THE INTERACTION OF ENVIRONMENTALLY RELEVANT POLLUTANTS WITH NUCLEAR HORMONE RECEPTORS OF EUROPEAN FLOUNDER (*Platichthys flesus)*

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

by
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Declaration

I hereby declare that this thesis has been composed entirely by myself and has not been submitted for any other degree or qualification. All information from other sources has been duly acknowledged.

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Abstract

Nuclear hormone receptors (NHRs) are ligand-activated transcriptions factors which transduce the effects of various hormones as well as nutritional and other environmental signals. They thus function to maintain physiological homeostasis by integrating the tissue expression of specific target genes to regulate a wealth of biological processes including reproduction, development, metabolism and environmental adaptation. Mounting evidence indicates NHRs are the target of endocrine disrupting compounds (EDCs), exogenous chemicals, often of anthropogenic origin, which disrupt NHRs and thus the processes under their control. EDCs can interfere with NHR signalling by activating receptors (agonists), by inhibiting the actions of the receptor (antagonists), or by disrupting endogenous hormone synthesis, secretion, transport or metabolism. Much of the focus to date has been on the risk of EDCs to reproductive functions, via estrogen and androgen NHRs in humans, and also in aquatic organisms. However environmental pollutants also have the potential to interact with other NHRs, particularly in aquatic environments, and cause dysregulation of other critical physiological processes, including energy homeostasis, immune functions and the stress response.

To address this possibility a reporter gene assay was developed, allowing the high-throughput screening of pollutants for their interactions with piscine NHRs with critical roles in energy homeostasis, stress reponse and immune functions, namely the peroxisome proliferator-activated receptors (PPARs) and corticosteroid receptors (CRs) from European plaice (*Pleuronectes platessa*) and European flounder (*Platichthys flesus*), respectively. Complementary DNA (cDNA) sequences encoding the ligand-binding domains of PPARs and CRs, critical for receptor-ligand

interactions and receptor activation, were ligated to the DNA-binding domain (DBD) of the yeast Gal4 transcription activator protein to create experimental expression plasmid constructs. Co-transfection of these expression plasmids into the fathead minnow (FHM) cell line with an upstream-activating sequence (UAS)-firefly luciferase reporter gene plasmid increased luciferase expression in the presence of known PPAR and CR ligands. Several aquatic pollutants including pharmaceuticals, industrial by-products and biocides were tested for their potential to disrupt PPAR and CR functions by interacting with these receptors in an agonistic or antagonistic manner.

Several fibrates, a group of pharmaceutical compounds used to treat dyslipidemia in humans by targeting the PPARs, were able to activate plaice Gal4-PPAR and Gal4-PPAR in the reporter gene assay, indicative of an interaction with PPAR receptors in non-target species. Fibrates which did not activate Gal4-PPAR were able to inhibit the activation of Gal4-PPAR by the PPAR -specific agonist, Wy14643, suggesting differential effects of fibrates on human and flounder PPARs. In addition some metabolites of widespread phthalate ester pollutants were also agonists of the Gal4-PPAR and Gal4-PPAR constructs. The Gal4-PPAR construct was unresponsive to almost all the compounds tested, including the mammalian PPAR agonist, rosiglitazone. The exception to this was the phthalate metabolite monobenzylphthalate, which induced a small increase in firefly luciferase in Gal4-PPAR transfected cells. All of the above effects required concentrations of at least 10 µM, which are unlikely to be encountered in the aquatic environment. In contrast bis(tributyltin) oxide (TBTO), a notorious environmental pollutant, inhibited Gal4-PPAR and Gal4-CR constructs at concentrations as low as 1 nM and 100 nM,

respectively. These concentrations are lower than those reported in aquatic environments, or in fish tissues, making TBTO a candidate endocrine disruptor in fish by inhibiting PPAR and CR signalling. A European flounder cDNA microarray was used to investigate the trasnscriptional responses of flounder hepatocytes to TBTO (10 nM) exposure.

Exposure to TBTO and Wy14643, both alone and in combination, indicated a TBTO-driven downregulation of several potential PPAR -target genes with functions in the immune system, the proteasome, and lipid metabolism, although, based on mammalian comparisons, some potential PPAR -target genes were also upregulated, indicating differences in mammalian and fish PPAR-target genes or reflecting the complexity of organisms at a higher organisational level than cell-based assay systems. However, the microarray-based approach was useful in formulating further hypotheses about the effects of TBTO on PPAR signalling.

Overall, these results indicate that exogenous chemicals entering the aquatic environment can interfere with NHRs with functions in energy homeostasis, immune functions and stress, in non-target organisms. The cell-based reporter gene assay is a useful tool for identifying potential endocrine disruptors which target PPARs and CRs and would be a useful method in a first tier testing approach, limiting the use of live animal models and enabling investigation into specific receptors which are targets of endocrine disrupting compounds. Although more work is required to confirm the physiological consequences of TBTO inhibition of PPAR , the results presented here indicate that organisms inhabiting TBTO-polluted environments may experience suppression of the immune system, an increase in non-functional or

misfolded proteins through suppression of genes involved in the ubiquitin/proteasome system and a disruption in lipid homeostasis.

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Abbreviations and Acronyms

Below is a list of the most commonly used abbreviations in the text. Other abbreviated terms are explained in the text.

ACO	Acyl-CoA Oxidase
AF	Activation Function
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary deoxyribonucleic acid
CR	Corticosteroid Receptor
DBD	DNA-binding domain
Dex	Dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
dNTP	Deoxyribonucleotide triphosphate
DPBS	Dulbecco’s phosphate buffered saline
ED	Endocrine Disruptor
EtOH	Ethanol
FBS	Fetal Bovine Serum
FHM	Fathead Minnow
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
HPG	Hypothalamus-Pituitary-Gonad
HPI	Hypothalamus-Pituitary-Interrenal
HRE	Hormone Response Element
L-15	Leibovitz-15 Medium

LBD	Ligand-binding domain
LXR	Liver X Receptor
MR	Mineralocorticoid Receptor
NF B	Nuclear Factor kappa B
NHR	Nuclear Hormone Receptor
PCR	Polymerase Chain Reaction
PPAR	Peroxisome Proliferator-Activated Receptor
PPRE	Peroxisome Proliferator-Response Element
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
rt-QPCR	real-time Quantitative Polymerase Chain Reaction
RXR	Retinoid X Receptor
TBTO	Bis(tributyl tin)oxide
TZD	Thiazolidinedione

Chapter 1. General Introduction

Since the early 19th century a dramatic increase in production levels of organic and inorganic compounds has occurred and by the 21st century production levels of synthetic organic chemicals in the USA alone exceeded 140 billion kg/annum (Baillie Hamilton, 2002). With such high production levels, it is inevitable that a fraction of these compounds are released into the environment, and ultimately enter aquatic ecosystems, and therefore concern has grown over the potential for these compounds to cause toxic effects in aquatic species. Over the last ten to fifteen years it has further become apparent that large numbers of these man-made chemicals can interact with the endocrine systems of aquatic organisms by specifically interacting with various endogenous hormone signalling pathways. This diverse group of chemicals has become known as endocrine disruptors and many different endocrine-disrupting compounds, particularly small lipophilic contaminants, are able to exert effects via interactions with cellular receptors for endogenous hormones. One of the largest and most studies classes of these cellular receptors are the nuclear hormone receptors.

1.1.1 Nuclear Hormone Receptors (NHRs)

The nuclear hormone receptors (NHRs) are a superfamily of transcription factors, regulating the expression of genes involved in key physiological processes such as cell growth, cell differentiation, development, reproduction, metabolism and homeostasis (Francis *et al*, 2003). To date 48 NHRs have been identified in humans, including those which are activated by thyroid hormones (thyroid receptor), sex steroid hormones e.g. testosterone (androgen receptors) and estradiol (estrogen

receptors), fatty acids (PPARs), vitamin D3 (vitamin D receptor), cortisol (glucocorticoid receptor), aldosterone (mineralocorticoid receptor) and retinoic acid (retinoic acid receptor) (le Maire *et al*, 2010). Some nuclear receptors remain as 'orphan' receptors, so-called because endogenous ligands have not yet been identified.

Nuclear hormone receptors are classified based on their sequence similarities and their positions in a phylogenetic tree connecting all known NHR sequences (Laudet, 1997; Nuclear Receptors Nomenclature Commitee, 1999). Thus, all NHRs discovered to date are placed into one of six evolutionary groups of unequal size. The distribution of receptors into six subfamilies correlates with their ability to bind DNA as monomers, heterodimers or homodimers, and to some extent with their ligand-binding specificities (Laudet, 1997). For example many nuclear hormone receptors which are able to heterodimerise with the retinoid X receptor (RXR) belong to the first or fourth subfamily while members of subfamily 3 are almost exclusively homodimeric, and bind to palindromic response elements.

Table 1-1. The 48 human nuclear hormone receptors (adapted from le Maire (2010)).

Subfamily /Group		NRNC	Name	Abbreviation	Ligand(s)	M,D,H*
1	A	NR1A1	Thyroid hormone receptor	TR	Thyroid hormone	H
		NR1A2	Thyroid hormone receptor	TR		
	B	NR1B1	Retinoic acid receptor	RAR	Retinoic acid	H
		NR1B2	Retinoic acid receptor	RAR		
		NR1B3	Retinoic acid receptor	RAR		
	C	NR1C1	Peroxisome proliferator-activated receptor	PPAR	Fatty acids (, ,), leukotriene B4 ( ), fibrates ( ), prostaglandin J2 ( ), thizaolidinediones ( )	H
		NR1C2	Peroxisome proliferator-activated receptor /	PPAR /		
		NR1C3	Peroxisome proliferator-activated receptor	PPAR		
	D	NR1D1	Rev-ErbA	Rev-ErbA	Heme	M, D
		NR1D2	Rev-ErbA	Rev-ErbA		
	F	NR1F1	RAR-related orphan receptor	ROR	Cholesterol ( ), Cholesteryl sulphate ( ), retinoic acid ( ), Orphan ( )	M
		NR1F2	RAR-related orphan receptor	ROR		
		NR1F3	RAR-related orphan receptor	ROR		
	H	NR1H3	Liver X receptor	LXR	Oxysterols, T0901317, GW3965	H
		NR1H2	Liver X receptor	LXR		
		NR1H4	Farnesoid X receptor	FXR	Bile acids, fexaramine	H
	I	NR1I1	Vitamin D receptor	VDR	Vitamin D, 1,25-dihydroxyvitamin D	H
		NR1I2	Pregnane X receptor	PXR	Xenobiotics, 16 -cyanopregnenalone	H
		NR1I3	Constitutive androstane receptor	CAR	Xenobiotics, phenobarbital	H
2	A	NR2A1	Hepatocyte nuclear factor 4	HNF4	Fatty acids	D
		NR2A2	Hepatocyte nuclear factor 4	HNF4		
	B	NR2B1	Retinoid X receptor	RXR	Retinoic acid (, ,)	D
		NR2B2	Retinoid X receptor	RXR		
		NR2B3	Retinoid X receptor	RXR		
	C	NR2C1	Testicular receptor 2	TR2	Orphan	D, H
		NR2C2	Testicular receptor 4	TR4		
	E	NR2E1	Homologue of the Drosophila tailless gene	TLX	Orphan	M, D
		NR2E3	Photoreceptor cell-specific nuclear receptor	PNR	Orphan	M, D

Table 1-1 cont…

Subfamily/ Group		NRNC	Name	Abbreviation	Ligand(s)	
2	F	NR2F1	Chicken ovalbumin upstream promoter-transcription factor I	COUP-TFI	Orphan	D, H
		NR2F2	Chicken ovalbumin upstream promoter-transcription factor II	COUP-TFII	Orphan	D, H
		NR2F6	V-erbA-related	EAR2	Orphan	M
3	A	NR3A1	Estrogen receptor-	ER	17 -estradiol (,), tamoxifen ( ), raloxifene ( ), various syntetic compounds ( )	D
		NR3A2	Estrogen receptor-	ER		
	B	NR3B1	Estrogen-related receptor-	ERR	Orphan ( ), DES (,), 4-OH tamoxifen (,)	M, D
		NR3B2	Estrogen-related receptor-	ERR		
		NR3B3	Estrogen-related receptor-	ERR		
	C	NR3C1	Glucocorticoid receptor	GR	Cortisol, dexamethasone, RU486	D
		NR3C2	Mineralocorticoid receptor	MR	Aldosterone, cortisol, dexamethasone, Spironolactone	D
		NR3C3	Progesterone receptor	PR	Progesterone, medroxyprogesterone acetate, RU486	D
		NR3C4	Androgen receptor	AR	Testosterone, Flutamide	D
4	A	NR4A1	Nerve Growth factor IB	NGFIB	Orphan	M, D, H
		NR4A2	Nuclear receptor related 1	NURR1	Orphan	M, H
		NR4A3	Neuron-derived orphan receptor 1	NOR1	Orphan	M
5	A	NR5A1	Steroidogenic factor 1	SF1	Phosphatidylinositol	M
		NR5A2	Liver receptor homolog-1	LRH-1	Phosphatidylinositol	
6	A	NR6A1	Germ cell nuclear factor	GCNF	Orphan	D
Misc.	A	NR0B1	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1	DAX1	Orphan	
		NR0B2	Small heterodimer partner	SHP	Orphan	H

*** M – Monomer; D – Homodimer; H – RXR heterodimer**

Below the nuclear hormone receptors are discussed in more detail with particular reference to PPARs (NR1C1, NR1C2 and NR1C3) and CRs (NR3C1 and NR3C2).

1.1.2 Structure of NHRs

All nuclear hormone receptors share a common domain structure, comprising an N-terminal A/B domain, a central DNA-binding C-domain, linked via a hinge region to a C-terminal ligand-binding E domain and the role of each domain in overall receptor function has been well defined (Figure 1-1).

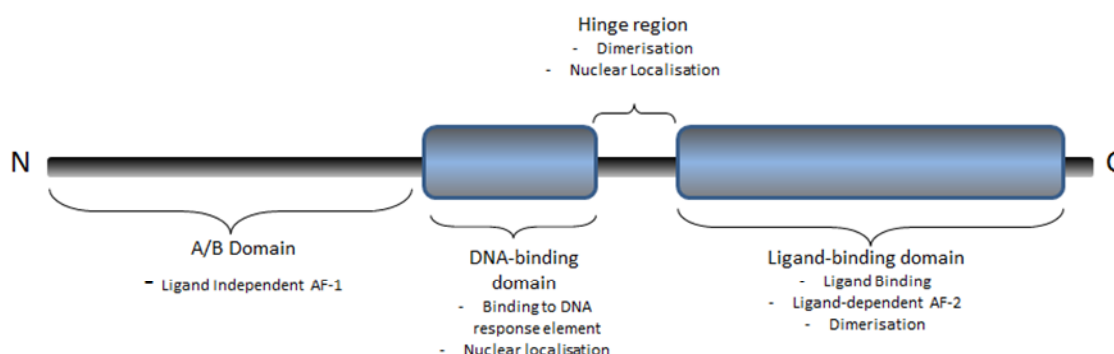


Figure 1-1. Schematic diagram of domain architecture of NHRs and the roles assigned to each of the domains.

1.1.3 A/B domain

The A/B domains of NHRs, unlike the DNA-binding domains and ligand-binding domains are relatively low in sequence conservation, even between different isotypes of the same receptor. For example the A/B domains of PPAR and PPAR from the amphibian Xenopus laevis share an amino acid identity of 11% and 21% respectively, with PPAR (Escher and Wahli, 2000), while the two glucocorticoid receptors of the marine medaka (*Oryzias dancena*) share an amino acid identity within their A/B domains of 17%, and an amino acid identity of less than 45% is generally observed between GR1 of this species and the GRs of other teleost GRs (Kim *et al*, 2011). As well as differing in sequence, the A/B domains of NHRs differ considerably in length. For example the N-terminal domain of the mineralocorticoid

receptor (MR) consists of 602 residues, while the glucocorticoid receptor (GR) and the estrogen receptor (ER) are 420 residues and 184 residues, respectively (Lavery and McEwan, 2005).

Regardless of the sequence diversity a common function for the A/B domain has been suggested following the identification of an activation function (AF-1) within this region of many receptors, including the glucocorticoid receptors (Sturm *et al*, 2010), the androgen receptor (Chamberlain *et al*, 1996), the progesterone receptor (Meyer *et al*, 1992), the estrogen receptor (Tora *et al*, 1989; Lees *et al*, 1989) and the PPARs (Werman *et al*, 1997; Juge-Aubry *et al*, 1999). A second activation function (AF-2) is present within the ligand-binding domain of receptors, whose role in receptor activation is dependent on a conformational change in helix 12 of the ligand binding domain (LBD), induced by interactions with an activating ligand. In some NHRs deletion of AF-2 has been reported to lead to a constitutively active receptor, leading researchers to conclude that the AF-1 functions in a ligand-independent manner (Hollenberg *et al*, 1987; Tora *et al*, 1989). Furthermore, deletion of the A/B domain of NHRs leads to a significant reduction in transactivation of the receptors (Hi *et al*, 1999; Sturm *et al*, 2010), indicating a synergy between the AF-1 in the A/B domain and AF-2 in the ligand-binding domain in full receptor activation.

Despite their importance in gene regulation, little is known about the mechanisms in which AF-1 functions. The structure of the A/B domain of several NHRs has been solved and all appear to be largely unstructured or intrinsically disordered (Baskakov *et al*, 1999; Kumar *et al*, 2001; Chandra *et al*, 2008), suggesting that the activation domains of these receptors may require a high degree of flexibility in order to function effectively (Garza *et al*, 2009).

It has been proposed that interactions of A/B domains with co-regulatory proteins give structure to the domain via an ‘induced-fit’ model (Kumar *et al*, 2004; Kumar and Litwack, 2009), and these interactions correlate *in vivo* with enhanced transcription of both co-transfected and endogenous genes (Warnmark *et al*, 2001; Copik *et al*, 2006). The strength of co-regulatory protein interaction may be further modulated via phosphorylation status (Passilly *et al*, 1999; Nordzell *et al*, 2004; Garza *et al*, 2010).

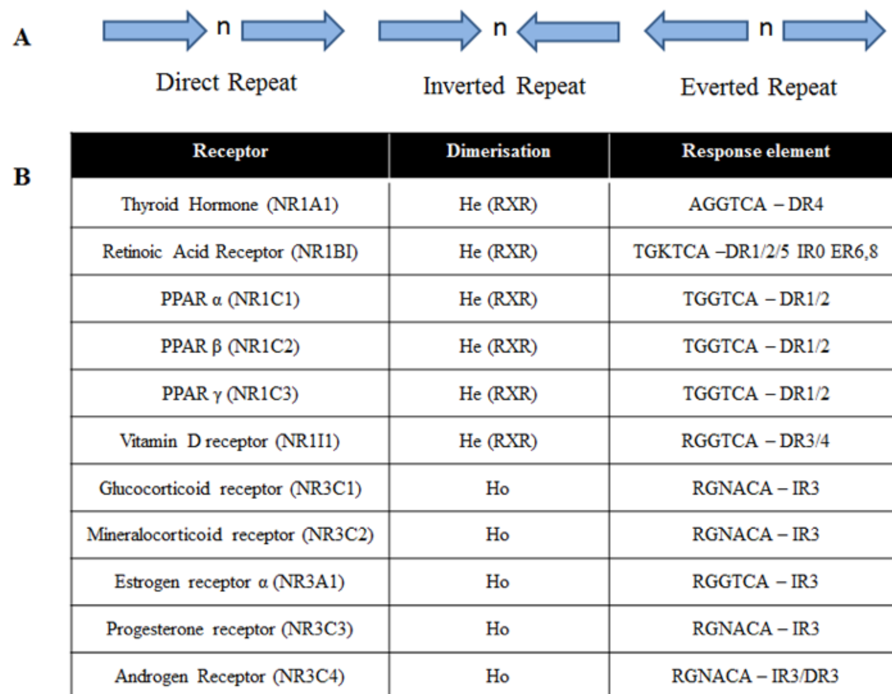
1.1.4 DNA-binding domain

Linked to the carboxyl end of the A/B domain is a highly conserved region in the vast majority of NHRs, the DNA-binding or C-domain. A comparison of the C-domains of glucocorticoid receptors from several teleost species and the human GR sees a similarity of no less than 80% (Kim *et al*, 2011) while between the three PPAR subtypes (, and) an amino acid identity of between 78 and 86% is observed (Dreyer *et al*, 1993). The DNA-binding domain is composed of two zinc finger structures, each of which is expressed by a separate exon (Huckaby *et al*, 1987; Luisi *et al*, 1991). A prerequisite for the regulation of target genes by NHRs is the binding of the NHR to a specific DNA sequence, termed a response element, within the promoter region of target genes and this contact is mediated by the central DNA-binding domain of NHRs.

Most nuclear receptors bind to DNA either as homodimers or as heterodimers with RXR. The former group includes the classic steroid hormone receptors, such as glucocorticoid receptors, estrogen receptors, progesterone receptors and androgen receptors, while the latter group includes the vitamin D receptor, the thyroid

hormone receptor and the peroxisome proliferator-activated receptors. In general homodimers recognise and bind to DNA sequences arranged as inverted repeat (IR) response elements, while receptors which heterodimerise with RXR bind to direct repeat (DR) response elements. Some receptors are also capable of binding to everted repeat sequences (ER) with various spacers separating the two half-sites (Mangelsdorf and Evans, 1995; Figure 1-2).

Figure 1-2. (A) Schematic diagram representing direct repeat (DR), inverted repeat (IR) and everted repeat (ER) hormone response elements, with each half-site of the HRE separated by 'N' base pairs. (B) The dimerization and recognised response element for a selection of human NHRs. He, heterodimer; Ho, homodimer; (RXR), indicates the receptors which heterodimerise with RXR; K, G/T; R, A/G; N, A/T/C/G.



Receptor	Dimerisation	Response element
Thyroid Hormone (NR1A1)	He (RXR)	AGGTCA – DR4
Retinoic Acid Receptor (NR1BI)	He (RXR)	TGKTCA –DR1/2/5 IR0 ER6,8
PPAR α (NR1C1)	He (RXR)	TGGTCA – DR1/2
PPAR β (NR1C2)	He (RXR)	TGGTCA – DR1/2
PPAR γ (NR1C3)	He (RXR)	TGGTCA – DR1/2
Vitamin D receptor (NR1I1)	He (RXR)	RGGTCA – DR3/4
Glucocorticoid receptor (NR3C1)	Ho	RGNACA – IR3
Mineralocorticoid receptor (NR3C2)	Ho	RGNACA – IR3
Estrogen receptor α (NR3A1)	Ho	RGGTCA – IR3
Progesterone receptor (NR3C3)	Ho	RGNACA – IR3
Androgen Receptor (NR3C4)	Ho	RGNACA – IR3/DR3

The recognition of specific hormone response elements by nuclear hormone receptors explains much, but not all, of their ability to alter gene transcription in a targeted manner and several residues within the DBD of receptors are implicated in the recognition of specific HREs (Umesono and Evans, 1989; Danielsen *et al*, 1989; Nguyen *et al*, 2001; Khorasanizadeh and Rastinejad, 2001).

The ability of NHRs to bind to specific response elements is also determined in part by the spacing between the two half-sites of the HRE; for example, while PPAR/RXR heterodimers recognise DRs with 1 base pair spacing (DR1), the vitamin D receptor/RXR heterodimer binds preferentially to DRs whose half sites are separated by 3 (DR3) or 4 (DR4) base pairs (Nishikawa *et al*, 1994; Quack and Carlberg, 2000; Tugwood *et al*, 1992).

Nuclear hormone receptors dimerise in a way that is specific to each dimer complex, resulting in optimal spatial arrangements for DNA binding. In the absence of a response element receptors have a limited capacity to dimerise due to the lack of dimerization contacts and the relatively small surfaces involved. In the presence of DNA, these monomers become aligned into dimers, resulting in a continuous surface with many DNA interaction-surfaces (Luisi *et al*, 1991; Zhao *et al*, 2000). To date, examination of protein-DNA complexes reveal discrete protein-protein interactions between the RXR and its partner monomers which ensure a precise match to the spacing between the direct repeats (Khorasanizadeh and Rastinejad, 2001).

Structurally, the C-domain consists of two ‘zinc finger’ such that four conserved cysteine residues are co-ordinately bound by a central divalent zinc ion (Figure 1-3). Each of the zinc fingers have been shown to have a distinct function. A proposed function for the second zinc finger is that of nuclear localization; Yu *et al* (1998) showed that deletion of the second zinc finger of the orphan nuclear receptor TR2 resulted in a receptor which was evenly distributed between the nucleus and the cytoplasm compared to a receptor which was exclusively nuclear when the second zinc finger was intact, suggesting the presence of a nuclear localisation signal within the second zinc finger. The second zinc finger region of PPAR has also been

implicated in nuclear localisation of the receptor, with a partial deletion of this region resulting in cytoplasmic localisation (Iwamoto *et al*, 2011).

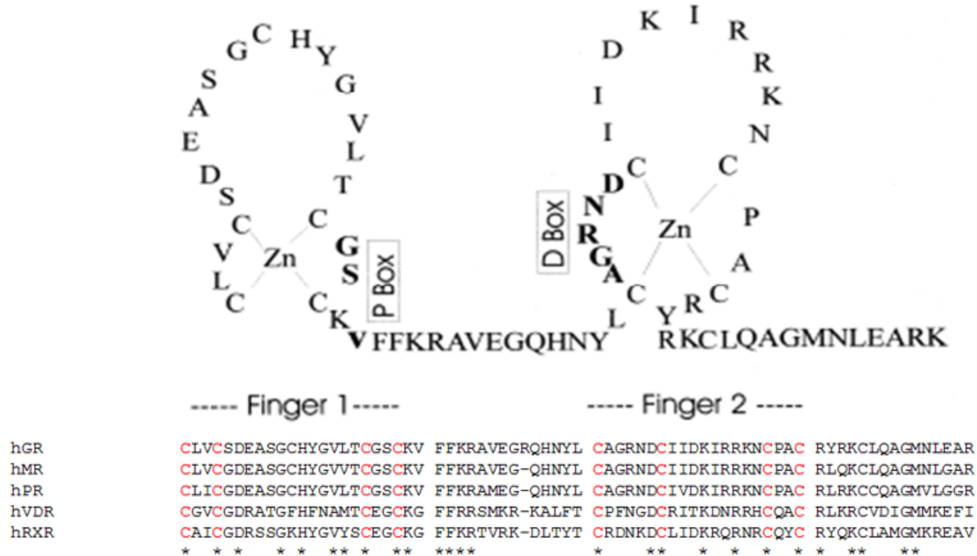


Figure 1-3. The DNA-binding domains of the NHRs are composed of two zinc finger structures, with each zinc molecule co-ordinated by four conserved cysteine residues. The P-box is involved in recognition of the response element of DNA to which NHRs bind, while the D-box has been implicated in dimerization (top; adapted from (Kumar and Thompson, 1999)). An alignment of the zinc finger regions of different NHRs (bottom) show the four conserved Cys residues (highlighted in red).

Thus, as well as mediating the binding of receptor dimers to hormone response elements within the promoter region of target genes, the DNA-binding domain is also functional in roles concerning localisation of the receptor to the nucleus and in receptor dimerization.

1.1.5 *Hinge region*

The hinge region (D-domain) of nuclear hormone receptors is located between the DNA-binding domain and the C-terminal ligand-binding domain and shows little conservation amongst various receptors. Studies in which D-domains are mutated or excluded demonstrate varying functions of this domain, depending on the receptor being studied. Within the 1 thyroid hormone receptor mutation of the D-domain

results in an E-domain unable to bind T3 hormone, and unable to release co-repressor proteins except at very high T3 concentrations (Lin *et al*, 1991; Safer *et al*, 1998). Similarly, a natural PPAR D-domain variant is associated with enhanced recruitment of the nuclear co-repressor NcoR, and a defective release of the co-repressor in the presence of PPAR ligands (Liu *et al*, 2008). Thus the hinge region appears to function in the proper association of coregulatory proteins with the ligand-binding region in several nuclear hormone receptors.

In contrast, a deletion of eight residues within the hinge region of the androgen receptor resulted in a stronger response in the presence of androgen hormone, which could be inhibited with anti-androgen compounds (Haelens *et al*, 2007). The dimerization function of the thyroid hormone receptor appears to rely on the hinge region since the ligand-binding domain alone of thyroid receptor is unable to homodimerise or heterodimerise with the RXR , while the inclusion of the D-domain allowed both homodimerisation and heterodimerisation (Miyamoto *et al*, 2001). In contrast to the thyroid hormone receptor, the D-domain of RXR does not have to be present in order for the receptor to dimerise (Miyamoto *et al*, 2001).

1.1.6 Ligand-binding domain

The ligand-binding domain (LBD) of nuclear hormone receptors, in which a ligand-dependent activation function (AF-2) is present, is found at the C-terminal end of nuclear hormone receptors and, as its name suggests, is involved in binding of ligand molecules to the receptor. Additionally the LBD contacts both co-repressor and co-activator proteins when in the inactive and active conformations, respectively, and together with the DNA-binding domain has a role in receptor dimerization.

Structure of the ligand-binding domain

The ligand-binding domains of nuclear hormone receptors have highly conserved tertiary structures, and are composed of 11 or 12 -helices arranged in an antiparallel 3-layer -helical sandwich, together with the presence of two -strands forming a hairpin structure (Renaud *et al*, 1995; Bourguet *et al*, 1995; Brzozowski *et al*, 1997; Egea *et al*, 2000), although 4 -strands have been described in the the glucocorticoid receptor (Bledsoe *et al*, 2002), PPAR (Xu *et al*, 1999) and the thryroid receptor, forming a mixed -sheet (Wagner *et al*, 1995).

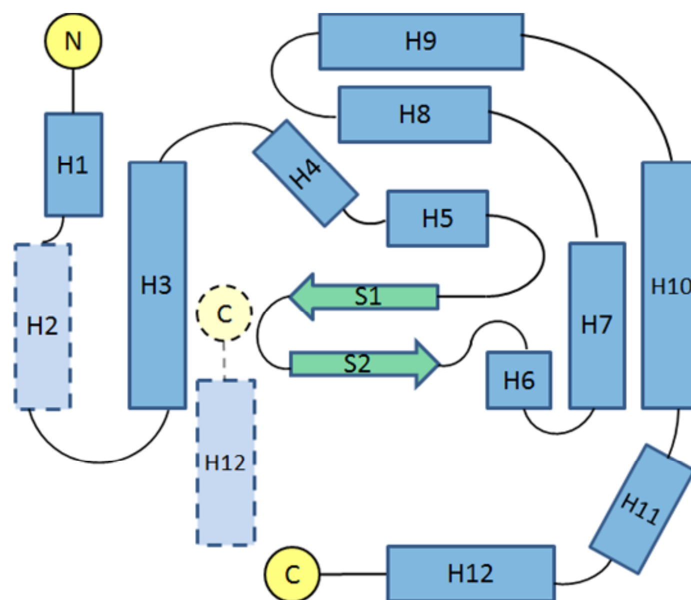


Figure 1-4. The topology of the 12 -helices found in the ligand-binding domain of the retinoid X receptor (RXR). Slight variations on this structure occur, for example Helix 2 does not exist in the retinoic acid receptor (RAR) and helix 12 together with the C-terminus is postioned perpendicularly to the same region in the RXR . The -sheet hairpin structure is represented by the two green arrows.Adapted from (Renaud *et al*, 1995).

Interactions between nuclear hormone receptors and their ligands occur within the ligand-binding domain and the 3-D structures of such interactions have been solved by x-ray crystallography for several receptors including the estrogen receptor (Brzozowski *et al*, 1997), the glucocorticoid receptor (Xu *et al*, 1999; Bledsoe *et al*, 2002), RAR- (Renaud *et al*, 1995), PPAR (Xu *et al*, 1999), and RXR (Egea *et al*, 2000). The overall structure of the ligand-binding domain of NHRs allows for the

presence of a hydrophobic pocket into which ligands can dock; all-trans retinoic acid, when bound to RAR- is found to bury into a pocket formed by residues located in helices 1, 3, 5, the -turm, loop 6-7, helix 11, loop 11-12 and helix 12 (Renaud *et al*, 1995) while the ligand-binding pocket of the glucocorticoid receptor is composed of residues from helices 3, 4, 5, 6, 7, 10 and 12 as well as residues from -strands 1 and 2 (Bledsoe *et al*, 2002). Receptors which bind with high affinity to a specific ligand generally have smaller ligand-binding pockets than more promiscuous receptors. For example both PPAR and PPAR have very large binding pockets of approximately 1300 Å3, of which only 40% of the latter is occupied by rosiglitazone, a PPAR agonist (Nolte *et al*, 1998; Xu *et al*, 1999) and the highly promiscuous pregnane X receptor has a ligand-binding pocket of 1150 Å3. This is in comparison to the ligand-binding pockets of the thyroid hormone receptor and the estrogen receptors which show high affinities for the thyroid and estrogen hormones, respectively, and which have ligand-binding cavities of 600 Å3 (thyroid hormone receptor) (Ribeiro *et al*, 2004) and 450 Å3 (estrogen receptor) (Brzozowski *et al*, 1997).

1.1.6.1 Helix 12 and AF-2

Agonist and antagonist ligands have been described for many NHRs, each of which make contact with the ligand-binding domain at the ligand-binding pocket (Brzozowski *et al*, 1997; Bledsoe *et al*, 2005). The importance of the C-terminal - helix in regulating the transcriptional activities of NHRs was underlined upon the discovery of a ligand-dependent activation function (AF-2) in this region (Nagpal *et al*, 1993; Barettino *et al*, 1994; Kraus *et al*, 1995; Bevan *et al*, 1999).

In their unliganded state, or when bound to antagonist molecules, nuclear hormone receptors are bound by co-repressor proteins which dissociate upon binding of agonists, allowing co-activator binding and receptor activation. It appears that the choice of co-regulatory protein, which is controlled by ligand-binding, is the ‘switch’ between a transcriptionally active receptor and one with no transcriptional activity. The interaction of co-activators with nuclear hormone receptors requires the most C-terminal helix (helix 11/12) to be in a specific conformation, which is obtained upon agonist binding. Binding of the potent agonist estradiol to the estrogen receptor results in a conformational change in helix 12, which results in the exposure of a conserved residue on this helix important in the efficient recruitment of co-activator proteins (Figure 1-5) (Henttu *et al*, 1997). Additionally, a hydrogen bond network within the mineralocorticoid receptor is required to stabilise the receptor and to re-orientate helix 12 to enable cofactor recruitment (Bledsoe *et al*, 2005). The antagonist spironolactone fails to make these hydrogen bonds, and thus lacks the ability to stabilise the receptor in order to re-orientate helix 12.

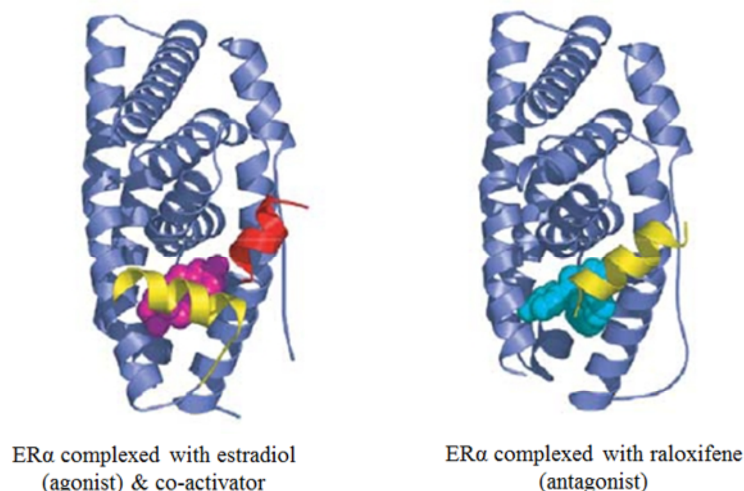


Figure 1-5. Binding of agonist (purple) + co-activator (red), and antagonist (blue) to the estrogen receptor (ER). Binding of agonist ligands leads to a conformational change in helix 12 (yellow) of the ER compared to the conformation when bound to antagonist molecules.

Interestingly co-repressor and co-activator proteins interact with nuclear hormone receptors on largely overlapping surfaces of the ligand-binding domain, within a hydrophobic groove situated between helices 3 and 4 (Nagy *et al*, 1999; Perissi *et al*, 1999; Marimuthu *et al*, 2002; Benko *et al*, 2003) and thus the question was raised as to how co-repressor proteins can bind to receptors when helix 12 is not in the correct orientation for co-activator binding. Several authors have reported the presence of multiple short interaction motifs (LxxLL) within co-activator proteins which bind to receptors through contacts with helices 3, 4 and 12 (Heery *et al*, 1997; Darimont *et al*, 1998). Similar, but longer, motifs have been described for co-repressor proteins (LxxxIxxx; L/I motif) (Nagy *et al*, 1999; Perissi *et al*, 1999; Webb *et al*, 2000) and it is hypothesised that the longer motif within the co-repressor proteins allows binding to nuclear hormone receptors in a way which does not require helix 12 to be in the active conformation (Nagy *et al*, 1999; Perissi *et al*, 1999).

In summary, in their un-liganded form most NHRs are in an inactive conformation by the binding of co-repressor proteins to a hydrophobic groove in their ligand-binding domain. Upon binding of agonists to the ligand-binding pocket, a network of bonds between the ligand and the receptor stabilise the receptor and re-orientate helix 12 to the 'active' conformation. This latter conformational change favours the dissociation of co-repressor proteins and the binding of co-activator proteins, rendering the receptor transcriptionally active.

1.1.6.2 Receptor dimerization

The ligand-binding domain, along with the DNA-binding domain, has a function in receptor dimerization. While dimerization within the DNA-binding domain is

important for contact with DNA (see 1.1.4 above), dimerization at the ligand-binding domain stabilises the receptor-DNA complex.

Several residues within helices 7, 9 and 10 of the ligand-binding domain have been shown, through mutation analysis, to function in receptor dimerization. The crystal structures of RXR in a homodimer complex and as a heterodimer with PPAR has been resolved and shows an unusual pi-helix (-helix) conformation within helix 7, caused by an additional glutamate residue at position 352 of the receptor (Gampe *et al*, 2000a and b). This unusual geometry results in the residue bulging out from helix 7 towards its partner monomer, improving the fit at the monomer-monomer interface. This additional glutamate residue, and the subsequent conformation of helix 7, appears to be unique to members of the nuclear receptor subfamily 2 (NR2) of which RXR , the orphan receptor hepatocyte nuclear factor 4 (HNF4), and COUP-TFI are members. Indeed the -helix conformation within helix 7 of HNF4 has also been described, the deletion or mutation of which fails to form homodimers, neither with wild-type HNF nor with other mutated HNF receptors and interferes with receptor interactions with co-factor proteins (Eeckhoute *et al*, 2003).

1.1.6.3 The Identity box (I-box)

Helices 9 and 10 of nuclear hormone receptors have been implicated in dimerization (Gampe *et al*, 2000a; Dhe-Paganon *et al*, 2002). A 40 amino acid region within helices 9 and 10 of the ligand-binding domains of RXR, RAR and TR, termed the identity box (I-box), has been shown to be critical for heterodimerisation (Perlmann *et al*, 1996). Within HNF4 , two residues one from each of helix 9 and 10, were shown to have a charge compatibility and the resulting reciprocal interactions were

important in stabilization of HNF4 homodimer complex (Bogan *et al*, 2000). This charge compatibility appears to be important in mediating dimerization not only of homodimers, but also of heterodimers; the residues within RXR and PPAR , corresponding of those in helices 9 and 10 of HNF4 , were mutated to result in a single charge reversal in either RXR or PPAR . As a consequence of these mutations, heterodimer formation of either receptor with its wild-type partner was blocked, but could be reinstated by reversing the charge of the residue in the corresponding wild-type partner (Chan and Wells, 2007). Additionally Nurr-1 and RXR heterodimerisation is inhibited following an amino acid substitution in one of the proteins (Perlmann *et al*, 1996), which further indicates the importance of interactions between residues on helix 9 and helix 10 of ligand-binding domains within nuclear hormone receptors.

1.2 Peroxisome proliferator-activated receptors (PPARs)

PPARs are members of the nuclear hormone receptor superfamily with functions in lipid, carbohydrate and protein metabolism, adipogenesis, development and the inflammatory response. To date PPARs in mammalian species have been extensively studied because they are targets for several pharmaceutical agents including hypolipidemic, non-steroidal anti-inflammatory and insulin sensitizing drugs (Forman *et al*, 1997; Lehmann *et al*, 1997; Rocchi *et al*, 2001). More recently the PPARs have been implicated in the human obesity epidemic, hypothesised to be targets of compounds which pollute the environment (Evans *et al*, 2004; Grün and Blumberg, 2006; Grün *et al*, 2006; Janosek *et al*, 2006; Desvergne *et al*, 2009; Janesick and Blumberg, 2011). Many nuclear hormone receptors, including the PPARs, are found in other species such as fish, birds and amphibians (Krey *et al*,

1993; Ruyter *et al*, 1997; Diot and Douaire, 1999; Andersen *et al*, 2000; Boukouvala *et al*, 2004; Leaver *et al*, 2005), although in general their interactions with endogenous and xenobiotic compounds, and their physiological roles is less studied.

Peroxisome proliferator-activated receptors share the domain structure common to almost all members of the nuclear hormone receptor superfamily, and function as heterodimers with the retinoid X receptor to alter the transcription of their target genes. Three PPARs (PPAR , PPAR and PPAR) have been identified, each the product of a separate gene and each with a distinct tissue distribution.

1.2.1 PPAR

PPAR , in accordance with its function in fatty acid oxidation in mitochondria and peroxisomes, is highly expressed in tissues with high rates of -oxidation such as the liver, heart, muscle and kidney. This is true in both mammalian (Braissant *et al*, 1996) and fish species, suggesting a conserved role for PPAR . Brown trout (*Salmo trutta*) and sea bream (*Sparus aurata*) show high PPAR expression in the liver and heart, with an increased expression also found in the white muscle of the former species and the red muscle of the latter (Leaver *et al*, 2005; Batista-Pinto *et al*, 2005). PPAR is highly expressed in the liver, proximal tubules of the kidney and in cells of the intestine of zebrafish (Ibabe *et al*, 2002).

Fatty acids were first identified as ligands of Xenopus PPAR by Keller *et al* (1993), who hypothesised that since known PPAR ligands shared a similar structure to fatty acids, and that high-fat diets and fatty acid analogues induced peroxisomal -oxidation of fatty acids, a role associated with PPARs, that fatty acids may be the endogenous ligands of these receptors. Indeed co-transfection experiments with

PPAR and RXR demonstrated the co-operative activation of the acyl CoA oxidase promoter which contains a peroxisome-proliferator response element (PPRE) when the receptors were linked as heterodimers in the presence of fatty acids. Furthermore a direct interaction between fatty acids and PPARs has been demonstrated (Kliewer *et al*, 1997; Murakami *et al*, 1999), suggesting that activation of the PPAR-RXR heterodimer by fatty acids is due to the interaction of PPARs with fatty acids and not a consequence of fatty acid interactions with the RXR heterodimer partner. This is a valid point considering 9-cis retinoic acid, the ligand for RXR, also led to acyl Co-A oxidase promoter activation (Keller *et al*, 1993), suggesting that both PPAR and RXR ligands can regulate the expression of PPRE-containing target genes via the activation of the PPAR-RXR heterodimer. Within several fish species fatty acids are ligands for PPAR , and activate PPAR of Japanese medaka (*Oryzias latipes*), gilthead sea bream and European plaice (*Pleuronectes platessa*) (Leaver *et al*, 2005; Kondo *et al*, 2010).

In addition to fatty acids themselves, various oxidised derivatives of fatty acids have also been shown to activate PPAR . Several transcription activation assays and gel shift studies indicate that derivatives of fatty acid metabolism, such as eicosanoids, are endogenous ligands for mammalian PPARs. Derivatives of arachidonic acid, including 20-hydroxyeicosatetraenoic acid (20-HETE) and several epoxyeicosatrienoic acids (EETs) and dihydroxyeicosatrienoic acids (DHETs) are able to activate PPAR and PPAR , as demonstrated in transactivation assays (Ng *et al*, 2007). Furthermore each of these compounds binds directly to PPAR , permitting a heterodimeric PPAR -RXR complex to form, which can subsequently bind to a PPRE (Ng *et al*, 2007). The eicosanoid 8S-HETE, produced as a result of

arachidonic acid entering the lipoxygenase pathway, has also previously been shown to interact with Xenopus (Krey *et al*, 1997; Kliewer *et al*, 1997) and mouse (Forman *et al*, 1997; Thuillier *et al*, 2002) PPAR .

The finding that fatty acids and their derivatives are able to activate PPARs has led to speculation of the existence of an autoregulatory feedback loop (Göttlicher *et al*, 1992; Krey *et al*, 1997). PPARs, upon binding to ligands and heterodimerising with RXR , upregulate genes involved in fatty acid oxidation and therefore by activating the PPAR receptors fatty acids and eicosanoids could mediate their own neutralisation and degradation. As yet the relative importance of the roles of fatty acids versus their oxidised derivatives *in vivo* has not been determined, and will depend on the physiological concentrations of each of these potential ligands in each tissue.

A number of synthetic PPAR agonists have been discovered or developed. Indeed the initial identification of PPAR was as the receptor for the hypolipidemic drug clofibrate (Issemann and Green, 1990). Since then several other members of the fibrate class of drugs, as well as the PPAR -specific ligand Wy14643, have been developed, in some cases specifically as ligands of PPAR (Willson *et al*, 2000). Several fibrates have since been tested in transcription assays for their ability to activate PPAR , and following transient transfection. Forman *et al* (1997) found a strong activation of PPAR , a weak activation of PPAR and no effect on PPAR when cells transfected with murine PPARs were treated with ciprofibrate, gemfibrozil and clofibrate. Fibrates are also activating ligands of amphibian and fish PPARs; interactions of bezafibrate and ciprofibrate with PPAR and PPAR of Xenopus laevis, respectively, has been demonstrated (Krey *et al*, 1997), and fibrates

administered to hepatocyte cells of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) resulted in an increased expression and activity of the PPAR target gene acyl-CoA oxidase (Donohue *et al*, 1993; Ruyter *et al*, 1997).

Prior to the discovery of the peroxisome proliferator-activated receptors, it was well established that treatment of rodents with various drugs, of which clofibrate was the archetype, caused a proliferation of peroxisomes which was associated with increases in the peroxisomal -oxidation of fatty acids (Lazarow and De Duve, 1976; Lazarow, 1978). Since the discovery of the PPARs and the finding that PPAR is highly expressed in tissues with high rates of fatty acid -oxidation, a well-established role for PPAR in fatty acid metabolism has been defined. Peroxisomal -oxidation genes which are targets of PPAR include acyl CoA oxidase (ACO) enzyme, which functions in the firstand rate-limiting step in the peroxisomal -oxidation pathway (Tugwood *et al*, 1992; Varanasi *et al*, 1996; Lambe *et al*, 1999) and enoyl-CoA hydratase/3-hydroxyacyl Co-A dehydrogenase bifunctional enzyme and ketoacyl-CoA, which function in the second step and the third steps (Zhang *et al*, 1992; Hansmannel *et al*, 2003).

Similarly, activation of PPAR mediates several steps in the microchondrial -oxidation pathway via the transcriptional regulation of genes involved in this pathway, including those with a function in the transport of fatty acids into the mitochondria and the major enzymatic steps in the -oxidation pathway. For example carnitine palmitoyl transferase expressed in the liver (CPT1a) and muscle (CPT1b) is involved in the translocation of long chain fatty acids across the mitochondrial membrane and an analysis of its promoter region reveals the presence of a functional PPRE (Brandt *et al*, 1998; Napal *et al*, 2005). Accordingly, hamsters treated with a

potent PPAR agonist saw a 2.3-fold increase in the transcription of CPT1 expression in muscle as well as hepatic effects (Minnich *et al*, 2001). PPAR has been shown to interact with the PPRE-containing promoter region of the medium chain acyl-CoA dehydrogenase gene, which encodes the enzyme responsible for the initial step in mitochondrial -oxidation (Gulick *et al*, 1994). Expression levels of both genes and proteins involved in both peroxisomal and mitochondrial fatty acid oxidation are induced upon activation of PPAR , and many of these genes have been shown to contain a peroxisome proliferator response element (PPRE) (Zhang *et al*, 1992; Hansmannel *et al*, 2003).

The role of PPAR in peroxisome proliferation and peroxisomal and mitochondrial fatty acid -oxidation is demonstrated further by animal models in which PPAR is non-functional. Rodents with a disrupted PPAR gene fail to increase the number and size of hepatic peroxisomes when treated with Wy14643 or clofibrate. Furthermore the increase in expression of peroxisomal -oxidation genes including acyl-CoA oxidase, bifunctional enzyme and 3-ketoacyl CoA thiolase, observed upon treatment with peroxisome proliferators, is abolished in PPAR -null mice (Lee *et al*, 1995). This failure to upregulate fatty acid -oxidation genes in PPAR knockout mice is associated with the accumulations of lipids in the liver and heart and changes in serum cholesterol (Peters *et al*, 1997). These results explain the efficacy of fibrate drugs in correcting, at least partially, dyslipidemia (Mukherjee *et al*, 1998; Fruchart and Duriez, 2006).

Taken together the evidence suggests that the physiological role of PPAR is to upregulate the oxidation of fatty acids for energy during periods of fasting or starvation.

1.2.2 PPAR

PPAR has been shown to be widely expressed amongst rodent tissues (Jones *et al*, 1995; Braissant *et al*, 1996). The findings in mammalian species agree with those in fish species where zebrafish, brown trout and sea bream show a widely expressed PPAR distribution. In addition to being the more widely expressed isotype, PPAR was found to be more abundant than PPAR in several tissues from both brown trout and sea bream (Leaver *et al*, 2005; Batista-Pinto *et al*, 2005).

Like PPAR , fatty acids have been demonstrated to activate PPAR (Xu *et al*, 1999; Fyffe *et al*, 2006), albeit in a differential manner (Yu *et al*, 1995). The authors showed that linoleic acid was able to preferentially activate human PPAR over PPAR and that PPAR was not activated by this fatty acid. In accordance with this, an overlap in ligand recognition amongst the three PPAR isotypes does occur, however both poly- and monounsaturated fatty acids are able to activate PPAR at a concentration up to 10-fold lower than that required for PPAR and PPAR activation (Krey *et al*, 1997). In fish species PPAR is activated by some fatty acids, although like mammals, the extent of its activation is less than that of PPAR (Leaver *et al*, 2005). Also like PPAR , and depending on species, PPAR is also activated by some fibrate drugs, although generally to a lesser extent than PPAR (Krey *et al*, 1997). Several PPAR subtype-specific synthetic ligands are available, for example GW501516 (Oliver *et al*, 2001), and there is some evidence that these also activate PPAR in fish (Leaver *et al*, 2007).

The physiological role of PPAR is less well established than for other PPARs. Although PPAR is widely expressed, it appears to be the predominant isotype

within skeletal muscle (Muoio *et al*, 2002) and as such has been implicated in the response of this tissue to exercise (Muoio *et al*, 2002; Sprecher, 2007). Target genes for PPAR appear to be in many cases the same as those for PPAR , and an *in vivo* human study, supported by *in vitro* cell culture experiments, has demonstrated the ability for the potent PPAR -selective agonist GW501516 to decrease serum triglyceride levels and enhance HDL, while upregulating the expression levels of carnitine palmitoyltransferase-1, CD36 and ABCA1 mRNA (Sprecher, 2007). These results suggest a role for PPAR , similar to PPAR in driving increases in fatty acid oxidation, but targeted to skeletal muscle, rather than liver.

1.2.3 PPAR

Within mammals PPAR exists as two forms originating from alternatively spliced transcripts (PPAR 1 and PPAR 2). PPAR 2 is mostly expressed in adipose tissue and organs with immune related functions (Braissant *et al*, 1996), whereas PPAR 1 is more widely expressed but generally at considerably lower level than other PPARs. The organs involved in immune function include the kidney, the spleen and the thymus. In accordance with this, a strong PPAR expression is found within the trunk kidney of brown trout suggesting that PPAR may play a similar role in this species as it does in mammals (Batista-Pinto *et al*, 2005). However, studies with different fish species suggest a differential expression of PPAR between species. Zebrafish tissues show little or no expression of PPAR (Ibabe *et al*, 2002), while almost all tissues of sea bream expressed PPAR , with levels appearing greater in the intestine and adipose tissue, and at a level similar or greater than the expression of PPAR (Leaver *et al*, 2005). There is no evidence of alternative PPAR polypeptides corresponding to the two mammalian forms in fish.

In mammals fatty acids are activators of PPAR , similar to PPAR , and the eicosanoid, 15-deoxy-D12, 14-prostaglandin J2 (15d-J2) has been identified as a possible specific endogenous agonist (Kliewer *et al*, 1995; Forman *et al*, 1995). A number of other lipid molecules have also been suggested as agonists and include lysophospholipids (McIntyre *et al*, 2003). Synthetic high affinity agonists include the thiazolidinediones (TZDs), which include troglitazone, pioglitazone, rosiglitazone and ciglitazone, and were identified as potentially useful drugs on the basis of stimulating the activation of rodent and human PPAR in transient transfection assays (Lehmann *et al*, 1995; Lambe and Tugwood, 1996). Furthermore TZDs have been shown to induce a conformational change in PPAR analogous to those produced by agonists of other nuclear hormone receptors (Berger *et al*, 1996) and inducing interactions of the CREB-binding protein (CBP) co-activator with PPAR (Mizukami and Taniguchi, 1997). TZDs have subsequently been used to treat non-insulin dependent diabetes mellitus (NIDDM). In addition to improving insulin sensitivity, these drugs have been reported to improve the lipid profiles of patients with NIDDM and have antioxidative, anti-inflammatory and antiapoptotic properties (Betteridge, 2007).

The identification of high affinity agonists for mammalian PPAR has enabled the discovery of a well-defined function for this receptor in adipose tissue, and more specifically in adipogenesis. Cultured fibroblasts expressing PPAR and treated with PPAR activators have been demonstrated to differentiate into adipocytes in a dose-dependent manner (Tontonoz *et al*, 1994). In undifferentiated preadipocyte cells PPAR expression is undetected (Chawla *et al*, 1994). However, upon addition of differentiation media or upon treatment with a PPAR -activating ligand PPAR

expression is quickly induced, concurrent with a differentiation of preadipocytes into mature adipocyte cells (Chawla *et al*, 1994). Much work has focussed on mammalian adipocyte differentiation and function and a complex system of signalling and transcriptional cascades has emerged (Farmer, 2006). Nevertheless the central and essential role of PPAR in the differentiation and maintenance of adipocytes is firmly established (Lowe *et al*, 2011).

Various PPAR target genes have been identified, several of which function in adipogenesis or are expressed in differentiated adipocytes. Importantly, following the addition of PPAR agonists, an increase in the expression of genes integral to the adipocyte phenotype are increased, including the adipocyte fatty acid binding protein (aP2), lipoprotein lipase, acyl-CoA synthase and fatty acid transport protein (Martin *et al*, 1998; Thompson *et al*, 2004). The modulation of PPAR has physiological consequences with particular relevance to type II diabetes. It has been demonstrated that accumulation of fatty acids in skeletal muscle is strongly associated with insulin resistance in type II diabetes (Ferrannini *et al*, 1983) and that glucose uptake within skeletal muscle in response to insulin is reduced in a dose-dependent manner with the levels of free fatty acids (Boden *et al*, 1994). Since PPAR promotes the uptake of free fatty acids into adipose tissue and thus away from skeletal muscle, known as 'free fatty acid steal', this action is likely to indirectly improve insulin sensitisation and glucose uptake in skeletal muscle. Indeed rosiglitazone (a thiazolidinedione) is able to improve the fatty-acid mediated resistance of insulin by increasing the uptake of fatty acids in adipocytes and reducing the uptake into the liver and skeletal muscle by 40% and 30%, respectively (Ye *et al*, 2004).

In addition to removing free fatty acids another mechanism, involving the modulation of adipocytokine levels, has been proposed that is likely to modulate glucose usage in skeletal muscle. Adipocytokines are cytokine proteins released specifically by adipocytes in response to extracellular stimuli or changes in metabolic status and include angiotensin, tumour necrosis factor (TNF) and adiponectin (Tarcin *et al*, 2007). Several lines of evidence support the hypothesis that adiponectin, the expression of which is regulated by PPAR , plays a part in insulin resistance. Levels of adiponectin protein in models of obesity, insulin-resistance and dyslipidemia, all of which predispose an individual to type 2 diabetes, are significantly decreased compared to ‘healthy’ individuals and administration of adiponectin ameliorates both hyperglycaemia and hyperinsulinemia (Arita *et al*, 1999; Yamauchi *et al*, 2001). Thiazolidinedione compounds increase the expression of adipophilin in a concentration dependent manner, an effect associated with a decrease in plasma free fatty acids, reduced hepatic fat content and an increase in hepatic insulin sensitivity in patients with type 2 diabetes (Bildirici *et al*, 2003; Bajaj *et al*, 2004). Since TZDs are potent activators of PPAR it has been speculated that adiponectin levels are increased in response to the activation of PPAR . Indeed, a PPRE has been identified within the human adiponectin gene (Iwaki *et al*, 2003) and co-transfection of a dominant negative PPAR diminished the increase in expression of adiponectin in response to TZDs (Maeda *et al*, 2001), providing evidence for the regulation of an adipocytokine by PPAR in a manner shown to improve insulin sensitivity.

1.2.4 PPARs in Inflammation and Immunity

Expression of PPARs in several cells of the immune system, including macrophages (Chinetti *et al*, 1998; Ricote *et al*, 1998), T and B lymphocytes (Jones *et al*, 2002) and dendritic cells (Gosset *et al*, 2001) have led to their being implicated in regulation of the immune system.

Several studies have identified PPAR , PPAR and PPAR , or their ligands, to have anti-inflammatory activities via the inhibition of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- B) signalling pathway (Chinetti *et al*, 1998; Jones *et al*, 2002; Planavila *et al*, 2005). The NF B family of proteins function as transcription factors whose activation and nuclear translocation, is associated with an increase in the transcription of genes encoding important components of the innate immune response, such as chemokines, cytokines, adhesion molecules, enzymes that produce secondary inflammatory mediators and inhibitors of apoptosis (Pahl, 1999). Both PPAR and PPAR ligands have been demonstrated to induce apoptosis in macrophage and T and B lymphocytes via inhibition of NF B signalling (Chinetti *et al*, 1998; Jones *et al*, 2002) while PPAR has been demonstrated to downregulate the expression of a NF B gene in the cardiac cells of rats via direct binding to NF B (Planavila *et al*, 2005). Both PPAR and PPAR are able to directly interact with the subunits of NF B (Delerive *et al*, 1999; Chung *et al*, 2000), with the former interaction occurring within the region which mediates NF B binding to DNA, dimerization and interactions with the NF B inhibitor, I B, and thus may inhibit NF B activity by blocking DNA-binding, dimerization and release of I B. Furthermore PPAR ligands including Wy14643 and fibrates have been found to

induce the expression of the NF B inhibitory protein, I B, in primary smooth muscle and hepatocyte cells in a PPAR -dependent manner (Delerive *et al*, 2000).

A mechanism for PPAR in anti-inflammatory effects has been demonstrated in monocytes/macrophages. Unliganded PPAR inhibits anti-inflammatory pathways by associating with the transcriptional co-repressor B cell lymphoma (BCL)-6, rendering it unavailable for anti-inflammatory actions, resulting in the upregulation of inflammatory proteins such as monocyte chemoattractant protein (MCP)-1, MCP-3 and interleukin-1 (Lee *et al*, 2003; Takata *et al*, 2008). Upon activation of PPAR , BCL-6 is released, allowing it to function in the repression of pro-inflammatory proteins.

PPAR also has important functions within keratinocytes whereas yet unidentified pro-inflammatory molecules released following skin injury activate PPAR . In these cells PPAR stimulates cell proliferation and inhibits apoptosis, thus aiding wound healing (Tan *et al*, 2001). The anti-apoptotic action of PPAR occurs via upregulation of two PPAR target genes, integrin-linked kinase (ILK) and 3-phosphoinositide-dependent kinase-1 (PDK1) (Di-Poi *et al*, 2002). These two kinase proteins phosphorylate and activate the Akt-1, central to the phosphatidylinositol-3 (PI3K) signalling pathway which results in the inhibition of pro-apoptotic regulatory proteins. The finding that PPARs have anti-inflammatory mechanisms has increased the interest in PPAR ligands as possible therapies for inflammatory diseases.

1.2.5 PPARs in Development

All three PPAR isotypes, together with the retinoid X receptor (RXR), the heterodimeric partner of PPARs, are present in the placenta of humans and rats

(Wang *et al*, 2002). Utilising PPAR -null mice a role for this receptor in the development of the labyrinth layer was suggested (Barak *et al*, 2001). This labyrinth of the placenta is involved in the exchange of material between foetus and mother, and PPAR -null mice saw labyrinth-layer defects including delayed differentiation and inappropriate vascular lake formation, impairing material exchange and leading to embryonic death.

Similar to PPAR , morphological defects are found in PPAR -null placentas with all three layers reduced in size, of which the giant-cell layer (trophoblast) is most severely affected. Cultured trophoblastic stem cells treated with the PPAR -specific agonist L165041 leads to their differentiation to giant cells while cells in which PPAR was silenced and treated with L165041 showed a significant decrease in differentiation (Nadra *et al*, 2006), implicating PPAR in the development of the placental trophoblastic layer.

1.3 Corticosteroid receptors (CRs)

Corticosteroid receptors, of which the glucocorticoid receptor and mineralocorticoid receptor are members, function as steroid ligand-activated transcription factors and have been identified in a variety of organisms including humans (Hollenberg *et al*, 1985; Arriza *et al*, 1987), rodents (Danielsen *et al*, 1986), amphibians (Csikos *et al*, 1995), birds (Kwok *et al*, 2007) and fish (Colombe *et al*, 2000; Marsigliante *et al*, 2000; Sturm *et al*, 2005; Takahashi *et al*, 2006; Schaaf *et al*, 2008; Pippal *et al*, 2011; Sturm *et al*, 2011; Kim *et al*, 2011). Similar to PPARs the glucocorticoid receptor is implicated in energy metabolism, as well as a plethora of other physiological functions including immune responses, the stress response and reproduction, while

the mineralocorticoid receptor has a well-studied role in osmoregulation, regulating salt balance in the kidney and intestine.

Like other members of the nuclear hormone receptor superfamily, ligand activation of the GR and MR induces binding to a glucocorticoid response element (GRE) within the promoter region of target genes. This receptor-DNA interaction induces conformational changes within the receptor which allow receptor dimerization and stabilization of heterodimeric complex, leading to the altered transcription of target genes (Härd *et al*, 1990; Luisi *et al*, 1991; Baumann *et al*, 1993). More recently, glucocorticoid receptor-mineralocorticoid receptor heterodimers have been described which use an alternative dimerization interface to the one used for homodimerisation, and induce a distinct transcriptional response pattern from that obtained with glucocorticoid receptor or mineralocorticoid receptor homodimers (Trapp *et al*, 1994; Savory *et al*, 2001; Nishi *et al*, 2004).

1.3.1 Glucocorticoid receptor (GR)

Within humans alternative splicing of the primary glucocorticoid receptor transcript gives rise to two, highly homologous protein isoforms, termed hGR and hGR , which differ in their carboxy-termini (Hollenberg *et al*, 1985). Using riboprobes specific to hGR and hGR each of these receptor isoforms have been shown to be widely distributed throughout human tissues (Oakley *et al*, 1999), with hGR being expressed most abundantly in terminal bronchiole epithelia of the lung, in the medulla of the thymus and in the lining of the bile duct within the liver (Oakley *et al*, 1997). Compared to hGR , hGR is expressed at much lower levels and increased expression of hGR is associated with glucocorticoid resistance, for example in

glucocorticoid insensitive asthma (Hamid *et al*, 1999; Sousa *et al*, 2000) and ulcerative colitis (Honda *et al*, 2000), potentially due to its function as a dominant negative inhibitor of hGR transcriptional activity (Bamberger *et al*, 1995). hGR is unable to bind glucocorticoids due to an altered ligand-binding domain (Hollenberg *et al*, 1985; Oakley *et al*, 1996), and unlike hGR has been found to reside preferentially in the nucleus of cells regardless of glucocorticoid availability (Oakley *et al*, 1997). The inhibitory effect of this GR isoform may be due to its competing with hGR for their common response element (Bamberger *et al*, 1995), its ability to bind to and thus compete for co-activators essential for full GR transcriptional activity (Charmandari *et al*, 2005), or its ability to interact directly with GR , forming GR -GR heterodimers and thus hindering the formation of transcriptionally active GR homodimer complexes (de Castro *et al*, 1996; Yudt *et al*, 2003).

The expression of the glucocorticoid receptor within teleost fish species largely reflects that found in mammals. Two glucocorticoid receptors, each the product of separate genes, have been identified in several teleost fish species (Greenwood *et al*, 2003; Bury *et al*, 2003; Kim *et al*, 2011), each of which are widely expressed. The two GR receptors (GR1 and GR2) from rainbow trout are expressed in most tissues including the intestine, liver, spleen, skin, heart and brain (Ducouret *et al*, 1995; Takeo *et al*, 1996; Bury *et al*, 2003). Within the cichlid fish species *Haplochromis burtoni* a higher expression of GR2, compared to either GR1 or MR, has been described in the heart, liver and spleen, with overlapping levels of expression in the gill (Greenwood *et al*, 2003).

1.3.2 *Mineralocorticoid receptor*

The expression of the MR has previously been identified in aldosterone-responsive tissues from humans including the distal parts of the nephron in the kidney, cardiomyocytes, enterocytes of the colonic mucosa, keratinocytes and sweat glands (Zennaro *et al*, 1997). Additionally a high expression of mineralocorticoid receptor has been identified within the hippocampus of both human and rodent brains (Chao *et al*, 1989; Seckl *et al*, 1991).

Within teleost fish species the mineralocorticoid receptor is widely expressed, although expression within the brain has been found to be significantly higher than in other tissues measured (Greenwood *et al*, 2003; Sturm *et al*, 2005). This led to the hypothesis that the mineralocorticoid receptor of teleosts may have roles in some aspects of their behaviour (Sakamoto *et al*, 2011). In support of this theory intracerebroventricular administration of cortisol or 11-deoxycorticosterone (DOC), the latter being a specific endogenous activator of the teleost MR, to the mudskipper (*Periopthalmus modestus*) altered the migratory behaviour of this species (Sakamoto *et al*, 2011), indicating the brain MRs to function in the preference for an aquatic habitat.

1.3.3 *Corticosteroid receptor ligands*

The adrenal gland in mammals and the homologous interrenal organ of the head kidney in teleost fish species are important in steroidogenesis, synthesising the corticosteroid hormones for which the glucocorticoid and mineralocorticoid receptors have a high affinity. While the adrenal gland produces two corticosteroids, namely cortisol (corticosterone in rodents) and aldosterone, teleost fish are thought to

contain only cortisol, with evidence for a mineralocorticoid hormone in teleost species lacking. Production and secretion of corticosteroid hormones is under the control of the highly conserved stress axis, involving the hypothalamus-pituitary-adrenal (HPA) axis in mammals, and the comparable hypothalamus-pituitary-interrenal (HPI) axis in teleost fish.

In response to stressors including salinity challenge, restraint, chasing and confinement, an increase in corticotropin-releasing hormone (CRH) from the pre-optic area of the hypothalamus has been observed in several fish species (Huising *et al*, 2004; Doyon *et al*, 2005; Wunderink *et al*, 2011). CRH is also found to be increased in mammals exposed to stress, as demonstrated by an increased expression of CRH in the hypothalamus of postnatal rats following prenatal undernutrition (Núñez *et al*, 2008). The hypothalamic axons of teleost fish species project directly to the pituitary gland, terminating in the area of the corticotrophic cells of the anterior pituitary (Flik *et al*, 2006). Within the cortitrophic cells of the pituitary, and in response to CRH, the large precursor protein proopiomelanocortin (POMC) is cleaved to adrenocorticotropic hormone (ACTH), which is released to the general circulation and binds to the melanocortin-2 receptors (MC2R) on the interrenal cells of the head kidney (Schiöth *et al*, 1996; Aluru and Vijayan, 2008). Similarly ACTH binds to MC2R on the adrenal gland of mammals. In response to ACTH-binding, interrenal MC2R of rainbow trout increases the mRNA expression levels of steroidogenic enzymes (Geslin and Auperin, 2004; Aluru and Vijayan, 2008), resulting in cortisol biosynthesis and release from the interrenal cells of the head kidney. Similarly MC2R, expressed on the adrenal gland of mammals, appears to mediate steroidogenesis in response to circulating levels of ACTH since MC2R

knockout mice have no detectable levels of corticosterone and an unresponsiveness to ACTH, despite the presence of measurable levels of the latter hormone (Chida *et al*, 2007).

