EXAMINING THE SUITABILITY OF MOLECULAR AND METABOLOMIC-BASED TECHNIQUES AS TOOLS FOR ASSESSING THE EFFECTS OF PHARMACEUTICALS IN THE AQUATIC ENVIRONMENT

A thesis submitted for the degree of Doctor of Philosophy

by

Paul David Eccles

Department of Biosciences, Brunel University,
Uxbridge, Middlesex. UB8 3PH

August 2009

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE

ANOVA	- Analysis of variance
APS	- Ammonium persulfate
ATF2	- Activating transcription factor 2
BAT	- Brown adipose tissue
BCF	- Bioconcentration factor
bp	- Base pairs
$CaCO_3$	- Calcium carbonate
cAMP	- Cyclic3'5'-adenosine monophosphate
cDNA	- Copy deoxyribonucleic acid
CE	- Capillary electrophoresis
cGMP	- Cyclic guanosine monophosphate
$CHCl_3$	- Chloroform
CHMP	- Committee for medicinal products for human use
CI	- Condition index
CNS	- Central nervous system
C_T	- Cycle threshold
CV	- Coefficient of variance
DEPC	- Diethylpyrocarbonate
DNA	- Deoxyribonucleic acid
dNTP	- Deoxyribonucleotide triphosphate
DO_2	- Dissolved oxygen
DWC	- Dilution water control
DWD	- Drinking water directive
EA	- Environment agency
EC_{50}	- 50% effective concentration
EDC	- Endocrine disrupting chemical
EDTA	- Ethylenediaminetetraacetic acid
EE_2	- Ethinyloestradiol
E_2	- Oestradiol
ELISA	- Enzyme-linked immunosorbant assay
EMEA	- European medicines agency
eNOS	- Endothelial constitutive NO synthase
EPA	- Environmental protection agency
ERA	- Environmental risk assessment
ERK	- Extracellular signal-related kinase
ESI-MS/MS	- Electrospray ionization-tadem mass spectrometry
ESR1	- Oestrogen receptor 1
ESR2	- Oestrogen receptor 2
EU	- European Union
FDR	- False discovery rate
FID	- Free induction decay
$F_{SS}PC$	- Fish stead state plasma concentration
FT-ICR-MS	- Fourier transform ion cyclotron resonance mass spectrometer

GC	- Gas chromatography
GC/MS	- Gas chromatography mass spectrometry
GDP	- Gross domestic product
GDP (ii)	- Guanine di-phosphate
GMP	- Good manufacturing practice
GPCR	- G-protein-coupled receptor
GRE	- glucocorticoid-responsive element
GRK	- G-protein coupled receptor protein kinase
GSI	- Gonadosomatic index
GTP	- Guanine tri-phosphate
H^+	- Hydrogen ion
HPLC	- High-performance liquid chromatography
HSL	- Hormone-sensitive lipase
H_TPC	- Human therapeutic plasma non-observed effect concentration
IMS	- Industrial methylated spirit
IPTG	- Isopropyl β-D-1-thiogalactopyranoside
ISO	- International organisation for standardization
JRES	- J-resolved
K_{oc}	- Organic carbon-normalised distribution coefficient
K_{ow}	- Water partition coefficient
k_{photo}	- Photolysis rate
L-broth	- Luria broth
LC/MS	- Liquid chromatography mass spectrometry
LC_{50}	- 50% lethal concentration
LDA	- Linear discriminate analysis
LOEC	- Lowest observed effect concentration
$LOEC^{survival}$	- Lowest observed effect concentration on survival
logP	- Blood: water partition coefficient
$MgSO_4$	- Magnesium sulphate
MCA	- Medicines control agency
MeOH	- Methanol
mRNA	- Messenger ribonucleic acid
MS	- Mass spectrometry
MS222	- Tricane methane sulhponate
Na^+	- Sodium ion
NAD+	- Nicotinamide adenine dinucleotide
NaOH	- Sodium hydroxide
NCBI	- National centre for biotechnology information
NO	- Nnitric oxide
NOEC	- Non-observed effect Concentration
NMR	- Nuclear magnetic resonance

NTC	- Non template control
OECD	- Organisation for economic co-operation and development
PAH	- Polycylic aromatic hydrocarbon
PCA	- Principal component analysis
PCR	- Polymerase chain reaction
PDE 2	- Phosphodiesterase 2
PDE 3	- Phosphodiesterase 3
PEC	- Predicted environmental concentration
$PEC_{surfacewater}$	- Predicted environmental concentration in surface water
PER	- Plasma Exposure Ratio
PGC-1α	- Peroxisome gamma coactivator 1 alpha
pJRES	- 1D skyline projections of J-resolved
pK_a	- Acid dissociation constant
PKA	- Protein kinase activation
PKA (ii)	- Protein kinase A
PKG	- cGMP-dependent protein kinase
PLSDA	- Partial least squares discriminate analysis
PNEC	- Predicted no-effect concentration
PPARγ	- Peroxisome proliferator-activated receptor-gamma
PPT	- Protein precipitation
p38 MAPK	- p38 mitogen-activated protein kinase
Q-PCR	- Quantitative polymerase chain reaction
QSAR	- Quantitative structure-activity relationship
RACE PCR	- Rapid amplification of cDNA ends polymerase chain reaction
RBC	- Red blood cell
RMSECV	- Root mean square error of cross-validation
RNA	- Ribonucleic acid
RSD	- Relative standard deviation
RT-PCR	- Real time polymerase chain reaction
SD	- Standard deviation
Src	- Cellular tyrosine kinase
SRM	- Selected reaction monitoring
SOC	- Super optimum broth with catabolite repression
SPE/LC	- Solid-phase extraction/liquid chromatography
STW	- Sewage treatment work
TBE	- Tris borate EDTA buffer
TEMED	- Tetramethylethylenediamine
TMD	- Transmembrane domain

TMSP	- Sodium 3-trimethylsilyl-1-2,2,3,3-d_4-propionate
tRNA	- Total ribonucleic acid
UCP1	- Uncoupling protein 1
USFDA	- The United States food and drug administration
UTR	- Untranslated region
UV	- Ultraviolet
VTG	- Vitellogenin
WAT	- White adipose tissue
^{1}H-NMR	- Proton nuclear magnetic resonance
1D	- One dimensional
2D	- Two dimensional
4R	- Tetraploid
βARK	- β-AR kinase
β-NHE	- β Na^+/H^+

DECLARATION

The work submitted in this thesis was conducted between 2005 and 2009 at Brunel University (Uxbridge, Middlesex) and Birmingham University (Edgbaston, Birmingham). This work was carried out independently and has not been submitted for any other degree.

ABSTRACT

Pharmaceuticals represent important and indispensable elements in modern society and their usage is considerable. Post consumption and body-elimination, pharmaceuticals are not completely removed in sewage treatment works (STWs) and as such, have been detected at low levels in STW effluents, surface waters, seawaters, ground waters and some drinking waters. Accordingly, pharmaceutical toxicity has been detected in several aquatic organisms. To date, environmental risk assessments (ERA) examine for toxicity using a series of chronic toxicity assays that examine for standard physiological responses in algae, *Daphnia* and fish and do not address pharmaceutical mode of action. Therefore, using the fathead minnow (*Pimephales promelas*) and the β-blocking pharmaceutical propranolol as the test-species and test-drug, respectively, the aim of this study was to establish an intelligent targeted 4-phased ERA using molecular, *in vivo* exposure, metabolomic and quantitative expression analytical techniques. The first phase established that the fathead minnow expressed the β_{3bi}-adrenergic receptor (AR), which is a target receptor for propranolol in humans. The *in vivo* pair-breeding assay suggested that at $1mgL^{-1}$ and $10mgL^{-1}$, propranolol levels in fish blood plasma exceeded the human therapeutic concentration and caused 80% and 100% mortality, respectively. The most likely causes of mortality were liver failure and central nervous system toxicity. It was not possible to identify a robust biomarker of propranolol exposure using proton nuclear magnetic resonance (^{1}H NMR) as there was considerable metabolic variation between male liver tissues within the same treatment groups. β_{3bi}-AR expression was significantly lower at $1mgL^{-1}$ in the brain and liver, which was most likely the result of desensitisation in response to elevated levels of epinephrine and cortisol. β_{3bi}-AR expression was significantly increased in the heart at the environmentally relevant concentration of $0.001mgL^{-1}$, however it was not possible to link β_{3bi}-AR expression to a toxic response. Propranolol is unlikely to pose a threat to the aquatic environment as the concentrations measured in the environment are approximately 1000-fold lower than those that induced a toxic response. The proposed ERA represents a marked improvement over the existing ERA as it addresses pharmaceutical mode of action and both subtle and physiological toxicity responses, however it still requires further validation studies to address both metabolomic and gene expression variation.

