β (also named PPAR δ in mouse and NUC1 in human) and PPAR γ (Michalik and Wahli, 1999). In mammals, the latter consists of two different proteins, γ 1 and γ 2, as a result of differential splicing of the same gene (Zhu *et al.*, 1995). Each of these receptors has a specific pattern of tissue expression.

Like other members of the superfamily, PPARs have six structural regions (A-F) in four functional domains, A/B, C, D and E/F (Schoonjans *et al.*, 1996; Michalik & Wahli, 1999; Vamecq & Latruffe, 1999). These receptors contain two activating functions, that is, the activating function 1 (AF-1) is localized in the poorly conserved A/B domain whilst the activating function 2 (AF-2), which has an amphipathic α -helical ligand-dependent activating function, is localized in the C-terminal part of E-domain (Schoonjans *et al.*, 1996). The highly conserved central DNA-binding domain (C domain) contains about 66 amino acids and it is stabilized by zinc atoms that bind to four invariant cysteine residues (Figure 1-2), giving these zinc finger-like complexes an α -helical structure for DNA-binding (Schoonjans *et al.*, 1996; Michalik & Wahli, 1999; Vamecq & Latruffe, 1999). The DNA binding domain contains two conserved sets of functionally important amino acids. The first conserved set, called the P-box is composed of amino acids (illustrated by circles in Figure 1-2) and two cysteine residues, and it determines specific contacts between receptor and DNA. As for the second conserved set, the D-box, is composed of amino acids residues (illustrated by squares in Figure 1-2) and it is believed to be involved in protein-protein interactions, such as receptor dimerization (Schoonjans *et al.*, 1996). In PPARs, the D-box has three amino acids and not five amino acids, and this distinguishes PPARs from other members of the nuclear hormone receptor family. The D-domain is a variable hinge region that allows conformational changes of the protein. The E/F domain, also known as the ligand-binding domain (LBD), is a multifunctional domain. Besides ligand binding, it is also responsible in transducing hormonal signals into transcriptional activation, involved in nuclear localization and

dimerization (Schoonjans *et al.*, 1996). The dimerization region is restricted in a region of nine heptad repeats (indicated by black stripes in Figure 1-2).

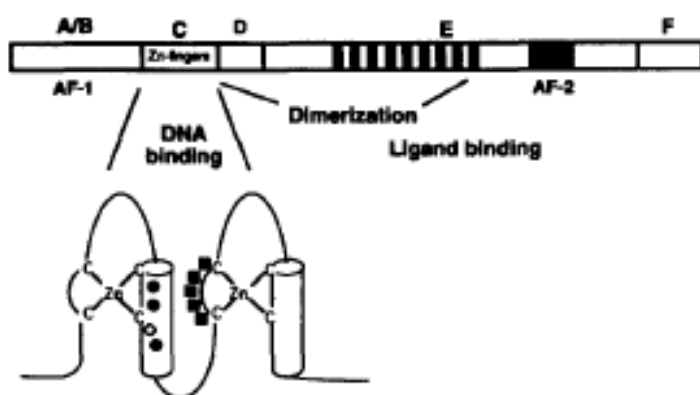


Figure 1-2 Functional domains of peroxisome proliferator-activated receptors (PPARs) (adopted from Schoonjans *et al.*, 1996)

In order for DNA binding to take place, PPAR is required to form a heterodimer with 9-*cis* retinoid X receptor (RXR) to form a complex, by interacting with a peroxisome proliferator responsive element (PPRE) with a consensus sequence of 5'-AACT AGGNCA A AGGTCA-3', located in the promoter of PPAR target genes (Ijpenberg *et al.*, 1997; Michalik and Wahli, 1999; Vamecq and Latruffe, 1999). The heterodimer can interact with and become activated either by PPAR ligands or by RXR ligands (Hihi *et al.*, 2002) and this in turn, induces conformational changes allowing the co-activators to become recruited and the co-repressors to be released, consequently stimulating the transcription of target genes that are implicated in various aspects of lipid and energy metabolism.

1.3.2 Discovery of piscine PPARs and their structure

Whilst the information of mammalian PPAR is abundant, reports for PPAR in teleost fish are very limited. PPARs in teleost fish have only been recently discovered and this include the zebrafish (*Danio rerio*) (Escriva *et al.*, 1997), the Atlantic salmon (*Salmo salar*) (Ruyter *et al.*, 1997; Andersen *et al.*, 2000), the plaice (*Pleuronectes platessa*) (Leaver *et al.*, 1998) and turbot (*Scophthalmus maximus*) (Robinson-Rechavi *et al.*, 2001). PPARs in teleost were initially found being expressed in the liver of Atlantic salmon (Ruyter *et al.*, 1997; Andersen *et al.*, 2000) and the first complete sequence and functional data for the three PPAR isotypes was reported on plaice and sea bream (Leaver *et al.*, 2005). A number of studies have been carried out to investigate the structural features of PPARs in teleost fish and mammals. In general, fish PPAR genes are up to ten times smaller than their mammalian counterparts, as observed in plaice and sea bream, due to the presence of much smaller introns (non-coding sections of DNA) in fish genes (Leaver *et al.*, 2005).

1.3.2.1 C-Domain or DNA-binding domain (DBD)

Amino acid sequences in PPAR proteins characterized in the Atlantic salmon, sea bass (*Dicentrarchus labrax*), sea bream (*Sparus aurata*) and plaice showed ≥ 90 % sequence identity with the correspondent sequences in humans within the core of the C-domain or DNA-binding domain (DBD) i.e. the two zinc fingers (Andersen *et al.*, 2000; Boukouvala *et al.*, 2004; Leaver *et al.*, 2005). Moreover, the DBD of salmon PPAR γ shares 78 % (out of 40 amino acid residues) sequence identity with rodents (Ruyter *et al.*, 1997). This proves that the DNA-binding properties of the DBD of fish and mammalian

PPARs are conserved, indicating that they may require very similar promoters for dependent transactivation activation (Leaver *et al.*, 2005). It is important to note that Atlantic salmon and plaice PPAR γ s share the characteristic feature of PPAR, having three amino acids between the two cysteines of the second zinc fingers in the DNA binding domain (Ruyter *et al.*, 1997; Leaver *et al.*, 1998) instead of five amino acids, as observed in all other members of nuclear receptor superfamily (Forman and Samuels, 1990). On the other hand, it was found that the DBD of Atlantic salmon PPAR γ only shares 53% sequence identity with the jawless lamprey (Escriva *et al.*, 1997), indicating that the DNA-binding properties of PPARs from representatives of ancestral fishes may have diverged somewhat.

1.3.2.2 E/F Domain or ligand-binding domain (LBD)

The LBDs of piscine PPARs also have significant levels of identity to mammalian PPARs, although there are insertions of an extra 20- and 25-amino acid residues in sea bass PPAR α and PPAR γ , respectively at the amino terminus of LBD (Boukouvala *et al.*, 2004), while in sea bream and plaice PPAR α , there is an insertion of an extra 21 residues in each receptor gene (Leaver *et al.*, 2005), and insertions of an extra 23- and 35-amino acid residues in the sea bass and plaice PPAR γ , respectively. These insertions may have resulted from the different number of exons encoded in fish and human PPAR α and PPAR γ , that is three exons in sea bass receptor proteins as opposed to two exons in humans. Thus, this structure is believed to be much larger and more hydrophilic in sea bream and plaice PPAR α and PPAR γ than in its mammalian counterpart. Nevertheless, significant identity (i.e. >70 %)

between sea bream/plaice and human PPAR α and PPAR γ was observed in this domain (Leaver *et al.*, 2005). Moreover, the LBDs of sea bass PPAR α and PPAR γ isotypes share 67 % and 66 % identity, respectively, with the corresponding sequences in humans (Boukouvala *et al.*, 2004) and part of D-domain and the LBD of PPAR α in brown trout showed 82 % and 78 % sequence identity in humans and rodents, respectively (Batista-Pinto *et al.*, 2005). In contrast, the PPAR α fragments in brown trout showed relatively higher levels of identity with corresponding sequences in goldfish (*Carassius auratus*) and plaice (90 % and 87 %, respectively), showing PPAR α gene is much more similar among fish species in comparison with mammalian PPAR α , as the number of amino acid residues in the deduced sequence of the protein varies only by one between fish and mammalian protein. It was also revealed that the LBD of PPAR γ in Atlantic salmon shares 63 % and 78 % sequence identity with human and rodent PPAR γ s, respectively (Ruyter *et al.*, 1997; Andersen *et al.*, 2000).

Evidently, PPAR β gene shows great homogeneity among fish species such as brown trout, plaice, and goldfish, and mammals as they have equal number of amino acid residues in the deduced sequence of the protein (Batista-Pinto *et al.*, 2009). The fragments of PPAR β in the D-domain and LBD in brown trout showed high levels of sequence identity with other fish species, i.e. the plaice (94 %) and Atlantic salmon (84 %). They also showed high levels of sequence identity with the correspondent PPAR β sequences of humans and rodents (i.e. 84 % and 82 %, respectively). It was observed that although the LBD in sea bass PPAR β which also contains three exons, containing equal number of amino acid residues as its human counterpart with only two exons, thus also

sharing relatively higher identity (i.e. 78 %) with human PPAR β (Boukouvala *et al.*, 2004).

1.3.2.3 A/B Domain

As for the A/B-domain, being the least conserved region in PPAR, is considerably longer in fish PPARs such as sea bass, sea bream and plaice, than that of mammalian (Boukouvala *et al.*, 2004; Leaver *et al.*, 2005), indicating that this domain has the lowest identity with its human counterpart. It was observed that PPARs in the fish species and mammals are of negative net charge. The A/B domain of mammalian PPAR β is only 42 amino acid residues long and is negatively charged due to the presence of 13 glutamate residues. 42 % of the residues and the net charge of this domain is maintained in sea bream PPAR β , whereas 39 % of the residues is found in plaice PPAR β and the net charge is less negative (Leaver *et al.*, 2005), indicating that teleost and mammalian PPARs have low similarity and this is also true for salmon PPAR γ (Andersen *et al.*, 2000). This gives an overall amino acid sequence identity of 43-48 % to mammalian PPAR γ .

1.3.2.4 D-Domain

The D-domains in sea bream/plaice and mammalian PPAR β and PPAR γ have the same number of amino acid residues i.e. 68 and 67 amino acids residues, respectively, and, are highly conserved, whereas PPAR α in sea bream and plaice possess a D-domain that is shorter by one residue when compared with mammals (i.e. 67 versus 68 amino acids) (Leaver *et al.*, 2005). Interestingly, according to Batista-Pinto *et al.* (2005), the D-domain of fish

PPAR γ shows high variability, not only in sequence, but also in length. PPAR γ in brown trout was observed to share 97 % sequence identity with PPAR γ in Atlantic salmon, while sharing only 46 % with plaice and <52 % with mammals. The lower identity between the salmonids and plaice PPAR γ s is due to the large insertion of amino acid residues in the D-domain in plaice PPAR γ that is absent in the salmonids, while the PPAR γ of the salmonids have a 10 amino acid segment that is not present in mammals (Batista-Pinto *et al.*, 2005).

1.3.3 Tissue-specific distribution of piscine PPARs

Recently, attention has been paid on the distribution patterns and expression of PPARs in tissues and cells of different organs in the Atlantic salmon (Ruyter *et al.*, 1997; Andersen *et al.*, 2000), the zebrafish (Ibabe *et al.*, 2002; 2005), gray mullet (*Mugil cephalus*) (Ibabe *et al.*, 2004), sea bass (Boukouvala *et al.*, 2004), sea bream, plaice (Leaver *et al.*, 2005) and brown trout (*Salmo trutta f fario*) (Batista-Pinto *et al.*, 2005). Ibabe *et al.* (2002) had carried out the first analysis on the tissue and cellular distribution of fish PPAR on zebrafish that was achieved by performing western blots and immunohistochemistry using commercially available antibodies against PPAR α , PPAR β and PPAR γ . Similar analyses were done with sea bass and sea bream using RNase protection assay (Boukouvala *et al.*, 2004; Leaver *et al.*, 2005), brown trout by conducting both semi-quantitative RT-PCR and real-time RT-PCR (Batista-Pinto, 2009) and the plaice by Northern blotting (Leaver *et al.*, 2005). These analyses of tissue distribution of the PPAR isotypes are crucial towards understanding of their physiological roles.

1.3.3.1 PPAR β

In mammals, a single gene for PPAR β encodes a transcription factor. However, teleosts have varying numbers of PPAR β genes, from one in the pufferfishes, *Takifugu rubripes* and *Tetraodon nigroviridis*, plaice and sea bream (Leaver *et al.*, 2005), and two in zebrafish (Robinson-Rechavi *et al.*, 2001). More recently, the sequencing of Atlantic salmon genome revealed that there are four PPAR β genes termed ssPPAR β 1A, ssPPAR β 1B, ssPPAR β 2A and ssPPAR β 2B, and at least two are functional PPAR β genes (Leaver *et al.*, 2007). The presence of these four PPAR β genes in Atlantic salmon supports theory that salmonids may be derived from a relatively recent autotetraploidization event (Allendorf and Thorgaard, 1984; Alexandrou *et al.*, 2013) and that a previous genome duplication may have occurred in early evolution of ray-finned fishes (Taylor *et al.*, 2003). This event is supported by the recent comparative syntenic analyses of zebrafish and pufferfish genomes suggesting that genes may have been lost or being retained through the divergence of different lineages of fish, resulting in different gene numbers between these species (Woods *et al.*, 2005).

Like its mammalian homolog, PPAR β being the most ubiquitous isotype, showed the strongest overall expression and is present in almost all tissues. Its widespread distribution was seen in fish species including sea bass (Boukouvala *et al.*, 2004), zebrafish (Ibabe *et al.*, 2002), sea bream, plaice (Leaver *et al.*, 2005) and brown trout (Batista-Pinto *et al.*, 2005). This subtype is more expressed in the liver of these fishes. However, the two functional Atlantic salmon PPAR β subtypes were expressed differently, with

PPAR β 1A expressed more in liver and PPAR β 2A in gill (Leaver *et al.*, 2007). Nevertheless, this subtype showed the strongest overall expression in almost all tissues when compared to that of PPAR α and PPAR γ .

Because of its great homogeneity among species and abundance, the role of PPAR β is less well understood. However, various studies suggest that this receptor plays a general role in controlling lipid homeostasis in mammals (Wagner and Wagner, 2010). It has been shown that PPAR β activates fatty acid oxidation in adipose tissues (Peters *et al.*, 2000) and that it plays an important role in β -oxidation as it directly controls lipid utilization through up-regulation of genes and energy uncoupling in the skeletal muscle cells (Dressel *et al.*, 2003). Besides functioning as a regulator in lipid metabolism, this receptor has significant roles in controlling cellular proliferation and differentiation (Burdick *et al.*, 2006).

1.3.3.2 PPAR α

PPAR α is encoded by a single gene in mammals and this isotype is highly expressed in tissues that catabolize large amounts of fatty acids, such as liver, kidney, heart, skeletal muscle and adipose tissue (Lemberger *et al.*, 1996). On the other hand, it was suggested that fish PPARs may exhibit two PPAR α subtypes, termed PPAR α 1 and PPAR α 2. The PPAR α subtype cDNAs that have been characterized from sea bream, sea bass and plaice (Boukouvala *et al.*, 2004; Leaver *et al.*, 2005), appear to have high identity to mammalian PPAR α . These proteins have been termed PPAR α 2 in sea bream and plaice and they are highly expressed in liver and heart, showing that they are structurally and functionally similar to the mammalian PPAR α (Leaver *et al.*, 2005). As for PPAR α 1, this subtype was found closely related to PPAR α present in genomes of pufferfish and zebrafish (Maglich *et al.*, 2003). Although its functional characteristics are not yet known, there was a PPAR α cDNA from Atlantic salmon that is evidently a homologue of the uncharacterised PPAR α 1 from pufferfish and zebrafish (Leaver *et al.*, 2005). It was suggested that since these two conserved PPAR α subtypes are found in zebrafish, pufferfish (Leaver *et al.*, 2005) sea bream, sea bass, and possibly salmonids, it is possible that the rest of teleost fish possess them too, phylogenetically (Leaver *et al.* 2008). Thus, this would further imply that PPAR α 1 and PPAR α 2 (and mammalian PPAR α) have different functions, though this hypothesis needs to be further justified.

In zebrafish, PPAR α was expressed in liver with higher intensity than PPAR β (Ibabe *et al.*, 2002). This is in agreement with the expression intensity in gray

mullet liver that PPAR α was the strongly expressed isotype compared to the other two isotypes (Ibabe *et al.*, 2004). PPAR α was also mainly found in kidney, intestine and pancreas of zebrafish, while in sea bream, plaice and brown trout, PPAR α is more expressed in heart and liver (Leaver *et al.*, 2005; Batista-Pinto *et al.*, 2005). Interestingly, although this isotype is more expressed in the liver of brown trout, it is expressed much weaker compared to its expression in mammals (Batista-Pinto *et al.*, 2005). In sea bass, while the highest expression PPAR α is detected in the red muscle and liver, this isotype is weakly expressed in intestine and spleen and none is found in kidney and adipose tissue (Boukouvala *et al.*, 2004).

PPAR α and PPAR β likely have similar functions to those interpreted in mammals. Like PPAR β , PPAR α directly controls the expression of key enzymes that regulate mitochondrial and peroxisomal fatty acid oxidation (Mandard *et al.*, 2004). It is hypothesised that PPAR α is responsible in the conversion and use of energy storage as its main function is to control the reversible induction of β -oxidation in specific tissues, especially in liver as a response to changing energy requirements and nutritional status. It was shown that mice that lack PPAR α are not able to up-regulate hepatic β -oxidation in response to starving (Kersten *et al.*, 1999; Leone *et al.*, 1999). Starvation stimulates lipolysis in the adipose tissue and releases fatty acids available, resulting in the development of fatty livers in mice lacking PPAR α . Besides this, PPAR α is also involved in amino acid metabolism, gluconeogenesis and inflammation (Mandard *et al.*, 2004).

1.3.3.3 PPAR γ

Mammals have a single gene encoding PPAR γ that is alternatively spliced to give two proteins, PPAR γ 1 and PPAR γ 2, each carrying different N-terminal sequence (Tontonoz *et al.*, 1994). PPAR γ 1 is highly expressed in gut where as PPAR γ 2 is abundant in adipose tissues compared to the other tissues (Escher *et al.*, 2001). While mammalian PPAR γ has two transcripts coding different proteins (Zhu *et al.*, 1995; Tsukahara, 2013), most teleost fishes only have one transcript (Maglich *et al.*, 2003; Boukouvala *et al.*, 2004; Ibabe *et al.*, 2005, 2004, 2002; Leaver *et al.*, 2005; Batisto-Pinto *et al.*, 2005; Tsai *et al.*, 2008; Oku and Umino, 2008; Cho *et al.*, 2009; Kondo *et al.*, 2010, 2007; Agawa *et al.*, 2012; He *et al.*, 2012; Li *et al.*, 2012)

PPAR γ , being the first PPAR isotype to be identified in Atlantic salmon and plaice (Ruyter *et al.*, 1997; Leaver *et al.*, 1998), exhibits restricted tissue distribution. This is in agreement with the tissue distribution analysis in sea bass in which PPAR γ is abundantly expressed in adipose tissue and gills, some in red muscle and intestine and only very small amounts are found in the liver (Boukouvala *et al.*, 2004). In the previous study done by Ibabe *et al.* (2002), because adipose tissue was not present in the preparation in the zebrafish examined, the expression of PPAR γ could not be justified in this species, however, the analysis has shown that this subtype is weakly expressed in other tissues, such as pancreatic cells, muscle and serous membrane of intestine and gonads of zebrafish. In contrast, in the liver of gray mullet *M. cephalus*, highest expression was observed in melanomacrophages (Ibabe *et al.*, 2004). Similar observations were found in

brown trout, where by the strongest PPAR γ expression was detected in trunk kidney where melanomacrophages were particularly abundant, and a weak expression found in liver where they were least found (Batista-Pinto *et al.*, 2005).

One of PPAR γ major roles is that it acts as a lipid regulator and is believed to play an important role in the accumulation of fat, particularly in adipocytes and lipid-accumulating macrophages (Fajas *et al.*, 2001; Rosen *et al.*, 1999). It is known to regulate the differentiation of adipocytes by detecting the availability of lipid and responding by adapting appropriate gene expression programs (Escher *et al.*, 2001).

1.3.4 PPAR activation

1.3.4.1 Endogenous (natural) ligands

Based on the reports that known ligands of PPAR shared similar amphipathic structure to fatty acids and that high-fat diets and some fatty acid analogues induced the peroxisomal β -oxidation of fatty acids, Keller *et al.* (1993), for the first time, successfully demonstrated that fatty acids were endogenous activators of *Xenopus laevis* PPAR α (xPPAR α). Transfection experiments with xPPAR α and RXR β and in the presence of fatty acids, indeed, have shown cooperative activation of the acyl CoA oxidase promoter through the binding of PPAR α -RXR β heterodimers to the peroxisome-proliferator response element (PPRE) of the promoter (Keller *et al.*, 1993). Because both PPAR and RXR ligands can regulate the expression of PPRE-containing target genes via the activation of the heterodimer, it was further demonstrated that the activation of PPAR-RXR β heterodimer by fatty acids was indeed through the direct interaction of fatty acids and PPARs and not due to the interaction between fatty acids and the RXR heterodimer partner (Kliwer *et al.*, 1997). Fatty acids are also observed to be activators of native PPAR α in fish species, for example, Japanese medaka (*Oryzias latipes*) (Kondo *et al.*, 2010), gilthead sea bream and European plaice (Leaver *et al.*, 2005). Furthermore, cell transient transfection experiments using fish cells co-transfected with Gal4-constructs of PPAR α and PPAR β demonstrated that these receptors are activated by fatty acids (Colliar *et al.*, 2011). In addition to fatty acids, transactivation assays and gel retardation studies have shown that derivatives of fatty acid metabolism such as eicosanoids are endogenous

ligands for mammalian PPARs (Kliewer *et al.*, 1997). Derivatives of arachidonic acid, including 20-hydroxyeicosatetraenoic acid (20-HETE) and several epoxyeicosatrienoic acids (EETs) and dihydroxyeicosatrienoic acids (DHETs) are activators of PPAR α and PPAR γ , as shown in transactivation assays (Ng *et al.*, 2007). Like PPAR α , fatty acids have been shown to activate mammalian PPAR β (Xu *et al.*, 1999), although the extent of its activation is less than that of PPAR α (Krey *et al.*, 1997). Evidently, piscine native PPAR β and Gal4-construct of PPAR β have also shown significant activation by some fatty acids in cell transactivation assays (Leaver *et al.*, 2005; Colliar, *et al.*, 2011). Also similar to PPAR α , fatty acids including eicosanoid, 15-deoxy-D12, 14-prostaglandin J2 (15d-J2) have been identified as potential specific endogenous agonists of PPAR γ (Forman *et al.*, 1995; Kliewer *et al.*, 1995). However, none of the fatty acids that activate mammalian PPAR γ is able to activate piscine PPAR γ .

1.3.4.2 Synthetic ligands

The hypolipidemic drug, clofibrate, was identified as the first activating compound for PPAR α (Issemann and Green, 1990). Since then several other members of the fibrate class of drugs, including the PPAR α -specific ligand WY-14,643, have been developed as ligands of PPAR α (Willson *et al.*, 2000). Several fibrates have been tested in transactivation assays using murine PPARs and revealed that ciprofibrate, gemfibrozil and clofibrate strongly activate PPAR α , weakly activate PPAR γ and did not activate PPAR β (Forman *et al.*, 1997). These compounds were also tested in amphibian and fish. Bezafibrate and ciprofibrate activate PPAR β and PPAR γ of *Xenopus*,

respectively (Krey *et al.*, 1997), and fibrate treatment was observed to increase the activity of the target gene acyl-CoA oxidase in Atlantic salmon hepatocytes (Ruyter *et al.*, 1997) and rainbow trout (*Oncorhynchus mykiss*) (Donohue *et al.*, 1993). Moreover, gemfibrozil and ciprofibrate were demonstrated to activate Gal4-PPAR α and bezafibrate activate Gal4-PPAR β in cell transient transfection experiments using fish cells (Colliar *et al.*, 2011). Several PPAR β subtype-specific synthetic ligands have been identified, for example, GW501516 (Oliver *et al.*, 2001) and this compound has been reported to activate PPAR β in fish (Leaver *et al.*, 2007; Colliar *et al.*, 2011). Thiazolidinedione (TZD) class of compounds, which include troglitazone, pioglitazone, rosiglitazone and ciglitazone, have been identified as potentially useful drugs based on their ability to activate rodent and human PPAR γ in transient transfection assays (Lehmann *et al.*, 1995; Lambe and Tugwood, 1996).

1.3.4.3 Transcriptional activation

PPARs target transcription of specific genes by binding to specific elements in gene promoter regions. Liganded PPARs activate transcription by binding co-activator proteins and/or releasing co-repressor proteins (Viswakarma *et al.*, 2010). Binding of ligand to PPAR causes a conformational change in the LBD region of the protein that leads to dissociation of co-repressor protein and recruitment of co-activator (McInerney *et al.*, 1998). Both co-activator and co-repressor proteins belong to families of transcriptional activators that have broad specificity with regard to interaction with DNA binding transcription factors, and act to integrate transcriptional responses within cells (Rosenfeld *et al.*, 2006). Thus, the activity of PPARs within particular cell types is not just dependent on the PPAR expressed, but also on the complement of co-activators and co-repressors.

1.4 Cell culture

In the growing aquaculture industry, cell cultures from a wide variety of fish species, including Atlantic salmon, Arctic charr, European whitefish, common carp, goldfish and zebrafish, have been developed with the aim to contribute towards increasing and/or improving fish production (Freshney, 2010). In *in vitro* studies, compared to *in vivo*, cell cultures are used as simple and manipulable analogs of animals, in order to reduce the number of sacrificed animals (Segner, 1998). These systems have several significant advantages as the basic characteristics of the more complex *in vivo* condition can be retained and experimental conditions can be controlled (Baksi and Frazier, 1990). The physical and chemical conditions surrounding the cells, such as temperature, pH, pCO₂ and ionic concentration, can be controlled over wide ranges to reveal fundamental mechanisms. Moreover, biological factors influencing cellular responses that cannot be measured individually and in combinations in *in vivo* studies, can be carried out using *in vitro* techniques. These systems also help in reducing variability between experiments, not only by controlling the environmental conditions, but also, the internal factors as these systems allow the incorporation of positive and negative control chemicals into the desired experimental design to calibrate the system for different experiments. Other advantages include the ability of repeating experiments, more time efficient and importantly, because of the use of small quantities of test chemicals, these systems are often cheaper and less toxic waste are disposed at the end of experiments.

A number of studies have been carried out using *in vitro* techniques in the investigation of peroxisome proliferation, ligand binding and activation of PPARs and changes in expression of PPARs (Ibabe *et al.*, 2005; Colliar *et al.*, 2011). In this study, permanent cell lines of Chinook salmon embryo 214 (CHSE-214) were used for *in vitro* investigations. Although permanent cell lines often have lost functional, structural or metabolic properties of the originating tissues or cells in comparison to primary cell cultures having many of *in vivo* features retained, cell lines can be in convenience in supply and propagated indefinitely in culture (Baksi & Frazier, 1990; Segner, 1998). These immortalized cultured cells can be transfected much more readily to higher efficiency as some parts of the cellular processes may have been altered during the process of transforming cells towards making them more amendable to culture conditions and thus, making them more susceptible to transfection (Hsu & Uludağ, 2012).

1.5 Thesis aims and objectives

There is strong evidence that PPARs play critical roles in lipid homeostasis based on the information gathered on the tissue distribution of PPARs and the identification of PPAR target genes. Although PPAR α and PPAR β may have similar functions to those described in mammals (Leaver *et al.*, 2005), the role of PPAR γ in fish still remain unclear. Because mammalian PPAR γ has a critical role in determining lipid uptake and storage, it is of particular interest in finfish aquaculture as farmed fish often accumulate excess visceral and hepatic fat especially when fed plant seed oil-based diets. This can affect the health and welfare of the fish, and also represents an economic waste of valuable feed that might otherwise be channelled into growth. Thus, this research may become relevant to feed formulation because at present most fish feed formulation is based on trial and error using ingredients that are either available locally or are economically feasible. By understanding the differences between the activities of fish and mammalian PPAR γ and the role PPAR γ in fish physiology, new diets can be designed with optimized quantities and qualities of lipid components from a variety of available ingredients.

The specific aims of this project were:

1. To optimise the transfection of plasmid DNA into an established fish cell line using a transfection reagent, branched polyethylenimine (bPEI), and range of DNA to reagent ratios (Chapter 3)
2. To prepare expression plasmid construct in which the ligand-binding domain of European plaice PPAR γ is ligated downstream of the Gal4 DNA-binding domain (Chapter 3)
3. To extract the total lipid from liver tissues of farmed fish, and to fractionate the lipid into lipid classes and test them with the Gal4-PPAR γ LBD expression construct in a series of luciferase assays (Chapter 4)
4. To identify molecular components within the lipid fractions that interact with plaice PPAR ligand-binding domain (Chapter 4)
5. To evaluate the transcriptional responses of potential molecular components identified in (4) on the cellular transactivation assay developed previously (Chapter 5)
6. To determine the tissue expression pattern of PPARs in Atlantic salmon (*Salmon salar*) and to construct phylogenetic trees comparing PPARs from different vertebrate species to determine whether or not the genetic divergence of Atlantic salmon PPARs exhibit functional divergence (Chapter 6)

2 General materials and methods

2.1 Preparation of media and buffers

2.1.1 Preparation of 50x TRIS/acetate/EDTA (TAE) buffer

A 50 x stock solution of TAE buffer was prepared using 121 g Tris base (2-amino-2-hydroxymethyl-propane-1,3-diol), 50 ml 0.5M Na₂EDTA (pH8.0) and 28.5 ml glacial acetic acid (100% acetic acid). First, 50 ml 0.5M Na₂EDTA was prepared by dissolving 9.3 g of EDTA in 50 ml of MilliQ water (Millipore) before this was stirred vigorously using a magnetic stirrer and the pH adjusted to 8.0 with sodium hydroxide (NaOH). Subsequently, 121 g Tris base was measured into 500 ml beaker containing about 350 ml of MilliQ water, stirred and the previously prepared Na₂EDTA and 28.5 ml glacial acetic acid were added to the mixture, stirred and MilliQ added to bring volume up to 500 ml.

2.1.2 Preparation of Luria-Bertani (LB) broth

A 400 ml of LB broth was prepared by dissolving 8 g of LB medium (USB, Ohio, USA) in 400 ml of MilliQ water in a 500 ml bottle before it was autoclaved and stored at room temperature.

2.1.3 Preparation of antibiotic ampicillin solution

Ampicillin solution (50 mg/ml) was prepared by dissolving 250 mg of ampicillin in 5 ml MilliQ before the solution was divided into 1 ml aliquots and stored at -20 °C.

2.1.4 Preparation of Luria-Bertani (LB) agar with ampicillin

LB agar was prepared by dissolving 12.8 g LB agar (USB, Ohio, USA) in 400 ml MilliQ in a 500 ml bottle and mixed before this was autoclaved and allowed to cool to 50 °C and stored at room temperature. To prepare the agar in plates, this was microwaved to melt the solid agar, and to 50 ml of molten LB agar, 100 µl of 50 mg/ml ampicillin solution was added. The agar was gently mixed and poured into two 100mm Petri dishes. Plates were allowed to set, and if not used immediately, stored at 4 °C.

2.2 Preparation of pure plasmid DNA

2.2.1 Transformation of plasmid DNA into *Escherichia coli* (*E. coli*)

This method involves the insertion of a foreign plasmid DNA into the bacteria using the traditional heat shock method. 0.5 µl of plasmid DNA was added into micro-tube containing competent *E. coli* Top 10 cells, previously prepared using the method employed by Inoue *et al.* (1990), and this was incubated in ice for 30 min. This mixture of chemically competent bacteria and DNA was then placed in a water bath at 42 °C for 1 min before it was placed back in ice to chill for 1-2 min. 900 µl of LB media was added to and the transformed cells were incubated at 37 °C for 1 hour with agitation. On LB agar plate containing ampicillin, 50 µl of the mixture was plated and this was incubated at 37 °C overnight.

2.2.2 Inoculation of bacterial cells containing plasmid DNA

A starter culture was prepared by inoculating 4 ml of LB medium (containing ampicillin) with a single colony picked from the agar plate prepared earlier. This was incubated at 37 °C for about 8 hours, shaking at 250 rpm. The inoculation of a large overnight culture was prepared by diluting the starter culture 1/1000 into 300 ml of LB medium (containing ampicillin) and this was incubated further 37 °C for 12-16 hours, shaking at 250 rpm.