To date, evidence for the presence of the mineralocorticoid hormone aldosterone within teleost fish is lacking. The p450 11 -hydroxylase (aldosterone synthase) is highly expressed in the rat adrenal gland and when transfected into a cell line confers the ability of the cells to convert 11-deoxycorticosterone (DOC) to corticosterone, 18-OH corticosterone and aldosterone through a series of hydroxylations (Zhou *et al*, 1995). Furthermore cells derived from the adrenal cortex of bovine, porcine and frog species containing aldosterone synthase have also been shown to have aldosterone synthesising activity, producing aldosterone from DOC (Nonaka *et al*, 1995). In contrast, aldosterone synthase enzyme isolated from the teleost Japanese eel has been found to lack aldosterone synthesising activity and is unable to convert DOC into aldosterone (Jiang *et al*, 1998).

Despite the absence of aldosterone, it has been suggested that other products of the steroidogenic pathway, such as DOC and other 11-deoxycorticosteroids, may act as endogenous activators for the teleost MR following the discovery that some of these compounds are able to bind the MR and enhance its transcriptional activity in a transient transfection assay (Colombe *et al*, 2000; Sturm *et al*, 2005).

Within tetrapods the glucocorticoids mediate their effects through both the glucocorticoid and mineralocorticoid receptors and often bind with a higher affinity to the latter receptor, while mineralocorticoids such as aldosterone bind exclusively to the mineralocorticoid receptor. Thus in cells expressing both GRs and MRs

glucocorticoids present at low concentrations are expected to bind to and exert their effects primarily through the mineralocorticoid receptor, while at higher concentrations will act via both receptors as the MR become saturated. Several mechanisms have been identified, both intracellular and extracellular, which limits the availability of glucocorticoid hormone to mineralocorticoid-responsive tissues, allowing the preferential binding of the mineralocorticoid hormone to the mineralocorticoid receptor.

11 -hydroxysteroid dehydrogenase (11 -HSD) is an enzyme with the ability to convert the active cortisol into its inactive form, cortisone, and vice versa (Buckingham, 2006). Two forms of 11 -HSD exist, 11 -HSDI and 11 -HSDII, responsible for activating and inactivating glucocorticoids, respectively. In aldosterone-responsive cells of the kidneys and the intestines, 11 -HSDII is highly expressed allowing preferential binding of the mineralocorticoid aldosterone to mineralocorticoid receptors. The 11 -HSDI isoform is mainly expressed in tissues in which the mineralocorticoid receptor is sparse but in which there is an abundance of the glucocorticoid receptor, such as the brain and the heart. This allows the generation of active glucocorticoids from inert cortisone and 11-dehydrocorticosterone, respectively, and thus allows glucocorticoids to be used as the primary ligands in these cells.

Corticosteroid-binding globulin (CBG), a member of the serine proteinase inhibitor (serpin) protein superfamily binds approximately 95% of total circulating glucocorticoids (Buckingham, 2006). According to the free hormone hypothesis only glucocorticoids unbound by CBG are able to enter cells and activate receptors and thus by altering the amount of hormone bound by CBG the level of free hormone

available to cells can be regulated (Breuner and Orchinik, 2002). CBG has also been suggested to have a degree of control over the localisation of delivery of glucocorticoids to the vasculature, for example to sites of inflammation (Pemberton *et al*, 1988). Corticosteroid-binding globulin is a clade A member of the serpin superfamily but, unlike most other members of this clade, CBG shows no proteinase inhibitory functions (Klieber *et al*, 2007). Instead it has been found that CBGs are a target for elastase, an enzyme released by neutrophils at sights of inflammation. Cleavage of CBG by elastase changes its conformation from a stressed state to a relaxed state, eradicating its steroid-binding ability. Therefore neutrophil release of elastase can mediate the localised release of steroid hormone from CBG at sights of inflammation.

P-glycoproteins are membrane bound proteins which are known to actively export both dexamethasone and cortisol out of cells. Glucocorticoid receptors have been shown to have a high affinity for the synthetic glucocorticoid, dexamethasone (Dekloet, 1975) but, unlike corticosterone, its administration to rats does not lead to its nuclear sequestration in the cells of the hippocampus. Disruption of the gene encoding P-glycoprotein leads to a greater sequestration of dexamethasone in the hippocampus of rodents compared to those containing a functional P-glycoprotein (Meijer *et al*, 1998), indicative of a role for P-glycoproteins in the active extrusion of dexamethasone from cells of the hippocampus. Recent evidence suggests that P-glycoprotein-mediated extrusion of glucocorticoids may also function in the placental glucocorticoid barrier, (Mark and Waddell, 2006) where disruption of the barrier results in placental apoptosis (Waddell *et al*, 2000), retarded foetal growth and alterations in postnatal phenotype (Smith and Waddell, 2000).

1.3.4 Function of CRs

1.3.4.1 Energy homeostasis

Addison's disease and Cushing's syndrome are two conditions resulting from the abnormal regulation of glucocorticoid levels within animal models, the underlying causes of these conditions being decreased and increased levels of glucocorticoids, respectively. Addison's disease is characterised by impaired stress responses, lymphoid tissue hypertrophy, weight loss and hypoglycaemia whereas Cushing's syndrome results in obesity, the increased breakdown of skeletal muscle mass, hyperglycaemia, liver steatosis, high cholesterol, immunodeficiency and insulin resistance. The pathologies of these two conditions highlights the importance of regulated glucocorticoid levels and also serves to underline the wide range of metabolic functions which are regulated by glucocorticoids.

In the liver the glucocorticoid receptor controls many aspects of energy metabolism, particularly protein and carbohydrate homeostasis. Glucocorticoids act as opposing hormones to insulin, with the ability to induce the transcription of genes involved in hepatic gluconeogenesis. This has been demonstrated in human subjects infused with cortisol over a four hour period, in which plasma glucose concentrations were increased compared to a control group in which no cortisol was administered (Khani and Tayek, 2001).

Reports of changes in plasma glucose levels as an indicator of the metabolic response of teleosts to cortisol have been mixed, with an increase reported in rainbow trout (*Oncorynchus mykiss*) (Barton *et al*, 1987), the sea raven (*Hemitripterus americanus*) (Vijayan *et al*, 1996) and coho salmon (*Oncorynchus kisutch*) (Vijayan and

Leatherland, 1989), no change reported in rainbow trout (Andersen *et al*, 1991) and brook trout (*Salvelinus fontinalis*) (Tam *et al*, 1988) and decreased in brook charr (*Salvelinus fontinalis*) (Vijayan *et al*, 1991), and American eels (*Anguilla rostrata*) (Foster and Moon, 1986). The inconsistencies in plasma glucose levels in response to cortisol may be attributed to differences in experimental methods or may be due to differences between species, developmental stage or nutritional status (Martinez-Porchas *et al*, 2009). Nevertheless, the consensus for teleosts is that cortisol enhances gluconeogenesis, with an increase in the expression of the main gluconeogenic genes including glucose 6-phosphatase (G6Pase), fructose 1,6-bisphosphatase and phosphoenol pyruvate carboxykinase (PEPCK) (Vijayan *et al*, 1996; Dziewulska-Szwajkowska *et al*, 2003; Sathiyaa and Vijayan, 2003). Furthermore glucocorticoid response elements (GREs) have been identified in the promoter regions of PEPCK and G6Pase (Scott *et al*, 1998; Vander Kooi *et al*, 2005), suggesting the regulation of such genes in response to cortisol is via the glucocorticoid receptor. Indeed, mice defective in hepatic glucocorticoid receptors are unable to upregulate the expression of genes coding for PEPCK and G6Pase, and as a result display a hypoglycaemic state under conditions of starvation (Opherk *et al*, 2004). Diabetic mouse models treated with an antisense oligonucleotide against hepatic glucocorticoid receptors in order to reduce their expression demonstrate a significantly greater inhibition of insulin-mediated hepatic glucose production and reduction of PEPCK and G6Pase activity, which resulted in significantly lowered blood glucose level (Liang *et al*, 2005), suggesting that glucocorticoids and their receptors may play a role in the hyperglycaemic phenotype of diabetes by upregulating genes involved in the gluconeogenic pathway. Since the glucocorticoid receptor has been shown to be upregulated in diabetic mouse models (Liu *et al*, 2005), downregulation of the

hepatic receptor, or an inhibition of its activity, may provide one mechanism by which the glucose profile of diabetics can be improved.

As well as a role in carbohydrate homeostasis, glucocorticoids have a role in lipid homeostasis as evidenced by Cushing's syndrome and the metabolic syndrome, two conditions in which obesity is a hallmark, and in which glucocorticoid levels are in excess (Walker, 2006). Furthermore the glucocorticoid receptor antagonist RU486 reverses the obese phenotype in rats (Langley and York, 1990), providing evidence that raised levels of glucocorticoids participate in the obese phenotype via the glucocorticoid receptor.

Within adipose tissue, overexpression of the enzyme 11 -hydroxysteroid dehydrogenase 1 (HSD1) and thus an excess of the active form of glucocorticoids, has been shown to increase abdominal fat deposits (Rask *et al*, 2002; Lindsay *et al*, 2003) contributing to the metabolic syndrome and promoting insulin-resistance. Glucocorticoids have also been shown to increase preadipocyte differentiation in primary and cell cultures (Gregoire *et al*, 1998) and the glucocorticoid receptor, acting as a transcription factor, can cooperate with other transcription factors and complexes to promote adipocyte differentiation. One such transcription factor whose expression is upregulated by the glucocorticoid receptor is the peroxisome-proliferator activated receptor (PPAR), which itself has been shown to promote adipocyte differentiation (Wu *et al*, 1996). Thus it would appear that that manifestation of the metabolic syndrome is mediated in part through increased levels of 11 -HSD1 together with the cooperative interaction of the glucocorticoid receptor with other transcription factors that promote adipocyte differentiation. Consequently, the inhibition of the 11 -HSD1 enzyme has emerged as a potential therapy in the

treatment of the metabolic syndrome and associated disorders (Tomlinson and Stewart, 2005).

As with the gluconeogenic pathway in teleosts the cortisol-mediated regulation of lipid metabolism in fish species is not without conflicting results, the root cause of which may be due to experimental or species differences (Mommsen *et al*, 1999). However, the prevailing evidence indicates a role for cortisol in peripheral and hepatic lipolysis. For example, an increase in peripheral lipolysis is observed in response to elevated cortisol levels in European eel and salmon parr (Lidman *et al*, 1979; Sheridan, 1989) while cortisol implantation in coho salmon caused reductions in total lipid concentration and triacylglycerol content in both the liver and muscle (Sheridan, 1986).

In addition to measured effects on lipid levels, effects on enzymes with a function in lipid homeostasis are reported to occur in response to cortisol. The activity of hydroxyacyl coenzyme A dehydrogenase (HOAD), an enzyme involved in catalysing the third step of fatty acid -oxidation, was shown to be higher in cortisol-treated sea ravens than in sham treated individuals and in brook charr reared at high stocking density (Vijayan *et al*, 1990; Vijayan *et al*, 1996).

Glucocorticoids have been proposed to function in protein and glucose metabolism in skeletal muscle. In both Cushing's syndrome and steroid therapy muscle atrophy (the loss of skeletal muscle mass) is common. The maintenance of muscular mass depends on the activation of protein synthesising pathways, such as the phosphatidylinositol-3 kinase (PI3K)/Akt signalling pathway, which leads to the promotion of glucose utilisation and protein synthesis while inhibiting protein

degradation. Several lines of evidence suggest a glucocorticoid-mediated inhibition of the PI3K signalling pathway thus inducing muscle atrophy. Several mechanisms as to how glucocorticoids inhibit this pathway have been proposed, including the inhibition of signalling proteins upstream of PI3K (Wang *et al*, 2007), an increase in the ratio of regulatory to catalytic PI3K subunits (Ueki *et al*, 2002; Brachmann *et al*, 2005) and, more recently a direct intracellular interaction between glucococorticoid receptors and the p85 subunit of PI3K (Zhaoyong *et al*, 2009).

1.3.4.2 Osmoregulation

Euryhaline fish species, which includes European flounder (*Platichthys flesus*), can tolerate a wide range of salinities from fresh to salt water. The gills, kidneys and the intestine of fish are the main organs involved in osmoregulation within teleosts. The gills contain chloride cells which in turn contain high concentrations of the enzyme sodium-potassium-activated adenosine triphosphate (Na+, K+-ATPase), which actively transports portassium into the cell while transporting sodium out to maintain the ionic gradient across the plasma membrane (Mobasheri *et al*, 2000). Two catalytic -subunit isoforms of Na+, K+-ATPase, 1a and 1b, have been identified in the gills of rainbow trout and Atlantic salmon which are differentially regulated during seawater transfer. While 1b is upregulated in response to seawater acclimation, 1a is downregulated, suggesting these isoforms to function in seawater and freshwater acclimation, respectively (Richards *et al*, 2003; McCormick *et al*, 2009). Evidence to date suggests a role for cortisol and the corticosteroid receptors in the regulation of Na+, K+-ATPase activity. Administration of cortisol to gilthead seabream (*Sparus aurata*) and freshwater tilapia (*Oreochromis mossambicus*) results in an increase in gill Na+, K+-ATPase activity and increased hypoosmoregulatory

capacity (Dang *et al*, 2000; Laiz-Carrion *et al*, 2002; Laiz-Carrion *et al*, 2003) while seasonal increases in circulating plasma cortisol levels in salmon species is associated with elevated Na +, K+, -ATPase activity and increases in intestinal fluid absorption, suggesting a role for cortisol during the parr-smolt transformation (Veillette *et al*, 1995; Veillette and Young, 2004). The regulation of Na+, K+-ATPase enzyme activity by corticosteroid receptors may occur via the upregulation of the Na+, K+-ATPase gene in a corticosteroid receptor-dependent manner, following the discovery of a functional glucocorticoid response element (GRE) within the promoter region of the 1 subunit of the human, rat and European eel Na+, K+-ATPase gene (Derfoul *et al*, 1998; Kolla and Litwack, 2000; Cutler *et al*, 2000; Hao *et al*, 2003).

An increase in the expression of corticosteroid receptors has been reported upon the transfer of fish species from freshwater to saltwater (Mizuno *et al*, 2001; Dean *et al*, 2003; Takahashi *et al*, 2006; Kiilerich *et al*, 2006; Yada *et al*, 2008). Within the intestine of the Mozambique tilapia (*Oreochromis mossambicus*), glucocorticoid receptors are localised to the posterior section where an increase in glucocorticoid receptor mRNA in freshwater-adapted individuals exposed to seawater has been reported, subsequent to an increase in plasma cortisol levels (Takahashi *et al*, 2006). Chronic cortisol treatment mimicked the effects of seawater adaptation, increasing glucocorticoid receptor mRNA, suggesting that increases in mRNA levels during seawater adaptation may be due to chronic release of cortisol from the interrenal tissue in euryhaline fish species.

Inhibitors of the glucocorticoid receptor can block the effects of cortisol in osmoregulation with observed decreases in activity and expression of ion

transporters, an inhibition of intestinal fluid absorption and a decreased tolerance to hyperosmotic conditions having been reported (Veillette *et al*, 1995; Veillette *et al*, 2007; McCormick *et al*, 2008; Kiilerich *et al*, 2011).

Although a role for the glucocorticoid receptor in osmoregulation is well documented, the role of the mineralocorticoid receptor is less clear. Changes in MR mRNA levels upon exposure to seawater are somewhat conflicting. While an upregulation of MR mRNA has been reported in the gills of Atlantic salmon and rainbow trout following seawater exposure (Yada *et al*, 2008; Nilsen *et al*, 2008), Kiilerich *et al* (2011) reported no change in the expression of gill MR mRNA during acclimation of rainbow trout to seawater, although an increase in kidney and intestine MR mRNA has been reported (Yada *et al*, 2008; Kiilerich *et al*, 2011). This may indicate that the response of the MR to hyperosmotic conditions is species- or developmental stage-specific.

A role for the mineralocorticoid receptor in acclimation to freshwater has been suggested following the observation that spironolactone, a specific inhibitor of the MR, could inhibit the proliferation of chloride cells in gill tissue (Sloman *et al*, 2001; Scott *et al*, 2005). The proliferation of chloride cells is believed to contribute to ionic homeostasis in freshwater fish by enhancing brachial ion uptake and evidence suggests this proliferation is mediated by cortisol; chronic administration of cortisol to rainbow trout increased sodium and chloride ion influx in the gills concomitant with an increase in the number and individual apical surface area of gill chloride cells per area of filament epithelium (Laurent and Perry, 1990).

Within mammalian and amphibious species, the mineralocorticoid hormone aldosterone has been found to increase expression of the serum glucocorticoid kinase (GSK) gene which has been found to strongly stimulate sodium transport in tight epithelia via the epithelial sodium channel (ENaC) (Chen *et al*, 1999; Pearce *et al*, 2000). Interestingly, the rodent glucocorticoid corticosterone was unable to induce GSK expression in adrenalectomised rats (Pearce *et al*, 2000), suggesting the mineralocorticoid is important in the regulation of GSK expression. Indeed, a glucocorticoid/mineralocorticoid response element has been found in the promoter region of the GSK gene (Webster *et al*, 1993; Maiyar *et al*, 1997), providing further evidence that the mineralocorticoid receptor can regulate the expression of this gene. More recently the expression of SGK in the euryhaline longjaw mudsucker (*Gillichthys mirabilis*) and the Killifish (*Fundulus heteroclitus*) has been shown to be altered in response to an osmotic challenge (Shaw *et al*, 2008; Evans and Somero, 2008). Furthermore in response to an increase in SGK, an increase in CFTR, a chloride-ion channel, was observed in the gills of the killifish (Shaw *et al*, 2008), suggesting a common role for SGK across a diverse range of vertebrate species in osmoregulation.

1.3.4.3 Inflammatory response

Like the peroxisome proliferator-activated receptors, glucocorticoid receptors are well recognised as functioning in the inflammatory response (Necela and Cidlowski, 2004), but this effect has been recognised for longer and is far more pronounced. As a consequence glucocorticoid hormones are widely prescribed for the treatment of inflammatory disorders such as rheumatoid arthritis, asthma and dermatitis, and in autoimmune diseases such as Crohn's disease (Barnes, 1998). The primary

mechanism through which glucocorticoids function to reduce the inflammatory response is to suppress, either directly or indirectly, the activation of pro-inflammatory cytokine genes (Barnes, 1998). Like PPARs the glucocorticoid receptor can inhibit the inflammatory response by affecting the actions of NF B, including causing upregulation of inhibitor of kappa B- (I B-) (Auphan *et al*, 1995; Scheinman *et al*, 1995), competition for essential NF B co-activator proteins (Sheppard *et al*, 1998) and direct protein-protein interactions between NF B and the GR (Adcock *et al*, 1999) (Figure 1-6).

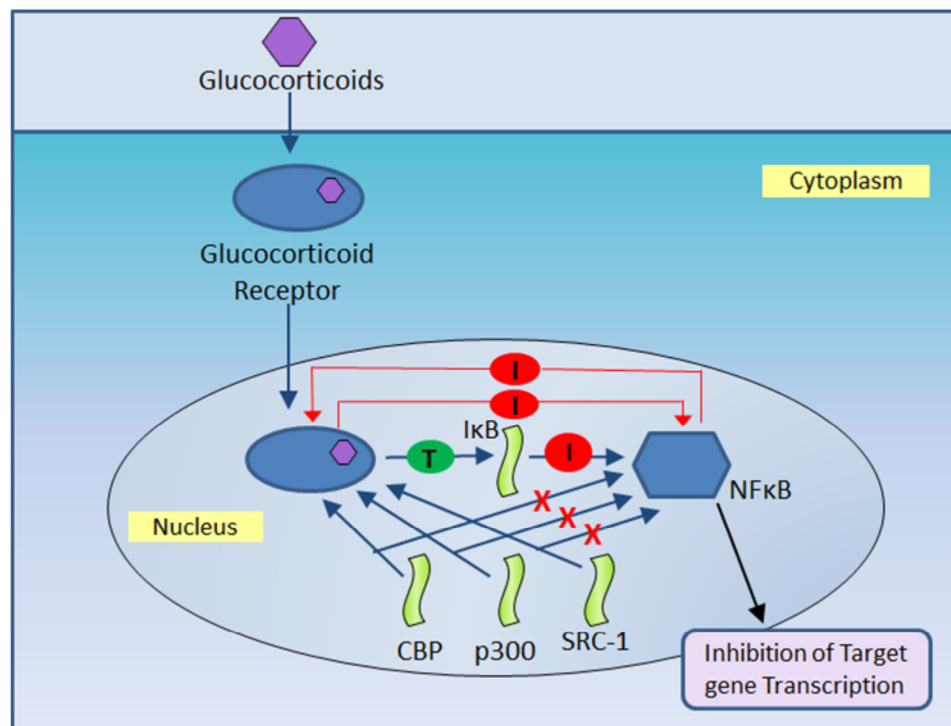


Figure 1-6. Glucocorticoid receptors are hypothesised to inhibit NF B activity by direct interactions with the pro-inflammatory transcription factor NF B, transcription of the NF B inhibitor I B and by competition for NF B co-activators (e.g. CBP, p300 and SRC-1).

I B- functions in resting cells to maintain NF B in the cytoplasm, thus impeding its ability to transcribe its target genes. Upon phosphorylation and subsequent ubiquitination, I B- is targeted for proteosomal degradation, leaving NF B free to

enter the nucleus and alter gene transcription. In 1995, two independent research groups reported an increase in I B- protein following administration of glucocorticoids (Auphan *et al*, 1995; Scheinman *et al*, 1995). However since these first reports, several research groups have failed to observe an increase in I B- protein following administration of glucocorticoids (Adcock *et al*, 1999; Pruett *et al*, 2003) or the observed increase was at a level insufficient to prevent its targeted degradation and to prevent the translocation of NF B to the nucleus (Auwardt *et al*, 1998). Furthermore a mutant glucocorticoid receptor with the inability to dimerise, and which does not increase levels of I B- , is still able to repress NF B (Heck *et al*, 1997). Together these results suggest that the mechanism of glucocorticoid-mediated increases in I B levels is restricted to certain cell types and is not a universal mechanism.

Glucocorticoid receptors have been hypothesised to compete with NF B for essential co-activator proteins, thus limiting the amount of co-activators available for NF B activation and therefore inhibiting activation of its transcriptional activities (Sheppard *et al*, 1998). The p65 subunit of NF B has been shown to interact with the co-activators CREB-binding protein (CBP), p300 and SRC-1 and co-regulatory proteins potentiate the p65-mediated transcription of reporter constructs (Gerritsen *et al*, 1997). Previously the glucocorticoid receptor has been observed to repress transcription of non-GR target genes via competition for limiting amounts of CBP and this repression was abolished upon expression of increasing levels of CBP (Kamei *et al*, 1996). Despite this finding, several lines of evidence suggest that competition for co-activator proteins is not the mechanism by which the GR represses NF B activity. For example while it is true that increasing levels of CBP

increases the transcription of several NF B-driven genes in the absence of glucocorticoids (Sheppard *et al*, 1998; De Bosscher *et al*, 2000), an overexpression of CBP, p300 or SRC-1 and the presence of glucocorticoids does not relieve the repression (De Bosscher *et al*, 2000). A lack of specificity in the model is another shortcoming of this competition model; both CBP and p300 interact with various transcription factors and thus each of these proteins, along with the glucocorticoid receptor, could be considered competitors and be expected to transrepress the activity of NF B, which is not the case. The third model proposed for glucocorticoid-mediated inhibition of NF B is one involving direct protein-protein interactions between the glucocorticoid receptor and NF B. Indeed such an interaction between NF B and the glucocorticoid receptor has previously been reported (Adcock *et al*, 1999) which resulted in decreased expression of the NF B target, interleukin-6 (IL-6) (Ray and Prefontaine, 1994), a cytokine overexpressed in rheumatoid arthritis (Papanicolaou *et al*, 1998). Multiple regions of the GR are required for effective transrepression of NF B; GR mutants lacking the DNA-binding domain, and more specifically either of the zinc finger regions, the steroid-binding domain or part of the amino-terminal A/B domain are unable to repress NF B activity (McKay and Cidlowski, 1998).

1.3.4.4 CRs in reproductive function

Within vertebrates the hypothalamic-pituitary-gonadal (HPG) axis controls reproduction with the actions of the main hormones, including gonadotropin releasing hormone (GnRH), gonadotrophins and gonadal sex-steroids having been extensively described. Within fish species, increasing evidence suggests a role for the corticosteroid hormones in the endocrine control of reproduction.

Several adverse effects on teleost reproductive performance have been reported in response to stress. Within females adverse effects included a reduction in vitellogenesis, oocyte size, impaired zonagenesis and a reduced progeny survival in Atlantic salmon and rainbow trout in response to thermal and confinement stress, respectively (Campbell *et al*, 1994; King *et al*, 2003). These effects may be related to cortisol levels since increased cortisol has been reported in response to stress (Barton and Peter, 1982; Pickering and Pottinger, 1989; Vijayan *et al*, 1997) and has been linked to a disruption of the HPG-axis, vitellogenesis, androgen and estrogen signalling (Teitsma *et al*, 1999; Pankhurst and Van Der Kraak, 2000; Lethimonier *et al*, 2000; Consten *et al*, 2002; Berg *et al*, 2004; Berg *et al*, 2004).

Despite these adverse effects, several lines of evidence also support a positive role for corticosteroids in fish reproduction. Gonads of both male and female fish species have the potential to produce corticosteroid hormones such as cortisol, 11-DOC, corticosterone and 11-deoxycorticosterone, possessing all the enzymes responsible for their synthesis (Kobayashi *et al*, 1998; Kusakabe *et al*, 2002; Kazeto *et al*, 2003; Li *et al*, 2003; Socorro *et al*, 2007; Zhou *et al*, 2007). 11-deoxycorticosteroids have been shown to be important products of ovarian and testicular steroidogenesis (Colombo *et al*, 1978; Tesone and Charreau, 1980; Kime *et al*, 1992) and the presence of cortisol and 11-DOC in the ovary, sperm and seminal fluid supports the theory of gonadal steroidogenesis (Scott *et al*, 1991a; Scott *et al*, 1991b). Corticosteroid receptors have been found to be expressed within both male and female fish gonads. In rainbow trout the glucocorticoid receptor has been detected in the ovary (Takeo *et al*, 1996; Milla *et al*, 2006) while both the glucocorticoid and the mineralcorticoid receptors have been detected in the testes of trout and other species

(Takeo *et al*, 1996; Park *et al*, 2007; Filby and Tyler, 2007; Milla *et al*, 2008). Furthermore, 11 -HSD2 expression, which prevents the elicit activation of the mineralocorticoid receptor by catabolising cortisol, is found in both male and female reproductive organs, particularly from gametogenesis to the final stages of maturation, and displays activity in the testes (Kusakabe *et al*, 2003). A role for the corticosteroid hormones in oocyte maturation has been suggested since treatment with luteinizing hormone (LH) leads to *in vitro* follicular and interrenal synthesis of DOC and cortisol (Colombo *et al*, 1973; Truscott *et al*, 1978; Sundararaj *et al*, 1979). More recently administration of the human chorionic gonadotropin, used extensively to induce ovulation in fishes, was demonstrated to increase corticosteroid production which was associated with oocyte maturation (Mishra and Joy, 2006). In agreement with a role for corticosteroid hormones in oocyte maturation, 11-DOC has been found to be the most effective hormone, both *in vivo* and *in vitro*, in germinal vesicle breakdown (GVBD), a measure of oocyte final maturation, in the cyprinid species *Chalcalburnus tarichi* (Unal *et al*, 2008) and in brook trout (Duffey and Goetz, 1980). This latter effect may be species-dependent however since corticosteroid hormones are relatively ineffective, except at high concentrations, in inducing oocyte maturation in Coho salmon and are not effective in rainbow trout (Jalabert *et al*, 1972; Sower and Schreck, 1982). However corticosteroids have been shown to enhance the actions of gonadotropin and steroids on the induction of GVBD in rainbow trout (Jalabert, 1975). Associated with oocyte maturation is the biological process of oocyte hydration, characterised by an increased oocyte volume and oocyte swelling due to water influx and the entry of inorganic ions (Greeley *et al*, 1991; Finn *et al*, 2002). Injection of a high dose of cortisol was demonstrated to increase hydration in muscle and ovarian tissues in the Nile tilapia (*Oreochromis nilotica*),

and while sodium ion content was found to decrease in muscle an increase in ovarian sodium content was observed (Babiker and Ibrahim, 1979). More recently, and in agreement with this previous study, Milla *et al* (2006) reported an increase in the wet mass of rainbow trout oocytes in response to incubation with cortisol, concurrent with an increase in the mRNA levels of GR2, suggesting cortisol may function via GR2 in the control of oocyte hydration of rainbow trout.

Corticosteroids have also been implicated in male reproduction, and more specifically in spermatogenesis. Cortisol at 0.1-100ng/mL has been shown to induce spermatogonial mitosis by increasing levels of 11-ketotestosterone, the primary androgenic hormone in fish species, in the testes of Japanese eel (Ozaki *et al*, 2006). In immature bronze featherback fish (*Notopterus notopterus*), cortisol administration increased the testicular-somatic index (a measure of sexual maturity in fish species) and the onset of spermatogenesis (Shankar and Kulkarni, 2000). In contrast, higher levels of cortisol (100ng/mL) have been shown to inhibit the effect of 11-ketotestosterone on spermatogenesis (Ozaki *et al*, 2006), suggesting excess cortisol to be deleterious to male reproduction. Indeed following stress or administration of cortisol, male gonadal development was inhibited, characterised by smaller gonads, retarded spermatogenesis and a lower sperm quality (Campbell *et al*, 1992). Furthermore in common carp administration of RU486, the glucocorticoid receptor inhibitor, was able to prevent the effects of cortisol on gonadal development and spermatogenesis delay (Goos and Consten, 2002), demonstrating cortisol is likely acting via the GR to mediate these effects.

An adverse effect on all components of the HPG axis in immature common carp (*Cyprinus carpio*) has been reported following long-term exposure to dietary cortisol

(Consten *et al*, 2001). A decrease in brain GnRH content, mRNA levels of LH and FSH in the pituitary, and plasma levels of LH was reported, as was a decrease in testicular androgen secretion. Although it could not be determined whether the effects on gonadotropins was a direct effect on the pituitary or the hypothalamus or whether the reduced expression and release was due to a decrease in testicular androgen release, a reduced expression of GnRH in the brain suggests the effect is likely due to decreased secretion of GnRH.

In addition to cortisol a role for DOC as a regulator of spermiation, the reproductive stage when hydrated sperm are released from the testis to the vas deferens, in the rainbow trout has been suggested. This suggestion came following several observations; firstly DOC is up to 50-times higher in mature males than in immature males, increasing rapidly at the end of the reproductive cycle, and secondly the expression of MR, although higher in immature individuals than in mature individual, increases rapidly during the period of spermiation, immediately after the increase in plasma DOC (Milla *et al*, 2008).

Thus it appears that corticosteroids, of which cortisol is the primary example in fish species, exerts both positive and negative effects on fish reproduction (Figure 1-7). Higher levels of cortisol appear to inhibit vitellogenesis in female species, while disrupting gonadal development and spermatogenesis in males. A disruption of the hypothalamic-pituitary-gonadal axis is observed in both males and females. In females, evidence suggests a positive role for cortisol in oocyte maturation while in males spermatogenesis may be positively influenced. This information implies a role for corticosteroid hormones in the endocrine control of fish reproduction and some of

the effects may be mediated by activation of the glucocorticoid and/or mineralocorticoid receptors.

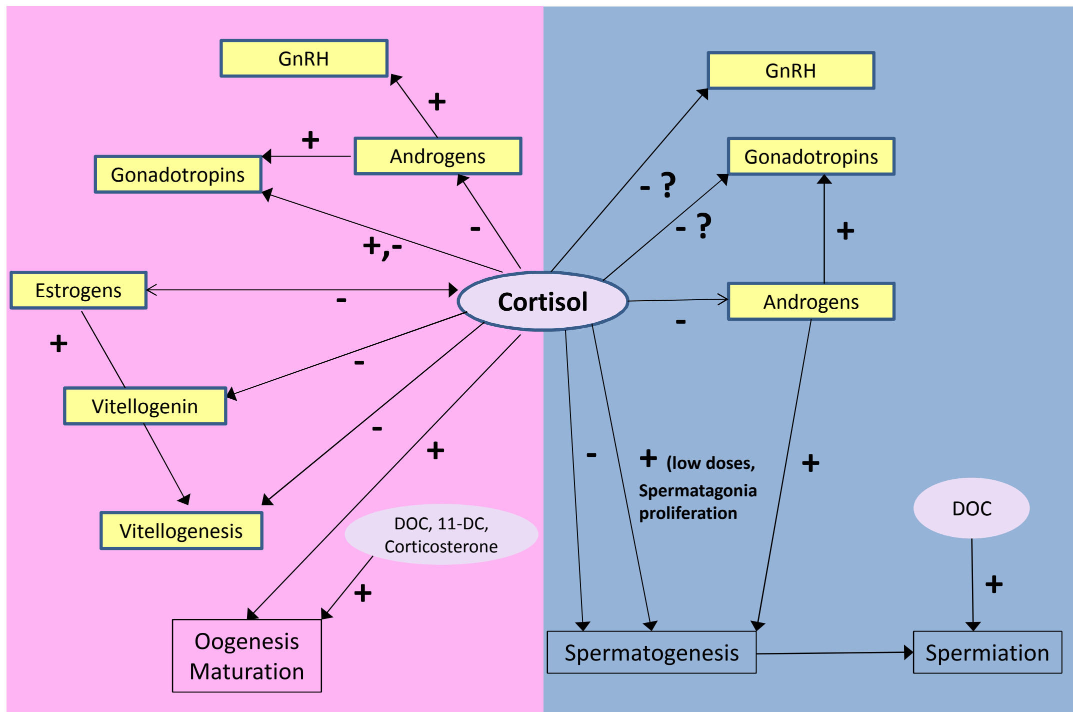


Figure 1-7. The implications of corticosteroids in female and male teleost reproduction. DOC – 11-deoxycorticosterone; 11-DC – 11-deoxycortisol; GnRH – gonadotropin-releasing hormone. + - indicates a positive influences of corticosteroids; - indicates a negative influences of corticosteroids; -? – indicates a suspected negative effect of corticosteroids. See text for details. Figure adapted from Milla et al (2009).

1.4 Endocrine disruption in aquatic organisms

Endocrine disruptors can be defined as exogenous substances which interfere with the endocrine system either by:

1. Mimicking the actions of a naturally-produced hormone, producing a similar response from the target receptor (agonist;)

2. Blocking the hormone receptor in target cells, preventing the actions of endogenous hormones (antagonist;)

3. Altering the synthesis, transport, metabolism and excretion of endogenous hormones, thus disrupting hormone homeostasis

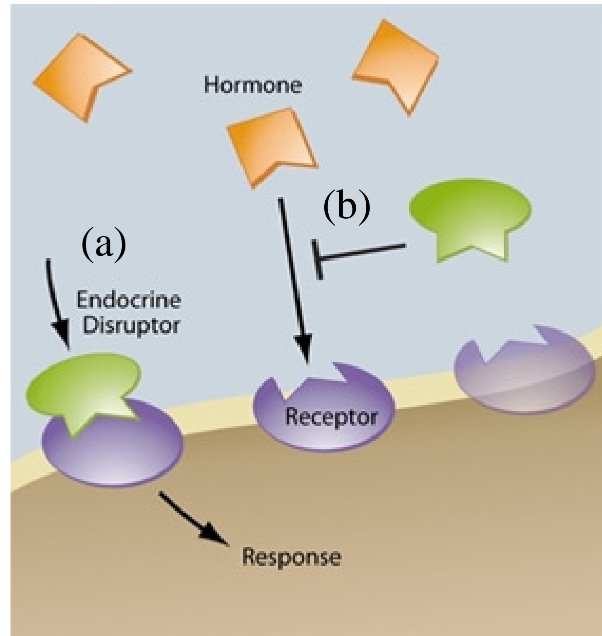


Figure 1-8. A diagrammatic representation of agonistic and antagonistic actions by endocrine disruptors. Endocrine disruptors (represented in green) can disrupt signalling pathways by a) binding to a receptor and mimicking the actions of a naturally produced hormone or b) block the hormone receptor, thus preventing the actions of endogenous hormones (represented in orange).

Behavioural, developmental, and reproductive effects have been reported in aquatic organisms in response to exposure to anthropogenic compounds which pollute aquatic environments. Such compounds include human pharmaceuticals, agricultural and industrial by-products.

1.4.1 Heavy metals

The increased use of heavy metals, such as cadmium and copper, in agricultural, chemical and industrial processes has resulted in their accumulation in the environment. Mounting evidence over the last decade suggests these metals to be endocrine disruptors in aquatic organisms.

Cadmium, widely used in pesticides including fungicides and insecticides and in commercial fertilizers, is a well-documented endocrine disruptor. The predator-avoidance behaviour of rainbow trout is eliminated upon a 7 day exposure to 2µg/L cadmium (Scott *et al*, 2003), characterised by a decrease in feeding and swimming and an increase in latency between first feed and sheltering time compared to unexposed control fish. These observations may be due to the effect of cadmium on cortisol synthesis. In response to alarm substance (the chemical signal released from epidermal cells of injured fish to warn others of danger), plasma cortisol levels in fish are rapidly elevated (Rehnberg and Schreck, 1987). However, brown trout (*Salmo trutta*) from the cadmium-contaminated Eagle River, Colorado, when placed under confinement stress, display a delayed elevation of plasma cortisol levels compared to fish from uncontaminated waters of the same river (Norris *et al*, 1999). Furthermore, raised cortisol levels could not be sustained suggesting that cadmium exposure impacts upon the hypothalamo-pituitary-interrenal (HPI) axis, resulting in a delayed or dampened response to stressors. Indeed, it has recently been demonstrated that cadmium suppresses ACTH-stimulated cortisol secretion in head kidney slices of rainbow trout, in parallel with decreased levels of steroidogenic enzymes important for corticosteroid biosynthesis (Sandhu and Vijayan, 2011).

Reproductive abnormalities have also resulted from the exposure of organisms to heavy metals. A significant decrease in the number of ovarian granulosa cells as well as a decrease in the binding of follicle stimulating hormone (FSH) and luteinising hormone (LH) to their receptors on the surface of granulosa cells has been reported in female rats exposed to either cadmium, lead or a combination of both (Nampoothiri and Gupta, 2006). The authors also found the membrane composition

of granulosa cells to be affected by exposure to cadmium as demonstrated by the decrease in cholesterol and phospholipids content and an increase in the fluidity of the membrane. This could be one explanation for the decrease in hormone binding since an alteration in the fluidity of the membrane could result in a conformational change in the membrane bound receptors, preventing the binding of the gonadotropin hormones. Not only does cadmium inhibit the binding of gonadotropin hormones to their receptors, thus inhibiting cholesterol uptake, but it also interferes with the progesterone biosynthesis pathway as demonstrated by the failure of the cytochrome P450 side-chain-cleavage (P450scc) enzyme to convert cholesterol to pregnenolone in the presence of cadmium in cultured human placental cells (Kawai *et al*, 2002).

Mercury, which enters the aquatic environment via atmospheric deposition from sources such as coal power plants, is found in fish predominantly in its most toxic form, methylmercury (Bloom, 1992). Studies concerning aquatic organisms have shown methylmercury to be an endocrine disruptor, altering the expression of genes within the liver, including those related to reproduction (Klaper *et al*, 2006). One such gene is that encoding for vitellogenin, a protein associated with egg growth and normally produced only in females. However a 142.8-fold increase in the levels of vitellogenin mRNA in male fathead minnows (*Pimephales promelas*) exposed to dietary methylmercury has been reported, while in females a downregulation in vitellogenin expression was reported (Klaper *et al*, 2006). The downregulation of vitellogenin mRNA in females could potentially reduce egg production and hence affect reproductive success in fathead minnows. As well as genes associated with reproduction, apoptotic genes are upregulated in female fathead minnows (Klaper *et al*, 2006). Ovarian follicular apoptosis increased after exposure to methylmercury,

resulting in a decrease in the production of 17- estradiol which subsequently led to impaired reproduction (Drevnick *et al*, 2006). The effects of methylmercury on reproduction are unlikely to be specific to female fish since male Wister rats exposed to methylmercuric chloride experience a decrease in testicular weight and sperm production and an increase in spermatocyte and spermatid (Homma-Takeda *et al*, 2001).

1.4.2 Pesticides

Pesticides encompass a wide range of chemicals including bactericides, fungicides, insecticides, herbicides and molluscicides. Many of these are classed as persistent chemicals, surviving in the environment long after their use has ceased. An example of such a chemical is the pesticide dichloro-diphenyl-trichloroethane (DDT), which is still detected in human breast milk and fat despite having been banned in the UK, USA and many other countries since the 1970s and 1980s. Traces of DDT can still be detected in individuals, irrespective of whether it was ever used in the country in which the individuals reside. This has been found to be the case in many areas of the Arctic where traces of DDT as well as hexachlorobenzene (HCB), and hexachlorocyclohexane (HCH) have been found in the indigenous people of the Russian Arctic areas, transported with the northward flow of rivers, oceans and atmospheric currents (Webster, 2004).

Many persistent organic pollutants have been shown to bring about adverse health effects in mammals, as well as in aquatic organisms. A link has been made between rotenone, a compound used in pesticides, and the development of Parkinson's disease-like symptoms in rats (Betarbet *et al*, 2000). Parkinson's disease is a

neurodegenerative disorder characterised by the death of dopaminergic neurons in the brain. Betarbet *et al* (2000) noted that constant administration of rotenone to rats resulted in the disruption of complex I, a part of the mitochondria electron transport system noted to be disrupted in Parkinson's disease patients (Keeney et al, 2006). This disruption of complex I was accompanied by the degeneration of dopamine-producing circuits and the appearance of Lewy bodies, abnormal protein deposits that aggregate in nerve cells and that are characteristic of Parkinson's disease. *In vitro* primary dopaminergic cell cultures exposed to rotenone have been shown to lose neurons in a progressive concentration and time-dependent manner while non-dopaminergic neurons and glial cells were less affected, demonstrating the importance of dopaminergic neurons in the sensitivity to rotenone pesticide exposure (Radad *et al*, 2008). In the human population more than 90% of cases of Parkinson's disease are considered to be sporadic, having no relation to genetic mutation. Further to this there is a strong correlation between the development of the disease and rural living, farming and well-water drinking, giving weight to the hypothesis that pesticide exposure increases the risk for the development of Parkinson's disease.

Organotins, which includes tributyltin chloride (TBTC), tributyltin oxide (TBTO) and bis(triphenyltin) oxide (TPTO), are compounds which have been in use since the 1950s as agricultural fungicides, rodent repellents and molluscicides, as well as being a component of antifouling paint used to treat marine vessels. Organotins are one of the most widely recognised endocrine disruptors in the aquatic environment and have been shown to induce irreversible sexual abnormalities in exposed aquatic organisms (Wibe *et al*, 2001; Garaventa *et al*, 2007). Although less studied in the human population, organotins have been suggested to act as obesognes, disrupting normal

adipocyte cell differentiation and resulting in a predisposition to obesity and related metabolic disorders (Kanayama *et al*, 2005; Grün and Blumberg, 2006; Grün *et al*, 2006).

Within the aquatic environment molluscs appear particularly susceptible to endocrine disruption by organotin compounds, with pseudohermaphroditism having been widely reported (Camillo *et al*, 2004; Pellizzato *et al*, 2004; Santos *et al*, 2006; Lee *et al*, 2009). Pseudohermaphroditism is the superimposition of male characteristics, such as a penis and vas deferens, on female organisms (imposex) (Garaventa *et al*, 2007). The mechanism by which organotins result in imposex has remained largely elusive despite the existence of several hypotheses to explain the phenomenon, including the inhibition of the P450-mediated aromatase enzyme which catalyses the conversion of androgens into estrogens (Bettin *et al*, 1996), the inappropriate release of the neuropeptide Ala-Pro-Gly-Trp-NH2 (APGW amide), a suspected penis-morphogenic factor (Oberdörster *et al*, 2005) and through interactions with the RXR and/or PPAR receptors (Nishikawa *et al*, 2004; Kanayama *et al*, 2005; Grün *et al*, 2006; Nishikawa, 2006; Castro *et al*, 2007).

1.4.3 Pharmaceuticals

Many human pharmaceutical products enter the aquatic ecosystem via their incomplete removal from wastewater treatment plants and several have been shown to disrupt endocrine systems within fish and other aquatic species. Particular emphasis has been put on the effects of synthetic sex steroid hormones, such as those present in contraceptives pills, due to their potential to disrupt reproductive functions in non-target organisms at environmentally relevant concentrations.

One of the first observations of reproductive dysfunction in the aquatic environment was in the wild roach (*Rutilus rutilus*) inhabiting UK rivers (Jobling *et al*, 1998). A high number of roach testes contained numerous female germ cells and oocytes, and experiments on rainbow trout initially indicated that alkylphenol detergent pollutants may be the cause (White *et al*, 1994). However subsequent wider investigations showed that in most cases the actual environmental concentrations of these compounds were likely insufficient to cause these effects and both natural and synthetic estrogens were to blame (Desbrow *et al*, 1998). Concentrations of the synthetic estrogen compound 17 -ethinyloestradiol (EE2) have been measured in UK freshwater systems at concentrations up to 3.4 ng/L (Williams *et al*, 2003). At concentrations of 3-5 ng/L a disruption in the development of the testes in genetically male zebrafish exposed to EE2 was observed, together with development of feminized gonads, inhibiting reproduction (Nash *et al*, 2004; Fenske *et al*, 2005). The inhibition of male gonadal differentiation may be due to the effects of estrogen on the doublesex and mab-3 related transcription factor 1 (Dmrt1) and anti-müllerian hormone (AMH) genes, the expression of which are correlated with the period of early gonadal differentiation (Schulz *et al*, 2007). Sex-dependent differences in the expression of these genes have been described in zebrafish previously, with an overexpression in males compared to females. This overexpression is inhibited by exposure to EE2, and was associated with an inhibition of male gonadal sex development (Schulz *et al*, 2007). A similar downregulation of Dmrt1 and AMH has been described in fathead minnow (*Pimephales promales*) following exposure to EE2 (Filby *et al*, 2007).

The induction of vitellogenin, an egg yolk protein expressed in females of almost every oviparous species including fish, amphibians, reptiles, birds, most invertebrates and the platypus (het Schip *et al*, 1987; Brown *et al*, 1997; Babin, 2008), has become a molecular marker for exposure to estrogenic endocrine disrupting compounds (EDCs) in male fish species, while the inhibition of vitellogenesis in female species can provide an indication of disruption in the female reproductive axis (Kime *et al*, 1999). A number of studies have used vitellogenin levels in male organisms to demonstrate the presence of estrogenic chemicals in the aquatic environment. For example rainbow trout placed downstream of sewage-treatment works and exposed to sewage effluent had vitellogenin levels 500-100,000 fold greater than fish upstream of sewage effluent (Purdom *et al*, 1994), while European flounder from the polluted Mersey Estuary, UK, displayed vitellogenin levels up to a million times greater than control fish from the relatively unpolluted Alde River estuary (Allen *et al*, 1999).

In addition to synthetic sex steroids, synthetic glucocorticoids administered in large quantities as anti-inflammatories, have been detected in surface waters and effluents in the ng/L range (Chang *et al*, 2007; Schriks *et al*, 2010). Within fish, relatively low concentrations (1 µg/L) of individual glucocorticoids can cause effects (Kugathas and Sumpter, 2011) and the effect of a mixture of glucocorticoids, as occurs in the environment, is unknown. Therefore synthetic glucocorticoids released to the environment may function as endocrine disruptors in non-target organisms.

1.5 Chemical Pollution and Environmental Risk Assessment

Many pollutants, including industrial byproducts (bisphenol A, polychlorinated biphenyls and alkylphenols), plant-derived phytoestrogens (isoflavones and lignans)

and pesticides (DTT and methoxychlor), have been described as xenoestrogens, mimicking the endogenous estrogen hormone 17 -estradiol and exerting effects by binding to the endogenous estrogen receptor (Korach *et al*, 1988; White *et al*, 1994; Kuiper *et al*, 1998; Yadetie *et al*, 1999; Matthews *et al*, 2000; Tollefsen *et al*, 2002; Uchida *et al*, 2010).

As well as estrogenic activities bisphenol A is able to disrupt endocrine functions in rodents by impairing the binding of thyroid hormone to the thyroid receptor, in part by recruiting the co-repressor N-CoR to the receptor (Moriyama *et al*, 2002). Similarly binding of the synthetic glucocorticoid dexamethasone to the glucocorticoid receptor and subsequent GR-mediated transcription of the phosphoenolpyruvate carboxykinase (PEPCK) gene is inhibited following exposure of rat hepatoma cells to non-toxic doses of arsenic (Kaltreider *et al*, 2001). Since nuclear translocation of the GR was unaffected, the authors deduced that the inhibitory effect of arsenic is due to its ability to interact directly with the glucocorticoid receptor thus blocking the access of dexamethasone to the ligand-binding site.

This brief list of examples summarizes a potentially much broader picture where many different pollutants, particularly small lipophilic contaminants, are able to exert effects via interactions with NHRs, emphasising the need for an establishment of methods to predict such interactions, improve risk assessment methods and thus limit the risk of exposure to organisms.

1.5.1 Environmental Risk Assessment (ERA)

ERA is defined as the procedure by which the likely or actual adverse effects of pollutants and other anthropogenic activities on ecosystems and their components are estimated with a known degree of certainty using scientific methodologies (Depledge and Fossi, 1994) and is the foundation for many national and international regulatory actions.

Well established methods are used to regulate the discharge of chemicals to the environment. In general these methods compare the dose response of single chemicals in laboratory toxicity tests on various organisms, to the predicted or measured environmental concentrations and thus provide discharge limits to minimize environmental effects (van Leeuwen and Vermeire, 2007). Such approaches can be criticised because they only consider chemicals in singlicate and they rely on standardised, usually short-term tests, neither of which scenario is usual in the environment. For aquatic systems fish have traditionally been used as the test organisms. In the United States the main test species have been fathead minnows and bluegill sunfish (*Lepomis macrochirus*) while within the UK much toxicity work has been performed with rainbow trout (Mason, 2001). The use of fish species to test the many hundreds of thousands of chemicals used by man requires an enormous number of tests on living organisms. Guidelines for fish toxicity testing, issued by the environmental protection agency (EPA) and the Organisation for Economic Co-operation and Development (OECD), which oversees the developoment of environmental risk assessment methods, specifies the use of at least seven fish (EPA prefer 10) at each test concentration and in controls, with at least five concentrations tested for each compound. Additionally the EPA recommends duplicate tanks per

test concentration, thus requiring a minimum of 70 fish per pollutant compound assessed. Thus with the many compounds requiring assessment the use of live animals has very negative ethical, logistic and financial implications. For these reasons more complex testing strategies have been proposed. Hutchinson (2008) has suggested an Intelligent Testing Strategy designed to prioritize chemicals for toxicity testing with the aim of reducing the number of tests and consequently the number of animals required. These types of strategies initially gather chemical information to establish likely persitence and bioaccumulative effects then assess toxicity using a "tiered" approach. Thus, the first tier of toxicological testing may include *in vitro* or *in cellulo* tests designed to establish whether a particular chemical is likely to have a specific toxic mode of action. In this regard the US Environment Protection Agency (EPA) launched the ToxCast screening programme in 2007 with the aim of developing an approach to predict potential toxicity and prioritize the many thousands of chemicals requiring further testing (www.epa.gov/ncct/toxcast/ [Accessed 27 Feb 2012]). Furthermore, in response to the finding that a variety of compounds can disrupt the endocrine system of animals in laboratory studies, the EPA instigated, as a first tier testing regime, the assessment of interaction of chemicals with mammalian NHRs including the estrogen receptor, androgen receptor and thyroid hormone receptor (http://www.epa.gov/endo/index.htm [Accessed 28 Feb 2012]). Further high throughput screening assays to detect pollutant interactions with a range of human NHRs, including PPARs and CRs are also under development in the USA (Huang *et al*, 2011). These tests are primarily aimed at assessing the risks of environmental chemicals to humans and to date tests targeted to aquatic organisms are lacking, although the OECD are currently in the early stages of reviewing possibilities for developing high-throughput *in vitro* tests for environmental

endocrine disruptor screening targeted to fish (www.oecd.org/dataoecd/22/8/48343294.pdf).

1.5.2 Pollutants in the Aquatic Environment

The requirement for high-throughput *in vitro* screening methods targeted to aquatic organisms comes from the finding that many compounds of anthropogenic origins have been detected in aquatic environments. The compounds used in the studies presented within this thesis have been detected in waters, sediments and/or the tissues of fish sepcies (Table 1-2, 1-3 and 1-4).

Table 1-2. Reported concentrations (ng/L) of selected pollutants in rivers and sewage effluent. In each case the place of sampling is provided.

Pollutant	Water concentration (ng/L)	References
	Fibrates (Lipid regulators)	
Gemfibrozil	790 (Max conc. in survey of 139 USA streams)	Kolpin et al (2002)
	66 (UK)	Kasprzyk-Hordern *et al* (2008)
Bezafibrate	Up to 4600 (German effluent)	Ternes (1998)
	Up to 3100 (German rivers and streams)	
	780 (French rivers)	Comoretto & Chiron (2005)
Clofibric Acid	5.5 (Swiss lake surface water)	Buser and Müller (1998)
	0.5-7.8 (North sea)	
	100 (French effluents)	Hernando *et al* (2006)
	24-35 (French Rivers)	
Fenofibrate	220-400 (Rio de Janeiro, Brazil)	Stumpf *et al* (1999)
	160 (Greece STP effluent)	Andreozzi *et al* (2003)
	Phthalates/Monopthalate esters (Plasticizers)	
Benzylbutylphthalate (BBzP)	n.d. – 61 (Tama River, Japan)	Suzuki *et al* (2001)
	3.5 (False Creek, Canada)	Sura (2007)
Dimethylphthalate (DMP)		
Diethylhexylpthalate (DEHP)	400-1900 (Manchester, UK)	Fatoki & Vernon (1990)
	18500 (max conc.); 9300 (Mean conc)	Yuan *et al* (2002)
Mono-1-methylhexyl phthalate	20 (Approx from graph data; False Creek, Canada)	Sura (2007)
	Bisphenol A	
Bisphenol A	n.d. – 330 (Marine/Estuarine waters, Netherlands)	Belfroid *et al* (2002)
	n.d. – 170 (Freshwaters of Netherlands)	
	Organotins/Pesticides	
TBT compounds	3 – 70 (Port of Osaka, Japan)	Harino *et al* (1999)
	n.d. – 0.47 (Xiamen area,China)	Wang *et al* (2008)
	2 – 200 (Corsica coast, West Mediterranean)	Michel *et al* (2001)
	3200 (Max conc., Singapore coastal water)	Basheer *et al* (2002)
Deltamethrin	n.d. – 6.28 (Guanting reservoir, Beijing)	Xue *et al* (2005)

Table 1-2 cont… Reported concentrations (µg/L) of selected pollutants in rivers and sewage effluent. In each case the place of sampling is provided.

Pollutant	Water concentration (ng/L)	References
Synthetic Surfactants		
Perfluorooctanoic Acid	0.1 – 456.41 (River and Pond Surface waters, Japan)	Saito *et al* (2004)
	1.90 – 447.74 (Coastal surface waters, Japan)	
	1.7 – 178 (Svitava and Svratka river, Czech Republic)	Kovarova *et al* (2012)

n.d. – not detected

Table 1-3. Reported concentrations (mg/Kg) of selected pollutants in sediments. In each case the place of sampling is provided in brackets.

Pollutant	Sediment concentration (mg/Kg)	Reference
Phthalates/Monopthalate esters (Plasticizers)		
Dimethylphthalate (DMP)	0.16 (South Africa river)	Fatoki *et al* (2010)
Benzylbutylphthalate (BBzP)	1.8 (max conc); 0.2 (Mean conc) (Taiwan river)	Yuan *et al* (2002)
	<0.05 – 3.1 (Taiwan rivers)	Huang *et al* (2008)
Diethylhexylphthalate (DEHP)	0.5-23.9 (range); 4.6 (Mean conc) (Taiwan river)	Yuan *et al* (2002)
	n.d. – 0.1 (Taiwan river)	Huang *et al* (2008)
Mono-1-methylhexyl phthalate	0.0004 – 0.0005 (d.w. False Creek, Canada)	Sura (2007)
Organotins/Pesticides		
TBT compounds	0.015 – 0.047 (Harbour area, Taranto, South Italy)	Cardellicchio *et al* (1992)
	0.0005– 1.3	Harino *et al* (1999)
	2.796 (max conc; Paranaguá Estuarine Complex, Brazil)	Santos *et al* (2009)
	n.d -0.573 (Gulf of Cadiz, Spain)	Garg *et al* (2009)
	n.d. – 0.026 (Xiamen area, China)	Wang *et al* (2008)
	0.007-0.173 (Mersey Estuary, UK)	Harino *et al* (2003)
	0.017 – 0.847 (Sao Paulo, Brazil)	Godoi *et al* (2003)
Deltamethrin	0.013 – 0.291 (Okavango delta, Botswana)	Daka *et al* (2006)
	78.6 – 301 (Guanting reservoir, Beijing)	Xue *et al* (2005)
Bisphenol A		
Bisphenol A	0.0012 – 0.022 (Urban area sediment concentration, Japan)	Kitada *et al* (2008)
	n.d. – 0.0068 (Rural area sediments, Japan)	

n.d. – not detected

Table 1-4. Reported concentrations of selected pollutants in tissues of fish species. The common species name and place of sampling is given in brackets .

Pollutant	Tissue concentration	Reference
Fibrates (Lipid regulators)		
Gemfibrozil	210 ng/ml (Rainbow trout blood plasma; Sweden)	Brown *et al* (2007)
Phthalates/Monopthalate esters (Plasticizers)		
Dimethylphthalate (DMPh)	n.d. – 0.17 mg/Kg (muscle of Nile tilapia; Taiwan) n.d. – 1.8 mg/Kg (muscle of greenback mullet; Taiwan)	Huang *et al* (2008)
Benzylbutylphthalate (BBzPh)	n.d. – 0.4 mg/Kg (muscle of Nile tilapia; Taiwan) n.d. – 26.8 mg/Kg (muscle of greenback mullet) n.d. – 28.2 (muscle of freshwater minnow; Taiwan)	Huang *et al* (2008)
Organotins/Pesticides		
TBT compounds	2.91 µg/g (muscle of sheepshead minnow)*	Ward *et al* (1981)
	4.19 µg/g (whole body burden of sheepshead minnow)*	Ward *et al* (1981)
	0.120 mg/Kg (average; Japanese sea perch)	Harino *et al* (2000)
	0.138 mg/Kg (average; white croaker)	
	0.157 mg/Kg (average; yellowtail)	
	0.166 mg/Kg (average; Bandfish)	
	0.182 mg/Kg (average; Silver whiting)	
	0.128 mg/Kg (average; Butter fish)	
	0.052 mg/Kg (File fish)	
	0.167 mg/Kg (Triped puffer)	
	0.011 mg/Kg (average; Grey mullet)	
	0.020 mg/Kg (average; Japanese barbel)	
	0.022 mg/Kg (average; Bluegill)	
Bisphenol A		
Bisphenol A	2-75 ng/g (European flounder liver, Netherlands)	Belfroid *et al* (2002)
	n.d. – 11 ng/g (European flounder muscle, Netherlands)	
	1-5 ng/g (Muscle of common bream, Netherlands)	
	0.69 – 3.01 ng/g (Perch muscle, Sweden)	Sternbeck & Forsgren (2007)
	1.44 - 4.74 ng/g (Cod muscle, Sweden)	
	<0.24 ng/g (Salmon muscle, Sweden)	
	<0.24 – 1.77ng/g (Cod liver, Sweden)	
Synthetic Surfactants		
PFOA	0.1-0.63 ng/ml (Chub (*Leuciscus cephalus*) blood plasma; Svitava and Svratka rivers, Czech Republic)	Kovarova *et al* (2012)

n.d. – not detected
*** Indicates concentrations derived from controlled laboratory studies**

European legislation provides for measures against chemical pollution of surface water. In 2000, the Water Framework Directive (WFD) was introduced by the European Commision (EC) (European Union, 2000). Article 16 of this directive, “Strategies against pollution of water”, required a list of priority substances to be selected from those presenting a significant risk to the aquatic environment, together with a proposal for their control in in order to reduce emissions, discharges and losses of all substances. Additionally a subset of priority hazardous substances (PHS), i.e. priority substances which are toxic, persistent and liable to bio-accumulate and other substances or groups of substances of high concern was to be identified (Annex X of the directive). The first list of such substances was adopted on 20 November 2001 (European Union, 2001) and consisted of 33 substances, 10 of which were identified as PHS and a further 15 of which were subject to a review for identification as possible PHS. Today, following reviews in 2008 (European Union, 2008) and a recently proposed amendment of the list in 2011 (European Commission, 2011), the list of priority substances consists of 48 substances, of which 21 are listed as priority hazardous substances (Table 1-5).

For substances in surface waters (river, lake, transitional and coast) environmental quality standards (EQS) have been set such that the concentrations stated are not expected to cause deleterious effects in aquatic organisms, provided they are not exceeded (European Union, 2008). Two types of EQS are set for priority substances: annual average concentrations and maximum allowable concentrations. The former protects against long-term chronic pollution problems, and the latter short-term acute pollution. Member States are responsible for monitoring the concentrations of priority substances in surface waters as part of their monitoring programmes and in

addition are authorised to set their own EQS for chemicals of concern and other chemicals. In addition to surface water EQS, 11 of the 48 priority substances have designated biota EQS. Table 1-6 provides, where available, the EQS for the compounds used within the present study.

Table 1-5. List of priority substances in the field of water policy. Taken from COM(2011)/876 (European commission, 2011).

Number	Priority Substance Name	Identified as PHS	Comments
1	Alachlor		
2	Anthracene	✓	
3	Atrazine		
4	Benzene		
5	Brominated diphenylethers	✓	Only Tetra, Penta, Hexa and Heptabromodiphenylether
6	Cadmium and its compounds	✓	
7	Chloroalkanes, C_{10-13}	✓	
8	Chlorfenvinphos		
9	Chlorpyrifos (Chlorpyrifos-ethyl)		
10	1,2-dichloroethane		
11	Dichloromethane		
12	Di(2-ethylhexyl)phthalate (DEHP)	✓	
13	Diuron		
14	Endosulfan	✓	
15	Fluoranthene		Indicator of other, more dangerous PAHs
16	Hexachlorobenzene	✓	
17	Hexachlorobutadiene	✓	
18	Hexachlorocyclohexane	✓	
19	Isoproturon		
20	Lead and its compounds		
21	Mercury and its compounds	✓	
22	Napthalene		
23	Nickel and its compounds		
24	Nonylphenols	✓	Includes isomers 4-nonylphenol and 4-nonylphenol (branched)
25	Octylphenols		Including isomer 4-(1,1',3,3'-tetramethylbutyl)-phenol
26	Pentachlorobenzene	✓	
27	Pentachlorophenol		
28	Polyaromatic hydrocarbons (PAH)	✓	Including benzo(a)pyrene, benzo(b)fluoranthene, benzo(g,h,i)perylene, benzo(k)fluoranthene, indeno(1,2,3-cd)pyrene,and excluding anthracene, fluoranthene and napthalene
29	Simazine		
30	Tributyltin compounds	✓	Including tributyltin-cation

Table 1-5 cont….

Number	Priority Substance Name	Identified as PHS	Comments
31	Trichlorobenzenes		
32	Trichloromethane (Chloroform)		
33	Trifluralin	✓	
34	Dicofol	✓	
35	Perfluorooctane sulfonic acid and its derivatives (PFOS)	✓	
36	Quinoxyfen	✓	
37	Dioxins and dioxin-like compounds	✓	Includes 7 polychlorinated dibenzo-p-dioxins (PCDDs), 10 polychlorinated dibenzofurans (PCDFs), 12 dioxin-like polychlorinated biphenyls (PCB-DL)
38	Aclonifen		
39	Bifenox		
40	Cybutryne		
41	Cypermethrin		Includes the eight isomers contributing to CAS 52315-07-8, and therefore also CAS 67375-30-8
42	Dichlorvos		
43	Hexabromocyclododecanes (HBCDD)	✓	This includes 1,3,5,7,9,11-Hexabromocyclododecane, 1,2,5,6,9,10- Hexabromocyclododecane, - Hexabromocyclododecane, - Hexabromocyclododecane and - Hexabromocyclododecane
44	Heptachlor and heptachlor epoxide	✓	
45	Terbutryn		
46	17 -ethinylestradiol		
47	17 -estradiol		
48	Diclofenac		The inclusion of these substances is without prejudice to Regulation (EC) 726/2004, Directive 2001/83/EC and Directive 2001/82/EC

Table 1-6. Environmental Quality Standards (EQS) set for several pollutants used in the present thesis. AA-EQS: environmental quality standard expressed as an annual average value; MAC-EQS: environmental quality standard expressed as a maximum allowable concentration.

Name	AA-EQS	MAC-EQS	Sediment-EQS
Benzyl butyl phthalate (1)	20 µg/L (Freshwater) 20 µg/L (Saltwater)	100 µg/L (Freshwater) 100 µg/L (Saltwater)	n/a
Dimethyl phthalate (1)	800 µg/L (Freshwater) 800 µg/L (Saltwater)	4000 µg/L (Freshwater) 4000 µg/L (Saltwater)	n/a
Tributyltin compounds (2)	0.0002 µg/L (Inland surface water) 0.0002 µg/L (Other surface water)	0.0015 µg/L (Inland surface water) 0.0015 µg/L (Other surface water)	n/a
Deltamethrin (3)	0.0003 µg/L (Water column) 9, 6, 4, 2, 1 ng/L following 3, 6, 12, 24, 48 hrs after release, respectively (Water column after release from aquaculture facilities)	n/a	0.33 µg/Kg (dry weight)

Table 1-6 cont…

Name	AA-EQS	MAC-EQS	Sediment-EQS
Fluorooctane sulfonic acid (FOSA) and its derivatives [2]	6.5×10^{-4} µg/L (Inland surface water) 1.3×10^{-4} µg/L (Other surface water)	36 µg/L (Inland surface waters) 7.2 µg/L (Other surface waters)	9.1 µg/Kg (wet weight)

[1] UK Non-statutory EQS (Proposed by Environment Agency)
[2] European commission (2011)
[3] Scottish Environment Protection Agency (SEPA), (2005).

1.6 Thesis aims and objective

Until recently the focus of endocrine disrupting compounds in the aquatic environment was on their interactions with the estrogen and androgen receptors of fish species, reflecting the importance of these receptors in reproductive functions. However, knowledge on the interactions of potential EDCs with other members of the NHR superfamily in aquatic organisms is lacking, despite involvement in numerous critical physiological processes. The objectives of the research presented here was to develop methods to screen environmental pollutants for their potential to interact with the PPARs of European plaice and the corticosteroid receptors of European flounder, both of which have roles in regulating energy homeostasis, stress and immune function. The choice of organisms was based on several considerations. Firstly, European flounder and plaice are already two of the choice species in determining the biological responses of fish to aquatic pollutants as part of the Oslo/Paris convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR) initiative (ICES, 2011). Several publications to date have also used European flounder and European plaice as indicator species to assess the health of estuarine habitats (Amara *et al*, 2009) or to identify biomarkers which reflect the exposure to environmental contaminants in coastal marine habitats (Eggens *et al*, 1995; Skouras *et al*, 2003), reflecting the sensitivity of these species to environmental contaminants. Secondly, benthic organisms which include plaice and

flounder are subjected to contaminants not only from the water column but also from sediments where pollutants can bind to sediment particles, accumulate to high concentrations and can persist for long periods of time and therefore the use of plaice and flounder as model organisms allows sediment concentrations, as well as water-column concentrations, to be considered.