ACKNOWLEDGEMENTS

I would like to firstly thank Dr Mariann Rand-Weaver for her supervision, continued support and encouragement throughout this PhD. Despite her many other commitments, Mariann always made time to help me and she is an inspiration to me, both professionally and personally. Thanks also goes to Professor John Sumpter and also to Dr Mark Viant at Birmingham University for his help and managing to teach me the fundamentals of NMR spectroscopy! I must also thank my surrogate "laboratory mum" Denise May for teaching me all the practical aspects of molecular biology and for her emotional support throughout my PhD, and to Dr Tamsin Runnalls for always making time to help and advise me with my molecular/exposure study work. I must also thank Emma, Steve, Julie and Sue for their help with the exposure studies and equally to Katie, Luigi, Jayne and Cath for their help on sampling days. To Dr Matthew Winter for kindly analysing the water and plasma samples and to Dr Evgeny Makarov for his help in building the polyacrylamide silver staining vessel and helping to analyse the RNA samples. To Adam and Andy at Birmingham University Environmental Metabolomics Research Laboratory for walking me through metabolomic preparation and analysis of samples and to Nadine for also helping me find a roof over my head! I could not have completed my metabolomics research without the help of Dr Huifeng Wu, who kindly guided me through NMR analysis and Helen Parsons for her help in understanding and interpreting the results. To my family of friends and "Science Alliance", who kept me going with their humour, encouragement, science-facts and days-of-fun breaks from the laboratory! To my "consultant mathematician" mum and dad for their love and encouragement throughout all my studies, without whom I would not have ventured into studying a PhD. Mum, your support really kept me going and dad, you're allowed to claim some credit for the PhD…after all, we share the same initials!

I finally want to thank my lucky charm and fiancé, Gemma, for her invaluable and unconditional love and support and patience. For putting up with piles of papers over the spare room, no evenings and holidays and for always picking me up at times when I felt low. I really could not have done it without her…….

……..it is to her and my family that this thesis is dedicated.

PUBLICATIONS

Below details the papers and presentations taken from the research reported in this thesis.

Giltrow, E., **Eccles, P.D**., Rand-Weaver, M., Hutchinson, T.H. & Sumpter, J.P (2009) β-adrenergic receptors in the fathead minnow (*Pimephales promelas*): part I – characterisation and identification. *Submitted to General and Comparative Endocrinology for publication*.

Giltrow, E., **Eccles, P.D**., Winter, M.J., McCormack, P.J., Rand-Weaver, M., Hutchinson, T.H. & Sumpter, J.P. (2009). Chronic effects assessment and plasma concentrations of the β-blocker propranolol in fathead minnows (*Pimephales promelas*). *Accepted for publication in Aquatic Toxicology*.

Eccles, P.D., Parsons, H., Wu, H., Giltrow, E., Sumpter, J.P., Rand-Weaver, M. & Viant, M.R. (2008). Examining the suitability of metabolomics in ecotoxicity studies. SETAC Europe, Warsaw, Poland.

Additional publications taken from research not reported in this thesis include:

Thorpe, K.L., Benstead, R., **Eccles, P.D.,** Maack, G., Williams, T. & Tyler, C.R. (2008). A practicable laboratory flow-through exposure system for assessing the health effects of effluents in fish. *Aquatic Toxicology*. **88(3)**. 164-172.

Allen, Y., Katsiadaki, I., Pottinger, T.G., Jolly, C., Matthiessen, P., Mayer, I., Smith, A., Scott, A.P., **Eccles, P.D.,** Sanders, M.B., Pulman, K.G.T. & Fesit, S. (2008). Intercallibration exercise using a stickleback endocrine disrupter screening assay. *Environmental Toxicology and Chemistry*. **27(2)**. 404-412.

CHAPTER 1: GENERAL INTRODUCTION

1.0 Pharmaceuticals in the environment

The United States Food and Drug Administration (USFDA) and European Union (EU) define a pharmaceutical drug as a substance intended to affect the structure or any "function of the body" for the purpose of cure, mitigation, treatment or prevention of a disease (Williams, 2006). Unsurprisingly, pharmaceuticals represent important and indispensable elements of modern life. They are employed in a variety of disciplines including human and veterinary medicine, agriculture and aquaculture. In 2002, the UK pharmaceutical industry alone contributed 0.6% of the total U.K gross domestic product (GDP) and accounted for 3% of world sales (£8.5 billion). It has been estimated that over 3000 active substances are licensed for use in the U.K and that usage exceeds 10 tonnes per annum for all of the top 25 compounds and 100 tonnes per annum for the top three compounds (paracetamol, metformin hydrochloride and ibuprofen) (Ayscough *et al*, 2000; Ernst & Young, 2003; Bound & Voulvoulis, 2004). These figures only represent prescription drug usage and do not include drugs sold over-the-counter, and and so actual usage could be considerably higher (Fent *et al*, 2006).

Coupled with their large volume production, pharmaceuticals are biologically active compounds that are designed to have a specific mode of action in the body. Furthermore, pharmaceuticals are usually lipophilic and often have a low biodegradability. These intrinsic properties pose potential for bioaccumulation and persistence in the environment (Christensen, 1998; Fent *et al*, 2006).

1.1 Routes of exposure

The potential sources of pharmaceuticals into the aquatic environment, excluding non-routine events such as potential transport accident, include release from pharmaceutical manufacturers, disposal of pharmaceutical from the supply chain (prior to distribution to patients), disposal of unused pharmaceuticals by patients or health care facilities in wastewater and patient excretion of an active pharmaceutical ingredient (Williams *et al*, 2006). The manufacture of pharmaceuticals in the UK is controlled by the Good Manufacturing Practice (GMP) regulations. Furthermore, sites are regularly inspected by the Medicines Control Agency (MCA) to ensure the quality and safety of products.

Therefore wastage from manufacturing sites is kept to a minimum due to the careful handling and packaging of the expensive pharmaceutical products (Ayscough *et al*, 2000). This therefore leaves use and disposal as key routes of pharmaceutical entry into the aquatic environment.

After consumption, most pharmaceuticals undergo complete or extensive Phase I and Phase II hepatic metabolism. Phase I reactions consist of oxidation, reduction and/or hydrolysis, whilst Phase II involves conjugation (e.g. the addition of glucuronic acid, sulphate, acetic acid or amino acid). These processes yield polar metabolites excreted in the urine, which normally exhibit insignificant pharmacological activity. However, in some instances, the parent-pharmaceutical-compound can be resistant to hepatic metabolism and can be excreted in its pure, biologically active, form (Ternes, 1998; Hirsch *et al*, 1999). Sewage is taken to sewage treatment works (STWs) where the majority of drugs are removed by either mineralisation by micro-organisms to carbon dioxide and water or adsorption to suspended solid sludge particles. Adsorption is dependent on both the hydrophobic and electrostatic interactions of the pharmaceutical with particulate matter. Acidic pharmaceuticals such as the non-steroidal anti-inflammatory drug (NSAID) acetylsalicylic acid do not adsorb readily to sludge and so elimination via mineralisation is most common (Ternes *et al*, 2004). STW elimination rates vary depending on the nature of the pharmaceutical, STW treatment technology and performance, temperature and weather (Vieno *et al*, 2007; Fent *et al*, 2006). Consequently, pharmaceutical elimination efficiencies range from 7 – 99%; for example, the elimination efficiency rate for carbamazepine was 7 to 8% whilst it was 99% for salicylic acid (Ternes, 1998; Heberer, 2002). Accordingly, a proportion of a pharmaceutical enters the aquatic environment via sewage effluent. In some cases STWs can activate pharmaceutical potency. There is evidence to suggest that conjugated pharmaceutical metabolites entering the STW may be hydrolysed to the parent drug through bacteria hydrolases, therefore reaching the environment in their active form (Panter *et al*, 1999; Jessica *et al*, 2007). For example metabolites of the pharmaceutical clofibric acid have been found to hydrolyse back to their parent compound (Daughton & Ternes, 1999). STWs represent the main route of pharmaceutical entry into the aquatic

environment; moreover they represent a constant source of pharmaceutical entry (Barceló & Petrovic, 2007).

Additional routes of entry include via veterinary medical applications. Unlike human pharmaceuticals, veterinary pharmaceuticals do not pass through STWs and so may enter the aquatic environment in their pure active state. Livestock manure, which may contain active pharmaceutical ingredients, is used as agricultural fertiliser and there is the potential for pharmaceuticals to enter the aquatic environment via leaching and run-off. STW sludge is also used as a soil conditioner and so human pharmaceuticals may enter the aquatic environment by similar means (Ternes, 1998; Jørgensen & Halling-Sørensen, 2000).