2.2.3 Purification of plasmid DNA

The following day, plasmid DNA was isolated from the overnight culture using using Nucleobond® Xtra kit (Macharey-Nagel), following the manufacturer's instructions. Briefly, the bacteria were harvested from the LB culture by centrifugation at 5,100 x *g* for 25min at 4°C and the supernatant was carefully decanted. The pellet of bacterial cells was re-suspended in 12 ml Resuspension Buffer RES (containing RNase A) by vortexing the cells, making sure that no clumps remain in the suspension for efficient cell lysis. To the suspension, 12 ml of Lysis Buffer LYS was added and was mixed gently by inverting the tube 5 times. At the stage, vortexing would have sheared and released contamination chromosomal DNA from cellular debris into the suspension. The mixture was left to incubate at room temperature (18-25 °C) for 5 min. To the suspension mixture, 12 ml of Neutralization Buffer NEU was added and was lysate was immediately mixed, gently by inverting the tube until a homogenous suspension containing an off-white flocculate was formed. The lysate was centrifuged at 5,100 x *g* at 4 °C for 10 min and the supernatant was then applied to the previously equilibrated Nucleobond®

Xtra Column Filter (with 25 ml of Equilibration Buffer EQU). This clarification step was extremely important to ensure that the column was not clogged by any residual precipitate. When the column was emptied by gravity flow, the Nucleobond® Xtra Column Filter and Nucleobond® Xtra Column were washed with 15 ml Equilibration Buffer EQU. The filter was then removed before the column was washed with 25 ml Wash Buffer WASH. The plasmid DNA was eluted with 15 ml Elution Buffer ELU and the eluate was collected in a 50 ml centrifuge tube. The eluted plasmid DNA was precipitated by adding 10.5 ml of room-temperature isopropanol, centrifuged at 5,100 x *g* at 10 °C for 25 min. The supernatant was carefully decanted before 4 ml room temperature 70 % ethanol was added to the pellet. This was centrifuged at 5,100 x *g* at room temperature (18-25 °C) for 5 min. The ethanol was carefully removed from the tube using a pipette tip and was allowed to dry at room temperature (18-25 °C) for 25 min. The pellet was then dissolved in 200 µl of 10 mM Tris-Cl pH8.0 and the concentration of the DNA was measured using the Nanodrop ND1000 spectrophotometer.

2.2.4 Determination of plasmid integrity by Agarose Gel Electrophoresis

2.2.4.1 Preparation of samples for Restriction Digestion

In this case, a Master mix was prepared depending on the number of plasmids and this consisted of all the reaction components except plasmid DNA. In a Master mix, 1 μ l NEB buffer was used to optimize the enzyme activity, 0.25 μ l each of two selected restriction enzymes, BamHI and Kpn1, were used to cut the DNA and 7 μ l of deionised water was added. In a microtube, 9 μ l of Master mix was prepared and 1 μ l of plasmid DNA was added. This was mixed gently by pipetting up and down. The mixture was incubated at 37 °C for 1 hour and into each tube, 2 μ l of 6 x loading dye was added and this was mixed by vortexing.

2.2.4.2 Preparation of Agarose Gel

Agarose gel electrophoresis is the easiest and commonest way of separating and analysing DNA. On a weighing boat, 0.25 g of agarose was weighed out and was decanted into 250 ml conical flask, into which 25 ml of 0.5 X TAE buffer was added. A 25 ml conical flask (inverted) was used as a lid and this was microwaved for 1 min, gently swirling that flask at regular intervals to help it dissolve. This was left to cool for 5-10 min until it reaches about 60 °C (just hand hot). To the flask, 0.25 μ l of ethidium bromide (5 mg/ml) was added and mixed by gentle swirling. A 6 cm x 7 cm gel casting tray was prepared by sealing the ends of the tray using masking tape and the required comb was placed into the position in the tray. The agarose gel was poured

slowly into the casting tray and was left to set for 1 hour. The tape was then removed from the tray and was placed in the electrophoresis tank, into which about 250 ml of 0.5 X TAE buffer was poured to submerge the gel to 2-5 mm depth, herein referred to as the running buffer. The gel was allowed to equilibrate in the buffer for 5-10 min.

2.2.4.3 Loading the gel

Using a fresh pipette tip for each sample, 1 μ l of sample was loaded alongside a 1 kb Hyper Ladder molecular weight marker (Fementas). The samples were run at 70 V for 45 min and the progress of the gel by the loading dye was monitored and stopped when the dye had run approximately $\frac{3}{4}$ of the way through the gel. The gel was then placed on the Syngene Transilluminator to observe DNA bands, indicating successful digestion.

3 Development of a luciferase reporter assay and the optimisation of transfection methods using branched polyethylenimine (bPEI) transfection reagent

3.1 Introduction

3.1.1 Development of a reporter gene construct

Peroxisome proliferator activated receptors (PPARs) are factors that activate transcription by interacting with peroxisome proliferator element (PPRE) of a target gene, and function as heterodimers with 9-*cis* retinoid X receptor (RXR). Gearing *et al.* (1993) had shown that endogenous PPARs expressed in rat cells, upon purification, were found to have lost their ability to bind to PPREs from acyl-CoA oxidase gene, and the binding was however restored by the addition of *in vitro*-translated RXR α . It was also observed that activation of the reporter gene depended on the interaction between PPAR and RXR, and this activation was limited to the levels of RXR. The formation of this heterodimeric complex with RXR brings about challenges particularly in determining activating compounds of PPARs as simultaneous exposure of the complex to clofibric acid, an activator of PPAR α , and 9-*cis* retinoic acid, a ligand of RXR α , resulted in the expression of acyl-CoA oxidase gene (Kliewer *et al.*, 1992). This showed that the PPAR-activated pathways in cells could also be influenced by the activation of RXR and that PPARs require RXR for proper functioning.

Thus, the identification of compounds that interact with PPARs can be hindered by the interference of compound that interact with the RXR. The removal of RXR, however, is not an option, as PPARs cannot function in the absence of RXR. Therefore, in order to overcome this, a fusion protein that consists of the Gal4 DNA-binding domain and the PPAR ligand-binding domain was developed for use in as reporter gene assay. Thus, the transcription of the reporter gene was depended on the activity of the activation function 2 (AF-2) located within the PPAR ligand-binding domain, without having to form a heterodimer complex. Binding of this ligand-dependent domain by an activating compound would enable the determination of PPAR activators and repressors with no interference or influence from the RXR.

3.1.1.1 The Gal4-PPAR/UAS assay system

Gal4 is a yeast transcriptional activator protein that binds to its specific recognition sequence, known as the upstream activation sequence (UAS) and activates transcription of target genes. Several studies have shown that the Gal4-UAS system has been demonstrated to be functional not only in yeast but also in various animal cells (Fischer *et al.*, 1988; Scheer and Campos-Ortega, 1999; Hartley *et al.*, 2002). The use of this system *in vitro* was developed by Kakidani and Ptashne (1988) to study the activation of gene expression in mammalian cells by Gal4 protein when the DNA-binding domain was inserted upstream of the mouse mammary tumor virus (MMTV) promoter. Similar system was adapted by Webster *et al.* (1988) to study the

activation of gene expression of a promoter region controlled by an oestrogen-responsive enhancer in human HeLa cells.

The development of the Gal4 transient assay provides several advantages and these include high throughput transactivation screen for fish PPAR activity, allowing the specific responses to be tested and the ability to define dose in a constant or controlled physio-chemical environment, without the interference of other receptors, hormones, signalling pathways and other *in vivo* complexities. The Gal4-UAS system is a powerful technique for studying gene expression, thus in this project, taking advantage of the functionality of the Gal4 DNA-binding domain as a fusion protein with an activation function-containing protein, Gal4-PPAR γ , was generated for use in fish cell line to identify activators and repressors of PPAR γ .

Two plasmid constructs were used in the Gal4-UAS system. The first plasmid construct is fusion protein consisted of amino acids 1-147 of the Gal4 gene, encoding the DNA-binding domain (Gal4-DBD). The Gal4-DBD was fused in-frame to the PPAR ligand-binding domain (PPAR-LBD), with the expression of the whole construct being driven by the cytomegalovirus (CMV) immediate early promoter (Figure 3-1A). The second plasmid construct consisted of five Gal4 UAS sequences upstream of a TATA box, which was upstream of the reporter gene, firefly luciferase (Figure 3-1B).

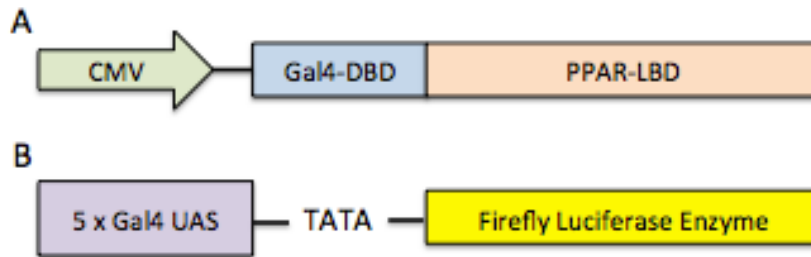


Figure 3-1 Schematic diagram of the two constructs in the Gal4-UAS system. A: The Gal4-PPAR construct consisted of PPAR-LBD cloned downstream of the Gal4-DBD, whose expression was driven by the CMV promoter. B: The Gal4UAS-luc construct consisted of 5 Gal4UAS sequences upstream of a TATA box, which was upstream of a firefly luciferase enzyme

The PPAR-LBD contains a crucial ligand-induced helical component, helix 12, that re-conforms to subsequently release the co-repressor proteins to allow the binding of co-activator proteins, and thereby activates transcription of the AF-2 (Moras and Gronemeyer, 1998). The interaction between the two plasmid constructs involves the binding of the Gal4UAS sequences with the Gal4-DBD, forming a complex, which remains transcriptionally inactive until binding of a ligand to the ligand-binding domain of the PPAR. This interaction leads to a conformational change in the Gal4-PPAR construct, releasing the co-repressor proteins and subsequently recruitment of co-activator proteins. As a result, AF-2 becomes activated and this ultimately an increase the luciferase expression (Moras and Gronemeyer, 1998). This whole process can be seen in Figure 3-2.

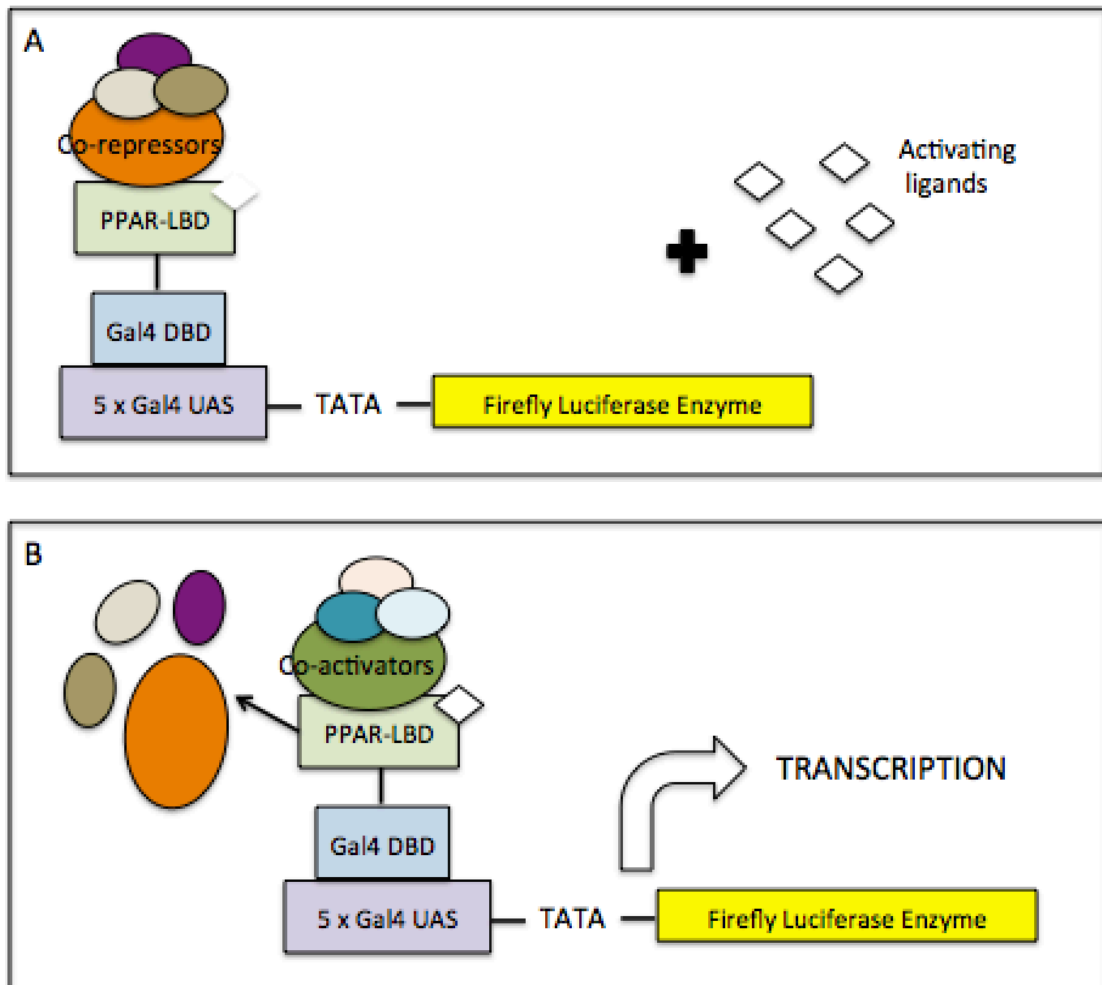


Figure 3-2 Schematic illustration of the Gal4-UAS assay system. A: The interaction between the two plasmid constructs involves the binding of the Gal4UAS sequences with the Gal4-DBD, forming a complex, which remains transcriptionally inactive due to the association of PPAR-LBD with co-repressor proteins. **B:** Binding of ligand to the ligand-binding domain of PPAR leading to a conformational change in the Gal4-PPAR construct, releasing the co-repressor proteins and subsequently, recruitment of co-activator proteins. AF-2 becomes activated and this ultimately results in an increase in the transcription of the firefly luciferase reporter enzyme.

3.1.1.2 Luciferase genes as reporter proteins

Luciferase reporter genes are commonly used in molecular and cell applications to monitor changes in transcriptional rate as bioluminescence that is instantaneous, sensitive and quantitative (van Lune and Bruggeman, 2006). Firefly (*Phonitus pyralis*) luciferase is a monomeric 61 kDa protein that yields a greenish yellow light at 560 nm, whereas *Renilla* luciferase is a 36 kDa protein isolated from the sea pansy (*Renilla reniformis*) that produces a blue light at 480 nm. The different light outputs, as well as, substrate and co-factor requirements are the characteristics that enable these enzymes to be used in the same assay system. This project made use of “homemade” luciferase assay buffers in a dual luciferase reporter assay in analysing the expression of the reporter genes, firefly luciferase and *Renilla* luciferase enzymes.

3.1.2 Transfection

Transfection is a non-viral delivery method of gene transfer and this involves an introduction of foreign nucleic acids into eukaryotic cells (Kim and Eberwine, 2010). Viral-mediated transfection, called transduction, involves the use of viral vectors derived from natural, usually partially disabled viruses such as adenovirus and retrovirus. Although viruses are efficient in transducing cells, their immunogenicity, cytotoxicity, limited gene-carrying capacity, potential pathogenicity and small-scale production have made non-viral delivery systems an attractive alternative (Li and Huang, 2000; Thomas, *et al.*, 2003).

Over the years, the development of the non-viral delivery systems has significantly improved and their application has been made a preferable option, depending on cell type, cellular context (*in vivo* or *in vitro*), desired efficiency as well as cost and time. There are two non-viral methods of gene delivery: physical and chemical methods.

3.1.2.1 Physical methods of transfection

Physical methods enable the direct transfer of plasmid DNA, through the cell membrane, into cytoplasm or nucleus by physical or mechanical means, which may (or may not) cause temporary micro-disruption to the cell membrane (Li and Huang, 2000; Wells, 2004). Some of the physical methods are described briefly below.

3.1.2.1.1 Electroporation

A frequently used physical method of gene transfer is known as electroporation and this involves an application of a series of electric pulses to overcome the barrier the cell membranes. The electric field creates a potential difference across the membrane subsequently inducing the formation of temporary hydrophilic pores in the cell membrane for plasmid DNA to pass through. The first successful gene transfer by electroporation was documented by Neumann *et al.* (1982), involving the transfection of circular plasmid DNA containing the herpes simplex thymidine kinase (TK) gene into TK-deficient mouse L cells. Since then, several studies have reported that electroporation can be done *in vitro* and *in vivo* involving the delivery of various molecules into eukaryotic cells, including ions (Saulis *et al.*, 2007), dyes (Mir *et al.*, 1988; Dinchuk *et al.*, 1992) oligonucleotides (Spiller *et al.*, 1998) and even anti-cancer drugs in patients (Sersa *et al.*, 2000).

3.1.2.1.2 Microinjection

Microinjection is another physical method of transfection and it is mainly used to manipulate single cells by injection of DNA, mRNA and proteins. It was recently reported by Michaelis *et al.* (2014) that *in vivo* microinjection was used to successfully transfect testicular mouse cells with a reporter vector pEGFP-C1 and this was illustrated by the green fluorescence expressed by the vector. Although this method is highly effective, it is however very time-consuming and is not suitable to transfect a large number of cells.

3.1.2.1.3 Particle bombardment or gene gun

Similarly, the particle bombardment, or gene gun, method, involves the acceleration of DNA-coated gold particles using a high-voltage electric discharge device, enabling efficient penetration of target organs, tissues or single cells (Yang *et al.*, 1990). Although this method causes minimum damage to the cells, the start-up cost is however very expensive.

3.1.2.2 Chemical methods of transfection

Chemical methods of transfection involves the interaction of negatively charged nucleic acids with positively charged carrier molecules (also known as transfection reagents), and this enables the positively charged nucleic acid chemical complexes to interact with negatively charged cell membranes consequently introducing the gene into the cell by endocytosis or phagocytosis, although the exact uptake mechanism is still unknown (Kim and Eberwine, 2010). These transfection reagents are cationic organic polymers, calcium phosphate or cationic lipids.

3.1.2.2.1 Cationic polymers

Diethylaminoethyl-dextran (DEAE-D) was the first non-viral transfection method proved by Vaheri and Pagano (1965). It was well-known for its simplicity and low costs, however, but is limited by low efficiency and reproducibility, and high toxicity. Over the recent years, various alternative cationic polymers with multiple functionalities have been documented to potentially achieve efficient gene transfection (Lin and Lou, 2012). Cationic polymers that have been frequently studied in gene delivery include

chitosan, PAMAM (polyamidoamine) dendrimer, and PEI (polyethylenimine). These polymers have the ability to self-assemble with negatively-charged DNA to form polymer/DNA complexes (polyplexes) and induce detectable gene transfection efficiency *in vitro*. PEIs with molecular weights above 2 kDa and dendrimers were reported to be very efficient in gene transfer (Remy *et al.*, 1998).

3.1.2.2 Calcium-phosphate

The calcium-phosphate co-precipitation method was first verified by Graham and van der Eb in 1973 and it was reported that adenovirus 5 DNA was successfully transfected into human KB cells. In principal, DNA is mixed with calcium chloride before this is added to a buffered saline/phosphate solution to form a precipitate. This consequently allows the DNA to be taken up by the cells, presumably by endocytosis or phagocytosis. Since then, this method has become one of the major methods for DNA transfer into mammalian cell lines (Loyter, *et al.*, 1982; Chen and Okayama, 1987; Jordan *et al.*, 1996; Jordan *et al.*, 1998) as the reagents are inexpensive and easily obtainable. This method, however, has low reproducibility and low transfection efficiencies, although recently, improvements have been made in increasing the transfection efficiency, for example by mixing ethidium monoazide and fluorescent peptide nucleic acid (PNA) labelling with the plasmid DNA (Batard, *et al.*, 2001). Results have revealed, for the first time, up to 100,000 plasmid molecules were successfully transfected into Chinese hamster ovary cells (CHO).

3.1.2.2.3 Cationic lipids

A successful *in vitro* transfection with cationic lipid was first reported by Felgner *et al.* in 1987 and this experiment made use of a synthetic cationic lipid, *N*-[1-(2,3-dioleyloxy)propyl]-*N*, *N*, *N*-trimethylammonium chloride (DOTMA). In principal, small unilamellar liposome vesicles of the synthetic cationic lipid interact with DNA to form lipid-DNA complexes. The synthetic lipid consequently facilitates the fusion of the complex with the cell membrane, resulting in the uptake of DNA into the cell. This method has a main advantage of successful delivery of DNA of all sizes into a wide range of cell types with high efficiency, but can exhibit high toxicity to cells.

3.1.3 Transfection of fish cell lines

Several studies have reported successful transfection of fish cell lines using the application of various methods described above including electroporation (Schiøtz *et al.*, 2011), cationic lipids (Romøren *et al.*, 2004) and cationic polymers (Bearzotti *et al.*, 1992; Leaver *et al.*, 2005; Colliar *et al.*, 2011). Because transfection efficiencies vary greatly among cell lines due to the differences in cell physiology and metabolic requirement that determines the uptake pathway, it is imperative that the transfection conditions are optimised for individual cell line (Hsu and Uludağ, 2012), depending on the transfection reagent of choice. Having said this, the DNA concentration, reagent concentration and the ratio of reagent to DNA must be determined to ensure optimal binding affinity between reagent-DNA complex and, effective release, consequently leading to successful transfection of cells.

This chapter focuses on:

- 1) The isolation of ligand-binding domain (LBD) of plaice (*Pleuronectes platessa*) from full receptors and clone the sequences encoding the LBD into the pBIND plasmid by polymerase chain reaction (PCR), to create Gal4-PPAR γ LBD construct (herein referred to as Gal4-PPAR γ).
- 2) Identifying the most suitable cell line by measuring the transfection efficiencies of two cell lines: Atlantic salmon (AS) and Chinook salmon embryo 214 (CHSE-214) cell lines, with the help of a transfection reagent, a cationic polymer, 25-kDa branched polythethylenimine (bPEI).

- 3) Assessment of a variety of reagent to DNA ratios through the measurement of luciferase enzyme activity in identifying the optimal conditions for use in subsequent experiments using three plasmids: Gal4-PPAR α LBD construct (herein referred to as Gal4-PPAR α), a reporter gene construct, Gal4UAS-luciferase plasmid (pGL4.31[*luc2P/Gal4UAS/hygro*]) and an internal control plasmid, *Renilla* luciferase plasmid (pGL4.75[*hRluc*/CMV]).
- 4) The measurement of luciferase enzyme activity from CHSE-214 cells transfected with Gal4-PPAR α and Gal4-PPAR γ and subsequently treated with their known PPAR agonists in mammals, WY-14,643 and rosiglitazone, respectively, and selected fatty acids.

3.2 Materials and methods

3.2.1 Construction of Gal4-PPAR γ expression plasmid

3.2.1.1 PCR amplification of PPAR γ ligand-binding domain

Sequences of cDNA encoding for PPAR γ isotype has previously been isolated from plaice (*Pleuronectes platessa*) and cloned into pcDNA3 vector (Invitrogen) (Leaver *et al.*, 2005). The tissue distribution and activation profile of plaice PPARs have been characterised previously and they are therefore a suitable starting point for the development of cellular transfection systems. The plaice PPAR α and PPAR β Gal4 constructs have previously been produced (Colliar *et al.*, 2011). The cDNA sequences encoding ligand-binding domain of plaice PPAR γ (amino acids 196 to 532) was amplified by polymerase chain reaction (PCR) and cloned downstream and in-frame with Gal4 DNA-binding domain (DBD) of the pBIND cloning vector.

A pair of primers was designed from the predicted coding regions of plaice PPAR gene around the nucleotide sequences corresponding to the amino acids above. The forward and reverse primer sequences for PPAR γ were 5'-TTG GAT CCG CAT GTC ACA CAA CGC TAT TCG TTT T -3' and 5'-AAG GTA CCC TCT AAT ACA AGT CCT TCA TGA TC -3', respectively. Within the forward and reverse primers were recognition sequences for restriction enzymes BamHI (5' -GGATCC- 3') and Kpn1 (5' -GGTACC- 3'), respectively, at their 5' ends underlined above. The selection of these enzymes were due to the presence of their recognition sequences within the multiple cloning region of

the pBIND plasmid into which the PPAR ligand-binding regions were to be cloned but absent in the ligand-binding domain of placenta PPAR γ . These restriction enzyme recognition sequences were to be incorporated into primers and were then incorporated into the amplified products, subsequently the directional cloning of the ligand-binding domain into the pBIND cloning vector, downstream of and in-frame with the Gal4-DBD.

Two PCR reactions were prepared in 50 μ l volumes in PCR tubes, each containing 5 ng DNA template (ppcDNA3-PPAR γ -1 or ppcDNA3-PPAR γ -2), 250 nM each of forward and reverse primer and 25 μ l 2 x My Taq HS mix (Promega). Both reaction tubes were cycled in a thermal cycler (Biometra T gradient) using the following conditions: one minute at 95 °C to activate the enzyme and break the DNA strand (initial denaturation), followed by 20 seconds at 95 °C to allow denaturation, 20 seconds at 55 °C to allow primers to anneal to DNA strands and one minute at 72 °C, an optimum temperature at which 1 kb fragment could be generated (extension). The denaturation, annealing and extension steps were repeated 35 x to get 2³⁵ copies.

3.2.1.2 Precipitation and restriction digestion of DNA

PCR products were precipitated out of enzymatic reactions using ethanol. To each PCR reaction 1/10 volume of 3 M sodium acetate (Sigma) and 2.5 volumes of 95 % ethanol were added. This was mixed thoroughly before incubating the reaction on ice (4 °C) for about 5 minutes, to allow the formation of precipitate. Each reaction was centrifuged at 15,000 x g for 10 minutes. The supernatant was carefully discarded and the DNA pellet was washed with 200 μ l 70 % ethanol before each reaction tubes was centrifuged

again for 5 minutes. The supernatant was removed from each tube and was left to dry at room temperature for 15 minutes to remove excess ethanol, leaving behind a pellet of purified DNA.

Each DNA pellet was re-suspended in 20 µl volumes digestion reactions containing final concentrations of 1 x multicore buffer (Promega), 5 units BamH1 restriction enzyme (Invitrogen) and 5 units Kpn1 restriction enzyme (Invitrogen). pBIND vector (1 µg; Promega) was digested in the same way in 50 µl volume, but whilst the digested reactions were incubated at 37 °C for an hour, the digested pBIND vector was incubated at 37 °C for 3 hours. The digested reactions were heated at 75 °C for 15 minutes to inactivate restriction nucleases. Purified DNAs were obtained from the final 2 digested reactions and a digested pBIND vector using QIAquick spin columns in a microcentrifuge, following the manufacturer's instructions in the QIAquick PCR purification kit protocol. The final products from this procedure obtained were purified DNAs, each eluted in 30 µl MilliQ water. A sample of each purified DNA was loaded onto a 1x TAE, 1 % agarose, 0.5 µg/ml ethidium bromide gel and electrophoresed at 75 V for 45 minutes alongside a 1 kb Hyper Ladder molecular weight marker (Fementas). Ethidium bromide-stained DNA was visualised by exposure of the gel to UV light.

3.2.1.3 Ligation of digested PCR products into digested pBIND vector

The amplified and digested ligand-binding domains were each ligated into pBIND vector in a 20 µl reaction volume, each containing an appropriate molar range of vector to insert of 1:3 that adds up to no more than 100 ng of DNA (volume was adjusted to 14 µl with MilliQ water), 1 µl QS ligase and 5 µl

of 4 x QS buffer. The ligation reactions were incubated at room temperature for 5 minutes.

3.2.1.4 Transformation of pBIND PPAR γ -LBD into Top10 *E. Coli*

Ligated DNA was transformed into Top10 chemo-competent *E. coli* cells. 100 μ l of *E. coli* cells were thawed on ice and 5 μ l of each ligation reaction containing plasmid was added. These were incubated on ice for 30 minutes before the cells were transformed by heat shock in a waterbath at 42°C for one minute. Into each tube of transformed cells, 0.9 ml of sterilised Luria-Bertani (LB) broth was added and incubated at 37 °C for one hour. 100 μ l of transformants were spread on selective LB agar plates (100 μ g/ml ampicillin). Plates were incubated at 37°C overnight. The colonies from each plate were inoculated into 4 ml cultures (LB media, supplemented with 100 μ g ampicillin) and these were incubated overnight at 37°C with agitation (~150 rpm). Purification of plasmid DNA from cultures was done using the ISOLATE II Plasmid Mini Kit, following manufacturer's instructions. DNA was eluted from columns using 70 μ l MilliQ water.

A restriction digest was performed on a sample of each eluate as described in Section 2.2.4.1. Each purified DNA was loaded onto a 1 x TAE, 1 % agarose, 0.5 μ g/ml ethidium bromide gel and electrophoresed at 75 V for 45 minutes alongside a 1 kb Hyper Ladder molecular weight marker (Fementas) to confirm the insertion of DNA fragments into the pBIND vector. Ethidium bromide-stained DNA was visualised by exposure of the gel to UV light. Successful cloning was indicated by the presence of at least one DNA band at 6 kb pBIND vector and another band about 1000 bp PPAR γ ligand-binding

domain insert before the plasmids were sent for sequencing to confirm this, using the selection of sequences in Table 3-1.

Table 3-1 Primer (sequences) used in the sequencing of pBIND vector containing ligand-binding domain of PPAR γ

Name of Sequencing Primer	Primer sequence (5' to 3')
pBINDseqF	CTC TAA CAT TGA GAC AGC
pBINDseqR	GGT TTG TCC AAA CTC ATC
PPAR γ LBDseqR	CCT CAG ATC TGC TGC TTC

3.2.2 Cell culture

3.2.2.1 Brief description of cell lines

The established cell lines of AS and CHSE-214 were obtained from the Laboratory of Disease and Virology, Institute of Aquaculture, School of Natural Sciences, University of Stirling, Stirling (UK). The AS cell line has a monolayer culture of fibroblast-like cells originally derived from a trypsin digest of heart, liver, kidney and spleen tissue of young Atlantic salmon (*Salmon salar*) (Nicholson and Byrne, 1973). Likewise, the monolayer culture of CHSE-214 has fibroblast-like morphology and this established cell line was initiated from Chinook salmon (*Oncorhynchus tshawytscha*) embryo (Lannan *et al.*, 1984).

3.2.2.2 Routine culture of cells

Cells were grown in 25 cm² tissue culture flasks (Sarstedt AG & Co. Laboratories, Nümbrecht, Germany) in the complete media, containing 5ml Eagle's Minimum Essential Medium (EMEM; Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS), 1 x non-essential amino acids (NEAA) and 2 mM L-glutamine, incubated at 22 °C in an atmosphere of 4 % carbon dioxide CO₂ (the lids of flasks were left slightly loose). All media were prepared under sterile conditions, kept at room temperature (20-22 °C) and supplemented weekly with 200 µM L-glutamine. Cells were passaged 1:3 once per week before reaching full confluence and the growth of the monolayers were observed under the inverted microscope (Olympus IMT-2).

All experiments were performed on cultures at 80-85 % confluence when the cells were still actively growing.

3.2.2.3 Harvesting of cells

Cells were harvested from the 25 cm² tissue culture flasks for the purpose of passaging, seeding and cell counting. The medium was removed from flasks by decanting and the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS; Gibco). Cells were harvested by adding 1 ml of 0.05 % trypsin/ethylenediaminetetraacetic acid (EDTA), with incubation for 2 min at room temperature. When the monolayer became opaque, excess trypsin/EDTA was decanted and the cells were dislodged by sharply tapping the side of flasks with the palm of the hand. Cells were then re-suspended in an appropriate volume of the complete media by pipetting up and down to ensure a homogenous cell suspension for further procedures depending on the purpose mentioned earlier.