The specific objectives of the current thesis were:

i. To optimise the transfection of plasmid DNA into an established fish cell line by assessing three different transfection reagents and a range of DNA to reagent ratios (**Chapter 2**)

ii. To prepare expression plasmids in which the ligand-binding domains of European plaice PPARs are ligated downstream of the Gal4 DNA-binding domain (**Chapter 3**)

iii. To test the expression constructs prepared in (ii) by co-transfection of a fish cell line with an expression plasmid in which the luciferase reporter gene is downstream of a Gal4-upstream activating sequence, treatment of transfected cells with known PPAR agonists, and measurement of the resulting luciferase activity (**Chapter 3**).

iv. To test a range of environmental contaminants with the Gal4-PPAR LBD expression constructs in a series of luciferase assays to identify pollutants which interact with plaice PPAR ligand-binding domains (**Chapter 4**).

v. To evaluate the transcriptional responses of European flounder hepatocytes to TBTO, and attempt to determine PPAR-specific responses to TBTO and a PPAR ligand (**Chapter 5**).

vi. To isolate corticosteroid receptors from European flounder and characterise the isolated receptors in terms of phylogenetic analysis and receptor expression throughout tissues of the European flounder (**Chapter 6**).

vii. To prepare expression plasmids for corticosteroid receptors in which the ligand-binding domains of European flounder corticosteroid receptors are ligated downstream of the Gal4 DNA-binding domain (**Chapter 7**).

viii. To test the corticosteroid receptor expression plasmids in (a) luciferase assays and (b) hormone binding assays, with known corticosteroid ligands and to test a range of environmental contaminants with the Gal4-corticosteroid receptor LBD expression constructs in a series of luciferase assays (**Chapter 7**).

Chapter 2. Optimisation of transfection methods and measurement of luciferase activities

2.1 Introduction

The introduction of foreign nucleic acids into eukaryotic cells using non-viral methods is defined as transfection. The capacity to efficiently transfect and express encoded proteins in eukaryotic cells *in vivo* using non-viral methods is an attractive prospect in gene therapy strategies for diseases such as cystic fibrosis (Welsh, 1999; Ferrari *et al*, 2002). In addition, successful transfection of foreign DNA into a variety of eukaryotic cell lines *in vitro* has provided researchers with a relatively simple means of studying promoter sequences, protein-protein interactions, protein-ligand interactions and trans-acting proteins e.g. transcription factors.

The introduction of negatively charged molecules, such as the phosphate backbones of DNA and RNA, into eukaryotic cells is problematic due to their negatively charged membranes. To overcome this, transfection vehicles are used to create neutral or overall positively charged molecules, thus facilitating the crossing of DNA across the cell membrane. These vehicles include both physical and chemical methods.

2.1.1 Physical methods of transfection

Physical methods of transfection facilitate the crossing of cell membranes by bringing molecules into closer proximity to the membrane and/or by temporarily disrupting the cell membrane (Wells, 2004). One such physical method of transfection in which the membrane is temporarily disrupted is that of electroporation (or electropermeabilisation), involving the application of an external electric field to cell membranes, resulting in the formation of hydrophilic pores through which

molecules can pass into the cell. The first application of this method was used to successfully transfect circular plasmid DNA carrying the thymidine kinase (TK) gene into TK-deficient mouse L cells (Neumann *et al*, 1982). Since then electroporation has been used *in vitro* to transfect mammalian cells with a variety of molecules including anti-cancer drugs (Mir, 2009) and fluorescent dyes (Mir *et al*, 1988). More recently results of *in vivo* clinical trials involving the use of electroporation to increase the uptake of chemotherapeutic drugs in tumours have been published with the results indicating electroporation to be a potentially promising development in the treatment of cancers (Mir *et al*, 1997). Electroporation is not limited to mammalian cells; the electro-transformation of bacterial cells with plasmid DNA is a widely used technique in molecular biology, with several strains of *E.coli* having been successfully transformed in this manner (Dower *et al*, 1988; Sawahel *et al*, 1993).

As well as electroporation, the direct insertion of DNA into cells by microinjection has been described as a method of transfection (Capecchi, 1980). This method offers the advantages of quantifying how much DNA is delivered to cells, the intracellular localisation of delivery and the minimisation of DNA degradation by nuclease activity in intracellular vesicles. This method however is impractical when transfecting a large number of cells.

A more recent development in transfection techniques is the particle bombardment, or gene gun, technique. This involves the accelerated delivery of DNA, coated with high density metal particles such as gold or tungsten, into cells using acceleration instruments. This technique has the advantage of low levels of cell damage and the

potential for *in vivo* transfection. However, the requirement for specialist machinery makes the initial costs of this technique an expensive option.

2.1.2 Chemical methods of transfection

Most chemical methods of transfection rely on the formation of a particle containing the DNA of interest complexed to a chemical, the formation of which relies on the interactions between the negatively charged DNA molecules with a positively charged moiety on the transfection reagent. Complexes are then internalised by cells using a variety of routes, most of which are not clearly understood. Three commonly used classes of reagents are cationic organic polymers, calcium phosphate and cationic lipids, each of which is described briefly below.

2.1.2.1 Cationic organic polymers

One of the first chemical reagents to be used for transfection of mammalian cells was diethylaminoethyl (DEAE) dextran, a high molecular weight cationic polymer. Due to relatively low transfection efficiencies, toxicity, non-biodegradability and reproducibility, this method has been largely superseded. More recently a variety of highly branched cationic organic polymers have been found to form complexes with DNA and these complexes can be efficiently taken up by cells. The mechanism of uptake is not fully understood but may involve endocytosis.

2.1.2.2 Calcium Phosphate

Calcium phosphate was first used to transfect adenovirus 5 DNA into the human KB cell line (Graham and van der Eb, 1973) and since then has been widely used to transfect many different mammalian cell lines including CV1, BHK-21 and HeLa

cells (Strain and Wyllie, 1984; Zhang and Kain, 1996; Howcroft *et al*, 1997). The popularity of this method is due to the availability and inexpensive cost of the reagents. Using this method DNA is mixed with calcium chloride and added to a buffered saline/phosphate solution to form a precipitate. Addition of the precipitate to cells in culture allows DNA to enter the cells via endocytosis or phagocytosis. One of the major disadvantages of the calcium phosphate precipitation method is, like the DEAE-dextran method, the relatively low transfection efficiencies and the low reproducibility between experiments.

2.1.2.3 Cationic lipids

Perhaps the most widely used transfection reagents are cationic lipids, of which there are several available. The cationic head groups of these compounds interact with the DNA of interest and the hydrophobic lipid tails then from a micelle. The resulting "liposomes", small complexes containing the DNA, are then thought to fuse with cellular membranes, thus resulting of delivery of DNA to the cell cytoplasm.

The successful transfection of fish cell lines using a variety of methods has been described previously (Leaver *et al*, 2005; Romøren *et al*, 2005; Schiøtz *et al*, 2011), and optimal conditions for transfection can vary widely for different cell types (Yamano *et al*, 2010) and thus must be optimised for the cell line and reagent of choice. The ratio of DNA for transfection-to-transfection reagent is one consideration for optimisation, since this determines the overall charge of the DNA-reagent complex being transfected, and ultimately the ability of DNA to cross the cell membrane, a critical step in successful transfection. Taking this into consideration, the present chapter focuses on the transfection of the fathead minnow (FHM) cell

line using three transfection reagents. A variety of DNA to reagent ratios are assessed with the aim of identifying the most suitable transfection reagent and the optimal conditions for use in subsequent experiments.

2.2 Materials and Methods

2.2.1 Harvesting of a fish cell line

2.2.1.1 Maintaining the FHM cell line

A cell line derived from the mass of tissue posterior to the anus of the northern fathead minnow (*Pimephales promelas*), designated as the FHM cell line (Gravell and Malsberger, 1965), was used in transient transfection assays. Cells were maintained in 1× Leibovitz's L-15 medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Biosera), 50 units/mL penicillin and 50 µg/mL streptomycin (Invitrogen) and passaged 1:3 once per week before reaching full confluence.

2.2.1.2 Seeding FHM cells

FHM cells were harvested and seeded into 96-well assay plates (Corning) 24 hours prior to transfection. Cells were washed twice with 1× Dulbecco's phosphate-buffered saline (DPBS; Invitrogen) before being dissociated from the surface of the flask by addition of 0.5 % trypsin/EDTA (Invitrogen). Cells were then resuspended in a volume of 1 × Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) containing 4.5g/L glucose, 10% (v/v) charcoal/dextran treated fetal bovine serum (Fisher Scientific) 50 units/mL penicillin and 50 µg/ml streptomycin (Invitrogen), herein referred to as complete media, and a cell count using the trypan blue exclusion

test for cell viability carried out. 100 µl cell suspension was diluted with an equal volume of with 0.4% trypan blue solution (Sigma Aldrich) and two chambers of an improved Neubauer hemocytometer filled with the solution by capillary action. The cells within each $0.1mm^3$ chamber were counted and the number of cells averaged over the two chambers. The average number of cells was then multiplied by 1000 to calculate the number of cells per mL suspension, and multiplied again by the volume of cell suspension to give the total number of cells. The suspension was diluted with complete DMEM media to a cell density of 2×10^6 cells/mL. 100 µl of cell suspension was added to the wells of 96-well assay plates (2×10^5 cells per well) and incubated overnight at 22°C in an atmosphere of 4% carbon dioxide (CO_2), ready for transfection the following day.

2.2.2 Transfection of FHM cells

Lipids have been demonstrated to be the endogenous activators of PPARs and thus, to avoid any possible interference by lipid-based transfection reagents three commercially available polycationic transfection reagents were each tested at varying reagent to DNA ratios for their efficiency to transfect plasmid DNA into FHM cells. Transfection efficiencies were assessed by measuring levels of a constitutively expressed *Renilla* luciferase protein, present in the transfected pGL4.75[hRluc/CMV] plasmid.

The manufacturers recommended ratios of Genejammer (Agilent) and Satisfection (Agilent) reagents to DNA was used as a starting point in the transfection of FHM cells. Both half and double the recommended ratio were also tested for each of the two reagents. The Polyfect transfection reagent (Qiagen) has been optimised for the

transfection of a variety of mammalian cell lines, each of which uses a different Polyfect to DNA ratio. Therefore for the transfection of the fish cell line a range of ratios covering those used for transfection of mammalian cell lines was used.

In total three DNA plasmids were co-tranfected into the FHM cells, seeded 24 hours previously into 96-well plates. These plasmids were:

- A plasmid containing the *Renilla* luciferase gene (pGL4.75[*hRluc*/CMV]), to be used as an indicator of transfection efficiency
- A plasmid containing the entire coding region of PPAR 2, isolated from plaice (*Pleuronectes platessa*)
- A plasmid into which the mouse Cyp4A6z peroxisome proliferator response element (PPRE) linked to the minimal mouse thymidine kinase promoter was cloned upstream of a firefly luciferase gene (pGL4.10TKPPRE)

Transfected cells were subsequently treated with Wy14643 (Sigma), an activator of PPAR 2, or a control treatment of ethanol vehicle, in order to assess the interaction between PPAR 2 and the PPRE of the reporter construct in response to a peroxisome proliferator.

2.2.2.1 Transfection of plasmid DNA using different reagents

According to the manufacturers handbook, the use of Polyfect reagent has been optimised for transfection of DNA into COS-7, NIH/3T3, HeLa, 293, and CHO cell lines derived from mammalian species, but no information is available for the transfection of fish cell lines. Therefore, transfection of DNA into the fathead minnow (FHM) cell line required optimisation. Manufacturer’s protocol

recommended altering both the quantity of DNA transfected and also the ratio of Polyfect reagent to DNA.

Nine DNA mixtures were prepared for transfection. Each transfection mix contained the pcDNA3-PPAR 2 plasmid, the pGL4.75[*hRluc*/CMV] internal control reporter plasmid and the reporter plasmid pGL4.10[TK/PPRE] in a ratio of 1:0.4:1, respectively. Three different amounts of DNA (40, 60 and 80ng) were used in three transfection mixes each. DNA was diluted to a total volume of 12 µl with DMEM containing no antibiotics or serum, vortex mixed and centrifuged briefly. A volume of Polyfect reagent (Qiagen) was then added to each tube, giving varying volume to weight (v/w) ratios of Polyfect reagent:DNA (Table 2-1). Tube contents were again mixed and briefly centrifuged before being incubated at room temperature for 10 minutes to allow for a complex between the Polyfect reagent and DNA to form. 80 µl of complete DMEM was then added to each tube.

Media in which FHM cells were seeded 24 hours prior to transfection was removed and replaced with 60 µl fresh complete DMEM. The nine DNA/transfection reagent mixes were aliquoted to four wells each (20 µl transfection mix per well). In addition to transfected cells, mock transfected cells into which no DNA was transfected was included as a negative control in order to quantify background levels of luminescence and to these cells 20 µl DMEM only was added.

Cells were incubated at 22°C in a 4% CO^2 incubator for a further 24 hours before treatment.

Table 2-1. The nine transfection mixes prepared using Polyfect transfection reagent. Each contained the *P. platesa* PPAR 2, pGL4.75[*hRluc*/CMV] and pGL4.10[TK/PPRE] at a constant ratio of 1:0.4:1, respectively. Polyfect reagent (Qiagen) was added before each of nine transfection mixes was split between 4 wells containing FHM cells.

	1	2	3	4	5	6	7	8	9
ppPPAR 2 (ng/well)	16.67			25			33.33		
pGL4.75[hRluc/CMV] (ng/well)	6.67			10			13.33		
pGL4.10[TK/PPRE] (ng/well)	16.67			25			33.33		
Total DNA (ng/well)	40.0			50.0			80.0		
Polyfect reagent (µl/well)	0.25	0.625	1.25	0.25	0.625	1.25	0.25	0.625	1.25
Polyfect (µl):DNA (µg)	6.25:1	15.6:1	31.25:1	4.2:1	10.5:1	20.8:1	3.13:1	7.8:1	15.6:1

For transfection of FHM cells using Genejammer and Satisfection reagents cells were harvested and seeded twenty four hours prior to transfection as described in 2.2.1.2 above.

To transfect cells with Genejammer reagent, a total of 2.5 µg plasmid DNA containing pcDNA3-PPAR 2 plasmid, pGL4.75[*hRluc*/CMV] and pGL4.10[TK/PPRE] in a 1:0.5:1 ratio, respectively, was diluted with DMEM (containing no serum or antibiotics), to a final concentration of 100 ng/µl. In separate tubes 25 µl DMEM (containing no serum or antibiotics) was combined with Genejammer transfection reagent (Table 2-2) and incubated at room temperature for 5 minutes. A volume of DNA (100 ng/µl) was then added to the mixture to give varying Genejammer reagent:DNA ratios, and incubated for 15 minutes at room temperature. Each transfection mix was then distributed between four wells each (5 µl per well).

For transfection with Satisfection reagent (Agilent), Satisfection reagent was added to three tubes and the volume made up to 37.5 µl with DMEM media containing no additives. In separate tubes plasmid DNA was combined in a ratio of 1:0.5:1 pCMV-PPAR 2, pGL4.75[*hRluc*/CMV] and pGL4.10[TK/PPRE], respectively, and diluted to final concentrations of 20 ng/µl and 40 ng/µl. 37.5 µl of DNA (either 20 ng/µl or 40 ng/µl) was added to each reagent/DMEM mix. The contents of all three transfection mixes were mixed and incubated at room temperature for 15 minutes before 15 µl of each transfection mixes was added to each of four wells of an assay plate. Mock transfected control wells in which no plasmids were transfected were also included on the assay plate and to these 15 µl complete DMEM was added. The assay plate was then incubated in a 4 % CO^2 incubator at 22°C overnight.

Table 2-2. Three DNA mixes each were prepared for transfection using Genejammer (Agilent) and Satisfection reagent (Agilent). Each contained the *P. platesa* PPAR 2, pGL4.75[*hRluc*/CMV] and pGL4.10[TK/PPRE] at a constant ratio of 1:0.5:1, respectively. DNA was added to Genejammer reagent (Agilent) or Satisfection reagent (Agilent), diluted with DMEM media, before each of the transfection mixes was split between 4 wells containing FHM cells.

	Genejammer			**Satisfection**		
Transfection Mix	1	2	3	4	5	6
ppPPAR 2 (ng/well)	20	20	40	60	60	120
pGL4.75[hRluc/CMV] (ng/well)	10	10	20	30	30	60
pGL4.10[TK/PPRE] (ng/well)	20	20	40	60	60	120
Total DNA (ng/well)	50	50	100	150	150	300
Polyfect reagent (µl/well)	0.15	0.3	0.15	0.22	0.44	0.22
Reagent (µl):DNA (µg)	3:1	6:1	1.5:1	1.5:1	3:1	0.75:1

2.2.3 *Application of treatment solution to transfected cells*

Twenty four hours following transfection of FHM cells with pCMV-PPAR 2, pGL4.75[*hRluc*/CMV] and pGL4.10[TK/PPRE] using either Polyfect reagent (Qiagen), Genejammer reagent (Stratagene) or Satisfection reagent (Stratagene), cells were treated with Wy14643 or ethanol vehicle.

2.2.3.1 Dilution of treatment into complete DMEM media

Cells were treated with [[4-chloro-6-[(2,3-dimethylphenyl)amino]-2-pyrimidinyl]thio]-acetic acid (Wy-14643), a compound previously shown to activate both mammalian and piscine, PPAR . A 5 molar stock solution of Wy-14643 compound was prepared in ethanol absolute and 5 µl diluted into 1 ml complete DMEM, giving a final concentration of 25 µM. A vehicle control treatment was also prepared, in which ethanol was diluted into DMEM at a concentration of 5 µl/mL.

2.2.3.2 Treatment of transfected cells

Transfection mixes were aspirated from the wells of the assay plates and 75 µl of diluted treatments added such that cells transfected with different reagents and at different ratios were each treated in duplicate with both Wy-13643 and ethanol.

Following treatment the assay plate was incubated at 22 °C, 5% CO_2 for 24 hours.

2.2.4 *Luciferase Assay and Data Normalisation*

2.2.4.1 Measurement of Firefly Luciferase

Cells were assayed for luciferase activity using the Dual-Glo™ Luciferase Assay System (Promega), following the manufacturer's instructions. Briefly 75 µl of Dual-

Glo™ Luciferase Reagent was added to each experimental well in the assay plate, and the contents of each well pipetted up and down four times to mix before being incubated at room temperature for ten minutes to allow complete cell lysis. The Dual-Glo™ Luciferase Reagent acts as a substrate for firefly luciferase, producing a luminescent signal which, after the ten minute incubation period, was quantified using the Wallac 1420 Victor 2 multilabel counter (Perkin Elmer).

2.2.4.2 Measurement of Renilla Luciferase

Subsequent to obtaining firefly luciferase values, 75 µl of Dual-Glo™ Stop & Glo® Reagent was added to each well of the assay plate, mixed and incubated as before. The Dual-Glo Stop & Glo reagent quenches firefly luminescence signal by at least 10,000-fold while providing the substrate for the *Renilla* luciferase enzyme. *Renilla* luminescence signal was measured using the same procedure as described for firefly luciferase.

2.2.4.3 Subtraction of Background Luminescence & Data Normalisation

Neither firefly nor *Renilla* luciferase enzyme is endogenously expressed in the FHM cell line, and therefore firefly luminescence and *Renilla* luminescence readings from mock-transfected cells in which no DNA had been transfected was an indication of background noise levels. Firefly and *Renilla* luciferase values from non-transfected cells 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. Since the results below

are based on one transfection experiment only, statistical differences were not calculated and thus the results are based only on graphical trends.

2.3 Results

2.3.1 Polyfect reagent

2.3.1.1 Transfection efficiency

The efficiency with which DNA is transfected into cell lines using Polyfect reagent is dependent on the overall net charge of the reagent-DNA complex, which in turn is dependent on the ratio of Polyfect reagent to negatively charged DNA. Therefore in optimising the transfection of FHM cells, various Polyfect to DNA ratios were used, altering both the amount of DNA transfected and the volume of Polyfect reagent used for transfection. Transfection efficiencies of different Polyfect to DNA ratios were determined by measuring the luciferase signal from the constitutively expressed *Renilla* luciferase gene, present in the transfected internal control plasmid pGL4.75[*hRluc*/CMV] (Figure 2-1). The efficiency with which plasmid DNA was transfected into FHM cells was dependent on the ratio of Polyfect to DNA, with *Renilla* luciferase values increasing with increasing Polyfect to DNA ratios, regardless of the amount of DNA transfected. A two-fold increase in the ratio of Polyfect reagent to DNA resulted in a 2.4-fold, 1.5-fold and 1.9-fold increase in *Renilla* luciferase signal when transfecting 40ng, 60ng and 80ng DNA, respectively. Increasing the ratio of Polyfect to DNA five-fold resulted in a fold- increase in *Renilla* luciferase of 23.6, 17.4 and 12.2 when transfecting 40, 60 and 80ng DNA, respectively. Maximum *Renilla* luciferase signal was obtained at a reagent to DNA

ratio of 31.25:1 while the lowest transfection efficiencies were observed at ratios of 6.25:1 and lower (Figure 2-1).

The effect of increasing the amount of DNA transfected while maintaining a constant ratio of Polyfect reagent to DNA was investigated. Doubling the amount of DNA transfected per well from 40ng to 80ng while maintaining the ratio of reagent to DNA at 15.6 µl to 1 µg resulted in a 1.45-fold increase in *Renilla* luciferase signal, suggesting that the amount of DNA transfected as well as the ratio of reagent to DNA could be optimised to give maximum transfection efficiency.

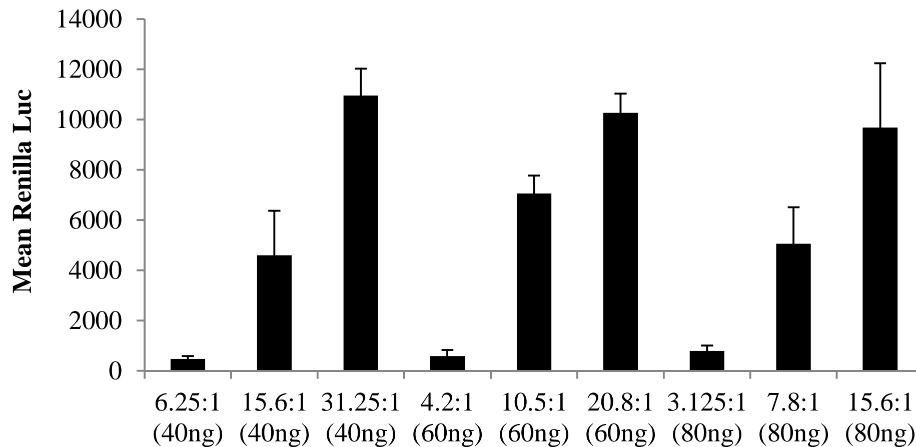


Figure 2-1. The effect of altering the Polyfect reagent to DNA ratio (µl to µg) on transfection efficiency. Varying ratios of Polyfect:DNA were used to transfect FHM cells before cells were assayed for Renilla luciferase signal, used as a measurement of transfection efficiency. Graph shows mean + SD of 1 experiment in which each transfection mix was used to transfect 4 wells each.

2.3.2 Genejammer Reagent & Satisfection Reagent

2.3.2.1 Transfection efficiency

For both Satisfection and Genejammer transfection efficiencies were assessed using ratios of reagent to DNA at the manufacturers recommendations and at both double and half that of the recommended amount. Doubling the ratio of Genejammer reagent

to DNA from 3:1 to 6:1 gave an increase in *Renilla* luciferase signal of 2.3-fold, while halving the ratio from 3:1 to 1.5:1 caused *Renilla* luciferase signal to decrease by half (Figure 2-2), suggesting transfection efficiency is proportional to the ratio of reagent:DNA.

Increasing the ratio of Satisfection transfection reagent to DNA did not change *Renilla* luciferase signal, while halving the ratio resulted in a two-fold increase in the signal from the control reporter (Figure 2-2).

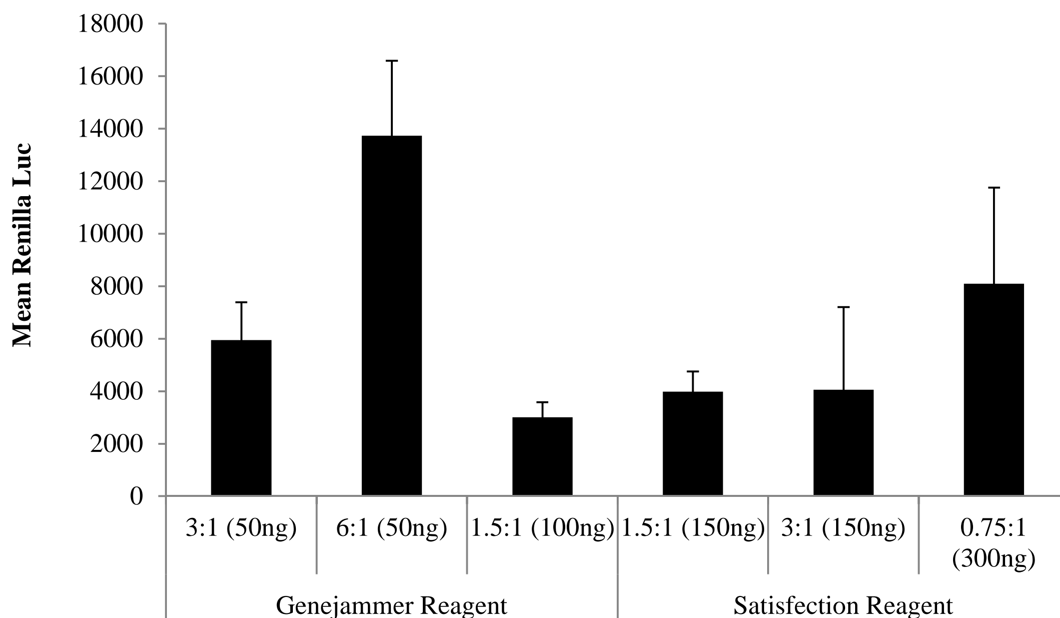


Figure 2-2. The effect of altering the Genejammer and Satisfection reagent to DNA ratio (µl to µg) on transfection efficiency. Varying ratios of reagent:DNA were used to transfect FHM cells before cells were assayed for Renilla luciferase signal, used as a measurement of transfection efficiency. Graph shows mean + SD of 1 experiment in which each transfection mix was used to transfect 4 wells.

2.3.3 Transactivation of PPAR by Wy-14643

Transfection of PPAR 2 and the experimental reporter pGL4.10[TK-PPRE] plasmid into FHM cells using Polyfect reagent, and subsequent treatment of transfected cells with 25 µM Wy-14643 saw an increase in reporter gene activity of between 3.09-fold

and 5.83-fold over treatment with ethanol vehicle, when normalised for transfection efficiency (Figure 2-3).

Transfection of DNA with Satisfection reagent and treatment of transfected cells with Wy14643 resulted in increases in firefly luciferase activity ranging between 3.2 and 3.8-fold over ethanol vehicle. Those cells transfected with Genejammer reagent and subsequently treated with Wy14643 or ethanol and assayed for luciferase luminescence signal saw the lowest fold-increases in firefly luciferase signal with signal more similar to those observed with ethanol vehicle. Compared to ethanol increases of between 1.3 and 1.7-fold were observed. Genejammer reagent was excluded as the transfection reagent of choice for future transfections due to the relatively low fold-induction in firefly luciferase when transfected cells were treated with a PPAR 2 activator.

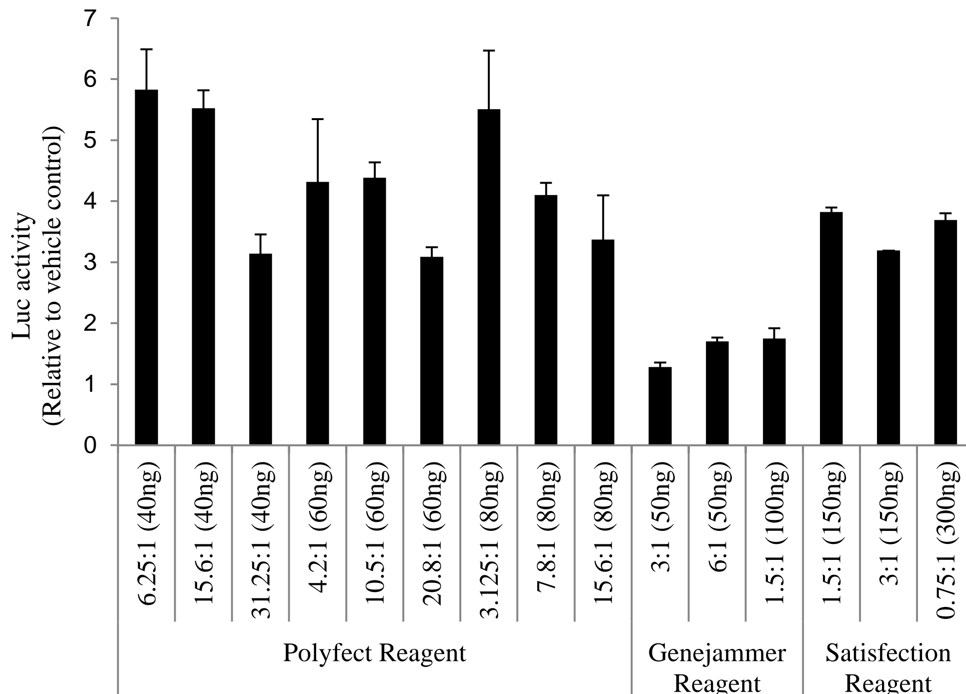


Figure 2-3. Firefly luciferase signal measured from cells transfected with Polyfect, Genejammer or Satisfection reagent at various reagent:DNA ratios and treated with the PPAR agonist Wy14643. The graph shows firefly luciferase activity, normalised for transfection efficiency, as a fold induction over treatment with ethanol vehicle. Data are the mean + SD of 1 experiment in which each Wy14643 and ethanol vehicle treatment was applied in duplicate.

2.4 Discussion

An assessment of the efficiency of three commercially available transfection reagents to transfect a cell line derived from the fathead minnow (*Pimephales promelas*) has been carried out. Transfection efficiency was increased with both Polyfect and Genejammer reagents with increasing ratios of reagent:DNA, while no obvious pattern was observed with Satisfection reagent. An increase in normalised firefly luciferase was noted in all transfected cells treated with Wy14643, in comparison to those treated with ethanol vehicle, suggesting PPAR to interact with the PPRE-containing firefly luciferase reporter gene construct (pGL4.10[TK-PPRE]) in response to PPAR ligands.

2.4.1 *Ratio of reagent:DNA and transfection efficiency*

As expected the efficiency of transfection using both Polyfect reagent and Genejammer was found to decrease with decreasing ratios of reagent to DNA. Decreases in transfection efficiencies are likely due to the lower concentrations of transfection reagents present, which may have resulted in the presence of excess, uncomplexed DNA which, by itself, has low transfection efficiency. Therefore in order to ensure efficient transfection of DNA in future experiments, a Polyfect to DNA ratio greater than 6.25 µl:1 µg should be used since efficiencies above this ratio were considered acceptable for the intended use in this study. GeneJammer transfection reagent, although providing an adequate level of transfection efficiency, was discounted from future use due to the very low inductions in firefly luciferase when transfected cells were treated with Wy14643 compared to ethanol (Figure 2-3).

Cells transfected with Satisfection reagent gave the lowest *Renilla* luciferase signal, when compared to Polyfect or GeneJammer reagent, suggesting this reagent to be the least effective in the transfection of FHM cells, perhaps due to sub-optimal transfection conditions (e.g. DNA:reagent ratio). Due to this low transfection efficiency Satisfection was also omitted as the transfection reagent of choice in future experiments.

Maintaining a constant Polyfect reagent to DNA ratio while doubling the amount of DNA in the transfection mix resulted in a doubling in the efficiency of transfection. It has previously been reported that increasing the amount of DNA while maintaining a constant transfection reagent to DNA ratio increases the number of rabbit corneal cells transfected up to a point, after which the number of rabbit corneal cells transfected with plasmid DNA decreases (Hudde *et al*, 1999). The authors hypothesised that the decrease in transfection efficiency was due to the toxicity of the dendrimer-DNA complex. In transfecting the FHM cell line as described above, maintenance of a Polyfect to DNA ratio of 15.6:1 while doubling the amount of DNA transfected per well from 40ng to 80ng resulted in an increase in transfection efficiency, suggesting that no dentrimer-DNA complex toxicity was occurring at 80ng DNA used in these experiments.

2.4.2 Activation of PPAR 2 by Wy14643 and interactions with a PPRE

PPAR -transfected cells treated with Wy14643 saw an increase in normalised firefly luciferase compared to ethanol vehicle treated cells. This increase in reporter gene expression in the presence of Wy14643 agrees with previous findings that PPAR 2

from plaice is activated by this compound. In addition these results indicate that PPAR 2 from plaice, when activated by Wy14643, interacts with the peroxisome proliferator response element to drive expression of firefly luciferase, thus suggesting the pGL4.10[TK-PPRE] reporter construct to be functional and suitable for use in studies involving peroxisome proliferator-activated receptor .

Previously Wy14643 has been shown to be a selective agonist of plaice PPAR , having no effect on PPAR and PPAR from the same species in transfection assays (Leaver *et al*, 2005). The levels of firefly luciferase activity in the present assay, although higher than vehicle controls, were lower than the levels of reporter gene protein observed by Leaver *et al* (2005) who observed a 35-fold increase in chloramphenicol acetyltransferase (CAT) compared to ethanol vehicle treatment. This is most likely due to differences in experimental protocol and assay technique; Leaver *et al* (2005) assayed for CAT protein levels using an enzyme-linked immunosorbent (ELISA) assay system while in the present study, an indirect measurement of luciferase protein levels was carried out based on enzyme activity, but may also be due to the use of different cell lines or differences in transfection efficiencies.

Differences in fold inductions of luciferase activity in the presence of Wy14643 was observed when different transfection reagents were used with the lowest fold inductions observed with GeneJammer reagent-transfected cells. Both Satisfection reagent and Polyfect reagent-transfected cells gave similar fold-inductions in response to Wy14643. Taking into consideration both the efficiency of transfection and the fold increase observed in transfected cells, Polyfect reagent was chosen as

the reagent of choice for future transfection experiments. In addition a reagent to DNA ratio of 10.5 µl:1 µg was selected as the future ratio for use in transfection.

Chapter 3. Development and validation of a luciferase reporter gene assay

3.1 Introduction

Peroxisome proliferator activated receptors (PPARs) are members of the nuclear hormone receptor superfamily and function as heterodimers with the retinoid X receptor (RXR). Gel retardation assays have revealed that binding of mouse PPAR and mouse RXR to the peroxisome proliferator-response element of the acyl CoA oxidase gene, a PPAR target gene, is weak when these two receptors are in isolation (Issemann *et al*, 1993). When combined however, a much stronger interaction with the PPRE of acyl-CoA oxidase is observed. Peroxisome proliferator-activated receptors from the rat, expressed in insect cells and subsequently purified, show a loss of activity to bind to DNA, a loss which can be restored upon addition of *in vitro*-translated RXR (Gearing *et al*, 1993). In searching for compounds which activate or repress PPAR activity the formation of this heterodimeric complex with RXR presents challenges, not least because it has been found that both clofibric acid, an activator of PPAR , and 9-cis retinoic acid, a ligand of RXR , can activate the expression of acyl-CoA oxidase gene (Kliewer *et al*, 1992). Thus not only can the PPAR/RXR heterodimer complex be activated by both PPAR and RXR ligands, it appears that PPARs cannot function without the presence of RXR.

3.1.1 *A reporter gene assay to identify PPAR-interacting compounds*

The identification of compounds which interact with PPARs is hindered by the interference of compounds that interact with the RXR, the heterodimeric partner of PPARs. The removal of RXR from a cellular system is not an option due to the

requirement of RXR for proper PPAR functioning. In order to overcome these problems a fusion protein, consisting of the Gal4 DNA-binding domain and the PPAR ligand-binding domain was generated for use in a reporter gene assay. This fusion protein was not dependent upon the formation of an active PPAR/RXR heterodimer complex for transcription of the reporter gene, but instead depended only on the activity of the activation function 2 (AF2) within the PPAR ligand-binding domain following binding of this domain by an activating compound, allowing PPAR activators and antagonists to be identified with no interference or influence from the retinoid X receptor.

3.1.1.1 The Gal4-PPAR/UAS assay system

The yeast transcription activator protein Gal4 activates the transcription of target genes by binding to upstream activating sequences (UAS) within their promoter region. Similar to NHRs, the Gal4 transcriptional regulator is composed of several distinct domains, including a DNA-binding domain and two transcriptional activation domains, termed ARI and ARII (Lohr *et al*, 1995). Furthermore the Gal4 DNA-binding domain has been found to be functional within a variety of cell lines other than yeast; when the DNA-binding domain of yeast Gal4 protein was cotransfected into mammalian cells alongside the Gal4 UAS linked upstream of the mouse mammary tumour virus (MMTV) promoter, the Gal4 protein was capable of activating the transcription of the MMTV promoter (Kakidani and Ptashne, 1988). Several authors have described the use of Gal4 fusion proteins in cellular assay systems to study the human steroid hormone receptors, with literature documenting the activities of mutated estrogen receptors (Bush *et al*, 1996), the assessment of the

estrogenic activity of various chemicals (Balaguer *et al*, 1996) and the modulation of the progesterone receptor (Rudakoff *et al*, 1999).

The present work took advantage of i) the functionality of the Gal4 DNA-binding domain as a fusion protein with an activation function-containing protein and ii) its functionality in cell types other than yeast. A fusion protein was created for use in a fish cell line for the development of a cellular transient transfection assay to identify both activators and repressors of the peroxisome proliferator-activated receptors. The fusion protein consisted of amino acids 1-147 of the Gal4 gene, encoding the Gal4 DNA-binding domain (Gal4-DBD). The Gal4-DBD was fused in-frame to the ligand-binding domain of PPARs with the expression of the whole construct being driven by the cytomegalovirus (CMV) immediate early promoter (Figure 3-1(A)). A second plasmid construct contained five Gal4 UAS sequences, to which the Gal4DBD could bind. These sequences were upstream of a TATA box which itself was upstream of a reporter gene, which in this case was firefly luciferase (Figure 3-1B).

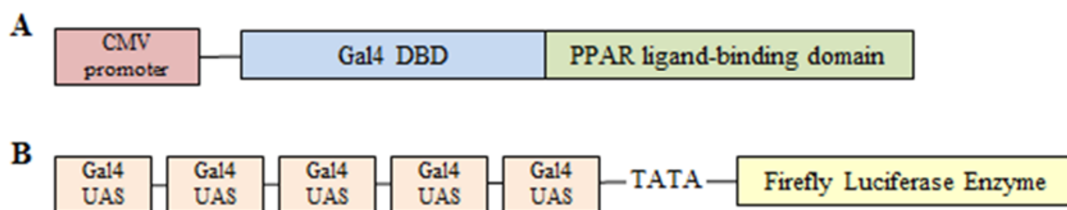


Figure 3-1. Schematic representations of the two constructs used in the Gal4-UAS assay system. A: the Gal4-PPAR construct in which the ligand-binding domain of PPARs were cloned downstream of the Gal4DBD whose expression was driven by the CMV promoter. B: The Gal4UAS-luc construct containing 5 Gal4UAS sequences upstream of a TATA box which itself was upstream of a firefly luciferase enzyme.

The PPAR ligand-binding domain contains an AF2 in which the ligand-induced reorientation of helix 12, and subsequent release of corepressor proteins and binding of coactivator proteins, is critical to its activation (Moras and Gronemeyer, 1998).

Thus although the DNA-binding domain of the Gal4-PPAR fusion construct can interact with the upstream activating sequences of the Gal4UAS-luc construct, this complex remains transcriptionally inactive until binding of a ligand to the ligand-binding domain of the PPAR, leading to AF2 activation and ultimately an increase in luciferase expression. The cascade of steps which ultimately results in an increase in the transcription of the firefly luciferase reporter gene is show in Figure 3-2. The use of the Gal4-PPAR fusion protein described above has a single major advantage over using full-length PPAR receptors in that the activity of the peroxisome proliferator-activated receptors can be assessed while uncoupled from its endogenous heterodimeric RXR partner. Furthermore it is foreseen that the assay can be adapted relatively easily to study other members of the hormone receptor superfamily.

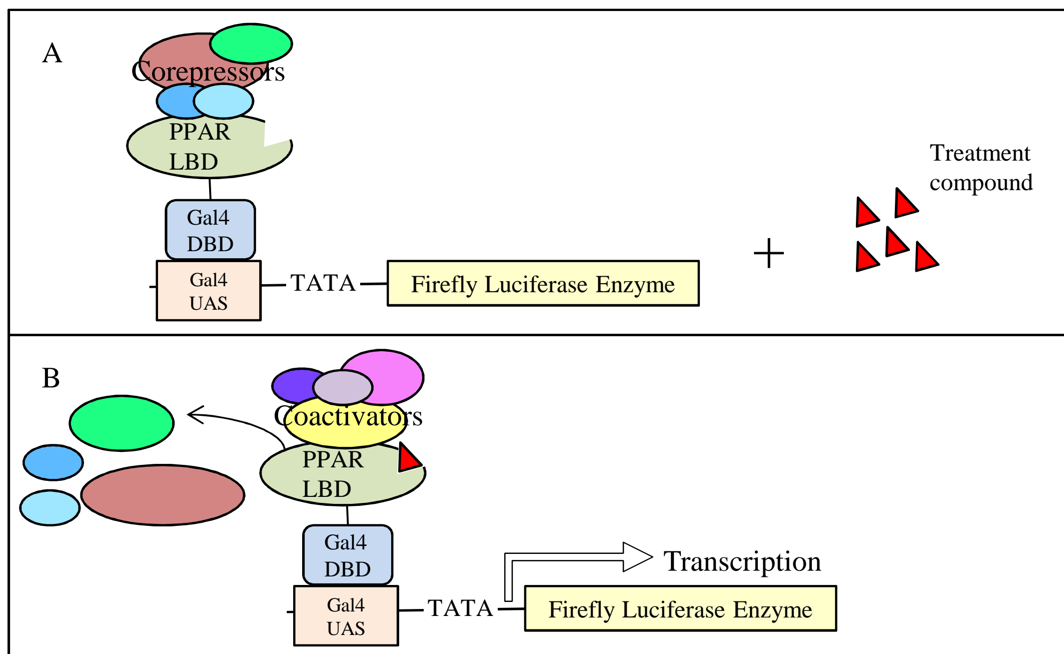


Figure 3-2. Schematic diagram of the Gal4-UAS assay system. A: The Gal4-PPAR and the Gal4UAS-luc constructs are co-transfected into a fish cell line. The fusion protein remains transcriptionally inactive due to the association of the PPAR LBD with corepressor proteins. Transfected cells are treated with a compound of interest. B: An interaction between the treatment compound and the PPAR ligand-binding domain leads to a conformational change in the Gal4-PPAR construct, a dissociation of corepressor proteins and the recruitment of the transcriptional machinery, leading to an increase in the transcription of the firefly luciferase reporter enzyme.

3.1.1.2 Luciferase genes as reporter proteins

Luciferase genes are widely used reporter genes due to their sensitivity and the relative ease in measuring the levels of the luciferase protein. *Renilla* luciferase is a 36kDa protein isolated from the sea pansy (*Renilla reniformis*) and differs from firefly luciferase both in its substrate and its cofactor requirements. In addition whereas firefly luciferase produces a greenish yellow light in the 550-570nm range, Renilla luciferase produces a blue light of 480nm. These differences in substrate, cofactor requirements and light output are characteristics which allow these enzymes to be used in the same assay system. Due to their wide usage several commercially available reagents, containing the substrates for luciferase proteins, are available which enables the quantification of luciferase protein levels in cell cultures. Generally, after producing luminescence, luciferase proteins undergo spontaneous inactivation producing the characteristic "flash"-type kinetics in which a very rapid decrease in luciferase signal occurs over a short time period. In order to overcome such a problem, several research groups have developed reagents in which the rate of luciferase inactivation is slowed by slowing the rate of catalysis, generating lower level of luciferase luminescence from samples but producing luminescence levels which remain stable over several hours. The most widely used commercial application of such reagents is the Dual-Glo® assay system developed by Promega. This has the advantage of allowing luciferase protein to be assayed over a longer time period. Despite the advantages of these reagents the costs can be prohibitively expensive, limiting the number of experiments which can be performed. Furthermore the compositions of commercial reagents are not available. However, literature documenting a formulation for non-commercial firefly and *Renilla* luciferase assay buffers (Dyer *et al*, 2000) along with several patents (Wood, 1994; van Lune and

Bruggeman, 2006) provide several methods for attenuating and prolonging luciferase signals. One finding is that the inclusion of a compound which competes with or which slightly inhibits the firefly luciferase enzyme can help sustain firefly luciferase signal for longer time periods. These compounds include thiol reagents such as coenzyme A (CoA) at concentrations between 0.1 and 1 mM and dithiothreitol (DTT) between 20 and 100 mM (Wood, 1994). Further to this, a patent exists (van Lune and Bruggeman, 2006) which describes the inclusion of phosphate and ammonium ions in a luciferase buffer, resulting in the sustainability of firefly luciferase signal with satisfactory results obtained in a reaction mixture comprising at least 250 mM Tris, accompanied with at least 125 mM phosphate. Using the information from these sources to produce non-commercial, “homemade” luciferase buffers would provide considerable cost-savings over commercial reagents.

The aims of this chapter were:

(i) to isolate, by polymerase chain reaction (PCR), the LBD of plaice PPARs from the full receptors and clone the LBD into the pBIND plasmid, thus creating the Gal4-PPAR LBD construct.

(ii) to test the response of the Gal4-PPAR LBD construct to known PPAR ligands. This aim will be fulfilled through the measurement of luciferase enzyme activity from FHM cells transfected with the Gal4-PPAR LBD construct, a reporter gene construct (Gal4UAS-luciferase plasmid) and an internal control plasmid (*Renilla* luciferase plasmid) and subsequently treated with Wy14643, GW501516, Bromopalmitate or Rosiglitazone, i.e. known PPAR agonists.

(iii) to use available patents and current literature to formulate and test non-commercial luciferase buffer preparations as a cheaper alternative to the commercially available Dual-Glo® luciferase assay system (Promega), while limiting the steps involved in luciferase measurements, thus also making the assay time-efficient.

3.2 Materials and Methods

3.2.1 Construction of the Gal4-PPAR expression plasmid

Three cDNA sequences, each encoding a separate peroxisome-proliferator activated receptor called PPAR 2, PPAR and PPAR , have previously been isolated from plaice (*Pleuronectes platessa*) and cloned into the pcDNA3 vector (Invitrogen) (Leaver *et al*, 2005). Accession numbers for each PPAR isotype from *P.platessa* are AJ539467.1, AJ536468.1 and AJ243956.2 for PPAR , PPAR and PPAR , respectively.

3.2.1.1 Amplification of PPAR ligand-binding domains

Ligand-binding domains of PPARs were amplified and cloned into the pBIND cloning vector, containing the Gal4-DBD. Amplification and cloning was performed such that the ligand-binding domains of PPARs were cloned downstream and in-frame with the Gal4-DBD. Corresponding to amino acids 153 to 474, 206 to 510 and 196 to 532 for PPAR , PPAR and PPAR respectively, the ligand-binding domains of PPARs were amplified by polymerase chain reaction (PCR). For each PPAR, a pair of primers was designed around the nucleotide sequences corresponding to the amino acids above (Table 3-1). Each forward and reverse primer included recognition sequences for restriction enzymes BamHI (5’ -GGATCC- 3’) and KpnI

(5' -GGATCC- 3'), respectively, at their 5' ends. The recognition sequences for these enzymes were selected due to their being present within the multiple cloning region of the pBIND plasmid into which the PPAR ligand-binding regions are to be cloned but absent in the ligand-binding domains of all three PPAR isotypes, confirmed using DNA restriction mapper tool, NEBcutter V2.0 (Vincze *et al*, 2003). Incorporation of these restriction enzyme recognition sequences into primers will allow their subsequent incorporation into the amplified PCR products and the directional cloning of the ligand-binding domains into the pBIND cloning vector, downstream of and in-frame with the Gal4 DNA-binding domain.

Table 3-1. Primer sequences used to amplify the ligand-binding domains of PPARs from *Pleuronectes platessa*. Recognition sequences of BamHI and KpnI are highligted in red.

PCR primer name	Primer sequence (5' → 3')
pBINDpp F	TT GGATCC GA ATG TCC CAC AAC GCC ATT CGG TTT
pBINDpp R	AA GGTACC TCA CTT CAG TAC ATG TCC CGG TAT
pBINDpp F	TT GGATCC GA ATG TCC CAT GAT GCG ATC CGA TAC
pBINDpp R	AA GGTACC GA ACA CTA ATA CAT GTC TTT GTA G
pBINDpp F	TT GGATCC GC ATG TCA CAC AAC GCT ATT CGT TTT
pBINDpp R	AA GGTACC CTC TAA TAC AAG TCC TTC ATG ATC

PCR reactions were prepared in 20 µl volumes each containing 5 ng template DNA (pcDNA3- PPAR , pcDNA3-PPAR or pcDNA3-PPAR), 1× *Pfu* polymerase buffer (Promega), 250 µM deoxynucleotide triphosphate (dNTP) mix, 250 nM each of forward and reverse primer and 2.5 units *Pfu* polymerase enzyme (Promega).

Reactions were cycled in a thermal cycler (Biometra T gradient) using the following conditions: one minute at 95°C (initial denaturation), followed by 20 seconds at 95°C

(denaturation), 20 seconds at 50°C (primer annealing) and 15 seconds at 72°C (extention). The denaturation, annealing and extention steps were cycled 30 × with a final extention step of three minutes at 72°C completing the cycle. A sample of each amplification product was loaded onto a 1× TAE, 1 % agarose, 0.5 µg/ml ethidium bromide gel and electrophoresed at 75 V for 30 minutes alongside a 1 kb DNA ladder (Fermentas). Ethidium bromide-stained DNA was visualised by exposure of the gel to UV light. Products of 974, 915 and 1014 base pairs were expected for the ligand-binding domains of PPAR , PPAR and PPAR , respectively.

3.2.1.2 Precipitation and restriction digestion of DNA from PCR reactions

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 ethanol absolute were added, reactions mixed thoroughly, incubated at -20°C for 1 hour and centrifuged at 16,000 × g for 15 minutes. The supernatant was discarded and the DNA pellet washed with 150 µl 70 % ethanol before a second centrifugation and decantation of the supernatant, leaving behind a pellet of purified DNA, which was dried at room temperature for 15 minutes.

Individual DNA pellets were resuspended in digestion reactions containing final concentrations of 1× multicore buffer (Promega), 5 units BamHI restriction enzyme (Invitrogen) and 5 units KpnI restriction enzyme (Invitrogen). pBIND vector (1µg; Promega) was digested in the same way. Reactions were incubated at 37 °C for one hour followed by inactivation of restriction nucleases by heating at 75 °C for 15 minutes. The digested pBIND vector was dephosphorylated, removing the 5' phosphate from the linearised vector to avoid any possibility of self-religation, using

1 unit of shrimp alkaline phosphatase enzyme (Boehringer Mannheim) and 1× dephophorylation buffer (Boehringer Mannheim). The reaction was incubated at 37°C for 15 minutes before the alkaline phosphatase enzyme was denatured by heating at 65 °C for 15 minutes. Products were then purified using the chloroform/phenol extraction method. This method involves the dilution of reactions to 100 μl with milliQ water before the addition of 100 μl phenol and 100 μl chloroform, and vortexed vigorously. Tubes were centrifuged at 16,000 × g for 2 minutes and the DNA-containing aqueous upper phase removed. To the aqueous phase 200 μl of chloroform was added and the centrifugation and removal of the upper phase process repeated. Ethanol precipitation then followed as previously described above.

3.2.1.3 Ligation of digested PCR products into digested pBIND vector

Ligation of the amplified and digested ligand-binding domains into the pBIND vector was done in a 10 μl reaction volume. Each of the precipitated DNA pellets were resuspended in 1 μl of the restriction-digested, dephosphorylated pBIND vector, 1× T4 DNA ligase buffer (Fermentas) and 0.5 μl (15 Weiss units) T4 DNA ligase enzyme (Fermentas). The volume was made up to 10 μl with Milli-Q water (Millipore) and ligation reactions incubated at room temperature overnight before transformation into chemocompetent *Escherichia coli (E.coli)* cells.

3.2.1.4 Transformation of pBIND-putative PPAR LBD into Top10 E.coli

Ligated DNA was transformed into Top10 chemocompetent *E.coli* cells. 100 μl of chemocompetent cells were thawed on ice and 5 μl of each ligation reaction added per treatment. Reactions were incubated on ice for 20 minutes before cells were

transformed by heat shocking in a waterbath at 42 °C for one minute, and replacing on ice for a further 20 minutes. Five hundred microlitres of Luria-Bertani (LB) media was added to each tube of transformed cells and subsequently incubated at 37 °C for one hour with agitation (~150 rpm).

Transformants were spread on selective LB agar plates (100 µg/ml ampicillin). Selection is based on the presence of the beta lactamase gene, conferring ampicillin resistance in the pBIND vector and thus in successfully transformed *E.coli* cells. Plates were incubated overnight at 37 °C overnight before four colonies from each plate were used to inoculate 4 ml cultures (LB media, supplemented with 100 µg ampicillin), followed by a further overnight incubation at 37°C with agitation (~150 rpm). Plasmid DNA was purified from cultures using the GenElute™ Plasmid Miniprep Kit (Sigma), following manufacturer's instructions. DNA was eluted from columns using 100 µl Milli-Q water (Millipore).

A sample of each eluate was subjected to a restriction digest as described in 3.2.1.2, the products of which were electrophoresed for 30 minutes at 75V on a 1× TAE, 1% agarose, 0.5 µg/ml EtBr gel alongside a DNA ladder to confirm the insertion of DNA fragments of expected size into the pBIND vector. At least one clone per ligation showed the expected restriction pattern, consisting of one DNA band at 6kb corresponded to the pBIND vector and a second band about 1000bp corresponded to the inserted PPAR ligand-binding domain.

3.2.1.5 Sequencing of the isolated plasmids

Plasmids were sequenced to confirm the identity of the insert using an autosequencer (CEQ8800, Beckman Coulter) in which capillary electrophoresis is used to separate

and detect dye-labelled DNA fragments (Swerdlow and Gesteland, 1990). Sequencing reactions were carried out in a 5µl volumes and each reaction contained 275 to 350 ng of plasmid DNA template, heated to 96°C for 1 minute before cooling rapidly on ice. The amount of template per reaction was dependent on the total size of plasmid plus insert as recommended in the sequencing protocol. Additionally 0.5 µM sequencing primer (Table 3-2) and 2 µl sequencing mix (GenomeLab DTCS Quick Start Kit, Beckman Coulter) was included. The strategy used for sequencing each pBIND-putative PPAR LBD construct is detailed in Figure 3-3. Reactions were placed in a thermocycler (Biometra TGradient) and cycled 35 times in the following sequence before post-reaction cleanup: 96 °C for 1 minute, 50 °C for 10 seconds and 60 °C for 4 minutes.

Table 3-2. Sequences of primers used in the sequencing of pBIND vectors containing putative ligand binding domains of PPAR , PPAR and PPAR .

Name of Sequencing Primer	Primer sequence (5' → 3')
PPAR LBDseqR	CCG GTG AGT ATG ACC TTC GCC
PPAR LBDseqR2	GCC GCC ACG TGC CAC CAG GAG GCC
PPAR LBDseqR3	TGG AGC TGC AGC GCC TGG AGA ATG
PPAR LBDseqR	GCC TGT CAG GTA CTG CGG GCC
PPAR LBDseqR2	TTT GGG CTC CAT GAT CTC ACT GAA
PPAR LBDseqR3	GAG CGT TCT CAG TAA CCA GCT GAC
PPAR LBDseqR	GGT CTT CCC AGA GAG GAT GGC
PPAR LBDseqR2	GGT CAT CTT CTG GAG CAG CTT GGC
pBINDseqF	CTT CAG TGG AGA CTG ATA TGC
pBINDseqR	CAC TGC ATT CTA GTT GTG GT

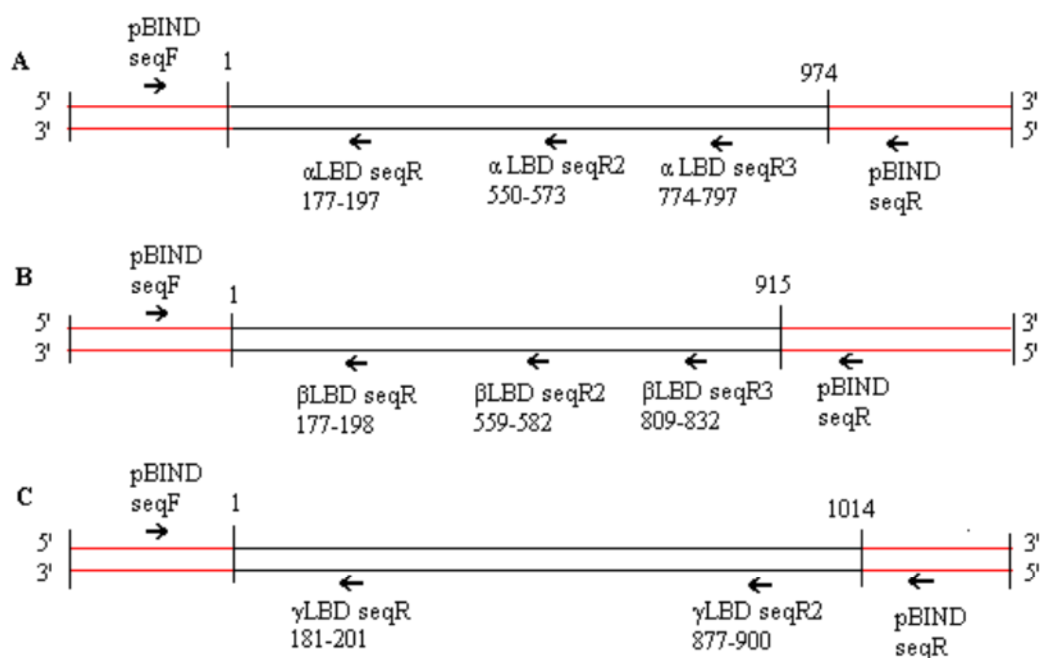


Figure 3-3. The annealing position of primers used to sequence the pBIND vectors containing putative PPAR (A), PPAR (B) and PPAR (C) ligand-binding domains. Lines represent the sense (5' ➔ 3') and antisense (3' ➔ 5') DNA strands of the plasmid. Red lines represent the pBIND vector sequence and black lines represent the PCR product ligated into the pBIND vector. Arrows indicate the direction in which sequencing will advance with numbers under primer names indicating the nucleotide positions within each ligand-binding domain to which the primer will anneal.

3.2.1.6 Post-reaction sequencing cleanup

To stop the sequencing reactions and precipitate DNA, 20 µl 150 mM sodium acetate (Sigma), 10 mM EDTA (Sigma), 1 µl glycogen and water was added along with 60 µl ice cold ethanol. Reactions were mixed thoroughly and centrifuged at 16,000 × g for 10 minutes in a microcentrifuge. The supernatant was pipetted off and the remaining DNA pellet washed with 150 µl 70% ethanol and centrifuged for 2 minutes before the ethanol was removed and the DNA pellet left to dry. Once all traces of ethanol was removed the DNA pellet was resuspended in a 35 µl volume of sample loading formamide solution (SLS formamide; GenomeLab), transferred to a sequencing plate and covered with a single drop of mineral oil (Beckman coulter).

The raw sequence data output was analysed using the Lasergene SeqMan programme (DNASTAR). Confident changes were made to the raw sequences where required and putative PPAR ligand-binding domain sequences compared to database entries for the European plaice PPAR ligand-binding domain sequences. Sequences were also analysed to confirm that the DNA insert in each construct was in the same open reading frame as the upstream yeast Gal4 DNA-binding domain found in the pBIND vector.

3.2.2 *Testing the Gal4-PPAR LBD constructs in a transactivation assay*

Following confirmation that the fragments ligated into the pBIND vector were the ligand-binding domains of PPAR , PPAR and PPAR , the response of the fusion proteins to well characterised PPAR ligands was assessed in a transient transfection assays.

3.2.2.1 Seeding of FHM cells

FHM cells, isolated from fathead minnow (Pimephales promelas) (Gravell and Malsberger, 1965) were seeded to 96 well assay plates in DMEM (supplemented with 10% charcoal/dextran treated FBS, 50 units per ml penicillin and 50 µg/ml streptomycin) as described in 2.2.1.2.

3.2.2.2 Transfection of FHM cells

Transfection occurred 24 hours after seeding of FHM cells. Cells were transfected with 25ng Gal4-PPAR LBD, Gal4-PPAR LBD, Gal4-PPAR LBD or pBIND plasmid containing the Gal4 DNA-binding domain only, together with the 10ng

internal reporter plasmid pGL4.75[*hRluc*/CMV] and 25 ng of the experimental reporter pGL4.31[*luc2P/Gal4*UAS/hygro]. For each well to be transfected DNA was diluted with DMEM, with serum and antibiotics absent, to a final volume of 3 µl. After mixing and brief centrifugation to collect DNA/DMEM mix in the bottom of the tube a volume of Polyfect reagent (Qiagen) was added to give a final Polyfect to DNA ratio of 10.5 µl:1 µg and transfection mixes incubated at room temperature for 10 minutes. Media in which cells were seeded was replaced with 60 µl 1 × Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) containing 4.5g/L glucose, 10% (v/v) charcoal/dextran treated fetal bovine serum (Fisher Scientific) 50 units/mL penicillin and 50 µg/ml streptomycin (Invitrogen) before 20 µl transfection mix was added to wells of the assay plate.

Transfected cells were incubated for 24 hours prior to treatment.

3.2.2.3 Treatment of transfected cells with mammalian PPAR agonists

Treatments were selected upon the basis that they had previously been shown to be mammalian and, in some cases, piscine PPAR activators (Leaver *et al*, 2005). Each compound was prepared in ethanol absolute at 200× the concentration to which the transfected cells would be exposed. Compounds and concentrations were as follows: 4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid (Wy14643; Sigma) prepared at 5 mM concentration, 2-methyl-4-((4-methyl-2-(4-trifluoromethylphenyl)-1,3-thiazol-5-yl)-methylsulfanyl)phenoxy-acetic acid (GW501516) prepared at 2 mM concentration, Bromopalmitate prepared at 2 mM concentration, Rosiglitazone prepared at 5 mM and fatty acids at 20 mM.

In 5mL bijoux tubes each compound was diluted into complete DMEM at a concentration of 5 µl per mL, giving final assay concentrations of 2 µM (GW501516 and bromopalmitate), 5 µM (Wy14643 and rosiglitazone) or 100 µM (fatty acids). In addition an ethanol vehicle control treatment was prepared using, per mL of DMEM, 5µl ethanol absolute. Diluted treatment compounds were mixed thoroughly before being added to transfected cells. Transfection mixes were removed from each well of the assay plate and cells washed once with 1 × DPBS (Invitrogen), after which 75 µl of treatment compounds added to cells. Treatments were administered in such a way that each Gal4-PPAR construct/empty Gal4 plasmid was treated in triplicate with each compound.

Cells were then incubated with treatment compounds for a further 24 hours at 22°C, 5% CO_2 before assaying for luciferase activity.

3.2.3 Luciferase assay, data normalisation and statistical analysis

To assay for luciferase activity in transfected cells treated with known mammalian PPAR agonists, the Dual-Glo® ™ luciferase assay system (Promega) was used, as described in the manufacturer's instruction manual. Both firefly and *Renilla* luciferase activites were measured. *Renilla* luciferase values were used to normalise for variabilities in transfection efficiencies between wells. This normalisation was achieved by dividing each firefly luciferase value by its corresponding *Renilla* luciferase value. Values from replicate wells were then averaged and expressed as mean normalised firefly luciferase activity. In each case data shown are the means of three independent experiments. The effects of treatments on transactivation were

tested by one way analysis of variance (ANOVA), followed by Dunnet's *post hoc* test to identify where significant differences lay. An assumption of ANOVA is that data are sampled from a population with equal variances. Therefore before carrying out ANOVA, homogeneity of variances was tested using the F-max test and where necessary data were log transformed to improve homogeneity. Results were considered statistically significant when the probability value (P) was less than 0.05 ($P < 0.05$).

3.2.4 *Formulation and testing of "homemade" luciferase buffers*

A draw-back in using the Dual-Glo® ™ luciferase assay system is that it is prohibitively expensive and the exact compositions of the buffers which allow extension and quenching of the luciferase signal are not available. However, a report of "homemade" luciferase buffers (Dyer *et al*, 2000) together with the valuable information available in several patent descriptions has been combined to formulate two buffers as an alternative to the Dual-Glo®™ luciferase assay reagents. The firefly reagent formulated contained, in addition to the required pH buffering and co-factors, high concentrations of phosphate, DTT and co-enzyme A, which partially inhibit firefly luciferase activity, leading to attenuation of light output but with a more stable luminescence signal over an extended time course. The *Renilla* reagent contained sodium azide and was buffered to pH 5.1, conditions which abolish (quench) firefly luciferase (Dyer *et al*, 2000), but still enable the measurement of *Renilla* luciferase activity.

Cells transfected with Gal4-PPAR and treated with Wy14643 (see 3.2.2), served to compare luciferase measurement methodologies between the Dual-Glo®™ luciferase assay system and the alternative luciferase assay buffers comparing the following parameters:

- Overall levels of firefly and *Renilla* luciferase signal
- Stability of firefly and *Renilla* luciferase signal over time
- The effectiveness of quenching firefly activity during the *Renilla* luciferase measurement

For each of the three conditions mentioned above, twelve wells were transfected with Gal4-PPAR , six of which were treated with Wy14643 and six with ethanol vehicle. In measuring luciferase activity half the wells of each treatment were assayed using the Dual-Glo luciferase assay system and the remaining half assayed using the "homemade" luciferase buffers.

3.2.4.1 Measurement of Firefly Luciferase Activity

Twenty four hours after treatment of transfected cells with 25 µM Wy14643 or 0.5% ethanol vehicle, a 2× concentrated volume of "homemade" firefly luciferase buffer or Dual-Glo® luciferase reagent was added to wells, the volumes of which were equal to the 75 µl volume of media already present in the wells. The final composition of the homemade buffer in wells was as follows; 1× passive lysis buffer (Promega), 16mM dithiothreitol (DTT; Sigma), 2mM magnesium sulphate, 25mM Tris Phosphoric acid (pH 8.0), 0.5mM Adenosine triphosphate (ATP; Sigma), 100 µM coenzyme A (CoA; Sigma), 1mM EDTA and 190 µM beetle luciferin (Promega).

The contents of the wells were pipetted up and down to mix and a 10 minute incubation given before measurement of luciferase activity on the Wallac 1420 Victor 2 multilabel counter (Perkin Elmer). One of the aims of this experiment was to measure the stability of firefly luciferase signal over time and therefore firefly luciferase activity was monitored in several wells every 20 minutes for 1 hour.

3.2.4.2 Renilla Luciferase Measurement

Following measurement of firefly luciferase, a 75 µl volume of 3× concentrated "homemade" *Renilla* luciferase buffer or 75 µl of Dual-Glo® Stop & Glo® reagent (Promega) was added. The final composition of the "homemade" *Renilla* luciferase reagent was 0.55M sodium chloride, 3.33mM EDTA, 0.33mg/mL BSA (Sigma), 0.67mM sodium azide (Sigma), 100mM sodium phosphate buffer (pH 5.1) and 1 µM coelenterazine (Promega). Several wells to which "homemade" *Renilla* luciferase buffer was added, was done so with coelenterazine absent with the volume of coelenterazine being replaced by MilliQ water. The purpose of these wells was to test the effectiveness of the *Renilla* buffer in quenching firefly luciferase signal, prior to measurement of *Renilla* luciferase luminescence. Coelenterazine acts as a substrate for *Renilla* luciferase protein, and upon its oxidation by *Renilla* luciferase, leads to the production of a measurable light emission. In the absence of Coelenterazine there is no light emission due to the activities of *Renilla* luciferase protein, therefore any luminescence produced after addition of *Renilla* buffer with no coelenterazine present will be due to the activity of firefly luciferase. In these wells minimal light emission is expected if the *Renilla* buffer is a good firefly luciferase quench. Following addition of *Renilla* luciferase buffer (with or without coelenterazine), or Stop & Glo® reagent, the contents of the wells were pipetted up

and down to mix and a 10 minute incubation given. *Renilla* luciferase activity was measured on the Wallac 1420 Victor 2 multilabel counter (Perking Elmer).

3.3 Results

3.3.1 Construction of the expression plasmid

3.3.1.1 Amplification of the ligand-binding domain from Pleuronectes platessa PPAR cDNA

Using primer pairs designed to the 5' and 3' end of the ligand-binding domains of each PPAR subtype, the polymerase chain reaction (PCR) was expected to amplify DNA fragments of 969, 915, and 1014 base pairs using the full open-reading frame of PPAR , PPAR and PPAR as a template, respectively. Analysis of PCR products using agarose gel electrophoresis revealed a single DNA product from each reaction. Furthermore these products were within the expected size range for the PPAR ligand-binding domains.