1.2 Pharmaceutical fate in the environment

The fate and transport behaviour of a pharmaceutical in a river/stream/water body very much depends on its physicochemical properties. For example the acid dissociation constant (pK_a) value will determine how much the pharmaceutical will dissociate at different environmental pH values, whilst the octanol and water partition coefficient (K_{ow}) values determines the likelihood of a pharmaceutical moving from a polar, water-soluble form into a non-polar fat-soluble form. A pharmaceutical with a high K_{ow} value is hydrophobic or lipophilic and is therefore more likely to accumulate in animal or plant tissues. Also, the organic carbon-normalised distribution coefficient (K_{oc}) value determines whether a pharmaceutical will interact and adsorb onto organic particle molecules during STW removal. Interactions with the environment will also determine the likely fate of a pharmaceutical. For example, the abiotic transformation of a pharmaceutical in surface waters may occur by hydrolysis and photolysis. Since pharmaceuticals are usually resistant to hydrolysis, the extent of this event is usually negligible. The photolysis rate (k_{photo}) value for a pharmaceutical determines the likelihood of transformation and degradation in the presence of sunlight (both ultraviolet (UV) and visible light) (Kümmerer *et al,* 2006). Direct photolysis of a compound is caused by direct absorption of solar light whereas indirect photolysis involves natural photosensitisers such as nitrate and humic acids. Pharmaceutical degradation due to both

direct and indirect photolysis has been experimentally demonstrated (Nikolaou *et al*, 2007). Therefore, the potency of the same pharmaceutical can differ hugely between different aquatic environments. There is also the potential for pharmaceuticals with a high degree of degradation resistance to accumulate in the aquatic environment.

1.3 Pharmaceutical concentrations in the aquatic environment

Arguably, our progressive understanding of pharmaceutical toxicity in the aquatic environment is driven by improvements and developments in water chemistry analysis (Sacher *et al*, 2001; Fent *et al*, 2006). To date, more than 50 pharmaceutical compounds have been detected in sewage, surface and ground waters throughout the U.K., Western and Eastern Europe, Asia and North and South America and Australasia. Table 1 provides a representative list of pharmaceuticals that have been detected in wastewater effluents and, in some cases, surface waters. This is not by any means a comprehensive list of all pharmaceuticals that have been identified, nor are the pharmaceuticals solely present at the identified locations.

In order to obtain a high degree of selectivity, which is extremely important when the water sample may contain a large number of closely-related compounds, chromatography followed by mass spectrometry allows for many pharmaceutical compounds to be identified and quantified with a high degree of accuracy (Ternes, 1998). This analytical and identification process has improved dramatically over the past decade, giving rise to several techniques. In some instances, load figures for the same pharmaceutical (e.g. the antibiotic trimethoprim) varied between different studies, which can either be the result of using different techniques (e.g. liquid chromatography followed by mass spectrometry [LC/MS] compared with gas chromatography followed by mass spectrometry [GC/MS]) or due to internal factors such as water matrix effects, which in turn can lead to either suppressed or enhanced measurements (Terzic *et al*, 2008). An additional source of variation between chemical measurements of drugs is unregulated drug prescriptions. For example levels can be higher in countries where prescription drugs can be purchased over the counter. Accordingly, the figures in table 1 merely provide an indication/estimation of pharmaceutical loads in the environment.

Unsurprisingly, the concentration of analgesics and anti-inflammatory drugs were relatively high compared with other pharmaceutical classes as would be expected for non-prescription over-the-counter drugs where use is considerably higher than prescription drugs (Reemtsma *et al*, 2006). Coupled with the fact that it is poorly metabolised and has a high K_{ow} value, ibuprofen in particular was found at considerably high levels (Ashton *et al*, 2004; Thomas & Hilton, 2004; Gros *et al*, 2006; Peng *et al*, 2008). Larsson and colleagues (2007) measured ciprofloxacin at $31mgL^{-1}$. This was unusually high compared to other pharmaceuticals; however Larsson and colleagues noted that over 90 pharmaceutical companies discharged into a single Indian STW, from which effluent was measured.

Table 1. Pharmaceuticals measured in effluent and (*) surface waters. ★ denotes unusually high quantity; Larsson *et al* (2007) reported that over 90 pharmaceutical companies discharged into a single STW.

Country	Class	Pharmaceutical	Concentration range (ngL^{-1})	Reference
U.K., Spain, Germany, South China	Analgesics and anti-inflammatory	Ibuprofen	<8 - 27,256*	Ashton *et al* (2004); Thomas & Hilton (2004); Gros et al (2006); Ternes (1998)
Spain		Ketoprofen	<28 - 1520	Gros *et al* (2006)
Spain, Germany, South China		Naproxen	<9 - 1550	Gros *et al* (2006); Ternes (1998); Peng *et al* (2008)
Spain		Indomethacine	<7 - 240	Gros *et al* (2006)
Spain, Germany, U.K.		Diclofenac	50 – 4200*	Gros *et al* (2006); Ternes (1998) Ashton et al (2004); Thomas & Hilton (2004)
Spain, U.K.		Mefenamic acid	<3 – 1440*	Gros *et al* (2006); Ashton et al (2004); Thomas & Hilton (2004)
U.K.		Dextropropoxyphene	<8 - 682*	Ashton et al (2004); Thomas & Hilton (2004)
Spain		Propyphenazone	<7 - 461	Gros *et al* (2006)
South China, Canada		Salicylic acid	24 – 35,000	Peng *et al* (2008); Brun *et al* (2006)
Spain	Psychiatric	Carbamazepine	120 - 1550	Gros *et al* (2006)
Spain		Fluoxetine	35	Gros *et al* (2006)
Spain		Paroxetine	6	Gros *et al* (2006)
Croatia, U.K.	Antibiotic	Trimethoprim	35 – 2550*	Senta *et al* (2005); Ashton et al (2004); Thomas & Hilton (2004)
U.K.		Sulfamethoxazole	<50 - 132*	Ashton *et al* (2004)
U.K.	Anti-cancer	Tamoxifen	<4 - 71	Thomas & Hilton (2004)
Croatia	Sulfonamide	Sulfadiazine	<1 - 132	Senta *et al* (2005)
Croatia		Sulfapyridine	<4 - 931	Senta *et al* (2005)
Croatia		Sulfamethoxazole	19 - 11600	Senta *et al* (2005)
Croatia, U.K.	Fluoroquinolone	Erythromycin	24 – 1842*	Senta *et al* (2005); Ashton *et al* (2004)
Croatia		Azithromycin	6 - 1140	Senta *et al* (2005)
Croatia		Josamycin	<8 - 16	Senta *et al* (2005)
Croatia		Roxithromycin	<1 - 50	Senta *et al* (2005)

Croatia	Fluoroquinolone	Norfloxacin	16 - 2940	Senta *et al* (2005)
Croatia, India		Ciprofloxacin	<7 – 31,000,000★	Senta *et al* (2005); Larsson *et al* (2007)
Croatia		Enrofloxacin	<2 - 18	Senta *et al* (2005)
Spain, Canada	Beta-blocker	Atenolol	<42 - 7560	Gros *et al* (2006); Lee *et al* (2007)
Spain, Canada		Sotalol	<29 - 1080	Gros *et al* (2006); Lee *et al* (2007)
Spain, Germany, Canada, U.S.A,		Metoprolol	<12 - 4680	Gros *et al* (2006), Ternes (1998); Lee *et al* (2007); Huggett *et al* (2003)
Spain, U.K., Germany, Canada, U.S.A		Propranolol	<12 - 255	Gros *et al* (2006) Ashton et al (2004); Thomas & Hilton (2004) Ternes (1998); Lee *et al* (2007); Huggett *et al* (2003a)
Canada		Acebutolol	<10 - 1090	Andreozzi *et al* (2003); Lee *et al* (2007)
Canada		Labetalol	64 - 279	Lee *et al* (2007)
Canada		Bisoprolol	20 - 74	Lee *et al* (2007)
Canada		Timolol	<7 - 7	Lee *et al* (2007)
Canada, U.S.A		Nadolol	26-360	Lee *et al* (2007); Huggett *et al* (2003a)
Canada		Salbutamol	<6 - 17	Lee *et al* (2007)
Spain	Anti-ulcer	Lansoprazole	14	Gross et al (2006)
Spain	Histamine H1 and H2	Loratidine	4	Gross et al (2006)
Spain	receptor agonist	Famotidine	<12 - 120	Gross et al (2006)
Spain		Ranitidine	<24 - 758	Gross et al (2006)
Spain, Germany	Lipid regulator and cholesterol	Gemfibrozil	<3 -1700	Gross et al (2006); Ternes (1998)
Spain, Germany	lowering drug	Bezafibrate	9-350	Gros *et al* (2006); Ternes (1998)
Spain		Pravastatin	<60 - 1170	Gross et al (2006)
U.K., Germany, South China		Clofibric acid	<20 - 248	Thomas & Hilton (2004); Ternes (1998); Peng *et al* (2008);
Germany		Fenofibric acid	45	Ternes (1998)
South China	Synthetic steroid	17 α-ethinyloestradiol	1 - 2	Peng *et al* (2008)
U.K.		(EE_2)	13.6 - 24	Johnson *et al* (1999); Aherne & Briggs (1989)
Germany & Russia			15	Rathner & Sonneborn (1979); Ternes *et al* (1999)
Canada			42	Ternes *et al* (1999)

1.4 Pharmaceutical effects on the aquatic environment

1.4.1 Mode of Action

Pharmaceuticals achieve their medical application via binding with a drug target, such as a receptor or an enzyme and initiating a response. Drug targets have been classified into 4 major groups: receptors, ion channels, enzymes and transporters (see figure 1) (Rang *et al*, 2003). In general, there are three requirements for a pharmaceutical to elicit a response in a body. The first is that the drug structure is bioactive, the second is that the organism has the necessary drug target (or site of action), and the third is that there must be a link between the drug target and a response mechanism (Williams, 2006). All commercially available pharmaceuticals meet these requirements in humans.