3.2.2.4 Counting and seeding of cells

Prior to seeding of cells, a cell count using the trypan blue exclusion method, was used to assess cell viability. Trypan blue solution was used to identify living and dead cells and with this solution, dead cells are permeable to take up the dye and are stained blue while the viable cells remain colourless do not take up impermeable dyes. Harvested cells were re-suspended in 3ml of fresh medium followed by gentle pipetting to ensure homogeneity. The cell suspension was then transferred to a fresh Bijoux sample container from which 100 µl was aliquoted and mixed with an equal volume of 0.4 % trypan

blue solution. A standard Neubauer chamber haemocytometer (improved Neubauer B.S. 748 Depth 0.1 mm or $1/400 \text{ mm}^2$) was used and a special haemocytometer coverslip 20 x 26 x 0.4 mm was pressed onto the slide to ensure good adherence between the coverslip and the haemocytometer and both chambers filled by capillary action with the stained cell suspension. Looking through the microscope under a low power objective, the viable, colourless cells within the 1 mm^2 square of the central portion of the ruled pattern were counted and the cell numbers were averaged over the two chambers. To calculate the number of cells per ml suspension, this average was multiplied by 1000 and again multiplied by the volume of cell suspension to give the total number of cells. To achieve a cell density of, the cell suspension was diluted with complete media to achieve a cell density of 2.0×10^5 cells/ml and to the wells of 24-well assay plates (Corning), 1ml of cell suspension was seeded, incubated overnight at 22 °C in an atmosphere of 4 % CO₂, ready for transfection the following day.

3.2.2.5 Freezing and preservation of cells

Cells were preserved for future use and subsequent experiments to ensure that all transfections were performed on cells with similar passage numbers. As described in Section 3.2.2.3, cells harvested from a 25 cm² tissue culture flask were re-suspended in 3 ml of growth medium by gently pipetting up and down. This cell suspension was collected in a universal container and a viable cell count was performed as described in Section 3.2.2.4. Cryovials were used to store the cells and each cryovial was prepared with the following details: cell line, passage number, date of freezing and number of

cells per ampoule. The total volume was calculated and adjusted using freezing medium (growth media + 10% dimethyl sulphoxide, DMSO) to produce a solution containing 3×10^6 cells per ml per cryovial. All cryovials were placed in $-20\text{ }^{\circ}\text{C}$ freezer, upright in a polystyrene box to avoid cell suspension freezing into lid, for 1 hour before they were being transferred to the $-70\text{ }^{\circ}\text{C}$ freezer for an additional 2 hours. Cryovials were then removed from the box, placed in labelled canes and immediately immersed in liquid nitrogen ($-196\text{ }^{\circ}\text{C}$).

3.2.3 Optimisation 1: Transfection of cell lines

AS and CHSE-214 cells were harvested and seeded into 24-well assay plates (Corning) 24 hours prior to transfection, as explained in Sections 3.2.2.3 and 3.2.2.4. The cells were incubated overnight at $22\text{ }^{\circ}\text{C}$ in an atmosphere of 4 % CO_2 .

3.2.3.1 Transfection of cell lines with plasmid DNA using bPEI reagent

According to Hsu and Uludağ (2012), the use of branched polyethylenimine (bPEI) reagent has been optimised for transfection of a variety of mammalian cells. In this project, optimisation was required for the transfection of DNA into fish cell lines by altering the ratio of bPEI reagent to DNA. The recommended ratio of bPEI (μg) to DNA (μg), 2.5:1 (Hsu and Uludağ, 2012) was used as a starting point in the transfection of the cells and that half, double and quadruple the recommended ratio were also tested.

In this first part optimisation experiment, plaice Gal4-PPAR α was used as a positive control and the transfection efficiencies were assessed by measuring

the levels of a constitutively expressed *Renilla* luciferase gene. A total of four transfection mixtures with bPEI:DNA ($\mu\text{g}:\mu\text{g}$) ratio of 1.25:1, 2.5:1, 5:1 and 10:1 were prepared using two-part mixing in salt-free buffer method (Hsu and Uludağ, 2012), to a volume enough for triplicates for both cell lines. Each transfection mix contained 1 μg of pDNA in equal amount (ratio 1:1:1): Gal4-PPAR α , a plasmid containing the firefly luciferase reporter gene pGL4.31[*luc2P/Gal4UAS/hygro*], used to report transcriptional activation, and a plasmid containing the *Renilla* luciferase gene pGL4.75[*hRluc/CMV*], used as an indicator of transfection efficiency. These plasmids were used because this combination reflects the experimental used in subsequent PPAR assays.

Pure bPEI (Sigma) stock solution was prepared by dissolving the powder in MilliQ water to a concentration of 5 mg/ml, sterile filtered through 0.22 μm membrane and stored at 4 °C. For transfection experiments, diluted bPEI of 1 mg/ml was used.

Table 3-2 The four transfection mixes prepared using two-part mixing in salt-free buffer method with bPEI transfection reagent, each containing the Gal-PPAR α , pGL4.31[*luc2P/Gal4UAS/hygro*] and pGL4.75[*hRluc*/CMV] at a ratio of 1:1:1 and different amounts of bPEI reagent. bPEI reagent was added before each of the four transfection mixes was split between 3 wells containing AS cells.

DNA Ratio (1 μ g)	1:1:1			
Gal4-PPAR α (ng)	333.3	333.3	333.3	333.3
pGL4.31[<i>luc2P/Gal4UAS/hygro</i>] (ng)	333.3	333.3	333.3	333.3
pGL4.75[<i>hRluc</i> /CMV] (ng)	333.3	333.3	333.3	333.3
bPEI:DNA (μ g: μ g)	1.25:1	2.5:1	5:1	10:1

A total of 1 μ g plasmid DNA containing Gal-PPAR α plasmid, pGL4.31[*luc2P/Gal4UAS/hygro*] and pGL4.75[*hRluc*/CMV] in a 1:1:1 ratio, respectively, was diluted with EMEM (containing no FBS or antibiotics), to a final concentration of 0.02 μ g/ml. In separate tubes, EMEM (containing no FBS or antibiotics) was combined with 1 mg/ml bPEI reagent (Table 3-2) and incubated at room temperature for 5 min. The diluted DNA solution was then added to the diluted polymer solution vortex mixed for 5 s before being incubated at room temperature for 25 min to allow for a complex between the bPEI reagent and DNA to form. This polyplex solution was then diluted in 400 μ l EMEM (containing no FBS or antibiotics) and was left at room temperature for an additional 5 min. The cell culture medium was removed

from each well of the 24-well plate by aspiration and 500 μ l of diluted polyplex transfection mixture was then added to the each well. The plate was gently agitated before it was centrifuged at 210 x g for 5 min at room temperature, forcing the complexes onto the cell surface at the bottom of the plate in a microplate adaptor rotor. The plate was then gently removed from the centrifuge, being careful not to disturb the medium and incubated overnight at 22 °C in an atmosphere of 4 % CO₂. The following day, the transfection mixture was removed from each well by aspiration and was replaced with 1ml complete media and incubated overnight at 22 °C in an atmosphere of 4 % CO₂. The aim of this experiment was to identify the most suitable and effective cell line for transfection using bPEI reagent.

3.2.3.1.1 Measurement of Firefly (*Photinus pyralis*) luciferase

After 24 hr incubation, the cell media were removed from the plate by aspiration and blotted on paper to remove any excess media. The cells in each well were washed using 1 x DPBS, twice. Cells were then lysed in 1 ml of 1 x passive lysis buffer (Promega) for 10 min, shaking. The cell lysate was then used in a dual luciferase assay system in which 75 μ l of cell lysate from each well was transferred to a black 96-well plate (Corning). Into each well, equal volume of a 2 x concentrated volume of “homemade” firefly luciferase buffer was added to wells and the final composition of the homemade buffer in wells was 15 mM potassium phosphate (K_xPO₄, pH 8.0), 25 mM glycylglycine (pH 8.0), 1 mM dithiothreitol (DTT, Sigma), 15 mM magnesium chloride (MgCl₂), 1 mM adenosine triphosphate (ATP), 100 μ M coenzyme A (CoA, Sigma) and 150 μ M luciferin (Promega). The contents of the wells were

pipetted up and down to mix and was incubated for 2 min before luciferase activity was measured on the Wallac 1420 Victor 2 multilabel counter (Perkin Elmer).

3.2.3.1.2 Measurement of *Renilla (Renilla reniformis)* luciferase

Following measurement of firefly luciferase, 75 μ l volume of 3x concentrated “homemade” *Renilla* luciferase buffer was added. The final composition of this reagent was 575 mM sodium chloride (NaCl), 100 mM sodium phosphate (NaPO₄, pH 5.1), 2 mM Ethylenediaminetetraacetic acid (EDTA), 1 mM sodium azide (NaN₃), 0.33 mg/ml BSA (Sigma) and 1 μ M coelenterazine (Promega). The contents were again pipetted up and down to mix and a 2 min incubation given. *Renilla* luciferase activity was measured on the Wallac 1420 Victor 2 multilabel counter (Perkin Elmer).

Both firefly and *Renilla* luciferase activities were assayed in order to accurately reflect the conditions for subsequent PPAR assays.

3.2.3.1.3 Data analysis

For this experiment, the *Renilla* luciferase values were averaged and these values would represent the transfection efficiencies of various bPEI to DNA ratios on transfected AS and CHSE-214 cells. The results are based only on graphical trends.

3.2.3.2 Transfection of AS cells with different amount of DNA

Renilla luciferase enzymes were expressed relatively higher in AS cell line than in CHSE-214 cell line at bPEI:DNA ratio of 2.5:1 (Figure 2-1). Another optimisation experiment was carried out by varying the amount of DNA plasmids in the 0.1 mg/ml DNA mix containing Gal4-PPAR α , pGL4.31[*luc2P/Gal4UAS/hygro*] and pGL4.75[*hRluc*/CMV] i.e. 1:1:1, 1:1:0.5 and 1:1:0.2, respectively and at a constant ratio of bPEI:DNA 2.5:1.

In this experiment, transfected cells were subsequently treated with 4-Chloro-6-(2,3-xylo-dino)-2-pyrimidinylthioacetic acid (WY-14,643; Sigma), a compound previously identified as a ligand, reported to activate mammalian and piscine PPAR α . A 2.5 mM stock solution of WY-14,643 compound was prepared in ethanol absolute and 10 μ l was diluted into 1 ml complete EMEM, giving a final concentration of 25 μ M. A control treatment of ethanol vehicle was also prepared, in which ethanol was diluted into EMEM at a concentration of 5 μ l/ml. This experiment aimed to determine the activation of Gal4-PPAR α by WY-14,643 by measuring the firefly luciferase signal at various amounts of DNA, in order to confirm that the PPAR assay worked under the transfection and luciferase assay conditions used.

Table 3-3 The three transfection mixes prepared using two-part mixing in salt-free buffer method with bPEI transfection reagent, each containing DNA mix of Gal4-PPAR α , pGL4.31[*luc2P/Gal4UAS/hygro*] and pGL4.75[*hRluc*/CMV] at different ratio 1:1:1, 1:1:0.5 and 1:1:0.2, respectively. bPEI reagent was added before each of the three transfection mixes was split between 3 wells containing AS cells.

bPEI:DNA ($\mu\text{g}:\mu\text{g}$)	2.5:1		
DNA Ratio (1 μg or 0.1 mg/ml)	1:1:1	1:1:0.5	1:1:0.2
Gal4-PPAR α (ng)	333.3	400.0	450.0
pGL4.31[<i>luc2P/Gal4UAS/hygro</i>](ng)	333.3	400.0	450.0
pGL4.75[<i>hRluc</i> /CMV] (ng)	333.3	200.0	100.0

3.2.3.3 Transfection of AS and CHSE-214 cells with pEGFP

The AS cell line showed instability in cell growth after a few passages and that the firefly luciferase activities were observed to have decreased at higher passage numbers when the transfected cells were treated with WY-14,643 (Figure 3-6). Thus, both AS and CHSE-214 cell lines were once again tested for optimisation but this time, enhanced green fluorescent protein plasmid (pEGFP) was transfected into the cells. This experiment was to determine the transfection efficiency by visually observing the expression of this plasmid in the transfected cells under a fluorescent microscope (Olympus). Six transfection mixes were prepared using a constant amount of pEGFP, 0.1 mg/ml (or 1 µg), and the bPEI:DNA (µg:µg) ratio set ups were 1:1, 2.5:1, 4:1 and doubling the amount of bPEI and DNA to the three ratios. These were added to the cells and were incubated overnight at 22 °C in an atmosphere of 4 % CO₂.

3.2.4 Optimisation 2: Transfection using CHSE-214 cell line

3.2.4.1 Transfection of CHSE-214 with plasmid DNA using bPEI

Transfected pEGFP was highly expressed in CHSE-214 cells when the amount of bPEI:DNA ratio of 2.5:1 was doubled (Figure 3-8). This was further tested in another optimisation experiment in the dual luciferase assay by measuring the expression of firefly and *Renilla* luciferase enzymes.

Table 3-4 The six transfection mixes prepared using two-part mixing in salt-free buffer method with bPEI transfection reagent, each containing the Gal4-PPAR α , pGL4.31[*luc2P/Gal4UAS/hygro*] and pGL4.75[*hRluc*/CMV] at ratio of 1:1:0.5, respectively. The bPEI:DNA ratio set ups were 1:1, 2.5:1, 4:1, and doubling the amount of bPEI and DNA to the three ratios. bPEI reagent was added before each of the six transfection mixes was split between 3 wells containing CHSE-214 cells.

DNA Ratio(1 μ g)	1:1:0.5			2 x (1:1:0.5)		
Gal4-PPAR α (ng)	400.0	400.0	400.0	800.0	800.0	800.0
pGL4.31[<i>luc2P/Gal4UAS/hygro</i>] (ng)	400.0	400.0	400.0	800.0	800.0	800.0
pGL4.75[<i>hRluc</i> /CMV](ng)	200.0	200.0	200.0	400.0	400.0	400.0
bPEI:DNA (μ g: μ g)	1:1	2.5:1	4:1	2 x (1:1)	2 x (2.5:1)	2 x (4:1)

3.2.5 Treatment of transfected CHSE-214 cells with PPAR ligands and fatty acids

The selected PPAR activators were 4-Chloro-6-(2,3-xylodino)-2-pyrimidinylthioacetic acid (WY-14,643) and rosiglitazone that had been previously reported to activate piscine PPAR α (Leaver *et al.*, 2005; Colliar, *et al.*, 2011) and mammalian PPAR γ respectively. Concentration of Wy-14643 (Sigma) was prepared at 2.5 mM stock concentration in ethanol and rosiglitazone at 5 mM. Selected fatty acids were prepared at 10 mM were docosahexaenoic acid (22:6; DHA), eicosapentenoic acid (20:5; EPA) and oleoylethanolamide (OEA).

In bijoux tubes, each compound was diluted into complete EMEM at a concentration of 10 μ l per ml, to give final assay concentrations of 25 μ M Wy-14643, 50 μ M rosiglitazone and 100 μ M fatty acids, enough to produce triplicates with each compound. An ethanol vehicle was also prepared as a control treatment at a concentration of 10 μ l ethanol absolute per ml of EMEM. The diluted treatment compounds were mixed thoroughly before being added to transfected cells. Transfection mixes were removed from each well of the 24-well assay plate by aspiration and the cells were washed once with DPBS, after which 1 ml of treatment compounds was added to cells. Cells were subsequently incubated with treatment compounds overnight at 22 °C in an atmosphere of 4 % CO₂ before assaying for luciferase activity.

3.2.5.1 Data normalisation and statistical analysis

Firefly and *Renilla* luminescence readings from three wells transfected cells in which no DNA had been transfected were taken as background levels. The background levels for the firefly and *Renilla* values were each averaged and subtracted from firefly and *Renilla* luciferase values from transfected cells, respectively. To account for differences in transfection efficiency between wells on an assay plate, the ratio of firefly to *Renilla* luminescence was calculated and the mean of replicate wells was obtained. To test for statistically significant differences between ethanol control treatment and treatment with PPAR agonists and fatty acids, T-test was carried out and results were considered statistically significant when the probability value (P) was less than 0.05 ($P < 0.05$).

3.3 Results

3.3.1 Construction of Gal4-PPAR γ expression plasmid

3.3.1.1 Amplification of PPAR γ ligand-binding domain

The cDNA encoding the LBD of PPAR γ were amplified by the polymerase chain reaction (PCR), using the primer pairs designed to the 5' and 3' of the ligand-binding domain of PPAR γ , yielding an expected DNA fragment of 1014 base pairs. This expected size of the PCR product was revealed in the agarose gel reflected the size for the PPAR γ ligand-binding domain.

3.3.1.2 Cloning of PCR-amplified DNA fragment into the pBIND vector

Following transformation of ligation product that was formed from DNA fragment being cloned in pBIND vector using restriction digestion and ligation reaction, into Top10 *E. coli* cells, a total of 23 colonies were formed on the agar plate. Following purification of plasmid DNA, a restriction digest was performed on a sample of each plasmid to confirm which, if any, of the purified plasmids contained the ligand-binding domain of PPAR γ . In 5 of 23 samples, two bands were visible following restriction digestions and these corresponded to the expected size of the PPAR γ -LBD insert of about 1000 base pairs and the pBIND vector of about 6 kb size, indicating successful cloning.