3.3.1.2 Cloning of PCR-amplified DNA fragments into the pBIND vector (Promega)

Before sequencing the putative ligand-binding domain products to confirm their identities, fragments were cloned into the pBIND vector (Promega) by means of a restriction digest and ligation reaction as described in 3.2.1.2 and 3.2.1.3. Following transformation of ligation product into Top10 *E.coli* cells, selection of colonies and purification of plasmid DNA, a restriction digest was carried out on a sample of each plasmid to confirm which, if any, of the plasmid preparations contained the putative ligand-binding domains. In five of the twelve samples, two bands were visible following restriction digest reactions, corresponding to the expected size of the

ligand-binding domain inserts and the 6 kb size of the pBIND vector. This was indicative that the ligation reactions had been successful and that each product amplified by PCR was present in at least one plasmid preparation.

3.3.1.3 Sequencing of the pBIND-PPAR ligand-binding domain plasmid

The five plasmid preparations which produced two fragments when subjected to a restriction digest (1, 4, 2, 3 and 3) were sequenced to confirm a) that the DNA inserts in the pBIND vectors are the ligand-binding domains of the PPARs and b) that the ligand-binding domain sequence and the Gal4 DNA-binding domain of the pBIND vector were in the same continuous reading frame. Assembly of the raw sequencing files for each plasmid resulted in a single continuous contig for each PPAR isotype. The sequences for each PPAR isotype from *P.platessa* are available from the Genbank database and comparisons of the cloned sequences with the sequences from the database revealed identical sequences. Importantly sequencing also confirmed that the Gal4 DNA-binding domain and the PPAR ligand-binding domains were in a continuous, single frame (Figure 3-4).

A

```
cagcatagaataagtgcgacatcatcatcggaagagagtagtaacaaaggtcaaagacag
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 L  T  V  S  P  E  F  P  G  I  R  M  S  H  N  A  I  R  F  G
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B