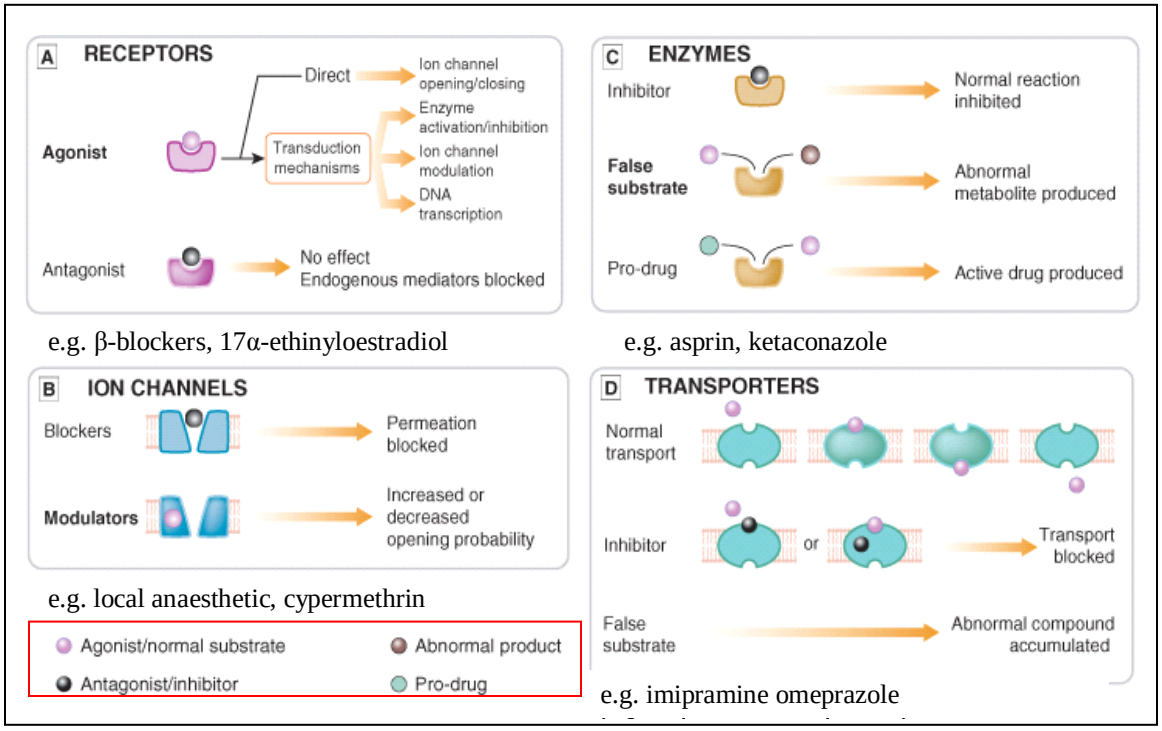


Figure 1. The four major groups of drug targets. Figure taken from Rang *et al*, 2003.

Advances in DNA sequencing and subsequent bioinformatics and mathematical modelling have led to the elucidation of full genomic sequences, and subsequent functional understanding (albeit limited compared to humans), for several test-species

including fish. A limited review of fish receptors and enzyme systems by Huggett *et al* (2003b) indicated that overall receptor and enzyme identities were 31 – 88% similar to mammalian targets. Therefore, it is possible that the therapeutic targets in humans may also be present in fish and, as such, pharmaceuticals present in the aquatic environment could interact with their target receptors and elicit a pharmacological response. Furthermore, some aquatic invertebrate taxa such as molluscs appear to contain many of the same enzyme and receptor systems expressed in mammals, however their pharmacological function is still poorly defined (Huggett *et al*, 2006).

There is also evidence to suggest that fish and aquatic invertebrates may express receptors in tissues different to that in humans, indicating that pharmacodynamic activities of certain drugs may also be very different (Nickerson *et al*, 2003; Laville *et al*, 2004). Furthermore, one cannot rule out potential cross-talk, where a drug designed to interact with a specific receptor in a human interacts with a structurally similar, but functionally very different, receptor in an aquatic organism. Finally, another interesting avenue to explore is whether a pharmaceutical present at the same pharmaceutical dose in the aquatic environment as administered in humans will elicit the same response in aquatic organisms. Currently, there is very little data available to answer that question.

1.4.2 Measurable effects

The first measurable effects of pharmaceuticals in the aquatic environment were observed over twenty years ago in the U.K. Male gonochoristic fish living downstream of STWs in the River Lea catchment (South-East England) were showing signs of intersex, or hermaphroditism. Further studies suggested that the principal component in the female contraceptive pill, α-ethinyloestradiol (EE_2), was present in STW effluent and was responsible for the feminisation of fish (Tyler & Routledge, 1998). Interestingly, although EE_2 has been measured in the aquatic environment (see table 1), it has not been found at considerably higher levels than other pharmaceuticals. However, EE_2 is highly lipophilic (log K_{ow} = 4.48) and so it is extremely likely to move from water into tissues and bioaccumulate. Although much attention has focused on the effects of EE_2, ecotoxicological studies have also examined the effects of other pharmaceuticals on

wildlife. Most notably, a population decline of >95% in the Oriental white backed vulture, *Gyps bengalensis*, was linked to the non-steroidal anti-inflammatory drug diclofenac (Oaks *et al*, 2004). By eating the carcasses of cattle treated with the drug, the vultures ingested diclofenac and subsequently suffered from renal failure and visceral gut. These two case studies alone have provided enough cause for concern to further examine pharmaceutical toxicity in the environment.

The possible effects of aquatic environmental exposure to pharmaceuticals can be classified into three groups, the first is a standard acute toxic response, the second is antibiotic resistance and third is endocrine disruption (or hormone mimicking) (Jørgensen & Halling-Sørensen, 2000).

1.4.2.1 Acute toxicity

The impact of a pharmaceutical can be on any level of biological hierarchy: cells-organs-organisms-population-ecosystems-ecosphere. At least 24 pharmaceuticals have been examined for their acute toxicity on aquatic wildlife (Ayscough *et al*, 2000). Measurable physiological effects include mortality, decreased heart rate and inhibition of growth for vertebrate fish (Henschel *et al*, 1997; Huggett *et al*, 2002; Larsson *et al*, 2006); growth inhibition, immobilisation and mortality for the invertebrate *Daphnia magna* water flea and mysid shrimp (*Mysidopsis bahia*) (Lilius *et al*, 1995; Henschel *et al*, 1997; Huggett *et al*, 2002); and growth inhibition, inhibition of luminescence and mortality for algae and bacteria (Sanyal *et al*, 1993; Kümmerer *et al*, 2000; Stuer-Lauridsen *et al*, 2000); and morphological changes in the cnidarian *Hydra attenuata* (Quinn *et al*, 2008). This list is by no means comprehensive, but it does give an indication as to the acute toxic effects observed in aquatic species exposed to environmentally relevant concentrations of pharmaceuticals.

1.4.2.2 Antibiotic resistance

The mechanism of action for antibiotics is very different from other pharmaceuticals because bacteria are the target organisms. The increased use of antibiotics over recent years has caused a genetic selection of more harmful bacteria that have become resistant

to antibiotics. The genetic pool of microorganisms within the aquatic environment has changed significantly, and one of the catalysts for this change is the introduction of xenobiotics. The development of antibiotic-resistance in aquatic ecosystems is favoured by low concentrations of antibiotics (Petersen *et al*, 1997) and therefore the surviving bacteria may be particularly harmful to other wildlife (Jørgensen & Halling-Sørensen, 2000; Williams, 2006). Antibiotic resistance as a result of pharmaceutical exposure is still poorly understood.

1.4.2.3 Endocrine disruption

To date, much attention has focused on this effects group because of the observed significant physiological impact. Following on from the initial work conducted by Tyler and Routledge (1998), hermaphrodite fish have also been observed in rivers below STW outlets in a number of countries including the U.K. (Purdom *et al*, 1994; Harries *et al*, 1997; Jobling *et al*, 1998) and the U.S.A (Folmar *et al*, 1996); there is a strong positive correlation between incidence and severity of feminisation of male fish and proportion of treated sewage effluent in receiving waters (Gross-Sorokin *et al*, 2006). Fish feminisation has also been measured in marine populations of flounder (*Platichthys flesus*) (Jobling *et al*, 2002) dab (*Limanda limanda*) (Scott *et al*, 2007) and cod (Scott *et al*, 2006). In recent years, there has been a surge of government- and academia-led activity in researching the effects of oestrogenic compounds on aquatic life and it has been demonstrated that EE_2 can inhibit fish growth and reproduction and can induce a female yolk protein, vitellogenin, in male livers, which has subsequently been used as a marker of oestrogenic exposure in male fish (Purdom *et al*, 1994; Jobling *et al*, 1996; Tyler & Routledge, 1998). Furthermore, it has been demonstrated that long-term exposure to EE_2 can cause mortality at the embryo, juvenile fry and adult stages (Schweinfurth *et al*, 1996). Consequent effects on reproductive hierarchies, sexual selection and group spawning have also been found to be influenced by exposure to EE_2 (Coe *et al*, 2008).