3.3.1.3 Sequencing of the pBIND-PPAR γ LBD plasmid

Successful cloning was indicated by the production of two DNA fragments as a result of restriction digestion. Each of the five purified plasmids prepared in Section 3.2.1.4, was sequenced to confirm (i) that the insert in the pBIND vector was the ligand-binding domain of PPAR γ and (ii) that the ligand-binding sequence and the Gal4 DNA-binding domain of the pBIND vector were in the same continuous reading frame. Using the software SeqManPRO, The raw sequencing files for each plasmid were assembled to a single continuous contig for PPAR γ and were compared to the PPAR γ sequences from plaice already available in the database, confirming that one out of five purified plasmids had identical sequences and that the Gal4 DNA-binding domain and PPAR γ -LBD were in a continuous, single frame (Figure 3-3).

```

:GAATCAAGGCTAGAAAAGACTGGAACAGCTATTTCTACTGATTTTTCTCGAGAAGACCTT
|-----|
E S R L E R L E Q L F L L I F P R E D L
GACATGATTTGAAAATGGATTCTTTACAGGATATAAAAGCATTGTTAACAGGATTATT
|-----|
D M I L K M D S L Q D I K A L L T G L F
:GTACAAGATAATGTGAATAAAGATGCCGTCACAGATAGATTGGCTTCAGTGGAGACTGAT
|-----|
V Q D N V N K D A V T D R L A S V E T D
ATGCCCTTAACATTGRGMCAGCATAGAATAAGTGCACATCATCATCGGAAGAGAGTAGT
|-----|
M P L T L X Q H R I S A T S S S E E S S
:AMCAAAGGTCAAAGACAGTTGACTGTATCGCCGGAATCCCGGGATCCGATGTCACAC
|-----|
X K G Q R Q L T V S P E F P G I R M S H
:AACGCTATTGTTTTGGCCGGATGCCGAGGCAGAGAAGGAGAAGCTTCTGGCCGGAGTTC
|-----|
N A I R F G R M P Q A E K E K L L A E F
TCGCTGACATGGAGCACATGCACCCGGAAGCAGCAGATCTGAGGGCCTTGGCCCGCCAT
|-----|
S S D M E H M H P E A A A D L R A L A R H
CTGTACGAGGCCTATCTGAAATACTTCCCCCTCACCAAGGCCAAGGCTAGAGCCATCCTC
|-----|
L Y E A Y L K Y F P L T K A K A R A I L
TCTGGGAAGACCGAGACAACGCGCCTTTGTTCATCCATGACATTAAGTCTCTAATGGAA
|-----|
S G K T G D N A P F V I H D I K S L M E
:GGAGAGCAGTTCATCAATTGCAGGCAGATGCCCATCCAGGAGCAGCAGCAGGCCTCTGTC
|-----|
G E Q F I N C R Q M P I Q E Q Q Q A S V
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|-----|
L T A T H R G L T E H H M G S D Y G V W
:GGAACGACGAGCATCAGCGGACAGSAGCCACAGAACGCTTTGGAGCTACGTTTCTTCCAA
|-----|
G T T S I S G Q E P Q N A L E L R F F Q
AGCTGTGTCAGTCACGCTCTGCTGAAGCAGTGAGGGAAGTACTGAGTTTGGCAAGAGTATC
|-----|
S C Q S R S A E A V R E V T E F A K S I
CCAGATTCACTGATCTGGATCTAAACGATCAGGTGACTTTGCTGAAGTATGGTGTGATA
|-----|
P G F T D L D L N D Q V T L L K Y G V I
GAGTCTTGATTATCATGATGTCCTCCTGATGAACAAGACGGGACCCCTGATCTCTTAC
|-----|
E V L I I M M S P L M N K D G T L I S Y
:GGCAGATCTTCATGACCGGGGAGTTCCTCAAGAGTCTCAGGAAACCTTTCTGTCAAATG
|-----|
G Q I F M T R E F L K S L R K P F C Q M
ATGGAACCGAAGTTCGAGTTCCTC GTTAAGTTC AACACGCTCGAGCTGGACGACAGTGAC
|-----|
M E P K F E F S V K F N T L E L D D S D
ATGGCGCTGTTTCTGGTTGTCATTATCCTCAGCGGGGACCGTCCAGGCCCTTCTGAACGTG
|-----|
M A L F L V V I I L S G D R P G L L N V
:AAACCCATCGAGCAGCTTCAGGAGACCGTGCTTCACTCAGCTGCAGCTGAAGCTG
|-----|
K P I E Q L Q E T V L H S L E L O L K L
AACCACCCGACTCTCTGCAGTGTTCGCAAGCTGCTCCAGAA GATGACCGACCTGCGG
|-----|
N H P D S L Q L F A K L L Q K M T D L R
:CAGATCGTCACGGACCAGTGCACCTCATCCAGCTGCTGAAGAAGACCGAGGTGGACATG
|-----|
Q I V T D H V H L I Q L L K K T E V D M
TGCCACACCCGCTGCTGCAGGAGATCATGAAGGACTGTATTAGAGGGTACC TGAATAA
|-----|
C L H P L L Q E I M K D L Y R V P E
:CTAAGGCCGCTTCCCTTTAGTGAGGGTTAATGCTTCGAGCAGACTTGATAAGATACTTTG
|-----|
L R P L P F S E G C F E Q T D T L

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Figure 3-3 Nucleotide and deduced amino acid sequences of Gal4-PPAR γ contigs assembled using SeqManPRO software. The first and stop codons of PPAR ligand-binding domains are boxed in blue. The BamH1 (GGATCC) and Kpn1 (GGTACC) enzyme recognition sites are boxed in red.

3.3.2 Transfection efficiency of bPEI reagent

Branched polyethylenimine (bPEI) was one of the first cationic polymers to be used as transfection reagent. Its transfection ability depends on the interaction between the positive charges of amine groups and the negatively charged phosphate backbone of DNA and its transfection efficiency depends on the overall net charge of the bPEI/DNA complex when bPEI condenses DNA, which consequently affects the binding ability of these complexes to the cell surfaces and DNA dissociation into the cell cytoplasm (Akinc *et al.*, 2005; Hsu and Uludağ, 2012).

In practical, the overall net charge of the complexes is dependent on the amounts of the reagent and the DNA, thus, in order to determine the most suitable cell line, different bPEI to DNA ratios were used to measure the transfection efficiencies and these were determined by measuring the luciferase signal from the constitutively expressed *Renilla* luciferase gene, present on the transfected internal control plasmid pGL4.75[*hRluc*/CMV] (Figure 3-4). Maximum *Renilla* luciferase signal, 40.5-fold over the control (mock transfected wells in which no DNA was transfected), was observed at bPEI to DNA ratio of 2.5:1, when plasmid DNA was transfected into AS cells. However, the efficiency decreased to 4.4-fold and 0.95-fold at bPEI to DNA ratios of 5.0:1 and 10.0:1, respectively. On the other hand, the efficiency with

which plasmid DNA was transfected into CHSE-214 cells saw the highest *Renilla* luciferase signal, 4.8-fold over the control at 1.25:1 bPEI to DNA ratio, and this decreased with increasing bPEI to DNA ratio, to 2.0-fold, 1.5-fold and 0.95-fold at bPEI to DNA ratios of 2.5:1, 5.0:1 and 10.0:1, respectively.

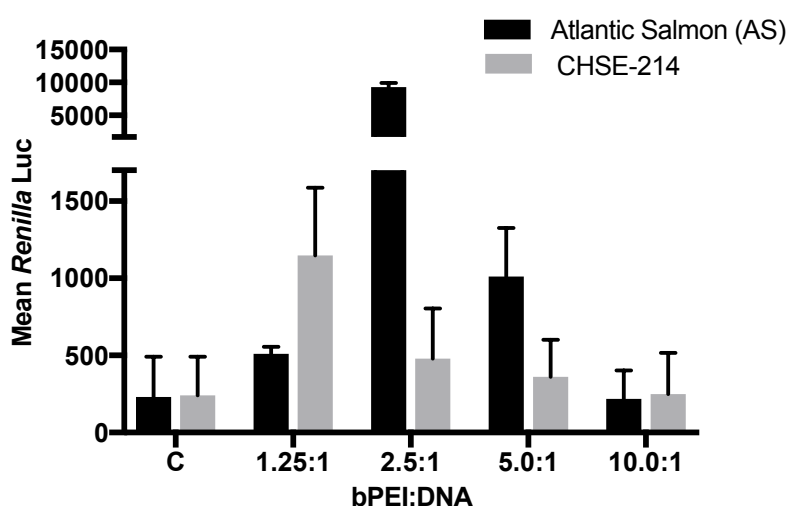


Figure 3-4 The mean transfection efficiencies (+ SD) at four different bPEI reagent to DNA ratios (μg to μg), containing equal amounts of plasmid DNA i.e. Gal4-PPAR α , pGL4.31[luc2P/Gal4UAS/hygro] and pGL4.75[hRluc/CMV]. Different ratios of bPEI:DNA were used to transfect AS and CHSE-214 before they were assayed for *Renilla* luciferase signal, used as a measurement of transfection efficiency.

The results have indicated that the AS cells had the highest transfection efficiency at bPEI:DNA ratio of 2.5:1, thus AS cell line and this optimised ratio were subsequently used to determine the effect of altering the amounts of plasmids transfected into the AS cells while maintaining a constant final concentration of 0.1 mg/ml DNA mix and the optimised amount of 2.5 μl bPEI (Figure 3-5). In this experiment, the PPAR α agonist, WY-14,643 compound,

was used to measure the firefly luciferase activity to determine Gal4-PPAR α activation. The highest firefly luciferase signal was observed at DNA ratio of 1:1:0.5, containing DNA mix of 400 ng Gal4-PPAR α , 400 ng pGL4.31[*luc2P/Gal4UAS/hygro*] and 200 ng pGL4.75[*hRluc*/CMV], with 9.4-fold increase over the ethanol control vehicle.

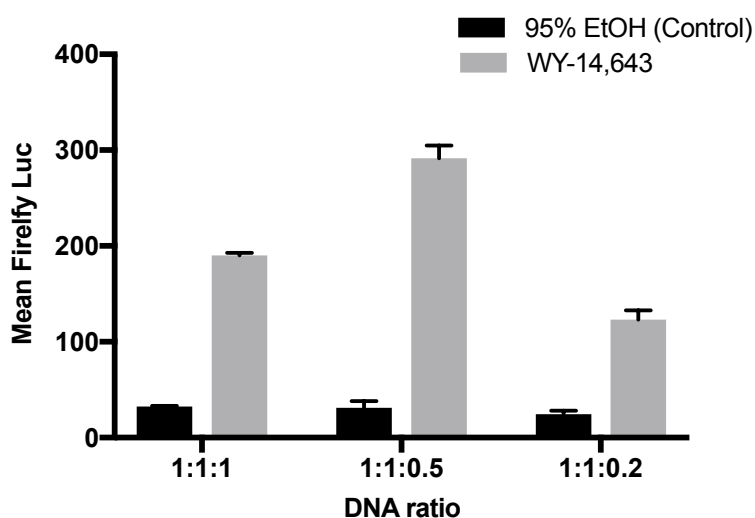


Figure 3-5 The mean firefly luciferase signals (+ SD) measured from transfected AS cells at 2.5:1, bPEI to DNA ratio, with different amounts of plasmid DNAs, Gal4-PPAR α , pGL4.31[*luc2P/Gal4UAS/hygro*] and pGL4.75[*hRluc*/CMV], respectively and treated with 95 % ethanol vehicle and PPAR α agonist, WY-14,643 compound.

3.3.3 Re-optimisation of AS and CHSE-214 cell lines

The AS cell line had shown instability in reproducibility when the same transfection experiment was repeated using a higher passage number i.e. p121 (labelled 'new' in Figure 3-6). When the transfected AS cells were treated with the PPAR α agonist, WY-14,643 compound, the firefly luciferase signals were significantly lower compared to that of the activity signals observed in the previous experiment, using AS cells with a lower passage number, p99 (labelled 'old' in Figure 3-6). Thus, re-assessment of the efficiency of bPEI to transfect AS and CHSE-214 cell lines were again conducted using enhanced green fluorescent protein plasmid (pEGFP) to determine the quality of the cells and the efficiency of the transfection reagent.

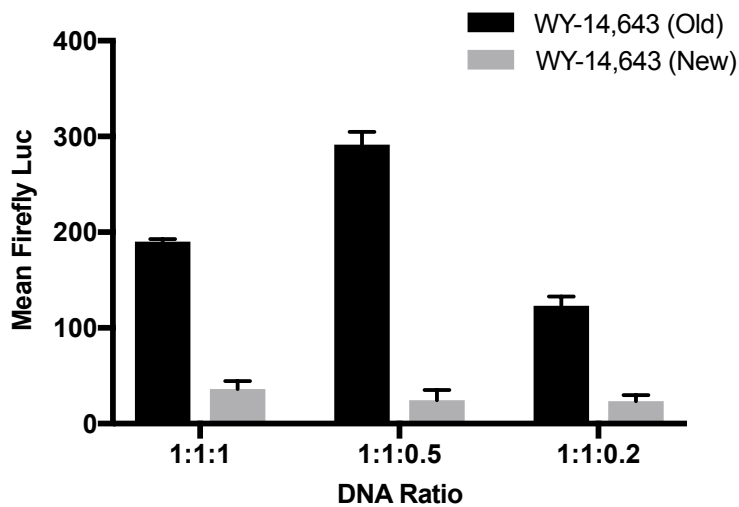


Figure 3-6 The mean firefly luciferase signals (+ SD) from the previous transfection experiment (labelled "old", from Figure 3-2), plotted against the latest firefly luciferase signals measured from the same experiment (labelled "new"). AS cells were transfected with different amounts of plasmid DNAs, Gal4-PPAR α , pGL4.31[*luc2P/Gal4UAS/hygro*] and pGL4.75[*hRluc/CMV*], at 2.5:1, bPEI to DNA ratio, and treated with 95 % ethanol vehicle and PPAR α agonist, WY-14,643 compound.

3.3.3.1 Transfection of cell lines with pEGFP

The effect of transfecting 1 μ g pEGFP into AS and CHSE-214 cell lines using different amounts of bPEI was investigated to determine the efficiency of the transfection reagent. The expression of the transfected plasmid DNA was visually observed under an inverted fluorescent microscope equipped with a digital camera and successful transfections of cells are indicated by green fluorescence. Results have shown that the green fluorescence AS cells. In the microscope images (Figure 3-7), no fluorescence was observed when the AS cells were transfected with 1:1, bPEI to DNA ratio, not even when the amount was doubled. Although the number of green AS cells increased when the amounts of bPEI and DNA were increased, the number of transfected cells, thus, transfection efficiency, was however, insignificant compared to the successful transfection of CHSE-214 cells observed (Figure 3-8). CHSE-214 cells treated with twice the amount of bPEI to DNA ratio, i.e. 2 x 2.5:1, showed the most numerous and brightest green fluorescent spots (Figure 3-8(D)) and this was in accordance with the increased transfection efficiency expressed by *Renilla* luciferase gene by 1268-fold over the control (mock transfected wells in which no DNA was transfected) (Figure 3-9). *Renilla* luciferase signals were observed to be about 5 times lower when the cells were treated with the recommended ratio of 2.5:1.

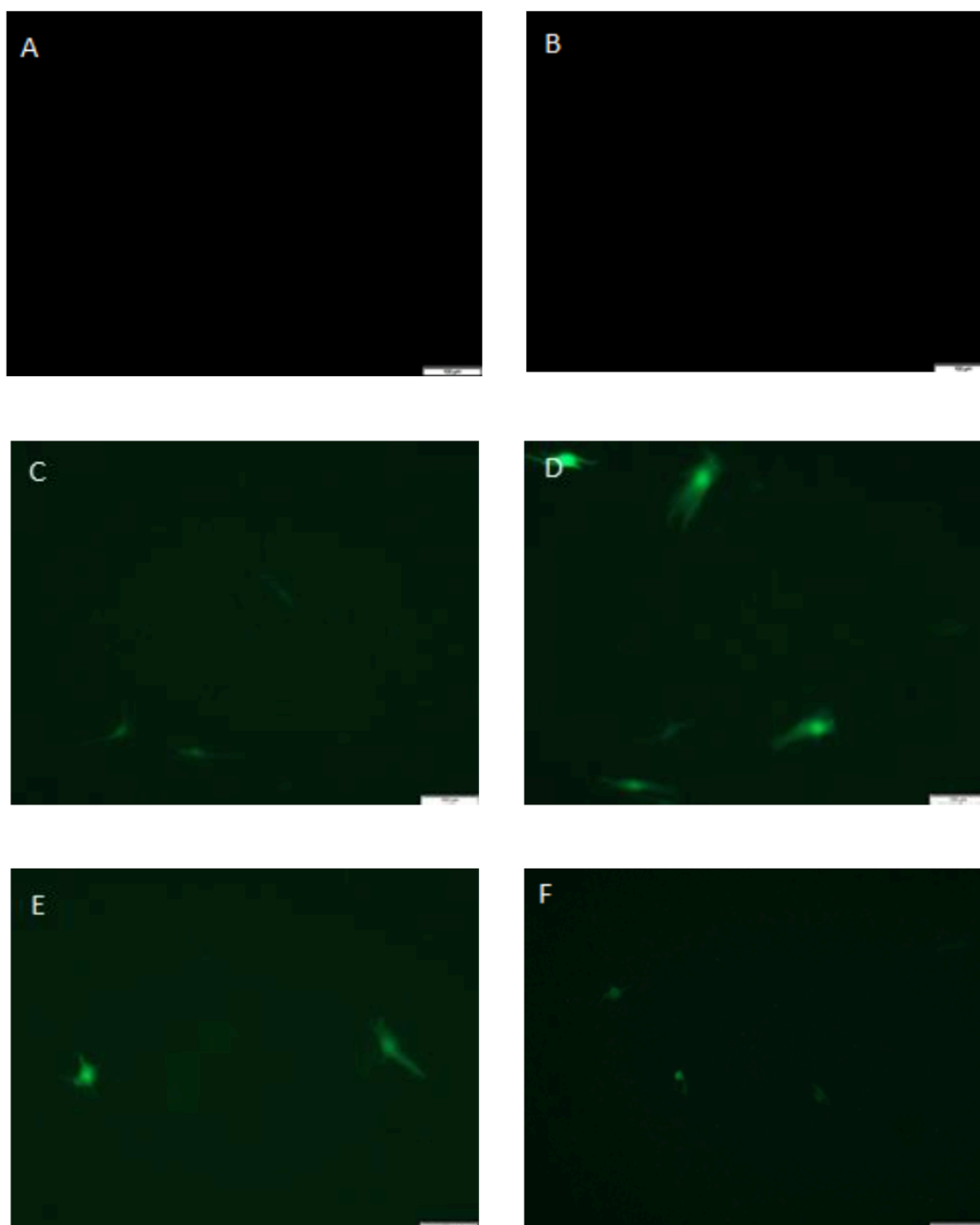


Figure 3-7 Morphology of “new” AS cells (at passage number 121) transfected with pEGFP overnight. Cells were transfected with the following bPEI:DNA ratios: (A) 1:1 (B) 2 x 1:1 (C) 2.5:1 (D) 2 x 2.5:1 (E) 4:1 (F) 2 x 4:1. Bar is approximately 100 μ m.

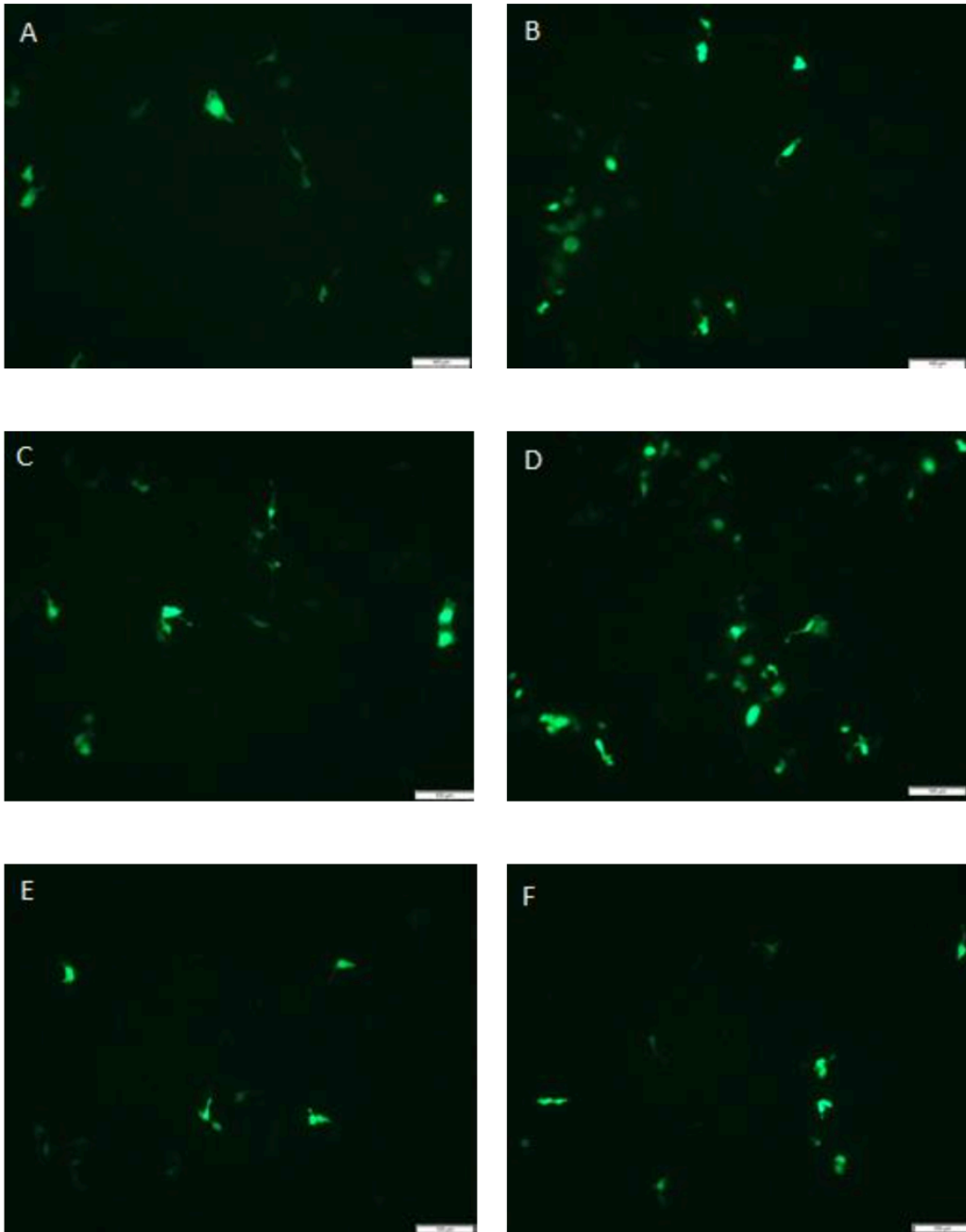


Figure 3-8 Morphology of CHSE-214 cells transfected with pEGFP overnight. Cells were transfected with the following bPEI:DNA ratio: (A) 1:1 (B) 2 x 1:1 (C) 2.5:1 (D) 2 x 2.5:1 (E) 4:1 (F) 2 x 4:1. Bar is approximately 100 μ m.

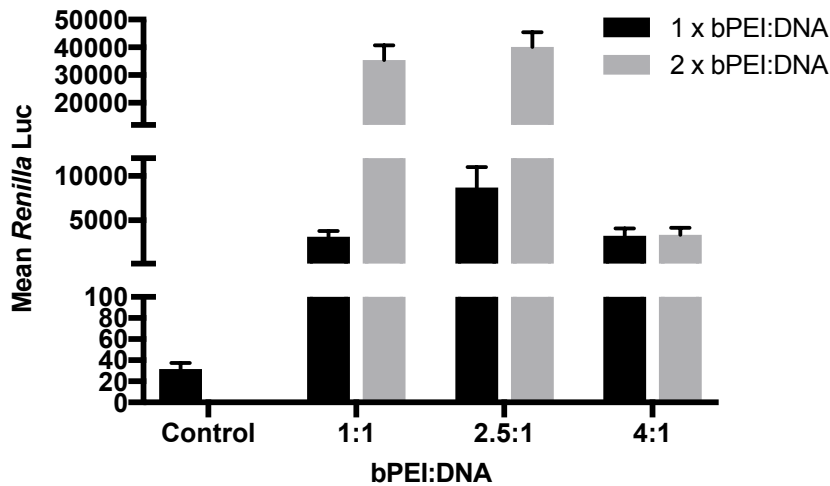


Figure 3-9 The mean transfection efficiencies (+ SD) at three different bPEI reagent to DNA ratios (μg to μg). Different ratios of bPEI:DNA were used to transfect CHSE-214 before they were assayed for *Renilla* luciferase signal, used as measurement of transfection efficiency.

When various amounts of bPEI to DNA ratio were transfected, cells transfected with the ratio 1:1 showed an efficiency of 100-fold over control and this increased by 11 times when the ratio was doubled. Transfection efficiencies at 4:1, bPEI to DNA ratio were also about 100-fold over the control, even when the ratio was doubled. Therefore, in the future transfections, the more stable CHSE-214 cells were chosen as the suitable cell line and doubling the amount of bPEI to DNA ratio, 2.5:1, was used as the optimised ratio for future transfections.

3.3.4 Transactivation of Gal4-PPAR with PPAR ligands and fatty acids

CHSE-214 cells transfected with Gal4-PPAR α and subsequently exposed to 25 μ M Wy-14,643 showed the highest luciferase activity which was about 6.7-fold over the ethanol control vehicle (Figure 3-10). This increase of induction in reporter gene expression by the PPAR α specific synthetic ligand, WY-14,643, has been previously shown in transient transfection assays using native full-length PPAR α from European plaice (Leaver *et al.*, 2005) and plaice Gal4-PPAR α (Colliar *et al.*, 2011). In contrast, transfection of the Gal4-PPAR γ construct and subsequent treatment with the compound rosiglitazone, a mammalian PPAR γ agonist, failed to induce an induction reporter signal. In CHSE-214 cells transfected with the Gal4-PPAR α construct, all the tested fatty acids at 100 μ M, significantly induced reporter gene expression, with increase induction of 2.3-fold, 2.0-fold and 2.7-fold over the ethanol control vehicle when exposed to docosahexaenoic acid (22:6; DHA), eicosapentenoic acid (20:5; EPA) and oleoylethanolamide (OEA), respectively. However, none of the fatty acids tested were able to significantly activate transcription from Gal4-PPAR γ .

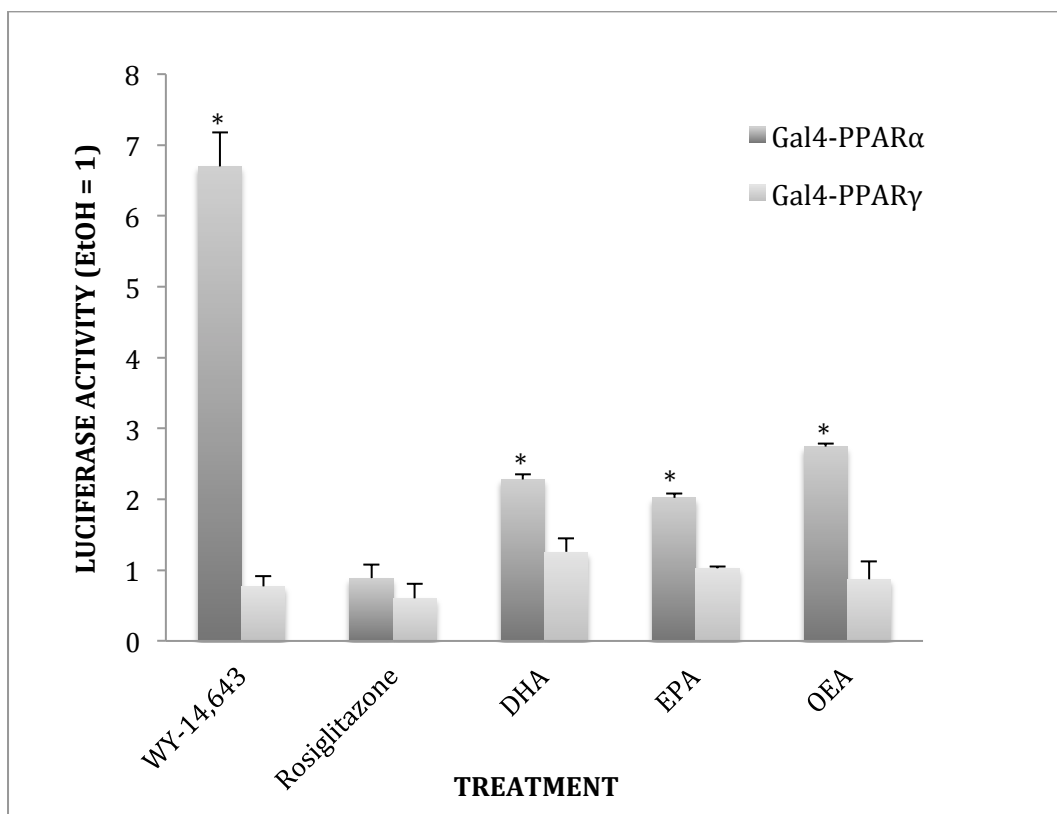


Figure 3-10 Transactivation of Gal4-PPAR α and Gal4-PPAR γ in response to PPAR activators and fatty acids. CHSE-214 cells were transiently transfected with Gal4-PPAR α firefly luciferase reporter plasmid pGL4.31 and an internal *Renilla* luciferase reporter used to correct for transfection efficiencies pGL4.75. Post-transfection cells were treated with 25 μ M Wy-14,643, 50 μ M rosiglitazone and 100 μ M fatty acids. Data are the mean (+ SD) in which each treatment was applied in triplicates, expressed as the fold increase over ethanol control of normalised firefly luciferase activity. Asterisks (*) indicate activities significantly different from those in ethanol-treated cells (EtOH=1).

3.4 Discussion

3.4.1 Determination of a suitable cell line

Two cell lines were transfected and used to assess the transfection efficiency of a cationic polymer, branched polythethylenimine (bPEI), previously shown to successfully transfect mammalian cells (Hsu and Uludağ, 2012). Maximum transfection efficiency of 40.5-fold over control (mock transfected wells) was observed at bPEI to DNA ratio of 2.5:1 when Gal-PPAR α , pGL4.31[*luc2P/Gal4UAS/hygro*] and pGL4.75[*hRluc*/CMV] were co-transfected into AS cells (Figure 3-4) compared to when the polyplex solution was transfected to CHSE-214 cells that has the transfection efficiency of 4.8-fold over the control at 1.25:1, bPEI to DNA ratio. The AS cell line was initially concluded suitable for future transfections as results have shown that highest firefly luciferase signal with 9.4-fold increase over the ethanol control vehicle observed when the transfected cells were treated with the PPAR α agonist, WY-14,643 (Figure 3-5), suggesting Gal4-PPAR α activates transcription from the promoter of the *Gal4UAS*-containing firefly luciferase reporter gene construct pGL4.31[*luc2P/Gal4UAS/hygro*] in response to PPAR α ligand. This transfection efficiency, however, decreased drastically in subsequent experiments, which may have been the result of changes in the characteristics of the cell line at increased passage number (Figure 3-6). The AS cell line was initially examined at low passage number of 99 and was examined in the later stages at a higher passage number of 121 to ensure reproducibility and reliable experimental results.

According to Jacobsen and Hughes (2007), passage number is one of the main factors that may influence the transfection efficiency in cellular transactivation. Increased passages may have led to undesirable differentiation in the cells resulting changes in characteristics and modified growth rates, affecting protein expression, for example expression of co-activator proteins, and causing the cells to respond differently to the same transfection conditions. The decrease in transfection efficiency in AS cells can be seen in Figure 3-7 when the AS cells were transfected with pEGFP at various bPEI to DNA ratio indicated by the presence of very few green fluorescent cells. The use of the enhanced green fluorescent protein plasmid (pEGFP) to evaluate the effectiveness of gene delivery using bPEI by examining the expression of the plasmid in both AS and CHSE-214 cell lines, indicated by the number of green cells (Zhang *et al.*, 1996). When CHSE-214 cells were transfected with pEGFP with the same bPEI to DNA ratios, double the amount of bPEI to DNA 2.5:1 ratio (2 x 2.5:1), showed the highest number of green fluorescent cells and this corresponded to the maximum transfection efficiency observed when Gal-PPAR α , pGL4.31[*luc2P/Gal4UAS/hygro*] and pGL4.75[*hRluc*/CMV] were co-transfected into CHSE-214 cells (Figure 3-8 and Figure 3-9). Although there has not been a direct method in determining the effect of passage number of a cell line, passage-related effects in cell lines have been demonstrated to be heavily dependent on the type of cell line (Hayes, 2010). Cited by Hayes (2010), based on an unpublished data, high passage Caco-2 cell lines had shown an increase in GFP expression levels following transfection, whilst the expression of GFP levels decreased in MCF7 cells with high passage number.

While CHSE-214 cell line was confirmed suitable for future transfections as this cell line has proven to give more reliable experimental results in terms of reproducibility, it was made sure that experiments were consistently performed within a minimum range of passage number following the optimization experiment, to prevent any passage-related effects above from influencing the transfections, thus, maintaining consistent cell performance.

3.4.2 Ratio of bPEI:DNA and transfection efficiency

Because each cell type respond differently to a transfection reagent, optimisation was necessary to obtain maximum results. It is generally understood that higher DNA concentration transfected into the cells, the higher the transfection efficiency. However, as far as the amount of bPEI was concerned, the amount of DNA that can be transfected depended on the final concentration on bPEI and the bPEI to DNA ratio. Thus, because each cell type responds differently to a transfection reagent, it was important to determine the optimal bPEI to DNA ratio to ensure the occurrence of full binding and full condensation (Hsu and Uludağ, 2012).

Doubling the recommended bPEI to DNA ratio, 2.5:1, exhibited relatively higher *Renilla* luciferase signals in the transfected CHSE-214 cells (Figure 3-9) compared to when the bPEI to DNA ratio of 1:1 was doubled. In solutions where the amount of bPEI was higher than the amount of DNA, would mean that the solution would have excess of free bPEI polymers and this would often be considered an advantage towards obtaining high transfection efficiency. These free, unbound, positive bPEI polymers had been documented essential for intracellular trafficking and for overcoming

the inhibitory effect of the polyanionic glycosaminoglycans (GAGs) (Ruponen *et al.*, 2004; Hanzlíková *et al.*, 2011; Hsu and Uludağ, 2012). GAGs are found highly abundant in the extracellular space and because they are negatively charged in nature, they become potential inhibitors of non-viral gene transfer. Thus, at the optimal of twice the amount of 2.5:1, bPEI to DNA ratio, the interactions between free positively charged bPEI polymers with negatively charged GAGs had possibly decrease the inhibitory effect of GAGs and therefore, enabling bPEI-DNA complexes to efficiently bind on the cell membrane, subsequently increase in cellular uptake, and as a consequence, exhibited the highest transfection efficiency. However, when the amount of bPEI was very high in relative to the amount of DNA, 4:1, even when the amounts were doubled, the transfection efficiencies decreased drastically and this may have been the result of cell toxicity from the presence of excessive unbound bPEI polymers, thus, reducing the overall cell viability (Hsu and Uludağ, 2012). Therefore, it can be concluded that double the amount of 2.5:1 bPEI to DNA ratio should be used to ensure efficient transfection of DNA in future experiments.

3.4.3 Response of Gal4-PPARs to PPAR agonists

There was an increase in normalised luciferase activity when the Gal4-PPAR α -transfected CHSE-214 cells (Figure 3-10) were treated with WY-14,643, indicating PPAR α -dependent transcription as reported for other fish cell lines. (Colliar *et al.*, 2011). In another study described by Leaver *et al.* (2005), when a full-length native PPAR α was isolated from two marine fish species, the plaice (*Pleuronectes platessa*) and gilthead seabream (*Sparus aurata*), PPAR α receptor was tested in a cell-based transactivation assay and it was observed that the expression of chloramphenicol acetyltransferase (CAT) reporter, under the control of promoter containing peroxisome proliferator-response element (PPRE), increased when the transfected fish cells were treated with WY-14,643, indicating that this compound is PPAR α agonist. This activation was not only seen in fish PPAR α , but also in mammalian PPAR α in an *in vivo* experiment conducted using mPPAR α -deficient mice. It was previously reported that mouse liver peroxisomes, when exposed to WY-14,643, increased in numbers significantly as a result of peroxisome proliferation (Moody and Reddy, 1978). However, in mice lacking PPAR α , no proliferation was observed when there were fed with the compound, indicating that this receptor mediates peroxisome proliferation in the presence of WY-14,643 (Lee *et al.*, 1995). These studies have therefore concluded that Gal4-PPAR α construct behaves similarly to native PPAR α . These results confirmed that the transfection system of the plasmid construct in CHSE-214 cells was performing as expected and required in terms of Gal4-PPAR fusion protein and luciferase expression, and in subsequent luciferase

assays. Thus, in this project, Gal4-PPAR α and its activating compound, Wy-14,643 were used as a positive control in cell-transactivation experiments.

Plaice Gal4-PPAR γ , on the other hand, was observed to be unresponsive to rosiglitazone, a member of the thiazolidinedione class of compounds, which had been identified as a known high affinity ligand for PPAR γ in mammals (Lehmann *et al.*, 1995). Previously isolated PPAR γ from the plaice and gilthead seabream (*Sparus aurata*) (Leaver *et al.*, 2005), and from olive flounder (*Paralichthys olivaceus*) (Cho *et al.*, 2009) showed significant but weak transactivation when exposed to rosiglitazone, indicating that these receptors may have structural differences contributing to this sensitivity. Whilst PPAR α within fish species, including plaice, sea bream, Atlantic salmon (*Salmon salar*) and *Fugu* (Andersen *et al.*, 2000; Maglich *et al.*, 2003; Leaver *et al.*, 2005), have identical key conserved residues to their human counterparts within the ligand-binding domain, suggesting similar structure and functions, PPAR γ has only one equivalent residue (H449) to one of three conserved residues (H323, H449 and Y473) in all mammalian, avian and amphibian PPAR γ proteins, identified to be critical for forming hydrogen bonds with the acidic head-group of PPAR ligands. The two residues H323 and Y473 were replaced by isoleucine and methionine (Maglich *et al.*, 2003; Leaver *et al.*, 2005). These residue substitutions may have altered the structure of peptide regions within the ligand-binding domain, which have consequently contributed to the sensitivity, affecting the binding of rosiglitazone or other ligands. It is possible that there are specific co-activator proteins required for PPAR γ activation that are not present in CHSE-214, or other previously tested fish cell lines. However, given that

PPAR α is robustly activated in these cells, and that PPAR γ and PPAR α share some co-activator proteins (Viswakarma *et al.*, 2010), this is unlikely to be the cause of non-activation of plaice PPAR γ .

3.4.4 Response of Gal4-PPARs to fatty acids

All of the three fatty acids tested induced transactivation of plaice Gal4-PPAR α , similar to the observations reported by Colliar *et al.* (2011). The highest signal observed in this study was the activation of Gal4-PPAR α by OEA, a fatty acid identified as a potent agonist of mammalian PPAR α , responsible in controlling feeding and fat-induced satiety (Scwartz *et al.*, 2008, Dipasquale *et al.*, 2010), suggesting that this fatty acid may have a similar function in both mammals and fish. In the contrary, the fatty acids tested failed to activate plaice Gal4-PPAR γ .

3.4.5 Use of non-commercial dual luciferase assay system

An alternative to the commercial assay system, a dual luciferase reporter assay was used to analyse the expression of reporter genes, by combining “homemade” buffers of both firefly (*Photinus pyralis*) luciferase enzyme and *Renilla* (*Renilla reniformis*) luciferase enzyme within the same reaction tube, sequentially. The development of luciferase-based protocols have been modified from Dyer *et al.* (2001) and these high throughput assays had been previously used in successful cell-transactivation experiments conducted by Colliar *et al.* (2011).

In the firefly luciferase buffer, luciferin was used as a substrate in the assay system as the firefly luciferase enzyme, in the presence of ATP, molecular oxygen and magnesium ions, catalyses luciferin to produce oxy-luciferin emitting light energy (bioluminescence). Sustaining the light intensity had previously become a major issue, there was a rapid increase in light intensity, characterised by a “flash” of light when substrates were added to the enzyme, which tend to decay rapidly to a constant low-level luminescence. This problem of the kinetics of the luciferase-luciferin reaction would have considerably reduced the time window in which a signal could be detected. The addition of the cofactor coenzyme A (CoA) in the firefly luciferase buffer had been reported to stabilise the luminescence signal and prolong light production at a higher level for a time period depending on the amount of CoA added, without having the initial peak of light intensity affected (van Lune and Bruggeman, 2006). Combining the use of CoA and dithiothreitol (DTT) in the reaction mixture had proven to sustain the half-life of the light

signal longer up to 300 to 500 seconds. A further improvement had been made to the reaction mixture was the addition of ammonium and phosphate ions present in the tribasic potassium phosphate produced a strong light signal that had even longer stabilising effect ranging from 30 minutes up to 8 hours.

Within the assay system, *Renilla* buffer was added to the reaction well to examine adequacy and kinetics of quenching the firefly luciferase activity. The *Renilla* luciferase activities were therefore used to normalise for differences in transfection efficiencies between wells on an assay plate. In the *Renilla* buffer, coelenterazine was used as a substrate, as the *Renilla* luciferase enzyme, in the presence of oxygen, catalyses coelenterazine oxidation leading to bioluminescence (Hori *et al.*, 1973). It had been shown that non-commercial *Renilla* buffer was able to quench the firefly luciferase activity as well as the commercial buffer, that the activity had exceeded 99.9 % (Dyer *et al.*, 2000), indicating this buffer to be efficient in this assay system.

3.5 Conclusions