```
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 R  Y  S  P  K  T  K  R  S  P  L  T  R  A  H  L  T  E  V  E
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 *  C  S  V  P  E  *  L  R  P  L  P
```

C

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E D L D M I L K M D S L Q D I K A L L T

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G L F V Q D N V N K D A V T D R L A S V

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E T D M P L T L R Q H R I S A T S S S E

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L N V K P I E Q L Q E T V L H S L E L Q

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L K L N H P D S L Q L F A K L L Q K M T

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D L R Q I V T D H V H L I Q L L K K T E

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V D M C L H P L L Q E I M K D L Y * R V

CCtgaataactaaggccgcttccctttagtgagggttaatgcttcgagcagacatgataa

P E * L R P L P F S E G * C F E Q T * *

gatacattgatgagtttggacaaaccacaactagaatgcagtgaaaaaaatgctttattt

D T L M S L D K P Q L E C S E K N A L F

gtgaaatttgtgatgctattgctttatttgtaaccattataagctgcaataaacaagtta

V K F V M L L L Y L * P L * A A I N K L

acaacaacaattgcattc

T T T I A F

Figure 3-4. Nucleotide and deduced amino acid sequences of Gal4-PPAR (A), Gal4-PPAR (B) and Gal4-PPAR (C) contigs produced from sequencing reactions described in 3.2.1.5. The DNA-binding domain of the Gal4 protein is underlined. The start and stop codons of the PPAR ligand-binding domains are boxed with the stop codon being indicated by an asterisk. The BamHI (GGATCC) and KpnI (GGTACC) enzyme recognition sites are highlighted in bold and italics.

3.3.2 Response of Gal4-PPAR LBD expression constructs to known PPAR ligands

Previously isolated from European plaice, transient co-transfection of native full-length PPAR with a reporter construct in which the peroxisome proliferator response element (PPRE) of mouse Cyp4A6z was attached upstream of a chloramphenicol acetyltransferase (CAT) reporter gene, has been shown to increase transcription of the CAT reporter gene following exposure to the synthetic PPAR -specific activator Wy14643 (Leaver *et al*, 2005). FHM cells transfected with Gal4-PPAR LBD and subsequently exposed to 25 μM Wy14643 showed similar behaviour to the full-length native receptor, inducing luminescence signal from the luciferase reporter construct 50-fold (Figure 3-5). Additionally, the induction in reporter gene expression by Wy14643 was specific to those cells transfected with Gal4-PPAR LBD, with inductions from Gal4-PPAR LBD- and Gal4-PPAR LBD-transfected cells being within the same range as that observed after ethanol vehicle treatment.

As well as Wy14643, GW501516 also increased Gal4-PPAR LBD mediated inductions in reporter gene expression, increasing luciferase activity by 20-fold. Developed as a selective modulator of PPAR , GW501516 was also able to increase reporter gene expression following transfection of the Gal4-PPAR LBD construct. The induction of luciferase luminescence from cells transfected with Gal4-PPAR LBD and subsequently treated with GW501516 was almost 2-fold greater than that from Gal4-PPAR LBD-transfected cells, with an induction of almost 37-fold being observed in the presence of Gal4-PPAR LBD.

Bromopalmitate, previously shown to be an activator of PPAR in Atlantic salmon (Leaver *et al*, 2007) was able to induce transactivation of Gal4-PPAR and Gal4-PPAR 32-fold and 35-fold, respectively.

Previously, native PPAR and PPAR from both sea bream (*Sparus aurata*) and plaice have been shown to respond to fatty acids, suggesting these compounds to be potential endogenous activators of piscine PPARs (Leaver et al., 2005). In FHM cells transfected with the Gal4-PPAR construct all of the tested fatty acids, with the exception of linoleic acid (18:2n-6), significantly induced reporter gene expression. In cells transfected with Gal4-PPAR there was no response after oleic (18:1n-9), linoleic (18:2n-6), linolenic (18:3n-3), arachidonic (20:4n-6) or docosahexaenoic acid (22:6n-3) treatments, whereas strong responses were observed with palmitoleic, -linoleic (18:2), stearidonic (18:4n-3) and eicosapentenoic acid (20:5n-3). N-oleoyl ethanolamine (OEA) and N-linoleyl ethanolamine (LEA) were effective agonists of both PPAR and PPAR . None of the fatty acids tested provoked a response with Gal4-PPAR .

FHM cells transfected with Gal4-PPAR LBD and Gal4-PPAR LBD, showed positive responses to treatment with activating compounds of the PPAR and PPAR receptors. In contrast transfection of the Gal4-PPAR LBD construct and subsequent treatment with the compound rosiglitazone, a member of the thiazolidinedione class of mammalian PPAR ligands, failed to induce an induction in reporter signal. In response to all compounds tested luciferase luminescence signal from Gal4-PPAR -transfected cells remained at levels similar to those observed in the presence of the ethanol control treatment.

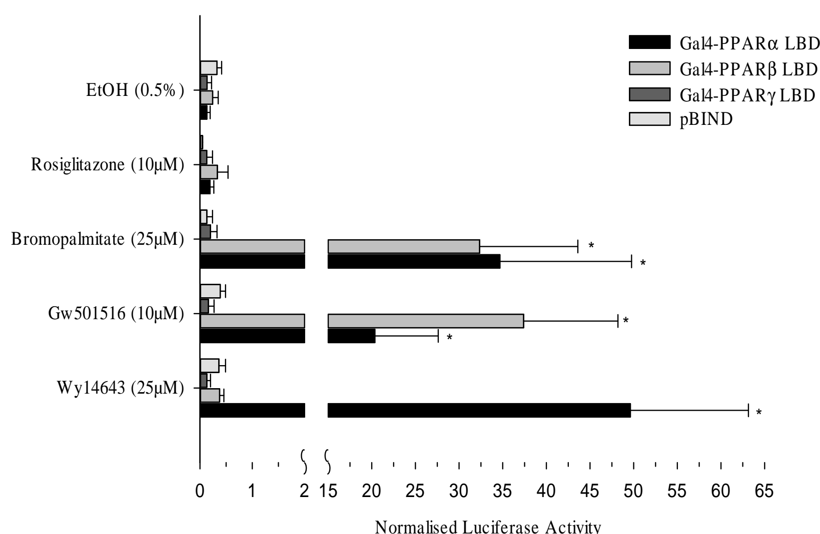

Figure 3-5. Transactivation of Gal4-PPAR constructs in response to PPAR activators. FHM cells were transiently transfected with with Gal4-PPAR constructs, firefly luciferase reporter plasmid pGL4.31 and an internal *Renilla* luciferase reporter used to correct for transfection efficiencies (pGL4.75). Post-transfection cells were treated with 25 µM Wy14643, 10 µM GW501516, 25 µM Bromopalmitate, 10 µM Rosiglitazone or 0.5% ethanol vehicle. Results are expressed as arbitrary units of firefly luciferase normalised to *Renilla* luciferase. Data represent the the mean ± SEM from three independent experiments. Asterisks indicate whether activities significantly differed (P<0.01) from those in ethanol-treated cells.

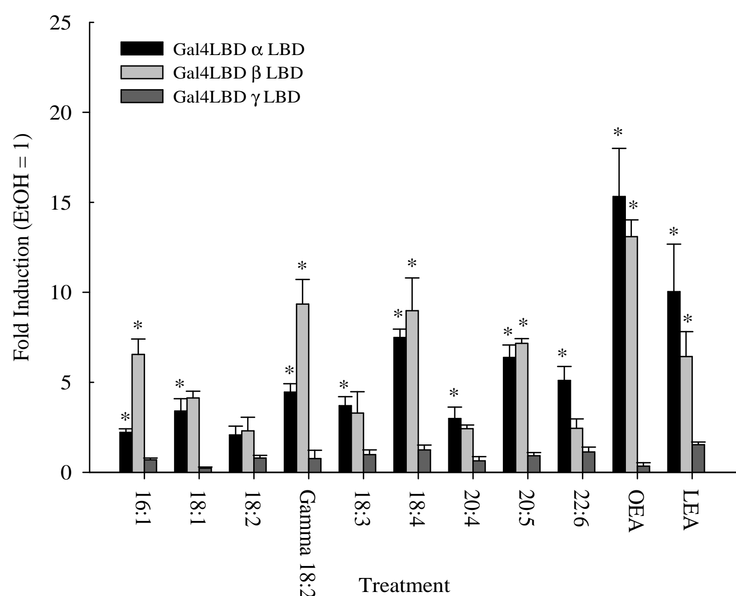

Figure 3-6. Transactivation of Gal4-PPAR constructs in response to fatty acids.FHM cells were transiently transfected as described in the legend of Figure 3-5. Post-transfection cells were treated with 100 µM of palmitoleic acid (16-1), oleic acid (18-1), linoleic acid (18-2), stearidonic acid (18-4), arachidonic acid (20-4), eicosapentenoic acid (20-5), docosahexaenoic acid (22-6), oleoylethanolamide (OEA or linoleoylethanolamide (LEA). Results are expressed as the fold increase over ethanol control of normalised firefly luciferase activity. Asterisks indicate whether activities significantly differed (P<0.01) from those in ethanol-treated cells.

Within this assay system the *Renilla* luciferase internal control reporter is constitutively expressed, and therefore the levels of luminescence from this enzyme can give an indication of transfection efficiency. The mean values obtained from measurement of *Renilla* luciferase luminescence reveals no significant differences in transfection efficiency between all four Gal4 constructs (Figure 3-7), indicating that the lack of activity from Gal4-PPAR did not originate due to low transfection efficiency of the construct into the FHM cell line.

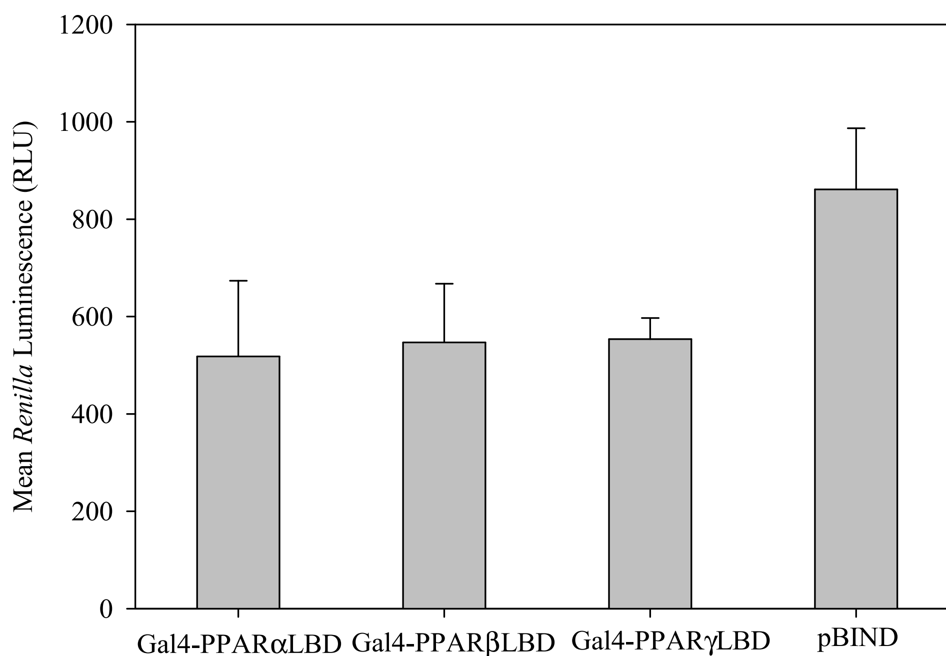

Figure 3-7. Levels of constitutively expressed *Renilla* luciferase luminescence. 10ng of plasmid DNA containing the *Renilla* luciferase gene (pGL4.75) was cotransfected with Gal4-PPAR and Gal4UAS-luc plasmids into FHM cells which were subsequently treated and assayed for firefly and *Renilla* luciferase activity. Data represents the the mean ± SEM from three independent experiments.

3.3.3 Dual-Glo® reagents vs. "Homemade" luciferase buffers

3.3.3.1 Stability of Firefly Luciferase Signal

The homemade firefly luciferase buffer, formulated using information from both patents and the non-commercial luciferase assay buffer described by Dyer *et al*

(2000) was compared to that of the Dual-Glo® assay system for its ability to sustain firefly luminescence signal.

Over a time period of 60 minutes, luminescence obtained using the Dual-Glo® luciferase reagent was very stable, losing only 10% of its luminescence by 60 minutes compared to time 0 (Figure 3-8). In comparison, the firefly luciferase signal obtained using the "homemade" firefly luciferase buffer was approximately 3.4-fold less stable, losing 34% luminescence over the 60 minutes. This was not deemed to be problematic due to the time scales involved in the measurements of luciferase activities in the experimental procedure described above. Specifically, measurement of firefly luciferase activity should occur 10 minutes after the addition of the firefly luciferase buffer (indicated as time 0 in Figure 3-8), with the multilabel counter used to measure luminescence taking approximately 2-3 minutes to read a full 96-well tissue culture plate in which assays are performed. Since the loss of activity over the 60 minute time period appears to be linear, with approximately 10% loss of signal every 20 minutes, it is predicted that in the time it takes to measure the luminescence from one whole assay plate, the loss of activity should be very small (approximately 1.5%).

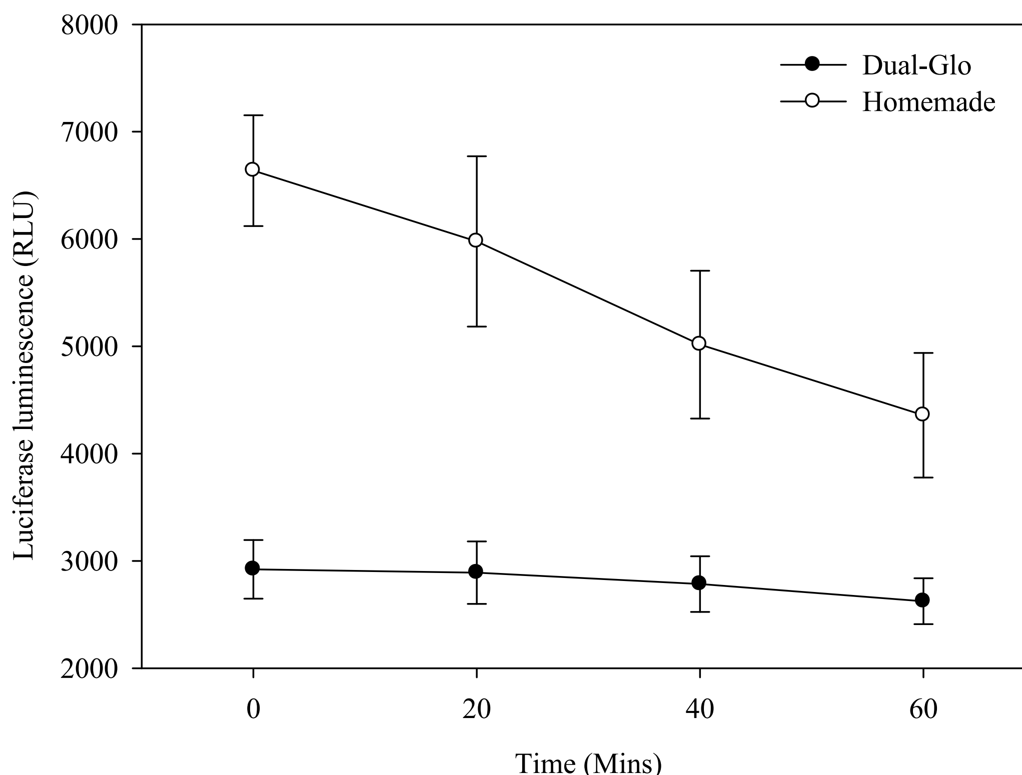

Figure 3-8. Comparison between Dual-Glo® Luciferase reagent (Promega) and homemade firefly luciferase buffer in sustaining the firefly luciferase signal over time. Cells transfected with Gal4-PPAR and treated with Wy14643 were assayed for luciferase activity 24 hours after treatment. Luciferase buffers were added and a 10 minute incubation given before the first measurement of firefly luciferase luminescence (T0). Firefly luciferase activity was measured thereafter every 20 minutes up to 1 hour.

3.3.3.2 Renilla luciferase buffer as a Firefly luciferase Quench

The next parameter of the assay to be tested was the ability of the "homemade" *Renilla* luciferase buffer to quench firefly luciferase activity. A sequential assay system in which firefly and *Renilla* luciferase activities are to be measured from the same sample requires that, prior to measurement of *Renilla* luciferase, firefly luciferase activity is completely quenched to allow *Renilla* luciferase activities to be quantified accurately. From Figure 3-9 it is evident that after addition of "homemade" *Renilla* luciferase buffer (T20), firefly activities produced from addition of both Dual-Glo® and "homemade" firefly luciferase reagents are quenched completely. Firefly activities measured after addition of Dual-Glo

luciferase reagent is quenched to 1.4% of its maximum (from 3391 units to 48 units) while firefly activities measured after addition of homemade firefly luciferase buffer is quenched to 0.24% of its original value (from 7422 units to 18 units). Mock transfected wells in which no DNA was transfected but in which firefly luciferase values were measured showed mean firefly luciferase values of 13 ± 3 units, indicating that the *Renilla* luciferase buffer quenches firefly signal down to a level which is very near that of background noise. Therefore the homemade Renilla luciferase buffer functions very well to quench firefly luciferase activities.

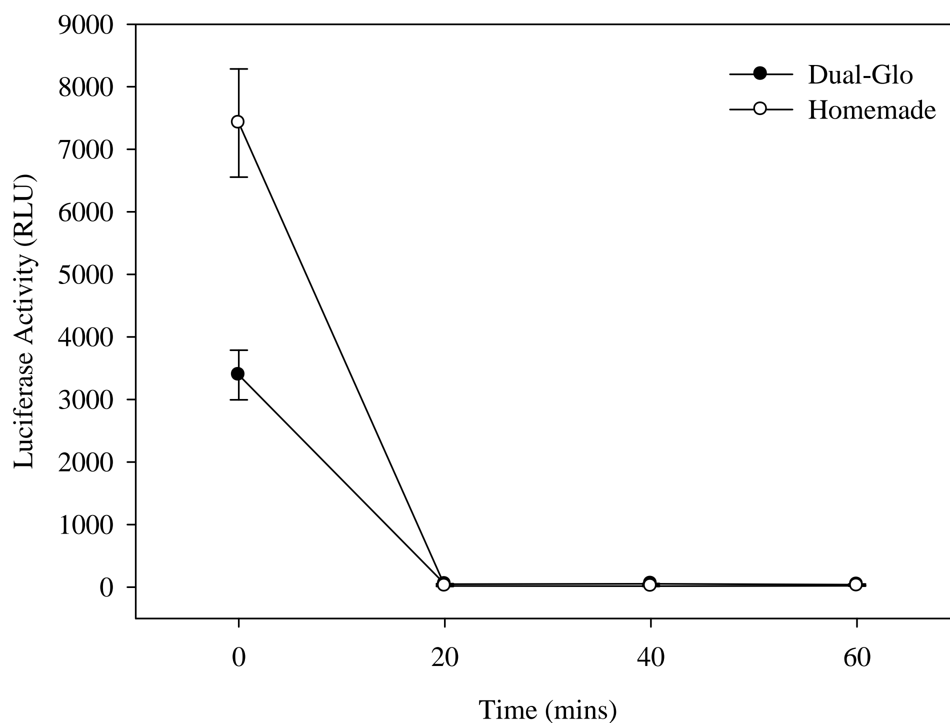

Figure 3-9. The functionality of homemade *Renilla* luciferase buffer as a quencher of firefly luciferase signal obtained by addition of Dual-Glo® reagent (Promega) or homemade firefly luciferase buffer. Cells transfected with Gal4-PPAR and treated with 25 µM Wy14643 were assayed for luciferase activity 24 hours after treatment. Firefly luciferase buffers were added and a 10 minute incubation given before measurement of firefly luminescence (T0). Homemade *Renilla* luciferase buffer with coelenterazine absent was then added, a 10 minute incubation given and luciferase activity measured (T20).

3.3.3.3 Firefly and Renilla Luciferase signals

Using both the homemade luciferase buffers, with coelenterazine included in the Renilla buffer, and the Dual-Glo® luciferase assay system, the levels of firefly and *Renilla* luciferase were compared between the two systems (Figure 3-10). The levels of firefly luciferase produced from use of the homemade luciferase buffer were approximately twice that of the Dual-Glo® luciferase reagent, in those cells transfected with Gal4-PPAR and treated with Wy14643. Levels of firefly luciferase produced from cells transfected with Gal4-PPAR and treated with ethanol vehicle was very low (5 and 29 units produced from Dual-Glo and Homemade firefly luciferase buffers, respectively) due to the inability of this compound to activate PPAR and consequently the repression of the AF2 function within the ligand-binding domain of this receptor and the inability of the Gal4-PPAR construct to transcribe the luciferase reporter gene. Upon quenching of the firefly luciferase signals by addition of Dual-Glo® Stop & Glo® (Promega) or *Renilla* luciferase buffer, *Renilla* luciferase luminescence is then produced due to the actions of this luciferase protein on its coelenterazine substrate which is present in the reagents. The Stop & Glo® reagent produces approximately 3-fold more *Renilla* signal than the homemade *Renilla* buffer (Figure 3-10), however the signal from the homemade buffer is still greater than 10-fold higher than that of background *Renilla* luminescence. Over the forty minute time period that *Renilla* luminescence was measured a decrease of 14% was observed when using the Dual-Glo® luciferase assay reagents while a decrease of approximately 25% was observed when using the "homemade" luciferase buffers. Similar to measurement of the firefly luciferase signal, the time taken to measure *Renilla* luciferase luminescence is a fraction of that measured above and therefore the decrease in *Renilla* luminescence over that time is

likely to be negligible. The results, taken together suggest that both the homemade firefly and *Renilla* luciferase buffers appear to function and are suitable for use in future luciferase reporter experiments.

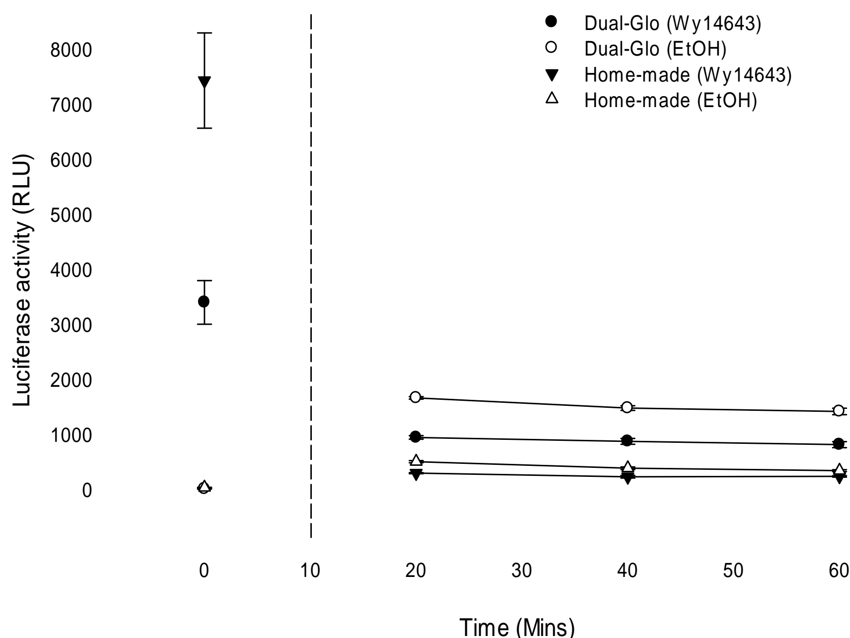

Figure 3-10. Firefly and *Renilla* luciferase luminescence obtained using either the Dual-Glo® luciferase reagents (Promega) or homemade luciferase buffers. Firefly reagents were added and a 10 minute incubation given before measurement of luciferase activity (T0). *Renilla* luciferase reagents were then added (indicated by the red dotted line) and a 10 minute incubation given before measurement of *Renilla* luciferase activities (T20). *Renilla* luciferase was then measured at 20 minute time intervals up to 60 minutes to assess the stability of *Renilla* luciferase signals. Data represents the mean ± SD of triplicate measurements made in a single experiment.

3.4 Discussion

3.4.1 Activation of Gal4-PPAR constructs by known PPAR agonists

3.4.1.1 Wy14643 as an activator of piscine PPAR

A Gal4-PPAR/Gal4UAS-luc assay was developed and validated using compounds previously shown to activate full-length native peroxisome proliferator-activated

receptors. The isolation of three PPAR isoforms from two marine fish species, the plaice (*Pleuronectes platessa*) and gilthead seabream (*Sparus aurata*), has previously been reported (Leaver *et al*, 2005). In testing each of these full-length receptors in a cell-based transactivation assay Wy14643 was found to increase the expression of the chloramphenicol acetyltransferase (CAT) reporter, under the control of the peroxisome proliferator-response element, in cells transfected with plaice and seabream PPAR . In these assays Wy14643 had no effect on the expression levels of CAT activity in cells transfected with PPAR and PPAR , despite these receptors having the ability to interact with the PPRE. As well as fish PPAR , PPAR from rodents has been found to be affected by Wy14643 *in vivo*; mice viable for PPAR , when fed a diet containing Wy14643 showed proliferation in the number and size of hepatic peroxisomes, typical of an activation of PPAR mice (Lee *et al*, 1995). This proliferation of peroxisomes in the liver was absent in mice in which PPAR was disrupted, suggesting the effects of Wy14643 to be mediated by PPAR . From this previous data in which PPAR from various species has been shown to be activated by Wy14643, the Gal4-PPAR construct appears to function similarly to native PPAR .

3.4.1.2 GW501516 activates both PPAR and PPAR

The Gal4-PPAR and Gal4-PPAR construct was able to increase expression of the firefly luciferase reporter gene in the presence of GW501516. GW501516 was developed, through combinatorial chemistry and structure-based drug design, as a potent subtype-selective PPAR agonist, interacting with the ligand-binding domain of the human PPAR to increase expression of a reporter construct within a reporter assay system (Oliver *et al*, 2001). GW501516 was able to increase the expression of

firefly luciferase reporter protein in those cells transfected with the Gal4-PPAR construct, confirming an interaction with the ligand-binding domain of PPAR in fish species as well as mammals. In their study Oliver *et al* (2001) reported that GW501516 had no effect on other nuclear hormone receptors, including human PPAR , which would appear to contrast with the findings with Gal4-PPAR in this study, which was activated by GW501516. It has been found that GW501516 is 1000 times more selective for PPAR than for PPAR , with EC50 values of 0.001µM and 1.1µM, respectively, in transactivation assays (Sznaidman *et al*, 2003). While Oliver *et al* (2001) used a maximum concentration of 1 µM GW501516 in their transactivation assays, a maximum concentration of 10 µM was used with the Gal4-PPAR constructs above. While 1 µM is approximately equal to the reported EC50 of GW501516 with PPAR , 10 µM is approximately 10-fold higher than the reported EC50 of this compound for PPAR and therefore an effect on PPAR will more likely be expected.

3.4.1.3 Fatty acids as PPAR agonists

Fatty acids, when tested at 100 µM, activated both PPAR and PPAR of flounder. This concentration is similar to that shown to activate mammalian and amphibian PPARs (G ttlicher *et al*, 1992; Krey *et al*, 1997). In line with mammals a number of fatty acids induced transactivation of plaice Gal4-PPAR , the most active of which were the fatty-acid like compounds OEA and LEA. In mammals OEA and LEA are potent agonists of PPAR and appear to control feeding and fat-induced satiety through PPAR interactions (Artmann *et al*, 2008; Schwartz *et al*, 2008; Dipasquale *et al*, 2010). The finding that OEA also activates PPAR of plaice indicates the potential for a similar function in fish species.

None of the fatty acids tested were able to activate the Gal4-PPAR . A previous study on native plaice PPARs indicated that docosahexaenoic acid (22:6; DHA) activated PPAR in preference to PPAR and PPAR (Leaver *et al*, 2005). However, neither plaice Gal4-PPAR nor Gal4-PPAR was affected by DHA. DHA has been reported to activate mouse RXR (de Urquiza *et al*, 2000), indicating that in the previous study on fish, DHA may have been acting on RXR, the endogenous heterodimeric partner for transfected PPAR.

3.4.1.4 Plaice PPAR is unresponsive to mammalian PPAR agonists

Peroxisome proliferator-activated receptor from mammals has been shown to be the target for the thiazolidinedione class of compounds (Lehmann *et al*, 1995; de Vos *et al*, 1996). This finding has been of particular interest due to the anti-diabetic and insulin-sensitising effects of these agents. Rosiglitazone (BRL49653) is a member of the thiazolidinedione class of compounds and in an assay system similar to the one described here were able to activate murine PPAR at a concentration of 10 µM, and with EC50 values of 30 nM and 100 nM for PPAR 1 and PPAR 2 isoforms, respectively (Lehmann *et al*, 1995). These findings are in contrast to those found here in which 10 µM Rosiglitazone failed to increase the expression of firefly luciferase reporter in cells transfected with the Gal4-PPAR construct. PPAR has recently been isolated from olive flounder (*Paralichthys olivaceus*) and its response to Rosiglitazone characterised (Cho *et al*, 2009). In these studies, PPAR was found to have weak transactivity for Rosiglitazone compared to murine PPAR , suggesting differences do exist in the sensitivity of PPAR from fish and mammalian species.

One reason for this difference in sensitivity has been suggested by Maglich *et al* (2003) and Willson *et al* (2000). Four conserved residues within the ligand-binding region of mammalian PPARs have been indicated as being important in ligand-receptor interactions and alignments between the human PPAR receptors and the orthologs in several fish species including plaice, salmon and fugu, reveal fish to have identical residues to their human counterparts at these key positions within PPAR (Maglich *et al*, 2003). Within PPAR of fish species, three of the four residues are identical to the human residues, however as Maglich *et al* (2003) states this single amino acid change would still be likely to allow binding of ligands to the receptor. In contrast to PPAR and PPAR , PPAR within fish species have two amino acid alterations at these key residues compared to the human PPAR isoform; His^{323} is changed to Iln and Tyr^{473} to Met. The thiazolidinediones make specific hydrogen-bonding interactions with these four conserved residues (Ser^{289}, His^{323}, His^{449} and Tyr^{473}) as well as with the Glycine residue at position 286 (position refers to human PPAR). In fish species including plaice, flounder, fugu and salmon two of these five residues are altered (Figure 3-11). These changes in key residues within PPAR may be enough to prevent binding of ligands to the PPAR receptor in fish species, thus reducing their sensitivity to the thiazolidinediones and other PPAR ligands.

Importantly, from previous studies and from the experiments reported here, it is apparent that fish PPAR does not have high levels of constitutive activity. In the Gal4 assay, reporter gene activity driven by plaice PPAR was very low and at background levels. The low constitutive activity of piscine PPAR , the high degree of conservation amongst fish species and the lack of response to fatty acids or

mammalian subtype specific ligands suggests that if endogenous and other ligands for piscine PPAR exist, they are likely to have some significant structural differences compared to fatty acids.

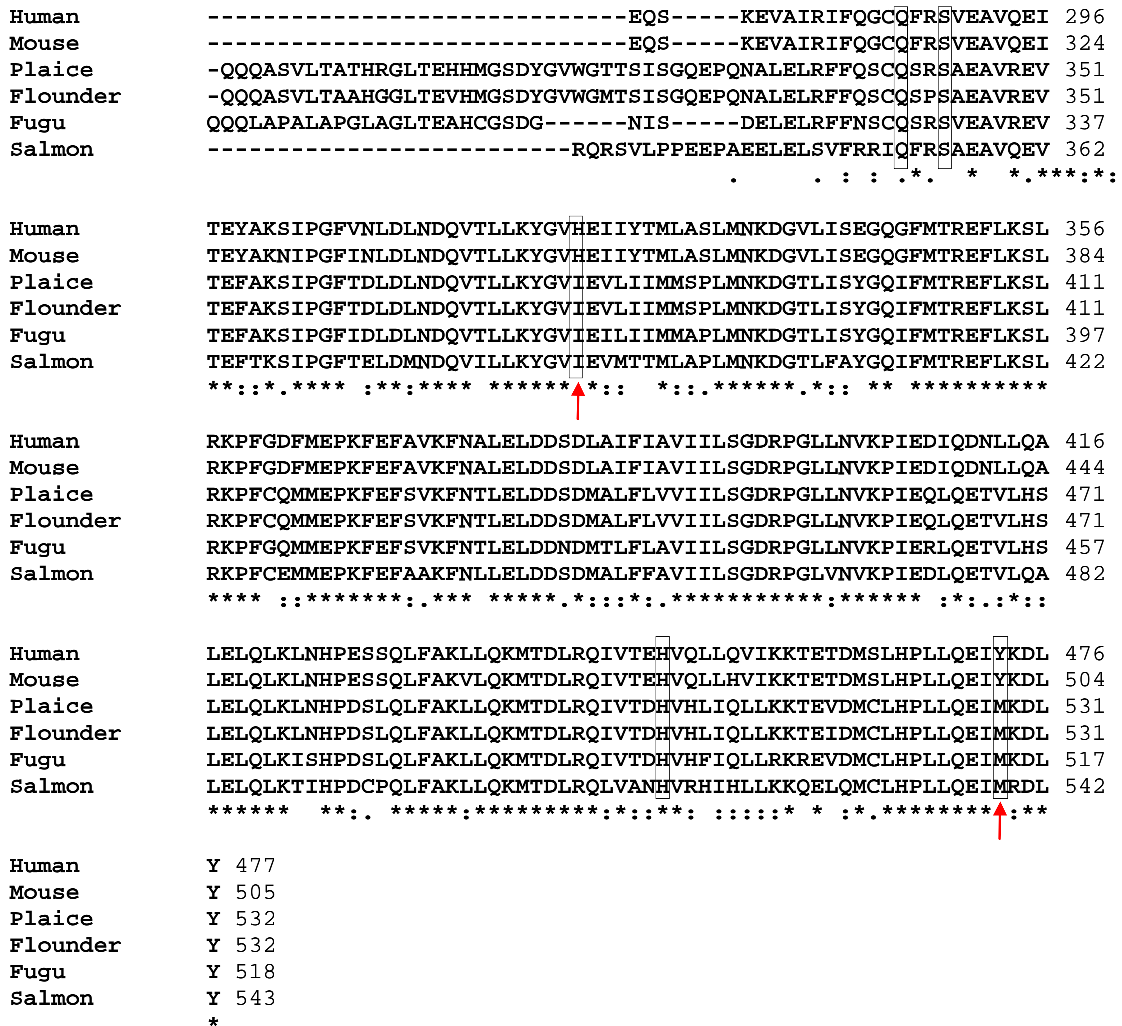

Figure 3-11. Alignment of the partial ligand-binding domains of mammalian and mouse PPAR . Residues important in the interaction between thiazolidinediones and the PPAR ligand-binding domain are shown in boxes. Those that are not conserved between human and piscine PPAR are indicated by a red arrow.

3.4.2 Evaluation of the Gal4-PPAR assay system

The use of cell-based assay systems as a replacement for animal testing is an attractive prospect, both in terms of ethical considerations and as a means of overcoming many of the limitations in using live animal models. The replacement of animals with cells or tissues *in vitro* is also in keeping with the internationally accepted Three Rs concept, which aims to Replace animal models with non-animal methods where possible, Reduce the number of animals used in experiments while obtaining comparable levels of information and Refine methods of testing so as to minimise or alleviate potential pain, suffering or distress and, where the use of animals persists, enhance animal welfare (Flecknell, 2002). These considerations are especially important in the context of assessing the risk from chemical released to the environment, and the use of *in vitro* receptor interaction assays may provide a first tier of assessment, allowing the prioritisation of chemicals for toxicological testing on animals. The development of the Gal4 transient transfection assay provides several advantages including high throughput screening of a number of relevant aquatic pollutants, allowing the specific responses of receptors to be tested and allows dosing of compounds at controlled concentrations and without interference from *in vivo* complexities such as other receptors, hormones, signalling pathways, bioaccumulation and depuration.

The use of fish cell lines in transfection experiments to evaluate environmental samples has been previously described. The zebrafish embryo-derived cell line, ZEM2S, transfected with a luciferase reporter constructs containing the aryl hydrocarbon response element (AHRE), the metal response elements (MRE) or the electrophile response elements (ERE) within its promoter region, saw an increase in

luciferase activity following treatment of transfected cells with aryl hydrocarbon compounds, cadmium, zinc, mercury and aluminium ions, and mercury, lead, arsenic, copper and cadmium ions, respectively (Carvan *et al*, 2000). The fathead minnow cell line employed in the present study has been previously reported to be a valuable tool for the study of *in vitro* cytotoxicity following the finding that *in vitro* toxicity studies with 49 chemicals correlated well with fish lethality data (Brandão *et al*, 1992). Furthermore the FHM cell line has recently been used to confirm transactivation data, previously obtained from transfected mammalian cells, for rainbow trout glucocorticoid receptors (Sturm *et al*, 2011), thus confirming the presence of co-regulatory proteins and other elements of the transcriptional machinery essential for the transcriptional activity of nuclear hormone receptors within this cell line.

The use of cell based assay also holds several advantages over other methods, e.g. computer-based *in silico* methods, which have been used to predict the toxicological effects of anthropogenic compounds via nuclear hormone receptors in mammals and other vertebrates (Jacobs, 2004; Vedani *et al*, 2007; Wu *et al*, 2009; Wu *et al*, 2010). One advantage over *in silico* predictive methods is that, while an interaction between a pollutant of interest and an endogenous receptor can be predicted, it is difficult to say whether the chemical will have agonistic or antagonistic effects upon the receptor. Furthermore several *in silico* studies have used experimental data from binding assays to validate the predicted *in silico* binding affinities due to the uncertainties associated with these methods (Wu *et al*, 2009; Wu *et al*, 2010;), and thus *in silico* methods may be better used in conjunction with *in vitro* studies rather than as an alternative method. *In silico* methods have been applied to identify ligands

of PPAR , and , using a virtual docking-system in compounds were docked into the ligand-binding domains of PPAR , and and a scoring system applied taking into account steric, hydrogen bonding, hydrophobicity, continuum electrostatics, and entropy terms to assign a score to the ligand reflecting the quality of the complex (Schapira *et al,* 2003). The authors of this study demonstrated that isotype selectivity was poorly represented, with PPAR -selective agonists being identified as agonists of all three PPAR subtypes while out of seven PPAR -selective agonists, five, four, and three were present in the top 1% PPAR , PPAR , and PPAR focused libraries, respectively. These results indicate that *in vitro* cell-based assay systems have the advantage of being able to discriminate between isotype-selective ligands of PPAR receptors, as demonstrated in the results of the current study.

In relation to the specific Gal4-NHR system employed in the present studies, the use of the Gal4 DNA-binding domain and its upstream-activating sequence (*UAS*) provides several advantages in itself. Previously a variety of receptor response elements, including the promoter region of receptor target-genes (Klotz *et al*, 1996; Tarumi *et al*, 2000) or the specific receptor response element (Legler *et al*, 1999; Rogers and Denison, 2000; Seinen *et al*, 1999; Yoon et al. 2001), have been incorporated into the reporter construct. The use of these promoter regions and response element constructs have been shown to display reduced responsiveness to the reporter gene through activation by non-NHR specific processes and the cross-talk with other signalling pathways. For example the PPAR/RXR heterodimer has been shown to inhibit transactivation activities of the estrogen receptor by binding to the estrogen response element (ERE) (Keller *et al*, 1995). Thus, the introduction of hybrid receptor and reporter constructs minimises the problem of cross-reactivity

described above since the Gal4-NHR ligand binding domain fusion recognises and activates the Gal4 DNA response element-reporter gene construct, which is not known to be bound by other factors within animal cells. The Gal4-PPAR expression constructs used in the present study utilised only the ligand-binding domain of the nuclear hormone receptors studied. Interactions between the ligand-independent AF1 in the A/B domain and the ligand-dependent AF2 in the ligand-binding domain of several nuclear hormone receptors have been described previously, and thus the omission of the whole amino terminus of the receptor may influence its transcriptional abilities *in vitro*. Using human PPAR in assay systems a comparison has previously been made between the full-length receptor and a truncated receptor, the latter using only the ligand-binding domain (Berbaum and Harrison, 2005). The authors reported that although there was a reduced affinity for co-activator proteins with the truncated receptor no difference in the potencies for agonists in cell-free and cell-based assays was observed. These results indicate that the expression plasmid used in the current study will be suitable for use in identifiying compounds which interact with PPAR receptors and that the use of a truncated receptor should not interfere with the potencies of such compounds.

3.4.3 Suitability of the "Homemade" luciferase assay buffers

Firefly and *Renilla* luciferase buffers have been formulated using data available in patents and in the description of the non-commercial buffers described by Dyer *et al* (2000). Firefly luciferase acts upon the substrate luciferin, oxidising it to oxyluciferin and light energy in the presence of cofactors such as ATP, oxygen and magnesium ions. For this reason beetle luciferin, ATP and magnesium sulphate were included in

the firefly luciferase buffer, the latter of which provided the necessary magnesium ions.

A common problem reported in measuring luminescence from luciferase-luciferin reactions is the "flash"-type kinetics associated with this reaction, characterised by an initial "flash" of light emission, which decays rapidly to a constant low level luminescence (Djouadi *et al* (1998); Figure 3-12).

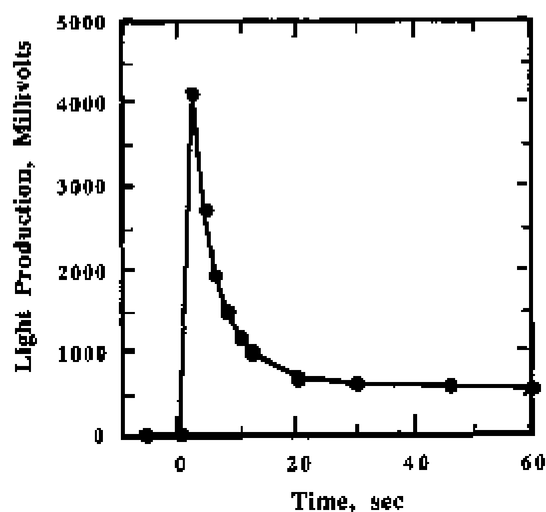

Figure 3-12. The "flash"-type kinetics associated with luciferase-luciferin reactions. Figure taken from Djouadi *et al* (1998).

Experimental difficulties can arise when we consider the delay that can occur between commencing the luciferase-luciferin reaction and starting the measurement of luminescence, by which time light levels may have already started to deteriorate. An early solution to this problem was to use an arsenate buffer, which inhibited the luciferase-luciferin reaction, altering the time-course of the reaction (DeLuca *et al*, 1979). More recently, it has been reported that coenzyme A (CoA) and dithiothreitol (DTT) can stabilise the luminescence signal of the luciferase-luciferin reaction, prolonging the signal and thus avoiding the "flash"-type kinetics associated with the reaction. CoA has been shown to stabilise light emission by converting

dehydroluciferyl-adenylate (L-AMP), a side product of the bioluminescence reaction and a potent inhibitor of the reaction, to dehydroluciferyl-coenzyme A (L-CoA), reducing the inhibitory actions of L-AMP (Fraga *et al*, 2005). As well as DTT and CoA having been identified as stabilisers of luminescence in luciferase-luciferin reactions, high concentrations of phosphate and ammonium ions, have also been shown to have a similar effect (van Lune and Bruggeman, 2006). From these results dithiothreitol (DTT) and coenzyme A (CoA) along with high concentrations of ammonium and phosphate ions, present as Tris-phosphoric acid, were included in the firefly luciferase buffer. This appeared to have a stabilising effect, with only a 34% loss of luminescence over a 60 minutes period. This is not perceived to be problematic in the current assay system because of the small lapse in time (10 minutes) between the addition of substrate and measurement of firefly luciferase, in which time only a 5% loss in luminescence signal is observed.

Within the experiments described above the firefly luciferase reagent contained a lysis buffer. One of the aims in the development of these alternative assay buffers was to limit the number of steps, and thus time, involved in the measurement of luciferase activities. A patent detailing a lysis reagent consisting of octoxynol (Triton-X 100), BSA and DDT has been described (Wood, 1997) and a concentrated lysis reagent thought to contain these components was available from Promega, which was added to the homemade firefly luciferase reagent in order to combine the cell lysis step and firefly luciferase reaction into one. In a further step to reduce assay time, measurements of luciferase activities were carried out in the presence of media which had been spiked with treatment compounds, in a similar method to that described for the Dual-Glo® luciferase assay system. Therefore the culture media

and any treatment compounds added made up 50% of the environment of the firefly luciferase reaction and one-third of the environment of the *Renilla* luciferase reactions. The effects of many of the treatment compounds on luciferase protein and other components of the assay system were largely unknown and therefore in subsequent experiments, the methodology was modified so that media and treatments were removed and cells washed prior to determination of luciferase activities. As the specific inhibition of firefly luciferase by long-chain fatty acids in the µM range has been reported previously (Matsuki *et al*, 1999), this additional step was considered a necessary precautionary measure for subsequent experiments, especially when fatty acids were being tested as potential PPAR activators.

Within this assay system *Renilla* luciferase activities were used to normalise for differences in transfection efficiencies between wells on an assay plate. *In vitro*, *Renilla* luciferase catalyses the oxidative carboxylation of coelenterazine in the presence of oxygen, producing *Renilla* luminescence, oxyluciferin and carbon dioxide (Hori *et al*, 1973). Coelenterazine was therefore present in the *Renilla* luciferase buffer. One of the requirements of the *Renilla* luciferase buffer was that it should quench the firefly luciferase signal down to background levels. Information contained within a patent show many reagents, including high concentrations of sodium chloride and sodium phosphate as well as EDTA quench beetle luciferase activity (Sherf *et al,* 1998). Additionally, it was stated that superior quenching was obtained with a combination of these reagents, several of which were included in the *Renilla* luciferase buffer formulated by Dyer *et al* (2000). The formulation of the "homemade" *Renilla* luciferase buffer described and used here was based roughly on that described by Dyer *et al* (2000) and included sodium chloride, sodium phosphate

and EDTA, shown by Sherf *et al* (1998) to effectively quench firefly luciferase activity by 91.7%, 90% and 99.87%, respectively. Using the *Renilla* luciferase buffer, firefly luciferase was quenched by more than 99%, and the *Renilla* luciferase-coelenterazine reaction was simultaneously activated, suggesting this buffer to be very effective in the dual-reporter assay system.

3.5 Conclusions

In order to find compounds that interact with the peroxisome proliferator-activated receptors (PPARs) a system is required in which PPARs are tested in isolation from their endogenous heterodimeric partner, the retinoid X receptor (RXR). A luciferase assay is described here which makes use of the interactions between the Gal4 DNA-binding domain and the Gal4 upstream-activating sequences to initiate transcription of the firefly luciferase reporter gene following compound interactions with the PPAR ligand-binding domain. The Gal4-PPAR and Gal4-PPAR constructs increased transcription of the firefly luciferase protein in response to known mammalian PPAR and PPAR agonists, respectively, leading to the conclusion that these constructs are appropriate to detect compounds which interact with PPAR and PPAR . The Gal4-PPAR construct was unresponsive to mammalian PPAR agonists. The unresponsiveness of full-length piscine PPAR to compounds which activate mammalian PPAR has previously been described, and therefore the unresponsiveness described for the Gal4-PPAR construct is not likely to be due to the truncation of the receptor.

Luciferase proteins are widely used as reporters, but their use has previously been hindered by the rate of their inactivation, which quickly reduces luminescence signal over a matter of seconds. Several commercial reagents have been described in which

the luminescence signal can be stabilised for longer time periods, allowing luminescence signal to be assayed over several hours. These reagents can be prohibitively expensive and therefore information from available literature was used to develop alternative firefly and *Renilla* reagents, the former of which initiated the luciferase-luciferin reaction and stabilised firefly luminescence signal for longer than was required to assay its activity and the latter of which both quenched firefly luciferase activity while generating *Renilla* luciferase activity.

Both the Gal4-PPAR/Gal4UAS-luc assay system and the reagents used to assay for luciferase protein can be combined to give a method for the high throughput screening of compounds which can interact with the ligand-binding domain of PPARs, separate from the influence of RXR.

Chapter 4. Response of piscine PPARs to environmental contaminants

4.1 Introduction

The production of synthetic organic and inorganic compounds has been increasing dramatically since the early 20th century, and in the USA alone production levels now exceed 140 billion kg/annum (Baillie Hamilton, 2002). This inevitably leads to a release of a fraction of the produced chemicals to the environment, with significant volumes ultimately entering aquatic ecosystems. Some of these pollutants have been shown to be endocrine disruptors in aquatic organisms via interactions with nuclear hormone receptors (NHRs). At present the interactions of pollutants with the estrogen receptor is relatively well studied, reflecting the importance of this receptor in reproductive function (Fent *et al*, 2006; Mortensen and Arukwe, 2007; Tollefsen *et al*, 2008). However much less is known of the effects of anthropogenic compounds on other nuclear hormone receptors (NHRs), many of which are known to function in endocrine and cellular signalling.

The peroxisome proliferator-activated receptors (PPARs) have been isolated from a variety of organisms, including mammals, fish and amphibian species (Lee, 1991; Göttlicher *et al*, 1992; Schmidt *et al*, 1992; Krey *et al*, 1993; Tontonoz *et al*, 1994; Kliewer *et al*, 1994; Aperlo *et al*, 1995; Andersen *et al*, 2000; Leaver *et al*, 2005) and function as regulators of energy homeostasis (Latruffe and Vamecq, 2002; Dressel *et al*, 2003; Wang, 2010). An emerging hypothesis concerning PPARs is that they are the targets of environmental pollutants known as 'obesogens', hypothesised to play a role in the increasing precedence of the metabolic syndrome and obesity in the human population, due to their interactions with the human PPARs. Such obesogenic compounds include phthalate plasticisers, bisphenol A and organotins (Desvergne *et*

al, 2009; Grun, 2010), all of which have been detected in the aquatic environment. Despite little being known about the interactions of such compounds with piscine PPARs or their physiological effects, some evidence exists to suggest that compounds which activate mammalian PPARs also interact with piscine PPARs, with a variety of physiological consequences. Examples of such effects are described in adult zebrafish and in Grass carp (*Ctenopharyngodon idella*) in which cholesterol levels are decreased in response to the pharmaceuticals bezafibrate and fenofibrate, respectively (Du *et al*, 2008; Velasco-Santamaria *et al*, 2011). Additionally plasma triacylglyceride, total whole body and liver lipid levels and tissue eicosapentanoic and docosahexanoic acid content was reportedly decreased in grass carp (Du *et al*, 2008), suggesting large alterations in lipid profiles in response to exogenous compounds. Since cholesterol is the precursor for all steroidogenic hormones it might be expected that steroid hormone synthesis would be decreased in response to decreasing levels of cholesterol. Indeed 11-ketotestosterone is decreased by bezafibrate and clofibric acid in the zebrafish (Runnalls *et al*, 2007; Velasco-Santamaria *et al*, 2011) and exposure of goldfish (*Carassius auratus*) to gemfibrozil also saw a reduction in testosterone (Mimeault *et al*, 2005) The purpose of the study described in this chapter was to assess a selection of persistent environmental pollutants for their ability to interact with PPARs from plaice (*Pleuronectes platessa*), a flatfish species which lives in close association with marine benthic sediments. Fourteen widespread pollutants, which are detected in benthic sediments were assessed, and included byproducts of the manufacturing industry, human pharmaceuticals and pesticides. The transient transfection assay developed in Chapter 3 was used within this study to identify:

i) pollutants with the ability to agonise the Gal4-PPAR LBD construct transfected into FHM cells, by increasing luciferase enzyme activity.

ii) pollutants with the ability to antagonise Gal4-PPAR LBD constructs transfected into FHM cells, identified as a decrease in luciferase enzyme activity when treatment is in combination with a PPAR agonist.

4.2 Materials and Methods

The Gal4-PPAR constructs described in Chapter 3 were used to test for agonistic and antagonistic effects of pollutants in cell-based transactivation assays.

4.2.1 Seeding and tranfection of FHM cells

FHM cells were harvested from near-confluent cell culture flasks and seeded at 2×10^5 cells per well of 96 well plates before transfection 24 hours later with with Gal4-PPAR , Gal4-PPAR , Gal4-PPAR or pBIND plasmid, pGL4.31[*luc2P*/*GAL4*UAS/Hygro] and pGL4.75[*hRluc*/CMV] as described in 3.2.2.1 and 3.2.2.2.

As a control, an experiment to assess the potential impact of environmental contaminants on PPAR-independent transcription within FHM cells was carried out using the Checkmate™ Positive Control vectors pBIND-Id and pACTmyoD (Promega). The Id and myoD proteins interact to generate a functional transcriptional complex and, when co-transfected together with pGL4.31[*luc2P*/*GAL4*UAS/Hygro], induce firefly luciferase proteins in a non-PPAR dependent manner. Per well 60ng DNA was transfected per well using 12.5ng pBIND-Id, 12.5ng pACTmyoD, 25ng of pGL4.31[*luc2P/GAL4*UAS/Hygro] and 10ng internal control vector

pGL4.75[*hRluc*/CMV] was transfected with 0.63 µl Polyfect reagent as described previously.

Cells were incubated with transfection mixes for 24 hours prior to treatment with compounds of interest.

4.2.2 Treatment of transfected FHM cells

Following transfection, cells were treated with various pollutants of the aquatic environmental. These compounds included fibrates (Gemfibrozil, Fenofibrate, Clofibric Acid, Bezafibrate and Ciprofibrate), phthalates and their metabolites (dimethylphthalate, benzylbutylphthalate, 1-monobenzyl-phthalate and mono-1-methylhexylphthalate), surfactant compounds (perfluorooctanoic acid and fluorooctane sulphonic acid), deltamethrin, bisphenol A and tributyltin oxide. The compounds were tested for their ability to interact with the PPAR-ligand binding domain as receptor agonists, i.e. chemicals capable of increasing the transcriptional activity of PPAR, or as receptor antagonists, i.e. molecules that are capable of blocking the action of an agonist. In experimental designs addressing antagonistic action, pollutants were tested in combination with pharmacological inducers of PPAR. Antagonism of Gal4-PPAR could not be addressed due to the lack of known activators of the Gal4-PPAR construct. Based on results obtained in Chapter 3, Wy14643 was used as an agonist for Gal4-PPAR , while GW501516 was used as an agonist for Gal4-PPAR . Compounds with antagonistic activitity on the PPAR will fully or partially reverse the stimulating effects of the above agonists on the transcription of the firefly luciferase gene, leading to lower luminescence compared to those cells treated with agonists alone.

Stock solutions of compounds were prepared in absolute ethanol at 200 times the exposure concentration. Directly prior to to the treatment of cells, stock solutions were diluted 1 in 200 into DMEM (supplemented with 10% charcoal/dextran treated FBS (Fisher Scientific), 50 units/mL penicillin and 50 μg/mL streptomycin (Invitrogen)). For antagonism experiments both the chosen agonist and the treatment compound of interest were diluted 1 in 200 into supplemented DMEM. Initially, two arbitrary concentrations, designated as 'high' and 'low', was chosen for each compound in activation experiments (see the legend of figure 4.1, figure 4.4 and figure 4.6 for concentrations), while a single concentration, equal to the 'high' concentration used in activation experiments, was chosen for inhibition experiments. Initial experiments with TBTO revealed cell toxicity at micromolar concentrations and therefore nanomolar concentrations of TBTO were used. On the day following transfection of cells, media was aspirated from the assay plate and cells washed once with 1× DPBS (Invitrogen) before addition of treatment compounds diluted in DMEM (75 μl/well). Each treatment was run in triplicate within each experiment. Control treatments included an ethanol vehicle treatment (0.5% or 1% ethanol for agonism and antagonism experiments, respectively). Wy14643 and GW501516 were used at final concentrations of 25 μM and 10 μM, respectively.

Cells were incubated with treatment compounds for a further 24 hours at 22°C, 5% CO_2, before being assayed for luciferase activity.

4.2.3 Luciferase activity, data normalisation and statistical analysis

4.2.3.1 Luciferase Assay

Firefly and *Renilla* luciferase activities were measured using the non-commercial luciferase assay buffers described in Chapter 3. Twenty four hours post-treatment, media containing treatment compounds was aspirated from assay plates and cells washed twice with 1× DPBS. Concentrated 5× Passive lysis buffer (Promega) was diluted down to a 1× concentration with MilliQ water (Millipore) and 75 µl added to cells. Cells were incubated with lysis buffer at room termperature for 10 minutes to allow for complete cell lysis, before adding 75 µl of 2× concentrated luciferase buffer to the lysate. The reagents making up the luciferase buffer and their final assay concentrations were as described in 2.2.4.1, except that the lysis reagent in the buffer was replaced by water because in these, and subsequent experiments, lysis and measurement of firefly luciferase activity was performed separately. The cell lysate and firefly luciferase buffer was mixed by pipetting up and down and firefly luciferase activity measured on Wallac 1420 Victor 2 multilabel counter (Perking Elmer).

Renilla luciferase was then measured using the non-commercial buffer described in 2.2.4.2. 75 µl of 3× concentrated *Renilla* luciferase buffer was added to lysate containing firefly luciferase buffer, mixed by pipetting and Renilla luciferase activity measured on Wallac 1420 Victor 2 multilabel counter (Perking Elmer).

4.2.3.2 Data Normalisation & Statistical Analysis

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 of replicate wells was calculated.

Experiments were repeated independently at least three times (i.e. with different flasks of cells seeded on different days). Data is reported as the mean fold inductions in firefly luciferase activites compared to ethanol vehicle controls. To test for statistically significant differences between ethanol control treatment and treatment with environmental pollutants a one way ANOVA was carried out followed by Dunnet's *post hoc* test. Where necessary data were log transformed to improve homogeneity of variances. Results were considered statistically significant when the probability value (P) was less than 0.05 (P <0.05).

4.3 Results

4.3.1 Environmental pollutants as Gal4-PPAR agonists

Of the tested compounds four were able to induce PPAR transcriptional activity, reflected by an increase in firefly luciferase activities of between 2-fold and 58-fold over ethanol controls (Figure 4-1).

4.3.1.1 Activation by Fibrates

Of the five fibrates tested (Gemfibrozil, Bezafibrate, Ciprofibrate, Fenofibrate and Clofibric Acid), two elicited a response from the Gal4-PPAR construct (Figure 4-1). At 100 µM, the presence of ciprofibrate and gemfibrozil increased firefly

luciferase luminescence 5.4-fold and 57-fold, respectively. A 2-fold increase in luciferase luminescence was seen in the presence of 100 µM Clofibric acid, however this was not statistically significant. The lower concentrations of of 10 µM failed to induce firefly luciferase activity. Bezafibrate and Fenofibrate at concentrations of 10 µM and 100 µM were not agonists of PPAR , indicated by similar firefly luciferase activities as observed with ethanol vehicle controls.

4.3.1.2 Phthalate metabolites

In addition to fibrates, the major metabolites of two phthalate compounds were shown to activate the Gal4-PPAR construct (Figure 4-1). At concentrations of 100 µM, both 1-monobenzylphthalate and mono-1-methylhexylphthalate increased reporter gene activity by 2-fold and 16.6-fold, respectively, over treatment with ethanol vehicle alone. No effect was observed at the lower 10 µM concentration. Two parent phthalate compounds which were also tested (benzylbutylphthalate and dimethylphthalate) had no effect on the transcription of firefly luciferase at concentrations up to 25 µM.

4.3.1.3 Perfluorochemicals chemicals, Pesticides & Bisphenol A

In the present study neither fluorooctane sulfonic acid (FOSA), perfluorooctanoic acid (PFOA), deltamethrin, TBTO nor bisphenol A at the concentrations tested induced any increase in firefly luciferase activity when incubated with the Gal4-PPAR (Figure 4-1).

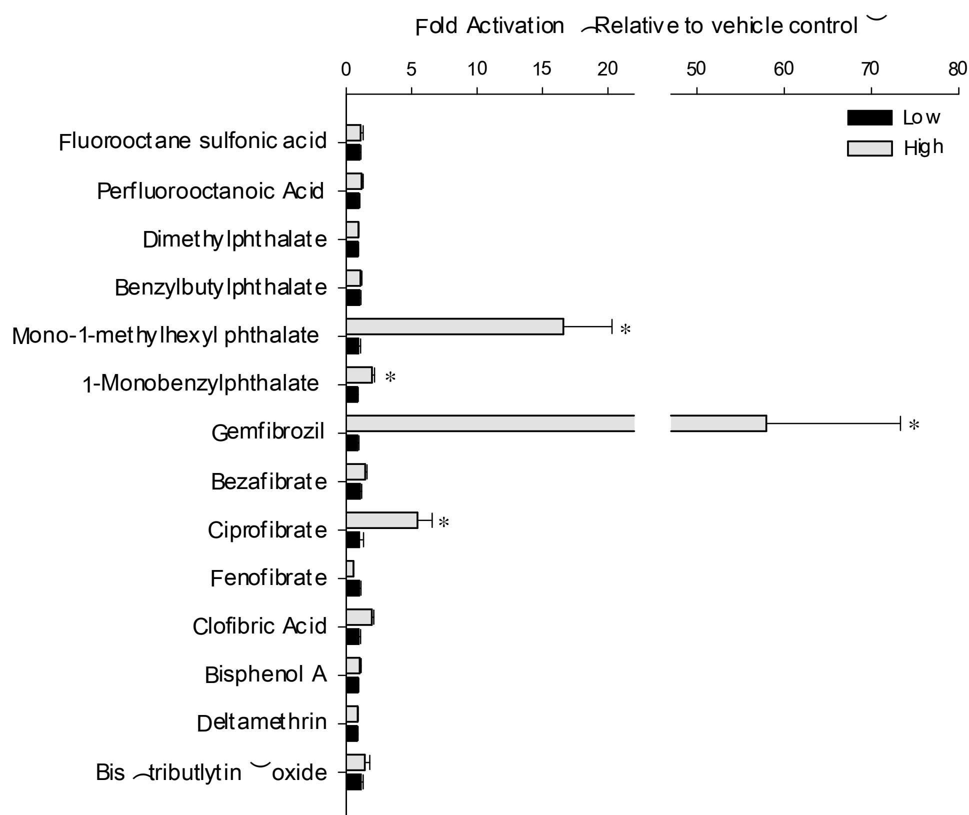

Figure 4-1. Activation of PPAR by aquatic contaminants. FHM cells were transfected and treated as described in materials and methods. Cells were treated with two concentrations per contaminant: Fluorooctanesulfonic acid, perfluorooctanoic acid, dimethylphthalate, benzylbutylphthalate, bisphenol A and deltamethrin were tested at 10 µM (LOW) and 25 µM (HIGH). Mono-1-methyl-hexyl-phthalate, 1-mono-benzylbutylphthalate, gemfibrozil, bezafibrate, ciprofibrate, fenofibrate and clofibric acid were tested at 10 µM (LOW) and 100 µM (HIGH). Bis(tributyltin)oxide (TBTO) was tested at 10 nM (LOW) and 50 nM (HIGH). Data are the means ± SEM of three independent experiments. Results are expressed as the fold increase over ethanol control of normalised firefly luciferase activity. Asterisks represent statistically significant differences to activities in ethanol controls (P<0.01).

4.3.2 Environmental Pollutants as antagonists of Gal4-PPAR

Antagonism of Gal4-PPAR by pollutant compounds reduced luciferase activity by up to 61.7%, compared to treatment by Wy14643 alone (Figure 4-2). Three compounds, (Bezafibrate, Fenofibrate and bis(tributyl tin) oxide (TBTO)), had statistically significant antagonistic effects. Antagonism of Gal4-PPAR by Bezafibrate and Fenofibrate was unexpected as fibrates have been described previously as activators of PPAR in mammalian and amphibian species. The

inhibition of Gal4-PPAR by TBTO is previously undescribed and antagonism occurred at concentrations 2000-fold lower than that of fibrates.

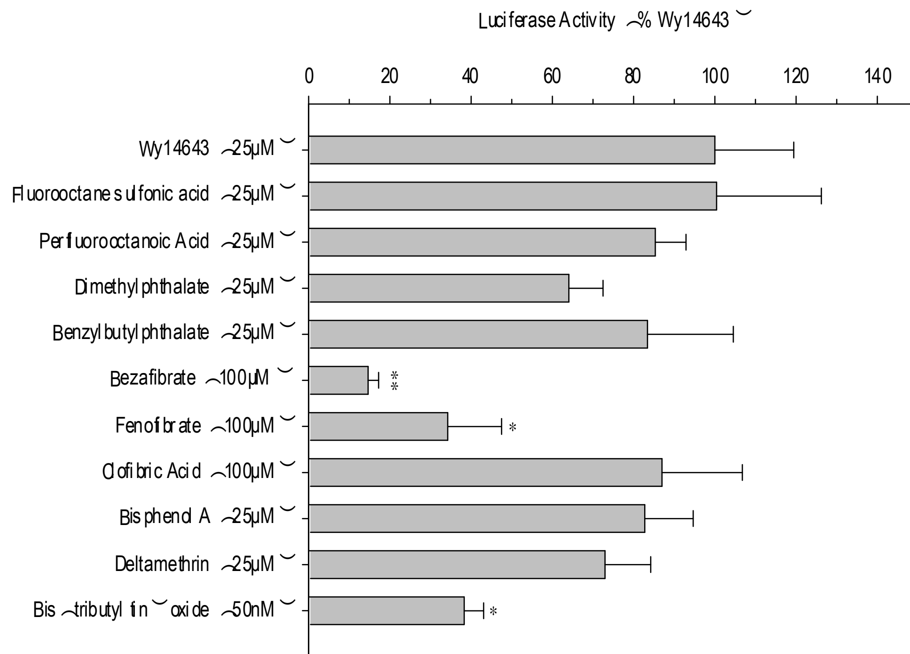

Figure 4-2. Antagonism of Gal4-PPAR by aquatic contaminants. Transfected FHM cells were treated with Wy14643 alone or in combination with aquatic contaminant, the concentration of which is indicated above. Data are represented as the mean ± SEM of 3 independent experiments and are expressed as the percentage of normalised firefly luciferase luminescence with respect to treatment with Wy14643 (25 µM) alone, set arbitrarily to 100%. Asterisks represent statistically significant differences to the treatment without inhibitor (* P<0.05, ** *P*<0.01).

Inhibitory effects of TBTO were further assessed over a range of concentration (1nM to 50nM) and were shown to significantly inhibit Wy14643-mediated luciferase activity at concentrations as low as 1nM (Figure 4.3).

Additionally, in order to assess whether this apparent antagonistic effect was due to specific interactions with the PPAR ligand-binding domain or due to non-specific effects on transcription, the effects of TBTO on luciferase activities were observed in FHM cells transfected with a positive control two hybrid construct system (pBIND ID and pACTmyoD, Promega) as described in 4.2.1. In these cells TBTO failed to reduce firefly luciferase activity (Figure 4.3), suggesting TBTO decreases luciferase

activity in Wy14643-treated Gal4-PPAR transfected cells in a PPAR dependent manner.

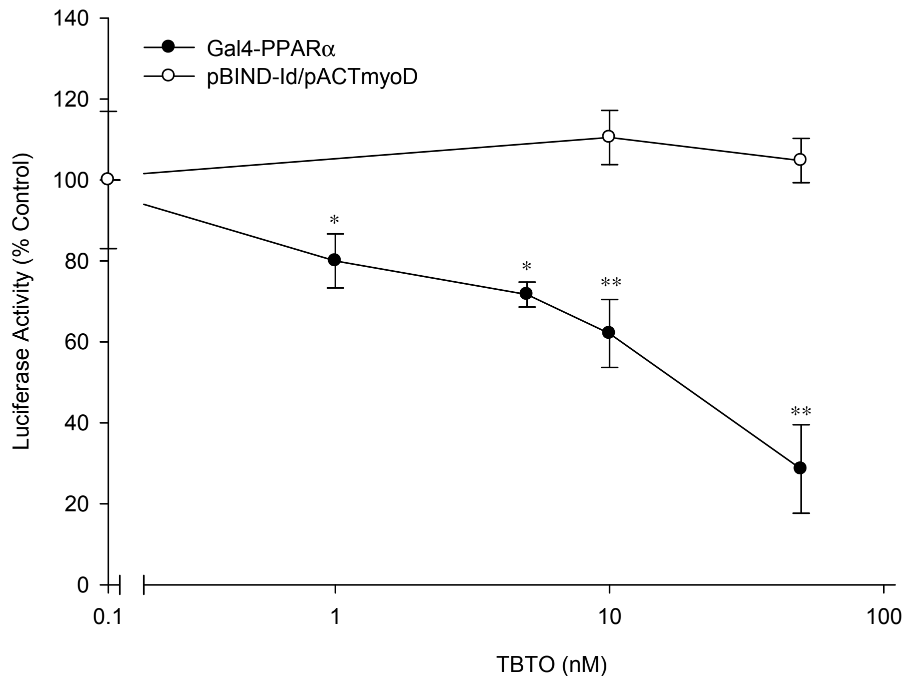

Figure 4-3. Concentration dependent effects of TBTO on plaice PPAR . FHM cells transfected with Gal4-PPAR were treated with Wy1463 either alone (Control) or in combination with various concentrations of TBTO. As an internal control, cells transfected with pBIND-Id and pACTmyoD, which resulted in constitutive luciferase expression, were included to test for general effects of TBTO on gene expression. Means ± SEM of three independent experiments are shown. Data are expressed as the percentage of normalised firefly luciferase luminescence observed in control treatments with Wy14643 (Gal4-PPAR transfected cells) or 0.5% ethanol (pBIND-Id/pACTmyoD-transfected cells) alone. Asterisks represent statistically significant differences to control treatments at *$P<0.05$* (*) or *$P<0.01$* (*).

4.3.3 Response of Gal4-LBD to environmental pollutants

4.3.3.1 Agonism of PPAR

Bezafibrate, a member of the fibrate class of pharmaceutical compounds, has previously been described as a dual agonist of PPAR and PPAR (Peters *et al*, 2003). Bezafibrate failed to activate plaice PPAR , but at both 10 and 100 µM concentrations this compound increased Gal4-PPAR -dependent transactivation by 2.4- and 59.8-fold, respectively, compared to ethanol vehicle.

Similar to the Gal4-PPAR construct the monophthalate esters mono-1-methylhexylphthalate and 1-monobenzylphthalate, at 100 µM, were able to significantly induce transcriptional activation of Gal4-PPAR by 2.9- and 2.3-fold, respectively (Figure 4-4).

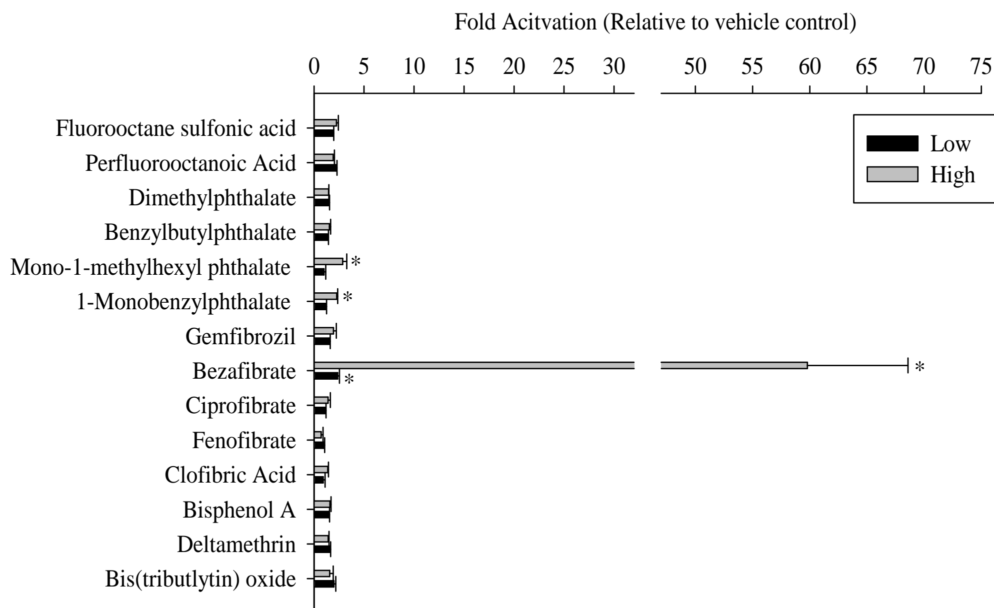

Figure 4-4. Activation of PPAR by aquatic contaminants. FHM cells were transfected and treated as described in materials and methods. Cells were treated with two concentrations per contaminant: Fluorooctanesulfonic acid, perfluorooctanoic acid, dimethylphthalate, benzylbutylphthalate, bisphenol A and deltamethrin were tested at 10 µM (LOW) and 25 µM (HIGH). Mono-1-methyl-hexyl-phthalate, 1-mono-benzylbutylphthalate, gemfibrozil, bezafibrate, ciprofibrate, fenofibrate and clofibric acid were tested at 10 µM (LOW) and 100 µM (HIGH). Bis(tributyltin)oxide (TBTO) was tested at 10 nM (LOW) and 50 nM (HIGH). Data are the means ± SEM of three independent experiments. Results are expressed as the fold increase over ethanol control of normalised firefly luciferase activity. Asterisks represent statistically significant differences to activities in ethanol controls (P<0.01).

4.3.3.2 Antagonism of Gal4-PPAR

None of the compounds tested were able to significantly reduce the luciferase activity in GW501516-treated cells transfected with Gal4-PPAR (Figure 4-5) although a slight (non-significant) inhibition by Bisphenol A (42.5% inhibition) and TBTO (27.3% inhibition) was observed.

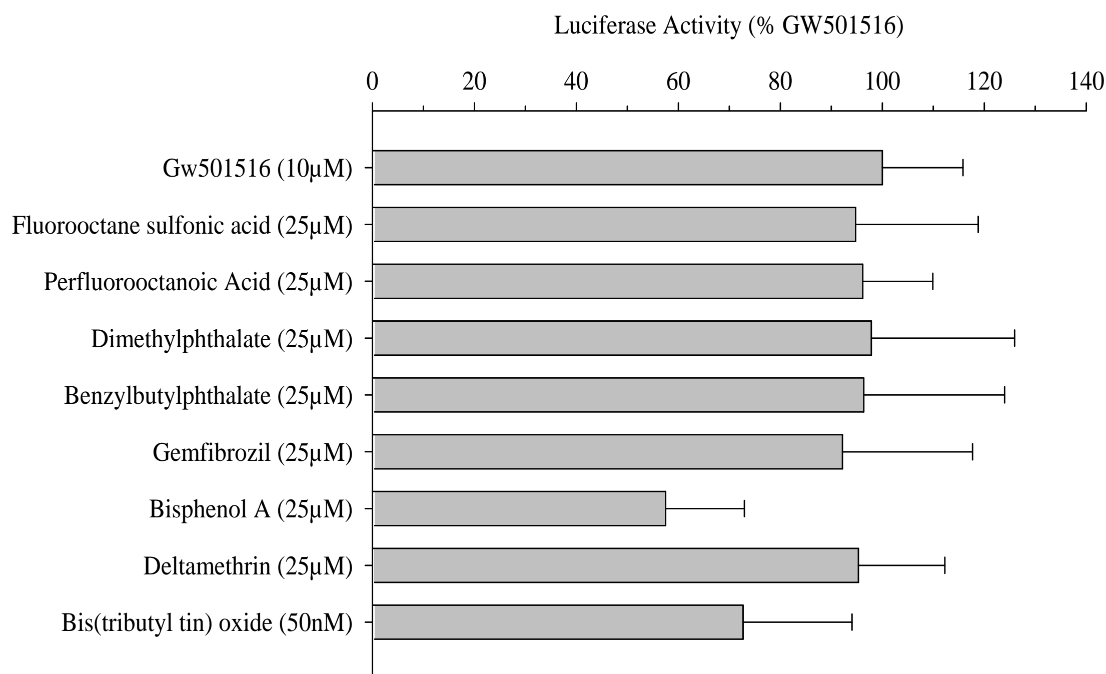

Figure 4-5. Antagonism of Gal4-PPAR by aquatic contaminants. Transfected FHM cells were treated with GW501516 alone or in combination with aquatic contaminanats, the concentrations of which are indicated above. Data are represented as the mean ± SEM of 3 independent experiments and are expressed as the percentage of normalised firefly luciferase luminescence with respect to treatment with GW501516 alone, set arbitrarily to 100%.

4.3.4 Activation of Gal4-PPAR by environmental pollutants

Aquatic contaminants incubated with Gal4-PPAR at low concentrations had no effect on the expression of firefly luciferase reporter gene. At high concentrations only one compound, 1-monobenzylphthalate, was able to increase expression of firefly luciferase over that observed with ethanol to a statistically significant level, although this increase was small at 1.72-fold (Figure 4-6).

Antagonism of Gal4-PPAR by the pollutant compounds could not be determined since no sufficiently potent plaice PPAR agonist was identified throughout this study.

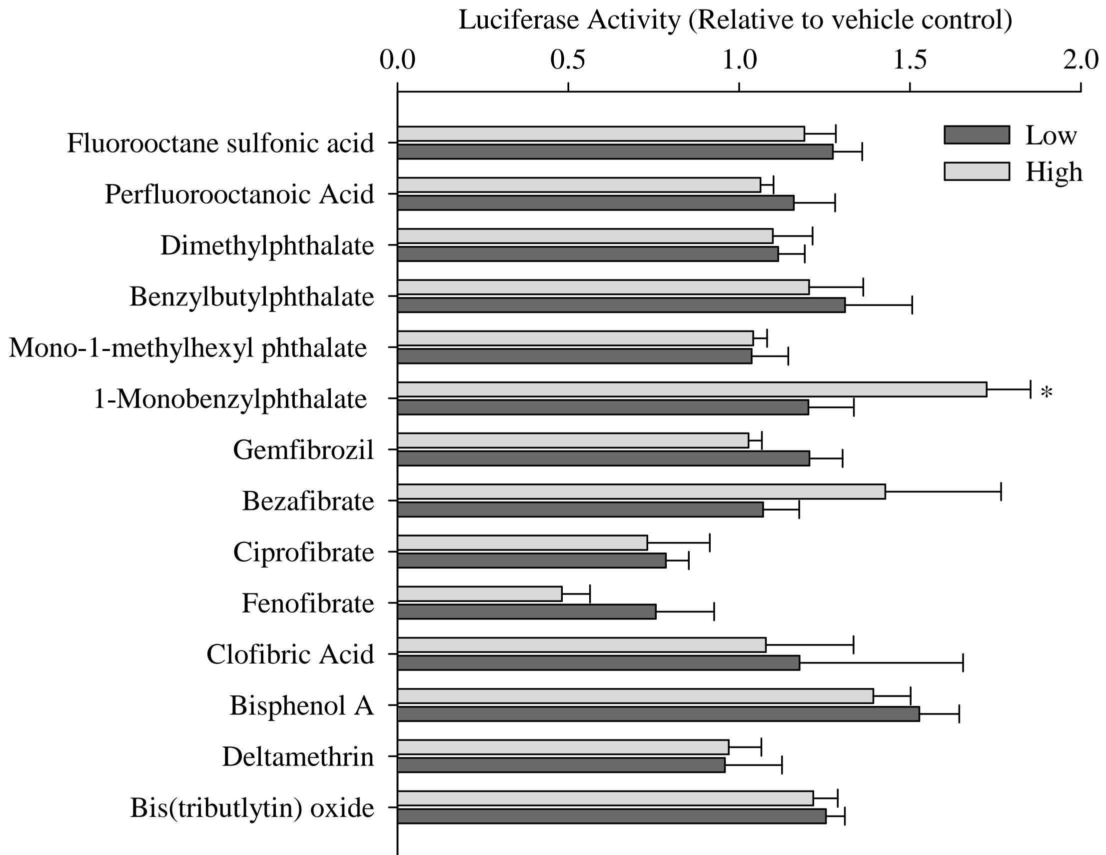

Figure 4-6. Activation of PPAR by aquatic contaminants. FHM cells were transfected and treated as described in materials and methods. Cells were treated with two concentrations per contaminant: Fluorooctanesulfonic acid, perfluorooctanoic acid, dimethylphthalate, benzylbutylphthalate, bisphenol A and deltamethrin were tested at 10 µM (LOW) and 25 µM (HIGH). Mono-1-methyl-hexyl-phthalate, 1-mono-benzylbutylphthalate, gemfibrozil, bezafibrate, ciprofibrate, fenofibrate and clofibric acid were tested at 10 µM (LOW) and 100 µM (HIGH). Bis(tributyltin)oxide (TBTO) was tested at 10 nM (LOW) and 50 nM (HIGH). Data are the means ± SEM of three independent experiments. Results are expressed as the fold increase over ethanol control of normalised firefly luciferase activity. Asterisks represent statistically significant differences to activities in ethanol controls (P<0.01).

4.4 Discussion

4.4.1 Fibrates

Several environmental pollutants, some of which have been reported to activate mammalian PPARs, were tested with the plaice Gal4-PPAR chimerae. Fibrates,

pharmaceutical agents used to treate dyslipidemia in humans and shown to function primarily via PPAR (Schoonjans *et al*, 1996; Staels *et al*, 1997), were amongst the compounds tested. Fibrates have been widely detected in the aquatic environment (Ternes, 1998; Buser and Müller, 1998; Andreozzi *et al*, 2003; Sanderson *et al*, 2003; Fent *et al*, 2006), with their incomplete removal from sewage influent being the main route of entry into such environments. Measurements of fibrate concentrations in sewage, following its treatment at a sewage treatment plant (STP), have shown that of initial concentrations detected in the sewage influent 66%, 54%, 55% and 50% of clofibric acid, gemfibrozil, fenofibric acid and bezafibrate, respectively, remains in the effluent (Stumpf *et al*, 1999). Thus there is some concern as to their effects on aquatic organisms, including fish species, which may come into contact with fibrate compounds.

Studies in mice and humans have shown clofibrate and fenofibrate to be at least an order of magnitude more potent for PPAR than for PPAR or PPAR while bezafibrate is an agonist of all three PPAR subtypes at similar potency (Willson *et al*, 2000). With regards to fish, evidence exists to suggest fibrates are also able to activate PPARs in several species. Donohue *et al* (1993) have previously shown that treatment of Rainbow trout hepatocytes with clofibric acid, from which clofibrate is metabolised, was effective in inducing the activity of the PPAR target gene fatty acyl-CoA oxidase (FACO), while the activity of the same enzyme also increased in culture hepatoctes from Atlantic salmon after treatment with clofibric acid and bezafibrate (Ruyter *et al*, 1997). *In vivo*, Yang *et al* (1990) and Du *et al* (2004), respectively showed that intraperitoneal injection with ciprofibrate and a diet containing fenofibrate, increased hepatic acyl-CoA oxidase activity in Rainbow trout.

In plaice neither clofibric acid, the metabolite of clofibrate, nor fenofibrate appear to interact with the ligand-binding domain of any of the PPARs. Although this appears contradictory to results obtained in rainbow trout and Atlantic salmon cell cultures, the concentrations of fibrates used in these latter experiments were in the ranges of 0.25mM to 3mM and therefore between 2.5 and 30-fold higher than the concentrations used here. Whether higher concentrations of fibrates could have induced the activities of the Gal4-PPAR constructs is unknown and was not tested due to higher concentrations not being considered relevant in an environmental context.

While bezafibrate is able to activate all three PPAR subtypes in mammalian species, it appears to be selective for PPAR in plaice, having no effect on PPAR or PPAR at concentrations up to 100 µM. Similarly bezafibrate has been shown to be selective for PPAR in the African clawed frog (*Xenopus laevis*) (Krey *et al*, 1997). Thus, data from this and earlier studies suggest that fibrates target PPARs, not just in humans but in other vertebrates including mammals, fish and amphibians. Although it appears that at least some of the fibrate drugs have the potential to exert toxic effects in plaice via PPAR-mediated pathways, the concentrations required to do so (>10 µM) far exceed measured concentrations of individual fibrates, which have been reported to be between 0.02 µM and 0.2 µM in various sewage treatment effluents (Krey *et al*, 1997; Ternes, 1998; Buser and Müller, 1998; Andreozzi *et al*, 2003; Sanderson *et al*, 2003). Measured or predicted environmental concentrations (PECs) of pharmaceuticals are often at least an order of magnitude lower than those levels shown to cause any effect in aquatic organisms (Corcoran *et al*, 2010). Despite this it has to be considered that in most exposure studies, organisms are exposed to

only one compound, while the situation within the environment is much more complicated. The finding that several fibrates can have an effect on fish PPARs suggests that the additive effects of these pharmaceuticals in the environment should be considered. Previously additive effects of chemicals in fish have been shown (Brian *et al,* 2005; Cleuvers, 2003; Thorpe *et al*, 2003) while some pharmaceuticals have been shown to modify the toxicity of others (Hasselberg *et al*, 2008).

It is expected that organisms will be chronically exposed to low-concentrations of these pharmaceutical drugs. Additionally plaice are a predatory benthic species, spending much of their time in contact with bottom sediments where they feed on invertebrate species including crustaceans, molluscs and worms. Many pollutants have been shown to bind to particles within sediments where they accumulate to much higher concentrations than are found in the water column (Peck *et al*, 2004; Martinez-Llado *et al*, 2007; Zeng *et al*, 2008). Thus the possibility exists that plaice may come into contact with higher concentrations of fibrate compounds than have been reported in the water column, by contact with the bottom sediments. In feeding on other organisms an opportunity exists for fibrates to bioaccumulate within plaice and other omnivorous fish species where they feed on organisms contaminated with fibrates.

One unexpected result of the experiments described above is the inhibition of the ligand-binding domain of PPAR by some fibrates, despite these pharmaceutical compounds being manufactured for their ability to activate PPAR . Notably the fibrates causing antagonism had no agonistic effects on PPAR when given alone.

4.4.2 Phthalates