1.5 β-blockers in the aquatic environment

β-blockers are prescribed to patients to treat migraines, essential tremors, stress, glaucoma and asthma and work by targeting β_1, β_2 and β_3-adrenergic receptors (β_1-ARs,

β_2-ARs and β_3-ARs, respectively). Because of their extensive use, β-blockers are in the top 200 prescribed medications in the U.S.A (Hernando *et al*, 2004). β-blockers oppose the excitatory effects of the neurotransmitters adrenaline and noradrenaline released from sympathetic nerve endings at β-ARs. There are at least 15 different beta-blockers available on the commercial market and they can be divided into two groups: specific and non-specific. β-blockers with a specific mode of action block one type of receptor, for example atenolol is a β_1-blocker and is prescribed to treat patients with hypertension-related illnesses. Non-specific beta-blockers, such as propranolol, interact with all three β-ARs (Gros *et al*, 2008).

The elimination of β-blockers after human consumption occurs via hepatic metabolism in the liver. Lipophilic β-blockers, such as propranolol (figure 2a) that has a logK_{ow} value of 3.78, are extensively metabolised and eliminated after glucuronidation, whilst the more hydrophilic β-blockers, such as atenolol (figure 2b) that has a log K_{ow} of 0.16, are almost exclusively excreted unchanged in urine (Lee *et al*, 2007). Due to their prevalence of use, β-blockers have been measured in environmental samples. As shown in table 2, at least 10 different β-blockers have been detected surface waters. It can be seen that β-blocker concentrations range from the ngL^{-1} to μgL^{-1} level, depending on both individual drug usage and STW removal efficiency rates; for example the removal rate of propranolol in a STW has been found to range from <10% (Roberts & Thomas, 2006) to 96% (Ternes, 1998), hence its presence in surface waters varies considerably. Regardless, β-blockers enter the aquatic environment and therefore there is the potential for β-blockers to directly interact with aquatic species and eliciting a toxicological effect.

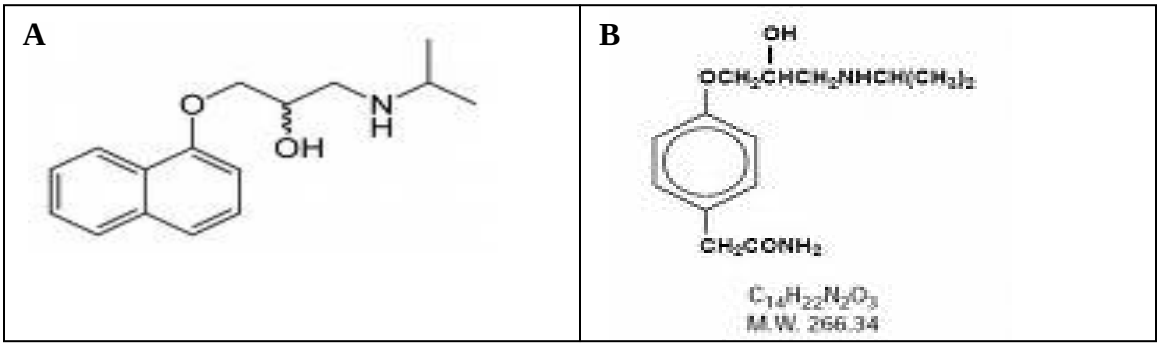


Figure 2. The chemical structure of propranolol (A) and atenolol (B).

Table 2. Minimum and maximum concentrations of β-blockers found in surface water samples throughout the world.

Compound	Surface water concentration (ngL^{-1})	Reference
Acebutolol	3 - 14	Vieno *et al* (2007)
Atenolol	3.44 - 859	Calamari *et al* (2003); Andreozzi *et al* (2003)
Betaxolol	28	Ternes, (1998)
Bisoprolol	2,900	Ternes, (1998)
Carazolol	110	Ternes, (1998)
Metoprolol	<3.8 - 2,200	Ternes (1998); Vieno *et al* (2006)
Propranolol	<10 - 590	Ashton *et al* (2004); Ternes, (1998)
Salbutamol	35	Ternes (1998)
Sotalol	<3.9-52	Vieno *et al* (2006)
Timolol	10	Ternes (1998)

1.5.1 Effects of β-blockers on aquatic organisms

Acute invertebrate toxicity studies have found that exposure to environmentally-relevant concentrations of β-blockers can inhibit algal (*Desmodesmus subspicatus, Cyclotella meneghiniana* and *Synechococcus leopolensis*) and *Lemna* (duckweed) growth and induce mortality and immobilize *Daphnia* water-flea (Cleuvers, 2004, Ferrari *et al*, 2004). However, of all the β-blockers, propranolol seems to pose the biggest threat to aquatic organisms. Propranolol is not found at the highest concentration compared to other β-blockers in the aquatic environment; however it has the highest log K_{ow} value and is therefore more likely to bioaccumulate in organism tissues. Furthermore, its Predicted Environmental Concentration/Predicted No Effect Concentration ratio (see section 1.6 for more details) is far higher than other β-blockers, suggesting it can elicit a toxic effect at much lower concentrations than other β-blockers (Cleuvers, 2004). Further fish-based studies have examined the effects of acute and chronic waterborne exposure to propranolol and have observed mortality (LC_{50} = 24.3mgL^{-1}), inhibition of growth

($0.5mgL^{-1}$) and reproduction ($<0.5mgL^{-1}$) and a decrease in heart rate ($1.5mgL^{1}$ via intravenous injection) (Huggett *et al*, 2002; Larsson *et al*, 2006).

1.5.2 β-blocker mode of action

In eukaryotes, communication between cells occurs via the release and synthesis of a signalling molecule and subsequent binding to a cell receptor. In mammals, the 'flight or fight' syndrome, or response to stress, is regulated by the catecholamines epinephrine (adrenaline) and norepinephrine (noradrenaline) (see figure 3). Norepinephrine is the major neurotransmitter in the peripheral nervous system, whereas epinephrine is the primary hormone secreted by the adrenal medulla in mammals. Both catecholamines are produced by chromaffin cells via the Blashko pathway from the amino acid precursor tyrosine (Reid *et al*, 1998). In times of stress (including exercise and trauma) secretion of these hormones is stimulated by acetylcholine release from preganglionic sympathetic fibres innervating the adrenal medulla. Following secretion into blood, the catecholamines bind loosely to, and are carried by, albumin and other serum proteins throughout the body to target tissues (Reid *et al*, 1998). Both catecholamines characteristically contain a catechol moiety and therefore their sudden presence in blood in response to a stress stimulus allows for a rapid response (Lodish *et al*, 2000). Both epinephrine and norepinephrine are involved in regulating a host of physiological functions such as peripheral excitation or inhibition of certain types of smooth muscles in different tissues and organs. Epinephrine is particularly important in mediating the body's response to stress, when all tissues have an increased requirement for glucose and fatty acids. Epinephrine triggers the breakdown of glycogen in the liver (glyocogenolysis) and triacylglycerol in the adipose tissues (lipolysis) and these principal metabolic fuels are rapidly supplied to the blood (Schwartz, 1996; Weaver, 2002).

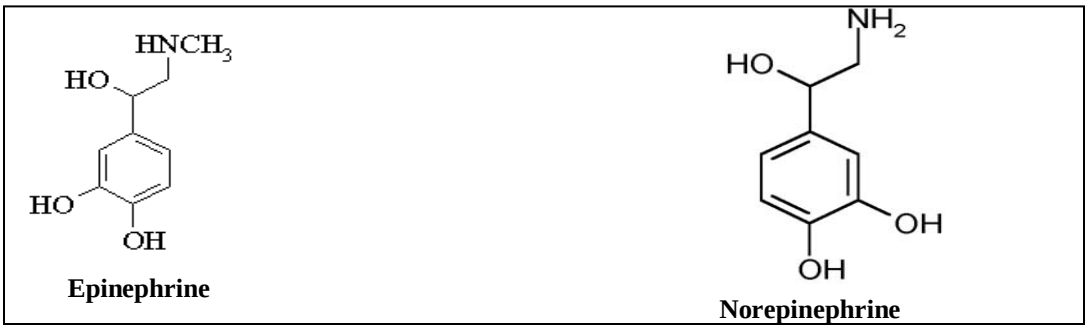


Figure 3. Chemical structures of epinephrine and norepinephrine. Image taken from www.biopsychiatry.com.