The determination of the most suitable cell line and its optimisation was imperative in the transfection experiments using the selected transfection reagent to maximise results and to ensure reproducibility and reliability. Because passage number may become an important factor for certain cell lines, they should be of relatively low and consistent passage number to avoid any changes or differentiation in cell structure or morphology which may in turn affect the overall performance. The health of the cell line and its maintenance should also be taken into consideration when it comes to transfection optimisation. CHSE-214 cell line has proven to be more reliable and robust compared to the AS cell line and that doubling the ratio to the amount of 2.5:1 bPEI (μl) to DNA (μg) ratio has shown to exhibit the highest transfection efficiency, which, therefore, being used as an optimal ratio in future transfection experiments. In the optimisation experiments, Gal4-PPAR α and Gal4-PPAR γ were tested in cellular-transactivation in response to their activating compounds in fish to ensure the effectiveness of transfection using bPEI and the efficiency of the dual-luciferase assay system using firefly luciferase and *Renilla* luciferase reagents. From the previous studies and from this study, because the full-length piscine PPAR γ and the Gal4-PPAR γ had been found to be unresponsive to fatty acids, PPAR γ has become a subject of interest, thus, the Gal4-PPAR γ construct was developed for use in subsequent experiments in identifying its activating ligands.

4 Lipid extraction and response of piscine PPAR γ to lipid fractions

4.1 Introduction

A major paradox and constraint to aquaculture is that much finfish aquaculture is dependent on feeds manufactured from wild fish, utilising products such as fish meal and oil from industrial fisheries which have now reached their maximum sustainable limit. Because of the expanding aquaculture sector, pursuing alternatives to its current dependence on these materials is becoming crucial. Terrestrial plant seed meals and oils derived from processed soybean or rapeseed have been used to substitute fish meal and oil in farmed fish diets (Bell *et al.*, 2001). However, the need to balance lipid storage and metabolism for growth, as well as ensuring health through the supply of essential nutrients such as polyunsaturated fatty acids must be addressed. Whilst the replacement of fish oil with terrestrial seed oils is an effective approach for some farmed species such as Atlantic Salmon, not all fish species tolerate such high levels of dietary seed oil inclusion (Bell *et al.*, 2001). It is understood that mammals and fish share the same pathways for biosynthesis and catabolism of fatty acids and these pathways are controlled by the similar molecular mechanisms (Ruyter *et al.*, 1997; Boukouvala *et al.*, 2004). In mammals, peroxisome proliferator-activated receptors (PPARs) have emerged as central factors in sensing fatty acid levels and in regulating fatty acid metabolism. Evidently, the PPARs in fish, generally, are the structural homologs of the mammalian PPARs, and are assumed to carry out similar functions. There is strong evidence that PPARs play critical roles in

fish lipid homeostasis and that PPAR α and PPAR β may have similar functions to those described in mammals for these receptors. This is based on having similar ligand activation profiles and tissue expression patterns as their mammalian counterparts (Leaver *et al.*, 2005). However, the role of PPAR γ in fish still remains unclear because piscine PPAR γ is not activated by compounds which activate mammalian PPAR γ (Maglich *et al.*, 2003; Leaver *et al.*, 2005). Piscine PPAR γ has some specific structural differences to mammalian proteins, particularly evident with regard to amino acid residues which are known to be critical for ligand binding in mammalian receptors (Ruyter *et al.*, 1997; Andersen *et al.*, 2000; Leaver *et al.*, 2005). Despite this, the overall structural similarity of piscine PPAR γ to all other PPARs strongly indicates that it is likely to be activated by a compound with structural similarities to the lipids and fatty acids which activate mammalian PPAR γ and other PPAR forms.

Therefore, because mammalian PPAR γ has a critical role in determining lipid uptake and storage, it is of particular interest in finfish aquaculture as farmed fish often accumulate excess visceral and hepatic fat especially when fed plant seed oil-based diets. This can affect the health and welfare of the fish, and also represents an economic waste of valuable feed that might otherwise be channelled into growth.

Therefore, the aim of this chapter is to discover fish lipid fractions, thus, molecular components, which activate piscine PPAR γ in cell transfection assays. An endogenous activator of fish PPAR γ would shed light on the roles

of this receptor, as well as providing tools for further function studies of lipid homeostasis in fish.

This chapter focuses on:

- 1) The extraction of total lipid from liver tissues of farmed Atlantic salmon (*Salmon salar*) and the determination of lipid classes by means of high-performance thin-layer chromatography (HPTLC) and lipid fractionation using thin-layer chromatography (TLC).
- 2) The discovery of the activating compounds of PPAR γ from the lipid fractions through a high-throughput cell-based transactivation screen using the transient transfection assay developed previously.
- 3) The identification of molecular components within the lipid fractions that activate PPAR γ using gas chromatography-flame ionisation detection (GC) and liquid chromatography-mass spectrometry (LC-MS).

4.2 Materials and methods

4.2.1 Lipid and fatty acid analyses

4.2.1.1 Sampling of salmon livers

This experiment was subjected to ethical review and approved by the University of Stirling through Animal and Welfare Ethical Review Body (AWERB). Atlantic salmon (*Salmon salar*) pre-smolts with weight range of 400-600 g were obtained from the University of Stirling Buckieburn Field Station, Scotland. Fish were sacrificed with an overdose of tricaine methanesulfonate (MS222) and a sharp blow to the head. Liver tissues, weighing about 1 g each, were sampled from 10 fish and immediately placed in a sample bottle containing 250 ml of chloroform/methanol (C:M) (2:1, v/v) and stored in -20 °C before lipid extraction was performed the following day.

4.2.1.2 Total lipid extraction from salmon liver tissues

The procedure for the lipid extraction was based on Folch method (Folch *et al.*, 1957), a non-destructive method for extracting total lipid from samples of animal tissues. In this project, liver was chosen because it contains all the important lipid classes and because of the central importance of this organ in lipid metabolism, and importantly because salmon and other fish PPARs are highly expressed in this tissue (Boukouvala *et al.*, 2004; Ibabe *et al.*, 2004; Ibabe *et al.*, 2005; Leaver *et al.*, 2005).

Folch bulk extraction of total lipid was conducted by first homogenising (Ultra Turrax™ rotating probe) tissue already collected in C:M (2:1, v/v). A quarter volume of 0.88 % (w/v) potassium chloride (KCl) was then added and mixed thoroughly, before transferring into a separating funnel and left to stand for one hour to allow the formation of two distinct layers. The bottom lipid layer was collected in a round-bottomed flask, leaving behind the top (aqueous) layer. The lipid layer was then concentrated and dried under vacuum in a rotary evaporator, to evaporate remaining solvent. The resulting concentrated lipid was dissolved in a small amount of C:M (2:1) and transferred to a pre-weighed test tube before the solvent was evaporated to dryness under a stream of oxygen-free nitrogen (OFN). Tubes with total lipid were left to desiccate in vacuum overnight. The following day, total lipid in each tube was weighed and was re-dissolved in C:M (2:1) + 0.01% (w/v) butylated hydroxytoluene (BHT) at a concentration of 10 mg/ml and stored in glass vials under nitrogen in a spark proof freezer at -18°C prior to lipid class and fatty acid analyses.

4.2.1.3 Determination of lipid class composition

Total lipid extracted was used to determine the lipid class compositions using single-dimension, double-development, high-performance thin-layer chromatography (HPTLC) and quantitative densitometry (Henderson and Tocher, 1992). HPTLC plates (10 cm x 10 cm x 0.25 mm, Merck, Darmstadt, Germany) were pre-washed, in order to remove impurities from the layer, with the first developing solvent methyl acetate/propan-2-ol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by volume),

before allowing the solvent to evaporate by air drying in a fume cupboard and leaving to desiccate under vacuum overnight. The washed plate was marked, by pencil, with six 3 mm origins at a distance of 1.2 cm between them. Four samples of 10 µg total lipid (i.e. 1 µl of 10 mg/ml), two lipid class standards (neutral lipid obtained from Sigma and cod roe total lipid and one blank) were applied to each 3 mm origin using a 10 µl Microliter™ glass syringe (Hamilton®, Bonaduz, Switzerland). In a development tank, the plate was developed to 5.5 cm in the first solvent mentioned above and this aimed to separate polar lipid classes. This was then removed and placed in the desiccator for at least 20 min to allow evaporation of excess solvent. The plate was then developed in the same direction in the second development containing iso-hexane/diethyl ether/acetic acid (80:20:1, by volume) to separate the neutral lipid classes. Plate was again placed in the desiccator for at least 5 min to evaporate excess solvent, and then sprayed with 3% cupric acetate in 8% phosphoric acid staining solution. Excess solution was drained and the plate was charred at 160°C for 18 min in an oven. Lipid classes on the plate were then examined by calibrated scanning densitometry using a Camag 3 TLC Scanner (Muttensz, Switzerland) and quantified using winCATS software.

4.2.1.4 Lipid fractionation into polar lipids and neutral lipids

Total lipid extracted in Section 4.2.1.2 was then fractionated into pure polar and neutral lipids using thin-layer chromatography (TLC) on 20 cm x 20 cm x 0.25 mm plates (Merck, Darmstadt, Germany). The plates were first marked, by pencil, with a 12 cm origin starting at 2 cm from the side and 1.5 cm from

the bottom of the plates, having the standards marked at 1 cm from both sides within the 12 cm origin. 2 ml of 10 mg/ml in C:M (2:1) + 0.01 % BHT of total lipid was evaporated to dryness under a stream of OFN on a nitrogen evaporator to obtain 20 mg of total lipid which was then reconstituted in 150 μ l C:M (2:1) (no BHT). This was applied to the origin using a 50 μ l Microliter™ glass syringe (Hamilton®, Bonaduz, Switzerland), drying the total lipid with OFN between every streak. The plates were developed to 10.5 cm in the first solvent system and were then placed in a vacuum desiccator for at least 1 hr with pump running to remove excess solvent. The plates were then fully developed in the second solvent system and were placed in a vacuum desiccator for a further 20 min. The lipid bands were revealed by lightly spraying the standard lanes at the sides of the plates with 1 % iodine in chloroform. The position of the lipid bands were then identified and marked in pencil before the silica containing the lipid classes were scraped off and transferred into fresh medium stoppered test-tubes. Each sample was dissolved in 20 ml C:M (2:1) and was placed overnight in a spark proof freezer at -25 °C. The following day, the silica samples were filtered through pre-washed filter papers and into fresh test tubes. The solvent from each test tube was evaporated under a stream of OFN to approximately 2 ml and was transferred to labelled and pre-weighed 2 ml glass vials. The remaining solvent was further evaporated and the lipid samples obtained were placed in the desiccator overnight to remove excess solvent. The next day, the glass vials were weighed again to measure the final amount of each lipid obtained, before they were reconstituted in the correct amount of C:M (2:1) to a concentration of 10 mg/ml.

4.2.1.5 Purification check of lipid fractions on HPTLC

Prewashed 10 cm x 20 cm x 0.2 mm HPTLC plates, marked by pencil, with 3 mm origins at a distance of 1.2 cm between them, enough to accommodate the lipid samples obtained previously. To each origin, 10 µg (i.e. 1 µl of 10 mg/ml) of sample was applied and the plates were developed to 5.5 cm in the first solvent to identify and ensure separation of pure polar lipids. The plates were placed in a vacuum desiccator for at least 20 min with pump running to remove excess solvent vapour. The plates were again placed in the same direction in the second developing solvent to identify and ensure separation of neutral lipids before they were placed in the desiccator again for at least 5 min to remove all the solvent vapour. The entire plates were then sprayed with 3 % cupric acetate in 8 % phosphoric acid staining solution before the excess solution was drained and plates were charred at 160 °C for 18 min in an oven.

4.2.1.6 Preparation of lipid fractions in absolute ethanol

Before each lipid fraction was reconstituted in absolute ethanol, C:M (2:1) solvent from each vial was evaporated under a stream of OFN and they were placed in the desiccator overnight to remove excess solvent. The following day, each lipid sample was weighed to get the final amount and was dissolved in the correct amount of absolute ethanol to a concentration of 5 mg/ml.

4.2.2 Treatment of transfected CHSE-214 cells with lipids

The Gal4-PPAR γ construct developed in Chapter 3 was used to test for agonistic effects of lipids obtained in cell-based transactivation assays, making use of Gal4-PPAR α and its activating ligand, WY-14,643 as positive control.

4.2.2.1 Seeding and transfection of CHSE-214 cells

CHSE-214 were harvested from near-confluent cell culture flasks and seeded at 2×10^5 cell per well of 24 well plates before transfection the following day with Gal4-PPAR α (treated with WY-14,643 tested for positive control only) and Gal4-PPAR γ as described in Sections 3.2.2.3 and 3.2.2.4.

4.2.2.2 Treatment of transfected cells with polar lipids and neutral lipids

Following transfection of cells with Gal4-PPAR γ using the optimised bPEI to DNA ratio determined in the previous chapter, cells were treated with polar lipids and neutral lipids obtained, identified from the two lipid class standards used earlier. The polar lipids include lysophosphatidylcholine (LPC), sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), unknown polar lipid (UNK1) and solvent front containing pigmented material (SF), and the neutral lipids identified are cholesterol (CHOL), free fatty acids (FFA), unknown neutral lipid (UNK2), triacylglycerols (TAG) and sterol esters (SE).

In bijoux tubes, each lipid with an initial concentration of 5 mg/ml was diluted into complete EMEM to give two final assay concentrations of 25

$\mu\text{g/ml}$ and $50 \mu\text{g/ml}$, enough to produce quadruplicates with each lipid. An ethanol absolute was also prepared as a control treatment at a concentration of $10 \mu\text{l}$ ethanol absolute per ml of EMEM. The diluted treatment compounds were mixed thoroughly before 1 ml of each mixture was being added to the transfected cells. Cells were subsequently incubated with treatment compounds for 24 hours at 22°C in an atmosphere of 4 % CO_2 before assaying for luciferase activity.

4.2.2.3 Luciferase activity, data normalisation and statistical analysis

Firefly and *Renilla* luciferase activities were measured using the luciferase assay buffers described in Chapter 3. Twenty-four hours post-treatment, media containing treatment compounds was aspirated from assay plates and cells were washed twice with 1x DPBS before they were lysed in 1 ml of 1 x passive lysis buffer (Promega) for 10 min, shaking. The cell lysate was then used in a dual luciferase assay system in which $75 \mu\text{l}$ of cell lysate from each well was transferred to a black 96-well plate (Corning). Into each well, equal volume of a 2 x concentrated volume of luciferase buffer was added to the wells. The contents of the wells were pipetted up and down to mix and was incubated for 2 min before luciferase activity was measured on the Wallac 1420 Victor 2 multilabel counter (Perkin Elmer). Following measurement of firefly luciferase, $75 \mu\text{l}$ volume of 3 x concentrated *Renilla* luciferase buffer was added and the contents were again pipetted up and down to mix, incubated for 2 min before *Renilla* luciferase activity was measured.

As previously described, *Renilla* luciferase values were used to normalise firefly activities for differences in transfection efficiency between wells. For

each well, the firefly luciferase value was divided by the corresponding *Renilla* luciferase value and the mean replicate wells was calculated.

Experiments were repeated independently twice (with different flasks of cells seeded on different days). Data is reported as the mean fold inductions in normalised luciferase activities compared to ethanol vehicle controls. To test for statistically significant differences between ethanol control treatment and treatment with lipid compounds, a one-way ANOVA was carried out followed by Dunnet's test on Minitab18 software. Results were considered statistically significant when the probability value (P) was less than 0.05 (P<0.05).

4.2.3 Identification of molecular components in lipid samples

4.2.3.1 Determination of fatty acids composition using GC

Lipid compounds that had significant luciferase activity with Gal4-PPAR γ transfected cells were further analysed to determine the fatty acid profile of lipid using fatty acid methyl esters (FAMES) by gas chromatography-flame ionisation detection (GC) (Christie, 2003). The FAMES were prepared by transmethylation. Following evaporation of ethanol, making sure that each sample contained 0.2-1 mg of lipid, each sample was reconstituted in 100 μ l C:M (2:1) before the samples were transferred into individual quick-test tube and solvent evaporated under a stream of OFN. One ml of toluene and 2 ml of 1 % (v/v) sulphuric acid (H₂SO₄) in methanol were added to each lipid sample. Samples tubes were flushed with N₂ to prevent oxidation, sealed with glass stoppers and small piece of paper tissue before they were incubated in a hot block (Techne Dri-block) at 50 °C overnight. The following day, after cooling, the FAMES were extracted by adding 2 ml of 2 % potassium bicarbonate (KHCO₃), followed by 5 ml of isohexane/diethyl ether (1:1, v/v) containing 0.01 % BHT. The contents were mixed by inverting the tubes followed by centrifugation at 400 g_{ave} for 5 min to separate the two phases. The organic upper layer was collected using a Pasteur pipette and transferred into a clean test tube. Further 5 ml isohexane/diethyl ether (1:1, v/v, without BHT) was added to the remaining lower layer and the extraction procedure repeated to ensure maximum recovery of FAMES. Following mixing and centrifuging at 400 g_{ave} for 5 min, the organic upper layer was collected and transferred to the test tube before. The organic layers were

placed under a stream of OFN to evaporate the solvent and the dry FAME extract re-suspended in 500 µl of isohexane containing 0.01 % BHT before purified FAME extracts were transferred to chromacol vials and stored under N₂ at -18 °C (spark proof), ready for GC. FAME samples were analysed using a Fisons GC-8160 (thermo Fisher Scientific, UK) gas chromatograph equipped with a 30 m x 0.25 mm i.d. x 0.25 µm ZB-wax column (Phenomenex, UK) and flame ionisation detector.

4.2.3.2 Determination of organic compounds using liquid chromatography-mass spectrometry (LC-MS)

With the help of GC, fatty acid composition in each lipid fraction above was analysed and identified. However, because many compounds are sometimes impossible to analyse with GC, the same lipid fractions (about 0.4 to 0.5 mg) were analysed using high resolution LC-MS with the help of experts in the Lipidomics Research Laboratory from the University of the Highlands and Islands in Inverness, Scotland.

Briefly, lipids were analysed by LC-MS using a Thermo Orbitrap Exactive mass spectrometer (Thermo Scientific, Hemel Hempstead, UK), equipped with as heated electrospray ionization (HESI) probe and coupled to a Thermo Accela 1250 UHPLC system. All samples were analysed in both positive and negative ion mode over the mass to charge (m/z) range 200-2000. The samples were injected on to a Thermo Hypersil Gold C18 column (2.1 mm x 100 mm, 1.9µm). Mobile phase A consisted of water containing 10mM ammonium formate and 0.1 % (v/v) formic acid. Mobile phase B consisted of 10:10 isopropanol/acetonitrile containing 10 mM ammonium formate and

0.1 % (v/v) formic acid. The initial conditions for analysis were 65%A/35%B. The percentage of mobile phase B was increased to 100 % over 10 minutes and held for 7 min before re-equilibration with the starting conditions for 4 min. The raw LC-MS data were processed with Progenesis CoMet v2.0 software (Non-linear Dynamics, Newcastle, UK) and searched against LIPID MAPS (www.lipidmaps.org) for identification.

4.3 Results

4.3.1 Lipid content and lipid class composition of liver tissues

The lipid content and lipid class composition of the liver tissues are shown in Table 4-1. A total of 26.15 mg of total lipid was extracted from the tissues and a total of eight polar lipids and five neutral lipids were present in the total lipid extracted. The percentage composition of total polar lipids was almost double of the percentage of the total neutral lipids. The HPTLC chromatograms showing the lipid classes and the purified polar lipids and neutral lipids can also be seen in Figure 4-1 and Figure 4-2.

Table 4-1 Lipid content and lipid class composition of liver tissues.

Lipid	
Lipid content (mg)	26.15 ± 0.5
Class composition (%)	
LPC	0.8 ± 0.2
SM	3.6 ± 0.4
PC	24.4 ± 1.6
PS	6.3 ± 1.2
PI	8.0 ± 0.6
PE	12.7 ± 1.7
UNK1	7.0 ± 0.4
SF	2.5 ± 0.8
Total Polar	65.3 ± 5.0
CHOL	10.9 ± 1.1
FFA	3.1 ± 0.8
UNK2	3.9 ± 0.7
TAG	16.4 ± 4.8
SE	0.3 ± 0.1
Total neutral	34.7 ± 4.3

Results are expressed as mean ± SD (n=4). Lipid class composition is given as a percentage of the total lipid content. Abbreviations for polar lipids: lysophosphatidylcholine (LPC), sphingomyelin (SM), Phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), unknown polar lipid (UNK1) and solvent front containing pigmented material (SF). Abbreviations for neutral lipids: the neutral lipids identified are cholesterol (CHOL), free fatty acids (FFA), unknown neutral lipid (UNK2), triacylglycerols (TAG) and sterol esters (SE).

PC was found to be the most abundant phospholipids, about 40 % of the total polar lipids, followed by PE and then by PI and PS. PI, PS and SM contents were much lower (≤ 8.0 %), and UNK1 and SF were found to be 7.0 % and 2.5 % of the total lipid content, while LPC was present at relatively low concentration only. The separation of SM and PC using the TLC plates was challenging, therefore these two lipids were pulled together as one lipid sample labelled SM/PC (Figure 4-2).

The major component of the total neutral lipids was TAG, followed by CHOL, making up about 50 % and 30 % of the total neutral lipid, respectively. FFA, UNK2 and SE were found to be lower than 4.0 % of the total lipid content.

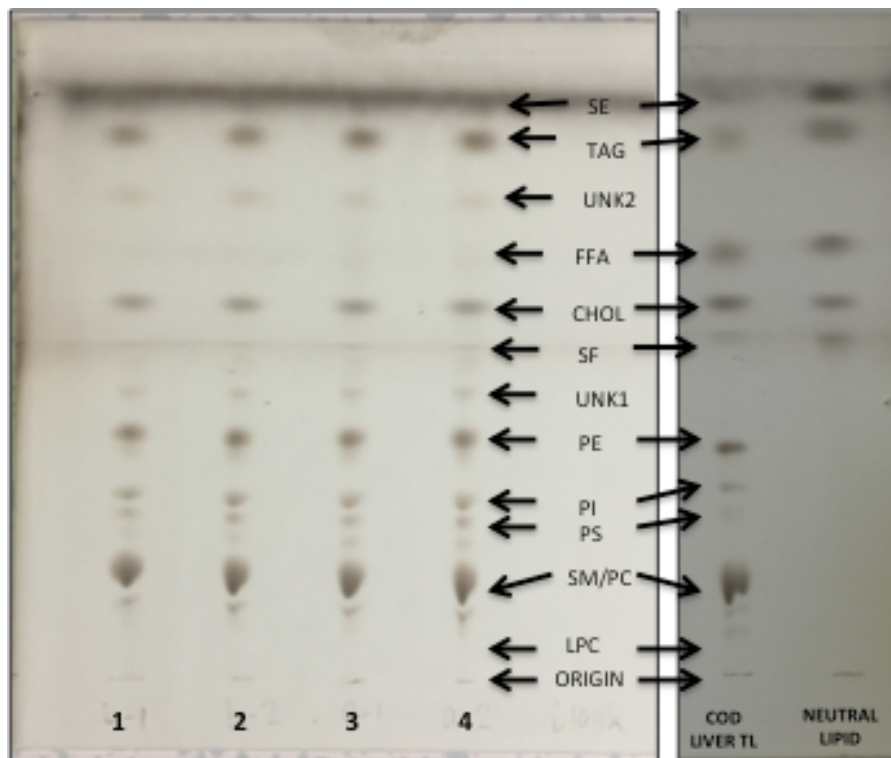


Figure 4-1 HPTLC chromatogram of lipid class compositions in single dimension, double development system of the total lipid extracted from liver tissues, as described in text. Total lipid was run with two lipid standards: cod roe total lipid (TL) and neutral lipids, to identify the location of each lipid class. The plate was stained with 3 % cupric acetate in 8 % phosphoric acid. Abbreviations refer to Table 4-1.

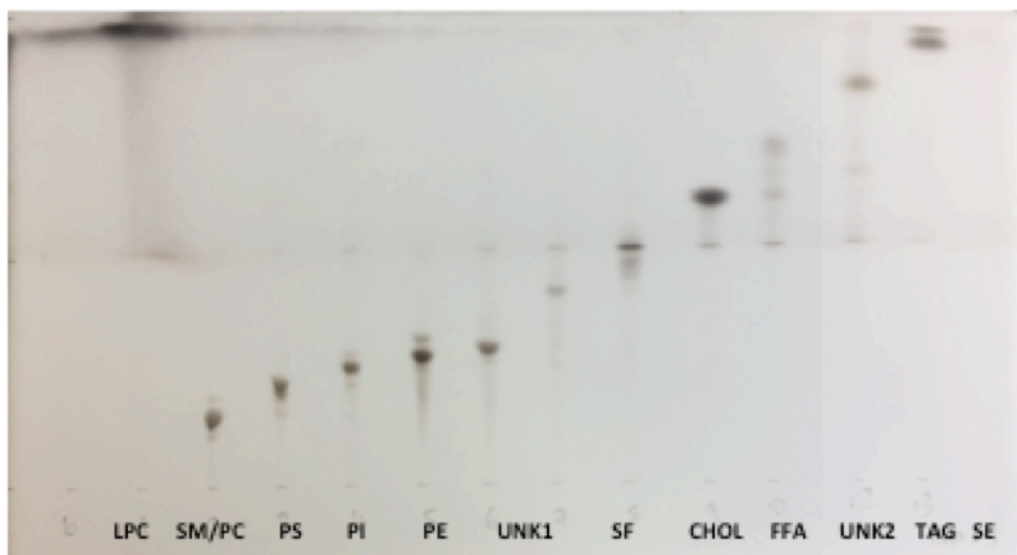


Figure 4-2 HPTLC chromatogram of pure polar and neutral lipids in 10 mg/ml C:M (2:1) prepared from TLC plates developed in single dimension, double development system, as described in text, before each lipid fraction was reconstituted in 5 mg/ml absolute ethanol. Abbreviations refer to Table 4-1.

4.3.2 Response of Gal4-PPAR γ to polar and neutral lipids

Of the tested lipid fractions, the unknown polar and solvent front lipid fractions at 50 $\mu\text{g}/\text{ml}$ were able to significantly induce transcriptional PPAR γ activity with 2.1-fold and 1.9-fold increase over ethanol control (Figure 4-3). However, none of the lipids at the concentration of 25 $\mu\text{g}/\text{ml}$ were able to induce PPAR γ activity.

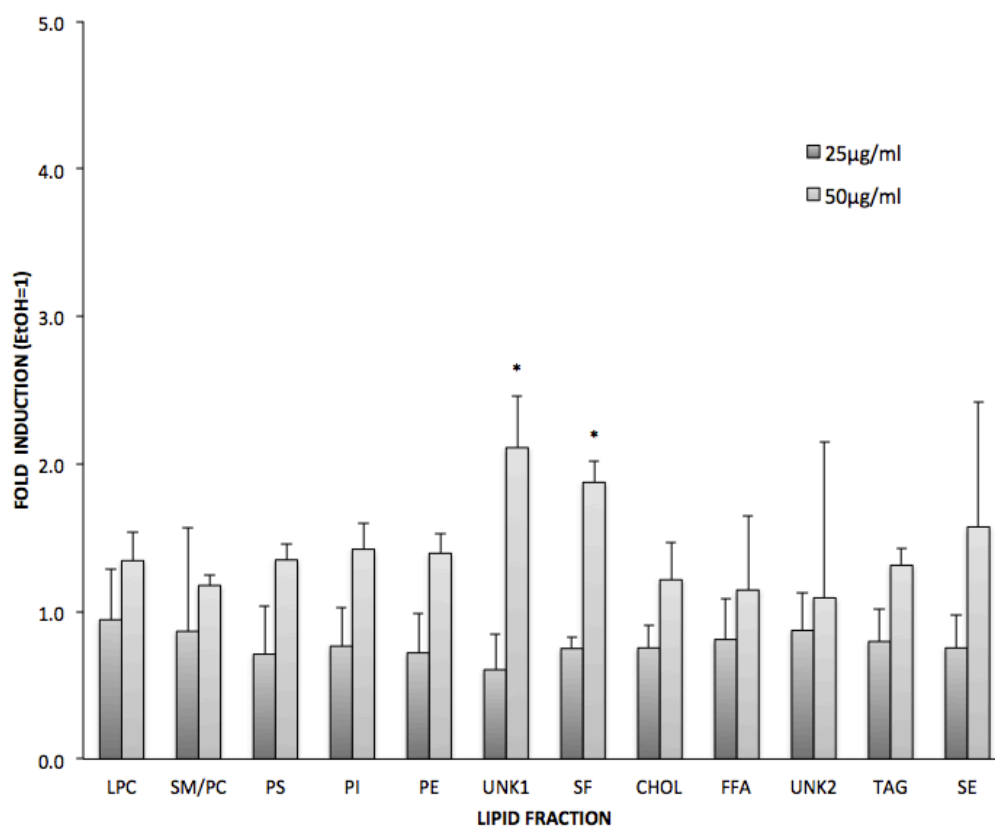


Figure 4-3 Response of PPAR γ to polar lipids and neutral lipids. Transfected cells were treated with two lipid concentrations: 25 $\mu\text{g}/\text{ml}$ and 50 $\mu\text{g}/\text{ml}$. Data are the means \pm SD of two independent experiments (n=4 for each experiment). Results are expressed as the fold increase over ethanol control of normalised firefly luciferase activity. Asterisk (*) represents statistically significant difference (P<0.05) to activities in ethanol controls.

4.3.3 Determination of molecular components in lipids

The two pure lipids fractions, the unknown polar lipid and solvent front, that were able to significantly induce PPAR γ activity, were analysed with GC to identify the molecular weights of the fatty acid components and omit artefacts. It was revealed that the unknown polar lipid fraction consisted of more than 50 % long-chain based saturated fatty acids, containing more than 50 % palmitic acid, 16:0 and about 20 % stearic acid, 18:0 (Table 4-2). The monoenoic (monosaturated) fatty acids were the second most abundant fatty acids in this lipid fraction, containing more than 50 % vaccenic acid, 18:1n-7. On the other hand, the solvent front fraction consisted of more than 50 % monoenoic fatty acids, comprising of almost 40 % oleic acid, 18:1n-9, and the second most abundant fatty acids in this lipid fraction was the n-3 polyunsaturated fatty acids (PUFA), consisting of high amounts of 22:6 docosahexaenoic acid (DHA).

Table 4-2 Fatty acid composition (%) of two pure lipid fractions UNK1 (unknown polar lipid) and SF (solvent front). Fatty acid is given as a percentage of the total fatty acid content. Abbreviation: polyunsaturated fatty acid (PUFA).

Fatty acid	Percentage (%)		Fatty acid	Percentage (%)	
	UNK	SF		UNK	SF
14:0	2.24	1.39	18:2n-6	2.70	4.45
15:0	0.69	0.22	18:3n-6	0.16	0.07
16:0	34.76	6.93	20:2n-6	0.61	1.25
18:0	11.64	2.71	20:3n-6	0.22	0.65
19:0	0.00	0.00	20:4n-6	0.34	2.47
20:0	0.75	0.21	22:4n-6	0.08	0.10
22:0	1.06	0.30	22:5n-6	0.27	0.39
24:0	0.43	0.00	Total n-6 PUFA	4.38	9.38
Total saturated	51.58	11.76			
16:1n-9	0.80	0.30	18:3n-3	0.42	0.86
16:1n-7	1.87	2.31	18:4n-3	0.06	0.18
16:1n-5	0.00	0.00	20:3n-3	0.00	0.23
18:1n-9	17.62	0.00	20:4n-3	0.12	0.14
18:1n-7	2.45	36.69	20:5n-3	1.42	2.18
20:1n-11	0.77	0.00	21:5	0.00	0.00
20:1n-9	4.91	0.72	22:5n-3	1.17	2.54
20:1n-7	0.55	0.27	22:6n-3	7.31	20.55
22:1n-11	2.25	3.07	Total n-3 PUFA	10.51	26.67
22:1n-9	0.60	0.65			
24:1n-9	1.39	7.93			
Total monounsaturated	33.23	51.93			

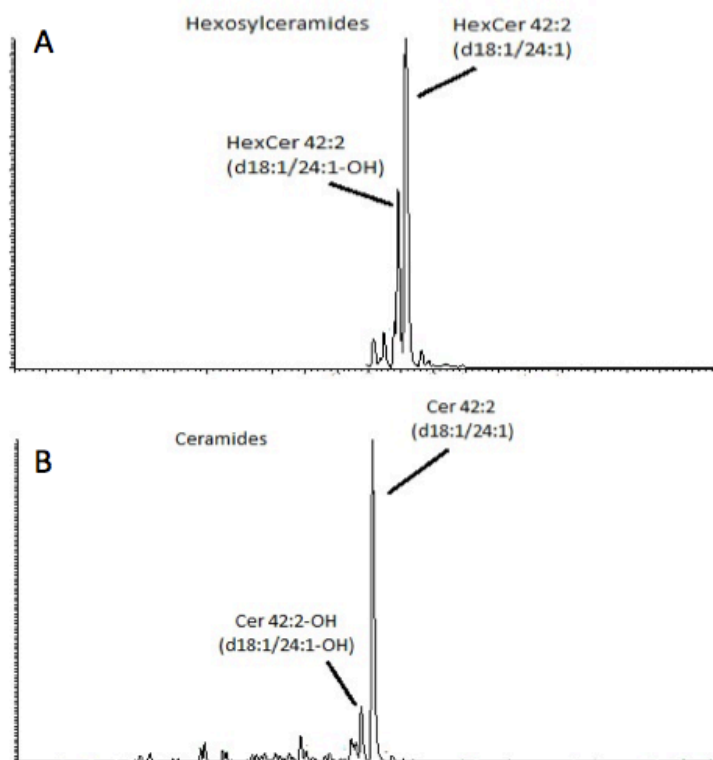


Figure 4-4 LC-MS analyses of the (A) unknown polar lipid (UNK1) and (B) solvent front (SF) previously selected as transfected CHSE-214 cells treated with these two lipids fractions showed significantly high PPAR γ activity.

The high resolution liquid chromatography-mass spectrometry (LC-MS) revealed that the major lipids found in the unknown lipid fraction were the molecular species of hexosylceramides and the predominant lipids in the solvent front fraction were ceramides (Figure 4-4).