Phthalates are compounds used in the manufacture of plastics, serving to soften PVC products and are also found in cosmetics, perfumes, industrial paints and solvents, and certain drugs. Phthalate compounds have been widely reported in the aquatic environment (Fatoki *et al*, 2010) and measured environmental concentrations can be similar to those causing reproductive disruption in laboratory experiments in some animals (Oehlmann *et al*, 2009). Previous studies concerning the interactions of phthalates with PPARs found interactions with all three subtypes. Diethylhexyl phthalate (DEHP) has been shown to induce PPAR -dependent activation of fatty acid catabolism in the liver of mice (Feige *et al*, 2010) and to disrupt the reproductive system of male zebrafish (*Danio rerio*), most likely via PPAR signalling pathways (Uren-Webster *et al*, 2010). Results from transactivation assays support an interaction between PPARs and phthalates, with both benzylbutyl phthalate and di-*n*-butylphthalate able to increase reporter gene expression when incubated with all three mouse PPAR subtypes (Lapinskas *et al*, 2005).

Despite phthalates having been shown to affect organisms in a PPAR-dependent manner, parent phthalates are usually subjected to biotic degradation within the environment. Abiotic degradation pathways, including hydrolysis and photo degradation have minor roles in the degradation of phthalate compounds compared to biotic degradation by microbes, both aerobic and anaerobic, and the metabolic pathway in aquatic organisms (Staples *et al*, 1998). Degradation by microbes and within higher aquatic organisms, results in parent phthalate metabolites being hydrolysed to a monoester phthalate and the corresponding alcohol (Barron *et al*, 1995), the former of which can be excreted or enter a further biotransformation

pathway. The monoester metabolites of phthalates have also been suggested as agonists of PPARs; the major metabolite of DEHP, mono(2-ethylhexyl) phthalate (MEHP), activated all three PPAR subtypes in a transactivation assay (Lapinskas *et al*, 2005) while monobenzyl phthalate, the monoester phthalate of benzylbutyl phthalate, has been shown to increase the expression of PPAR in COS cells, with a half-maximal response (EC50) at 21 μM (Hurst and Waxman, 2003).

Much of the literature documenting the effects of phthalates and their metabolites on PPARs is focused on the mammalian receptors, while little information is available concerning the effect of these compounds on fish PPARs. In this study both parent phthalate compounds (dimethyl phthalate and benzylbutyl phthalate) and their monoester metabolites (mono-1-methylhexyl phthalate and 1-monobenzyl phthalate) were tested with the Gal4-plaice PPAR constructs to assess the potential impact of these compounds on fish PPARs. Both dimethyl phthalate and benzylbutyl phthalate failed to increase reporter gene expression when incubated with PPAR constructs. In contrast, mono-1-methylhexyl phthalate and 1-monobenzyl phthalate had effects on both Gal4-PPAR and Gal4-PPAR while 1-monobenzyl phthalate was able to activate Gal4-PPAR , however these effects were only seen with phthalate monester concentrations of 100μM. Within the aquatic environment parent phthalate compounds have been detected at levels up to 33.5 μgL^{-1} in UK rivers (Fatoki and Vernon, 1990) while levels of up to 97.8 μgL^{-1} (0.25μM) have been detected in German surface waters (Fromme *et al*, 2002). Within sediments, concentrations of phthalates tend to be higher; within German sediments concentrations of DEHP ranged from 0.21-8.44 mg kg^{-1} (0.54-21.61 μM) while concentrations of dibutyl phthalate (DBP) was between 0.06-2.08 mg kg-1 (0.21-7.473 μM). The presence of

monoesther phthalate metabolites within environmental samples are much less reported, however MMP, MBP and MEHP have been detected in the Tama River, Tokyo at concentrations of 30-34 (0.17-0.19 nM), 10-480 (0.039-1.87 nM), and 10-1300 (0.037-4.68 nM) ng L-1, respectively (Suzuki *et al*, 2001). These concentrations would be unlikely to have an effect on the activation of PPARs in plaice in the environment, and therefore the threat of phthalate compounds to energy homeostasis in this species is small.

4.4.3 FOSA & PFOA

Previous studies on fish PPARs have shown PFOA to transactivate the PPAR/RXR heterodimers (Leaver *et al*, 2005) and in mice and humans both PFOA and PFOS act as weak agonists in a similar Gal4-PPAR assay to that used here (Takacs and Abbott, 2007). Perfluoro-based chemicals have been described as activators of human and mouse PPAR (Shipley *et al*, 2004) with fluorooctane sulfonic acid (FOSA) having an EC_{50} of approximately 13-15 μM. PFOA has been shown to increase the mRNA levels of the PPAR target gene acyl-CoA oxidase (ACO) in the livers of Japanese medaka (Yang, 2010), suggesting PFOA to activate PPAR .

However, plaice Gal4-PPAR was not affected by PFOA or FOSA, which suggests that these compounds may be targeting RXR in fish. Even in mice the effects of PFOA on PPAR-target genes appear to be, to some extent, independent of PPAR . Rosen *et al* (2008) showed that PFOA can regulate the expression of genes involved in amino acid homeostasis and fatty acid -oxidation, functions typically modulated via PPAR , in PPAR -null mice, indicating that other mechanisms of action should be investigated.

4.4.4 Bis(tributyl tin)oxide (TBTO)

Organotins have previously been widely used as antifouling biocides for ships and fishing nets as well as agricultural fungicides and rodent repellents, and although banned by International Maritime Organisation (IMO) since 2003, their persistence means they remain a common contaminant of aquatic environmnents, both in sediments and fish tissues (Antizar-Ladislao, 2008). Previous reports into the effects of organotin compounds on aquatic organisms has been largely focussed on female gastropods, in which the imposition of male sex organs on females (imposex) in response to TBT has been found (Rilov *et al*, 2000; Hung *et al*, 2001; Gravel *et al*, 2006). More recently the masculinisation of fish species in response to TBT has also been reported (McAllister and Kime, 2003; Shimasaki *et al*, 2003). The mechanism by which imposex occurs remains largely elusive but several theories have been suggested, such as the inhibition of the P450-mediated aromatase enzyme responsible for catalysing the conversion of androgens, e.g. testosterone, to female sex hormones such as estradiol and estrone (Bettin *et al*, 1996) and the inappropriate release of the neuropeptide Ala-Pro-Gly-Trp-NH_2 (APGWamide) (Oberdörster and McClellan-Green, 2000). More recently a third target for organotin compounds has been suggested – the retinoid X receptor (RXR) (Kanayama *et al*, 2005; Grün *et al*, 2006; Nishikawa, 2006; Castro *et al*, 2007). Kanayama *et al* (2005) first discovered that tributyltin and triphenyltin were potent activators of the RXR/PPAR receptor complex in mammals, being high affinity ligands for both proteins. The PPAR /RXR heterodimer is involved in adipogenesis and in the storage of lipids in adipocytes (Chawla *et al*, 1994; Martin *et al*, 1998) and organotin compounds have been shown to induce adipogenesis both *in vitro* and *in vivo,* further confirming a role for the

RXR/PPAR heterodimer in mediating the effects of organotins (Kanayama *et al*, 2005; Grün *et al*, 2006; Kirchner *et al*, 2010). With the discovery that organotins are high affinity ligands for both the RXR and the PPAR receptors in mammals and are able to induce imposex in invertebrates, came the hypothesis that organotins promote imposex via binding to the RXR receptor. PPAR receptors have been shown to have evolved relatively late in the evolution of receptors (De Mendonca *et al*, 1999) and may not even be present in invertebrate organisms and thus the target receptor for organotins in promoting imposex in gastropods is more likely to be the retinoid X receptor, which evolved earlier in time, being present even in insects and nematodes (Nishikawa, 2006). Indeed, it has been shown that injection of 9-*cis* retinoic acid, the natural ligand of RXRs in humans, into female rock shells (*Thais clavigera*) and into the neogastropod *Nucella lapillus*, is sufficient to induce imposex in these organisms and is also able to increase the penis length in male *T. clavigera* (Nishikawa *et al*, 2004; Castro *et al*, 2007). Despite organotin compounds having no resemblance, neither in structure nor chemical properties, to known RXR and PPAR ligands (retinoids and thiazolidinediones, respectively) tributyl tin (TBT) has been shown to bind to the human retinoid X receptor and promote the recruitment of PGC-1 , a PPAR coactivator peptide (le Maire *et al*, 2009). In contrast TBT interacts poorly with PPAR , leading to a poor recruitment of PGC-1 (le Maire *et al*, 2009). Further to this the crystal structure of RXR bound to TBT has been resolved, providing unambiguous evidence that TBT interacts with the RXR, rather than PPAR (le Maire *et al*, 2009). This agrees with the findings here in which TBTO was found not to interact with the Gal4-PPAR construct. Little evidence is available describing the interaction of TBT with PPAR and PPAR in fish species, although an increase in the mRNA expression of all three PPAR subtypes in Atlantic salmon (*Salmo salar*)

has been found when fed a diet containing tributyltin (Pavlikova *et al*, 2010). Furthermore, an increase in whole body lipid content has been measured in chinook salmon (*Onchorynchus tshawytscha*) fed a TBT-contaminated diet, consistent with the hyposthesis that TBT acts as an obesogen and as a PPAR /RXR agonist (Meador *et al*, 2011). In the studies here TBTO was found to antagonise the activation of Gal4-PPAR by the model activator Wy14643 at low nanomolar, environmentally relevant concentrations. Thus results from earlier studies and those presented here indicate that TBTO may function as an agonist of the RXR receptor while antagonising PPARs. In PPAR / -null mice, an RXR homodimer complex has been shown to compensate for PPARs by binding to PPREs (IJpenberg *et al*, 2004). Thus PPAR inhibition by TBTO may be ameliorated by the activation of other signalling pathways.

Tributyltin has been detected in aquatic environments worldwide with sediment concentrations ranging from ng/g to µg/g (Godoi *et al*, 2003; Harino *et al*, 2003; Santos *et al*, 2009; Garg *et al*, 2009) while concentrations within the water column have been reported at concentrations from 200ng/L in the Mediterranean Sea (Michel, 2001) to 3.20 µg/L in Singapore (Basheer *et al*, 2002). Organisms inhabiting TBT-contaminated environments have been shown to accumulate the compound within their tissues, with most research focussed on molluscs and crustaceans due to their being important seafood resources. Marine bivalves have been shown to accumulate TBT to high levels due to efficient transfer of TBT into their tissues via the pump activity used during filter feeding (Laughlin *et al*, 1986). Additionally the low efficiency of their metabolic systems causes TBT to be degraded slowly (Lee, 1991). Within fish species, TBTO and related organotin

compounds have been found to accumulate to the μg kg^{-1} range (Hoch, 2001; Antizar-Ladislao, 2008). These concentrations are comparable to the concentrations which were shown to inhibit PPAR - 10 nM is equivalent to approximately 6 μg L^{-1}.

4.5 Conclusion

In conclusion plaice PPAR and PPAR exhibit both similarities and differences to their mammalian counterparts. Some fibrates act as activators of plaice PPARs as they do in mammals, whilst other fibrates which are agonists of mammalian PPARs are not effective in activating fish PPARs, but in the current study were antagonists of PPAR . In addition to fibrates phthalate compound metabolites were also agonists of all three plaice PPAR subtypes. However, these aforementioned compounds were effective at concentrations higher than that recorded in the aquatic environment. Of the environmental pollutants tested, only TBTO had effects at environmentally relevant concentrations. Although environmental concentrations of TBT have been decreasing in many parts of the world following legislation to limit their use, they are still detectable in sediments and fish tissues in quantities (Antizar-Ladislao, 2008) that would, based on the results reported here, inhibit ex vivo piscine PPAR . Given the potency of this contaminant and its persistent and ubiquitous presence in estuarine environments, these effects should be further studied *in vivo*, to establish what, if any, are the physiological consequences and knock-on ecological effects of exposure.

Other than the compounds mentioned above, no other compound tested had any agonistic or antagonistic effects on Gal4-PPAR LBD constructs from plaice.

Chapter 5. Transcriptomic response of European flounder hepatocytes to TBTO

In Chapter 4 it was shown that fenofibrate, bezafibrate and TBTO inhibit the transcriptional activity of peroxisome-proliferator activated receptor alpha (PPAR). In an environmental context, the study of TBTO is perhaps most relevant since, as discussed in chapter 4, the concentrations at which TBTO is able to inhibit PPAR activity are found within the environment, while the necessary concentrations of fibrates are unlikely to be encountered. As a preliminary investigation into this apparent PPAR antagonism by TBTO, and to identify genes whose expression has the potential to be regulated by PPAR , a transcriptomic approach was taken, comparing the resulting gene expression profiles using a flounder cDNA microarray.

5.1 Microarray

DNA microarrays are used as a means of measuring the expression of many genes in parallel. First used to measure the expression of 45 cDNAs from the small flowering plant *Arabidopsis thaliana* (Schena *et al*, 1995), the use of microarray technology for expression profiling has since developed to allow measurement of thousands of genes in parallel and platforms have been developed for many organisms including several piscine species.

As part of the GENIPOL consortium a European flounder cDNA array has been developed, consisting of 3336 unique genes (Williams *et al*, 2006). This array has been enriched with transcripts induced by a wide range of model toxicants, including a polycyclic aromatic hydrocarbon (PAH), a mixture of polychlorinated bipheyls (PCBs), a peroxisome proliferative agent, a heavy metal, an organic hydroperoxide and estrogen, by using a cDNA library derived from mRNA obtained from the livers of individuals following intraperitoneal injection with the aforementioned

compounds (Diab *et al*, 2008). Previously this array has been used to determine the responses of flounder to disease (*Aeromonas salmonicida*) aquatic toxicants (17 -estradiol, cadmium and cadmium salts, polycyclic aromatic hydrocarbons and a chlorinated hydrocarbon pesticide) and hormone (Williams *et al*, 2006; Williams *et al*, 2007; Falciani *et al*, 2008; Diab *et al*, 2008).

Using a reporter gene assay designed to identify compounds which interact, either as agonists or antagonists, with the peroxisome proliferator activated receptors, bis(tributyl tin) oxide (TBTO) was identified as an aquatic contaminant with the potential to inhibit the alpha subtype of these receptors (Chapter 4). Within the present study, a preliminary study into this apparent antagonism of PPAR was carried out using a microarray-based approach. The transcriptomic responses of European flounder hepatocytes to the PPAR -specific agonist Wy14643, TBTO or a combination of both compounds was measured in order to identify

(i) Genes whose expression is altered upon PPAR activation with Wy14643

(ii) Genes whose expression is altered upon exposure to TBTO

(iii) Genes whose expression is upregulated by Wy14643 but downregulated by a combination of Wy14643/TBTO, thus indicative of a PPAR inhibition.

5.2 Materials and Methods

5.2.1 *Hepatocyte isolation and treatment*

5.2.1.1 Isolation of liver hepatocyte cells

A single male juvenile flounder (*Platichthys flesus*), obtained from the Forth estuary and acclimated in seawater for six months in an aquarium, was killed by a blow to the head and the liver removed. The liver was immediately washed in chilled phosphate buffered saline (1×) and chopped into fine dice which were then placed into 1× Hanks' Balanced Salt Solution (HBSS; Gibco), supplemented with 200 units kanamycin, 100 units penicillin, 100 µg streptomycin, 1g bovine serum albumin and 5mM EDTA and incubated on ice for 30 minutes with gently agitation. Liver fragments were allowed to settle before HBSS was removed, liver pieces washed twice with HBSS supplemented as above but excluding EDTA, and incubated at 15°C for 3 hours in HBSS supplemented with 200 units kanamycin, 100 units penicillin, 100 µg streptomycin, 1g bovine serum albumin and 0.2g collagenase type IV (Sigma).

Digested liver pieces were passed through a course filter (250µm) before being filtered through a 100µm filter into a sterile 50 ml centrifuge tube. The solution was centrifuged at 100 ×*g* for 2 minutes at 4°C, supernatant removed and the pellet containing hepatocyte cells resuspended in Leibovitz's L15 medium (Invitrogen) supplemented with 10% fetal bovine serum, 200 U kanamycin, 100 U penicillin and 100 µg streptomycin. A second centrifugation was then done at 100g for 5 minutes at 4°C before supernatant was removed and the pellet resuspended in a 20 mL volume of L15 media, supplemented as described above.

5.2.1.2 Seeding of Hepatocytes and treatment

Using a hemocytometer, the density of hepatocyte cells in 20 ml suspension was determined and adjusted to 5×10^5 cells per ml by addition of L15 medium. Cells were then seeded into 6-well tissue culture plates in a volume of 2 mL per well (1×10^6 cells) and incubated for 2 hours at 15°C before being treated.

Four different treatments were administered to cells in total volumes of 5 µl and each treatment was in triplicate. Treatments and the final treatment concentrations used were as follows:

- 0.25% Ethanol (vehicle)
- 25 µM Wy14643
- 10 nM TBTO
- 10 nM TBTO + 25 µM Wy14643

Each treatment was prepared by diluting concentrated stock solutions in absolute ethanol. Concentrated stocks of Wy14643 were prepared at 10mM and 20 mM concentrations and TBTO was prepared at 4 µM and 8 µM concentrations. Treatments were added directly to the media in which cells were seeded in a total volume of 5 µl. The 10 nM TBTO + 25 µM Wy14643 treatment was achieved by addition of 2.5 µl of 20mM Wy14643 and 2.5 µl 8 µM TBTO to the media while 10 nM TBTO and 25 µM Wy14643 treatments were achieved by addition of 5 µl 4 µM TBTO and 5 µl 10 mM Wy14643, respectively. Tissue culture plates were then swirled gently to achieve a homogenous treatment mixture. An additional a control treatment of ethanol vehicle was administered by addition of 5µl ethanol to cell culture media. Treatment levels were chosen based on the results of Chapter 3. Thus a concentration of 25 µM Wy14643 was shown to maximally increase PPAR

transactivation in a transient transfection assay, while 10 nM TBTO was shown to be the concentration at which the maximal transactivation by 25 μM Wy14643 was halved, and therefore these concentrations were considered suitable to study gene expression changes.

Treated cells were incubated for a further 40 hours at 15 °C before being harvested for RNA extraction.

5.2.2 *Extraction, amplification and labelling of RNA from treated hepatocytes*

5.2.2.1 Extraction of RNA from treated hepatocyte cells

Hepatocyte cells and media were collected by aspiration from each well of the tissue culture plates following 40 hours of incubation and placed into 2ml microcentrifuge tubes. Cells were pelleted by centrifugation for 2 minutes at 100 × *g* and 4 °C, supernatant removed and cell pellets resuspended in 500 μl TRIpure isolation reagent (Roche). TRIpure isolation reagent was used to extract RNA as described in the TRIpure isolation reagent protocol. Briefly, cells were incubated with TRIpure reagent for 5 minute to allow for complete cell lysis, after which chloroform was added and the mixture shaken vigorously for 15 seconds. The tubes were then incubated at room temperature for a further 15 minutes before being centrifuged at 12,000 × *g* for 15 minutes to effect phase separation. The top aqueous phase was transferred to a fresh tube and isopropanol added to precipitate RNA. After mixing, the sample was incubated for 10 minutes at room temperature and centrifuged for 15 minutes at 12,000 ×*g*. Supernatant was removed, being careful not to disturb the

visible RNA pellet, and the pellet washed in 75% ethanol. After removal of ethanol, the pellet was left to dry slightly before being resuspended in 15 µl of MilliQ water.

RNA concentrations and purity was assessed spectrophotometrically on a Nanodrop ND1000 spectrophotometer, while RNA integrity was confirmed electrophoretically using a 1% agarose, 1× TAE, 0.5 µg EtBr gel to resolve 1 µg of each RNA sample after denaturation at 75 °C for 5 minutes.

5.2.2.2 RNA amplification

Spectrophotometric analysis revealed RNA yields which were less than that required for direct labelling for microarray experiments and therefore messenger RNA was amplified using the Amino Allyl MessageAmp™ II aRNA amplification kit (Ambion). Amplification of mRNA using this kit is based on the RNA amplification protocol developed by Van Gelder *et al* (1990) in which RNA is first used in reverse transcription with a modified oligo(dT) bearing the phage T7 RNA polymerase promoter to create first strand cDNA. Following second strand synthesis and cleanup the obtained cDNA is used as a template for *in vitro* transcription (IVT) with T7 RNA polymerase, producing many copies of antisense RNA for each mRNA in the original sample.

For first strand cDNA synthesis 1 µg total RNA was combined with 1 µl Oligo(dT) and incubated at 70 °C for 10 minutes before placing on ice. A mastermix of reagents, sufficient for each reaction was prepared using, per 20 µl reaction, 1× First strand buffer, 4 µl dNTP mix, 10 units RNase inhibitor and 1 µl Arrayscript™ reverse transcriptase enzyme. All reagents were provided with the Amino Allyl MessageAmp™ II aRNA amplification kit (Ambion). Reactions were mixed and

centrifuged briefly before being incubated at 42 °C for 2 hours. Following incubation reactions were placed on ice ready for second strand cDNA synthesis.

A second strand cDNA synthesis mastermix was prepared containing, for a single reaction, 1× second strand buffer, 4 µl dNTPs, 2 µl DNA polymerase and 1 µl RNase H and milliQ water to a volume of 80 µl. 80 µl of the mastermix was added to the first-strand cDNA synthesis reaction, mixed and incubated for 2 hours at 16 °C. cDNA was then purified using cDNA filter cartridges provided with the amplification kit. Briefly 250 µl cDNA binding buffer was added to each tube and the contents mixed gently. The sample was then placed onto the centre of the cartridge and centrifuged for 1 minute at 10,000 ×*g*. The flow through was discarded and the cartridge washed with wash buffer. Excess wash buffer was removed with a further centrifugation at 10,000 ×*g* for 1 minute and cDNA eluted with 20 µl milliQ water heated to 55 °C.

Eluted cDNA was then used for *in vitro* transcription to aminoallyl-labelled amplified RNA (aRNA). A mastermix for *in vitro* transcription was prepared, containing per reaction, 2.5 µl 50mM aminoallyl-labelled UTP, 3.5 µl 50mM unlabelled UTP, 12 µl ATP, GTP, CTP mix (25 mM each), 1× T7 buffer and 4 µl T7 enzyme mix. The mastermix was vortexed and briefly centrifuged before 26 µl was added to purified cDNA, mixed and incubated for 18 hours at 37°C. After 18 hours aRNA was purified using aRNA filter cartridges supplied with the Amino Allyl MessageAmp™ II aRNA amplification kit and eluted in 60 µl milliQ water, preheated to 55°C. Concentrations of aRNA were checked spectrophotometrically on Nanodrop ND1000 spectrophotometer.

5.2.2.3 Labelling of aRNA with Cy-dyes

Cyanine reagents have previously been shown to be readily conjugated to antibodies, avadin, modified DNA and other amino-group containing molecules (Mujumdar *et al*, 1993; Yu *et al*, 1994) and have been shown to be useful in the labelling of nucleic acid substrates for microarray experiments (Kimura *et al*, 2005; Staal *et al*, 2005). A pooled reference design (Novoradovskaya *et al*, 2004) was employed for the microarray experiments. Thus a sample of each aRNA sample was labelled with Cy3 dye, while a pool of all aRNA samples was labelled with Cy5 dye. A single vial of Cy3 and Cy5 dyes (GE Healthcare) were each resuspended in 38 µl high-purity, anhydrous dimethyl sulfoxide (DMSO; Sigma) and brought to room temperature in the dark.

For labelling with Cy3 dye, 3 µg of aRNA was added to 1.5ml centrifuge tubes and made up to a 10 µl volume with MilliQ water. The sample was placed on a dri-block (Techne) at 70°C for 2 minutes and cooled to room temperature. 3 µl 0.5M sodium bicarbonate (Sigma) was added to each tube together with 2 µl of Cy3 dye. For labelling with Cy5 dye an aRNA pool was made using 250 ng of aRNA from each sample, giving a total of 24 µg aRNA for labelling. aRNA was made up to a volume of 80 µl with milliQ water, which was subsequently split into 8 × 10 µl aliquots (3 µg/aliquot). Sodium bicarbonate and Cy5 dye was added to each reaction as described for the Cy3 reaction. Dye-labelling reactions were incubated at 25°C for 1 hour, after which labelled aRNA was purified from unincorporated dyes by column purification using Illustra Autoseq G50 dye terminator spin columns (GE Healthcare) following manufacturer's instructions.After pooling of Cy5 labelled aRNA, dye incorporation was meausured on Nanodrop ND1000 spectrophotometer. Additionally

0.4 µl of each dye-labelled aRNA product was electrophoresed on a thin 1% agarose, 0.5× TAE gel. Following electrophoresis the gel was scanned on a Typhoon Trio scanner (GE Healthcare).

5.2.3 Microarray hybridisation, washing and scanning

The array used in these experiments was the GENIPOL European flounder 13K cDNA microarray, (EMBL ArrayExpress Accession no. A-MAXD-12), which has been described in detail elsewhere (Williams *et al*, 2006; Diab *et al*, 2008). This array consists of 13,000 cDNAs spotted in duplicate. After contig clustering, the 13000 cDNAs represent 3336 unique genes. The array design along with accession numbers is available through EMBL ArrayExpress (http://www.ebi.ac.uk/microarray- as/aer/lob?name=adss&id=1308203622).

5.2.3.1 Microarray slide Presoak and Pre-hybridisation

A sodium borohydride pre-soak of the microarray slides was performed to block the glass surface, preventing binding of labelled aRNA to the glass platform thus reducing non-specific fluorescence. A pre-soak has previously been shown to reduce auto fluorescence on glass slides with and without DNA arrays, the reduction of which remained unaltered for up to 6 months (Raghavachari *et al*, 2003). A 2× SSC, 0.05% SDS, 0.25% sodium borohydride pre-soak buffer was prepared and preheated to 42°C before twelve European flounder 13K microarray slides were incubated with mild agitation (~125 rpm) for 30 minutes at 42°C. Slides were rinsed in 2× SSC twice before being transferred to pre-hybridisation buffer. The pre-hybridisation buffer was prepared fresh containing 25% deionised formamide, 5× SSC, 0.1% SDS and 10mg/ml BSA (fraction V; Sigma) and was preheated to 42°C before slides were

incubated at 42°C in pre-hybridisation buffer for 2 hours. Following pre-hybridisation slides were dipped twice in milliQ water, followed by a dip in ethanol and dried by centrifugation at 2000 rpm for 10 minutes at room temperature. Pre-hybridised slides were then used immediately for hybridisation with labelled aRNA.

5.2.3.2 Hybridisation of labelled aRNA to microarray slides

Hybridisation buffer was prepared using 1.2× ULTRAhyb® ultrasensitive hybridisation buffer (Ambion), 1.2× saline-sodium citrate (SSC) buffer (Sigma), 0.89mg/ml polyadenylic acid (PolyA; Sigma), 0.44mg/ml single stranded Herring sperm DNA (Sigma) and 2.2mg/ml UltraPure BSA (Ambion). The volume of hybridisation buffer was aliquoted into 1.5ml microcentrifuge tubes, enough for 1 per hybridisation to be performed, with 225 µl per tube. Hybridisation buffer was kept at 60°C until required. For hybridisations, Cy3-labelled aRNA and pooled aRNA labelled with Cy5 dye was combined such that 40 picomoles of each dye were present and the volume made up to 25 µl with MilliQ water. The samples were then denatured at 95°C for 3 minutes, centrifuged briefly and added to a 225 µl volume of hybridisation solution and incubated in the dark at 60°C until ready for hybridisation.

Hybridisation reactions were done on a Lucidea™ SlidePro Hybridiser (Amersham Biosciences). Once samples were injected, chambers were heated to 70°C for 10 minutes followed by heating at 42°C for hybridisation and washing stages. The hybridisation stage was carried out for 17 hours with a pulse mix of 25 µl at 5 µl per second every 15 minutes. Following the hybridisations, wash stages were carried out. The first of these flushed 800 µl of 1× SSC, 0.1% SDS solution over each slide at 8 µl per second. A second wash consisted of 800 µl of 0.3× SSC, 0.2% SDS being

flushed over each slide, also at 8 µl per second, after which slides were transferred to an EasyDip container containing the second wash buffer preheated to 45°C. Slides were washed twice for 3 minutes each in an orbital incubator set at 45 °C with shaking at 125 rpm. A further three washes of 2 minutes each in 0.2× SSC buffer were carried out before slides were dipped for 20 seconds in 0.1× SSC and centrifuged at 500×g for 5 minutes at room temperature to dry. Throughout all manual wash steps and the final drying stage caution was taken to avoid exposure of the slides to light sources, thus preventing degredation of the fluorescence signal from Cy3 and Cy5 dyes.

Microarray slides were scanned using the Axon 4200A Scanner and GenePix® software version 6.1. To calculate the optimum conditions at which Cy5 and Cy3 dyes are detected, the red and green lasers were selected and an auto-PMT for a single array on each slide carried out to fine the optimum scan conditions. The full slide was then scanned using the calculated PMTs.

5.2.4 Microarray analysis

Following hybridisation and scanning, images were first analysed by Bluefuse software (BlueGnome Ltd, UK) which was used to identify features, fuse replicates and quantify Cy3 and Cy5 fluorescent signals from each feature. Bluefuse data were then analysed in Genespring GX7.3.1. (Agilent). To normalise data, all features with less than 0.01 fluorescence units were first discarded, then a Lowess per spot, per chip normalisation was carried out. Features whose sequence data formed contigs (Williams et al., 2006) were treated as on-chip replicates in Genespring.

Pair wise T-tests were carried out using Genespring GX7.3.1 to identify list of genes whose expression was changed between particular treatments. Genes were considered as significantly altered at $P<0.05$. To test the hypothesis that TBT reversed the WY16463-dependent effect on gene expression, a list comprising genes which were significantly changed after comparison of WY16463 alone and WY16463 plus TBT was first generated. To further explore the central hypothesis, lists of genes whose expression increased after WY16463 and decreased after TBT treatment were also generated.

5.3 Results

An overview of the genes whose expression changed in each treatment is provided in Table 5-1. These lists were generated by T-tests of selected pairwise comparisons, designed to explore the hypothesis that TBTO antagonises the effects of Wy14643 on PPAR . Application of false discovery correction (Bonferroni and Hochberg) returned very few genes in any of the comparisons, and therefore it is likely that the uncorrected lists contain false positives.

Gene ontology (GO) analysis of these lists (Figure 5-1) provided further lists of physiological processes which were over-represented in up- or down-regulated outputs from each of the treatment comparisons. In both TBTO and Wy14643, “proteolysis” was increased and “xenobiotic metabolism” decreased, otherwise, in terms of GO biological process categories, there were few overlaps between treatments.

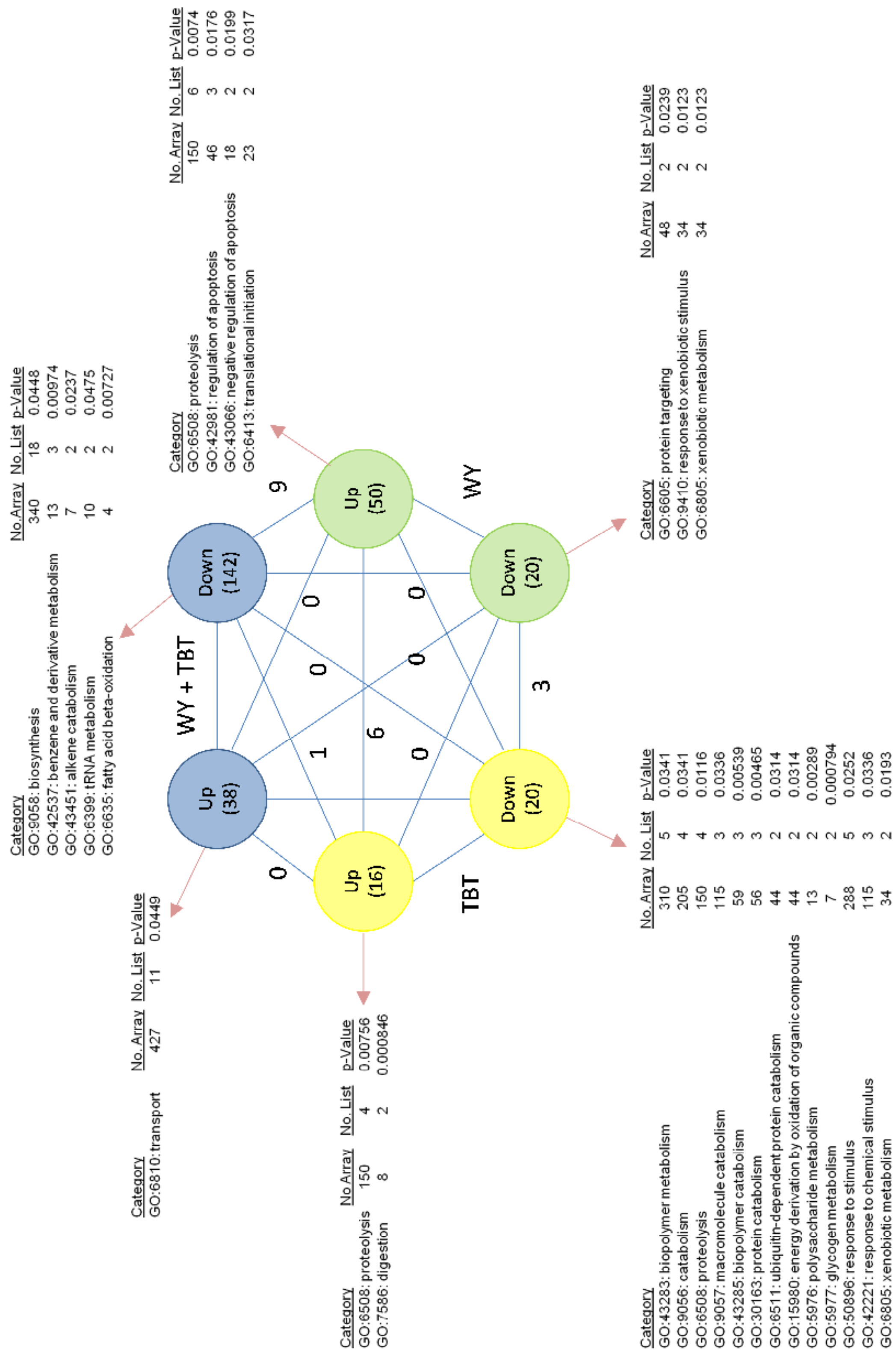

Figure 5-1. Overview of gene expression changes in hepatocyte treatments. Lists of genes whose expression is upregulated (UP) or downregulated (DOWN) is represented by blue nodes when comparing Wy14643 treatment with Wy14643 +TBT, yellow nodes when comparing TBT with vehicle control (Ethanol) and green nodes when comparing Wy14643 with vehicle control. Numbers linking nodes indicate the numbers of genes common to the two lists. Red arrows link to statistically over-represented Gene Ontology (GO) categories in each of the lists, as calculated by Genespring GX from the total number of representative genes on the array (No. Array), and the number of genes in an output list (No. List).

In Chapter 4, it was hypothesised that TBTO inhibited the actions of Wy14643 and therefore those genes downregulated by exposure to Wy14643 and TBTO in combination might be expected to be upregulated by treatment with Wy14643 alone and potentially downregulated by TBTO alone, and thus an overlapping pattern of expression would be expected. This was not reflected in GO categories, although the subset of 9 genes showing increased expression in Wy14643 and decreased expression in TBTO with Wy14643, was the largest overlapping group of any pair of output lists. To explore these results at the gene level, further manual annotation of the output lists was performed. The original microarray annotation was achieved automatically using BLASTtoGO and resulted in identification and annotation of between 60 and 70% of features (Williams *et al*, 2006). It was possible to identify and annotate approximately a futher 10% of these genes by manual comparison with Genbank/EMBL EST databases, followed by BLASTX to non-redundant protein databases.

Genes whose expressions were altered by the treatments with Wy14643 and TBTO, either alone or in combination, are listed in Table 5-1. Thus, after additional manual annotation, 14 genes were identified whose expressions were upregulated by Wy14643 and who were also downregulated by treatment with Wy14643 in combination with 10nM TBTO (Table 5-2), compared to the original 9 found after automatic annotation. Additionally, of the 20 genes shown to be downregulated by treatment with TBTO alone, 4 were also downregulated by TBTO in Wy14643 treatment. Of these overlapping genes those with a function in mitochondrial oxidative phosphorylation and proteasomal-dependent protein degradation formed the largest functional groups, with all genes in these groups being downregulated by

TBTO in Wy14643 treatment. Genes with functions in carbohydrate metabolism and protein biosynthesis were also similarly affected, being largely decreased. One such gene decreased by TBTO in Wy14643, glyoxylase 1, was also increased by Wy14643 alone. Genes involved in lipid metabolism, a function associated with PPAR , were of particular interest and showed both increases and decreases depending on the gene, when treated with a combination of TBTO and Wy14643. Notably, GO biological process "fatty acid β-oxidation", the archetypal target of PPAR action (Cook *et al*, 2000) was decreased in this group (Figure 5-1). The genes in this β-oxidation group whose expression decreased included PPARβ and mitochondrial delta 3,5-delta 2,4-dienoyl-CoA isomerase. Notably, a flounder gene, glutathione S-transferase A1 was similarly changed and has been implicated in fatty acid metabolism and is a potential PPAR target (Leaver et al, 1997). Two other potential mammalian PPAR target genes, L-3- hydroxyacyl-Coenzyme A and G0s2 G0/G1 switch gene 2 (Zandbergen *et al*, 2005; Zhang *et al*, 1992) were upregulated following treatment with Wy14643 and TBTO.

Table 5-1. Genes whose expressions are changed by treatment with 25 µM Wy14643 + 10nM TBTO, Wy14643 alone and TBTO alone in *P.flesus* hepatocytes. Upregulated genes are indicated in red while downregulated genes are indicated in green

BLAST	FOLD change	*P* value	Function
Genes whose expressions are significantly altered by treatment with a combination of Wy14643 + TBTO compared to Wy14643 alone			
Aquaporin 8	2.536	0.0349	water balance
Pre-mRNA splicing factor ATP-dependent RNA helicase PRP16	2.226	0.0157	RNA processing
Survival motor neuron domain containing 1	2.684	0.0272	
Bile acid binding protein (FABP10)	2.182	0.000829	lipid transport and metabolism
Retinol binding protein II, Cellular (CRBP-II)	1.215	0.0235	
TBT-binding protein 2 (lipocalin)	1.325	0.0134	
L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain	1.839	0.0201	
G0s2 G0/G1 switch gene 2	2.475	0.038	
Alpha amylase	1.091	0.0237	Carbohydrate metabolism
Trafficking protein particle complex subunit 1	2.155	0.0219	protein transport and secretion
component of oligomeric golgi complex 4	1.903	0.011	
similar to RAB5 interacting protein 2	2.065	0.00437	
Vesicle-associated membrane protein 8 (endobrevin)	1.706	0.00259	
SLC6A8 neurotransmitter transporter, creatine	1.987	0.0393	creatine uptake
slc35d1 UDP-glucuronic acid/UDP-N-acetylgalactosamine transporter	2.241	0.00514	chondroitin sulphate synth
heat shock protein gp96	1.807	0.0327	stress
copper/zinc superoxide dismutase	1.229	0.00853	
POMP proteasome maturation protein	1.8	0.0205	proteasome
IgE receptor gamma	1.764	0.0144	Immune
Protein phosphatase 6, catalytic subunit	1.759	0.0397	cell cycle

Table 5-1 cont…

Blast	Fold Change	*P* value	Function
RNA polymerase II, polypeptide H	1.489	0.0358	Transcription
similar to SMT3 suppressor of mif two 3 homolog 2	1.441	0.0244	Sumoylation
calmodulin 1	1.379	0.0341	Ca signalling
FAM162A family with sequence similarity 162, member A	1.85	0.0276	apoptosis
NOD3 protein; CARD15-like	1.329	0.000829	
glutamate carboxypeptidase (Darmin)	2.267	0.0485	metabolism
60S ribosomal protein L11	1.071	0.0216	Translation
40S ribosomal protein S11 (poor seq)	1.1	0.0197	
ATPase inhibitor, mitochondrial precursor	0.852	0.0138	Mitochondrial electron transport and oxidative phosphorylation
H+ transporting F1 ATP synthase epsilon subunit	0.73	0.0184	
NADH dehydrogenase B14.5b chain	0.722	0.0145	
NADH dehydrogenase subunit 4	0.597	0.0428	
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4	0.764	0.045	
NADH-ubiquinone oxidoreductase subunit B17.2	0.576	0.0192	
cytochrome b-c1 complex subunit Rieske, mitochondrial	0.527	0.00778	
cytochrome c oxidase	0.576	0.0404	
slc25a12 solute carrier family 25 member 12	0.76	0.0216	Mitochondrial
Mitochondrial ribosomal protein L22	0.626	0.0117	
UXT protein	0.605	0.0261	
Glutaredoxin	0.814	0.0265	Redox homeostasis
SPC18 protein, signal peptidase complex (18kD)	0.666	0.0182	Protein Processing
calreticulin	0.83	0.0208	

Table 5.1 cont…

Blast	Fold Change	*P* value	Function
C100 chaperonin subunit 2	0.713	0.0127	Protein processing
dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 4	0.704	0.00619	
ERP27 endoplasmic reticulum protein 27	0.81	0.00344	
proteasome subunit beta 7	0.789	0.00885	Proteasome and Ubiqutination
PSMA8 proteasome subunit, alpha type, 8	0.715	0.0157	
Proteasome subunit beta type 1 precursor (EC 3.4.25.1)	0.603	0.00189	
Proteasome beta-subunit C5	0.693	0.0175	
FBXO48 F-box protein 48	0.638	0.013	
Thioredoxin-like 1 (proteosomal protein)	0.79	0.0215	
possible de-ubiquitinylating peptidase	0.71	0.0405	
Vitamin K epoxide reductase complex subunit 1	0.857	0.00739	blood coagulation
similar to coagulation factor 10; coagulation factor X	0.81	0.0454	
PPAR beta	0.524	0.0492	lipid transport and metabolism
TBT-binding protein 1 (lipocalin)	0.758	0.0467	
APOD apolipoprotein D	0.725	0.00682	
inter-alpha (globulin) inhibitor H4 isoform 1	0.766	0.0162	
Delta3,5-delta2,4-dienoyl-CoA isomerase, mitochondrial	0.804	0.0169	
Probable ergosterol biosynthetic protein 28	0.751	0.00735	
inositol(myo)-1(or 4)-monophosphatase 1	0.618	0.00315	
glutathione S-transferase A1 (rho class)	0.511	0.0428	

Table 5-1 cont…

Blast	Fold Change	*P* value	Function
Glyoxalase 1	0.63	0.0081	Carbohydrate metabolism
Fructose-1,6-bisphosphatase 1	0.808	0.0419	
Fucosidase, alpha-L-1, tissue	0.634	0.037	
xylulokinase homolog	0.785	0.0279	
protein phosphatase 1, regulatory (inhibitor) subunit	0.653	0.0242	
carboxymethylenebutenolidase homolog	0.776	0.0498	Other metabolism
pnpo pyridoxine 5'-phosphate oxidase	0.623	0.00595	
Spermidine/spermine N1-acetyltransferase	0.618	0.029	
histidine ammonia lyase	0.593	0.0458	
Copper homeostasis protein cutC homolog	0.744	0.0316	
Warm-temperature-acclimation-related protein (65kDa)	0.75	0.0117	
chloride channel protein	0.596	0.0188	ion balance
slc29a3 nucleoside transporter	0.682	0.0263	nucleoside transport
GTP cyclohydrolase 1	0.414	0.0254	tetrahydrofolate biosynthesis
Vitronectin	0.801	0.00659	Cell adhesion, Growth, Motility
Hgfac hepatocyte growth factor activator	0.622	0.0356	
Hyaluronic acid binding protein 2	0.621	0.0221	
Developmentally regulated GTP binding protein 1	0.793	0.034	
Receptor-binding cancer antigen expressed on SiSo cells	0.428	0.0393	
Epithelial protein lost in neoplasm	0.73	0.0106	
sorcin isoform b	0.72	0.0411	Calcium binding

Table 5-1 cont…

Blast	Fold Change	*P* value	Function
isg12-like	0.882	0.0104	immune function
isg12-like	0.804	0.033	
isg12-like	0.766	0.0397	
Ifit interferon-induced protein with tetratricopeptide repeats	0.754	0.0416	
MHC class IIb antigen	0.858	0.0157	
myeloid differentiaton factor 88, myD88	0.671	0.039	
Heterogeneous nuclear ribonucleoprotein A/B	0.733	0.0174	RNA processing
nucleolar protein family A, member 2	0.717	0.0138	
processing of precursor 5, ribonuclease P/MRP subunit	0.673	0.0325	
Splicing factor 3 subunit 1	0.609	0.0123	
NOL12 nucleolar protein 12	0.791	0.0461	
nucleolin	0.879	0.00196	
Density-regulated protein	0.72	0.011	
Aspartyl-tRNA synthetase	0.698	0.00292	
Elongation factor 2	0.843	0.0467	Protein Biosynthesis
60S ribosomal protein L7a	0.924	0.0107	
60S ribosomal protein L28	0.793	0.00968	
60S ribosomal protein L13a	0.758	0.0482	
eukaryotic translation initiation factor 5	0.71	0.00756	
ATPase family, AAA domain containing 1	0.851	0.00312	Unknown function
ATPase family, AAA domain containing 4	0.486	0.000748	
Transmembrane protein 93	0.715	0.0235	
rhamnose binding lectin STL2	0.711	0.0317	
Oral cancer overexpressed 1	0.677	0.0205	

Table 5-1 cont…

Blast	Fold Change	*P* value	Fuction
LIM motif-containing protein kinase 2 (Limk2)	0.515	0.0051	Unknown Function
EF hand containing family 1A (Smhs2)	0.652	0.0384	
Cytochrome b5 domain containing 2	0.584	0.0374	
Genes whose expression is upregulated by exposure to 25 µM Wy14643			
IDH3A isocitrate dehydrogenase 3 (NAD+) alpha		1.664	Mitochondrial electron transport and oxidative phosphorylation
NADH-ubiquinone oxidoreductase 30 kDa subunit		1.505	
cytochrome b-c1 complex subunit Rieske, mitochondrial		1.549	
cytochrome c oxidase subunit VIb precursor		1.443	
brain protein 44-like; HSPC040 protein		1.281	mitochondrial
importin 9		1.709	protein processing
intraflagellar transport protein 20		1.295	
Reticulon-1		1.322	
prefoldin 1		1.944	
COMM domain containing 7		1.863	Transcription
CLEC4E C-type lectin domain family 4 member E		1.831	immune
PPDPF pancreatic progenitor cell differentiation and proliferation factor		1.354	Cell adhesion, Growth, Motility
transducer of ERBB2, 1b (tob1b),		1.256	
CRIPT cysteine-rich PDZ-binding protein		1.486	
Microtubule aggregate protein		3.552	
Vitronectin		1.526	
glyoxalase 1		1.203	carbohydrate metabolism
PPAR gamma		1.518	lipid transport and metabolism
Vacuolar ATP synthase subunit H (V-ATPase H subunit)		1.266	ATP synthesis

Table 5-1 cont…

Blast	Fold Change	Function
anticoagulant protein C precursor (PROC)	1.431	blood coagulation
similar to basic leucine zipper and W2 domains 1	**1.199**	**transcription**
Heterogeneous nuclear ribonucleoprotein A/B	1.285	RNA processing
splicing factor arginine/serine-rich 3	1.361	
splicing factor, arginine/serine-rich 2 (SC-35)	1.421	
PRPF8 PRP8 pre-mRNA processing factor 8	1.205	
Proteasome (Prosome, macropain) subunit, beta type, 8	1.32	Proteasome and Ubiqutination
FBXO48 F-box protein 48	1.486	
ubiquitin specific peptidase 50	1.315	
Chymotrypsinogen 2	1.182	proteolysis
elastase 2-like protein	1.17	
elastase 2 precursor	1.16	
pancreatic elastase 1	1.094	
Ribosomal Protein S3A	1.154	Protein Biosynthesis
18S ribosomal RNA gene	1.314	
Genes whose expressions are downregulated by treatment with 10nM TBTO		
acetyl-Coenzyme A acyltransferase 2 (mitochondrial)	0.718	Lipid metabolism
Liver glycogen phosphorylase	0.668	carbohydrate metabolism
protein phosphatase 1, catalytic subunit, gamma isoform	0.653	
proteasome, 26S, non-ATPase regulatory subunit 6	0.405	proteasome
PABPC3 poly(A) binding protein, cytoplasmic 3	0.43	RNA processing
hepcidin precursor	0.841	immune
CLEC10A C-type lectin domain family 10, member A	0.506	
alpha-2-macroglobulin	0.702	
fetuin B	0.409	ossification
Derlin-2 (Degradation in endoplasmic reticulum protein 2)	0.675	Protein processing
18S ribosomal RNA	0.64	protein biosynthesis

Table 5-2. Genes, or genes which are similar, whose expression is downregulated by treatment with Wy14643/TBTO combination and also either upregulated or downregulated in response to Wy14643 and TBTO alone, respectively.

BLAST	Function
Genes upregulated by Wy14643 alone and downregulated by Wy14643 + TBTO	
NADH-ubiquinone oxidoreductase 30 kDa subunit cytochrome b-c1 complex subunit Rieske, mitochondrial cytochrome c oxidase subunit VIb precursor	Mitochondrial electron transport and oxidative phosphorylation
CLEC4E C-type lectin domain family 4 member E	immune
Vitronectin	Cell adhesion, Growth, Motility
glyoxalase 1	carbohydrate metabolism
Heterogeneous nuclear ribonucleoprotein A/B	RNA processing
Proteasome (Prosome, macropain) subunit, beta type, 8 FBXO48 F-box protein 48	Proteasome and Ubiqutination
18S ribosomal RNA gene	Protein Biosynthesis
Genes dowregulated by Wy14643 + TBTO treatment and downregulated by TBTO alone	
protein phosphatase 1, catalytic subunit, gamma isoform	Carbohydrate Metabolism
proteasome, 26S, non-ATPase regulatory subunit 6	proteasome
CLEC10A C-type lectin domain family 10, member A	Immune
18S ribosomal RNA	Protein Biosynthesis

5.4 Discussion

The liver is an organ in which PPAR is highly expressed in fish species (Leaver *et al*, 2005) and is also the main organ of biotransformation of contaminants, making it an ideal organ in which to study gene expression changes by an anthropogenic compound. Here we have preliminarily identified several European flounder genes whose expression in hepatocytes is altered after exposure to a PPAR agonist, Wy14643, in combination with TBTO and compared to those exposed to Wy14643 alone. Additionally several genes have been identified as being upregulated by Wy14643 and downregulated by TBTO in isolation. Although this evidence is preliminary and requires confirmation by quantitative PCR (rt-qPCR), this is the first indication that TBT inhibits the effects of Wy14643 in European flounder.

5.4.1 Differential gene expression changes between species

Wy14643, a PPAR -specific agonist (Leaver *et al*, 2005; Seimandi *et al*, 2005), has previously been shown to alter 6.5% of all genes within wild-type mouse liver (Anderson *et al*, 2004). Of the 815 Wy14643-regulated genes only 2 were regulated in a PPAR -independent manner, being regulated by Wy14643 in PPAR -null mouse models. Regarding PPAR -regulated genes several species-specific differences have previously been noted; a mostly divergent set of genes has been found to be regulated by PPAR in mouse and human hepatocytes (Rakhshandehroo *et al*, 2009) and fewer genes have been found to be affected by treatment with Wy14643, with a lesser maximal response, in human hepatoma cells compared to rat hepatoma cells (Vanden Heuvel *et al*, 2003). This latter effect is likely due to the greater abundance of PPAR in rodent liver than in human liver, but other qualitative differences between species, such as the presence or absence of transcriptional activators/repressors, the existence of dominant negative PPAR variants, and differences in the PPRE sequence of target genes, have been suggested as possible factors contributing to the differences in response to peroxisome proliferators (Holden and Tugwood, 1999). Thus, despite the number of clear parallels which exist between the flounder data described here and the many studies on PPAR effect in rodents and humans, it may not be feasible to directly compare results from other species in order to fully elucidate the effects of contaminants which are suspected to disrupt PPAR in flounder.

5.4.2 Alterations in proteome maintenance genes

Wy14643, being a highly potent PPAR agonist, was expected to upregulate the expression of PPAR target genes in the hepatocytes of European flounder. In total

50 genes were upregulated within hepatocyte cells of European flounder, three of which were components of, or associated with the proteasome. Of the genes whose expression decreased following treatment with Wy14643 plus TBTO, 7 genes were associated with the proteasome, and two of these (PSMB8 and FBOX48) were identical to genes increased in the Wy14643 only treatment. PSMB8 was also decreased in the TBTO only treatment. Previously genes encoding proteins which function in the maintenance of the proteome, including components of the proteasomal protein degradation pathway, have been reported to be upregulated in response to treatment with Wy14643 in mouse hepatocytes (Anderson *et al*, 2004; Ren *et al*, 2010) and also upregulated in response to ciprofibrate, another PPAR agonist, in a primate species (Cariello *et al*, 2005). The hypothesis that PPAR is important in the regulation of proteasome maintenance genes was strengthened by the finding that PPAR -null mice display defects in the expression of proteins that protect the proteome from chemical damage. Thus, the patterns of expression of these genes are consistent with a hypothesis that TBTO antagonises the activity of PPAR in regulating proteosomal genes in flounder.

5.4.3 Mitochondrial oxidative phosphorylation

As well as changes in the expression of genes encoding proteins involved in the proteasome and in ubiquitination, changes in the expression of genes encoding proteins with a function in mitochondrial oxidative phosphorylation were also highly represented. All of the genes in this category displayed the same pattern of expression across treatments. They were increased in Wy14643 (4 genes), and decreased by TBTO in Wy14643 treatment (8 genes). Three of these genes were the same in both lists. The increased expression of oxidative phophorylation genes by

both PPARα and PPARβ has been consistently observed in mammalian studies although, an indirect mechanism has been inferred (Kelly and Scarpulla, 2004).

Many of the regulatory functions of hormone receptors are mediated via their interactions with co-activators and co-repressors. PPAR gamma co-activator 1 (PGC-1), so called due to its first being identified as a PPAR -interacting protein (Puigserver *et al*, 1998), is a co-activator whose interactions with all three PPAR subtypes has since been described. Vega *et al* (2000) demonstrated a ligand-influenced interaction between PGC-1 and PPAR , which increased the expression of mitochondrial fatty-acid oxidation (FAO) genes, while transient transfection assays have revealed PGC-1 to be a potent co-activator for all three PPAR subtypes (Røst *et al*, 2009). The identification of a peroxisome proliferator response element (PPRE) within the promoter region of the human PGC-1 gene (Schuler *et al*, 2006) has also led to the suggestion of a feedback loop between PGC-1 and PPARs with PPARs increasing the expression of PGC-1 , which in turn increases the expression of PPAR-target genes via its function as a co-activator (Hock and Kralli, 2009). Thus PPARs may indirectly modulate the expression of proteins involved in mitochondrial oxidative phosphorylation by increasing the expression of PGC-1 , which itself has been shown to modulate the expression of genes involved in oxidative phosphorylation via interactions with other transcription factors (e.g. nuclear respiratory factor (NRF) 1 and GA binding protein (GABP)) (Wu *et al*, 1999).

5.4.4 TBT-binding proteins (TBT-bp)

The lipocalin protein family functions in the transport of small hydrophobic molecules (Flower, 1996). Two lipocalin-like proteins, tributyltin-binding protein (TBT-bp) 1 and TBT-bp 2, were found to be differentially regulated in response to

the addition of TBTO in Wy14643 treatments. An upregulation of TBT-bp2 was observed when compared to treatment in which TBTO was absent. Although a small upregulation of TBT-bp2 was observed in treatments with TBTO with Wy14643 treatments, there was no indication that this gene was upregulated in European flounder hepatocytes treated with TBTO alone. TBT-bp1 was found to be downregulated after treatment with TBTO in Wy14643. This pattern of expression in which TBT-bp2 is upregulated and TBT-bp1 downregulated, has previously been found in Japanese flounder (*Paralichthys olivaceus*) injected with tributyltin (Nassef *et al*, 2011). Furthermore medaka exposed to TBT (Nassef *et al*, 2011) and *K. marmoratus* exposed to Bisphenol A (Lee *et al*, 2007) also showed a downregulation of TBT-bp1 while an upregulation was seen in medaka in response to TCDD (Volz *et al*, 2005). Therefore it appears that the response of European flounder to TBTO may reflect that of other fish species. Although the mechanisms relating to the downregulation of TBT-bp1 following TBT exposure is currently unknown, it has been suggested that the response of TBT-bp1 may be dependent on the exposure chemical (Nassef *et al*, 2011). TBT injected intraperitoneally in Japanese flounder and dab (*Limanda Yokohamae*) was found to accumulate within blood serum at a higher concentration than muscle, liver, spleen, bile and kidneys (Oshima *et al*, 1998), and this accumulation has been found to correlate with the appearance of TBT binding proteins (Shimasaki *et al*, 2002). It has since been suggested that TBT-binding proteins might be secreted into the skin mucus of fish as a complex with TBT as a means of excretion, thus protecting fish species from the toxicological effects of such a compound (Satone *et al*, 2008).

5.4.5 Lipid metabolism and transport

As discussed in Chapter 1 one of the main functions of PPAR is in regulating lipid metabolism. Genes involved in lipid transport and metabolism were both upregulated and downregulated by the presence of TBTO in Wy14643 treatments, several of which were identified as PPAR target genes. Consideration of these genes indicates that their patterns of expression are not entirely consistent with the hypothesis that TBTO antagonises the activity of PPAR . Both, mitochondrial delta3,5-delta2,4-dienoyl-CoA isomerase (a β-oxidation enzyme) and glutathione-S-transferase A1, have been suggested as PPAR-targets, with their expression increasing in response to an activation of PPARs (Berger *et al*, 2002; Leaver *et al*, 2007; Williams *et al*, 2008). Since they are both decreased by TBTO in Wy14643 treatment, this pattern of expression would be consistent with the TBTO antagonism hypothesis. Another β-oxidation gene, acetyl-Coenzyme A acyltransferase 2 (mitochondrial ACAT2) is reduced in TBTO treated hepatocytes. However, two potential PPAR targets are also present in the list of genes increased by TBTO in Wy16463 treatment. These are L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain (HADH, a short chain fatty acid β-oxidation enzyme) andG_0/G_1 switch gene 2 (G0S2). HADH catalyses the third step in the mitochondrial -oxidation of short chain fatty acids and a peroxisome proliferator response element has previously been found in the promoter region of the rodent gene (Zhang *et al*, 1992). Previously HADH has been found to be upregulated in response to TBTO in thymocytes of rats (Baken *et al*, 2007). G0S2 has previously been identified in mouse hepatocytes as a PPAR -target gene, containing a functional peroxisome proliferator-response element (PPRE) and being upregulated by the PPAR agonist Wy14643 (Zandbergen *et al*, 2005). In addition

the G0S2 gene has also been shown to be a direct target of PPAR , increasing in expression during adipocyte differentiation (Zandbergen *et al*, 2005).

Although these results are clearly equivocal, it is also true that TBTO may have other effects than the antagonsim of PPARα. For example in mammals organotins are potent agonists of RXR (Cui *et al*, 2011), the heterodimeric partner of PPARs, and of PPARγ. Although effects on PPAR by TBTO has not been examined, since no potent agonist has been discovered for this receptor from fish species, in treatment with Wy14643 alone, PPAR was found to be one of the genes upregulated. Also the HADH gene, as well as being a PPAR target, has been shown to be upregulated in response to the liver X receptor (LXR) ligand T0901317 (Hu *et al*, 2005), suggesting HADH to be regulated by more than one nuclear hormone receptor. Furthermore the LXR also partners with RXR to form a heterodimer, indicating other potential mechanisms of regulation.

5.4.6 TBT downregulates the expression of genes involved in immune functions

TBTO in rodents has been previously shown to be an immunotoxicant, decreasing the thymus volume and the expression of genes with a function in cell division, pointing to immunosuppression via cell cycle arrest (Baken *et al*, 2008). A reduction in thymus volume has also been reported after TBTO exposure in European flounder, leading to the suggestion that the immunocompetence of the animals may be compromised (Grinwis *et al*, 1998; Grinwis *et al*, 2009).

In flounder hepatocytes in response to treatment with TBTO only, of the twenty genes downregulated, three have immune function. Six immune function genes were

also downregulated in the treatment with TBTO in Wy14643. These genes were also well represented in the treatment with TBTO in Wy14643.

One of the TBTO down-regulated genes, alpha 2 macroglobulin (2M) is an important acute-phase proteins whose hepatic expression has been found to be modified by PPAR agonists in a PPAR -dependent manner, being significantly downregulated in rats treated with the PPAR agonist fenofibrate (Carmen González *et al*, 2009). Despite no PPRE having been identified within the promoter region of 2M, Carmen González *et al* (2009) suggested that PPAR may be negatively regulating 2M through negative regulation of STAT3 or NF-kB. Furthermore it has also been suggested that PPAR may have a role in the regulation of 2M expression; Kino *et al* (2007) found that GW501516, a PPAR agonist, interfered with interleukin-6 (IL-6)-induced acute phase response by inhibiting the transcriptional activity of STAT3 thus inhibiting 2M expression. Within the study here a small downregulation of 2M was noted in response to TBTO, possibly due to PPAR mechanisms. This decrease in 2M expression, although small, is unexpected since TBTO is thought to inhibit both PPAR and PPAR and would therefore diminish PPAR-dependent inhibition of pathways leading to 2M downregulation. However, in agreement with a downregulation of 2M, the expression of 2M of the small abalone (Haliotis diversicolor) was found to be downregulated upon exposure to TBT (Jia *et al*, 2011).

5.5 Conclusions

Several genes have been discussed whose expression is altered by TBTO in the presence of Wy14643. Although the results are not entirely clear with respect to some of the known effects of PPARs, for example fatty acid -oxidation, the products of the most highly represented classes of genes belong to several functional

groups including those involved in the maintenance of a healthy proteome, mitochondrial oxidative phosphorylation, excretion of toxins and lipid homeostasis. These groups are consistently decreased in expression by TBTO, increased by Wy14643 and decreased by TBTO in Wy14643-treated cells. Several of these genes have been described previously as PPAR -target genes or genes indirectly regulated by activated PPARs. Due to the constraints of time, confirmation of the changes in gene expression, for example by application of rt-qPCR, was not done and thus these results should be regarded tentatively. However the overall pattern of gene expression indicates that TBTO has the potential to alter normal gene expression patterns in European flounder via PPAR . Of the genes amenable for further study, glutathione S-transferase A1 (GSTA1) would be the most informative due to its prior characterisation from flounder/plaice (Leaver *et al*, 1993; Henson *et al*, 2000) and the presence of a PPAR-binding PPRE within its promoter region (Leaver *et al*, 1997; Leaver *et al*, 2005), indicating that it is likely regulated by PPARs.

Chapter 6. Isolation of corticosteroid receptors from European flounder (Platichthys flesus).

6.1 Introduction

Corticosteroid receptors, of which the glucocorticoid (GR) and the mineralocorticoid receptor (MR) are distinguished, are members of the superfamily of nuclear hormone receptors and mediate the effects of the corticosteroid hormones. These steroid hormones, produced by the adrenal cortex in tetrapods and the homologous interrenal cells of the head kidney of teleost fish (Phillips and Mulrow, 1959) have been implicated in a wide range of physiological functions such as energy metabolism, anti-inflammatory and immune responses (Amsterdam *et al*, 2002; Stolte *et al*, 2008), development (De Jesus *et al*, 1991), osmoregulation (McCormick, 2001; Kiilerich *et al*, 2011) and reproduction (Brann and Mahesh, 1991; Milla *et al*, 2009).

Mammals possess two main corticosteroid hormones, the glucocorticoid cortisol (corticosterone in rodents) and the mineralocorticoid aldosterone. While cortisol and corticosterone can bind to and activate both GR and MR, aldosterone is, at physiological levels, limited to binding to MR only. In teleost fish, the glucocorticoid cortisol is the main product of interrenal cells, and has traditionally been regarded as the main functional corticosteroid hormone (Mommsen *et al*, 1999). The ability to synthesise aldosterone may have evolved relatively recently in the lineage leading to the tetrapods given that this mineralocorticoid is absent in teleosts (Jiang *et al*, 1998), elasmobranchs (Simpson and Wright, 1970; Nunez and Trant, 1999), and agnathans (Bridgham *et al*, 2006). The emergence of aldosterone as a mineralocorticoid appears to be due to a modification of cytochrome P450 hydroxylase enzyme which is found in all jawed vertebrates and whose ancestral function appeared to be the hydroxylation of DOC in cortisol synthesis. Only in tetrapods does this enzyme have

the ability to hydroxylate corticosterone to aldosterone (Nonaka *et al*, 1995; Bülow and Bernhardt, 2002). Several authors have described the inability of this enzyme from teleosts to synthesis aldosterone (Jiang *et al*, 1998; Nelson, 2003). Despite the lack of aldosterone, an MR has been identified and characterised in rainbow trout (Colombe *et al*, 2000; Sturm *et al*, 2005) and other teleost species (Greenwood *et al*, 2003; Stolte *et al*, 2008; Pippal *et al*, 2011). In addition two glucocorticoid receptors have been identified in several teleosts (Greenwood *et al*, 2003; Bury *et al*, 2003; Stolte *et al*, 2006; Stolte *et al*, 2008).

Since these receptors have a role in a wide range of physiological processes in fish species a disruption of their transcriptional abilities, for example by environmental contaminants, might be expected to negatively impact fish species in polluted environments. European flounder (*Platichthys flesus*) is a sentinel species used to monitor the biological effects of contamination in inshore/estuarine waters of the OSPAR maritime area. The aim of this study was to isolate cDNAs for corticosteroid receptors from flounder as a necessary first step in determining the effects of environmental contaminants on corticosteroid receptor function in flounder. Three corticosteroid receptors from this species and their tissue distributions are described here for the first time.

6.2 Materials and Methods

6.2.1 Total RNA isolation from tissues of European flounder