Catecholamines are unable to pass through cell plasma membranes and therefore, to initiate their action, bind to specific protein receptors, termed adrenergic receptors, located on the surface of cell membranes. Adrenergic receptors belong to the large family of integral membrane proteins termed the G-protein-coupled receptors (GPCRs). GPCRs are membrane receptors that are coupled to intracellular effector (response) systems via a Guanine-binding protein. Using large-scale phylogenetic analysis, GPCRs have been divided into 5 distinct families in the human genome: (G) Glutamate, (R) Rhodopsin, (A) Adhesion, (F) Frizzled/Taste2 and (S) Secretin, which has been aptly named the GRAFS classification system (Schiöth & Fredriksson, 2005). Adrenergic receptors belong to the largest Rhodopsin family. Subsequent studies examining agonist and antagonist binding have further divided the adrenergic receptors into two principal groups: α and β (Fredriksson *et al*, 2003). In mammals, nine different subtypes exist; α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C} and β_1, β_2, and β_3. Each subtype carries out a particular function and has been identified as a separate subtype based on the receptor's pharmacology, location and relative binding affinity for adrenaline and noradrenaline (Owen *et al*, 2007).

GPCRs are membrane-bound serpentine receptors and consist of a single polypeptide chain of up to 1100bp; their characteristic structure, as shown in figure 4, comprises seven hydrophobic transmembrane domain (TMD) α-helices which span the membrane in an anti-clockwise manner with an extracellular amino terminus (N-terminal domain) of varying length and an intracellular C-terminal domain that contains several sites for phosphorylation by protein kinase A and β-adrenoceptor kinase (Strosberg, 1993). There are over 800 different human GPCRs, all of which share this 7 TMD characteristic arrangement (Lodish *et al*, 2000; Schwartz, 1996; Warne *et al*, 2008). These transmembrane regions are crucially involved in the binding of ligands (Lefkowitz & Caron, 1988). The β-ARs are not fixed in a linear fashion within the membrane; instead through a series of amino-acid bonds between TMDs, they exist as a complex cylindrical 3-dimensional (3D) structure. The 3D structure produces a "pocket", which enables ligand binding. For example in the β_3-AR, bonding between TMDs 3, 5 and 7 and between TMDs 4, 5 and 6 produces an adrenaline-binding pocket (Blin *et al*, 1993; Strosberg & Pietri-Rouxel, 1996).

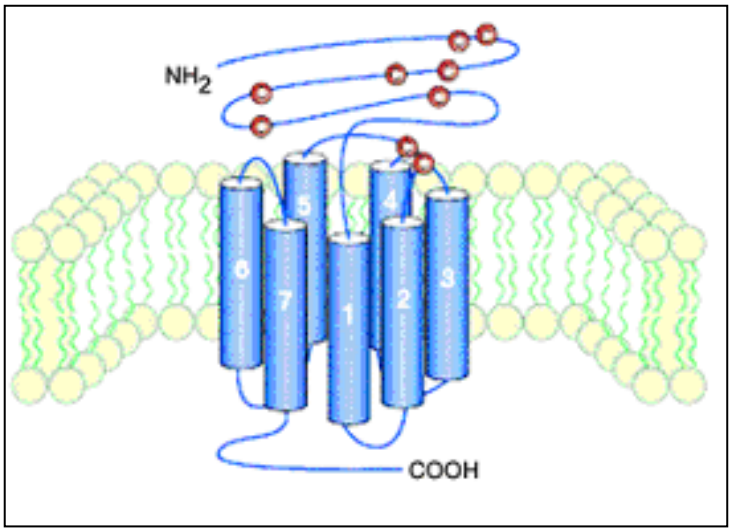


Figure 4. Three dimensional structure of a typical GPCR with noted TMD regions within the membrane lipid bilayer, external N-terminus and internal C-terminus. Image taken from Strosberg & Nahmias (2007).

G-proteins represent the level of middle management, intervening between the receptors and the effector enzyme or ion channel gate (Weaver, 2002). These proteins are so called G-proteins because of their interaction with the guanine nucleotides guanine tri-phosphate (GTP) and di-phosphate (GDP(ii)). The G-protein consists of three subunits α, β and γ and is freely diffusible in the membrane plane. The G_{α}-subunit is a GTPase switch protein that alternates between an active (on) state with bound GTP and an inactive (off) state with bound GDP(ii). The G_{α}-subunit has three different subtypes: $G_{\alpha q}$, to stimulate phospholipase C and $G_{\alpha s}$- and $G_{\alpha i}$- to stimulate or inhibit adenylyl cyclase, respectively (Lodish *et al*, 2000). Figure 5 illustrates the response of the G_{α}-subunit to receptor binding to a ligand. In a 'resting state', (stage 1), the G-protein exists as an unattached αβγ-trimer, with GDP(ii) occupying the site on the α-subunit. Upon stimulation by a ligand, (stage 2), a conformational change occurs in the cytoplasmic domain of the receptor causing it to acquire higher affinity for the αβγ-trimer. Association of the αβγ-trimer with the receptor causes the bound GDP(ii) to dissociate and be replaced with GTP, which in turn causes dissociation of the G-protein trimer, releasing α-GTP and βγ-subunits. The α-GTP subsequently binds to and activates the effector, (stage 3), which in the case of β_1 and β_2-ARs, and sometimes in β_3-ARs (depending on the tissue) is the enzyme adenylyl cyclase (Lodish *et al*, 2000). Once

activated, adenylyl cyclase catalyses the synthesis of the second messenger, cyclic3'5'-adenosine monophosphate (cAMP). cAMP is a nucleotide that regulates many aspects of cellular functioning including enzymes involved in energy metabolism, cell division and cell differentiation, ion transport, ion channels and the contractile proteins in smooth muscle. These varied effects are brought about by the activation of protein kinase A by cAMP. Protein kinases regulate the function of different cellular proteins by catalysing the phosphorylation of serine and threonine residues, using ATP as a source of phosphate. The binding of epinephrine to β-ARs induces a rise in cAMP production, which in turn activates enzymes involved in glycogen and fat metabolism in liver, fat and muscle cells. The overall result is a coordinated response to noradrenalin/adrenaline in which stored energy in the form of glycogen and fat is made available as glucose to fuel muscle contraction and enable the individual to respond to the stress-stimulus (Alberts, 1994; Watson & Arkinstall, 1994). The activation process is terminated when the hydrolysis of GTP to GDP(ii) occurs through the GTPase activity of the α-subunit. The resulting α-GDP(ii) then dissociates from the effector enzyme and reunites with the β-complex, (1) (Watson & Arkinstall, 1994; Lodish *et al*, 2000).