4.4 Discussion

4.4.1 Phospholipids (polar lipids) and neutral lipids composition

Seven fractions of polar lipids were found and the principal phospholipids in the total lipid extracted from liver tissues were PC and PE. These were quantified by the densitometer, which can be seen in Table 4-1, and the dark stains on the HPTLC plate in Figure 4-1 corresponded to the relatively high polar lipid concentrations.

From the results, it can be deduced that the phospholipid class composition of salmon liver corresponded to the content of phospholipid classes in the fish liver tissues observed in previous studies. For example, the polar lipids in liver tissues of two species of cottoid fish (*Cottocomephorus grewingki* and *C. inermis*) (Kozlova, 1998) and bogue (*Boops boops*) (Kapoulas and Miniadis-Meimaroglou, 1985) were found to consist about 50 % of phosphatidylcholine. While PC was the dominant phospholipid observed in liver tissues, this was also true for other fish tissues, such as in ovaries of the two species of cottoid fish containing about 58-78 % of phosphatidylcholine, in ovaries of Atlantic salmon was 94 % (Cowey *et al.*, 1985) and in ovaries of Atlantic herring (*Clupea harengus*) was about 90 % (Tocher *et al.*, 1985). It was also observed that PC was the most abundant class in other tissues, such as in testes and muscles of the two species of cottoid fish (Kozlova, 1998), and in head and skin of bogue (Kapoulas and Miniadis-Meimaroglou, 1985). PC is the most abundant phospholipid in animals and is the major phospholipid component of the plasma membrane bilayers. Moreover, the

presence of this lipid in relatively high concentration in tissues is apparently essential to produce phosphatidic acid that is responsible in the activation of the main enzyme, phosphatidylinositol 5-phosphate 5-kinase, to generate polyphosphoinositides, responsible in the metabolism and signalling functions (Christie, 2010).

PE, being the second most abundant phospholipid, amounted to about 20 % of liver phospholipids. Similar results were documented that the polar lipids consisted of about 20-30 % of PE in liver tissues of two species of cottoid fish (Kozlova, 1998) and bogue (Kapoulas and Miniadis-Meimaroglou, 1985), and this also agreed with the other tissues such as in ovaries, testes and muscles of the two species of cottoid fish, with a range of about 20-32 %, 25-40 % and 25-42 % of PE, respectively. Although this lipid is sometimes associated with PC in biological systems, its chemical and physical properties are significantly different from that of PC, and therefore possess different functions in biochemical processes (Christie, 2010). While both lipids are key components of membrane bilayers with equal importance to many cellular functions, PE interacts with other lipids in the bilayer and exerts a lateral pressure to maintain membrane curvature and stabilize the optimal conformations of membrane proteins.

Similar results were also observed for the other classes of phospholipids in liver tissues of the two species of cottoid fish (Kozlova, 1998). In this study, the solvent front was obtained in small amounts and this lipid belongs to the more polar lipid class i.e. phospholipids.

Five fractions of neutral lipids were quantified, TAGs being the most predominant in salmon liver tissues, a qualitative characteristic in most tissues, especially in the main lipid storage organ i.e. liver, in an overwhelming majority of fish (Lee *et al.*, 1975; Henderson and Tocher, 1987; Neighbors, 1988; Eastman, 1988; Kozlova, 1998; Sargent *et al.*, 2002).

FFA and LPC were found to be present only in small amounts, indicating that the lipids were extracted efficiently. Presence of high amounts of FFA in lipid extracts would have indicated tissue disruptions and consequently, affecting the overall amounts of the main lipid components (Christie and Han, 2010). In the previous studies, it was observed that rapid freezing and pulverized animal tissues at -70 °C prior to extraction, yielded very low levels of free fatty acids in comparison to more conventional procedures, in which the lipid extracts were extracted from the animal tissues directly with a homogenizer of the rotating blade type at 0 °C (Kramer and Hulan, 1978). Also, improper storage of tissue sample may cause lipases present in the tissues to breakdown of some lipids, causing artefactual formation of free fatty acids, even at -20 °C, and this activity will only accelerate during the thawing process, prior to extraction (Christie, 2018), which may lead to the losses of essential unsaturated fatty acids and intact lipids (Christie and Han, 2010). In this experiment, the artefactual hydrolysis of lipids in the liver tissues producing high amounts of free fatty acids was successfully avoided by the immediate transfer of the tissues into C:M (2:1) during sampling and stored in -20 °C before total lipid extraction was performed the following day.

As seen in the results, this method is particularly very effective in extracting most of the lipids and substantially decreasing the losses of lipids incidental to freezing and thawing process. This also has led to the advantage of detecting various unidentified lipid classes (such as UNK1 and UNK2) using lipid fractionation by TLC (Fuchs *et al.*, 2011).

4.4.2 Activation of Gal4-PPAR γ by polar lipids

Gal4-PPAR γ transfected cells were treated with two concentrations of lipid fractions dissolved in absolute ethanol, 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$, aiming to detect any PPAR γ activity at both low and high concentrations. At low lipid concentration, none of the lipid fractions were able to activate PPAR γ activity, suggesting that this concentration may have not been sufficient to produce an effect on the transcription of the firefly luciferase. When the lipid concentration was doubled, two of the polar lipid fractions, unknown polar and solvent front, were able to activate PPAR γ activity, while no PPAR γ activity was detected when the transfected cells were treated with the neutral lipid fractions. It can be suggested from this experiment that these lipids may contain molecular components that have the ability to bind with PPAR γ ligand-binding domain and activate transcription of firefly luciferase activity.

4.4.3 Identification of polar lipid fractions

Initially, these two lipids were analysed with gas chromatography-flame ionisation detection (GC) to identify the fatty acid components in the fractions. The fatty acids identified to be present in the lipid fractions and in

high amounts were palmitic acid, stearic acid, oleic acid and DHA. These fatty acids have been identified to activate mammalian PPAR γ (Walkey and Spiegelman, 2008) and have been tested on piscine PPAR γ but none of these fatty acids were able to activate the receptor (Kondo *et al.*, 2007; Colliar *et al.*, 2011). The molecular components in the lipid fractions were further investigated using high resolution liquid chromatography-mass spectrometry (LC-MS) to identify individual molecular species of lipids that consisted of the previously identified fatty acid compositions (Table 4-2). Both of the lipid fractions consisted of mainly ceramide species in general. According to Christie and Han (2010), the nature of fatty acids and long-chain bases in ceramides are commonly saturated and monoenoic (monosaturated) and this agrees with the high amounts of saturated fatty acids (mainly palmitate, 16:0) in the unknown polar lipid fatty acid composition and the high amounts of monosaturated fatty acids (mainly vaccenic acid, 18:1n-7) in the solvent front fraction. These findings suggested that the individual fatty acids within the lipid fractions are not ligands or activators of piscine PPAR γ due to their inability to activate the receptor, but it was the intact lipid structure comprising of these long-chain bases fatty acids that may have been able to bind with the receptor and activate transcription of the firefly luciferase. However, it should be noted that the activation was weak, and it is possible that these compounds could be affecting the transcription of the reporter genes in a non-PPAR dependent manner, or that low concentration of unidentified minor components of the fractions may be causing the observed effects. Nevertheless, these lipid fractions are one of the few, or possibly only examples showing activating effects on piscine PPAR γ . It was also discussed

in the previous chapter that alterations in the peptide regions within the ligand-binding domain due to residue substitutions (Leaver *et al.*, 2005) may have contributed to the ability of certain ceramide species to bind with the receptor of PPAR γ . Thus, the ability of LC-MS to detect the unknown compounds was very imperative in this project to further identify the potential organic compounds that may have binding specificity with PPAR γ ligand-binding domain and the possibility of activation of this receptor in particular.

4.5 Conclusions

Total lipid extraction from salmon liver and the efficient separation and detection of various lipid classes was achieved. This is, as far as can be found in the literature, the only example of preparative lipid fractionation from fish tissues. Potential activators of PPAR γ in cell transfection assays were present in two unidentified lipid fractions. GC and LC-MS showed that the major molecular species in these fractions were ceramides and hexosylceramides and this information was used as a prerequisite in conducting the experiments that followed in the next chapter.

5 Response of fish PPAR γ to sphingolipids

5.1 Introduction

Sphingolipids, together with sterols and glycerolipids, are three major classes of lipids, recognised as essential components in eukaryotic cell membranes found in various species from fungi to mammals and in some bacteria and viruses (Merrill, 2008). In addition to their well-established roles as structural components of cell membranes, they have become a major topic of interest more recently since they have been recognized as signaling molecules (Hannun and Obeid, 2008; Maceyka and Spiegel, 2014) and essential components of 'membrane-rafts' (Simons and Gerl, 2010). Sphingolipids are composed of characteristic long-chain or sphingoid bases linked to a fatty acid via an amide bond (Christie and Han, 2010). Sphingolipids may be divided into several major classes (Table 5-1, Figure 5-1) and these include: the sphingoid bases and their simple derivatives (e.g. the 1-phosphate), the sphingoid bases with an amide-linked fatty acids (e.g. ceramides), and more complex sphingolipids with head groups via phosphodiester linkages (the phosphosphingolipids), via glycosidic bonds (the glycosphingolipids, and other groups e.g. phosphono- and arseno-sphingolipids (Fahy *et al.*, 2005).

Table 5-1 Sphingolipids classes and subclasses. Adopted from Fahy *et al.* (2005)

Sphingoid bases
Sphing-4-enines (sphingosines)
Sphinganines
4-Hydroxyphinganines (phytosphingosines)
Sphingoid base homologs and variants
Phingoid base 1-phosphates
Lysophingomyelins and lysoglycophingolipids
N-Methylated phingoid bases
Sphingoid base analogs
Ceramides
N-Acylsphingosines (ceramides)
N-acylsphinganines (dihydroceramides)
N-Acyl-4-hydroxysphinganines (phytoceramides)
Acylceramides
Ceramide 1-phosphates
Phosphosphingolipids
Ceramide phosphocholines (sphingomyelins)
Ceramides phosphoethanolamines
Ceramide phosphoinositols
Phosphonosphingolipids
Neutral glycosphingolipids
Simple Glc series (GlcCer, LacCer, etc)
GalNAc β 1-3Gal α 1-4Gal β 1-4Glc- (globo series)
GalNAc β 1-4Gal β 1-4Glc- (ganglio series)
Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc- (lacto series)
Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc- (neolacto series)
GalNAc β 1-3Gal α 1-3Gal β 1-4Glc- (isoglobo series)
GalNAc β 1-2Man α 1-3Man β 1-4Glc- (mollu series)
Gal β 1-4GlcNAc β 1-3Man β 1-4Glc- (athro series)
Gal- (gala seires)
Other
Acidic glycosphingolipids
Gangliosides
Sulfoglycosphingolipids (sulfatides)
Glucuronosphingolipids
Phosphoglycosphingolipids
Other
Basic glycosphingolipids
Amphoteric glycosphingolipids
Arsenosphingolipids
Other

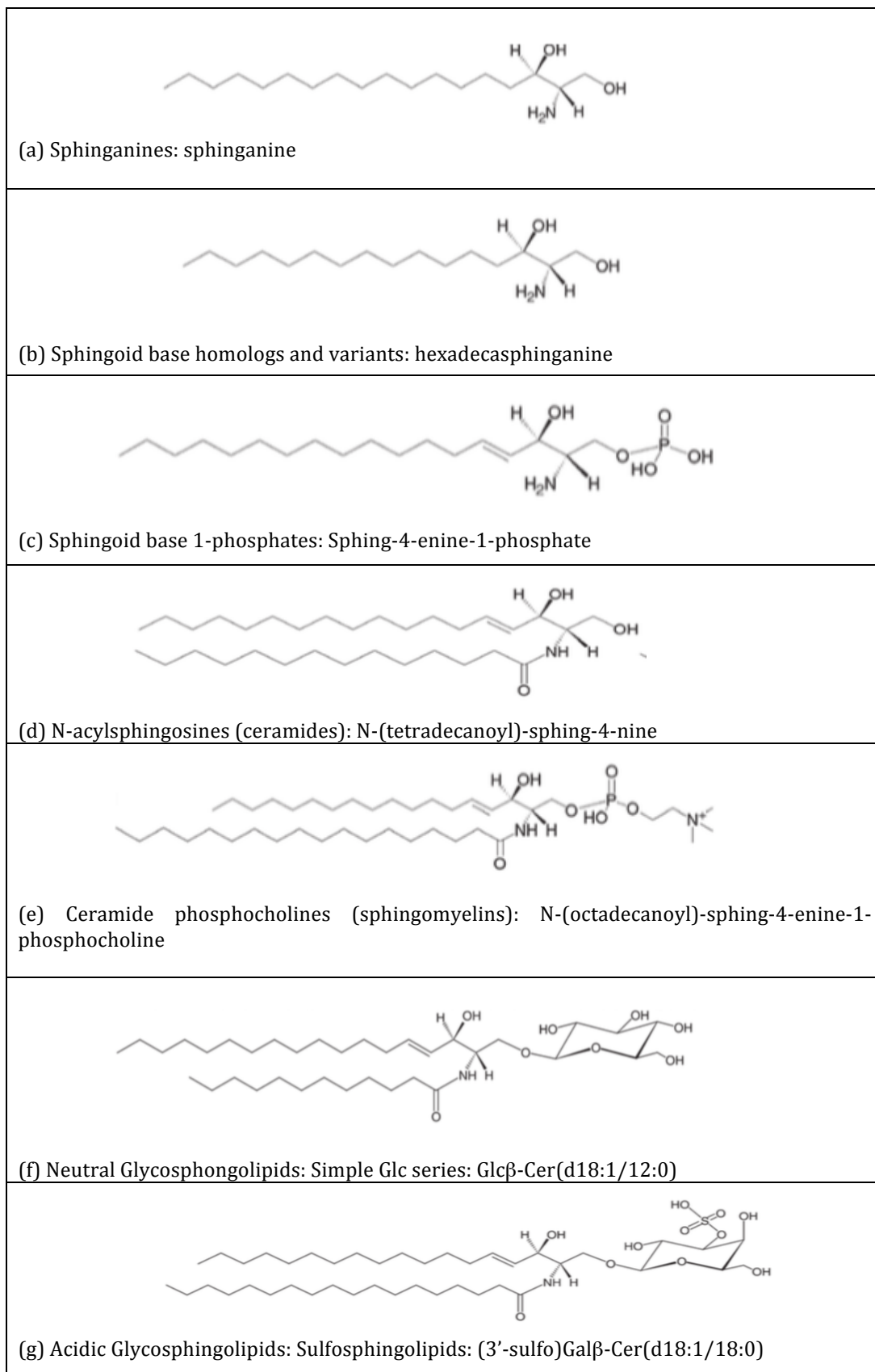


Figure 5-1 Some representative structures for sphingolipids. Adopted from Fahy *et al.* (2005).

Sphingolipid metabolites, mainly ceramide, S1P and C1P, are important lipid mediators that function to regulate cellular activities including cell growth, survival, migration, immune cell trafficking, angiogenesis, inflammation and cancer (Hannun and Obeid, 2002; Kihara *et al.*, 2007; Tani *et al.*, 2007; Bartke and Hannun, 2009; Parham *et al.*, 2015; Kleuser, 2018). Ceramides are also known as key intermediates in the biosynthesis of the above complex sphingolipids in the sphingolipid metabolic pathways (Hait and Maiti, 2017). Sphingolipid metabolism involves ceramide as the central sphingolipid molecule which may be produced via three major pathways: by de novo synthesis with a series of enzymatic reactions in the endoplasmic reticulum and mitochondria; by a salvage pathway in which ceramide is generated through the acylation of sphingosine; and by the hydrolysis of sphingomyelin through the action of sphingomyelinase (Figure 5-2).

The de novo synthesis of sphingolipid commences with the condensation of serine and palmitoyl-CoA, catalysed by serine palmitoyl transferase to produce 3-keto sphingosine, which is subsequently reduced by a reductase to form sphinganine (dihydrosphingosine). Sphinganine is N-acylated by (dh)ceramide synthases to form dihydroceramide or ceramide. Ceramide is converted to sphingomyelin by sphingomyeline synthase and this reaction can be reversed by sphingomyelinase to regenerate ceramide. Ceramide can also be converted to a hexosylceramide, usually glucosylceramide (GlcCer), by glucosylceramide synthase, to sphingosine by ceramidase and to C1P by ceramide kinase. Sphingosine can be converted to S1P by sphingosine kinase enzymes, S1P can be converted back to sphingosine by S1P phosphatase and S1P lyase irreversibly degrades S1P. C1P can also be converted back to

ceramide by C1P phosphatase and GlcCer back to ceramide by glucosylceramidase (Figure 5-2; Hannun and Obeid, 2008; Bartke and Hannun, 2009; Aguilera-Romero *et al.*, 2014; Hait and Maiti, 2017).

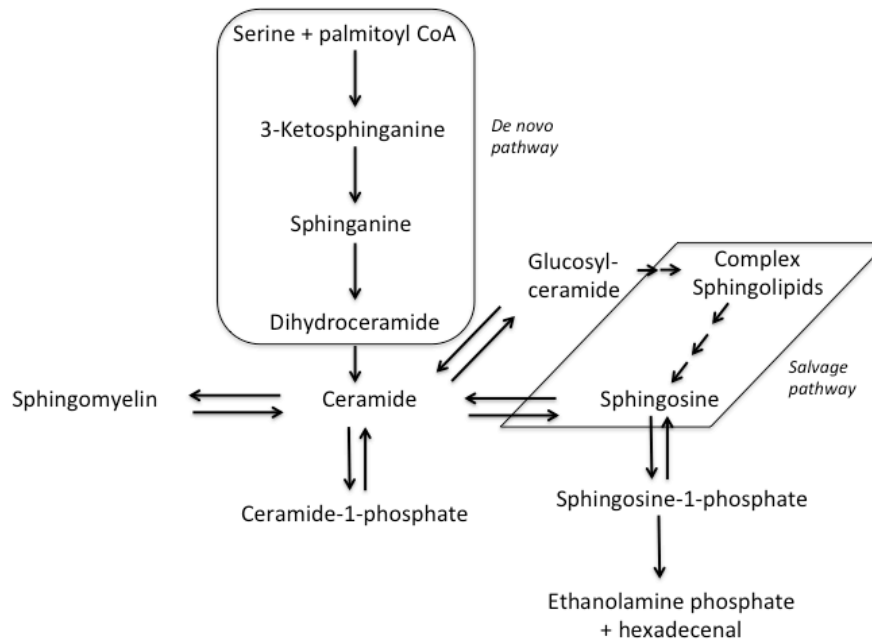


Figure 5-2 Sphingolipid metabolic pathways. Adopted from Hait and Maiti (2017)

Based on the results from LC-MS analysis (in Chapter 4), lipid fractions which increased reporter activity on Gal4-PPAR γ transfected cells contain as major components, ceramides and hexosylceramides. These compounds, together with other metabolites involved in the reversible metabolic pathways in Figure 5-2, were tested on transfected cells in the subsequent experiments.

This chapter, therefore, focuses on the application of pure sphingolipid compounds above to test the response of Gal4-PPAR γ construct using the transient transfection assay developed previously.

5.2 Materials and methods

Six pure sphingolipid compounds were tested on Gal4-PPAR γ transfected CHSE-214 cells for luciferase enzyme activity: ceramide, sphingosine, glucosylceramide (GlcCer), sphingosine-1-phosphate (S1P), ceramide-1-phosphate (C1P) and sphingomyelin (SM). Pure compounds of SM and S1P were purchased from Sigma, C24: Ceramide (d18:1/24:1(15z) and C24: C1P (d18:1/24:0) from Stratech, and N-Hexanoyl-glucosyl ceramide and D-erythro-C17-Sphingosine were obtained from Universal Biologicals.

The stock solutions of these compounds were prepared in three different concentrations in absolute ethanol: 5 μ M, 10 μ M and 500 μ M, and were used to dilute complete media EMEM to give final concentrations per ml: 5 nM, 10 nM, 50 nM, 100 nM, 1 μ M, 5 μ M, 10 μ M and 20 μ M, to be tested on transfected CHSE-214 cells.

5.2.1 Seeding and transfection of CHSE-214 cells

CHSE-214 were harvested from near-confluent cell culture flasks and seeded at 2×10^5 cell per well of 24 well plates before transfection the following day with Gal4-PPAR α (treated with WY-14,643 tested for positive control only) and Gal4-PPAR γ as described in Sections 3.2.2.3 and 3.2.2.4.

5.2.2 Treatment of transfected cells with sphingolipids and their metabolites

Following transfection, cells were treated with the above compounds to test for their ability to interact with the PPAR γ -ligand binding domain as receptor agonists, i.e. molecules capable of increasing transcriptional activity of PPAR γ . Media was aspirated from the assay plate and cells were washed once with 1 x DPBS (Invitrogen) before the addition of the diluted treatment compounds prepared previously. Each treatment was run in triplicates within each experiment with an ethanol absolute as a control treatment at a concentration of 10 μ l ethanol absolute per ml of EMEM. Cells were incubated with treatment compounds for a further 24 hours at 22°C in an atmosphere of 4% CO₂ before being assayed for luciferase activity.

5.2.3 Luciferase activity, data normalisation and statistical analysis

Firefly and *Renilla* luciferase activities were measured using luciferase assay buffers described in Chapter 3. Twenty-four hours post-treatment, media containing treatment compounds was aspirated from assay plates and cells were washed twice with 1x DPBS before they were lysed in 1 ml of 1 x passive lysis buffer (Promega) for 10 min, shaking. The cell lysate was then used in a dual luciferase assay system described in Chapter 3.

As previously described, *Renilla* luciferase values were used to normalise firefly activities for differences in transfection efficiency between wells. For

each well, the firefly luciferase value was divided by the corresponding *Renilla* luciferase value and the mean replicate wells was calculated.

Experiments were repeated independently three times (with different flasks of cells seeded on different days). Data is reported as the mean fold inductions in normalised luciferase activities compared to ethanol vehicle controls. To test for statistically significant differences between ethanol control treatment and treatment with lipid compounds, a one-way ANOVA was carried out followed by Dunnet's test on Minitab18 software. Results were considered statistically significant when the probability value (P) was less than 0.05 ($P < 0.05$).

5.3 Results

Of all the sphingolipids incubated with Gal4-PPAR γ , only one compound, GlcCer at a treatment concentration of 5 μ M, was able to increase expression of firefly luciferase over that observed with ethanol to a statistically significant level of $P < 0.05$, although this increase was small at 1.91-fold (Figure 5-3). Pure sphingomyelin compound caused no increase in luciferase activity, and this agrees with the observation previously documented in Chapter 4 in which the lipid fraction sphingomyelin/phosphatidylcholine (SM/PC) (and its molecular components) also failed to increase luciferase activity, therefore confirming that this compound does not act as an activator of PPAR γ . As for the other sphingolipids tested, sphingosine (at 20 μ M), ceramide (at 100 nM and 1 μ M), S1P (at 5, 10, 20 μ M) and C1P (at 20 μ M) were observed to significantly suppress the PPAR γ activity.

When the cells transfected with empty pBIND only were treated with 5 μ M of the sphingolipids being tested above, there was no significant increase in luciferase activity over ethanol (Figure 5-4).

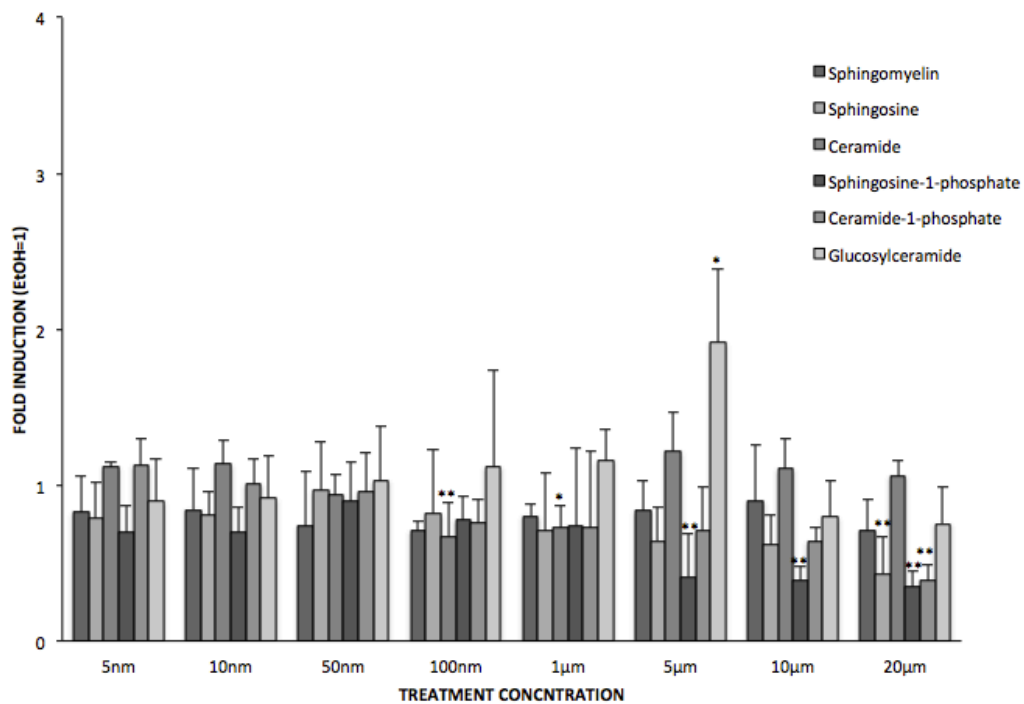


Figure 5-3 Response of PPAR γ to sphingolipids at different concentrations. Data are the means \pm SD of three independent experiments. Results are expressed as the fold increase over ethanol control of normalised firefly luciferase activity. Two asterisks () represent statistically significant difference ($P < 0.01$) and one asterisk (*) represents statistically significant difference ($P < 0.05$) to activities in ethanol controls.**

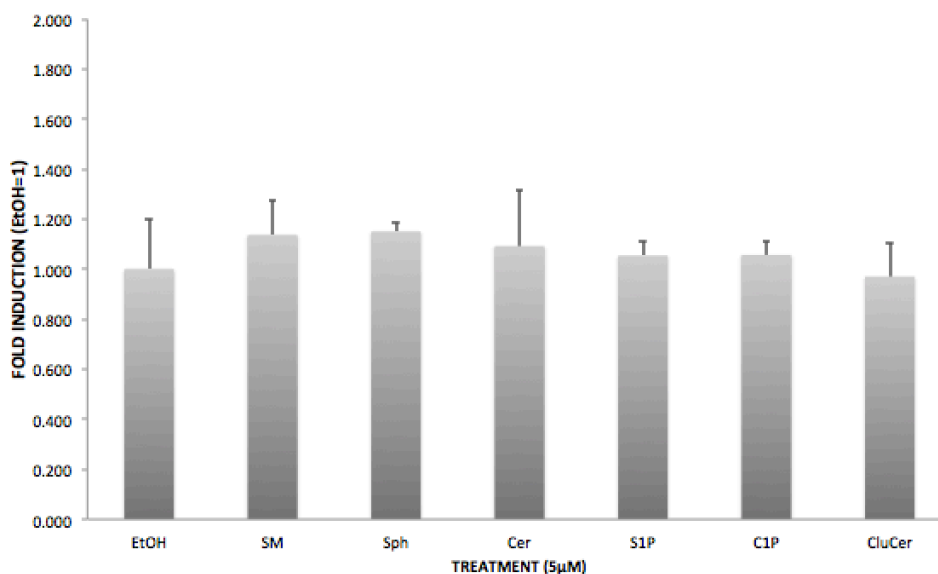


Figure 5-4 Response of empty pBIND to sphingolipids at 5 μ M. Data are the means \pm SD and results are expressed as the fold increase over ethanol control of normalised firefly luciferase activity.

5.4 Discussion

Sphingolipids are bioactive lipids that are important in signal transduction (Mathias *et al.*, 1998; Pyne and Pyne, 2000; Chalfant and Spiegel, 2005, Kihara *et al.*, 2007). Ceramide is one of the main sphingolipid metabolites and, along with S1P and C1P, are lipid mediators that function to regulate cellular activities including cell growth, survival, migration, immune cell trafficking, angiogenesis, inflammation and cancer (Hannun and Obeid, 2002; Kihara *et al.*, 2007; Tani *et al.*, 2007; Bartke and Hannun, 2009; Parham *et al.*, 2015; Kleuser, 2018). Because ceramide is the central sphingolipid molecule in sphingolipid metabolism and the key intermediate in the biosynthesis of complex sphingolipids, and because of its essential role in differentiation and in apoptosis (Futerman and Hannun, 2004; Hannun and Obeid, 2008), ceramide has emerged to become the most well-studied class of

sphingolipids. Reports on the relationship between mammalian PPAR γ and sphingolipids are very limited, and to date, there are no published information that links PPAR γ in fish and sphingolipids. The present study, for the first time, suggested a potential activating ligand of piscine PPAR γ by indicating the involvement of sphingolipid metabolites.

5.4.1 Ceramide and its precursors

In mammals, ceramide has been reported to induce apoptosis in cancer cells, which required the activation of PPAR γ pathway. Furthermore, ceramide was able to activate PPAR γ in a dose dependent manner (Wang *et al.*, 2006). This activation, however, is contradictory to the results observed in this study when Gal4-PPAR γ transfected CHSE-214 cells were treated with ceramide. In fact, ceramide suppressed piscine PPAR γ activity. These results are more consistent with a previous report showing that PPAR γ expression in cultured mouse preadipocytes (3T3-L1) was downregulated by ceramide, resulting in the inhibition of adipogenesis (Spratt, *et al.*, 2002). Similarly, sphingomyelin downregulated mammalian PPAR γ expression in cultured preadipocytes (3T3-F442A) *in vitro* (Al-Makdissy *et al.*, 2001) and adipocytes *in vivo* (Zeghari *et al.*, 2000). This down-regulation of PPAR γ expression was evidently dependent on the length of the acyl chain of sphingomyelin such that longer acyl chains down-regulated PPAR γ to a greater extent (Al-Makdissy *et al.*, 2001). In this study, sphingomyelin failed to activate piscine PPAR γ activity at various concentrations. Nevertheless, being the most abundant sphingolipid, sphingomyelin together with the enzyme sphingomyelinase provide the greatest contribution to ceramide production

(Kihara *et al.*, 2007) and thus, the production of other important sphingolipids metabolites.

Similar to ceramide treatment of Gal4-PPAR γ transfected cells, other precursors of ceramide, sphingosine, S1P and C1P, also suppressed the activity of piscine PPAR γ in a dose dependent manner, although it is not possible to rule out toxic effects. Though the context of PPAR γ signaling in fish remains unknown, the role of piscine PPAR γ in fish lipid metabolism could be affected by these sphingolipid metabolites. This appears to be in contrast to the role of S1P with regard to PPAR γ in mammals. S1P is known to be a bioactive lipid mediator important in various physiological and pathological cellular processes (Hla, 2004) and its functions include regulating cell survival, proliferation, migration, signaling and angiogenesis (Hannun and Obeid, 2008). Levels of S1P are affected by the amount of S1P lyase present in liver cells as this enzyme is responsible for the irreversible degradation of S1P (Bektas *et al.*, 2010). In murine liver tissues where S1P lyase was deficient, PPAR γ expression was observed to be upregulated. And S1P lyase deficiency affected the levels of multiple sphingolipid metabolites including sphingosine, ceramide, sphingomyelin and C1P (Bektas *et al.*, 2010), suggesting this enzyme is central to the regulation of the sphingolipid metabolic pathway (Serra and Saba, 2010). Moreover, overexpression of sphingosine kinase, an enzyme responsible in the production of S1P from sphingosine, increased lipid accumulation in mouse liver and PPAR γ expression, similarly to treatment with S1P (Chen *et al.*, 2016). These studies suggest that because ceramide and its metabolites are mutually convertible, it is desirable for cells to regulate the total amount of each metabolite in

order to maintain the overall balance in cellular levels (Kihara *et al.*, 2007). More recently and in complete contrast to the piscine results reported herein, S1P has been identified as a ligand of mammalian PPAR γ in the regulation of neoangiogenesis (Parham *et al.*, 2015). The interaction between S1P and mammalian PPAR γ was predicted to have occurred with the formation of hydrogen bonds between the phosphate group of S1P and H323 and H449 of Helix 12 within PPAR γ LBD. However, as previously discussed in Chapter 3, one of these amino acids in piscine PPAR γ LBD, i.e. H323, has been replaced by isoleucine in the PPAR γ LBD, while H449 remains equivalent with PPAR γ LBD in mammals (Leaver *et al.*, 2005). This again confirms that the alteration in the structure of the peptide regions within the LBD in fish, may be responsible for the differences between mammalian and piscine ligand specificity.

5.4.2 Glucosylceramide as a potential activating compound of PPAR γ

While none of the sphingolipids tested above had the ability to activate PPAR γ in fish, treatment of Gal4-PPAR γ transfected cells with glucosylceramide (GlcCer), one of the metabolites of ceramide in the synthesis of more complex membrane glycosphingolipids, resulted in significant increase in luciferase activities. Glucosylceramides are glucosylated lipids with simple structures and functions critical for cellular homeostasis and cellular activities (Ishibashi *et al.*, 2013). Recent studies evidently suggested that GlcCer functions in fat metabolism in *Drosophila* (Kohyama-Koganeya *et al.*, 2011). The *Drosophila* fat body is an organ

previously shown to be equivalent to both liver and white adipose tissue in mammals (Kohyama-Koganeya *et al.*, 2004; van Eijk *et al.*, 2009). As previously mentioned and shown in Figure 5-2, glucosylceramide synthase (GlcT-1) is the enzyme responsible for the production of GlcCer from ceramide. A recent study documented that there was an improvement in insulin sensitivity and normalization of adipogenesis in obese mice with the inhibition of GlcCer synthesis by GlcT-1-specific inhibitor, N-(5-adamantane-1-yl-methoxyl)-pentyl-1-deoxynojirimycin (AMP-DNM) (van Eijk *et al.*, 2009). The normalization of adipocytes was associated with an increase expression of a number of genes including PPAR γ , suggesting that reduced amounts of GlcCer in the adipose tissue, improved lipid metabolism in dyslipidemic mammalian models. Overexpression of GlcT-1 in the *Drosophila* fat body, increased fat storage, and the reduction of GlcT-1 decreased storage, suggesting that GlcCer has an important role in energy storage in this species (Kohyama-Koganeya *et al.*, 2011). However, the molecular mechanism that links GlcCer with the regulation of energy metabolism is still not clearly understood in *Drosophila* and given that insects do not possess PPAR genes, and the exact relevance to vertebrates is not clear. It has been noted that relatively small amounts of dietary GlcCer can vastly improve human skin conditions, and this may be due to PPAR γ activation, but involving GlcCer metabolites, 4-8-sphingadienine and 4-hydroxy-8-sphingenine (Shirakura *et al.*, 2012).