For the purpose of receptor isolation total RNA was extracted from eight tissues of a single juvenile male European flounder (*Platichthys flesus*) obtained from the Forth estuary and acclimated to seawater in an aquarium for six months. The fish was killed by a single blow to the head, after which samples of the brain, gills, heart,

small intestine, kidneys, liver, white muscle and gonads were removed and homogenised in 10 volumes of TRIpure isolation reagent (Roche). Six hundred microlitres of homogenate were centrifuged for 2 minutes at 16,000 $\times g$ to remove any remaining lumps of tissue and 500 µl of the resulting supernatant transferred to a clean tube. RNA isolation was continued as described in the TRIpure isolation reagent instruction manual (Roche), with RNA finally being resuspended in MilliQ water (Millipore) to a final concentration of 1mg/mL.

RNA concentration and purity were determined spectrophotometrically on Nanodrop ND1000 spectrophotometer. In addition, the integrity of total RNA samples was confirmed. 1 µg total RNA from each tissue was heated at 75°C for 2 minutes and loaded onto a 1 × TAE, 1% agarose, 0.5 µg/ml EtBr gel for electrophoresis at 75V for 30 minutes. RNA was visualised by exposure of the gel to UV light and RNA integrity confirmed by the presence of 28S and 18S rRNA bands at an intensity ratio of approximately 2:1 (28S:18S).

RNA samples and excess tissue homogenates were stored at –70°C until further use.

6.2.1.1 Reverse transcription

Total RNA isolated from each tissue, was reverse transcribed using either the commercially available SMART™ (Switching Mechanism At 5' end of the RNA Transcript) RACE cDNA amplification kit (Clontech) or an oligo(dT)-anchor primer according to an in-house protocol. Furthermore two separate reverse transcription reactions were prepared using the SMART™ RACE cDNA amplification kit (Clontech), one of which would allow subsequent rapid amplification of 3' cDNA ends (3' RACE) and the other which would allow rapid amplification of 5' cDNA ends (5' RACE).

Regardless of which method of reverse transcription was used, an oligo(dT) primer was included in each reaction (see Table 6-1). The oligo(dT) portion of the primer compliments the polyA tail at the 3' end of mRNA molecules. An anchor sequence is included at the 5' end of both the in-house RACE RT oligo(dT) and the 3' RACE CDS primer A (Clontech). Reverse transcription from the polyA tail of these oligo(dT)-anchor primers results in first-strand cDNA with the anchor sequence incorporated into the 5' end, the presence of which is important in subsequent 3' RACE. In order to be used for 5' RACE, first-strand cDNA requires a similar anchor sequence incorporated into the 3' end of reverse transcription products. Reverse transcription using the SMART™ RACE cDNA kit (Clontech) to produce 5' RACE ready cDNA makes use of both the terminal transferase activity of the Moloney murine leukemia virus reverse transcriptase (MMLV RT) enzyme and a so-called 'template-switch' oligonucleotide primer during first-strand cDNA synthesis (Matz *et al*, 1999). MMLV RT is capable of adding 3-5 non-template residues, preferentially dC, to the 3' end of the reverse transcription product. The SMART template switch oligonucleotide primer, included in the reverse transcription reaction, hybridises to the dC residues via a terminal stretch of guanosine residues, and serves as an extended template for reverse transcription, the end product of which is first strand cDNA with the additional SMART sequence at the 3' end.

Table 6-1. Oligo(dT) primers used during reverse transcription of total RNA into first-strand 3' RACE ready cDNA. V = A, G or C; N = A, T, C, G

Primer Name	Primer sequence
In-house RACE RT oligo (dT)	CAG CTG CAG GTA CCG GAT CCT C GAG AAG C (T)19
3' RACE CDS primer A (Clontech)	AAG CAG TGG TAT CAA CGC AGA GTA C(T)30 V N
5' RACE CDS Primer (Clontech)	(T) 25 V N

Per reaction 1 µg total RNA and oligo(dT)-anchor primer at a concentration of 1 µM was used. Additionally, 1 µM of SMART II oligonucleotide (5'- AAG CAG TGG TAT CAA CGC AGA GA CGC GGG -3'), the 'template-swich' oligonucleotide described above, was included in the reaction aiming to produce 5' RACE ready cDNA. Reactions were then made up to 5 µl with water and incubated at 70°C for 5 minutes before being rapidly cooled on ice for 2 minutes. 1× first strand buffer, 1mM of each dNTP and either 1 µl M-MLV RT(-)H point mutant reverse transcriptase enzyme (Promega) for use with the In-house oligo(dT) and 3' CDS primer A (Clontech) or 1 µl Powerscript reverse transcriptase (Clonech) for use with the 5' CDS primer were. A 2mM concentration of dithiothreitol (DTT) was also included in the reverse transcription yielding 5' RACE-ready cDNA. Reactions were gently mixed and placed in a thermocycler (Biometra Tgradient) at 42°C for 90 minutes after which each 10 µl reaction was diluted to 110 µl by the addition of Tricine EDTA buffer. Reverse transcriptase enzyme was inactivated by heating at 70° for 10 minutes and first-strand cDNA stored at -20°C until further use.

6.2.2 Amplification of corticosteroid receptors by PCR

A partial cDNA for GR2 from the European flounder (*Platichthys flesus*; Genbank accession number AJ867607.1) has previously been cloned (Lu *et al*, 2007). When aligned to GR2 sequences of burton's mouthbrooder (*Astotilapia burtoni*) and gilthead seabream (*Sparus aurata*) the GR2 of European flounder corresponds to

amino acids 430-759 of *A.burtoni* and 434-764 of *S.aurata* GR2, encompassing the D-domain and the majority of the ligand-binding E-domain of the receptor, with the most C-terminal 20 amino acids absent. In databases, no GR1 or MR sequences were available for *P.flesus*. To isolate partial sequences of these receptors, a PCR approach was used, based on the design of primers taking into account available MR and GR1 from other fish species.

6.2.2.1 Primer design and Polymerase Chain Reaction (PCR)

MR sequences from other fish species (Japanese pufferfish, *Takifugu rubripes*; zebrafish, *Danio rerio*; rainbow trout, *Onchorynchus mykiss*; Burton's mouthbrooder, *Haplochromis burtoni*) were aligned using ClustalW (Larkin *et al*, 2007) and primers derived from highly conserved regions. Two gene-specific pairs of MR primers were designed (Table 6-2) that were expected to amplify two overlapping MR fragments.

A nucleotide sequence for the GR1 of Japanese flounder (*Paralichthys olivaceus*) was available within the Genbank database, (Accession no. AB013444). Similar to the above strategy for MR, two GR1 primer pairs were made in order to amplify overlapping partial European flounder GR1 sequences. All primers used for PCR amplification are described in Table 6-2. PCR amplification reactions for each primer pair was done in 20 µl volumes containing 2.5 µl first-strand cDNA from kidney, liver and brain for GR1, GR2 and MR, respectively, 200nM forward and reverse primer each, 200 µM of each dNTP, DNA polymerase enzyme (0.4 µl Pfu fusion II polymerase (Stratagene), 1 × Advantage II polymerase mix (Clontech) or Expand long template polymerase (Roche) for GR1, GR2 and MR, respectively) and a 1× concentration of corresponding polymerase buffer. Single primer and no-

template controls were included for each reaction, the former of which contained only a single primer with water replacing the second primer and the latter of which contained water instead of first-strand cDNA template. No-template controls ensure contamination of reagents has not occurred and that primers are specific for their target sequence and do not amplify in a non-specific manner while the single primer controls ensure the amplified products are not due to the mis-priming of an individual primer.

Table 6-2. Primer sequences used to amplify partial cDNAs of each of the corticosteroid receptors from European flounder (Platichthys flesus). The given name of each primer is shown alongside its nucleotide and amino acid sequence. Primer annealing positions refers to amino acid positions of European flounder GR1, GR2 and MR, which have been added to the GenBank database with Accession nos. JF951960, JF951961 and JF951959, respectively.

Primer name	Primer sequence (5' → 3')	Amino Acid sequence (5' → 3')	Annealing Position
P.fGR1F1	ATG-GAT-CAG-GGT-GGA-CTG-AAG-CG	M-D-Q-G-G-L-K	1-6
P.fGR1R1	CAG-GTT-ACG-ACA-CCG-TAG-TGG-CAT-C	C-H-Y-G-V-V-T	455-461
P.fGR1F2	GTG-GGC-CTA-CAA-GAC-CAG-AA	V-G-L-Q-D-Q	364-369
P.fGR1R2	CAC-GCT-GAG-GGT-TTT-ATT-CAC	V-N-K-T-L-S-V	771-777
P.fGR2F1	CAC-TAC-GGC-GTT-CTC-ACC-TG	H-Y-G-V-L-T	77-82
P.fGR2R1	GTC-CTC-CAA-CCA-TCT-CAT-GC	H-E-M-V-G-G	372-377
P.fMRF1	ATG-GAG-AC(CT)-AAA-AGA-TAC-C(AC)A-AGT-T(AGT)C	M-E-T-K-R-Y-Q/P-S-Y/C/F	1-9
P.fMRR1	A(CT)G-A(AG)T-GTC-C(AG)C-C(GT)G-AAT-TAA-C(AGT)C	V-N-S-G-G-H-S	527-533
P.fMRF2	TGG-TAT-CTT-AGG-ACC-CCC-TGT	g-i-l-g-P-P	477-482
P.fMRR2	GAC-TCC-ACC-TTT-GGT-ATC-TGG	Q-I-P-K-V-E	972-977

Cycling conditions for each PCR reaction included an initial denaturation step of 2 minutes at 94 °C followed by 35 cycles of denaturation, primer annealing and extension steps (Table 6-3). Following each PCR amplification reaction, amplified products were visualised by agarose gel electrophoresis, as described in 3.2.1.1, and subcloned into plasmid vectors for further analysis.

Table 6-3. PCR cycling conditions for amplification of partial GR1, GR2 and MR fragments using primers detailed previously in Table 6-2.

	GR1		GR2	MR	
Forward Primer	F1	F2	F3	F4	F5
Reverse Primer	R1	R2	R3	R4	R5
Polymerase enzyme used	Pfu fusion II (Stratagene)	Expand Long template (Roche)	Advantage II mix (Clontech)	Expand Long template (Roche)	Expand Long template (Roche)
Cycling Parameters					
Denaturation	94 °C 20 secs	94 °C 10 seconds	94 °C 20 secs	94 °C 10 secs	94 °C 10 seconds
Primer annealing	64 °C 30 secs	55 °C 30 seconds	57 °C 30 secs	60 °C 30 secs	59 °C 30 seconds
Extension	72 °C 60 secs	68 °C 2 minutes	72 °C 60 secs	68 °C 4 mins	68 °C 2 minutes
Final extension	72 °C 3 minutes	68 °C 7 minutes	72 °C 5 minutes	68 °C 7 minutes	68 °C 7 minutes

6.2.2.2 Cloning methods

Amplicons from PCRs were ligated into one of three different cloning vectors, selected due to their availability and the method of cloning required.

The Pfu fusion II enzyme produces amplicons with polished ends, reflecting the enzyme's 3' → 5' exonuclease proofreading. Accordingly, blunt-ended cloning was used to subclone the putative GR1 fragment generated with this enzyme. All other amplicons were generated with DNA polymerases adding single 3'-Adenine overhangs, and thus were subcloned into linearised vectors containing single 3' – terminal thymidine residues at both ends.

The blunt-end GR1 fragment was purified from the enzymatic reaction using the QIAquick PCR purification kit (Qiagen), following manufacturer's instructions,

followed by ethanol precipitation as described in Chapter 3 (3.2.1.2). The resulting DNA pellet was resuspended in a ligation reaction containing 0.3 µl EcoRV-digested pBluescript vector (Stratagene), 1× T4 DNA ligase buffer (Fermentas), 15 weiss units T4 DNA ligase enzyme (Fermentas) and water to a volume of 10 µl. The ligase reaction was allowed to proceed at room temperature overnight.

For TA cloning either the pCR®2.1-TOPO (Invitrogen) or pGEM® T-easy (Promega) cloning plasmid was used. Use of the TOPO TA cloning® method avoids the need for post-PCR cleanup, allowing PCR enzyme reactions to be used directly in TA cloning in the case of amplification of a single, discrete band. The putative GR2 fragment was ligated into this vector in a 6 µl reaction volume in which 4 µl of the PCR product were incubated with 1 µl of salt solution (1.2M sodium chloride/0.4M magnesium chloride; Invitrogen) and 10ng of pCR® 2.1-TOPO vector at room temperature for 30 minutes.

All other products of PCR were cloned into the pGEM® T-easy cloning vector. Prior to ligation, DNA was first purified from the PCR reaction using ethanol precipitation or the QIAquick PCR purification kit (Qiagen), following manufacturers protocol. DNA purified using the latter method was further purified using ethanol precipitation. Precipitates were then resuspended in a 10 µl ligation reaction containing 1× Rapid ligation T4 DNA ligase buffer (Promega), 50 ng pGEM® T-easy vector (Promega) and 3 Weiss units of T4 DNA ligase (Promega) and the reactions incubated at 4 °C overnight.

6.2.2.3 Transformation of chemocompetent E.coli cells with ligation reaction product and plasmid purification

Following ligation, an aliquot of each reaction was transformed into chemocompetent *E.coli* (prepared in-house from the DH5 strain (described below), or commercially obtained JM109 high efficiency cells provided with the Promega's pGEM® T-easy vector system kit).

In-house *E.coli* cells were prepared by inoculation of 5ml SOB media with a single colony of DH5 and incubation overnight at 37°C. A 200ml volume of SOB, pre-warmed to 37°C, was then inoculated with 200 µl of the starter culture and incubated at 37°C until the OD600 0.4, determined spectrophotometrically using SOB media only as a blank. Bacterial growth was stopped by placing the flask on ice and the 200 ml volume aliquoted to 50ml sterile centrifuge tubes before incubating on ice for a further 10 minutes. Cells were pelleted by centrifugation (1000 ×g for 15 minutes at 4 °C) and SOB media discarded. Cells were resuspended in 67ml RF1 (100mM RbCl, 50mM Magnesium chloride, 30mM potassium acetate, 10mM calcium chloride, 15% (w/v) glycerol, pH5.8) before pelleting as described above. The supernatant was discarded and cells resuspended in 16ml RF2 (10mM MOPS, 10mM RbCl, 75mM calcium chloride, 15% (w/v) glycerol). Cells were incubated on ice for 15 minutes before being aliquoted to sterile pre-chilled tubes, snap frozen and stored at -70 °C.

For transformation, an aliquot of chemocompetent *E.coli* cells, thawed on ice (100 µl per reaction), were mixed with 5 µl of the products of the above ligation reactions and incubated on ice for 30 minutes before heat shocking for 45 seconds at 42 °C, followed by incubation of ice for 2 minutes. Luria broth (LB) media was then added

to transformed cells in a volume of 500 µl (DH5 *E.coli*) or 950 µl (JM109 high efficiency *E.coli*) and the mixtures incubated for 1 hour at 37°C with shaking at 150 rpm.

LB agar plates (90mm diameter containing 20ml agar) supplemented with 0.1 mg/ml ampicillin were treated with 2µg/plate 5-bromo-2-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) and 100 µl/plate 10 mM isopryl -D-1-thiogalactopyranoside (IPTG). Transformed *E.coli* cells were then spread and the inoculated plate incubated overnight at 37 °C. The inclusion of ampicillin provided a selection for cells transformed with plasmids conferring ampicillin-resistance, while the use of X-gal allows for a further colorimetric distinction (blue/white screening) of cells transformed with plasmids containing an insert (white colonies) from those containing empty vector (blue colonies), based on the cleavage of X-gal by -galactosidase, an enzyme encoded by the LacZ gene present in the cloning plasmids. Cleavage of X-gal results in the production of galactose and 5-bromo-4-chloro-3-hydroxyindole, the latter of which is further metabolised to the insoluble blue-coloured product, 5,5'-dibromo-4,4'-dichloro-indigo. Plasmids in which the PCR product is successfully ligated results in a disruption of the LacZ reading frame and thus a production of white colonies.

Individual white colonies from each plate were selected, used to inoculate small scale cultures (4 ml of LB media, supplemented with ampicillin at 0.1mg/ml), which were grown overnight at 37°C and 150 rpm agitation. Plasmid DNA was isolated from cultures using the GenElute™ Plasmid Miniprep kit (Sigma), following manufacturer's instructions, with plasmid DNA being eluted in 50 µl MilliQ water.

The obtained plasmid preparations were characterised by restriction mapping using enzymes releasing the insert. Plasmids derived from pGEM® T-easy and pCR®2.1-TOPO vectors were digested with EcoRI, using 2 µl plasmid preparation, 2 units of EcoRI enzyme (Invitrogen), 1× MULTI-CORE™ digestion buffer (Promega) and water to a volume of 10 µl. Plasmids derived from pBluescript were digested by HindIII and EcoRV, using 2 µl plasmid preparation, 1.3 units HindIII enzyme (Invitrogen), 1.3 units EcoRV enzyme (Invitrogen), 1× REact 2 digestion buffer (Invitrogen) and water to make the volume to 10 µl. Reactions were incubated at 37 °C for 1 hour before 5 µl of digested plasmid product was electrophoresed at 75V on a 1 × TAE, 1% agarose, 0.5 µg/mL EtBr gel alongside a 1 kb DNA ladder (Biogene), and the gel exposed to UV light in order to visualise digestion products. Plasmid preparations in which digestion yielded two DNA bands of the expected sizes, one corresponding to the plasmid and the other to the corticosteroid receptor fragment, were sequenced to confirm the identity of the insert.

6.2.3 *Rapid Amplification of 3' and 5' cDNA ends (3' RACE & 5' RACE)*

RACE-PCR was performed in order to amplify the 5' and 3' ends of each corticosteroid receptor cDNA, facilitating the isolation of the full open-reading frame of GR1, GR2 and MR from European flounder. As described in 6.2.1.1, reverse transcription reactions were done using either a modified oligo(dT) with an anchor sequence attached, designed to produce a reverse transcription product with the anchor incorporated at the 5' end, suitable for 3' RACE, or with both an oligo(dT) and a template switch oligonucleotide in which products of reverse transcription

would have the template switch oligonucleotide sequence incorporated at the 3' end, making it suitable for 5' RACE.

6.2.3.1 3' RACE using in-house oligo(dT) and anchor primer

For both the MR and GR2, 3' RACE was carried out using cDNA reverse transcribed from RNA using the in-house RACE anchor primer using a semi-nested strategy with primers described in Table 6-4.

First round PCR reactions used 2.5µl RACE-ready liver cDNA, 1 × PCR buffer IV (Abgene), 200 nM of forward and reverse primer each, 200 µM dNTPs, and 1 unit DNA taq polymerase (Abgene). The reactions were cycled for 2 minutes at 95°C followed by 25 cycles of dentaturation, primer annealing and extention steps at 95°C 30 seconds, 55°C (MR) or 62.5°C (GR2) for 30 seconds and 72°C for 2 minutes, respectively. A final extension of 72°C for 5 minutes completed the PCR programme. After analysis on a 1% agarose, 1 × TAE, 0.5 µg/mL EtBr gel, no amplification product was visible and therefore a second round of semi-nested PCR was performed. The reaction from first round PCR was diluted 1 in 100 with Milli Q water and 2.5 µl used as the template in a second round of PCR. All reagents and concentrations described above for first round PCR were the same for semi-nested PCR. The forward gene specific primers used were located internal to the original forward primers. Cycling conditions were as described for first round PCR, with the annealing temperatures altered to 57 °C and 63 °C for 3' RACE of GR2 and MR, respectively, to ensure compatibility with the internal forward primers.

6.2.3.2 3’ RACE using the SMART RACE cDNA kit (Clontech)

The SMART™ RACE cDNA amplification kit (Clontech) was used to amplify the 3’ region of GR1, employing a GR1-specific primer.

3’ RACE-ready cDNA, produced from reverse transcription using the 3’ CDS primer A (see 6.2.1.1) was used in 3’ RACE PCR at a volume of 2.5 µl per reaction together with 1 × Advantage II PCR buffer, 200 µM dNTP mix (Clontech), 1 × Universal primer mix (oligo(dT) kit primer), 200 nM gene specific forward primer and 1 × Advantage II polymerase mix (Clontech). The reaction was cycled using a touchdown cycling programme as recommended in the SMART™ RACE kit protocol. Touchdown PCR involves an increased annealing temperature at the beginning of the cycling programme such that annealing is at, or just above, the expected annealing temperature. During cycling the annealing temperature is decreased in increments to a temperature at which primers will anneal in a manner which is least permissive of non-specific binding, thus increasing the likelihood of amplifying the targeted sequence (Don *et al*, 1991; Hecker and Roux, 1996). Here, cycling was performed using 5 cycles combined annealing/extention step at 72 °C for 3 minutes, following an initial denaturation step at 94 °C for 30 seconds and followed by 5 cycles of denaturation, annealing and extention steps at 94 °C for 30 seconds, 70 °C for 30 seconds and 72 °C for 3 minutes, respectively. The programme was completed using 25 cycles of denaturation, annealing and extention steps at 94 °C for 30 seconds, 68 °C for 30 seconds and 72 °C for 3 minutes.

Table 6-4. Gene specific primers and primers designed to anneal to the anchor sequence at the 3' extremity of cDNA used in first and second-round PCR to amplify 3' RACE products for GR1, GR2 and MR.

Primer Name	Primer Sequence (5' → 3')	Amino Acid sequence (annealing position)	Additional Information
P.fGR13'F	TGC ATG AAG GTC CTG TTA CTG CTG AGT	C-M-K-V-L-L-L-L-S (695-703)	Primers used in first-round RACE PCR
P.fGR23'F	CTT TCG GTC TGG GCT GGA GGT CTT ACC	F-G-L-G-W-R-S-Y (252-259)	
P.fMR3'F	AAC CCT TTG TTG AGC CTA ATT TC	N-P-L-L-S-L-I (556-562)	
P.fGR2 3'F nested	GCA TGA GAT GGT TGG AGG AC	H-E-M-V-G-G (372-377)	Primers used in second-round semi-nested PCR
P.fMR 3'F nested	GGC GGC GTT CGA GGA GAT GAG AGT	A-A-F-E-E-M-R (895-901)	
Universal primer mix (UPM; Clontech)	CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT AND CTA ATA CGA CTC ACT ATA GGG C	Reverse primer for 3'RACE; sequence used in PCR of GR1	
RACE(+GSP)	GCA GGT ACC GGA TCC TCG AGA AG	Reverse primer for 3' RACE; used in first and second-round PCR of GR2 & MR	

6.2.3.3 5' RACE PCR

For each of the three corticosteroid receptors 5' RACE was undertaken using the SMART™ RACE cDNA amplification kit (Clontech). A gene specific reverse primer was designed to be used in PCR reactions with the Universal primer mix (UPM) contained in the SMART™ RACE cDNA amplification kit (Table 6-5).

Table 6-5. Gene-specific primers used in conjunction with the Universal primer mix to amplify the 5' cDNA ends of corticosteroid receptors.

Primer Name	Primer Sequence (5'→3')	Amino acid sequence (5'→3') & annealing position
P.fGR1 5'R	TTG-ATG-TGC-TGG-GGG-AAA-AGG-TTG	N-L-F-P-Q-H-I (142-148)
P.fGR2 5'R	GCT-GTC-GTA-GCC-GGC-AAA-GAT-GGT-GT	T-I-F-A-G-Y-D-S (189-196)
P.fMR 5'R	AGT-GGC-GTT-GGA-CTG-CTG-AAA-ACC-A	G-F-Q-Q-S-N-A-T (161-168)

RACE PCR reactions were prepared in 20 µl volumes, each containing 2.5 µl 5' RACE-ready cDNA, prepared as described in 6.2.1.1, 1× UPM (Clontech), 200 nM gene-specific primer, 1× Advantage II PCR buffer (Clontech), 200 µM dNTP mix and 1× Advantage II polymerase mix (Clontech). PCR reactions were cycled using a touchdown PCR cycle, starting with a combined annealing and extension temperature of 72°C, followed by cycles using successively lower annealing temperatures. Cycling parameters were as follows – 5 cycles at 94°C for 30 seconds and 72° for 3 minutes, 5 cycles at 94°C for 30 seconds, 70°C for 30 seconds, 72°C for 3 minutes and 25 cycles of 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 3 minutes.

Products amplified by 3' and 5' RACE were visualised by gel electrophoresis at 75 V for 30 minutes on a 1 × TAE, 1 % agarose, 0.5 µg/ml EtBr gel alongside a 1kB DNA ladder (Biogene). In order to view any amplified products the gel was exposed to UV light allowing the visualisation of EtBr stained DNA. Single bands of interest were obtained for each corticosteroid receptor and therefore DNA within the remaining reaction volumes was purified, cloned and sequenced to determine their identity.

6.2.3.4 Ligation of RACE amplicons into cloning vector

DNA from 5' and 3' RACE reactions were each purified from agarose gel and subsequently ligated into the pGEM®-T easy cloning vector. Purifications were performed using either the NucleoSpin extract II PCR clean up gel extraction kit (Machery Nagel) or the Illustra GFX PCR DNA and Gel band Purification kit (GE Healthcare). Both kits are based on the principle of binding DNA to a silica

membrane in the presence of chaotropic salts. The membrane is then washed with an ethanol-based wash solution to remove salts and other contaminants and DNA is eluted in a low- or salt- free solution in a small volume. In each case, following purification, the concentration of eluted DNA was checked spectrophotometrically using a Nanodrop 1000 spectrophotometer and a 3:1 molar ratio of PCR product to vector was used in the cloning reactions. The amount of DNA required for ligation was calculated as follows:

$$\text{ng insert DNA} = \left[\frac{\text{mass of vector (50ng)} \times \text{size of inster (kb)}}{\text{size of pGEM®Teasy (3.0kb)}}\right] \times \text{Insert:Vector molar ratio}$$

To the vector and insert DNA, Rapid ligation buffer (Promega) and T4 DNA ligase (Promega) were added to a final concentration of 1× and 3 weiss units, respectively. Cloning reactions were left overnight at 4°C to produce the maximum number of transformants before being transformed into chemocompetent DH5 *E.coli*, which were then spread on LB agar plates selective for ampicillin resistance and blue/white screening. White bacterial colonies were selected from each plate and grown further in LB media before purification of plasmid DNA using the GenElute™ Plasmid Miniprep kit (Sigma). Transformation, growth of bacterial colonies and plasmid purifications were performed as described previously in 6.2.2.3. As described in 6.2.2.3 samples of each plasmid preparation were digested with EcoRI enzyme and those plasmids containing ligated RACE amplicons were sequenced.

6.2.3.5 Sequencing of plasmids containing putative RACE PCR fragments

Sequencing and post-reaction cleanup of plasmid DNA was done as described in 3.2.1.5. Primers used for sequencing are detailed in Table 6-6 with the sequencing strategy, including annealing positions of primers, detailed in Figure 6-1.

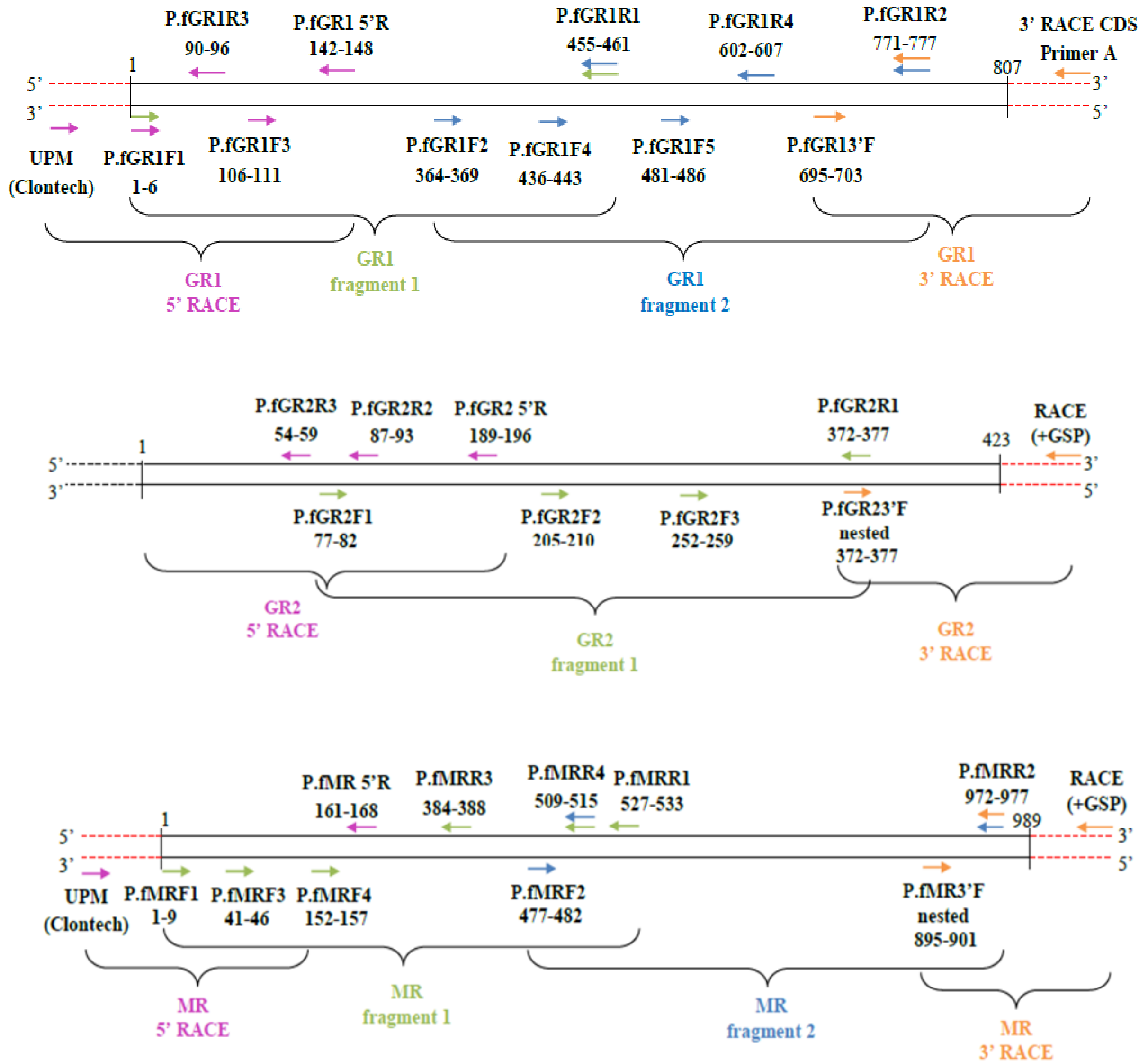

Figure 6-1. Strategy used to sequence PCR fragments of corticosteroid receptors from European flounder. Primers are indicated by arrows, colour co-ordinated to identify the fragment for which they were designed to sequence. Numbers associated with primers indicate the amino acid positions to which they were designed to anneal and correspond to amino acids for GR1 (top), GR2 (middle) and MR (bottom) sequences in GenBank database.

Table 6-6. Primers used to sequence PCR fragments and their nucleotide sequences. The positions to which they are designed to anneal are given in Figure 6-1.

Sequencing primer name	Primer sequence (5’ → 3’)
Primers designed to RACE anchor	
RACE(+GSP)	GCA GGT ACC GGA TCC TCG AGA AG
Primers designed to cloning plasmids	
M13R	CAG GAA ACA GCT ATG AC
M13F	GTA AAA CGA CGG CCA G
T-easy F	TAA AAC GAC GCC CAG TGA AT
T-easy R	CTC AAG CTA TGC ATC CAA CG
Primers designed to PfGR1	
PfGR1F1	ATG-GAT-CAG-GGT-GGA-CTG-AAG-CG
PfGR1R1	CAG-GTT-ACG-ACA-CCG-TAG-TGG-CAT-C
P.fGR1F2	GTG GGC CTA CAA GAC CAG AA
P.fGR1R2	CAC GCT GAG GGT TTT ATT CAC
P.fGR1F3	GAG GAC AAT ATG CCG TTG CT
P.fGR1R3	CGG TGC TGT CGT TTC TGC AAT CTG
P.fGR1F4	CCA AGC CCA GTG GTC AGA CCC ATA AGA T
P.fGR1R4	GCC CAC TTG ACT GCA GAG AT
P.fGR1F5	AAC A CA GAT GGC CAA CAC AA
P.fGR13’F	TGC ATG AAG GTC CTG TTA CTG CTG AGT
P.fGR15’R	TTG ATG TGC TGG GGG AAA AGG TTG
Primers designed to PfGR2	
P.fGR2F1	CAC TAC GGC GTT CTC ACC TG
P.fGR2R1	GTC CTC CAA CCA TCT CAT GC
P.fGR2F2	CCT CAT GAC CAC CCT GAA CA
P.fGR2R2	CAC TGC TCT CTT GAA GAA GAC TTT
P.fGR2F3	CCT CAT GTC TTT CGG TCT GG
P.fGR2R3	TCT TTG CCT GCG CTA AGG AT
P.fGR25’R	GCT GTC GTA GCC GGC AAA GAT GGT GT
P.fGR23’F nested	GCA TGA GAT GGT TGG AGG AC

Table 6-6. continued…

Sequencing primer name	Primer sequence (5’ → 3’)
Primers designed to PfMR	
P.fMRF1	ATG-GAG-AC(CT)-AAA-AGA-TAC-C(AC)A-AGT-T(AGT)C
P.fMRR1	A(C/T)G A(A/G)T GTC C(A/G)C C(G/T)G AAT TAA C(G/A/T)C
P.fMRF2	TGG TAT CTT AGG ACC CCC TGT
P.fMRR2	GAC TCC ACC TTT GGT ATC TGG
P.fMRF3	GTG AT(A/G) T(G/C)C T(G/C)A TGG ATA T(A/T/C)G
P.fMRR3	TTG AAC ACC TCC CCG TCA G
P.fMRF4	CAC CAA GAG CCC CAA GAT AA
P.fMRR4	TTC TCT TGG TA(A/G) TAG CTG CC(A/G) TC
P.fMR3’F nested	GGC GGC GTT CGA GGA GAT GAG AGT
P.fMR5’R	CTG CAG GAC AGG GAT GAG ACC AGC AT

6.2.4 *Confirmation of corticosteroid receptor fragments by long PCR*

Individual DNA sequences from all PCR and RACE reactions were assembled into contigs and using the Lasergene SEQman software (DNASTAR). Original trace files and raw nucleotide sequences were compared and where necessary confident changes within the raw nucleotide sequence were made to match the trace file. The corrected raw nucleotide sequence was translated in all three 5’ to 3’ reading frames to its corresponding amino acid sequence using the ExPASy DNA to protein ‘Translate’ tool (Gasteiger *et al*, 2003). Per receptor, the reading frame with the longest predicted amino acid sequence uninterrupted by stop codons was selected and using the corresponding nucleotide sequence, primers were designed to amplify a single fragment covering the whole open-reading frame.

6.2.4.1 Amplification of GR1

For confirmation of the GR1 sequence a forward (5'- GTG GAC GCT GCG TAA TTT CT -3') and reverse (5'-TTG TGC TCT TGG AGG AGG AT -3') primer was made, designed to the 5' and 3' untranslated regions, respectively. These primers were then used in a 25 µl PCR reaction which included 5 µl cDNA from gills, 0.2 µM forward and reverse primer each, 200 µM of each dNTP, 1 × polymerase buffer and 1.25 units PrimeSTAR HS DNA polymerase (Takara). PCR reactions were given an initial incubation step for 30 seconds at 98°C, before being cycled 20 times at 98°C for 10seconds (denaturation), 55°C for 15 seconds (annealing) and 72°C for 3 minutes. A final extension at 72°C for 5 minutes completed the PCR. The product resulting from this first PCR (PCR-1) was diluted 10-fold and used as a template in a subsequent PCR reaction (PCR-2). PCR-2 was a nested PCR, using a second set of primers designed to amplify a region within the expected product of the PCR-1. The forward primer (5'- GAT GGT ACC ATG GAT CAG GGT GGA CTG AAG -3') was designed to the first 21 nucleotides of the GR1 open-reading frame with the KpnI restriction enzyme recognition site incorporated (underlined). The reverse primer (5'- GAT GGA TCC TCA TTT CTG ATG AAA CAG CAG AGG -3') was designed to the 5' end of the GR1 open-reading frame with the BamHI restriction enzyme recognition site incorporated (underlined). PCR-2 included 5 µl template (1/10 dilution of first PCR product), 0.2 µM forward and reverse primer each, 200 µM of each dNTP, 1 × polymerase buffer and 1.25 units PrimeSTAR HS DNA polymerase (Takara). Cycling conditions were as described for PCR-1 except denaturation, annealing and extension steps were cycled 35 times and the extension step reduced by 30 seconds to 2.5 minutes.

6.2.4.2 Amplification of GR2 and MR

The GR2 and MR sequences were confirmed using a single PCR reaction each. The component of each PCR reaction were as described for the PCR-1 of GR1, except primers were specific to the corticosteroid receptor being amplified. For GR2 the forward and reverse primer sequences were 5’- TGT ATC TCC CAG TGA CGA CCAT -3’ and 5’- AGA CCA CGT GGA TGT TAG CC -3’, and designed to the most 5’ end of the incomplete open-reading frame and the 3’ untranslated region, respectively. The forward and reverse primer sequences for the MR were 5’- GAT GGT ACC ATG GAG ACC AAA AGA TAC CAA AGT TTC -3’ and 5’- GAT GGA TCC TCA CTT CTT GTG GAA GTA GAT TGT G -3’, respectively. Similar to the GR1 primers the forward and reverse primer designed to the MR was designed to the 5’ and 3’ end of the open-reading frame and incorporated the KpnI and BamHI restriction enzyme recognition sites, respectively. Cycling conditions to amplify GR1 and GR2 were as for PCR-1 of GR1 except denaturation, annealing and extension steps were cycled 35 times.

6.2.4.3 Ligation and purification of plasmids containing inserts

The amplified products of each PCR were visualised by electrophoresis on a 1× TAE, 1% agarose, 0.5 μg/ml EtBr gel and subsequent exposure to UV light. The remaining reaction volume was purified and subcloned into pGEM® T-easy (Promega) as described in 6.2.3.4. Ligated plasmids were transformed into chemocompetent DH5 plasmids, grown, and purified as described in 6.2.2.3. As described in 6.2.2.3 samples of each plasmid preparation were digested with EcoRI enzyme and of those plasmids containing the putative corticosteroid receptors, at least three were sequenced per receptor. Following confirmation of the corticosteroid receptor sequences, they were

submitted to the Genbank database and assigned accession numbers JF951960 (GR1), JF951961 (GR2) and JF951959 (MR).

6.2.5 *Phylogenetic analysis*

Amino acid sequences of corticosteroid receptors from European flounder and other vertebrates were aligned by ClustalW. Due to the GR2 of European flounder being incomplete at the 5’ end, all sequences selected for phylogenetic analysis were trimmed at their 5’ ends so as to align with the first available amino acid of European flounder GR2. Thus phylogenetic analysis was done on receptors spanning a partial section of A/B domain and the whole DNA- and ligand-binding domains. GRs and MR of European flounder were compared to corticosteroid receptors of other mammalian (chicken, mouse and human), amphibian (frog) and fish (Burton’s mouthbrooder, zebrafish, Japanese medaka) vertebrates.

Phylogenetic analyses, using Mega4 (Tamura *et al*, 2007), utilised the neighbour-joining method (Saitou and Nei, 1987) and the reliability of the nodes of the tree tested using the bootstap test with 5000 replicates (Felsenstein, 1985).

6.2.6 *Real-time quantitative PCR analysis*

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) was used to examine the tissue distributions of mRNA of the three corticosteroid receptors isolated from European flounder.

Five European flounder, obtained from the Forth estuary and adapted in an aquarium to seawater for six months, were killed and samples of eight tissues were collected from each individual. Tissues were homogenised in Tri-reagent (Roche) and stored at -70°C

until proceeding to RNA extraction. Tissues were sampled from the brain, gill, testes (all five individuals were male), heart, small intestine, kidney, liver and white muscle.

RNA extraction was done as described in 6.2.1 and RNA quantity assessed spectrophotometrically on a NanodropND1000 spectrophotometer. The ratio of absorbances at 260nm and 280nm and 230nm and 260nm was also used to assess RNA purity. In addition 1 µg RNA was heated at 75°C for 5 minutes and run on a 1% agarose, 1× TAE, 0.5 µg/ml EtBr gel to assess RNA integrity. Both the 28S and 18S rRNA bands were visible at an approximate intensity ratio of 2:1 (28S:18S), indicating good RNA quality, and therefore reverse transcription reactions were proceeded with.

6.2.6.1 Reverse Transcription

Reverse transcription reactions were done using either the ImPromII™ Reverse transcription system (Promega) with a blend of oligo(dT) and random primers or the High Capacity cDNA reverse transcription kit (Applied Biosystems) using random primers only.

6.2.6.2 High Capacity cDNA Reverse Transcription kit

Reverse transcription using the High Capacity cDNA reverse transcription kit was done in 20 µl reaction volumes. A mastermix containing reverse transcription buffer, dNTPs, RT random hexameric primers and Multiscribe™ Reverse transcriptase, all supplied with the kit, was prepared to give enough reagents for all reverse transcription reactions. Aliquots of this mastermix were then added to 0.2 ml PCR tubes containing 1 µg total RNA each, such that per reaction 1× Reverse transcription buffer, 4mM dNTP mix, 1× Random primers and 50 units Multiscribe™ Reverse transcriptase was present. Reactions were gently mixed and centrifuged briefly before being placed in a thermal

cycler (BiometraT gradient) and cycled at 25°C for 10 minutes, 37°C for 2 hours and 85°C for 5 minutes. In addition to reverse transcription reactions, a control reaction in which a sample of RNAs were pooled in a total of 1 µg and incubated with all above reagents except for the enzyme was included. This control reaction is to serve as a control in qPCR, testing for the potential interference from genomic DNA contamination.

Once reverse transcription was completed each reaction was diluted 10-fold, to 200 µl, with tricine EDTA and stored at -20°C until further use.

6.2.6.3 ImPromII™ Reverse transcription system

Reverse transcription with the ImPromII Reverse transcription system was done in 20 µl reaction volumes. cDNA was prepared using 1µg of total RNA, denatured at 70°C for 5 min and placed on ice. A volume of reverse transcription mastermix was then added to total RNA to a volume of 20 µl containing 300 ng of random hexamers, 125 ng of anchored oligo-dT, 0.5 mM dNTPs, 1× ImProm-II™ Reaction Buffer and 1µL of ImProm-II™ Reverse Transcriptase (Promega). The synthesis reaction was carried out at 42 °C for 1 hour and then stopped by heating at 75°C for 10 min. Finally, cDNA reactions were diluted to 200 µL total volume (dilution 1:10) with MilliQ water and stored at -20°C until required for QPCR.

6.2.6.4 Design of qPCR primers

Primers for qPCR were designed, taking into account amplicon length and target sequence. In general, amplification efficiencies are increased with shorter amplicons and therefore primers were designed to give products less than 200 base pairs in length.

From an alignment of corticosteroid receptors from European flounder a greater amino acid identity was observed between the DNA-binding C-domains and the ligand-binding E-domains of the glucocorticoid receptors and mineralocorticoid receptor. Therefore to minimise the likelihood of primers amplifying non-target corticosteroid receptor sequences, the forward primer of a pair was designed on the A/B domain which shows little sequence conservation. In addition primers were either designed over exon boundaries or the forward and reverse primers of a primer pair designed on two separate exons. Exon boundaries were identified from an alignment of European flounder CRs with those of other fish species (zebrafish; fugu, Japanese pufferfish and green spotted pufferfish), whose sequences, showing exon boundaries, are available from the Ensembl database (http://www.ensembl.org). Design of primers over exon boundaries, with the 5' end of a primer on one exon and the 3' end of the same primer on the adjacent exon, allows amplification of products from cDNA template reverse transcribed from messenger RNA but avoids interference from any contaminating genomic DNA. Messenger RNA is processed to remove introns in a process called RNA splicing, leaving behind only exons, however genomic DNA contains both introns and exons, the introns of which are located between different exons. Therefore primers designed over exon boundaries are complimentary to cDNA reverse transcribed from mRNA but not to genomic DNA. Where it was not possible to design primers over exon boundaries, they were designed on two separate exons, with the forward primer on the exon encoding the A/B domain and the reverse primer on the adjacent exon which encodes the 3' end of the A/B domain and part of the DNA-binding domain. This allowed products amplified from cDNA to be differentiated from those amplified from contaminating genomic DNA since the latter product would be greater in length due to the amplification of intron sequences, absent in product from cDNA template.

Reference genes are usually included in experimental plans for quantitative RT PCR to provide a loading control, and differences in the efficiency of reverse transcription. A requirement of the housekeeping gene is that its expression levels remain unchanged between treatments, i.e. tissue type in the experiments described here. As a housekeeping gene that is expressed equally amongst all tissues, demonstrated by gel electrophoresis, 18S ribosomal RNA (18S rRNA) was used (Figure 6-2).

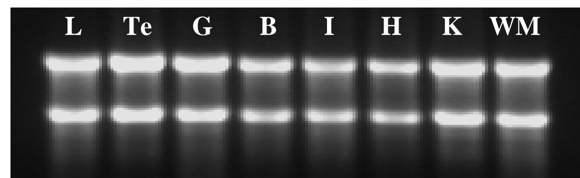

Figure 6-2. The expression of 18s rRNA expressed across tissues used in qPCR. 1 µg of RNA from each tissue was electrophoresed on an agarose gel as described in 6.2.6

Primers for European flounder 18S rRNA have previously been designed to amplify a product of 100 base pairs (Williams *et al*, 2006) and these primer sequences were used in the present study. All primers used in qPCR are described below in Table 6-7.

Table 6-7. Primers used in RT-qPCR. Primers were designed to amplify a small fragment of GR1, GR2 and MR from European flounder. A primer pair was also used to amplify a fragment of 18S rRNA, the expression of which was to be used as a normalising gene.

Primer Name	Sequence	Amino acid sequence/Annealing position	Expected product (no. base pairs)
P.f18S rRNAF	GCC TGA ATA CCG CAG CTA GG		100
P.f18S rRNAR	TAG CGG CAC AAT ACG AAT GC		
P.fGR1 R1qPCR	CAG GCA GAT CTT ATG GGT CTG A	Q-T-H-K-I-C-L (440-446)	102
P.fGR1 F1qPCR	GCC AAC TTC TGC AGC TCA TCT	A-N-F-C-S-S-S (414-420)	
P.fGR2 F1qPCR	TGT ATC TCC CAG TGA CGA CCA T	Y-L-P-V-T-T (1-6)	135
P.fGR2 R1qPCR	CGA GGT AGA ACT TGC AAA GCT G	S-F-A-S-S-T-S (39-45)	
P.fMR F1qPCR	CCA ACC CTT TGT TGA GCC TAA T	N-P-L-L-S-L (556-561)	150
P.fMR R1qPCR	GGA GGA CGG CTG CCT CAG	L-R-Q-P-S-S (600-605)	

6.2.6.5 Real time quantitative PCR (RT-qPCR) reactions

RT-qPCR reactions were done in 20 µl volumes using, per reaction, 2 µl cDNA, reverse transcribed from total RNA as described in 6.2.6.1, 70 nM forward and reverse primer each and 1× ABsolute QPCR SYBR green mix (Thermo). Each plate included several negative control reactions comprising no template controls in which cDNA was replaced by water, and no reverse transcriptase controls in which the template was a pool of RNA run through the reverse transcription transcription protocol but omitting the enzyme as described in 6.2.6.2. The former control was included to detect potential unspecific products amplified due to primer dimer formation while the latter was used to detect potential genomic DNA contamination. A cDNA reference pool dilution series was also included on each plate in order to calculate the efficiency of the PCR reaction. Dilutions were done so as to give cDNA dilutions of 1 in 3, 1 in 9, 1 in 27, 1 in 81 and 1 in 243 in water. The undiluted cDNA reference pool was aliquoted in small volumes and stored at -20°C and dilutions freshly prepared on the day of use. All cDNA samples, controls and pooled-dilutions were present in triplicate on each plate. Plates were sealed using Clear Seal lids (KBiosciences) and briefly centrifuged to collect reactions in the bottom of plate wells. Reactions were then cycled in a mastercycler ep realplex (Eppendorf) at 95°C for 15 minutes in order to activate the polymerase enzyme followed by 45 cycles of denaturation at 95°C for 15 seconds, primer annealing at 57°C for 30 seconds and extension at 72°C for 30 seconds to amplify products of 99, 137, 152 and 100 base pairs for GR1, GR2, MR and 18S rRNA, respectively. Fluorescence was detected during the 72°C extension step during each of the 45 cycles and crossing points calculated by the software of the qPCR cycler.

At the end of the cycling programme the temperature was increased from 65°C to 95°C in 0.25°C increments every 10 seconds in order to produce a dissociation curve, used to identify the products of any non-specific amplification. The point at which double stranded DNA products denatured and separated into single strands was visualised by a rapid decrease in fluorescence. The presence of multiple products can be detected at this point since products of different lengths and base composition will have different melt temperatures. In the case of primers for amplification of GR1, GR2, MR or 18S rRNA only a single product was detected each time, indicating the primers were specific for the target sequence. In addition six randomly selected samples per primer pair were run on a 1× TAE, 1% Agarose, 0.5 µg/ml EtBr gel at the end of cycling and amplified products visualised by exposure of the gel to UV light. Gel electrophoresis of qPCR product revealed the presence of only one DNA band around 100-200 base pairs depending on primers used.

6.2.6.6 Calculation of Relative Expression Ratios

Relative expression ratios (R) of each corticosteroid receptor was calculated using the method of (Pfaffl, 2001) whereby 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. The equation used to derive R was as follows:

$$R = (E_{target})^{CPtarget(control - sample)} / (E_{ref})^{CPref(control - sample)}$$

The relative expression of each corticosteroid receptor target was calculated for each tissue from each of the five fish using, as the control, the reference cDNA diluted 1 in 9.

For each receptor, expression was compared across tissues using one way ANOVA, followed by post-hoc comparisons using Tukey's test.

6.3 Results

6.3.1 Isolation of Corticosteroid receptors from P.flesus

Using both reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) PCR the full open-reading frame of GR1 and MR was isolated from the European flounder (*Platichthys flesus*). A partial sequence for the GR2 was isolated, extending from the 3' end of the N-terminal A/B domain through to the stop-codon of the ORF and into the 3' untranslated region. Each of these sequences showed high sequence similarity to the corresponding receptors from other fish species.

6.3.1.1 PCR and RACE reaction products

Both GR1 and MR were isolated using two primer pairs each, which generated two overlapping partial cDNA fragments per receptor. A single cDNA fragment was isolated using a single primer pair specific to the GR2. The 5' and 3' extremities of the open-reading frames of GR1 and MR were isolated using 5' and 3' RACE PCR, respectively. While 5' RACE of GR2 produced an incomplete fragment which did not extend all the way to the start codon, 3' RACE allowed the full 3' extremity of this receptor to be isolated. Assembly of the various fragments, together with longer PCRs to confirm fragment assembly and sequencing, resulted in the full ORF of GR1 and MR, and a partial ORF for GR2. The strategy used to isolate cDNAs for each receptor is shown in Figure 6-3.

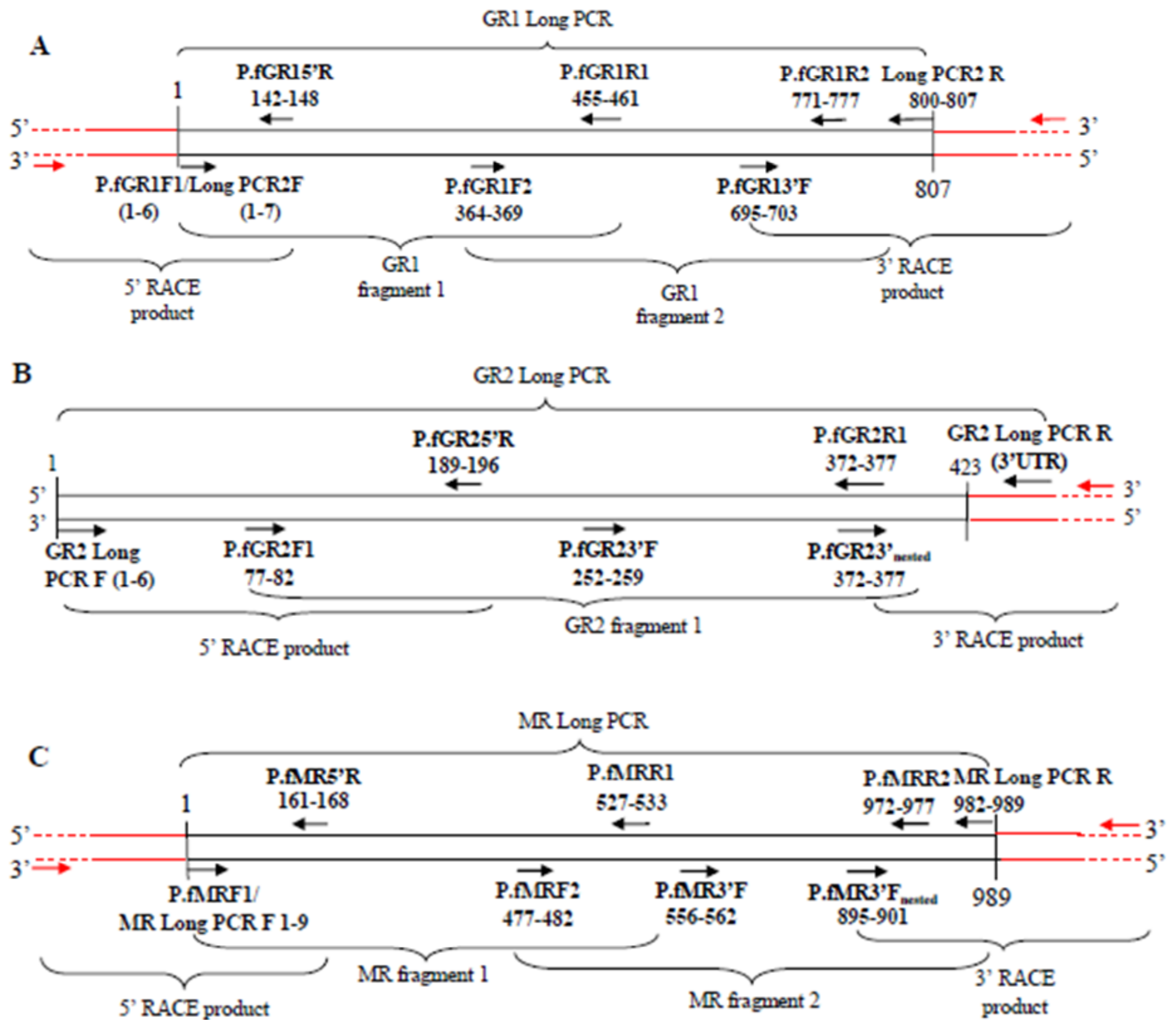

Figure 6-3. The annealing positions of primers used to amplify the GR1 (A), GR2 (B) and MR (C) from European flounder (*Platichthys flesus*). Sense and anti-sence DNA strands are represented by black lines while red lines represent the 3' and 5' untranslated regions. Primers are represented by arrows with arrow heads indicating the direction in which amplification proceeded, with numbers under primer names indicating the amino acid positions to which the primer is expected to anneal, with positions complementary to those detailed in GenBank database for GR1, MR and GR2.

6.3.1.2 Glucocorticoid Receptor 1

For GR1, an open reading frame of 807 amino acids was obtained. This amino acid sequence, when subjected to a blast search revealed a high sequence identity to the GR1 of other fish species. An 89% sequence identity was found with the glucocorticoid receptor 1 of Japanese flounder (*Paralichthys olivaceus*; Accession no. BAA25997), while an identity of 80% was found with the glucocorticoid receptor of European sea

bass (*Dicentrarchus labrax*; Accession no. AAS48459) and 76% with glucocorticoid receptors from both the Burton's mouthbrooder (*Haplochromis burtoni*; Accession no. AAM27889) and European perch (*Perca fluviatilis*; Accession no. ACF75335).

6.3.1.3 Glucocorticoid Receptor 2

The GR2 sequence was obtained by assembling three fragments generated by a single PCR reaction and 3' and 5' RACE. The product of 5' RACE failed to extend to the initiation codon of the receptor but did extend past the sequence obtained with PCR to allow further deduction of the GR2 sequence.

The assembled contig gave a fragment, uninterrupted by stop codons, 423 amino acids in length and corresponding to a partial A/B domain, the full DNA-binding domain and the full ligand-binding domain up to the stop codon at the 3' end of the receptor. A protein blast search of this sequence revealed it to have a high amino acid identity to the glucocorticoid receptor 2 of the Japanese medaka (*Oryzias latipes*; Accession no. NP_001156605), Gilthead sea bream (*Sparus aurata*; Accession no. ABF30967) and Burton's mouthbrooder (*Haplochromis burtoni*; Accession no. AAM27887), with amino acid identities of 87% each.

6.3.1.4 Mineralocorticoid Receptor

Assembly of the individually sequenced partial DNA fragments for the MR of European flounder resulted in the complete open-reading frame being deduced. The MR sequence was 989 amino acids long and showed high amino acid identity with the mineralocorticoid receptor of other fish species (90%, 86%, 70% and 69% with the MR of Burton's mouthbrooder (*Haplochromis burtoni*; Accession no. AAM27890) Japanese medaka (*Oryzias latipes*; Accession no. NP_001156601) zebrafish (*Danio rerio*;

Accession no. ABS00395) and common carp (*Cyprinus carpio*; Accession no. CAH03995), respectively).

6.3.1.5 Structure and Sequence similarity between the corticosteroid receptors of European flounder

A highly conserved domain structure, comprised of an N-terminal A/B domain, a central DNA-binding C domain, a hinge region (D-domain) and a C-terminal ligand-binding domain E domain, has been described for nuclear hormone receptors, including the receptors for corticosteroid hormones. Each of the corticosteroid receptors isolated from European flounder shared this domain architecture with domain regions being defined according to Greenwood *et al* (2003) and Bury *et al* (2003), who has previously reported the domain architecture for the GR1, GR2 and MR of the cichlid species *Haplochromis burtoni* and the GR2 of rainbow trout (*Onchorynchus mykiss*), respectively. Sequence identity between each domain of the three receptors was compared (Figure 6-5) using the needle algorithm (European Bioinformatics Institute; http://www.ebi.ac.uk), revealing the A/B domains of GR1 and MR to have the least conservation with an amino acid identity of 14.6%. Since the A/B domain of the GR2 receptor was incomplete the A/B domain of this receptor was excluded from comparisons. The highest amino acid identity was seen within the DNA-binding domains, with identities between the receptors of greater than 90%. Within GR1 an additional 27 nucleotides (9 amino acids) were present between the two zinc fingers of the DNA-binding domain which were absent in the glucocorticoid 2 and mineralocorticoid receptors (highlighted in Figure 6-4).

Within the ligand-binding E-domains the two glucocorticoid receptors, GR1 and GR2, showed high amino acid identity (89.3%), however less conservation was seen between the E-domain of the mineralocorticoid receptor and those of the GR1 and GR2, with identities of 57.1% and 56.7%, respectively.

Figure 6-4. Alignment of deduced amino acid sequences of glucocorticoid 1, glucocorticoid 2 and mineralocorticoid receptors isolated from European flounder (*Platichthys flesus*). DNA-binding and ligand-binding domains are shown. Due to the low sequence conservation within the A/B domain, this domain is omitted. The 9 amino acid insert found between the two zinc fingers of the the DNA-binding domain of GR1 is shown boxed in red.

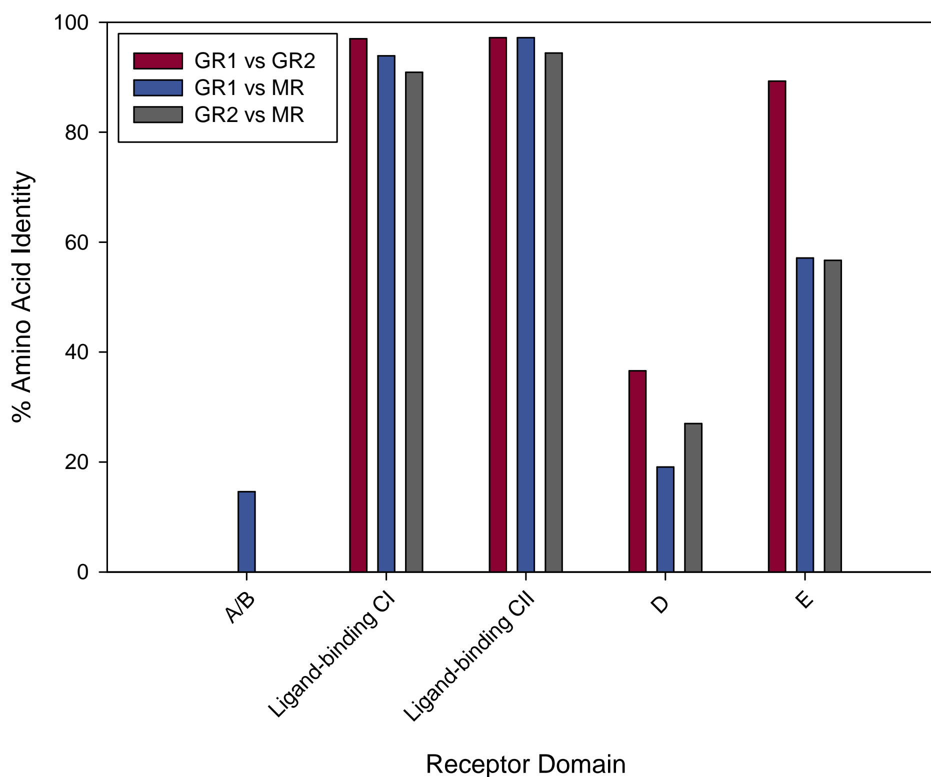

Figure 6-5. Percent amino acid identity between European flounder corticosteroid receptors as a function of receptor domains. Receptor domains, defined following Ducouret et al. (1995), were compared between receptors using the needle algorithm as implemented on the website of the European Bioinformatics Institute (http://www.ebi.ac.uk/). The full sequence of the A/B domain of GR2 was not available, precluding comparisons.

6.3.2 *Phylogenetic Analysis*

To infer evolutionary relationships of GR1, GR2 and MR from European flounder to those of other vertebrate species a bootstrap-consensus phylogenetic tree was constructed using the neighbour-joining method and 5000 bootstrap replicates (Figure 6-6).

Within several fish species two glucocorticoid receptors have been identified. When analysed phylogenetically it was found that these receptors formed two distinct clusters, with one glucocorticoid receptor per species in each cluster. The glucocorticoid receptors of European flounder were grouped with those of other fish species with high

bootstrap support. Additionally the fish mineralocorticoid receptors grouped separately from the glucocorticoid receptors from the same species and also grouped in a separate cluster from tetrapod mineralocorticoid receptors (Figure 6-6).

6.3.1 Tissue distribution of Flounder corticosteroids

All three corticosteroid receptors showed a wide distribution throughout the tissues of European flounder (Figure 6-7). There was a tendency for all three receptors to be expressed at higher levels within the testes although the mRNA expression levels of GR1 and GR2 within this tissue were not significantly different to any other tissue. The high variability in GR2 in the testes of European flounder was due to one single fish having very large relative levels of GR2 in this tissue compared to other individuals. The reason for this is unknown and no difference in the stage of maturity in this individual compared to the others was observed during tissue sampling. In contrast the mRNA expression of MR within the testes was significantly higher to that in the liver, kidney and white muscle in which low mRNA levels of this receptor was expressed. Similarly an increased expression of MR was also observed in the gills and brain, with the expression levels significantly higher than in the liver, kidney and white muscle.

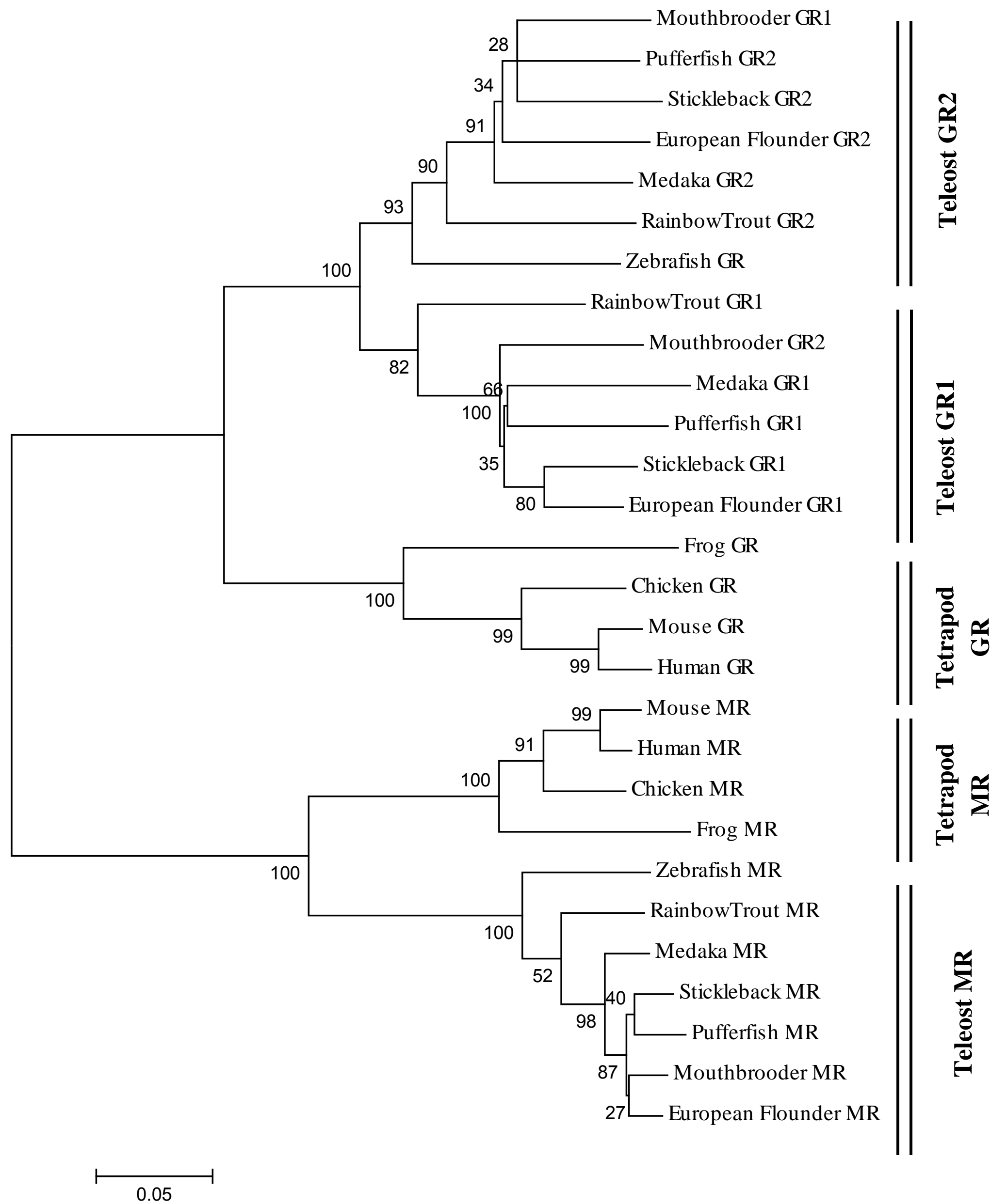

Figure 6-6. Phylogenetic tree.. A comparison between the glucocorticoid and mineralocorticoid receptors of vertebrates. The tree was generated using MEGA version 4 software using the neighbour-joining method. Bootstrapping, using 5000 replicates, assessed the reliability of the tree with figures representing the bootstrap value in percentage. Receptor sequences used were mouseMR (NP_001077375), HumanMR (NP_000892), ChickenMR (ACO37437), FrogMR (NP_001084074), ZebrafishMR (NP_001093873), Rainbow troutMR (NP_001117955), MedakaMR (NP_001156601), SticklebackMR (ENSGACP00000022714), PufferfishMR (ENSTRUP00000037995), MouthbrooderMR (AAM27890) and European flounderMR (not submitted to database). GR sequences used were Mouthbrooder GR1 (AAM27887), Pufferfish GR2 (ENSTRUP00018413), Stickleback GR2 (ENSGACP00000024074), European flounder GR2 (not submitted to database), MedakaGR2 (NP_001156605), Rainbow trout GR2 (NP_001117954), Zebrafish GR (NP_001018547), Rainbow trout GR1 (NP_001118202), Mouthbrooder GR2 (AAM27889), Medaka GR1 (ENSORLP00000001939), Pufferfish GR1 (ENSTRUP00015645), Stickleback GR1 (ENSGACP00000027400), European flounder GR1 (not submitted to database), Frog GR (NP_001081531), Chicken GR (NP_001032915), Mouse GR (NP_032199), Human GR (CAJ65924).

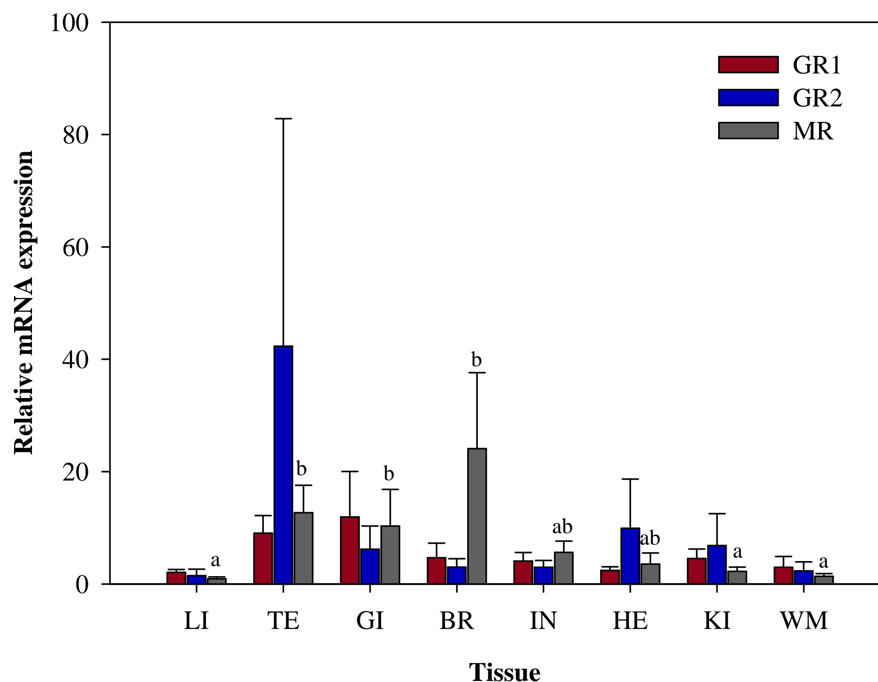

Figure 6-7. mRNA expression of GR1, GR2 and MR in body tissues of male European flounder (*Platichthys flesus*). Quantitative real-time PCR was performed on tissues from sea water adapted flounder. LI, liver; TE, testes; GI, gills; BR, brain; IN, intestine; HE, heart; KI, kidney; WM, white muscle. Expression of corticosteroid receptors was normalised to 18S RNA levels and is expressed as relative to a reference sample. Data are the average and SEM of results from five fish. Expression levels of GR1 and GR2 did not vary significantly between tissues. For MR expression levels are significantly different (P<0.05) when columns are not labelled by the same letter.

6.4 Discussion

Corticosteroid receptors have previously been isolated from several fish species including rainbow trout (*Onchorynchus mykiss*) (Ducouret *et al*, 1995; Bury *et al*, 2003), Burton's mouthbrooder (*Haplochromis burtoni*) (Greenwood *et al*, 2003), zebrafish (*Danio rerio*) (Schaaf *et al*, 2008; Pippal *et al*, 2011) and the common carp (Stolte *et al*, 2008; Stolte *et al*, 2008). With the exception of zebrafish in which only one glucocorticoid receptor and one mineralocorticoid receptor has been isolated (Alsop and Vijayan, 2008), each of these species contains three functional corticosteroid receptors, comprising a single mineralocorticoid receptor and two glucocorticoid receptors. The isolation of three corticosteroid receptors from the European flounder (*Platichthys flesus*) is described here for the first time. Sequence analysis reveals two of these

receptors to be glucocorticoid receptors while the third receptor was most similar to a mineralocorticoid receptor.

6.4.1 Corticosteroid receptor structure

The domain structure of corticosteroid receptors from humans, rodents and fish species has been described previously (Hollenberg *et al*, 1985; Danielsen *et al*, 1987; Carlstedt-Duke *et al*, 1987; Rogerson *et al*, 2003; Rogerson *et al*, 2004; Pippal *et al*, 2011). Within the present study the complete open-reading frames of GR1 and MR, and a partial sequence for GR2 (missing the first 2/3rds of the N-terminus), from European flounder have been isolated and analysis of their sequences reveals them to share the common modular structure previously described. The GR1, GR2 and MR of European flounder each have an N-terminal A/B domain (partially isolated in the case of GR2), a DNA-binding C-domain and a C-terminal ligand-binding domain.

6.4.1.1 A/B domain

Previously, and in contrast to the DNA-binding and ligand-binding domains, the A/B domains of corticosteroid receptors from several fish species have been shown to be low in sequence conservation (25%) (Greenwood *et al*, 2003; Sturm *et al*, 2010). This limited sequence homology however has been shown not to impact on the functionality of the A/B domains since those of rainbow trout GR1 and GR2 can be swapped with little effect on receptor transactivation (Becker *et al*, 2008; Sturm *et al*, 2011), suggesting a functional equivalency.

6.4.1.2 DNA-binding domain

The DNA-binding C domain of corticosteroid receptors consists of two zinc fingers, the molecular shape of which are determined by the co-ordinate binding of four cysteine residues to a divalent zinc ion (Freedman *et al*, 1988; Luisi *et al*, 1991; Low *et al*, 2002). Similar to the DNA-binding domains of other fish species, such as rainbow trout and *Haplochromis burtoni* in which amino acid identities greater than 90% have been described (Greenwood *et al*, 2003; Bury *et al*, 2003), the C-domain of European flounder GR1, GR2 and MR was also found to be highly conserved. The highly conserved sequence and resulting tertiary structure is likely to be optimal for specific DNA-binding to the hormone response element (HRE) within the promoter region of target genes, a prerequisite for the regulation of gene expression by steroid hormone receptors.

Despite a large degree of sequence conservation in the C-domain, the number of amino acid residues in the interfinger region (the region between zinc finger 1 and 2) has been found to differ between species. An extra one amino acid was found within the GR of a primate species (Brandon *et al*, 1991), while an additional 4 amino acids have been found in the interfinger region of human and rat MR (Bloem *et al*, 1995). Within several teleost species, in which duplicate GRs have been found, the DNA-binding domains of GR1 have been found to contain a nine amino acid insert between the two zinc fingers, which is absent in GR2. These additional nine amino acids appear to be highly conserved in the GR1 of fish species, having been described in rainbow trout (Ducouret *et al*, 1995; Takeo *et al*, 1996), Japanese flounder (Tokuda *et al*, 1999), Burtons mouthbrooder (Greenwood *et al*, 2003), brown trout (AY863149), European sea bass (AY549305) and fugu (*Takifugu rubripes*). Through amplification and sequencing of rainbow trout glucocorticoid receptor genomic DNA it has been found that the presence

of this insert is due to an additional exon, 27 nucleotides (9 amino acids) in length, located within the intron between exons three and four which code for the two zinc fingers (Tujague *et al,* 1998; Lethimonier *et al*, 2002). The impact of these additional amino acids on DNA-binding and receptor dimerization, two functions associated with the zinc finger region, of GR1 has been previously suggested. Modelling of the rat GR with that of the common carp (*Cyprinus carpio*), the latter of which contains the 9 amino acid insert, reveals the nine amino acids results in an extention of the loop after the interfinger -helix, leading to a loop which protrudes outside of the protein and which was shown not to interfere with receptor DNA-binding and receptor transactivation (Stolte *et al*, 2008). A similar model was predicted using the GR1 of rainbow trout (Lethimonier *et al*, 2002; Wickert and Selbig, 2002). Although DNA-binding and receptor transactivation appears not to be affected by the additional exon between the two zinc fingers, a suggestion that the association of the GR1 with coactivator corepressor proteins and may be affected has been suggested (Stolte *et al*, 2008).

6.4.1.3 Ligand-binding domain

The ligand-binding domain, as the name suggests, is responsible for binding of ligand molecules which results, in the case of the holo (agonist bound) receptor, in a conformational change in the ligand-binding domain structure, leading to the activation of the receptor and to an alteration in the transcription of target genes. A role for the LBD in receptor dimerization has also been shown. Similar to the DNA-binding domain, and in agreement with the situation in other fish species (Greenwood *et al*, 2003; Bury *et al*, 2003), the ligand-binding domain of European flounder GR1, GR2 and MR showed a large degree of amino acid sequence conservation.

Despite the high sequence similarity, mineralocorticoid receptors and glucocorticoid receptors of European flounder show a difference in their response to the mineralocorticoid hormone aldosterone; while the mineralocorticoid receptor is activated by both glucocorticoids and mineralocorticoids, the glucocorticoid receptor is inactive in the presence of aldosterone, suggesting a difference in the ligand-binding region of the corticosteroid receptors which confers ligand-specificity. Previously several amino acids within the ligand binding domains of the GR and MR have been suggested to be important in steroid selectivity (Baker *et al*, 2007). An example of such an amino acid is the highly conserved serine residue within the mineralocorticoid receptor, corresponding to the serine residue at amino acid position 949 in human MR and found within the loop between -helix 11 and -helix 12, responsible for positioning the AF-2 domain for binding of coactivator and corepressor proteins. This amino acid has been found to be lacking in almost all glucocorticoid receptors, with the exception of the skate (*Raja erinacea*) GR (Baker *et al*, 2007). An alignment of the corticosteroid receptors of European flounder with the human mineralocorticoid receptor reveals the serine residue to be conserved in the mineralocorticoid receptors but lacking in the glucocorticoid receptors (Figure 6-8). Thus we would expect both GR1 and GR2 of European flounder to be transcriptionally inactive in the presence of aldosterone. The skate GR is transcriptionally active in the presence of both aldosterone and cortisol, in which the serine residue is present, suggesting this residue is important for aldosterone specificity since glucocorticoid receptors lacking this serine residue, or those in which the serine residue is deleted, are unresponsive to aldosterone (Bridgham *et al*, 2006). Additionally, mutants within the loop region can decrease or eliminate transcriptional activity of the mineralocorticoid receptor, even when the receptor retains

high affinity for the steroid, indicating this region to be important in defining transcriptional activity (Hellal-Levy *et al*, 2000; Hultman *et al*, 2005).