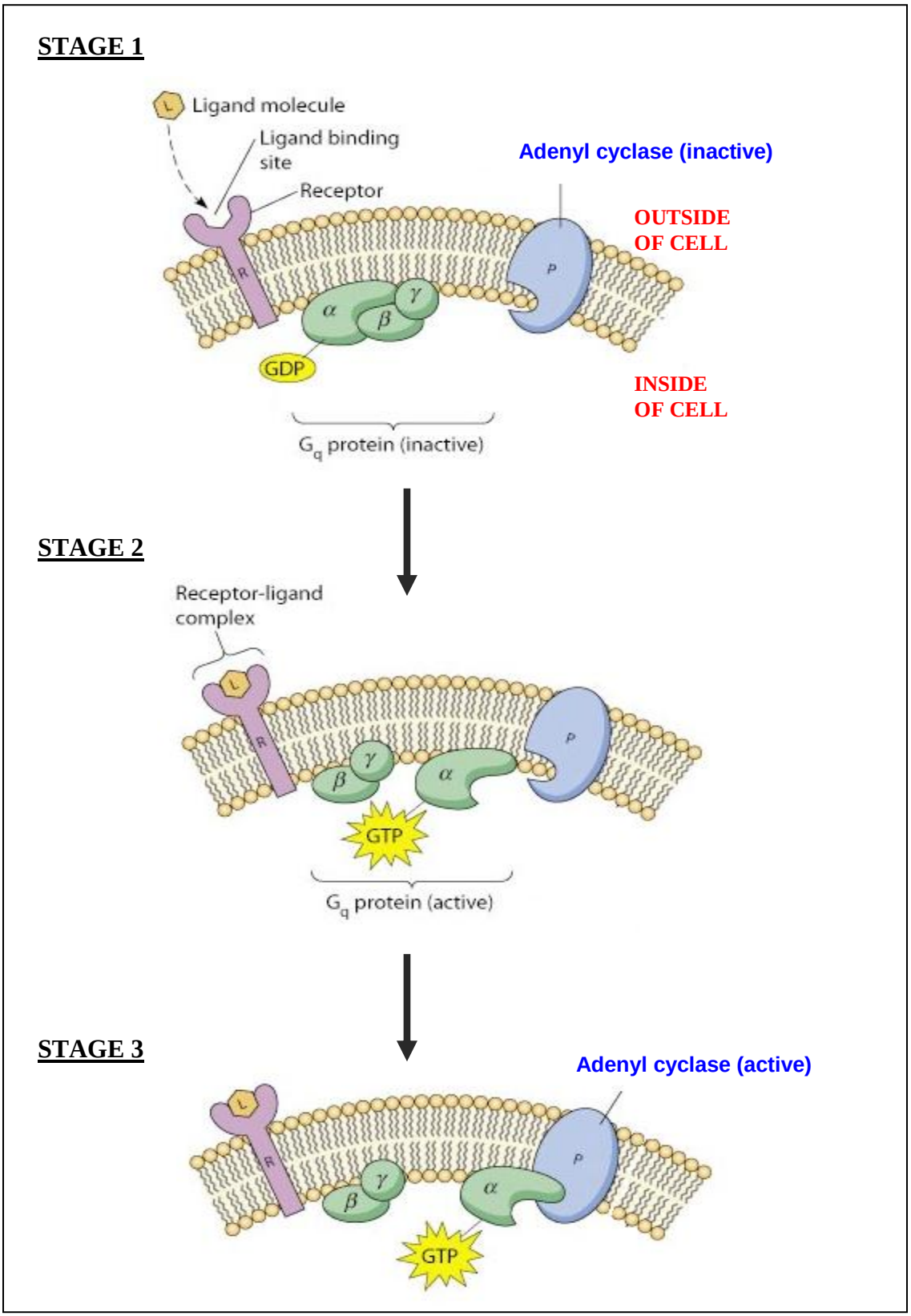


Figure 5. Schematic illustration of the response of the G_{α}-subunit to receptor binding to a ligand. Stage 1 refers to the receptor complex in a "resting state"; stage 2 illustrates ligand binding to the GPCR; and the replacement of α-GDP with α-GTP and stage 3 shows how α-GTP displaces from the βγ-subunit and moves to the effector. Image amended from Becker *et al* (2008).

High intracellular concentrations of cAMP ranging from tens of thousands to millions of molecules per cell are required to initiate a cellular response. However, approximately only a thousand β-ARs are present on the outer membrane of a cell. Therefore, the hormone signal is amplified in order to generate sufficient numbers of cAMP. This is achieved by two methods, the first is by 'signal amplification' where a single GPCR complex can activate up to 100 inactive $G_{\alpha s}$-molecules, and the second is by the synthesis of several hundred cAMP molecules by adenylyl cyclase before the receptor-complex dissociates (Lodish *et al*, 2000).

Beta-blockers, also referred to as β-AR antagonists, bind to the β-AR complex without initiating a G-protein response, which firstly minimises adrenaline and noradrenaline binding, and secondly reduces the usual physiological activity of the hormones (Lodish *et al*, 2000).

1.5.3 β-AR subtype locations and pharmacology

The three β-AR subtypes in humans have been classified according to their location and binding affinities with adrenergic ligands. β_1- and β_2-ARs are generally located in the cardiovascular system; β_1-ARs are primarily located in cardiac muscle and are responsible for inducing heart rate and contractility and subsequently blood flow when activated by adrenergic ligand catecholamines, whilst β_2-ARs are present in smooth muscle cells lining the bronchial passage, and when active enable the relaxation of smooth muscle, allowing the bronchioles to open (Lodish et al., 2000). Diseases related to elevated levels of adrenaline and noradrenaline and subsequent activation of β_1- and β_2-ARs include hypertension, angina, pectoris, hypertrophy, arrhythmia, bradycardia, tachycardia and heart failure (Watson & Arkinstall, 1994). Therefore, the majority of non-specific β-blockers (such as propranolol) are primarily designed to interact with both β_1- and β_2-ARs as to modulate heart function; selective β_1-AR blockers (such as atenolol) are also available and may be preferentially used by patients where there are fewer side effects, such as bronchial constriction, which may occur as a result of β_2-AR blocker activity in the lung (Warne *et al*, 2008).

In humans, the β_3-AR has been identified in a variety of tissues. They have been found to inhibit contractile activity of the ileum and colon (Manara & Bianchetti, 1990), modulate neuronal bronchomotor inducing relaxation of airway smooth muscle, produce peripheral vasodilation which is predominant in skin, reduce contractile force in human ventricular muscle, stimulate L-type calcium current in human atrial myocytes and regulate vasodilation in the human internal mammary artery (Krief *et al*, 1993; Skeberdis, 2004; Rozec *et al*, 2005). The β_3-AR mRNA is also expressed in the human brain, especially in very young infants; the role of this, however, has not yet been confirmed (Rodriguez *et al*, 1995). However, the β_3-AR has been found to be most highly expressed in the brown and white adipose tissues (BAT and WAT, respectively) where it is likely to regulate norepinephrine-induced changes in energy metabolism and thermogenesis and thus play an important role in obesity and type II diabetes (Muzzin *et al*, 1991; Krief *et al*, 1993). The ratio of β_1, β_2 and β_3-AR expression in the BAT and WAT is 3:1:150, respectively (Collins *et al*, 1994). The essential features of BAT and WAT is their capacity to stimulate the mobilisation of lipids from the white fat cells, oxidise fat and produce heat without synthesising ATP from ADP (Arch, 1989; Collins *et al*, 1994; Arner & Hoffstedt, 1999).

There is still some debate as to whether the β_3-AR is a separate β-AR subtype or is actually a sub-species of a β_1-AR. Pharmacological analysis suggests that the human β_1-AR and β_3-AR a more similar to each other across the transmembrane domains (76%) than to the β_2-AR (71% and 68% for the β_1- and β_3-AR, respectively) (Machida *et al*, 1990; Skeberdis, 2004; Owen *et al*, 2007). Additionally, the most potent catecholamine for both β_1-AR and β_3-AR is noradrenaline, whilst it is adrenaline for the β_2-AR (Strosberg & Pietri-Rouxel, 1996). However, there are some notable differences between the β_1- and β_3-AR. Firstly, it has been suggested that the β_3-AR has either three exons and two introns in its sequence (as in humans) or two exons and one intron (as found in dogs and monkeys) (Bensaid *et al*, 1993; Watson *et al*, 1997); whereas there are no introns in the β_1-AR. Secondly, there is data to suggest that unlike the β_1- and β_2-AR which only interact with the $G_{\alpha s}$-proteins, the β_3-AR interacts with a multitude of proteins, as shown in figure 6. In adipocytes, the β_3-AR is coupled to the two heterotrimeric proteins $G_{\alpha s}$-

and $G_{\alpha i}$ (Soeder *et al*, 1999). This dual coupling permits two parallel signaling pathways to be activated: (i) cAMP generation and camp-dependent protein kinase activation (PKA), and (ii) cellular tyrosine kinase (Src) recruitment to the receptor and activation of extracellular signal-related kinase (ERK). In the first case (A), stimulation of the β_3-AR leads to the sequential stimulation of adenylyl cyclase and cAMP and PKA, which in turn activates a specific protein kinase cascade culminating in the activation of p38 mitogen-activated protein kinase (p38 MAPK) and activation of a subset of transcription factors including activating transcription factor 2 (ATF2), peroxisome proliferator-activated receptor-gamma (PPARγ) and –gamma coactivator 1 alpha (PGC-1α). These events allow for a sustained thermogenic response by increasing uncoupling protein 1 (UCP1) expression. In the second case, the PKA and ERK pathways can also lead to the phosphorylation of hormone-sensitive lipase (HSL), which mediates lipolysis (Robidoux *et al*, 2006). The β_3-AR-mediated signalling pathways in the human atrial myocytes (B) are very different to those in the adipocytes. Activation of the β_3-AR leads to the phosphorylation of calcium channels and an increase in I_{ca}. β_3-AR agonists exert a negative inotropic effect (weakens the force of muscular contractions) on human ventricular muscles and this is related to the activation of inhibitory $G_{\alpha i}$-proteins (Skeberdis, 2004). The stimulation of $G_{\alpha i}$ does not induce cAMP but instead activates the nitric oxide (NO) pathway via the activation of endothelial constitutive NO synthase (eNOS). The production of NO causes an increase in intracellular cyclic guanosine monophosphate (cGMP) and subsequent activation of cGMP-dependent protein kinase (PKG) and possible inhibition of phosphodiesterase 3 (PDE 3) and/or activation of phosphodiesterase 2 (PDE 2), which can reduce the contraction force usually stimulated through the cAMP pathway (Skeberdise, 2004). In contrast to the heart that uses calcium channels, the β_3-AR mediated relaxation effect on the rat aorta involves several potassium channels (C), whilst in the portal vein myocytes (D), the activation of β_3-AR stimulates L-type calcium channels through a $G_{\alpha/s}$-induced stimulation of the cAMP/PKA pathway leading to the subsequent phosphorylation of the those channels (Rautureau *et al*, 2002). Therefore, considering their different modes of activation, it is more likely that the β_3-AR as an individual β-AR subtype, rather than a β_1-AR subtype.