Application of GlcCer to transfected cells indicated a clear activation effect at 5 μ M as was observed with lipid fractions whose major constituents were GlcCer compounds (Chapter 4). However, at 10 μ M and above, GlcCer did not

have a significant effect. Whilst this might suggest random effects that do not represent bona fide activation, it is notable that this was the only compound tested that showed significant activation, and was also one of the major components of the only lipid fractions that showed effects. Therefore, the possibility of GlcCer interaction with piscine PPAR γ is worthy of further investigation. However, it should also be noted that in the cell transfection assays herein, it is not possible to conclude whether GlcCer or one of more of its metabolites, as suggested for mammalian PPAR interactions described above, was able to activate piscine PPAR γ . It is also possible that piscine PPAR γ is not ligand activated, but acts as a repressor of gene transcription when bound to its cognate promoter, in which case target gene expression would be controlled by expression levels of PPAR γ . This hypothesis and ligand activation hypothesis will only become testable after genuine gene targets for PPAR γ are identified in fish. This presents a conundrum, as the mechanism of PPAR γ may have to be determined before experiments can be performed to identify target genes in fish. Nevertheless, the lack of response of piscine PPAR γ to fatty acids observed previously in the experiments reported in Chapter 3 and from the previous studies (Leaver *et al.*, 2005; Colliar *et al.*, 2011), and the ability of a more complex sphingoid lipid structures in the activation of the receptor in both mammals and fish, suggest there may be common endogenous activators of mammalian and piscine PPAR γ .

5.5 Conclusions

Piscine PPAR γ is annotated based on its structurally close similarity to PPAR γ in mammals. Despite this similarity, piscine PPAR γ is not activated by typical lipid or synthetic ligands of mammalian PPAR γ . This may be due to a small number of critical and phylogenetically invariable amino acid substitutions in the ligand binding domain of piscine PPAR γ . However, both ceramide and GlcCer were identified as major components of lipid fractions that were capable of activating piscine PPAR γ in cellular transactivation assays. Here, it is shown that of these two pure sphingolipids, only GlcCer showed activation of piscine PPAR γ . Other sphingolipids, sphingosine, ceramide, S1P and C1P, which may have been present at lower levels in these lipid fractions, were not activators and in some cases appeared to inhibit PPAR γ . There is a limited wider literature on sphingolipid interactions with PPARs and to date, there is evidence that GlcCer metabolites might activate mammalian PPAR γ , but this does suggest that these metabolites should be tested on piscine PPAR γ .

6 Phylogeny and tissue distribution of peroxisome proliferator-activated receptors (PPARs) in Atlantic salmon (*Salmon salar*)

6.1 Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-inducible transcription factors that belong to the nuclear hormone receptor superfamily. Since their initial discovery as transcription factors, PPARs have been intensively studied and the three PPAR isotypes, PPAR α , PPAR γ and PPAR β (or δ) have been identified and functionally characterised in mammals (Kliewer *et al.*, 1992; Issemann *et al.*, 1993), amphibians (Dreyer *et al.*, 1992) and birds (Diot and Douaire, 1999). Each isotype is encoded by a single gene and each has different functions, reflected by distinct patterns of tissue distribution (Desvergne and Wahli, 1999; Escher *et al.*, 2001; Hihi *et al.*, 2002; Boukouvala *et al.*, 2004; Leaver *et al.*, 2005).

PPAR α functions in fatty acid oxidation in mitochondria and peroxisomes, thus, is highly expressed in tissues with high rates of β -oxidation such as the liver, heart, muscle and kidney. The role of PPAR α is conserved in both mammalian (Braissant *et al.*, 1996) and fish species. Although only one PPAR α has been identified in mammals, most fish investigated so far express two PPAR α subtype genes, termed PPAR α 1 and PPAR α 2, (Maglich *et al.*, 2003; Leaver *et al.*, 2005).

Like its mammalian homolog, piscine PPAR β being the most ubiquitous isotype, showed the strongest overall expression and is present in almost all

tissues, thus, the physiological role of PPAR β is less well established than for other PPARs. While there is only a single gene that encodes for PPAR β in mammals, the numbers of PPAR β subtypes varies in fish species. Similar to mammals, pufferfishes exhibit only one PPAR β gene (Maglich *et al.*, 2003) and this is also evidently true for plaice and sea bream (Leaver *et al.*, 2005). However, it has previously been reported that more than one gene for PPAR β may be present in zebrafish (Robinson-Rechavi *et al.*, 2001). Multiple PPAR β genes have been have also been identified in Atlantic salmon, termed ssPPAR β 1A, ssPPAR β 1B, ssPPAR β 2A and ssPPAR β 2B, at least two of which appear to be functional (Leaver *et al.*, 2007).

Mammals have a single gene encoding PPAR γ that is alternatively spliced to give rise to two proteins, PPAR γ 1 and PPAR γ 2, each carrying different N-terminal sequence (Tontonoz *et al.*, 1994). PPAR γ 1 is highly expressed in gut where as PPAR γ 2 is abundant in adipose tissues compared to the other tissues (Escher *et al.*, 2001). From the available genome information of zebrafish, stickleback, medaka, pufferfish, sea bream, sea bass and plaice, it was observed that fish also possess a single PPAR γ gene with no indications of alternatively spliced PPAR γ proteins (Maglich *et al.*, 2003; Boukouvala *et al.*, 2004; Leaver *et al.*, 2005).

The differences in the number of genes in different fish may have resulted from the occurrence of ancestral genome duplications after the split between mammalian and teleost lines. These genes may have been lost or retained during the divergence of different lineages of fish (Woods *et al.*, 2005). Whilst the previous genome duplication may have occurred in early evolution of

ray-finned fishes (Taylor *et al.*, 2003), the presence of multiple PPAR β genes in Atlantic salmon supports the theory that salmonids may be derived from a relatively recent autotetraploidization event (Allendorf and Thorgaard, 1984; Alexandrou *et al.*, 2013). The recent availability of the salmon genome sequence (Lien *et al.*, 2010) now enables the identification of the full complement of PPAR genes in this species.

This study aimed:

- (1) To assess the phylogenetic relationship of Atlantic salmon PPAR genes and the PPAR genes in other fish species and other vertebrates,
- (2) To determine the PPAR distribution in various tissues of Atlantic salmon through the investigation of the gene expression of the different isoforms of PPAR.

6.2 Materials and methods

6.2.1 Sampling

This experiment was subjected to ethical review and approved by the University of Stirling through Animal and Welfare Ethical Review Body (AWERB). Five juvenile pre-smolt specimens of Atlantic salmon (*Salmon salar*) with weight range of about 300-400 g were obtained from the University of Stirling Buckieburn Field Station, Scotland. Fish were sacrificed with an overdose of tricaine methanesulfonate (MS222) and a sharp blow to the head. Approximately 50-100 mg of different tissue samples including heart, brain, spleen, intestine, kidney, ovary, liver, gill and muscle were collected from five female fish. The samples were preserved overnight in RNA later (stabilisation buffer) at 4 °C and subsequently stored in -70 °C freezers till required.

6.2.2 RNA extraction

Total RNA was extracted following the RNA tri Reagent (Sigma-Aldrich, USA) extraction protocol. About 50 mg tissue samples fixed in RNAlater were homogenised in 1 ml TRI Reagent in 2 ml screw cap microtubes using a Mini-Beadbeater (Bio Spec Products Inc., Bartlesville, USA). Homogenised samples were incubated at room temperature for 5 min before they were centrifuged at 14,000 x g in a microcentrifuge for 5 min. In the fumehood, the supernatants were transferred into fresh Eppendorf tubes and 1/10 volume of 1-bromo-3-chloropropane (BCP) was added. The contents were mixed by vortex before they were incubated at room temperature for 15 min and

centrifuged at 14,000x g for 10 min. The clear, upper aqueous phase was carefully transferred to fresh Eppendorf tubes in 200 µl aliquots. Half the volume (per aqueous phase volume) of isopropanol and half the volume of RNA precipitation solution, consisting of 1.2 M sodium chloride (NaCl) and 0.8 M sodium citrate sesquihydrate ($C_6H_6Na_2O_7 \cdot 1.5H_2O$), were added to precipitate the RNA. The mixtures were subsequently mixed thoroughly before the tubes were centrifuged at 14,000 x g for 15 min. The RNA precipitate formed gel-like pellets on the bottom of the tubes. The supernatant was removed carefully by pipetting and pellet was washed with 1 ml of 75 % ethanol in MilliQ water (v/v). The pellets were lifted from the bottom of the tube by flicking and inverting the tubes a few times to make sure the entire surface of the pellets was properly washed before they were incubated at room temperature for an hour for the pellets to become clearly visible. The tubes were then centrifuged at 14,000 x g for 5 min and the ethanol was carefully removed and discarded. The RNA pellets were air dried at room temperature until all visible traces of ethanol were gone. Subsequently, RNA pellets were re-suspended in an appropriate amount of MilliQ water of 40-400 µl depending on the size of the RNA pellet. RNA solutions were incubated at room temperature for 30-60 min with gentle flicking of the tubes every 15 min to aid resuspension. The concentration and quality of RNA were assessed spectrophotometrically using the NanoDrop® (Labtech International ND-1000 spectrophotometer). The quality and integrity of RNA samples were further assessed by electrophoresis on 1 % agarose gel, as described in Section 2.2.4. The RNA solution were then stored at -70 °C until further analysis.

6.2.3 Cloning of Atlantic Salmon PPAR γ L cDNA

RNA was extracted (as describe in 6.2.2) from a whole Atlantic salmon alevin. Synthesis of cDNA was carried out with 2 μ g of total RNA, and a Nanoscript2 synthesis kit (Primer Design, UK), using the oligo dT primer supplied with the kit. The database entry for mRNA sequence for Atlantic salmon PPAR γ L (XM_014168483) is a prediction based on the genome sequence and during alignment with other salmon PPARs sequences the deduced amino acid sequence showed some unlikely irregularities. Therefore, primers were designed (GGACCTGGCAGAGATGGACAAC, CCCACTACTCTAGTACAGGTCCCT) to regions encompassing the predicted start and stop codons and applied to the salmon alevin cDNA sample in a PCR reaction containing 10 μ l 2 x MyTaqHS mastaermix (Bioline, UK), 200 nM each primer, and 1 μ l of cDNA synthesis reaction. Cycling parameters were 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 30 s. PCR products were purified from reactions using silica membrane-based spin columns (Qiagen), and ligated to pGEMTeasy PCR product cloning vector (Promega), according to the manufacturer's instructions. Plasmids containing cDNA inserts were sequenced commercially (GATC Biotech).

6.2.4 Complementary DNA (cDNA) synthesis

Complementary DNA (cDNA) was synthesised using the Precision nanoScriptTM2 Reverse Transcription Kit (Primer Design Ltd, UK), following manufacturer's instructions. The reverse transcription kits and the RNA were allowed to thaw on ice. A total of 10 μ l of RNA solution containing 1 μ g RNA and 1 μ l reverse transcriptase oligo dT primers in MilliQ water was prepared

in 0.2 ml PCR tubes. These were heated in a Biometra thermocycler for 5 min at 65 °C to denature RNA and placed on ice at 4 °C immediately to cool the tubes. The cDNA reverse transcriptase master mix was prepared according to manufacturer's instructions, multiplied by the number of samples available. A volume of 10 µl of the cDNA reverse transcriptase mix containing 5 µl nanoScript2 4x buffer, 1 µl dNTP mix 10 mM, 1 µl nanoScript2 enzyme and 3 µl nuclease-free water was added to the 10 µl solution of denatured RNA, mixed briefly by vortexing followed by a pulse spin. These tubes were put in a thermocycler set at 25 °C for 5 min, 42 °C for 20 min, 75 °C for 15 min and 4 °C for 4 min, after which the cDNA samples were diluted to 80 µl total volume (1:4) with MilliQ water and stored at -20 °C until required for quantitative polymerase chain reaction (qPCR).

6.2.5 Quantitative polymerase chain reaction (qPCR) primers

Atlantic salmon PPAR mRNA sequences (Table 6-1) were identified by a combination of text searches of current annotations of the salmon genome on NCBI, and BLAST searches of the salmon genome using previously characterised fish PPAR sequences. This resulted in the identification of eight (8) genes, and corresponding cDNAs with high similarity to PPARs across vertebrates. As these salmon mRNAs are to some extent the result of computer predictions based on genomic sequence, they were also compared to previously deposited salmon sequences and inspected for possible errors or ambiguities in order to exclude regions of ambiguity when designing amplification primers. In the case of PPAR γ L, substantial problems with the database mRNA sequence prediction necessitated cDNA cloning (described above in Section 6.2.2). Primers were derived using PrimerBLAST (www.ncbi.nlm.nih.gov), setting requirements for amplicon lengths between 150 and 250 base pairs, and primer melting temperatures (T_m) between 59 and 61 °C. Primers were checked for specificity against all other Atlantic salmon sequence deposited on Genbank_nr.

Table 6-1 Nucleotide sequences of primers used for PCR amplification of cDNA fragments encoding ssPPARs

Name of Sequencing Primer	Forward Primer sequence (5' to 3')	Reverse Primer sequence (5' to 3')	Amplicon size (base pairs)
ssPPAR α	GGCGTCTACGAGGCACT GTT	GCGAACTGAAACTTGG GCTC	152
ssPPAR α L	CAGTCGAGTAACGGCTC TGG	GGCGAAAGAAACCCTT GCAG	214
ssPPAR β 1A	GCCAACCACACTGACTC AAAG	CTCCCAATCCCAGAAA CCAGG	234
ssPPAR β 1B	TCACATTACCTCTTCCCC AAGC	ACTCACAGGAGTGAGT GAACAG	223
ssPPAR β 2A	CAAACCTTCCACATCCCC TGAA	TTCCATGATACCCTTG AGATGGG	175
ssPPAR β 2B	CCCTTGCTGTCAGAGCA GTT	GCTCTCTCACAGCGCTC ATA	188
ssPPAR γ	TCATCCTCAGTGGAGAC CGT	CTGCTTCTTGAGCAGG TGGA	206
ssPPAR γ L	GCACATTGGACCTGGCA GAGA	GGACTGTTGAGGCTGT GCTCT	112
18S rRNA	ACCACATCCAAGGAAGG CAG	CACCAGACTTGCCCTCC AAT	159

6.2.6 Tissue expression analysis

The expression of each target gene mentioned in Section 6.2.5 was measured by real time quantitative PCR (qPCR) using Eppendorf Mastercycler® *ep realplex* (Germany). The qPCR reactions were prepared in 10 μ l volumes in MilliQ water using, per reaction, 2 μ l cDNA, prepared in Section 6.2.4, 5 μ l SensiFAST™ SYBR® No-ROX mix (Bioline) and 0.2 μ l (10 μ M) forward and reverse primer each. The qPCR reaction for the reference gene, 18S rRNA, was also prepared in the same way. Each plate included negative controls (no template control, NTC) containing no cDNA to ensure contamination has not occurred. A cDNA reference pool dilution series was also included on each plate to calculate the efficiency of the PCR reaction. Each cDNA (4 μ l each) from all the tissues of sampled fish were pooled and was diluted to give cDNA dilutions of 1 in 2, 1 in 5, 1 in 10, 1 in 50 and 1 in 100 in MilliQ water. The undiluted cDNA reference pool was prepared in aliquots and stored at -20 °C and dilutions were freshly prepared on the day of use. The cDNA samples were run in duplicates, and the controls and pooled-dilutions in triplicates on each plate. Plates were sealed using Clear Seal lids (KBiosciences) and briefly centrifuged to collect reactions in bottom of plate wells. Reactions were run at 95 °C for 2 min to activate the polymerase enzyme followed by 40 cycles of denaturation at 95 °C for 5 seconds, primer annealing at 60 °C for 10 seconds and extension at 72 °C for 30 seconds. After amplification, the temperature was increased from 60 °C to 95 °C in 0.5 °C increments every 15 seconds to produce a dissociation curve, used to confirm a single product in each reaction.

6.2.7 Data normalisation and statistical analysis

Relative expression ratios (R) of each PPAR was calculated using a mathematical 'delta-delta' method (Pfaffl, 2001) where by the relative expression of a target gene is calculated based on the amplification efficiency (E) and the crossing point (CP) deviation of an unknown sample versus a control and expressed in comparison to a reference gene, using the equation below:

$$R = \frac{(E_{target})^{\Delta CP_{target}(control-sample)}}{(E_{ref})^{\Delta CP_{ref}(control-sample)}}$$

The relative expression of each PPAR target was calculated for each tissue from the best four of five fish using the reference gene, 18S rRNA, and the expression of each PPAR was normalised to PPAR expression in muscle. To test for statistically significant differences, for each receptor, expression was compared across tissues using one-way ANOVA, followed by post-hoc comparisons using Tukey's test at a significance level of $P < 0.05$, using Minitab 18 software.

6.2.8 Phylogenetic analysis

Amino acid sequences of PPAR receptors from Atlantic salmon and other vertebrates obtained from the NCBI/EMBL/UNIPROT databases were aligned using Mega 7 software. Phylogenetic analysis was done on the full receptors and ligand-binding domains. The PPARs of Atlantic salmon were compared to those of other mammalian (human), bird (chicken), amphibian (frog) and fish (Northern pike, gilthead seabream, European plaice and zebrafish) vertebrates. The phylogenetic analyses, using Mega 7 Software, utilised the maximum likelihood method (Zuckerkandl and Pauling, 1965) and the reliability of the nodes of the tree tested the bootstrap test with 1,000 replicates.

6.3 Results

6.3.1 PPAR γ L mRNA sequence

The results of cDNA cloning and sequencing of products for PPAR γ L indicated substantial differences from the predicted mRNA on GenBank, but included an open reading encoding a protein with a sequence much more closely similar to salmon PPAR γ (Figure 6-1). This enabled design of primers for tissue expression analysis as in Figures 6-4 to 6-8.

6.3.2 Phylogenetic analysis

The phylogenetic analyses of full-length and LBD of PPARs were performed by constructing trees comparing the different PPAR isoforms from a variety of vertebrate species. The topology of the trees from both analysis showed similar results with two clades: one consisting of PPAR α and PPAR β and the other consisting of PPAR γ (Figure 6-2 and Figure 6-3). The PPAR γ proteins themselves were separated into two clusters, one group consisting of fishes whereas the other consisted of PPAR γ of the higher vertebrates i.e. human, frog and chicken. Similar observations were seen for PPAR α and PPAR β . The level of statistical support in the full-length PPAR phylogeny was relatively higher (92%) in the way PPAR α isoforms of the teleost group are positioned compared to level of confidence in the PPAR LBD phylogeny (75%). In the phylogeny tree for full-length PPAR, all teleost PPAR β strongly clustered (99%) separately from the higher vertebrates (chicken and human) whilst the level of confidence for teleost PPAR LBD was relatively lower i.e. 83%.

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ssPPARa1a 166 IRFGRMPQSEKLLK-AEILTGD-REVEDPQQADQKTLARHIYEAYLKNFNMNKAKARTI
ssPPARa1b 171 IRFGRMPQSEKLLK-AEILTGD-REVEDPEQADQKTLARHIYEAYLKNFNMNKAKARTI
hsPPARa    171 IRFGRMPRSEKAKLK-AEILTCE-HDIEDSETADLKSLAKRIYEAYLKNFNMNKVKARVI
ssPPARb1a 139 IRYGRMPEAEKRKLV-AGLLAGERAPTTPNGSDLKSLAKEVNNAYLKNLNMTKKKARSI
ssPPARb1b 234 IRYGRMPEAEKRKLV-AGLLAGDTAPTTPNGSDLKSLAKRVNNAYLKNLNMTKKKARSI
ssPPARb2b 222 IRYGRMPEAEK-KLM-AGLLAEE-LDAQHGGGLDLKTLAKQVYQAYLKNLIMTKKKARNI
ssPPARb2a 223 IRYGRMPGAEEKKLV-AGLLAEE-LDPHHLGGSDLKTLAKQVYQAYLKNLIMTKKKARSI
hsPPARb    143 IRFGRMPEAEKRKLV-AGLTANE-GSQYNPQVADLKAFSKHIYNAYLKNFNMTKKKARSI
ssPPARg    237 IRFGRMPQVEREKLLQAEFM--D-VEPRNPESADLRALSRLCLSYHRHFPLTKSKAKAI
ssPPARgL   207 IRFGRMPQEEREKLL-AEFM--G-VSPRSPESADLRALSRLCLSYHRHFPLTKSRARAI
hsPPARg    180 IRFGRMPQAEKEKLL-AEISS-D-IDQLNPESADLRALAKHLYDSYIKSFPLTKAKARAI

ssPPARa1a 224 LTGKTS-TP-----PFVIHDMETLQLAEQTLVAKM-----VGTAGSHLLEKEAEVR
ssPPARa1b 229 LTGKTS-TP-----PFVIHDMDTLQLAEQTLVAKM-----VGTAGSHLLEKEAEVR
hsPPARa    229 LSGKASNNP-----PFVIHDMETLQMAEKTIVAKL-----VANG---IQNKEAEVR
ssPPARb1a 198 LTGKTSPPVEYYPFVIHDMDSLQCAENGLVWKQ-----LINGT--TPNKEIGVH
ssPPARb1b 293 LTGKNSSSP-----PFVIHDMDSLHQAENGLVWNQ-----LINGT--PPNKEIGVH
ssPPARb2b 279 LTGKTSCTS-----TFVIHDVDTLWQAERGLVWNQ-----LVPGA--PLTKEIGVH
ssPPARb2a 281 LTGKTSCTS-----PFVIHDVDTLWQAESGLVWNQ-----LIPGA--PLTKEIGVH
hsPPARb    201 LTGKASHTA-----PFVIHDVDTLWQAERGLVWKQ-----LVNGL--PPYKEISVH
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hsPPARg    237 LTGKTTDKS-----PFVIYDMNSLMGEDIKFKH-----ITPLQ--EQSKEAIR
<-----
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hsPPARg    281 IFQGCQFRSVEAVQEI TEYAKSI PGFVNLNDQVTLKYGVEI IYTMLASLNMKDGVL

ssPPARa1a 329 VAYGSGFITREFLKS LRRPFS DMMEPKFQFAMKFNGLELDDSDLALFVAAI ICGDRPGL
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ssPPARb2a 385 VANGKGFVTRREFLRS LRPFS EIMEPKFEFAVKFNALELDDSDLALFVAI ILCGDRPGL
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-----
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ssPPARb2b 503 SEISLHPLLQEIYKDMY
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hsPPARb    425 TETSLHPLLQEIYKDMY
ssPPARg    527 LQMCLHPLLQEIIMRDLY
ssPPARgL   496 LEMCLHPLLREIMRDLY
hsPPARg    461 TDMSLHPLLQEIYKDL
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Figure 6-1 Alignment of deduced amino acid sequences of salmon and human PPARs. Ligand-binding domains are shown.

Four PPAR β genes and corresponding cDNAs were identified in Atlantic salmon. Within the PPAR β phylogeny based on full-length protein sequences (Figure 6-2), the four sequences were grouped in two branches containing two PPARs each. Each of these two branches was mostly closely attached to two PPARs present in Northern Pike. Thus, these salmon PPAR β forms have been provisionally denoted as ssPPAR β 1A, ssPPAR β 1B, ssPPAR β 2A and ssPPAR β 2B. It is also notable that although the zebrafish possess two PPAR β subtypes, they do not resolve in the same branches as the Atlantic salmon and Northern pike isoforms. Nevertheless, the two zebrafish PPAR β subtypes seemed to be more closely related to the Atlantic salmon PPAR β 1s than to the PPAR β 2s and PPAR β of plaice and sea bream.

Within the teleost group of PPAR α , zebrafish, sea bream and Northern pike seemed to possess two PPAR α lineages. These have been denoted as PPAR α 1 and PPAR α 2. Whilst Atlantic salmon also has two PPAR α genes, it appears that both of these belong to the PPAR α 1 subgroup as they resolve in the same branch as the Northern pike PPAR α 1, although their positions in the LBD phylogeny are not well supported (29%). Salmon do not seem to possess PPAR α 2 form present in many other teleost species.

The Northern pike PPAR γ was observed to possess two PPAR γ forms, which is in contrast to other teleost species examined so far, which only contain a single gene. Atlantic salmon also possess two PPAR γ genes with one form strongly clustered (100 %) with one of the pike forms and the other form strongly clustered (95 %) with the other PPAR γ form of pike and of other teleost species.

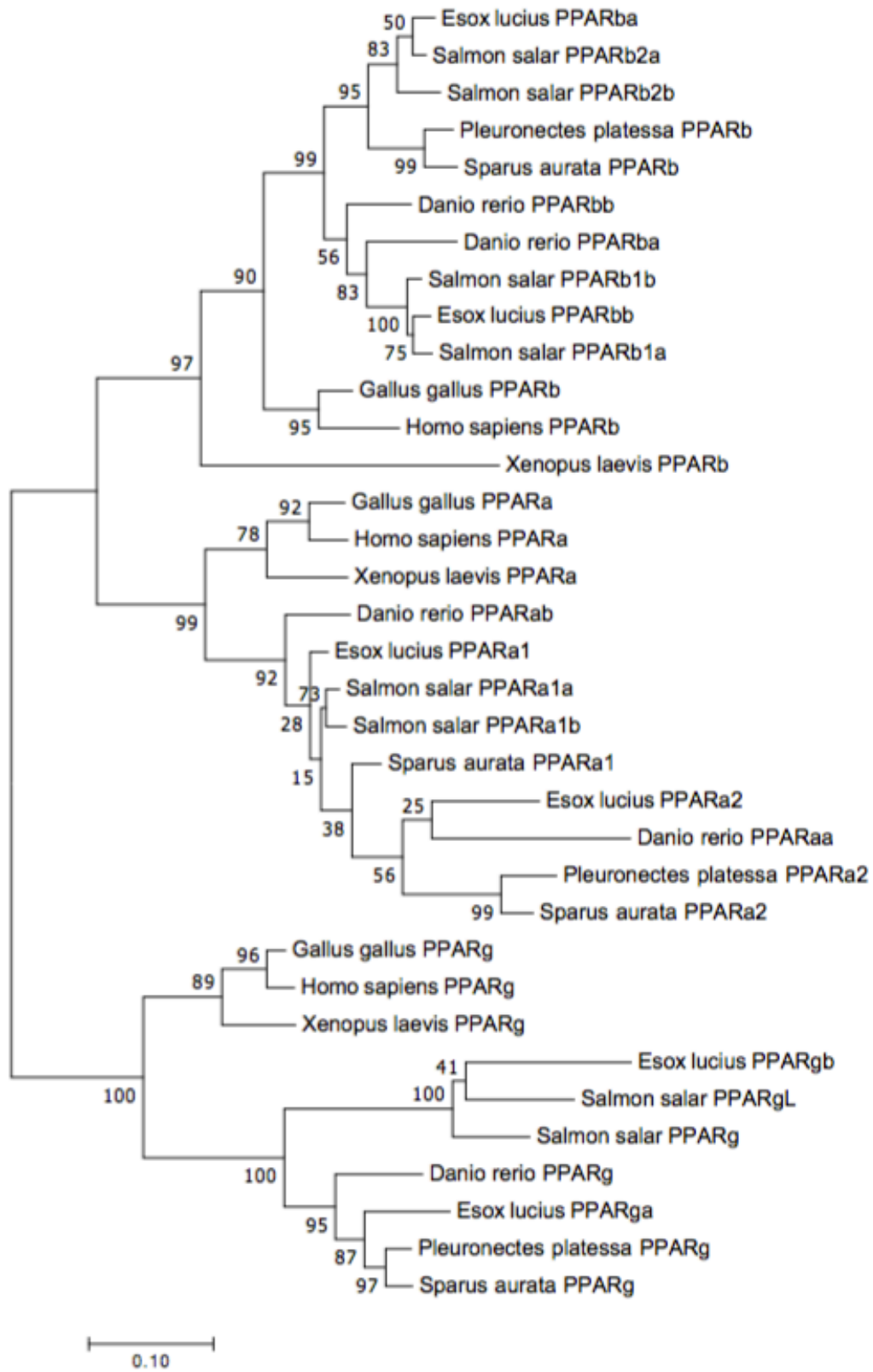


Figure 6-2 Phylogenetic tree for the full-length PPARs from diverse species, generated using MEGA 7 software using maximum likelihood method. Bootstrapping, using 1000 replicates, assessed the reliability of the tree with figures representing the bootstrap value in percentage (%). Receptor sequences used were: (1) *Gallus gallus* (chicken) PPARa (NP_001001464), PPARb (NP_990059), PPARg (NP_001001460); (2) *Homo sapiens* (human) PPARa (NP_005027), PPARb (NP_006229), PPARg (NP_005028); (3) *Xenopus laevis* (African clawed frog) PPARa (P37232), PPARb (NP_001081310), PPARg (XP_018095920) (4) *Esox Lucius* (Northern pike) PPARa1 (XP_010882200), PPARa2 (XP_010885449), PPARba (XP_010891645), PPARbb (XP_012993730), PPARga (XP_010872599), PPARgb (XP_010900626); (5) *Sparus aurata* (gilthead seabream) PPARa1 (not submitted to database), PPARa2 (AAT85613), PPARb (AAT85615), PPARg (AAT85618); (6) *Pleuronectes platessa* (European plaice) PPARa2 (CAD62447), PPARb (CAD62448), PPARg (CAB51618); (7) *Danio rerio* (zebrafish) PPARaa (NP_001154805), PPARab (NP_001096037), PPARba (XP_699900), PPARbb (NP_571543), PPARg (NP_571542); (8) *Salmon salar* (Atlantic salmon) PPARa1a (PPARa, NP_001117032), PPARa1b (PPARaL, XP_014025332), PPARb1a (Q1XE69_SALSA), PPARb1b (Q1XE68_SALSA), PPARb2a (NP_001117031), PPARb2b (XP_014002724), PPARg (XP_014000887), PPARgL (not submitted to database).

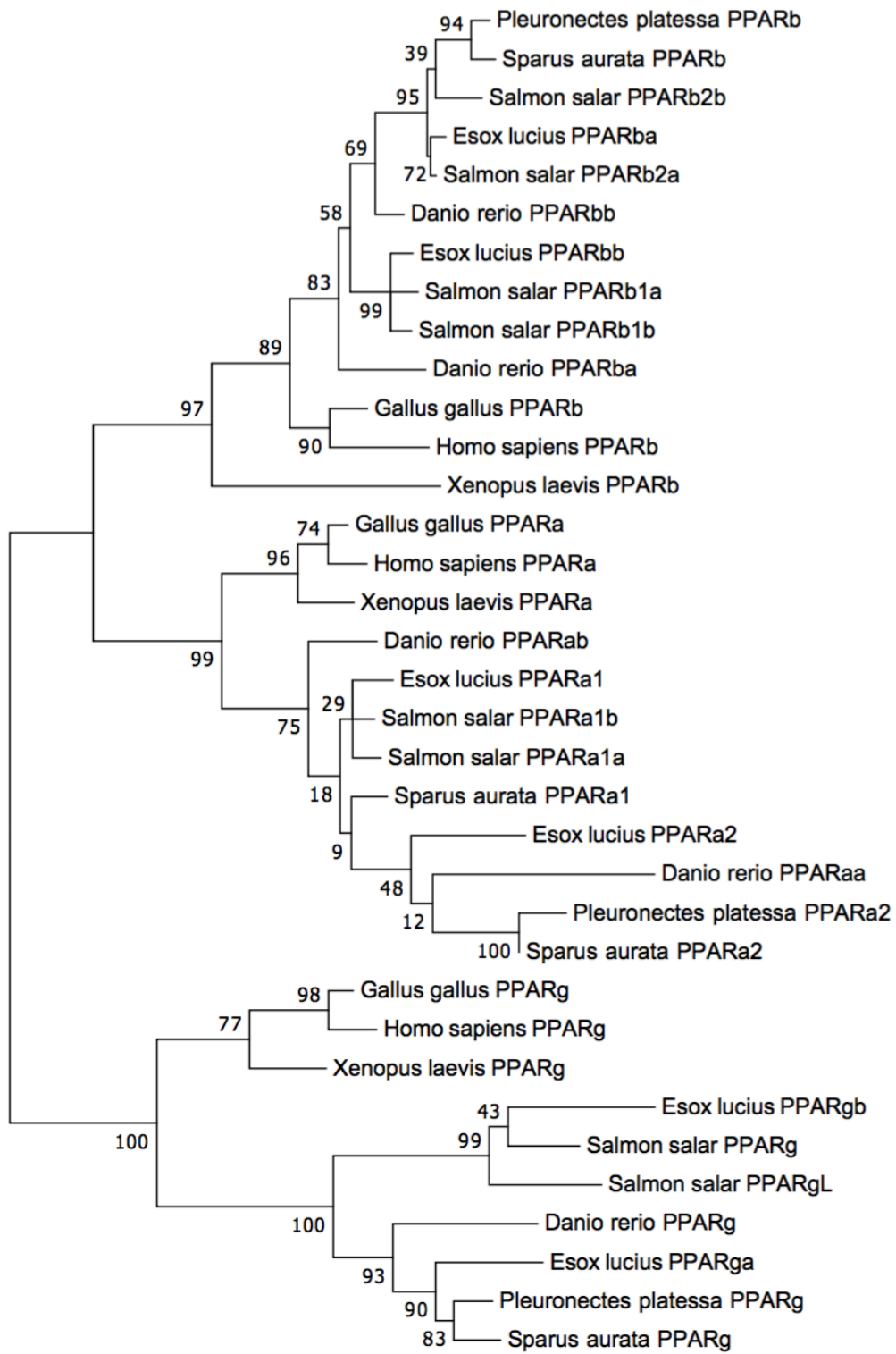


Figure 6-3 Phylogenetic tree for the ligand-binding domain (LBD) of PPARs from diverse species, generated using MEGA 7 software using maximum likelihood method. Bootstrapping, using 1000 replicates, assessed the reliability of the tree with figures representing the bootstrap value in percentage (%). Receptor sequences used were: (1) *Gallus gallus* (chicken) PPARa (NP_001001464), PPARb (NP_990059), PPARg (NP_001001460); (2) *Homo sapiens* (human) PPARa (NP_005027), PPARb (NP_006229), PPARg (NP_005028); (3) *Xenopus laevis* (African clawed frog) PPARa (P37232), PPARb (NP_001081310), PPARg (XP_018095920) (4) *Esox Lucius* (Northern pike) PPARa1 (XP_010882200), PPARa2 (XP_010885449), PPARba (XP_010891645), PPARbb (XP_012993730), PPARga (XP_010872599), PPARgb (XP_010900626); (5) *Sparus aurata* (gilthead seabream) PPARa1 (not submitted to database), PPARa2 (AAT85613), PPARb (AAT85615), PPARg (AAT85618); (6) *Pleuronectes platessa* (European plaice) PPARa2 (CAD62447), PPARb (CAD62448), PPARg (CAB51618); (7) *Danio rerio* (zebrafish) PPARaa (NP_001154805), PPARab (NP_001096037), PPARba (XP_699900), PPARbb (NP_571543), PPARg (NP_571542); (8) *Salmon salar* (Atlantic salmon) PPARa1a (PPARa, NP_001117032), PPARa1b (PPARaL, XP_014025332), PPARb1a (Q1XE69_SALSA), PPARb1b (Q1XE68_SALSA), PPARb2a (NP_001117031), PPARb2b (XP_014002724), PPARg (XP_014000887), PPARgL (not submitted to database).

6.3.3 Distribution patterns of Atlantic salmon PPARs