```
                                                                    ↓
HumanMR         NNSGQSWQRFYQLTKLLDSMHDLVSDLLEFCFYTFRESHALKVEFPAMLVEIISDQLPKV
EfMR            NNSGQTWQRFFQLTKLLDAMHDLVGNLLDFCFYTFRESRALKVEFPEMLVEIISDQIPKV
EfGR1           ENASQNWQRFYQLTKLLDSMQEMVEGLLQICFYTFVN-KTLSVEFPEMLAEIITNQIPKF
EfGR2           ENSSQNWQRFYQLTKLLDSMHEMVGGLLNFCFYTFVN-KSLSVEFPEMLAEIISNQLPKF
                :*:.*.****:*******:*:::* .**::***** : ::*.**** **.***::*:**.
                           H10/11                                  H12

HumanMR         ESGNAKPLYFHRK                                     AF-2
EfMR            ESGLTHTIYFHKK
EfGR1           KDGSVKPLLFHQK
EfGR2           KAGSVKPLLFHQR
                . *  . . **..
```

Figure 6-8. Alignment of the ligand-binding domain of human MR with European flounder corticosteroid receptors. ClustalW was used to align the sequences. The AF-2 domain is underlined while the position of the conserved serine residue in the mineralocorticoid receptor, which is absent in the glucocorticoid receptors, is indicated by an arrow.

Further regions in the ligand-binding domain of the MR have been implicated in the specificity of this receptor for aldosterone. Using chimeric structures of MR and GR ligand-binding domains from human receptors in a cell-based reporter assay system, amino acids 804-874 of the human MR were identified as being crucial for aldosterone binding specificity (Rogerson *et al*, 1999). This region was further narrowed down to amino acids 820-844, where twelve of the sixteen amino acids that differ between the GR and MR were critical in conferring responsiveness to aldosterone (Rogerson *et al*, 2007). Of these 25 amino acids described by Rogerson *et al* (2007), 19 are conserved between the human MR and European flounder MR, of which eight are also conserved in GRs and eight differ between MRs and GRs (Figure 6-9). These residues lie on the surface of the MR molecule and thus are not part of the ligand-binding domain pocket and quite how they are able to influence the specificity of steroid binding in such a way is unknown. Nevertheless it has been found that in other receptors, such as the androgen and the glucocorticoid receptor, the region corresponding to amino acids 820-844 of the

MR, is critical for binding of natural and synthetic ligands, providing further evidence that this region may influence ligand specificity in the GR and MR (Vivat *et al*, 1997; Robin-Jagerschmidt *et al*, 2000).

Human MR	820	KHT**N**SQFLY**FAPDL**VF**N**EEK**M**HQSA	844
P.flesus MR		KHT**N**GQMLY**FAPDL**IF**N**EDR**M**QQSA	
P.flesus GR1		EQC**N**GNMLC**FAPDL**VI**N**QER**M**KLPF	
P.flesus GR2		QQC**N**GNMLC**FAPDL**VI**N**EER**M**KLPY	
		* * * * * ***	

Figure 6-9. A comparison of the sequence of amino acids 820-844 of the human MR which has been implicated in aldosterone hormone selectivity. Amino acids which are identical within the MR and GRs are shown in bold while those that are conserved in the MR but differ in GRs are indicated by an asterisk.

In addition to residues which confer ligand-selectivity, residues of the ligand-binding domain which make contact with the ligand molecule have been described using structural analysis of the glucocorticoid and mineralocorticoid receptors (Figure 6-9).

Maximal activation of the MR by aldosterone has been shown to involve an intricate network of hydrogen-bonds mediated by ligand-binding (Bledsoe et al, 2005). Helix 3 of the ligand-binding domain in the human receptor contains several residues, including aspargine at position 770 (Asn770) and a serine residue at position 767 (Ser767), which have been shown to form hydrogen bonds with aldosterone or with other residues of the MR, thus stabilising and activating the receptor (Bledsoe et al, 2005; Hellal-Levy et al, 2000). In addition to residues in helix 3 of the MR, both aldosterone and deoxycorticosterone (DOC) have been found to be contacted by helix 10 of the MR via hydrophobic interactions with the threonine residue at position 945 (Thr945) (Bledsoe et al, 2005). It has been hypothesised that ligands which promote a hydrogen bonding network and interact with helix 10 of the MR via Thr945 induce stabilisation of helix 3

and movement of the AF-2, allowing cofactor recruitment and ultimately activation of target gene transcription. Since all of the residues described above are conserved in European flounder MR, it is possible that the same mechanism of receptor activation occurs for both mammalian and teleost species.

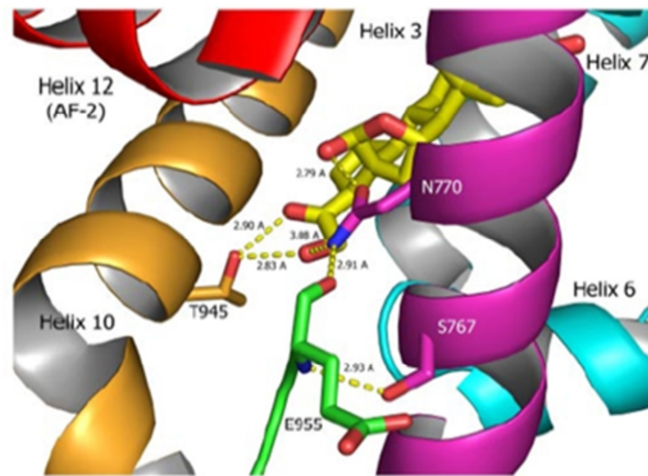

Figure 6-10. The contact points of aldosterone with the ligand-binding domain of MR and the intramolecular associations. Residues Asn770 and Ser767 of helix 3 (purple) form H-bonds (yellow dashed lines) with the Glu955 within the loop preceding the AF-2 (red). Asn770 also forms H-bonds with C18-OH and C21-OH. Thr945 on helix 10 (orange) hydrogen bonds to C20 carbonyl and C21hydroxyl of aldosterone (yellow). Image taken from Bledsoe et al (2005).

Similar to the MR, a network of hydrophilic and hydrophobic interactions between ligand molecules and the GRs have been described. Twenty four amino acids within the ligand-binding domain of the human GR have been described by to interact with dexamethasone (Bledsoe *et al*, 2002), likely explaining the high affinity binding of dexamethasone by the GR. Of these residues only four, corresponding to methionine, alanine, tyrosine and isoleucine at positions 601, 605, 747 and 735 of the hGR, respectively, differ in GR1 and GR2 of European flounder. A substitution of residues from phenylalanine to tyrosine (as in Tyr747 to Phe of GR2) has previously been shown to have no effect on the rat GR and its affinity for dexamethasone (Ray *et al*, 1999). Additionally, each of the substitutions described above for the European flounder GRs

have been previously described for rainbow trout GRs (Bury *et al*, 2003) and a comparison of the substitution positions and residues reveals them to be identical, suggesting these residues which differ between human and European flounder GRs are conserved across teleost species.

Several residues in the ligand-binding domain have been demonstrated, through mutation analysis, to be critical in GR functioning. Glutamine at position 642 of the human GR (Gln642) has been proposed to interact with the 17 -OH group of steroids such as cortisol, 9 -fluorocortisol, prednisolone and dexamethasone. This suggestion was made following the discovery that the affinity of the GR for these steroids decreased after mutation of Gln642 to alanine, a decrease not observed with steroid molecules lacking the 17 -OH group (Lind *et al*, 2000). The elucidation of the crystal structure of human GR bound to dexamethasone strengthened this suggestion when Gln642 was indeed found to bind to the 17 -OH group of the dexamethasone molecule (Bledsoe *et al*, 2002).

A second residue shown to have an important function in GR ligand binding and transactivation is the asparagine at position 564 (Asn564) of the human GR (Bledsoe *et al*, 2002; Hammer *et al*, 2003), which forms two hydrogen bonds with the C11-OH group and the C21-OH group of dexamethasone ligand. Mutation of this residue results in a loss of binding affinity for dexamethasone as well as a pronounced loss of transactivation function, suggesting this Asn564 to be important not only for steroid binding but also for transactivation. The destruction of GR function by mutations in this residue has been suggested to be due the influence of the residue on interactions of the GR with coactivator proteins since helix 3, to which Asn564 belongs, together with

helices 4, 5 and 12, have been shown to form a hydrophobic groove important for coactivator binding in the related estrogen receptor (Brzozowski *et al*, 1997).

A high degree of amino acid conservation is seen in the ligand-binding domains of human and European flounder corticosteroid receptors. Residues which have previously been identified to have a function in ligand-binding and receptor transactivation are especially conserved, suggesting a common mechanism in which these receptors interact with their ligands, ultimately leading to receptor transactivation.

6.4.2 Multiple Corticosteroid Receptors within European flounder

Steroid hormone receptors within jawed vertebrates evolved, through several duplication and divergence events, from a single ancient steroid receptor most closely related to that of the estrogen receptor (ER) (Eick and Thornton, 2011). The estrogen receptor is thought to have undergone an initial duplication and divergence event to give rise to the progesterone receptor (PR) which was itself duplicated, and underwent divergence, to give rise to the corticosteroid receptors (CRs). Following the divergence of the jawed (gnathostomes) and jawless vertebrates, the ER, PR and CR were duplicated in a whole-genome duplication event within the gnathostome lineage and diverged, giving rise to the six steroid receptors currently found in jawed vertebrates (ER , ER , GR, MR, PR and AR) (Thornton, 2001).

Here, the isolation of a single mineralocorticoid receptor and two glucocorticoid receptors from European flounder is described. Within fishes the presence of two separate genes encoding glucocorticoid receptors appears to be unique to, and widespread amongst, actinopyterygii species, having been isolated from rainbow trout

(Bury *et al*, 2003), Burton's mouthbrooder (Greenwood *et al*, 2003), green spotted puffer fugu (Stolte *et al*, 2006), common carp (Stolte *et al*, 2008) and sea bass (Terova *et al*, 2005; Vizzini *et al*, 2007). The presence of these two GRs, which are encoded by separate genes, have been hypothesised to have arisen by a second whole-genome duplication event, following the divergence of the teleostomi into the actinopterygii and sarcopterygii lineages, the former of which European flounder belong (Jaillon *et al*, 2004). Therefore, due to the duplication of the actinopterygii genome, the presence of two glucocorticoid receptors within European flounder is not surprising. Along with corticosteroid receptors, other steroid hormone receptors have been identified to occur in multiple forms in fish species; two androgen receptors have been discovered in the cichlid fish species *Haplochromis burtoni* (Vagell *et al*, 2001; Harbott *et al*, 2007) while three estrogen receptors have been identified in the Atlantic croaker (*Micropogonias undulatus*) (Hawkins *et al*, 2000; Hawkins and Thomas, 2004).

Phylogenetic analysis of GR1 and GR2 from European flounder, together with those of other fish species and several tetrapod species, showed that the glucocorticoid receptors of flounder cluster with those of other teleost species and separately from those of other vertebrates. Additionally two separate sub-clusters formed from fish glucocorticoid receptors, with each sub-cluster containing one glucocorticoid isoform from each species. A 989 amino acid sequence was isolated from European flounder, with high amino acid identity with mineralocorticoid receptors of other vertebrate species. The isolation of mineralocorticoid receptors from fish species has been surprising since fish are thought to lack aldosterone, the typical mineralocorticoid hormone present within other vertebrate species (McCormick *et al*, 2008). Since the discovery of a mineralocorticoid receptor it has been found that cortisol, typically considered to be a

glucocorticoid receptor agonist, can act as an activator for both the glucocorticoid and mineralocorticoid receptors in fish species (Greenwood *et al*, 2003; Stolte *et al*, 2008).

6.4.2.1 Expression of corticosteroid receptors in European flounder

The tissue expression profiles of the two glucocorticoid receptors and the mineralocorticoid receptor was determined in nine tissues from European flounder. The expression of GR1 and GR2 was found to be widely expressed in European flounder, although there was a tendency for GR1 to be more highly expressed in the gills and the gonads while GR2 had a tendency for an increased expression in the gonads, although these higher expressions were not significant. Mineralocorticoid receptor expression was highest in the brain of European flounder, with an elevated expression also being observed in the testes and the gills. Studies in rainbow trout and carp have previously described tissue expression patterns similar to those observed for European flounder; the two GRs from rainbow trout and carp were expressed ubiquitously across a wide range of tissues while the MR of rainbow trout was found to be expressed at significantly higher levels in the brain than in other tissues examined (Bury *et al*, 2003; Sturm *et al*, 2005; Stolte *et al*, 2008). The lean towards a more pronounced expression of MR in the brain may reflect a neuroendocrine function for this receptor while a more pronounced expression of all three corticosteroid receptors in the testes of European flounder may demonstrate a function in male reproduction. Indeed Milla *et al* (2008) has previously demonstrated that MR expression in the testicular tissue and the vas deferens increases in mature testis during the initiation of sperm production in rainbow trout and may be involved in male reproduction by altering the ionic concentration of seminal fluid during the release of sperm. The gills of European flounder displayed a tendency for higher expression of GR1 compared to other tissues examined, which may

indicate a role for the corticosteroids and their receptors in osmoregulation, which has been demonstrated previously in other fish species (Dang *et al*, 2000; Laiz-Carrion *et al*, 2002; Dean *et al*, 2003; Laiz-Carrion *et al*, 2003; Takahashi *et al*, 2006).

6.5 Conclusions

Several fish species from which corticosteroid receptors have been isolated have been shown to differ from other vertebrates in terms of the number of functional corticosteroid receptors they contain. While a single glucocorticoid receptor and a mineralocorticoid receptor has been identified in humans and other mammals, fish species of the actinopterygii lineage have two glucocorticoid receptors and a single mineralocorticoid receptor. The two glucocorticoid receptors have been shown to be products of separate genes as opposed to products of alternative splicing of the same gene. In the present study three sequences from the European flounder which show high sequence identity with the two glucocorticoid receptors and mineralocorticoid receptor of other teleost species has been isolated. This is the first report of three corticosteroid receptors from European flounder. Phylogenetic analysis of deduced European flounder corticosteroid receptor sequences with the same receptors of other fish and tetrapod species reveals the European flounder receptors were more similar to those of other teleost species than to mammalian, avian or amphibian species, which is to be expected.

Expression of the three corticosteroid receptors found a tendency for the receptors to be expressed more highly in the gills and the testes compared to other fish species. Additionally the mineralocorticoid receptor was highly expressed in the brain, as described previously for other fish species. The expression patterns of corticosteroid receptors in European flounder agree with previous fish species and indicate common functions to those described for other species, namely osmoregulation, reproduction and neuroendocrine functions.

The functionality of the corticosteroid receptors of European flounder is, as yet, uncharacterised and therefore it cannot be said with certainty that the three receptors described here are functional. However an analysis of the ligand-binding domain of the glucocorticoid and mineralocorticoid receptors of European flounder and the orthologous human corticosteroid receptors reveal that the residues which contact the ligands and those with a function in positioning of the ligand-dependent activation function within the LBD for receptor activation are highly conserved. Thus the European flounder receptors are expected to bind to ligands. Having isolated the full ligand-binding domains from each of the receptors, the next step will be to characterise their response to model activators and repressors of corticosteroid receptors in the Gal4-NHR assay described in Chapter 3. Additionally since these receptors are speculated to have a large variety of functions within fish species, the response of these receptors to a persistent environmental contaminant will shed some light on the potential for endocrine disruption in European flounder via the glucocorticoid and mineralocorticoid receptors.

Chapter 7. Corticosteroid Receptors & their interactions with environmental pollutants

7.1 Introduction

Several lines of evidence exist to suggest that environmental pollutants may impair the estrogen- and androgen-signalling pathways in fish species. For example, atrazine, a widely used herbicide has been shown to suppress levels of plasma androgens in male goldfish (*Carassius auratus*) while elevating levels of 17 -estradiol (Spanò *et al*, 2004), likely due to its aromatase-inducing effects (Sanderson *et al*, 2001). Moreover, several environmental pollutants such as perfluoro alkyl acids which are widely used industrial and commercial chemicals, have been shown to directly interact with the estrogen receptor to disrupt signalling (Benninghoff *et al*, 2011). However while a relatively large number of studies have assessed endocrine disruption via the estrogen and androgen receptors, relatively little is known about endocrine disruption of other endocrine axes, such as the corticosteroid receptors.

In Chapter 6 the isolation of two glucocorticoid receptors and a mineralocorticoid receptor from European flounder (*Platichthys flesus*) was described. These receptors mediate the effects of the corticosteroid hormones, produced by the adrenal cortex of mammals (Nanki *et al*, 1967) and the homologous interrenal cells of the kidney in teleosts (Bern, 1967). Tetrapods possess two types of corticosteroids, glucocorticoids and mineralocorticoids. In mammals, the main glucocorticoid is cortisol (corticosterone in rodents), which interacts with both glucocorticoid and mineralocorticoid receptors, while the main mineralocorticoid is aldosterone, which specifically interacts with MR with no agonistic effects on the GR at physiological concentrations. In contrast, in teleosts the main corticosteroid is cortisol. Whether or not aldosterone is present in teleosts has long been a debated subject, with the current consensus being that fish lack

the aldosterone hormone and the enzyme required for its synthesis (Jiang *et al*, 1998; Nelson, 2003). Despite the absence of aldosterone, a homologue of the mineralocorticoid receptor has been isolated from several teleost species (Colombe *et al*, 2000; Greenwood *et al*, 2003; Stolte *et al*, 2008; Pippal *et al*, 2011). In both mammals and teleost species the glucocorticoid receptor is selectively activated by cortisol, while the mineralocorticoid receptor is less selective, showing transactivation in the presence of different gluco- and mineralocorticoids (Sturm *et al*, 2005).

Apart from their lack of aldosterone, teleosts further differ from mammals in terms of the number of glucocorticoid receptors they possess. While mammals have only a single glucocorticoid receptor, two glucocorticoid receptors encoded by distinct genes have been identified in several teleost species, for example in rainbow trout (Bury *et al*, 2003), Burton's mouthbrooder (Greenwood *et al*, 2003), Green puffer, Japanese pufferfish and common carp (Stolte *et al*, 2006; Stolte *et al*, 2008).

Fish corticosteroid receptors are involved in a wide range of physiological functions and thus the disruption of corticosteroid signalling might be expected to have a negative impact on the fitness of individuals, with the potential to disrupt their normal physiology. Evidence to-date indicates a disruption of corticosteroid signalling in fish by pollutant compounds, resulting in an inhibited stress response (Cericato *et al*, 2008; Hontela *et al,* 1992). A lack of cortisol elevation has been found to correlate with the impaired response of interrenal tissue to ACTH (Brodeur *et al*, 1997). This in turn may be a result of impaired interrenal steroidogenesis, since a diminished cortisol response to ACTH has been found in salicylate-exposed trout, congruent with a decrease in the expression of key proteins involved in corticosteroidogenesis (Gravel and Vijayan, 2006). Furthermore corticosteroid receptors are implicated in osmoregulatory functions

in fish species and therefore it might be expected that fish inhabiting polluted environments and which come into contact with pollutants with an inhibitory effect on corticosteroid receptors, will display a limited capacity to acclimate to different osmotic conditions. This will likely affect euryhaline species, such as salmonids and European flounder, which move between osmotic gradients in order to reproduce.

Several compounds have been suggested to bind directly to corticosteroid receptors. Methylsulfonyl PCBs were shown to antagonise both human and mouse GR by direct interactions with the receptor, to which it has been shown to have an affinity (Johansson *et al*, 1998). Additionally these compounds have been shown to affect dexamethasone-induced tyrosine aminotransferase activity in a rat hepatoma cell line (Johansson *et al*, 2005). Gumy *et al* (2008) demonstrated the ability of an organotin compound to bind directly to the human glucocorticoid receptor and antagonise its transcriptional activity while human pharmaceuticals have been implicated in corticosteroid-mediated endocrine disruption in fish, with ten of the most prescribed corticosteroids in the UK each able to transactivate the glucocorticoid receptors of trout (Kugathas and Sumpter, 2011).

All the aforementioned studies demonstrate the potential for environmental pollutants to disrupt corticosteroid receptor signalling in human and other vertebrate species. In the present study a reporter gene assay based on recombinant fusion proteins combining the ligand-binding domain of flounder corticosteroid receptors with the DNA binding domain of the yeast transcription factor Gal4 has been developed to allow screening of aquatic pollutants for their potential to interact with the ligand-binding domain of corticosteroid receptors from European flounder. The reporter gene assay was first

validated by elucidating its response to classical corticosteroid receptors agonists and antagonists, before selected chemical pollutant were tested.

7.2 Materials and Methods

7.2.1 Constructs expressing the corticosteroid ligand-binding domains

During the cloning of corticosteroid receptors from European flounder (*Platichthys flesus*), cDNAs comprising the putative full-coding sequences of MR and GR1 were obtained, while a partial cDNA sequence was isolated for GR2. To study the transcriptional activation of these receptors by cognate hormones, and the potential of chemicals to interfere with this receptor function, chimeric constructs were made combining the DNA-binding domain of the yeast transcription factor GAL4 with the ligand-binding domain of the individual flounder corticosteroid receptors. To this end, the commercial plasmid pBIND was used, which contains a sequence encoding GAL4 upstream of a multiple cloning site. Using primers containing suitable restriction sites, cDNAs of receptor ligand-binding domains were generated by PCR and inserted into pBIND using suitable enzymes.

7.2.1.1 Polymerase chain reaction

The polymerase chain reaction was employed to amplify the ligand-binding domains of each corticosteroid receptor before ligation into the pBIND vector. A primer pair was designed for each receptor. The forward primer was positioned at the start of the ligand-binding domain while the reverse primer was positioned at the stop codon of the open-reading frame and the end of the ligand-binding E-domain. Recognition sequences for BamHI and KpnI restriction enzymes were placed at the 5’ ends of the forward and

reverse primers respectively, to allow the directional cloning of ligand-binding domains into the cloning vector while maintaining the reading frame of the plasmid region encoding GAL4. Primer pairs are described in Table 7-1.

Table 7-1. Primer sequences used to amplify the ligand-binding domains of corticosteroid receptors from *Platichthys flesus*. Recognition sequences of BamHI and KpnI are highligted in red

Primer Name	Primer sequence (5' → 3')
$GR1_{LBD}F$	TT GGATCC TG CCC CAG CTC GTG CCC ACC AT
$GR1_{LBD}R$	AA GGTACC AG TCA TTT CTG ATG AAA CAG CAG AGG CTT G
$GR2_{LBD}F$	TT GGATCC GC ATG CCT CAA TTG TCC CCA CAA TG
$GR2_{LBD}R$	AA GGTACC AG TCATCT CTG GTG AAA GAG GAG GGG CTT GAC
$MR_{LBD}F$	TT GGATCC TT CTG CCG CCC TCC ATC T
$MR_{LBD}R$	AA GGTACC AG TCA CTT CTT GTG GAA GTA GA

Polymerase chain reactions were in 20 µl reaction volumes containing, per reaction, 2.5 µl 3' RACE ready cDNA, 0.2 µM of each of the forward and reverse primers, 0.2 mM dNTP, 1× Advantage II PCR buffer (Clontech) and 1× Advantage II polymerase mix (Clontech). To generate the template 3' RACE-ready cDNA 1 µg RNA from kidney, liver and intestine was reverse transcribed using the SMART rapid amplification of cDNA ends kit (Clontech) for amplification of ligand-binding domains of GR1, GR1 and MR, respectively. Reverse transcription of RNA using this kit is described in Chapter 5.

Cycling conditions for each receptor included an initial denaturation of 2 minutes at 95°C, followed by 35 cycles of denaturation (95°C for 30 seconds), primer annealing (65 °C for GR1 and MR and 67°C for GR2 for 30 seconds) and extension (72°C for 2 minutes). A final extension step of 72°C for 5 minutes completed the PCR cycle.

An aliquot of each PCR product was electrophoresed on a 1× TAE, 1% agarose, 0.5µg/mL EtBr gel alongside 1kb DNA ladder, confirming amplification of a product of

expected size. The remainder of the PCR products were purified from enzymatic PCR reactions and ligated into the prepared pBIND cloning vector (Promega). Purification of PCR products and their restriction digest, along with restriction digest of pBIND plasmid was done using ethanol precipitation as described previously in 3.2.1.2.

Following digestion, DNA was precipitated and a 10 µl ligation reaction prepared for each receptor. For GR1 receptor 30ng digested GR1 LBD DNA was ligated into 50 ng digested pBIND vector using 1× T4 DNA ligase buffer (New England Biolabs) and 200 cohesive-end units of T4 DNA ligase (New England Biolabs). The ligand-binding domain of GR2 was ligated into pBIND using 50ng digested pBIND plasmid, 1× T4 DNA ligase buffer (Promega) and 7.5 weiss units of T4 DNA ligase (Promega) in a total of 10 µl. The digested ligand-binding domain of the mineralocorticoid receptor was ligated into the digested pBIND plasmid using 100ng pBIND vector, 35ng putative ligand-binding domain, 1.5 units T4 DNA ligase enzyme (Promega) and 1× T4 DNA ligase buffer (Promega).

All reactions were incubated overnight at 4°C before being transformed into chemocompetent DH5 *E.coli* cells. Transformants were spread on selective agar plates and small scale cultures were grown from single colonies and plasmid DNA isolated. The method used for these procedures was the same as described for transformation of *E.coli* Top10 and isolation of plasmid DNA in 3.2.1.4.

7.2.1.2 Sequencing of pBIND-corticosteroid ligand-binding domain constructs

Plasmids resulting from the above ligation reactions were characterised by restriction mapping. A 2 µl sample of each plasmid preparation was incubated with 5 units BamHI, 5 units KpnI, 1× Universal restriction buffer and water to a volume of 10 µl at 37°C for 1 hour. Digested plasmids were then electrophoresed on a 1× TAE, 1% agarose, 0.5

µg/ml EtBr gel to identify those plasmids in which the digestion released a fragment, corresponding to the size of the ligand-binding domains of GR1, GR2 and MR. At least two plasmid preparations per corticosteroid receptor showed this expected band pattern and were sequenced to confirm the identity and correct insertion of the insert. Sequencing was carried out as previously described (see 3.2.1.5) using primers detailed in Table 7-2

Table 7-2. Primers used for the sequencing of the Gal4-corticosteroid ligand-binding domain plasmids.

Primer Name	Primer sequence (5' → 3')	Amino acid sequence
pBINDseqF	CTT CAG TGG AGA CTG ATA TGC	n/a
pBINDseqR	CAC TGC ATT CTA GTT GTG GT	n/a
P.fGR1seqF	GCT CCT GGC TCT TTC TCA TG	S-W-L-F-L-M
P.fGR1R2	CAC GCT GAG GGT TTT ATT CAC	V-N-K-T-L-S-V
pBIND GR1R	AA GGTACC AGT CAT TTC TGA TGA AAC AGC AGA GGC TTG	L-F-H-Q-K-*
pBIND GR1F	TT GGATCC TGC CCC AGC TCG TGC CCA CCA T	P-Q-L-V-P-T
P.fGR1seqR2	GCC CAC TTG ACT GCA GAG AT	I-S-A-V-K-W
pBIND MRF	TT GGATCC TTC TGC CGC CCT CCA TCT	L-P-P-S-I
pBIND MRR	AA GGTACC AGT CAC TTC TTG TGG AAG TAG A	Y-F-H-K-K
P.fMR 3'F	GGC GGC GTT CGA GGA GAT GAG AGT	A-A-F-E-E-M-R
P.fGR2 5'R	GCT GTC GTA GCC GGC AAA GAT GGT GT	T-I-F-A-G-Y-D-S
P.fGR2 GSP1	CCT CAT GAC CAC CCT GAA CA	L-M-T-T-L-N
P.fGR2 F3	GAT GAG ATT CGG ATG GCC TA	D-E-I-R-M-A

7.2.2 Hormone-binding assays

Hormone binding assays with Gal4-corticosteroid receptor ligand-binding domain constructs were performed in COS-7 cells derived from kidney cells of African green monkey, which lack endogenous expression of corticosteroid receptors.

7.2.2.1 Transfection of COS-7 cells

COS-7 cells were maintained in DMEM (Invitrogen), supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 2mM glutamine and 10% denatured fetal calf serum in a humidified atmosphere with 5% CO_2. For transfection media from a flask of almost-confluent COS-7 cells was emptied and the cells washed twice with 1 × DPBS (Gibco, Invitrogen). Trypsin/EDTA was added to cells in order to release them from the flask and subsequently resuspended in DMEM, supplemented with 10% charcoal/dextran treated FBS.

COS-7 cells were counted using a hemocytometer and the method described for FHM cells in paragraph 2.2.1.2, and diluted to a final density of 16×10^4 cells/ml. Cells were then seeded into 24 well plates at a volume of 0.5 ml per well (8×10^4 cells) in DMEM. Plates were incubated overnight at 37 °C, 5% CO_2. The following day media was aspirated from cells and washed with 1 × DPBS and 0.3 ml DMEM with penicillin streptomycin and serum added to final concentrations of 50 µg/ml, 50 units/ml and 10% respectively.

For each plasmid to be transfected, a tranfection mix was prepared. For each 24 well plate, 7.5 µg plasmid DNA was diluted to a volume of 500 µl with DMEM (free of serum and antibiotics) and 50 µl polyfect reagent was added. Polyfect-DNA mixture was incubated for 10 minutes at room temperature to allow the DNA complexes to form then 3ml DMEM (supplemented with antibiotic and serum as above) was added to the tube containing the DNA and the Polyfect, and the resulting transfection solution mixed by gently pipetting. To each well of a 24 well assay plate 142 µl of the transfection solution was added (300 ng DNA per well) and plates incubated for a further 24 hours

at 37 °C, 5% CO_2 before replacing transfection solutions with fully supplemented media.

7.2.2.2 Preparation of tritiated and cold hormone treatments

Stock solutions of tritiated hormone solutions ($[^3H]$-dexamethasone (6,7-3H(N), Perkin Elmer, specific activity 2.072 Bq/fmol) and $[^3H]$-cortisol (1,2,6,7(N), Perkin Elmer, specific activity 2.6788 Bq/fmol)) were diluted to 200-times the final assay concentration in absolute ethanol. Stock concentrations were then added to DMEM media containing no supplements in a ratio of 1 µl tritiated hormone:1 µl ethanol:200 µl DMEM, giving final assay concentrations of 1.56, 3.12, 6.25, 12.5, 25 and 50 nM tritiated dexamethasone and 0.28, 0.85, 2.56, 7.67, 23 and 69 nM tritiated cortisol. To measure non-specific hormone binding a second treatment was prepared in which the tritiated hormone was present with a 500-fold excess of unlabelled hormone. Unlabelled hormone stocks were diluted into ethanol to 200-times the final assay concentration. Tritiated hormone and unlabelled hormone, both at 200-times the final required concentrations, were diluted into a volume of DMEM in a ratio of 1 µl tritiated hormone:1 µl unlabelled hormone:200 µl DMEM giving the same tritiated hormone concentrations as described above, each with a 500-fold excess of unlabelled hormone (0.78, 1.56, 3.12, 6.25, 12.5 and 25 µM dexamethasone and 0.125, 0.425, 1.28, 3.84, 11.5 and 34.5 µM cortisol.

7.2.2.3 Treatment of transfected COS-7 cells

Supplemented media supplied to cells during transfection were removed from the assay plate and cells washed twice with DPBS. Media containing the appropriate concentrations of radiolabelled and unlabelled hormones were then added to cells in a volume of 250 µl. All treatments were performed in duplicate within a given

experimental repeat. Cells were placed in a 37°C incubator for 1 hour after which media was removed and cells washed twice with DPBS. Radiolabelled hormone retained by the cell monolayer was then extracted from cells by adding 130 µl of ethanol absolute to each well, incubating the plate for 5 minutes and removing the ethanol quantitatively into a 6ml scintillation vial (Perkin Elmer). The extraction was repeated and the second extract placed into the same vial as the first extract. 4 ml of scintillation fluid was added to each tube and contents vortex mixed before being subjected to scintillation counting.

7.2.2.4 Calculating specific binding, Kd and Bmax

Following scintillation counting, disintigrations per minute (DPM), calculated automatically by the scintillation counter, were converted to Becquerel (Bq) units by dividing each DPM value by 60 (1 Bq = 1 DPS and therefore 1Bq=60 DPM). Using the specific activity of each hormone (2.6788 Bq/fmol for [^{3}H]-cortisol and 2.072 Bq/fmol for [^{3}H]-dexamethasone), Bq units were then converted to fmol/well by division by the specific activity and further converted to nanomoles (by multiplying by 4 and dividing by 0.004). Specific and non-specific binding, as well as the ratio of bound to free hormone was calculated. Specific binding of the hormone by Gal4-CR-transfected cells was calculated as the difference between total binding, i.e. cells treated with tritiated hormone only, and non-specific binding, i.e. cells exposed to tritiated hormone with a 500-fold excess of unlabelled hormone.

Specific hormone binding in receptor transfected cells followed Michaelis-Menton type saturation kinetics in accordance with the presence of a single binding site; however, the upper asymptote of replicates differed significantly between experiments, probably reflecting the differences in transfection efficiency. This was accounted for by fitting a Michaelis-Menton-type non-linear least squares model of Binding (B) versus substrate

concentration (Uij) for measuring j in experiment i. This model allowed for a different asymptote (Bmax) for each experiment, *i*, but with a single kinetic parameter (Kd). The error term (ij) was assumed to be normally distributed:

$$B_{i,j} = \mathrm{B}_{max,i}\,([U_{ij}]/([U_{ij}] + K_d)) + \;_{ij}$$

Curvilinear regressions according to the above model were calculated using the R modelling environment (http://www.r-project.org/).

7.2.3 Transactivation assay

Transactivation assays were performed in order to characterise the response of glucocorticoid and mineralocorticoid receptors to model agonists cortisol, dexamethasone and aldosterone, respectively. In addition mifepristone (RU486) and spironolactone were assessed for their ability to antagonise the receptors.

Transactivation assays were carried out in transiently transfected fathead minnow cells using the same procedures as described for PPAR transactivation assays in 3.2.2.

7.2.3.1 Seeding and transfection of FHM cells

Twenty four hours prior to transfection FHM cells were seeded into 96 well black assay plates with clear bottoms (Corning) at a density of 2×10^5 cells per well as described in 3.2.2.1.

Transfection of plasmid DNA into cells was carried out using Polyfect transfection reagent (Qiagen). Per well, 60ng DNA was transfected consisting of 10 ng Gal4-corticosteroid ligand-binding domain, 10ng of control reporter plasmid pGL4.75[*hRluc*/CMV] and 40ng experimental reporter plasmid pGL4.31[*luc2P/Gal4*UAS/hygro]. DNA was diluted with DMEM, lacking serum and

antibiotics, such that 0.05 µl DMEM was added per 1ng of DNA. After mixing and brief centrifugation a volume of Polyfect reagent (Qiagen) was added to give a final Polyfect to DNA ratio of 10.5 µl:1 µg and transfection continued as detailed in 3.2.2.2.

Transfection of cells continued for 24 hours before cells were treated with compounds of interest.

7.2.3.2 Treatment of transfected cells

To study the hormone dependency of the transactivation of Gal4-corticosteroid constructs, cells transiently transfected as above were treated with different concentrations of aldosterone, cortisol and dexamethasone. Concentrated ethanolic stocks of each compound were prepared using absolute ethanol and diluted 1 µl per mL into complete DMEM media to give final assay concentrations. Antagonism of Gal4-corticosteroid constructs was assessed by combining receptor agonists dexamethasone (GR1 and GR2) or aldosterone (MR) at a concentration of 10^{-6}M with different concentrations of mifepristone and spironolactone, with appropriate single compound controls. Concentrated stocks of agonists and antagonists were added to DMEM, each at 1 µl per 1000 µl DMEM, to give final stated assay concentrations.

Transfection mixes were removed from wells of the assay plate and cells washed once with 1× PBS (Invitrogen) before adding 75 µl of media containing treatments to each well of the assay plate. Each treatment was performed in triplicate within a given experiment. In addition a control treatment was included in which absolute ethanol was diluted into complete DMEM at a final concentration of 0.1% or 0.2% for agonism and antagonism experiments, respectively. A second control was included on plates in which antagonism was studied, which consisted of dexamethasone or aldosterone only at a concentration of 10^{-6}M, and was used as a positive control against which all other

treatments were compared. On each assay plate non-transfected cells were included to assess for background luciferase.

Cells were incubated with treatments for 24 hours before being assayed for luciferase activity

7.2.3.3 Luciferase Assay & Statistical Analysis

At the end of experiments, media was removed from the plates and cells washed twice with 1× PBS (Invitrogen). Firefly and *Renilla* Luciferase activities were measured using buffers adapted from Dyer *et al* (2000), as described in Chapter 3.

Statistical analysis was carried out using Graphpad InStat version 3.05 to determine statistically significant differences between control treatments and hormone treatments in transactivation experiments. The F-max test was used to confirm the homogeneity of variances and, where necessary, data were log transformed before performing repeated measures ANOVA followed by *post hoc* comparisons with Dunnett's test. Differences were considered statistically significant when the probability value (P) was less than 0.05.

7.3 Results

7.3.1 Construction of Gal4-Corticosteroid LBD constructs

From previous isolation of the three corticosteroid receptors (see 0) polymerase chain reaction with primers specific to the ligand-binding region of GR1, GR2 and MR was expected to produce products of 756 (MR), 762 (GR2) and 759 (GR1), respectively. Additionally, when cloned into the pBIND vector containing the DNA-binding domain of Gal4 protein, the ligand-binding domain of the corticosteroid receptors and the Gal4-

DBD were expected to be in the same reading frame. Sequencing analysis revealed the ligated DNA fragments to be the ligand-binding regions of GR1, GR2 and MR and that all three DNA-binding domains appeared in the same reading frame as the Gal4 DNA-binding domain as expected.

7.3.2 Steroid-binding assays

Ligand-binding affinities of Gal4-GR1, Gal4-GR2 and MR for dexamethasone and cortisol was assessed using whole cell binding assays using COS-7 cells transiently expressing Gal4 constructs. Specific binding of the tritiated hormone to the Gal4 constructs were obtained as the difference between total binding and non-specific binding, as exemplified in Figure 7-1. A comparison of hormone binding in transfected and non-transfected cells revealed total binding in non-transfected cells to be equivelant to the non-specific binding in transfected cells for both dexamethasone and cortisol, confirming the absence of endogenous corticosteroid receptors in the COS-7 cell line (Figure 7-1(A) and (B)).

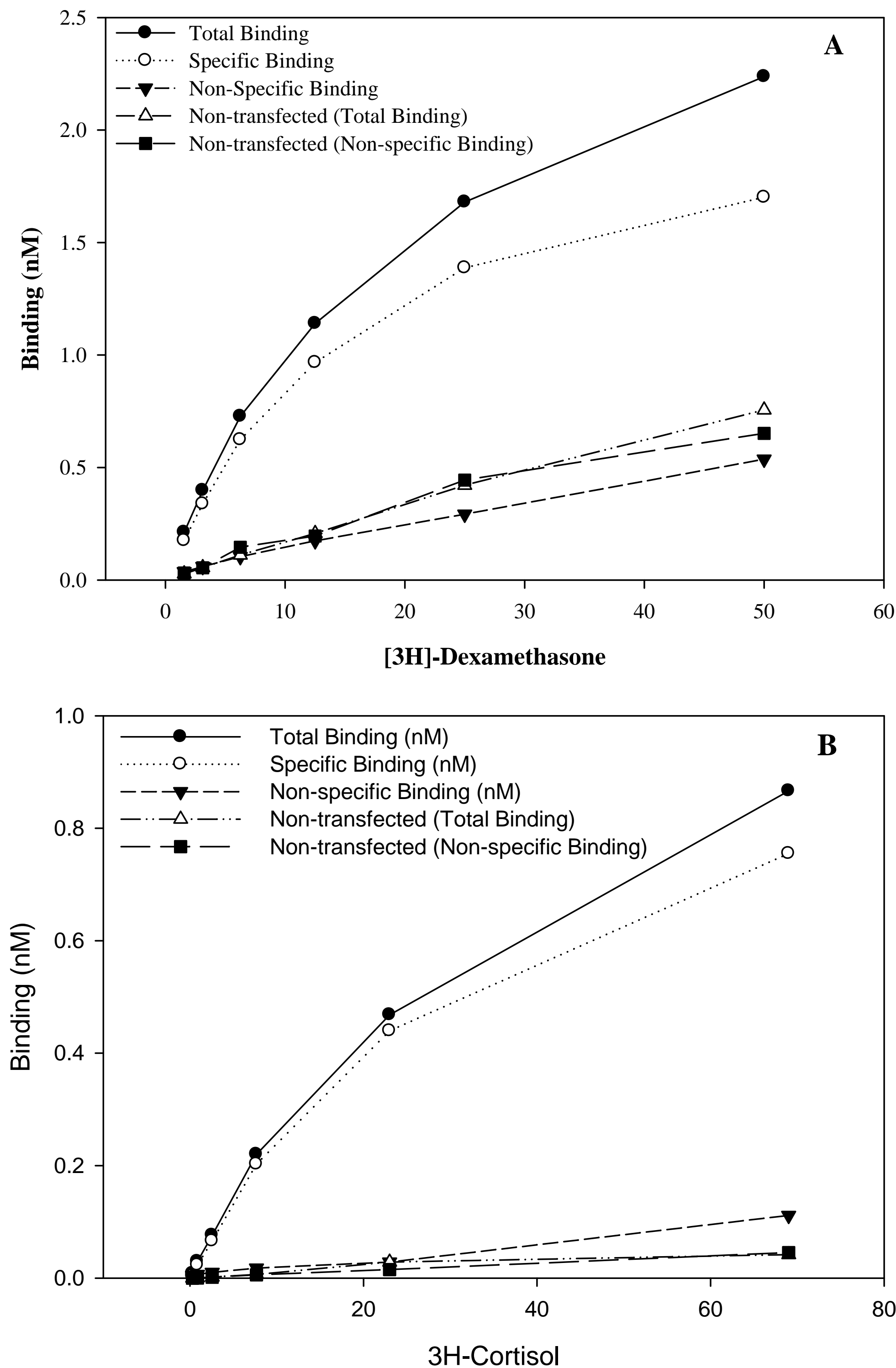

Figure 7-1. Binding of [3H] Dexamethasone (A) and Cortisol (B) to Gal4-GR2 construct and in cells transfected with empty pBIND plasmid only. COS-7 cells transiently expressing Gal4-GR2 or pBIND were treated with 1.56 - 50 nM [3H]-Dexamethasone or 0.28–69nM [3H]-Cortisol either in the presence of (non-specific binding) or the absence of (specific binding) a 500-fold excess of unlabelled hormone. Specific binding was calculated as the total binding minus the non-specific binding.

Binding data followed Michaelis-Menten kinetics in accordance with the presence of a single binding site. Figure 7-2 shows typical binding results, expressed as linearized scatchard plots, from cells transfected with Gal4-GR1, Gal4-GR2 and Gal4-MR in the presence of both [3H]-dexamethasone and [3H]-cortisol.

For all Gal4-flounder CR constructs, specific binding followed saturation kinetics allowing a dissociation constant (Kd) and an estimate of the number of binding sites (Bmax) (Table 7-3). Bmax varied between transfections but did not differ between receptors. Thus, Gal4 construct expression was similar between all receptors.

In terms of hormone affinity, Gal4-GR1 and Gal4-GR2 had a higher affinity for dexamethasone than cortisol, while Gal4-MR had a similar affinity for both hormones.

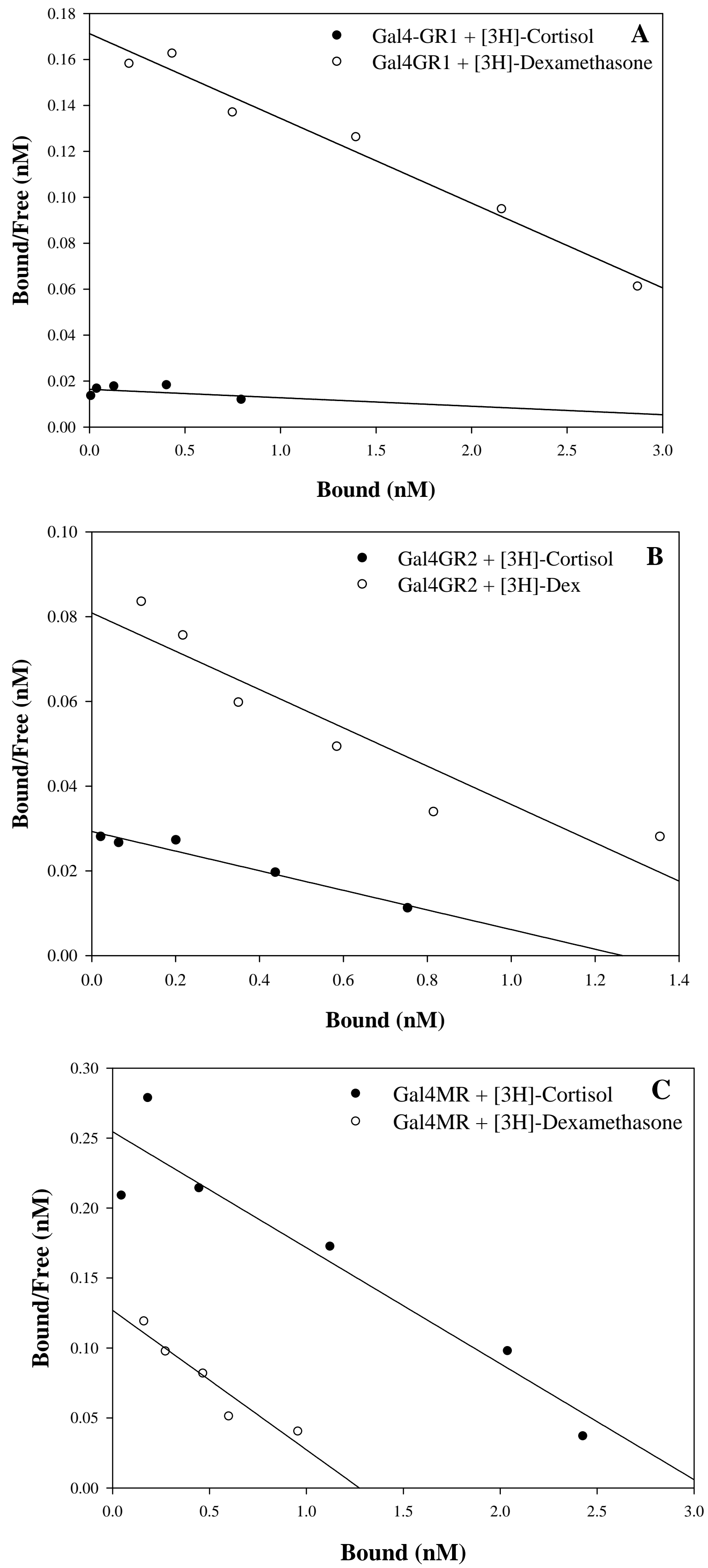

Figure 7-2. Typical results of [3H]-dexamethasone and [3H]-cortisol binding assays for Gal4-GR1 (A), Gal4-GR2 (B) and Gal4-MR (C) represented as scatchard plots.

Table 7-3. Binding affinity (Kd with 95% confidence intervals in brackets) and number of binding sites (Bmax ± S.D.) estimated from whole cell binding assays with Gal4-GR1, Gal4-GR2 and Gal4-MR constructs.

Receptor Construct	Hormone	Kd (nM)	Bmax (nMol/well)
Gal4-GR1	Cortisol	70.9 (51.6-103.0)	1.45 ± 0.94
Gal4-GR2		63.8 (50.6-82.7)	1.08 ± 0.53
Gal4-MR		11.9 (10.5-13.5)	1.33 ± 0.45
Gal4-GR1	Dexamethasone	19.0 (15.9-22.9)	3.79 ± 0.02
Gal4-GR2		17.5(14.5-21.2)	1.82 ± 0.32
Gal4-MR		13.1 (10.0-17.4)	0.71 ± 0.67

7.3.3 Activation of corticosteroid receptors by model agonists

Each of the constructs described in 7.3.1 were used in transient transfection assays to assess the ability of model activators of corticosteroid receptors to activate the mineralocorticoid receptor and the two glucocorticoid receptors from European flounder. The potent synthetic glucocorticoid hormone dexamethasone, as well as the naturally occurring glucocorticoid hormone cortisol, was able to activate all three corticosteroid receptors, indicated by an increase in luciferase activity (Figure 7-3). The mineralocorticoid aldosterone failed to activate the glucocorticoid receptors of European flounder, but as expected had an agonistic effect on the mineralocorticoid receptor.

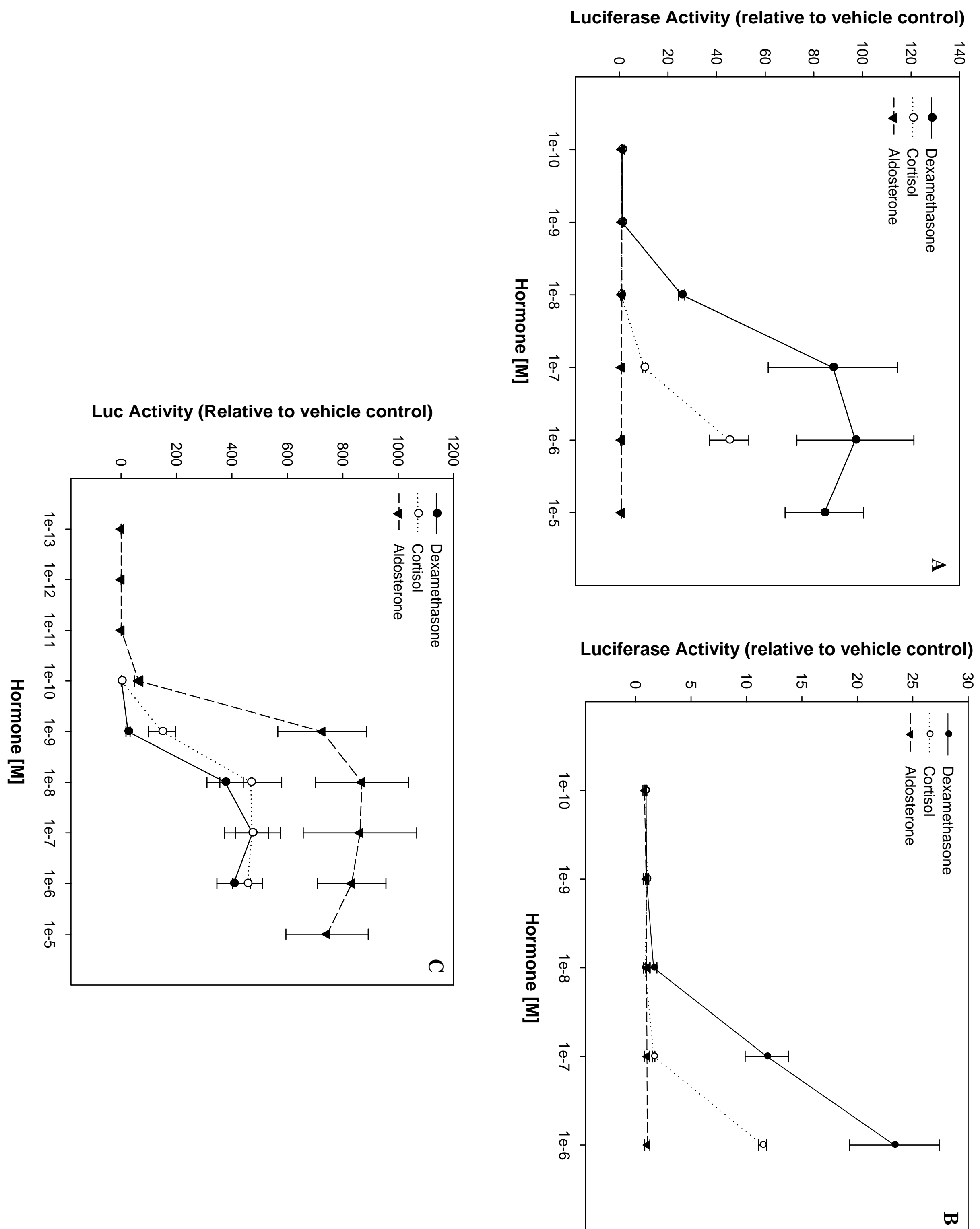

Figure 7-3. Activation of Gal4-GR1 (A), Gal4-GR2 (B) and Gal4-MR (C) ligand-binding domain constructs by dexamethasone, cortisol and aldosterone. FHM cells were transfected as described in 7.2.3.1 and treated with increasing concentrations of dexamethasone, cortisol or aldosterone before being assayed for luciferase activity as previously described. Data are represented as the fold-activation in luciferase activity, relative to treatment with ethanol vehicle only. The mean ± SEM of three independent experiments are shown for each receptor construct. Asterisks represent luciferase activities significantly different to control (* P<0.05; ** P<0.01).

Cortisol appears to exhibit similar potencies on the two glucocorticoid receptor constructs, with luciferase increasing at a concentration of 1×10^{-7} M for both receptors. Dexamethasone, a highly potent synthetic glucocorticoid receptor, was a more potent agonist of both GR1 and GR2 compared to cortisol, as indicated by a shift of the curve to the left for dexamethasone as compared to cortisol. Aldosterone failed to activate either of the glucocorticoid receptors at any of the concentrations tested.

All three corticosteroids tested were able to activate the transcriptional activity of the mineralocorticoid receptor. Both cortisol and dexamethasone were more potent activators of the mineralocorticoid receptor than the glucocorticoid receptor, indicated by their effectiveness at lower concentrations. Aldosterone was the most potent activator of mineralocorticoid receptor transactivation.

7.3.4 Antagonsim of corticosteroid receptors by model antagonists

Antagonism of the Gal4 corticosteroid constructs was assessed using Gal4-CR constructs activated by either 10^{-6}M dexamethasone (Gal4-GR1 and Gal4-GR2) or 10^{-6}M aldosterone (Gal4-MR) both alone and in the presence of increasing concentrations of an antagonist.

Mifepristone (RU486), an anti-progesterone and an anti-glucocorticoid almost completely abolished dexamethasone-induction of GR1 and GR2 when at equimolar concentrations with dexamethasone (10^{-6}M) (Figure 7-4(A) & (B)). In contrast to the effects of mifepristone on the glucocorticoid receptors, this compound had no effect on the aldosterone-induction of the mineralocorticoid receptor (Figure 7-4(C))

Spironolactone is a compound which has been shown to inhibit the mineralocorticoid receptor. Spironolactone failed to inhibit dexamethasone-activated GR1 and although a slight inhibition of GR2 appeared to occur, this inhibition was statistically not significant (Figure 7-4(A) & (B)). Spironolactone partially inhibited aldosterone-activated MR when at equimolar concentrations with aldosterone (Figure 7-4(C)).

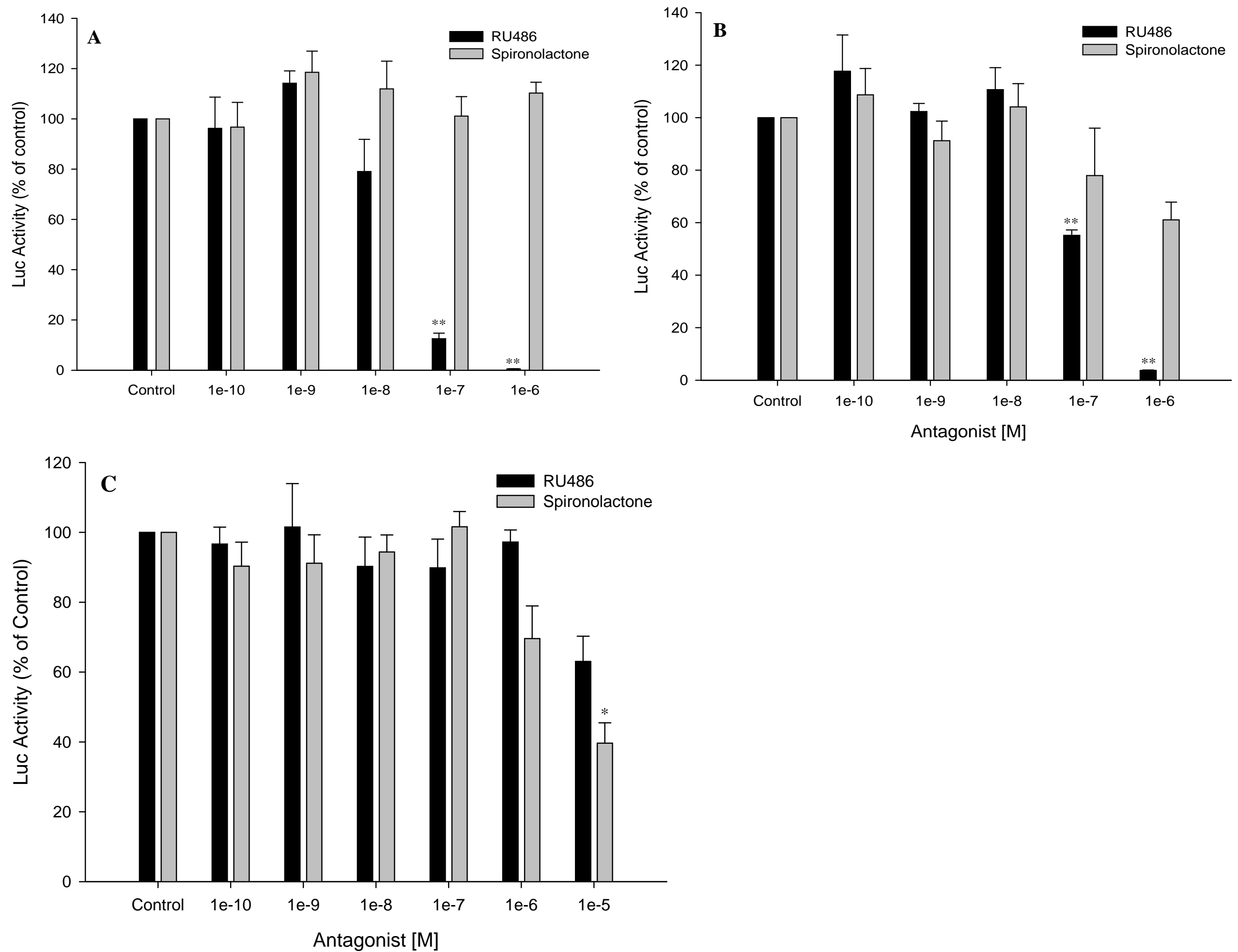

Figure 7-4. Antagonism of GR1 (A), GR2 (B) and MR (C) of European flounder by mifepristone and spironolactone. FHM cells were transfected as described in 7.2.3.1 and treated with increasing concentrations of antagonist in the presence of 1×10^{-6}M dexamethasone (GR1 & GR2 transfected cells) or 1×10^{-6}M aldosterone (MR transfected cells) before being assayed for luciferase activity as previously described. Data are expressed as a percentage of the GR1, GR2 or MR activities measured in the presence of 10^{-6}M dexamethasone or aldosterone only. The mean ± SEM of three independent experiments are shown for each receptor construct with asterisks representing statistical significance (* $p<0.05$ & ** $p<0.01$; one way ANOVA with Dunnett's post hoc test).

7.3.5 Response of corticosteroid receptors to Pollutants

It has been reported previously that the ligand selectivity of corticosteroid receptors is conserved in Gal4 constructs (Bridgham *et al*, 2006). In experiments with model agonists and antagonists Gal4-MR and Gal4-GR showed specificities very similar to those previously described from teleost and tetrapod GR and MR. The constructs thus appear suitable to identify agonist and antagonist compounds amongst environmental chemicals. An environmental contaminant, bis(tributyl tin) oxide (TBTO), shown to interact with other nuclear hormone receptors was tested for its ability to both activate and inhibit the corticosteroid receptors of European flounder.

7.3.5.1 Bis(tributyl tin) Oxide (TBTO)

TBTO caused no increase in luciferase activity, indicating that TBTO does not act as an agonist of CRs (Figure 7-5). At 250 nM TBTO a significant drop in luciferase activity compared to ethanol treatment was observed, which may have been due to cell toxicity.

In antagonism experiments, an inhibition of Gal4-GR1, Gal4-GR2 and Gal4-MR was observed and this occurred in a dose dependent manner with statistically significant inhibition at concentrations above 100nM (Figure 7-6), although similar to the agonism experiment, at 250nm, the inhibition may have occurred due to cell toxicity. At 100 nM the activity of dexamethasone-activated constructs was inhibited by 63%, 88% and 82% for Gal4-GR1, Gal4-GR2 and Gal4-MR, respectively.

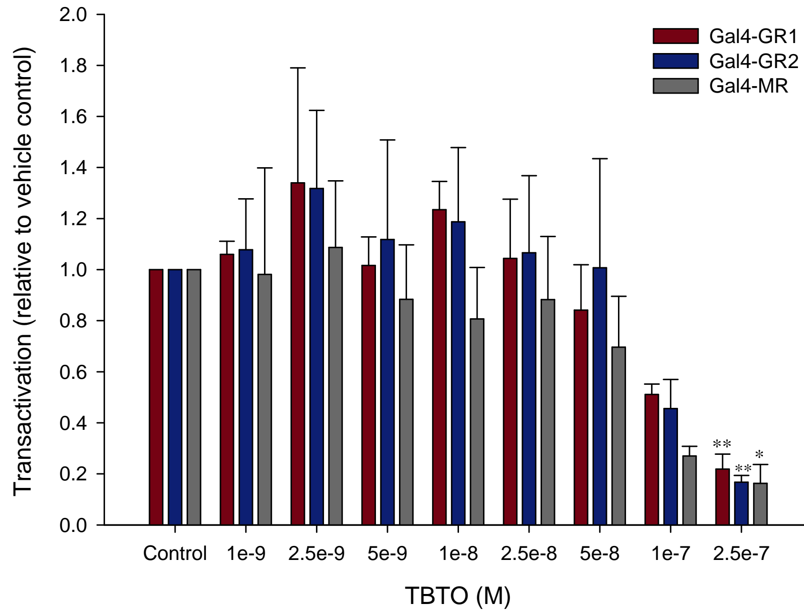

Figure 7-5. TBTO as a potential agonist of Gal4-CR constructs. FHM cells transiently expressing Gal4-GR1, Gal4-GR2 or Gal4-MR constructs were treated with increasing concentrations of TBTO (1-250 nM). Luciferase activity is expressed relative to treatment with ethanol vehicle only (control). The graph shows the mean ± SEM of three independent experiments. Asterisks represent values which differ significantly from the control treatment (* *P*<0.05, ** *P*<0.01).

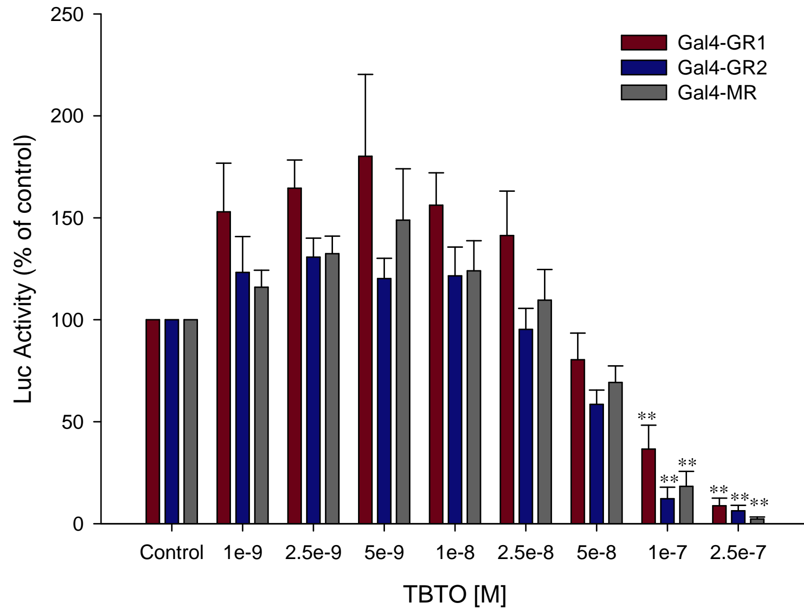

Figure 7-6. Antagonism of Gal4-GR constructs with an environmental contaminant. FHM cells transiently expressing Gal4-GR1, Gal4-GR2 or Gal4-MR constructs were treated with 10^{-6}M dexamethasone in combination with concentrations of TBTO between 1 and 250nM. Luciferase activity are expressed as a percent of a control treatment of 10^{-6}M dexamethasone only, which was assigned the arbitrary value of 100%. The graph shows the mean ± SEM of three independent experiments. Asterisks represent values which differ significantly from the control treatment (*P*<0.01).

7.3.5.2 Bisphenol A and Phthalate compounds

Bisphenol A and phthalate compounds are used in the manufacturing industry as plasticisers. In the present study bisphenol A (BPA), benzylbutylphthalate (BBP) and dimethylphthalate (DMP) were tested as agonists and antagonists with the Gal4-CR constructs. None of the compounds were agonists for Gal4-CR transactivation (Figure 7-7).

While dimethylphthalate had no antagonistic activity, bisphenol A and benzylbutylphthalate were partial antagonists of Gal4-GR1 and Gal4-GR2, although antagonism by the latter compound with Gal4-GR2 was not significant (Figure 7-8).

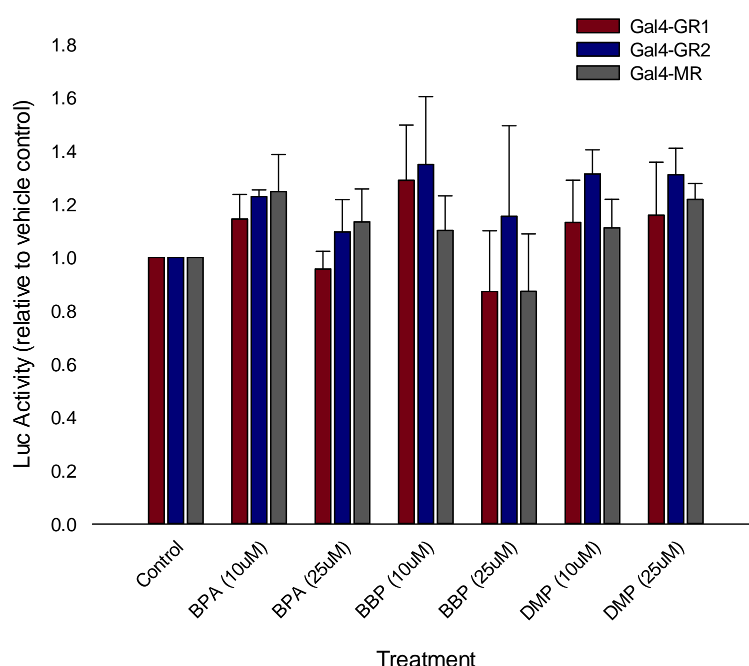

Figure 7-7. Agonism of Gal4-CR constructs by bisphenol A (BPA), benzylbutylphthalate (BBP) and dimethylphthalate (DMP) at 10 µM or 25 µM. FHM cells were transiently transfected with Gal4-CR constructs and reporter plasmids as described in materials and methods. Cells were then treated with either 10 µM or 25 µM of each compound and transactivation activities determined by measuring firefly luciferase normalised to internal *Renilla* luciferase controls. Data are expressed as the fold activation over ethanol vehicle controls and are the mean and SEM of at least three independent experiments.

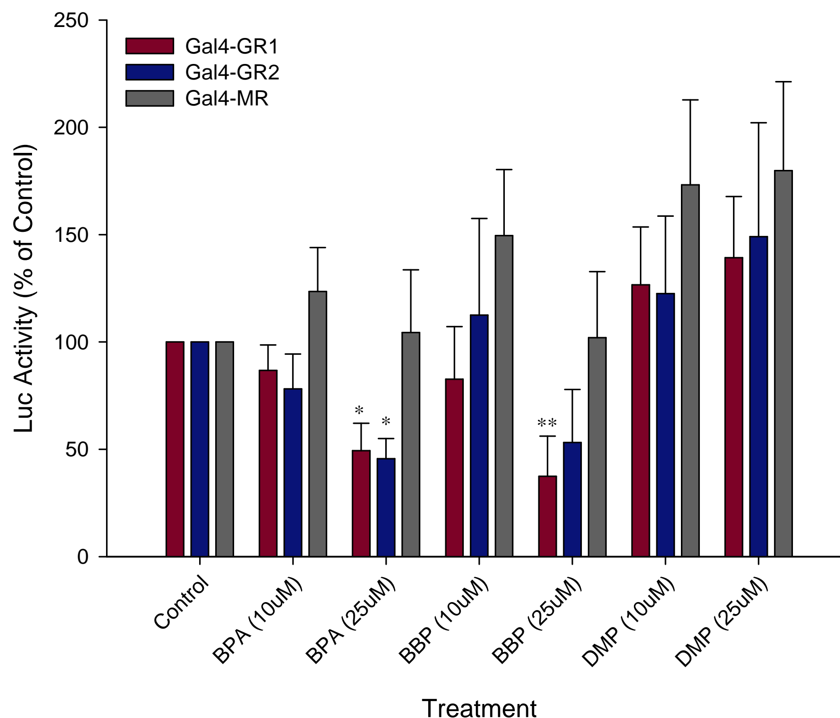

Figure 7-8. Antagonism of Gal4-CR constructs by bisphenol A (BPA), benzylbutylphthalate (BBP) and dimethylphthalate (DMP) at 10 µM or 25 µM. FHM cells were transiently transfected with Gal4-CR constructs and reporter plasmids as described in materials and methods. Cells were then treated with either 10 µM or 25 µM of each compound in combination with 10^{-6}M (Gal4-GR1 and Gal4-GR2) or 10^{-8}M (Gal4-MR) cortisol and transactivation activities determined by measuring firefly luciferase normalised to internal *Renilla* luciferase controls. Data are expressed as transactivation as a percentage of that observed with cortisol alone and are the mean and SEM of at least three independent experiments. Treatments which are significantly different to cortisol only controls are indicated by asterisks (*, *P*<0.05; **, *P*<0.01).

7.4 Discussion

In order to generate a reporter gene assay suitable for detecting interactions of environmental pollutants with the corticosteroid receptors from European flounder, recombinant fusion proteins were made linking the ligand-binding domains of the corticosteroid receptors to the DNA binding domain of the yeast transcription factor Gal4, and a reporter gene assay based on these constructs was developed. The assay was validated by testing the effects of well-known hormonal and pharmacological agonists and antagonists of corticosteroid receptors. The assay was further used to analyse the *in vitro* effects of selected common water pollutants. The persistent aquatic pollutant TBTO was shown to be a potent antagonist of all flounder corticosteroid receptors,

while two of three studied plasticisers were moderately active antagonists of GR1, one of which was also an antagonist of GR2. The possibility that these common environmental contaminants might interfere with corticosteroid signalling in wild fish from polluted environment, and consequently might adversely affect the biological processes under the control of corticosteroid signalling, deserves further enquiry.

In agreement with previous studies (Colombe *et al*, 2000; Greenwood *et al*, 2003; Bury *et al*, 2003; Pippal *et al*, 2011) cortisol and dexamethasone were able to activate both the glucocorticoid receptors and mineralocorticoid receptor of European flounder while the mineralocorticoid aldosterone, was an agonist of the mineralocorticoid receptor only. Furthermore cortisol stimulated activity of the mineralocorticoid receptor of European flounder at lower levels than required to elicit effects on either of the two glucocorticoid receptors. A similar situation has been described for Burton's mouthbrooder (*H.burtoni*) in which the concentration of cortisol required for half maximal activation of the mineralocorticoid receptor was approximately 180-fold less than for the GR1 and 270-fold and 105-fold less for the GR2a and GR2b receptors, respectively (Greenwood *et al*, 2003). These findings parallel those in mammals in which mineralocorticoid receptors are activated by cortisol at lower concentrations than are required by glucocorticoid receptors (Rogerson and Fuller, 2000).

Aldosterone, the mineralocorticoid hormone found in mammals, is thought to be absent in most teleosts, who are thought to lack the P450 11 -hydroxylase (P450(11)) enzyme that results in aldosterone biosynthesis (Jiang *et al*, 1998). Cortisol is the most abundant corticosteroid in circulation in teleost species, and has been reported to function as both a mineralocorticoid and glucocorticoid hormone. Current research has hypothesised that 11-deoxycorticosterone could function as a mineralocorticoid in fish species (Sturm *et*

al, 2005; Kiilerich *et al*, 2011) but further research and evidence is required before a definitive conclusion can be drawn. Despite the lack of aldosterone in teleost's it has previously been found that this hormone retains the ability to activate the mineralocorticoid receptor in zebrafish (Pippal *et al*, 2011) and rainbow trout (Sturm *et al*, 2005). In agreement with these previous studies and reminiscent of the human mineralocorticoid receptor (Hellal-Levy *et al*, 1999), aldosterone was found to activate the transactivation properties of the Gal4-MR receptor with greater potency than either of the glucocorticoid hormones, but with no effect on the glucocorticoid receptor constructs and thus it appears the Gal4-corticosteroid receptor ligand-binding domain constructs retains the ligand specificity previously observed with full-length fish corticosteroid receptors.

As well as classical agonists, two well characterised corticosteroid antagonists were tested with the Gal4-CR constructs. Spironolactone has been shown to be an antagonist of the human mineralocorticoid receptor and is used as an MR antagonist in the treatment of patients with cardiac failure (Fuller and Young, 2005). In contrast to the mammalian mineralocorticoid receptor, spironolactone has been demonstrated to be an agonist of teleost mineralocorticoid receptors (Sturm *et al*, 2005; Pippal *et al*, 2011). Spironolactone was not tested as an agonist of the European flounder mineralocorticoid receptor but was shown to antagonise the MR significantly at an equimolar concentration (10^{-6}M) with aldosterone. It is possible that spironolactone acts as a partial agonist of the European flounder below 10^{-6}M, since no inhibition was seen at these concentrations but further work would be required to confirm this. Importantly spironolactone was specific for the MR receptor, having no antagonistic effect on the glucocorticoid receptors of European flounder.

Mifepristone (RU486) is a glucocorticoid receptor-specific inhibitor, having been shown to inhibit the actions of glucocorticoids both in mammals and fish (Bury *et al*, 2003; Lee *et al*, 2009). The ligand-binding domain of the glucocorticoid receptor has previously been shown to be important in RU486-induced antagonism, with binding of RU486 to this domain inducing a conformational change in which helix 12 of the ligand-binding domain covers the coactivator binding site, preventing receptor activation (Kauppi *et al*, 2003). Additionally the C-terminal AF1 region of the glucocorticoid receptor has also been shown to be important in RU486-induced GR antagonism (Lee *et al*, 2009). Here we have demonstrated that the presence of the ligand-binding domain is enough for RU486-induced antagonism of the European flounder GRs. Similar to spironolactone-inhibition of the mineralocorticoid receptor, inhibition by RU486 was specific for the glucocorticoid receptors.

The above results from these transactivation and repression studies reveal the Gal4-corticosteroid constructs to retain their expected ligand-specificity, with dexamethasone and cortisol activating both the glucocorticoid and mineralocorticoid receptors, with greater potency for the latter receptor, while aldosterone increases the transactivation function of the mineralocorticoid receptor with no effect on the glucocorticoid receptors. Spironolactone and RU486 were specific inhibitors of the mineralocorticoid and the glucocorticoid receptors, respectively. Since they retained ligand specificity, the Gal4 receptor constructs were considered suitable to study the activation and repression of corticosteroid receptors by several environmental pollutants.

Organotin compounds have already been demonstrated to interfere with the glucocorticoid receptor of humans, with interactions between dibutyltin and the ligand-binding domain having an inhibitory effect at concentrations of 250nM and

above (Gumy *et al*, 2008). The inhibitory effects of TBTO on European flounder corticosteroid receptors was observed at concentrations within the range of 100-250nM, a similar concentration at which dibutyltin was observed to bind to the human glucocorticoid receptor. Thus organotins are inhibitory to both GR1 and GR2 at similar concentrations to the model anatagonist, RU486, while inhibition of the MR by TBTO occurs at concentrations lower than that observed with spironolactone.

As well as interactions directly with the glucocorticoid receptor a disruption of the human 11 -HSD2 enzyme by organotins, including tributyltins, has been reported (Hellal-Levy *et al*, 1999; Atanasov *et al*, 2005), suggesting the ability of organotin compounds to disrupt corticosteroid signalling at various levels. 11 -HSD2 functions in mineralocorticoid-specific tissues to convert active cortisol into the inactive 11-keto form cortisone, protecting the MR from activation by glucocorticoids and rendering the specificity of the receptor for aldosterone. More recently, this enzyme has been isolated from fish species in which aldosterone is absent and has been postulated to protect tissues expressing this enzyme from the effects of stress-induced cortisol during key developmental stages (Kusakabe *et al*, 2003; Jiang *et al*, 2003). With the discovery of this enzyme in fish species it is possible that organotin compounds may inhibit this enzyme in teleosts as well as mammals, leading to an overexposure of 11 -HSD2-expressing tissues to cortisol.

The inhibitory effects of TBTO on the corticosteroid receptors in European flounder are observed at concentrations greater than 100nM. Despite the ban on the use of organotin compounds, TBT still persists within the aquatic environment due to its slow degradation in sediments (Stewart and de Mora, 1990; de Mora *et al*, 1995; Viglino *et al*, 2004). Organotin compounds have been found to accumulate to high concentrations

within tissues of aquatic organisms; 35 ng TBT/g has been detected in the liver of European flounder (Albalat *et al*, 2002), while 67 ng TBT/g wet weight has been detected in Korean mackerel (Oh, 2009). These concentrations equate approximately to 59 nM and 112 nM, respectively, the latter of which is above the level in which effects were observed in the present study.

As well as TBTO, three compounds used as plasticizers in the manufacturing industry were tested in transfection assays for their ability to alter transcriptional activities of the corticosteroid receptors. Two of these compounds, bisphenol A (BPA) and benzylbutylphthalate (BBP) were able to antagonise GR1 of European flounder, while GR2 was antagonised by BPA. Previously the ability of BPA to bind to the human glucocorticoid receptor has been reported (Prasanth *et al*, 2010) and the high sequence similarity seen between the ligand-binding domain of human GR and teleost GRs makes it likely that BPA will also bind to GRs of the latter. As a receptor antagonist BPA is not very potent, with antagonism observed only at concentrations of 25 µM. Within the environment, BPA is not considered a persistent pollutant due to its rapid biodegradation in wastewater treatment plants and receiving wasters, with a reported half-life of 2.5 to 4 days (Staples *et al*, 1998). Reported concentrations of BPA in water samples are typically well below the 25 µM concentration at which an antagonistic effect on GR was observed in the transient transfection assay; Fromme *et al* (2002) found levels of BPA in surface waters of rivers, lakes and channels across Germany of between 0.0005 µg/L to 0.229 µg/L (2.19 pM – 1nM). While isolated examples of higher BPA concentrations (up to 21 µg/L, equivalent to ~92nM) have been reported in the Netherlands (Belfroid *et al*, 2002), these concentrations are still well below 25 µM. Nevertheless, with the ever-increasing demand for bisphenol A (global demand is predicted to grow from 3.9 million tons in 2006 to approximately 5 million tons in 2010

(Tsai, 2006)), and the detection of bisphenol A in fish tissues (Mita *et al*, 2011) the chronic input of this compound into receiving waters and its accumulation in the tissues of fish species warrants consideration.

Previously, benzylbutyl phthalate has been shown to have estrogenic effects in fathead minnow (*Pimephales promelas*) exposed to the phthalate compound at 100 μg/L (Harries *et al*, 2000), consistent with the mode of action *in vitro* in which benzylbutyl phthalate was able to bind weakly to the estrogen receptor in fish cell cultures, eliciting weak estrogenic activity (Jobling *et al*, 1995). These studies used very high phthalate exposure concentrations, which are unlikely to be present within the water column. Indeed, concentrations of BBP have been reported in the river Rhine at concentrations up to 10 μg/L (approx. 32nM) and therefore well below the concentrations at which estrogenic effects in fathead minnow and inhibition of the GRs of European flounder were observed. Within the muscle of different fish species, levels of different phthalate compounds have been measured and benzylbutylphthalate levels found to reach up to 9.4mg/kg dw (Huang *et al*, 2008), equating to a concentration of approximately 30 μM. Thus BBP is able to accumulate within the tissues of fish species and may lead to concentrations at which BBP was observed to antagonise GRs of European flounder.

7.5 Conclusion

A reporter gene assay has been developed to screen aquatic contaminants for their potential to cause endocrine disruption by interactions with the ligand-binding domains of the corticosteroid receptors in European flounder. The ligand-binding domains, using classical agonists, have been shown to confer ligand specificity with the Gal4-GR ligand-binding domain constructs being activated only by glucocorticoids (cortisol and dexamethasone) and the Gal4-MR construct being activated by both glucocorticoids and the mineralocorticoid hormone present in mammalian species (aldosterone). Thus, these

constructs are suitable for identifying interactions between corticosteroid receptors and potential ligands, such as environmental pollutants. Several phthalate compounds, namely bisphenol A and benzylbutylphthalate were observed to antagonise one or both of the GRs of European flounder at concentrations of 25 μM. Such a high concentrations is unlikely to be found within aquatic environments but BBP may accumulate to such levels in the tissues of fish species. Of more environmental relevance bis(tributyl tin) oxide was shown to inhibit all three corticosteroid receptors of European flounder at concentrations above 100 nM. To further confirm the interaction between TBTO and corticosteroid receptors and to determine the likely effects of such an interaction further studies are warranted at an organisational level higher than the *in cellulo* level (such as *ex vivo* or *in vivo*) in order to elucidate the impact of exposure in whole organisms.

Chapter 8. General Discussion

8.1 Key Findings

In the current thesis a cell-based reporter gene assay system has been developed and used to the study of the potential for aquatic pollutants to disrupt endocrine signalling in fish via interactions with two NHRs - the peroxisome proliferator activated receptors and the corticosteroid receptors. To this end three corticosteroid receptors have been cloned for the first time from European flounder and, together with PPAR , and previously cloned from the closely related European plaice, were used to construct receptor plasmid constructs. The ligand-binding domains (LBDs) of each receptor was ligated downstream of the DNA-binding domain of yeast Gal4, and when co-transfected into a fish cell line (FHM) with a reporter plasmid, containing the upstream activating sequence (UAS) of yeast Gal4 ligated upstream of the firefly luciferase reporter gene, could be used to detect pollutant-receptor LBD interactions in cells to which pollutants of interest were administered. Using cells transfected with PPAR and corticosteroid receptor constructs, the system was demonstrated to respond in a predictable fashion to known agonists and was therefore considered a suitable method in which to study pollutant-nuclear hormone receptor interactions.

The transient transfection assay identified bis(tributyl)tin oxide (TBTO) as an inhibitor of both PPAR and corticosteroid receptors at low nanomolar concentrations. In a very preliminary study to attempt to appreciate the wider consequences of the former inhibition, a cDNA microarray analysis was undertaken using hepatocyte cells of European flounder to which a PPAR agonist was administered, either alone or in combination with TBTO, and gene expression profiles compared. Despite failing to provide definitive conclusions, the results of the microarray analysis hinted at possible

effects on proteasomal pathways, mitochondrial oxidative phosphorylation, immune functions and lipid homeostasis.

The newly cloned European flounder corticosteroid receptors were studied further with sequencing, tissue expression profiles and transactivation assays allowing the characterisation of each receptor. Cortisol, the endogenous fish glucocorticoid, and dexamethasone activated the transcriptional activities of all three corticosteroid receptors while aldosterone, the tetrapod mineralocorticoid hormone, was specific for the mineralocorticoid receptor. Additionally the abilities of RU486 and spironolactone to function as an anti-glucocorticoid and anti-mineralocorticoid respectively, was demonstrated. Steroid binding assays demonstrated a direct binding of cortisol and dexamethasone to the Gal4-CR constructs.

8.2 *In vitro* versus *In vivo* studies

In the context of environmental screening, a shift towards the use of *in vitro* assay systems to further understand the molecular and pathway perturbations caused by chemical pollutants is occurring. Knowledge from such assay systems may be used to prioritise chemicals for *in vivo* screening. Indeed such methods are being initiated by the OECD and the US environmental protection agency (EPA), the latter of which has introduced the ToxCast programme, with the aims of (a) identifying *in vitro* assays that can reliably indicate perturbations in biological processes relevant to *in vivo* toxicity, (b) develop prediction models based on multiple assays and chemical properties, a combination of which can achieve higher predictive power than each of these methods alone and (c) use the combination of *in silico* and *in vitro* assay-based signatures to screen large numbers of untested environmental chemicals (Judson *et al*, 2010).

The use of *in vitro* transactivation assays to screen chemical pollutants presents both advantages and disadvantages compared to *in vivo* methods of screening. One major advantage is the ability to screen many chemicals in a cost effective and timely manner, compared to *in vivo* screening which can be both time-consuming and costly. The use of *in vitro* assays also provides the ability to study individual pathways and their perturbation by chemicals of interest, thus indicating a chemicals mode-of-action.

However, compared to *in vivo* methods, cell-based systems may be over simplistic and ultimately it is the emergent properties of tissues and organisms which are key determinants of whether a compound will elicit endocrine-disrupting or toxic effects. Furthermore difficulties also arise when considering the timescale of *in vitro* assays, which usually run over hours to days, and how this relates to processes which can lead to *in vivo* toxicity end point, such as cancer, over longer timescales of months to years. Finally, the production of metabolite compounds which occurs upon parent compound biotransformation, and which can be more or less active than the parent compound clearly must be considered in the assay or modeling treatment. Indeed within the present study, while the parent phthalate compounds had no effect on PPAR signalling, the monoesther metabolites were able to activate PPAR constructs (Chapter 4). It is clear therefore that the development of assays in which realistic levels of biotransformation occur *in vitro* may be required in order to assess the complete suite of active metabolites, otherwise the testing of metabolites will have to be a consideration. Despite these drawbacks in comparison to *in vivo* testing, assay-based sceening methods can be useful in providing a first tier approach to screening, allowing chemicals which show the highest likelihood of disruptive effects *in vivo* to be prioritised for screening. This tiered screening approach will reduce the number of organisms required for *in vivo* screening and thus is in keeping with the three Rs concept (Flecknell, 2002).

8.3 TBTO as an inhibitor of PPARs and CRs

Using the transient transfection assay developed in this study (Chapter 3) several environmental pollutants were found to agonise or antagonise the PPAR and corticosteroid receptor constructs, resulting in increased or decreased luciferase activity, respectively. Most of the compounds were only effective at concentrations unlikely to be encountered in the aquatic environment. Fibrates have been measured in sewage effluents at concentrations between 20-200 nM (Krey *et al*, 1997; Ternes, 1998; Buser and Müller, 1998; Andreozzi *et al*, 2003; Sanderson *et al*, 2003), phthalate metabolites have been detected at concentrations of <5 nM (Suzuki *et al*, 2001) and concentrations of bisphenol A are generally at concentrations of <1nM (Fromme *et al*, 2002). Effects of these compounds in transfected cells were not seen at concentrations of less than 10 µM and therefore, in an environmental context, were considered insignificant.

8.3.1 PPAR

TBTO significantly inhibited PPAR at concentrations as low as 1nM and inhibited the transcriptional activities of the corticosteroid receptors at 100nM and above. Within the environment levels of TBT have been reported at concentrations of 200ng/L (0.34 nM) to 3.2 µg/L (5.4 nM) in the water column (Michel *et al*, 2001; Basheer *et al*, 2002), and at higher concentrations in sediments (44 nM in coastal environment of Xiamen, China (Wang *et al*, 2008); up to 1.42 µg in São Paulo, Brazil (Godoi *et al*, 2003) and 961 nM in the Gulf of Cádiz, Spain (Garg *et al*, 2009)). Moreover, due to the lipophilic nature of tributyl tin compounds, these are readily accumulated in the tissues of aquatic organisms with concentrations of 35ng/g (58.71 nM TBTO), 114ng/g (191.24 nM TBT) and 30ng/g (50.33 nM) having been reported in the liver, digestive tract and gills of European flounder, respectively, suggesting multiple routes of uptake including the diet

and directly from the water and accumulation at concentrations which could potentially affect both PPAR and CR signalling.

In response to the recognised impacts of organotins on gastropod species, many countries, including the USA, France, and the UK, banned the use of tributyltin-based antifouling paints on smaller shipping vessels in the late 1980s, and the International Maritime Organization (IMO) called for a complete ban in the application of such antifoulants by 2008. Despite this legislation it is thought that TBT may persist in the aquatic environment for tens of decades (Dowson *et al*, 1996) due to their being retained in sediments, their slow rates of degradation, their continued use as material and wood preservatives and their continued use by countries not covered by IMO regulation. Therefore, due to the likelihood that TBTO will remain as a relevant environmental pollutant for the foreseeable future, it is important to decipher its effects and modes of actions in aquatic organisms. Previously the main focus of TBTO has been as an endocrine disruptor in female gastropods, due to the disruptive effects of this compound on the reproductive system of females, inducing the development of male reproductive organs resulting in sterility (Rilov *et al*, 2000; Blackmore, 2000; Camillo *et al*, 2004; Nishikawa, 2006; Gravel *et al*, 2006; Garaventa *et al*, 2007; Lima *et al*, 2011). A proposed target for TBTO in gastropods is the retinoid X receptor, the heterodimeric partner of PPARs. TBT binds covalently to human RXR and in a stable transfection assay was shown to activate all three RXR -PPAR , and heterodimers, via interactions with RXR (le Maire *et al*, 2009), although several studies have also reported TBT to be an agonist of human PPAR *in vitro*, but showed no agonist effect on human PPAR and PPAR (Kanayama *et al*, 2005; Grün *et al*, 2006). Despite being an agonist of human PPAR , TBTO was unable to activate the transcriptional activity of plaice PPAR , although this is not necessarily surprising given that PPAR of fish

species appears to be relatively insensitive to compounds which activate mammalian PPAR (Leaver *et al*, 2005). In agreement with the studies with human PPAR and PPAR , TBTO was not an agonist of PPAR or PPAR from plaice. The finding that TBT activates the RXR-PPAR heterodimers via interactions with the RXR partner in humans provides an interesting quandary, since it is demonstrated in the present study that TBTO significantly inhibits PPAR of plaice. Further investigations into the effects of TBTO on RXR in fish species may prove interesting and would provide a more thorough understanding into the likely effects of TBTO *in vivo* with regards to PPAR signalling, and signalling via other RXR heterodimers.

As a preliminary experiment to investigate effects of TBTO on PPAR -target gene expression, a microarray analysis was carried out using hepatocyte cells of European flounder (Chapter 5). PPAR is highly expressed in the liver of fish species, including sea bream and plaice (Leaver *et al*, 2005), making it an ideal tissue in which to study PPARs. One of the main functions of PPAR -target genes is in mitochondrial and peroxisomal fatty acid -oxidation, thus it was predicted that such genes which are upregulated upon activation of PPAR may be downregulated upon inhibition of PPAR by TBTO. As discussed in Chapter 5 genes which have previously been identified to function in lipid metabolism were both downregulated and upregulated by the presence of TBTO and the PPAR activator, Wy14643. Although the downregulation is in keeping with the predicted outcome, an upregulation of PPAR target genes was unexpected and suggests other factors to function in the regulation of these genes. Roles for other NHRs in regulating PPAR -target genes have been reported which, along with the as yet untested possibility of TBTO affecting fish RXRs, may complicate these analyses. For example, a role for the liver X receptor (LXR) in hepatic peroxisomal -oxidation has been described for rodents in response to an LXR

activator in PPAR -null mice, indicating LXR regulatory mechanisms, independent of PPAR (Hu *et al*, 2005). Three genes, acyl CoA oxidase (ACO), L-3-hydroxyacyl-Coenzyme A dehydrogenase and 3-ketoacyl-CoA thiolase, all previously identified as PPAR target genes, were upregulated in mice hepatocytes in response to the LXR agonist T0901317. A similar role for the LXR in fatty acid metabolism has been described in Atlantic salmon after cholesterol was found to simultaneously increase the expression of acyl CoA oxidase (ACO) and LXR in Salmon head kidney (SHK) cells (Minghetti *et al*, 2011). The ACO enzyme is the first, and rate-limiting, step in the -oxidation pathway and has previously been cloned from humans and rodents (Tugwood *et al*, 1992; Varanasi *et al*, 1996; Lambe *et al*, 1999). Analysis of the promoter region of the ACO gene reveals a DR-1 PPRE, which is functional in the rodent gene, being activated by the potent PPAR ligand, Wy14643. An upregulation in the expression of the ACO gene following treatment of human hepatocytes with Wy14643 was detected using microarray analysis (Rakhshandehroo *et al*, 2009), suggesting PPAR may also regulate the transcription of the human ACO gene. Functional PPREs have also been characterised in the enzymes participating in the second (enoyl-CoA hydratase/3-hydroxyacyl Co-A dehydrogenase bifunctional enzyme) and third steps (ketoacyl-CoA) of the peroxisomal -oxidation pathway (Zhang *et al*, 1992; Hansmannel *et al*, 2003). The LXR response element (LXRE) typically consists of a direct repeat of the sequence TGACCT separated by 4 nucleotides (DR-4), and a search of each of these three genes from rodents described above revealed a putative LXRE sequence, further implicating the LXR in mitochondrial -oxidation (Hu *et al*, 2005).

As well as fatty acid homeostasis, genes with functions in proteasome maintenance were observed to be downregulated by TBTO. In response to chemical and physical stressors, proteins can become damaged or misfolded caused, in part, by the effects of

oxidative stress. Several pathways are activated in response to stress to restore cellular homeostasis including the stabilisation of unfolded proteins and removal of damaged proteins. The proteasome prevents the accumulation of non-functional or potentially toxic proteins through their ubiquitin-dependent degradation (Goldberg, 2003). Previously genes encoding proteins which function in the maintenance of a healthy proteome, including components of the proteasomal protein degradation pathway, have been reported to be upregulated in response to PPAR agonists in rodents and primates (Anderson *et al*, 2004; Cariello *et al*, 2005), suggesting a PPAR -dependent regulation of protein associated with the proteasome. The hypothesis that PPAR is important in the regulation of proteasome maintenance genes was strengthened by the finding that PPAR -null mice display defects in the expression of proteins that protect the proteome from chemical damage. A role for PPAR in the regulation of proteasomal proteins is also indicated in the results of the microarray presented in Chapter 5, in which multiple genes encoding subunits of the proteasome are upregulated by the PPAR agonist Wy14643, while being downregulated by treatment with TBTO in Wy14643, consistent with an inhibition of PPAR . A direct binding of organotin compounds to the proteasome has been reported previously (Shi *et al*, 2008), indicating that organotins can inhibit the proteasome by (i) inhibiting PPAR -dependent regulation of proteasome subunit expression and (ii) direct binding to the assembled proteasome complex, thus inhibiting its cellular activity.

8.3.2 Corticosteroid Receptors

In accordance with several other teleost species, European flounder appear to express three corticosteroid receptors, having two glucocorticoid receptors and a single mineralocorticoid receptor (Chapter 6). The presence of two GRs is unique to fish

species, being absent in other vertebrate lineages. The success of the vertebrate lineage is thought to have been driven by two rounds of whole genome duplications early in their evolution (Dehal and Boore, 2005; Kasahara, 2007). A consensus has emerged in support of a third whole genome duplication event in fish species, following the divergence of the teleostomi into the actinopterygii and sarcopterygii lineages, the European flounder belonging to the former group (Jaillon *et al*, 2004; Meyer and Van de Peer, 2005). The result of this additional whole genome duplication is the presence of duplicated genes, present only in fish. Whole genome duplication can give rise to duplicated copies of genes which can be lost (non-functionalisation), undergo neo-functionalisation in which the duplicated copy acquires a new function, or sub-functionalisation in which both copies of the duplicated gene loses part of the ancestral function and both are required to maintain the full function. The presence of two glucocorticoid receptors within European flounder, although not proven, is likely to have arisen following the actinopterygii-specific genome duplication. Both glucocorticoid receptors were found to be responsive to cortisol, the endogenous ligand of corticosteroid receptors in fish, with similar affinities and concentrations being required to stimulate transactivation of both Gal4-GR1 and Gal4-GR2 constructs, suggesting a sub-functionalisation event occurred in the European flounder to result in the maintenance of two glucocorticoid receptors. Previously neo-functionalisation has been reported for the duplicated glucocorticoid receptors from the midshipman fish (*Porichthys notatus*), on the basis of the responsiveness of the GR2 to aldosterone, which was absent in GR1 of the same species (Arterbery *et al*, 2011). Neither of the European flounder GRs was activated by aldosterone and the sensitivity of glucocorticoid receptors to mineralocorticoid hormone is not consistent with their phylogenetic relationship; midshipman fish GR2 is aldosterone sensitive, in contrast to

GR1 of the same species, while the GR1 of the daffodil chichlid is responsive to aldosterone while the GR2 is not (Arterbery *et al*, 2011), leading the authors to suggest that novel amino acid substitutions permit aldosterone binding to otherwise cortisol specific receptors. Nine amino acid residues within the ligand-binding domains of mineralocorticoid-sensitive and insensitive glucocorticoid receptors were suggested to function in mineralocorticoid sensitivity, due to their differing in aldosterone-sensitive and insensitive GRs. Two of these residues proved to be particularly strong candidates due to their proximity to bound corticosteroids, or due to the predicted structural changes induced in the receptor protein. Both GR1 and GR2 of European flounder had a serine residue at position 49, consistent with the serine residue at the same position in aldosterone-insensitive GRs of other species. At the position of the second residue however (amino acid 204), GR1 of European flounder matched that of the other aldosterone-insensitive GRs while the equivalent residue in GR2 was the same as that in aldosterone-sensitive GRs. Since GR2 showed no sensitivity to aldosterone it may be that the substitution at amino acid 49 is more influential in conferring aldosterone sensitivity than that at position 204, or a double substitution at both positions 49 and 204 may be necessary to confer transcriptional activation by aldosterone.

In the search for aquatic pollutants with the potential to interfere with transcriptional activities of corticosteroid receptors, TBTO was identified as a potential antagonist at concentrations greater than 100 nM. Antagonism of the human glucocorticoid receptor by dibutyltin has previously been reported, blocking access of glucocorticoid hormone to the ligand-binding site and inhibiting transcriptional activation (Gumy *et al*, 2008). Furthermore tributyltin can inhibit the 11 -HSD2 enzyme, leading to a disruption in 11 -HSD2-dependent glucocorticoid inactivation (Atanasov *et al*, 2005). This inhibitory action of organotin compounds is likely to increase glucocorticoid hormone

concentrations in aldosterone-sensitive tissues where 11 -HSD2 is highly expressed and, during pregnancy, increase the exposure of the foetus to maternal glucocorticoids. The effects of corticosteroid receptor inhibition in response to TBTO *in vivo* requires further investigation but as the corticosteroid receptors function in the stress response, osmoregulation, energy homeostasis, the immune system and inflammatory response, it might be assumed that numerous harmful effects will be observed.

8.4 Predicted *in vivo* effects of TBTO exposure in fish species

Having observed a TBTO-mediated inhibition of PPAR and corticosteroid receptors and having preliminarily identified genes whose altered expression in response to TBTO may impact on particular physiological functions in European flounder, a final concluding discussion on the possible impacts of TBTO exposure in fish species is presented below.

The microarray experiment in Chapter 5 identified several genes with functions in lipid metabolism and fatty acid -oxidation which may be altered in response to TBTO. *In vivo*, alterations in the obesogen response of chinook salmon to TBT exposure have previously been described (Meador *et al*, 2011). Consistent with the obesogen response, an increase in body mass, plasma triacylglycerols, cholesterol and lipase were found in the salmonid species. However other parameters associated with the obesogen response in small mammals differed in the fish species upon exposure to TBT, including an increase in serum glucose, free fatty acids and triglyglycerids. Although intuitively enhanced growth and lipid storage may appear beneficial, Meador *et al* (2011) described numerous potentially negative consequences for fast growing salmon, including inappropriately timed migration patterns (early smolting during outmigration and an early return to streams and rivers), inapporopriate seasonal behaviours (e.g. an

increase in appetite during winter) and increased activity resulting in higher metabolic demands.

From previous studies involving the inhibition of proteasome complexes, several *in vivo* effects may be predicted upon exposure of plaice to TBTO. An inhibition of proteasome functions through a decrease in the expression of proteasome subunits may result in an accumulation of misfolded and non-functional proteins. In addition inhibition of proteasome may lead to inappropriate levels of apoptosis as demonstrated in cells to which a proteasome inhibitor is added (Shinohara *et al*, 1996). Within fish species a proteasome complex has been detected in the flagella of sperm, the inhibition of which reduces sperm motility (Inaba *et al*, 1993; Inaba *et al*, 1998). Similarly the proteasome is involved in female oocyte maturation in many species, including fish and amphibians. It has been found that the meiotic cell cycle of mature oocytes of many species is arrested in metaphase II until eggs are fertilised, after which progression into anaphase is dependent on the degradation of cyclin B protein in a ubiquitin-dependent proteolytic manner which involves the 26S proteasome (Josefsberg *et al*, 2000; Horiguchi *et al*, 2006). A disruption of the proteasome complex has been demonstrated to prevent germinal vesicle breakdown (GVBD), disrupt meiotic apparatus organisation, prevent cell cycle progression and inhibit entry of the sperm into the oocyte (Sun *et al*, 2004). Taken together these results suggest that an inhibition of proteasome functions may reduce gamete quality by interfering with sperm motility and fertilisation ability while preventing the final maturation of oocytes.

Results from the microarray also revealed a downregulation in genes which encode proteins with roles in immune functions following treatment with TBTO. There are many examples within the literature which document the immunosuppressive effects of

organotin compounds in fish species and since both PPARs and corticosteroid receptors have roles in immune responses and an *in vitro* inhibition of both receptors was observed in the present work some of the immunosuppressive effects observed in fish species may be due to interactions of TBT with these receptors. A dose-dependent lymphocytic depletion in the spleen has been reported in rainbow trout exposed to TBTO (Schwaiger *et al*, 1994), and a significantly reduced resistance to a bacterial *Aeromonas hydrophila* challenge has been reported in rainbow trout yolk fry exposed to both TBT and dibutyltin (De Vries *et al*, 1991). Using medaka and guppy, a comparative study showed the induction of thymus atrophy in the guppy (Wester and Canton, 1987; Wester et al., 1990), while a dose dependent decrease in phagocyte activity has been reported in Atlantic croaker (*Micropogonias undulatus*), hogchoaker (*Trinectes maculatus*) and oyster toadfish (*Opsanus tau*) exposed to TBT at 0.04 to 400 µg/L (Wishkovsky et al., 1989). Similarly TBT and dibutyltin have been shown to reduce lymphocyte number in Murray cod (*Maccullochella peelii*) and rainbow trout with a reduction in phagocytic activity also reported in the former species (O'Halloran *et al*, 1998; Harford *et al*, 2007). A suppressive effect on the non-specific immune system, indicated by a decrease in non-specific cytotoxic cells (NCC), and a deleterious effects on the thymus have been described in European flounder exposed to TBTO, leading to the possibility of a TBTO-induced immunocompetence (Grinwis *et al*, 1998; Grinwis *et al*, 2000; Grinwis *et al*, 2009).

In addition to immune functions, one of the major functions of corticosteroid hormones in fish species is in osmoregulatory functions. Evidence from previous literature suggests the disruption of corticosteroid signalling impacts the osmoregulatory capacity of fish species. For example the survival of summer flounder (*Paralichthys dentatus*) larvae in saltwater (30ppt) was significantly reduced when immersed into the

glucocorticoid receptor blocker RU486, compared to larvae in which no RU486 was present, indicative of a role for cortisol and the glucocorticoid receptors in the seawater tolerance of larval marine teleosts (Veillette *et al*, 2007). European flounder share a similar life cycle to that of summer flounder in that they hatch in offshore waters and therefore must tolerate hyperosmotic conditions. From the results described for summer flounder, an inhibition of the glucocorticoid receptors of European flounder, for example by the pollutant compounds in the present study, is likely to impact on the survival of larvae and thus has the potential to impact population numbers in heavily contaminated areas. A further example of the impact of organotin compounds on the osmoregulatory capacity of flounder was provided by Hartl *et al* (2001) who measured alterations in mechanisms which maintain osmotic homeostasis, including membrane permeability and hydromineral fluxes, in freshwater-adapted juvenile European flounder exposed to sediment-associated organotins. While an unexposed control group of flounder demonstrated behaviours of healthy freshwater-acclimated osmoregulators, producing large volumes of dilute urine and drinking only occasionally in order to maintain blood osmolality, flounder exposed to organotins demonstrated behaviours more similar to seawater-adapted organisms, increasing their drinking rates and reducing urine production. The authors hypothesised that these alterations in behaviour were to compensate for increased blood osmolality, caused by organotin-dependent reductions in membrane water permeability and increased passive sodium efflux (Hartl *et al*, 2001). Thus exposure of flounder to organotin compounds may exhibit significant disruptions in osmoregulatory capacity.

The corticosteroid receptors also have a role in the stress response in fish. A study of brook charr (*Salvelinus fontinalis*) in which the glucocorticoid receptor was blocked by RU486 found the stressor-related increase in plasma glucose concentrations, which was

seen in groups of brook charr in which RU486 was not administered, was absent (Vijayan and Leatherland, 1992). Conversely rainbow trout show little evidence of an altered stress response following RU486-mediated glucocorticoid blockade, but the post-stressor cortisol profiles in RU486 treated trout were more erratic than control fish in which RU486 was not administered leading authors to suggest a disturbance of normal interactions of components of the HPI axis (Reddy *et al*, 1995). Alterations in the stress responses in fish have previously manifested in an altered response to secondary stressors. For example a stress response has been shown to occur in response to exposure of rainbow trout to municipal wastewater effluents, which results in an inhibited response to a further stress event, such as handling (Ings *et al*, 2012). This inhibition of a secondary response may result in a greater susceptibility to disease as shown in Atlantic salmon exposed to hyperoxygenation which had increased susceptibility to infectious pancreatic necrosis virus (IPNV) (Sundh *et al*, 2009) and decreased predator-avoidance behaviour, demonstrated in rainbow trout exposed to cadmium (Scott *et al*, 2003). Thus, in response to TBTO an impaired stress response may be expected, which might then further impact on the normal behaviour of exposed organisms to further stressors such as predation or pathogens.

8.5 Conclusions

In summary the results of the present work indicate that:

1. The peroxisome proliferator-activated receptor and corticosteroid receptor ligand-binding domains, in the presence of previously identified agonists, are able to adopt a transcriptionally active conformation resulting in a change in luciferase reporter expression. Therefore, the assay developed using these constructs is considered a suitable system in which to screen pollutants present in the aquatic environment which disrupt PPAR and CR functions of

European flounder (*Platichthys flesus*). The adoption of an *in vitro* assay system is in keeping with recent trends to reduce the use of live animal models for environmental toxicological screening.

2. The European flounder contains three distinct corticosteroid receptors (two glucocorticoid receptors and a single mineralocorticoid receptor), each most likely expressed by a separate gene. Both GR1 and GR2 are responsive to cortisol, the endogenous teleost corticosteroid hormone, and dexamethasone while the mineralocorticoid receptor is responsive to cortisol, dexamethasone and the mammalian mineralocorticoid hormone, aldosterone. Transfection of COS-7 cells with the Gal4-CR ligand-binding domain constructs, and treatment with tritiated hormones confirmed the expression of Gal4-CR protein and the binding of corticosteroid hormones.

3. The transient transfection assay identified several environmental compounds which act as PPAR and CR ligands. Fibrate compounds were both transcriptional activators (Gemfibrozil, Ciprofibrate) and inhibitors (Fenofibrate, Bezafibrate) of PPAR , while Bezafibrate activated PPAR . In response to phthalate monoesters, mono-1-methylhexyl phthalate and 1-monobenzylphthalate, an increase in transcriptional activation was observed with both PPAR and PPAR . 1-monobenzylphthalate was the only compound found to illicit any increase in transcriptional response from PPAR throughout the research presented here. Bisphenol A was demonstrated as an inhibitor of GR1 and GR2 while benzylbutyl phthalate inhibited GR1 only. The above responses generally required between 10 and

100 µM of compound before a change in reporter gene activity was observed and thus were considered irrelevant in an environmental context.

4. TBTO at low (up to 10 - 100 nM) concentrations is an inhibitor of PPAR and all three corticosteroid receptors. Inhibition occurs in a dose dependent and receptor-dependent manner; no inhibition was observed when the transcriptionally active pBIND ID-pACTmyoD complex was transfected and treated with up to 50nM TBTO, indicating that the effect was specific for the transfected NHR

5. A comparison of the gene expression profiles of hepatocytes exposed to Wy14643 either in the presence or absence of TBTO suggested differential expression of genes previously shown to be PPAR -targets in mammals. These genes encode proteins with functions in lipid metabolism/homeostasis, immune functions, mitochondrial functions and proteasome maintenance. The presence of TBTO resulted in a significant downregulation of several of these genes, consistent with an inhibition of PPAR by Wy14643. The conclusions drawn from this microarray experiment are made tentatively, however, since the study was done on hepatocytes from a single fish only and with false discovery correction omitted, and thus false positive results are likely to be present.

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Publications and Conferences Attended

Publications

- **Colliar, L**., Sturm, A. & Leaver, M.J. (2011) Tributyltin is a potent inhibitor of piscine peroxisome proliferator-activated receptor and . *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology*, 153 (1), pp. 168-173

- Sturm, A., **Colliar, L.,** Leaver, M.J. and Bury, N.R. (2011) Molecular determinants of hormone sensitivity in rainbow trout glucocorticoid receptors 1 and 2. *Molecular and Cellular Endocrinology*, 333(2), pp 181-189

In preparation:

- **Colliar, L.,** Leaver, M.J. and Sturm, A. Chemical disruption of corticosteroid receptor function in European flounder (*Platichthys flesus*).

Conference contributions

Institute of Aquaculture PhD Research Conference (2008), Stirling, UK.

- **Colliar, L.,** Leaver, M.J. and Sturm, A. Effects of chemical pollutants on energy homeostasis in fish: Interaction with peroxisome proliferator-activated receptors and glucocorticoid receptors - **Poster**

Meeting of the Society for Experimental Biology (2009), Glasgow, UK

- **Colliar, L.,** Leaver, M.J. and Sturm, A. A reporter gene assay to test for pollutant activation of fish peroxisome proliferator-activated receptors – **Poster**

15th International Symposium on Pollutant Responses in Marine Organisms (2009), Bordeaux, France

- **Colliar, L.,** Leaver, M.J. and Sturm, A. Interaction of organic pollutants with flounder peroxisome proliferator-activated receptors and corticosteroid receptors – **Poster**

Institute of Aquaculture PhD Research Conference (2010), Stirling, UK

- **Colliar, L.,** Leaver, M.J. and Sturm, A. A persistent environmental pollutant as an endocrine disruptor via the peroxisome proliferator-activated receptor (PPAR) – **Oral presentation**