Quantitative PCR (qPCR) of salmon PPAR expression across a range of tissues indicated there were differences in relative expression levels (Figure 6-4, Figure 6-5, Figure 6-6, Figure 6-7, Figure 6-8). ssPPAR α and ssPPAR α L were most expressed in heart with 26- and 23-fold over muscle, respectively, whilst ssPPAR γ L was most expressed in heart and ovary and ssPPAR β 2B in ovary, with about 30-fold over muscle. ssPPAR γ was most highly expressed in most tissues, i.e. intestine, liver, kidney and gill with 311-fold, 538-fold, 107-fold and 234-fold over muscle expression, respectively and was least expressed in spleen with equal expression to muscle. In the ovary, the predominant isoform found was ssPPAR β 1B and this isoform has the significantly highest overall relative gene expression relative to muscle of more than 600-fold. The significantly second highest relative gene expression in the ovary observed was ssPPAR β 2A with gene expression of about 85-fold over muscle, whilst ssPPAR β 1A has the third highest relative gene expression in the ovary of about 41-fold over muscle. Lowest expression levels for the PPARs were recorded in spleen and brain.

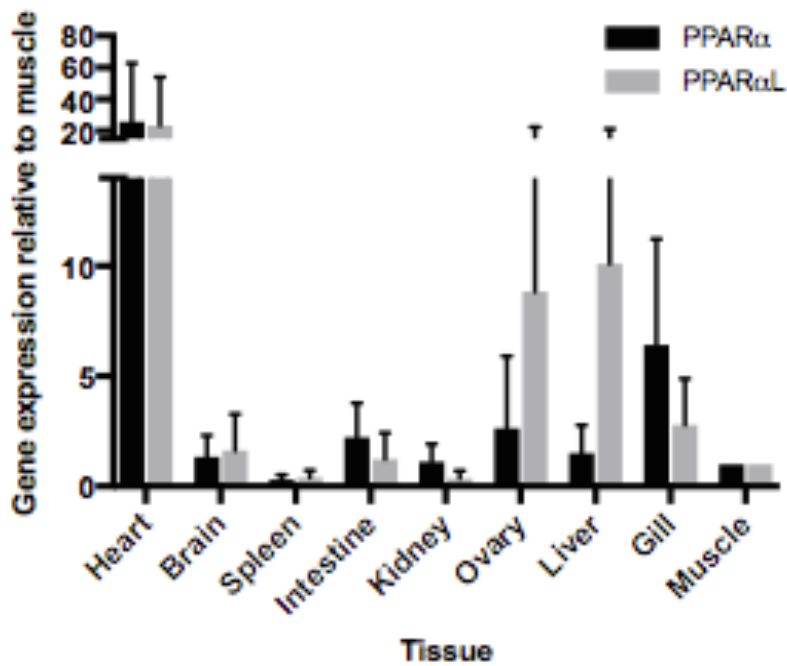


Figure 6-4 Tissue expression profile of female Atlantic salmon PPAR α isotypes. Relative expression of each PPAR was normalised to PPAR expression in muscle. Data are the means \pm SD of results from four individual salmon fish.

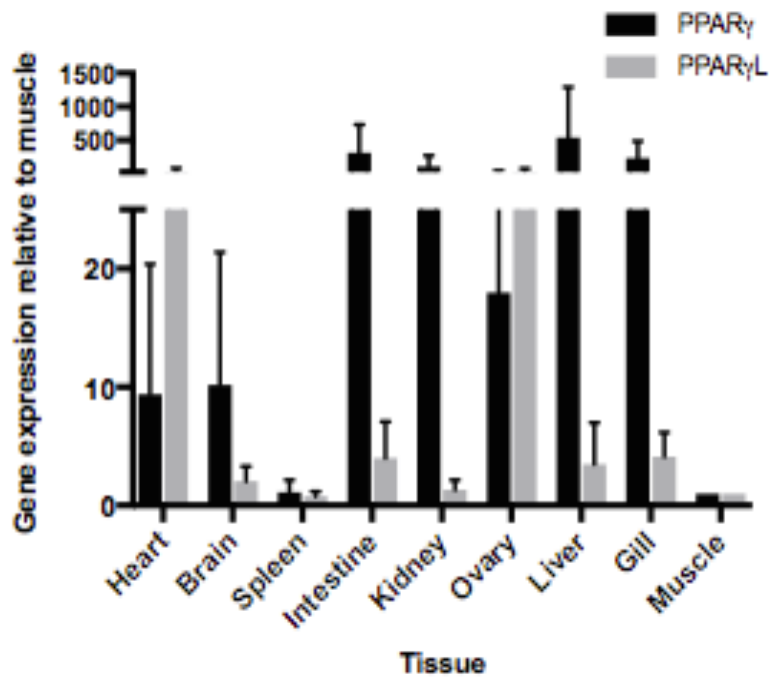


Figure 6-5 Tissue expression profile of female Atlantic salmon PPAR γ isotypes. Relative expression of each PPAR was normalised to PPAR expression in muscle. Data are the means \pm SD of results from four individual salmon fish.

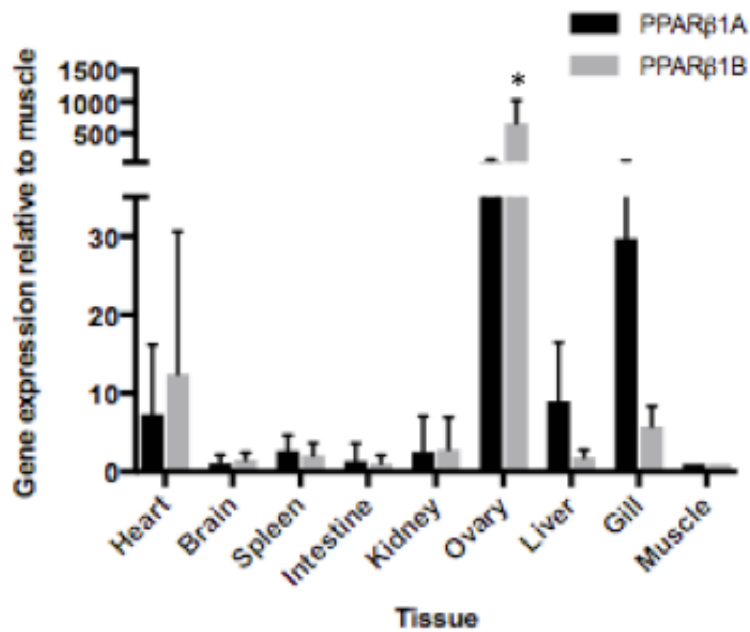


Figure 6-6 Tissue expression profile of female Atlantic salmon PPARβ1 isotypes. Relative expression of each PPAR was normalised to PPAR expression in muscle. Data are the means ± SD of results from four individual salmon fish. Asterisk (*) represents PPAR expression value, which differs significantly from muscle (P<0.05).

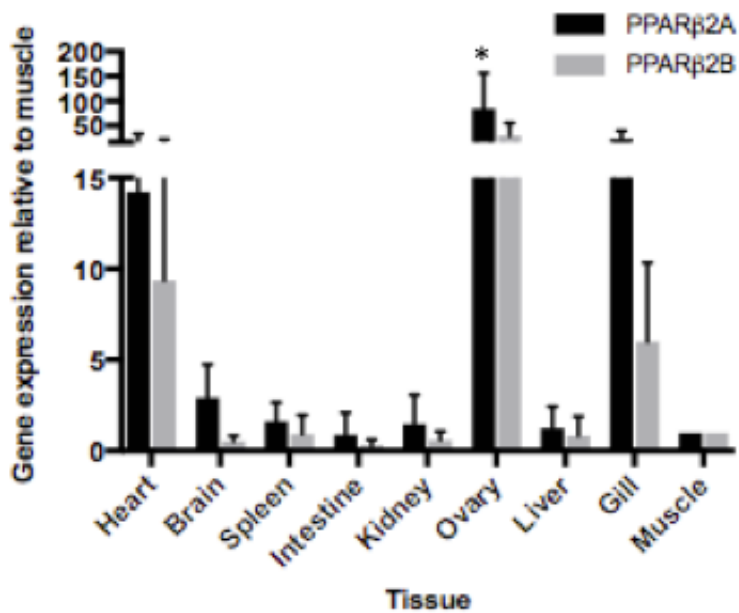


Figure 6-7 Tissue expression profile of female Atlantic salmon PPARβ2 isotypes. Relative expression of each PPAR was normalised to PPAR expression in muscle. Data are the means ± SD of results from four individual salmon fish. Asterisk (*) represents PPAR expression value, which differs significantly from muscle (P<0.05).

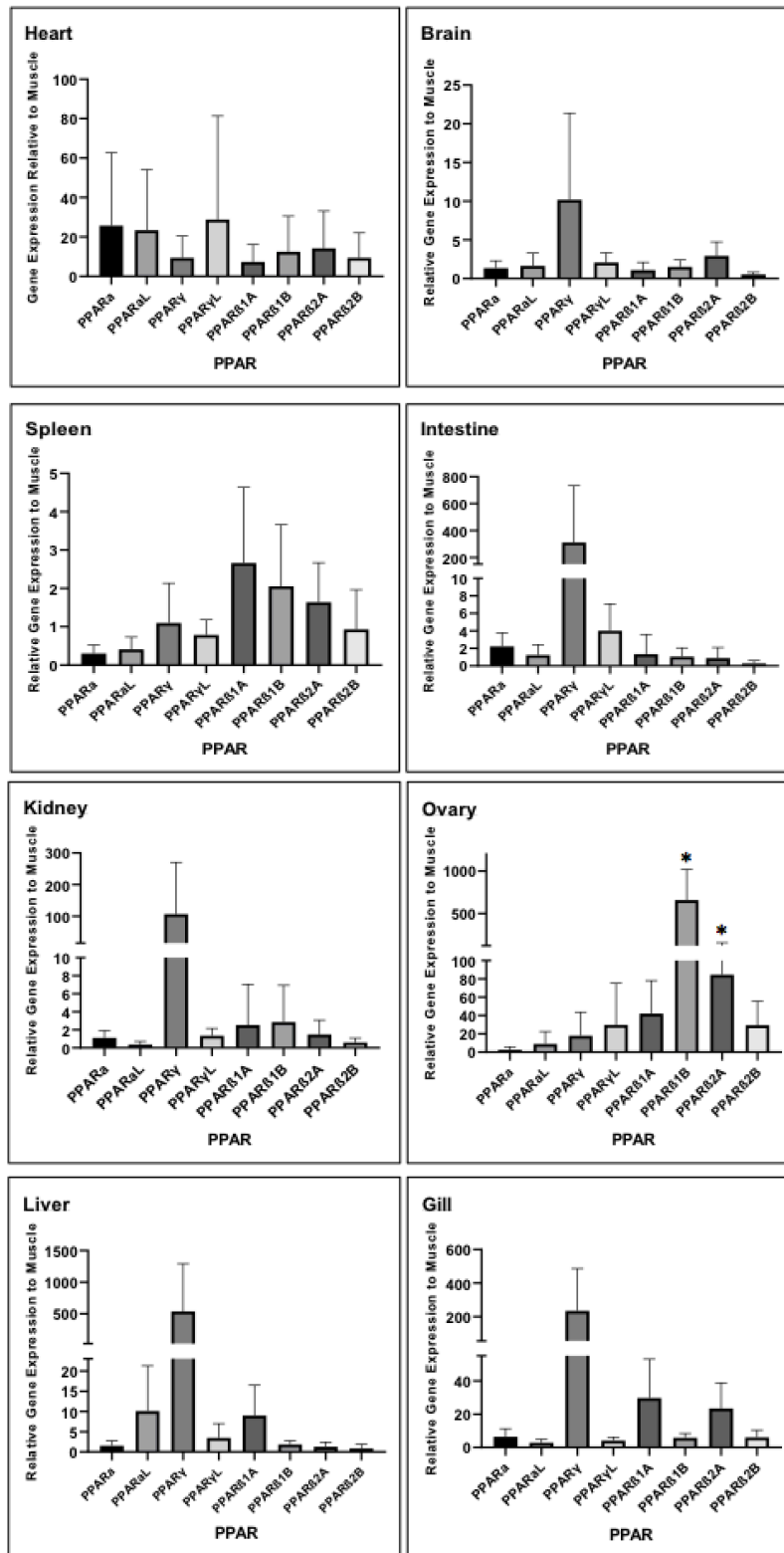


Figure 6-8 Gene expression of PPARs in each tissue where the data is normalised to muscle. Data are the means \pm SD of results from four individual salmon fish. Asterisk (*) represents PPAR expression value, which differs significantly from muscle ($P < 0.05$).

6.4 Discussion

PPARs have been previously identified and characterized in various tissues of several fish species including zebrafish (*Danio rerio*) (Escriva *et al.*, 1997; Ibabe *et al.*, 2005), Atlantic salmon (*Salmo salar*) (Ruyter *et al.*, 1997; Andersen *et al.*, 2000; Robinson-Rechavi *et al.*, 2001), plaice (*Pleuronectes platessa*) (Leaver *et al.*, 1998, 2005), gilthead sea bream (Leaver *et al.*, 2005), liver of gray mullet (*Mugil cephalus*) (Ibabe *et al.*, 2004), sea bass (*Dicentrarchus labrax*) (Boukouvala *et al.*, 2004), turbot (*Scophthalmus maximus*) (Robinson-Rechavi *et al.*, 2001), Japanese pufferfish (*Fugu rubripes*) (Maglich *et al.*, 2003), brown trout (*Salmo trutta* f. *fario*) (Batista-Pinto *et al.*, 2005, 2009), thicklip grey mullet (*Chelon labrosus*) (Raingeard *et al.*, 2009) and red sea bream (*Pagrus major*) (Oku and Umino, 2008). Although PPARs are present in all fish species, their expression levels vary in different tissues and it is assumed that the expression patterns of PPARs reflect their different physiological functions.

PPAR sequences have been previously applied to model the functional role of genome duplications (Escrivá García *et al.*, 2003), and because these receptors are dispersed in the genome, they are strong phylogenetic markers (Laudet *et al.*, 1992) due to the highly conserved DNA- and ligand-binding domains consisting of large numbers of amino-acid sites that can be compared, to allow robust phylogenetic construction (Escrivá García, Laudet and Robinson-Rechavi, 2003).

Because gene and genome duplication are believed to have become the driving force in shaping the evolution of organisms (Ohno, 1970), the in-

depth study of gene families can be illuminating in linking duplications to functional adaptations. Therefore, in this study, the full receptors and the ligand-binding domains of PPARs of various vertebrates were used to construct phylogenetic trees, with the aim of determining the origin of Atlantic salmon PPARs, to identify genetic divergence of these receptors and possibly infer functional adaptations. The availability of genome information of fish such as Northern pike, zebrafish, plaice, sea bream and other vertebrates including human, chicken and frog, has shown that similar isoforms clustered together which is consistent with studies reporting that fish PPARs share high degrees of sequence similarities to their higher vertebrates counterparts. For example, Atlantic salmon PPAR has shown to share 44-49 % overall sequence identity with PPAR α and PPAR β of higher vertebrates, and 56 and 47 % identity with PPAR γ of human and frog, respectively (Ruyter *et al.*, 1997). Moreover, PPARs of sea bream and plaice also share the sequence identity of more than 70 % in the DNA-binding and ligand-binding domains of their higher vertebrates counterparts (Leaver *et al.*, 2005). In addition, sea bass shares more than 90 % PPAR sequence identity to their human counterparts in the DNA-binding domain and that 67, 78 and 66% common residues in the ligand-binding domain of the α , β and γ isoforms (Boukouvala *et al.*, 2004).

The PPAR phylogeny supports the teleost fish as a separate group within the vertebrates, with very high support, and also indicates the divergence of PPARs from an ancestral gene before the evolutionary divergence of fish and higher vertebrates about 500 million years ago (Laudet *et al.*, 1992; Taylor *et al.*, 2003).

The presence and phylogenetic pattern of four PPAR β genes in Atlantic salmon supports the theory that salmonids may be derived from a relatively recent autotetraploidization event (Allendorf and Thorgaard, 1984; Alexandrou *et al.*, 2013) and that a previous genome duplication may have occurred in early evolution of ray-finned fishes (Taylor *et al.*, 2003). In the phylogeny trees, Northern pike PPAR β b formed a group with ssPPAR β 1A and ssPPAR β 1B with very high support, and similarly, the other β form of Northern pike, PPAR β a, is more closely related to ssPPAR β 2A and ssPPAR β 2B. The four PPAR β genes grouped into two subfamilies as a result of duplication of two PPAR β subtypes from an ancestral diploid salmonid, with high support in both phylogeny trees. This has previously been noted and was suggested to have enabled some functional divergence (Leaver *et al.*, 2007). However, the tissue expression results for salmon PPAR β forms argues against a major functional difference as it indicates a broadly similar pattern amongst all four forms, with the exception of heart, where the PPAR β 2 forms are relatively higher than PPAR β 1 expression. These results differ from a previous study, which concluded that ssPPAR β 1A and ssPPAR β 2A have distinct tissue expression profiles, based on differences in expression liver and gill (Leaver *et al.*, 2007). It should be noted that these differences are also evident in the results presented here, but across the broader range of tissues and PPAR β subtypes in this study, it is clear that there is more similarity than difference in overall PPAR β expression profile.

However, the origin of the salmon PPAR β genes, consistent with genome duplication hypotheses, may not be the case for Atlantic salmon PPAR α and PPAR γ . Many widely diverged fish species possess two forms of PPAR α ,

apparently diverging early and subsequently conserved sufficiently to demonstrate a strong phylogenetic signal. Although Atlantic salmon possesses two PPAR α genes, they are clearly the result of a salmonid-specific duplication of only one of the piscine forms, denoted PPAR α 1. Since the Northern pike, possess the two piscine PPAR α forms, it would appear that salmonids have lost the second gene following their emergence and more recent salmonid-specific genome duplication event. Despite this recent PPAR α 1 duplication in salmonids, the two Atlantic salmon genes show some differences in tissue expression pattern, with PPAR α L being relatively more highly expressed in ovary and liver than PPAR α . This could represent some sub-functionalization at least at the level of gene regulation. PPAR γ of the Atlantic salmon shows similar pattern to PPAR α , with duplication of one ancestral gene clearly represented in Northern pike. However, it is notable that, unlike any other fish, Northern pike possess two distinct PPAR γ genes, only one which is present as a duplicate in Atlantic salmon. The Atlantic salmon have presumably lost the other PPAR γ gene, but notably the gene retained and duplicated in the same lineage as the novel PPAR γ , one also existing in the Northern pike. The two salmon PPAR γ forms have quite distinct tissue expression patterns. ssPPAR γ L had a tendency for increased expressions in the heart and ovary and its expression in other tissues were relatively very low. On the other hand, ssPPAR γ had very high expression levels in most tissues including liver, intestine, gills, kidney and ovary. Thus, salmon PPAR γ is highly unusual and it is not known whether the unique PPAR γ represented in the Atlantic salmon and the Northern pike is functionally different from other PPAR γ , or indeed whether the duplicates of

this gene in salmon have functionally diverged by their distinct expression patterns.

Clearly PPAR biology is complex in vertebrates, and particularly complex in Atlantic salmon and probably salmonids generally. Salmonids appear to have gained genes for PPAR β and PPAR γ and both gained and have lost a pan-vertebrate PPAR α subtype, but duplicated a teleost specific PPAR α . Whilst it is not possible at the moment to definitively conclude that this has functional consequences for lipid and energy homeostasis in salmonids, there is some evidence here that PPAR α and PPAR γ duplicates in Atlantic salmon have diverged in terms of expression profile. It should be noted that tissue expression was only measured in pre-smolts in freshwater and a full analysis of tissue expression would require measurement at various development stages and life history transitions in salmon. Functionally the consequences of duplicated PPAR genes in salmon must await the discovery of gene-specific activation compounds, or genetic knock-out/knock-in studies, which would enable the genes these proteins regulate to be identified in a tissue, stage and nutrition-specific manner.

6.5 Conclusion

Several fish species from which PPARs have been isolated have shown to differ from other vertebrates in terms of the number functional PPARs they contain. Phylogenetic analyses deduced that sequences of Atlantic salmon PPARs corresponding to that of other fish species and higher vertebrates reveals that the Atlantic salmon PPARs are more similar to those of other fish species than to mammalian, bird or amphibian species, as expected. From the phylogenetic evidence, whilst the four PPAR β s in Atlantic salmon may be the result of autotetraploidization in the salmonid lineage, to the distribution of duplicated PPAR α and PPAR γ genes cannot be simply explained by this genome duplication model. Tissue expression evidence suggests that duplicated salmon PPAR α and PPAR γ have functionally diverged to a greater extent than the PPAR β forms, and the potential identification of ligands for the receptors will enable the physiological relevance of this divergence to be investigated. Given the unique complement of PPAR genes in Atlantic salmon and their presumed roles in lipid homeostasis, it is interesting to understand whether this extends to a unique energy and lipid metabolism in this species, especially in an aquaculture context.

7 General discussion and conclusions

7.1 Introduction

Studies have proven that mammals and fish share the same pathways for biosynthesis and catabolism of fatty acids and these pathways are controlled by similar molecular mechanisms (Ruyter *et al.*, 1997; Boukouvala *et al.*, 2004). In mammals, PPARs have emerged as central factors in sensing fatty acid levels and in regulating fatty acid metabolism. Evidently, the PPARs in fish, generally, are structural homologs of the mammalian PPARs, therefore assumed to carry out similar functions. Thus, there is a reasonable evidence that PPARs play critical roles in fish lipid homeostasis and because PPAR α and PPAR β have similar ligand activation profiles and tissue expression patterns as their mammalian counterparts (Leaver *et al.*, 2005), these receptors may have similar functions to those described in mammals. However, because studies have reported that compounds that activate mammalian PPAR γ do not activate piscine PPAR γ (Maglich *et al.*, 2003; Leaver *et al.*, 2005), the role of PPAR γ in fish still remains unclear. Piscine PPAR γ has some specific structural differences to mammalian proteins, particularly evident with regard to amino acids, which are known to be critical for ligand binding in mammalian receptors (Ruyter *et al.*, 1997; Andersen *et al.*, 2000; Leaver *et al.*, 2005). Despite this, the overall structural similarity of piscine PPAR γ to all other PPARs strongly indicates that it is likely to be activated by a compound related to the lipids and fatty acids that activate mammalian PPAR γ . For this reason, the critical role of mammalian PPAR γ in determining lipid uptake and storage has led to this particular

study with the aim of characterising PPAR distribution in fish and identifying the activating ligands of piscine PPAR γ .

7.2 Suitable cell line for cellular transactivation assay

A cell-based reporter gene assay system was developed and used to study the potential activating ligands of PPAR γ in fish. To achieve this, PPAR γ was cloned from European plaice and was used to develop a receptor plasmid construct. The ligand-binding domain of PPAR γ was ligated downstream of the DNA-binding domain of yeast Gal4 to produce Gal4-PPAR γ LBD. The cationic polymer, branched polyethylenimine (bPEI), was successfully applied as a transfection reagent at an optimal of double the amount of bPEI to DNA ratio 2.5:1 (2x 25:1) for CHSE-214 cell line. Notably, the AS cell line was optimally transfected at a ratio of 2.5:1 as well but at 1 x concentration.

Between the Atlantic salmon (AS) and Chinook salmon embryo 214 (CHSE-214) cell lines tested, the AS cell line was initially concluded suitable for future transfections, however, this cell line failed to show reproducibility and reliability in the transfection experiments, indicated by significant decrease in firefly luciferase activity, which may have been due to undesirable differentiation in the cells at higher passage numbers, resulting changes in characteristics and modified growth rates, affecting protein expression and causing cells to respond differently to the same transfection conditions. For these reasons, therefore, the present study utilised the CHSE-214 cell line, as its cells are fast growing, easy to subculture and maintain and it has also been widely used in other studies (Jensen *et al.*, 2002; Jørgensen *et al.*, 2007; Herath *et al.*, 2009). It was also ensured that transfection experiments were

consistently performed within a minimum range of passage number following the optimization experiment, to prevent any passage-related effects from influencing the transfections, thus, maintaining consistent cell performance. While mammalian studies have shown that certain cell lines are particularly useful for particular PPAR studies, the availability of fish cells is much more restricted. In this study, it was assumed that a salmonid cell line would be preferable to one from another group, because the presence of compatible co-activators/repressors would be more likely. However, it would be potentially useful to screen a range of available salmonid and other cell lines for optimizing the transfection assays.

7.3 Lipid extraction from liver tissues

Salmon liver tissue was selected for total lipid extraction as it contains all the important lipid classes (Christie and Han, 2010) and because of its central importance in lipid metabolism (De Silva and Anderson, 1995), and also most importantly because PPARs are highly expressed in this tissue of salmon and other fish (Boukouvala *et al.*, 2004; Ibabe *et al.*, 2004; Ibabe *et al.*, 2005; Leaver *et al.*, 2005).

To avoid lipid degradation, as observed by the high free fatty acid content of extracts stored frozen, liver tissues samples were immediately transferred into chloroform/methanol solvent (2:1) after removal to allow maximum extraction of lipids present in the tissues with minimum breakdown of lipids by hydrolysis. This method yielded very low levels of free fatty acids in comparison to the initial procedure above. Also, this method facilitated separation of various lipid classes, including the unknown polar and

unknown neutral lipids, in addition to the common polar and neutral lipid classes. While this method is applicable for maximum extraction of lipids, and when rapid extraction is not feasible, tissue samples can also be frozen as rapidly as possible with dry ice or liquid nitrogen, stored in sealed glass containers at -20 °C in an atmosphere of nitrogen (Natio and David, 1984). The samples should then be homogenized and extracted with solvent at the lowest temperature practicable, without being allowed to thaw to avoid any breakdown of lipids by active enzymes. Storing tissue samples in bags, vials or other plastic containers should also be avoided as plasticizers will leach out and contaminate extracts.

7.4 Piscine PPAR γ and its potential ligand

In the optimisation experiments, Gal4-PPAR α was used as a positive control, tested in cellular transactivation in response to its known agonists in fish to ensure effectiveness of transfection using bPEI and the efficiency of the dual-luciferase assay system using firefly luciferase and *Renilla* luciferase reagents. From the previous studies and from this study, because the full-length piscine PPAR γ and the Gal4-PPAR γ had been found to be unresponsive to fatty acids (Leaver *et al.*, 2005; Kondo *et al.*, 2007; Colliar *et al.*, 2011), PPAR γ has become a subject of interest, thus, the Gal4-PPAR γ construct was developed to identify its potential activating ligands.

Polar and neutral lipids fractionated from the total lipid extracted from salmon liver using thin-layer chromatography (TLC), were tested in cellular transactivation assay. None of the neutral lipid fractions induced PPAR γ activity while two polar lipid fractions, containing unknown polar

compounds and unresolved compounds present at the solvent front were able to significantly increase PPAR γ activity at 50 $\mu\text{g}/\text{ml}$, suggesting that these lipid fractions contain molecular components or metabolites that have the ability to bind with PPAR γ ligand-binding domain and activate transcription of firefly luciferase activity. It was deduced by LC-MS that these lipid fractions mainly contain hexosylceramides and ceramides. According to Christie and Han (2010), the nature of fatty acids and long-chain bases in ceramides are commonly saturated and monoenoic (monosaturated) and this agreed with the high amounts of saturated fatty acids (mainly palmitate, 16:0) in the unknown polar lipid fatty acid composition and the high amounts of monosaturated fatty acids (mainly oleic acid, 18:1n-9) in the solvent front fraction. These fatty acids have been tested on piscine PPAR γ but none of them were able to activate the receptor (Kondo *et al.*, 2007; Colliar *et al.*, 2011). These findings suggested that the individual fatty acids within the lipid fractions are not ligands or activators of piscine PPAR γ due to their inability to activate the receptor, but it was the intact lipid structure containing esterified fatty acids that constitute the activating compound. These assays do not indicate whether such compounds bind with the receptor and activate transcription of the firefly luciferase. It is possible that they give rise, directly metabolism, or indirectly by the stimulation of endogenous ligand formation, to other true ligands.

When Gal4-PPAR γ transfected CHSE-214 cells were treated with ceramide, PPAR γ activity was suppressed in a dose dependent manner. Precursors of ceramide which have been reported to activate mammalian PPAR γ , sphingosine, S1P and C1P also suppressed luciferase output in Gal4-

transfected cells. It is not clear whether this suppression is physiologically relevant, or simply represents increasing cellular toxicity in the assay at higher treatment levels.

While none of the sphingolipids tested above has the ability to activate PPAR γ in fish, treatment of Gal4-PPAR γ transfected cells with glucosylceramide (GlcCer), a major component of one of the activating lipid fractions, resulted in significant increase in luciferase activities. Glucosylceramides are glucosylated lipids with simple structures and functions critical for cellular homeostasis and cellular activities (Ishibashi *et al.*, 2013). Glucosylceramide synthase (GlcT-1) is the enzyme responsible for the production of GlcCer from ceramide. Interestingly, insulin activity and adipocyte profile has been shown to be improved in obese mice by the inhibition of GlcT-1 (van Eijk *et al.*, 2009). Moreover, dietary GlcCer has been shown to dramatically improve certain skin conditions in humans, and there is evidence that this effect is mediated by GlcCer metabolites through activation of PPAR γ (Shirakura *et al.*, 2012). As GlcCer did not have direct effect on PPAR γ in this human study, it is possible that the effects observed with GlcCer on piscine PPAR γ transactivation might also be mediated by GlcCer metabolites produced in the CHSE-214 cells, or which are present at low levels in the extracts. Future studies should focus on testing these metabolites if they can be obtained in pure form.

7.5 Phylogeny and tissue expression patterns of Atlantic salmon PPARs

From the phylogenetic evidence, it is clear that PPARs in vertebrates have diverged into three forms (α , β and γ) before the evolutionary divergence of fish and higher vertebrates about 500 million years ago (Laudet *et al.*, 1992; Taylor *et al.*, 2003).

In this study, the phylogeny of Atlantic salmon PPARs was examined, because salmon is an important aquaculture species and because they possess at least 8 intact PPAR genes, possibly due to the fact that they have a recently duplicated genome. It was clear that salmon have two PPAR α genes, four PPAR β genes, and two PPAR γ genes. The four PPAR β genes are clearly the result of the salmonid genome duplication, forming highly supported groupings with the two forms from Northern pike, the nearest relative of salmon with an unduplicated genome. However, it was observed that the two PPAR α and PPAR γ forms in salmon are not the direct result of salmonid genome duplication. Northern pike clearly have two PPAR γ forms as well, which form clear groups with the salmon forms, indicating that the gene duplication event, which generated these two PPARs occurred before the salmon whole genome duplication. In all other fish examined so far, only one PPAR γ form is present. Whilst other fish species, including pike possess two forms of PPAR α , when compared again to pike, the Atlantic salmon appears to have duplicated only one form of PPAR α , and appears to have lost the other completely.

Tissue expression analysis revealed that the four β -subtypes in Atlantic salmon generally have high expression levels in heart, ovary and gill, and overall the tissue expression profile of all four isotypes is similar. Similarly, both duplicated genes of PPAR α in the Atlantic salmon exhibited a similar expression pattern, with PPAR α L displaying a tendency for slightly higher expression in liver. However, the two PPAR γ genes were clearly differently expressed across tissues, with the novel PPAR γ L form showing higher expression than PPAR γ in heart and ovary. In all other tissues, PPAR γ was the more highly expressed gene. Given the relatively early duplication of the gene in the Northern pike lineage, the conservation of both genes in salmon, and these distinct expression differences, this suggested that there may be some functional divergence with regard to function. It is notable that many salmonids have an unusual life history, with early life spent in nutrient-poor freshwaters where growth can be slow, and later life spent at sea with much faster growth. This period is then followed by return to freshwater to spawn, during which time gonads mature and fish cease to eat, existing entirely on lipid and protein reserves. These switches in lifestyle and extreme changes in metabolism may have evolved in tandem with the enabling molecular regulatory mechanisms and could explain the retention and divergence of PPAR duplicate in salmonids.

However, in order to gain a further understanding of the different tissue expressions of PPARs, functional characterization of each of these subtypes is required to further determine if activating compounds differ, and also to determine if the expression differs in adipose tissue, which was not identifiable in the young fish sampled for this experiment.

7.6 Conclusions

In summary, the results of the present study indicated that:

- a) CHSE-214 cell line has proven to be more reliable and robust compared to the AS cell line and the use of bPEI as transfection reagent has proven to successfully transfect fish cell lines in this study.
- b) The CHSE-214 cell line, and Gal4-PPAR luciferase transactivation system is suitable for the screening of potential activating compounds as well as cell extracts.
- c) Screening of cell lipid extracts demonstrated that it was possible to identify fractions with piscine PPAR γ -induce activity and LC-MS confirmed that the predominant lipids present in these fractions were ceramides and glucosylceramides.
- d) Of all the ceramide precursors tested in the cellular transactivation assay, glucosylceramide increased transcriptional activity suggesting that this lipid molecule, or its metabolites could be a biologically relevant endogenous activator of piscine PPAR γ .
- e) In the important aquaculture species, Atlantic salmon, there are two PPAR γ genes, which have both been conserved since their duplication before the salmonid whole genome duplication. These PPAR γ forms have different tissue distributions, which suggest some functional divergence that may be some relevance given the importance of dietary lipid quality and quantity and the issue of excessive adiposity in farmed fish.

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