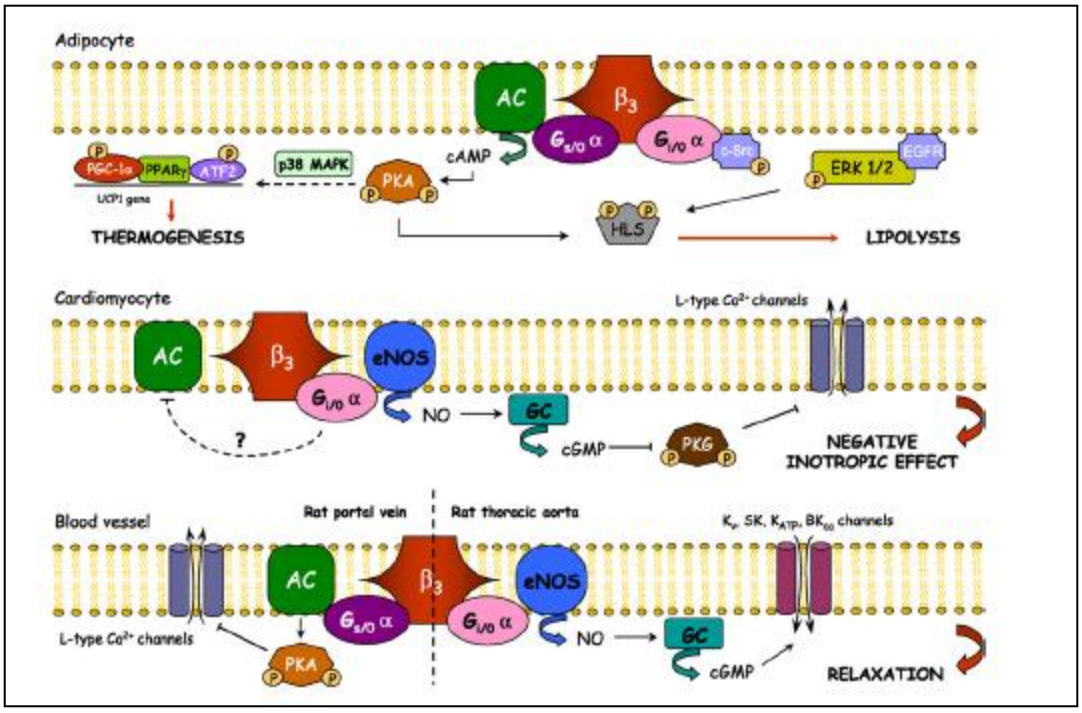


Figure 6. Signaling pathway of β_3-AR in adipocytes, cardiomyocytes and blood vessels. In adipocytes (A), the β_3-AR is coupled to both G_s and G_i leading to the activation of PKA and ERK 1/2 pathways, respectively. PKA subsequently activates p38 MAPK, which in turn activates ATF2, PPARγ and PGC-1α which control for prolonged thermogenesis, whilst ERK 1/2 and PKA activate HSL, which controls for lipolysis. (B) β_3-AR is not coupled to the G_s protein but only to G_i, which leads to the activation of eNOS. The downstream production of NO activates the soluble GC isoform leading to an increase in cGMP concentration, which in turn activates PKG. (C) β_3-AR mediates relaxation through activation of a NO synthase pathway and subsequent increase in cGMP concentration. (D) Activation of β_3-AR stimulates L-type calcium channels. G_s = stimulatory G-protein; $G_{i/o}$ = $G_{i/o}$ protein; AC = adenylyl cyclase; cAMP = cyclic adenosine monophosphate; cGMP = cyclic guanosine monophosphate; PKA = cAMP-dependent protein kinase; HSL = hormone-sensitive lipase; PGC-1α = peroxisome proliferator-activated receptor; PPARγ = peroxisome proliferator-activated receptor-gamma coactivator-1; UCP1 = uncoupling protein 1; ERK = extracellular signal-related kinase; p38 MAPK = p38 mitogen-activated protein kinase; eNOS = endothelial nitric oxide synthase; GTP = guanosine triphosphate; GC = guanylyl cyclase; NO = nitric oxide; Ca^{2+} = calcium; K^+ = potassium. Figure taken from Ursino *et al* (2009).

1.5.4 β-ARs in fish

Similarly to humans and higher vertebrates, fish respond to environmental or physiological stress via the secretions of the catecholamines adrenaline and noradrenaline. Like humans, fish also possess α and β adrenergic receptors and the

transduction pathway of GPCR interaction and subsequent cAMP activation is the same (Randall & Perry, 1992). However unlike humans, adrenaline, like noradrenaline, is synthesized in both adrenergic neurons and chromaffin cells and thus both catecholamines can act as neurotransmitters. Additionally in fish, the chromaffin tissue is located within the anterior or head kidney, often associated with the walls of the posterior cardinal veins (Nilsson, 1983). Furthermore, levels of adrenaline and noradrenaline in the chromaffin tissue vary between teleost (bony) and elasmobranch (cartilaginous) fish species (Hathaway & Epple, 1989). Catecholamine activation in fish leads to numerous physiological responses, both direct and indirect, all of which either increase or maintain energy turnover via glyogenolysis or gluconeogensis and oxygen supply under adverse conditions (Randall & Perry, 1992).

To date, the β_1- and β_2-ARs have been characterised in a handful of aquatic species including the African clawed frog *Xenopus laevis*, where the β_1-AR mRNA was expressed in the mature oocytes and embryos (Devic *et al*, 1997); the channel catfish *Ictalurus punctatus*, where both β_1- and β_2-AR were identified in membranes isolated from the head kidney and spleen leukocytes (Finkenbine *et al*, 2002) and the rainbow trout *Oncorhynchus mykiss*, where the β_2-AR was identified in the spleen, white muscle, red muscle, liver and kidney of adult rainbow trout (Nickerson *et al*, 2001). It has been proposed that the β_1-AR is involved in maintaining the immunostatus of carp, however its function in other fish and aquatic organisms is unknown (Jozefowski & Plytycz, 1998; Owen *et al*, 2007). Meanwhile, the function of the liver β_2-AR in rainbow trout and other species of teleosts has been found to be involved with the mobilisation of hepatic glycogen (Nickerson *et al*, 2001).

The complete β_3-AR has been solely characterised in the rainbow trout, *Oncorhynchus mykiss* (Nickerson *et al*, 2003). Two β_3-AR subtypes were identified as β_{3a}- and β_{3b}-AR. The β_{3a}-AR was found in the heart and gills of adult rainbow trout, whilst the β_{3b}-AR was found in the red blood cells (RBC). Interestingly, neither subtype was found in the adipose tissue; fish are poikilothermic and do not regulate their own body temperature and are therefore not likely to require a thermogenic response mechanism. Nickerson and

colleagues suggested that the catecholamines were able to augment haemoglobin/oxygen-binding affinity by increasing RBC intracellular pH through β_3-AR signalling. Activation of the trout the β_3-AR leads to accumulation of cAMP and activation of PKA that in turn activates the β Na^+/H^+ (β-NHE) exchange system. Once activated the β-NHE extrudes H^+ from the RBC in exchange for Na^+, resulting in alkalisation of the cytoplasm. This increase in RBC intracellular pH enhances the affinity of haemoglobin for O_2 and allows for the increased oxygen transport by the blood. This suggests the roles of both β_{3a}- and β_{3b}-AR are interestingly novel in the trout and possibly fish. Maximum likelihood analysis of aligned vertebrate β-AR sequences placed the trout β_{3a}- and β_{3b}-AR in the mammalian/avian β_3-AR group with strong statistical support (97%) and further ligand binding assays revealed both β_3-ARs had a stronger affinity for norepinephrine than for epinephrine, matching the preferential catecholamine affinity identified in human β_3-ARs.

A partial β_{3b}-AR sequence has also been identified in the black bullhead (*Ameiurus melas*) (Dugan *et al*, 2008). Along with identifying the β_2-AR, Dugan and colleagues identified a β-AR sequence that was phylogenetically most similar to other vertebrate β_3-ARs and spanned from TMD4 to TMD7. The proposed β_{3b}-AR sequence was identified in the liver, however its function is not known and one cannot rule out the possibility that the β_{3b}-AR mRNA detected was a result of residual blood in the hepatic tissues.

Fish and humans both express β-adrenergic receptors, albeit in different tissues and their exact functions in fish still unknown or unconfirmed. Fish and humans are separated by approximately 450 million years of evolution, though they both belong to the same vertebrate lineage and therefore share important developmental pathways, such as the evolution of adrenergic receptors (Froschauer *et al*, 2006). It has been suggested that two genome duplications occurred at the origin of vertebrates 500-800 million years ago, termed the 2R hypothesis (Ohno, 1970), hence explaining why humans are diploid. However, further fish-specific genome duplications have occurred leading to most ray-finned fish being tetraploid (4R) (Froschauer *et al*, 2006). More recent tetraploidisation (and even octoploidisation) events have occurred independently through different

mechanisms in several lineages of teleost fish and it is these events that are responsible for the huge species diversity observed in this group (Ruuskanen *et al*, 2005). Duplicated genes can also lead to functional divergence, where one duplicate might acquire a novel, positively selected function (termed neofunctionalisation) and it is therefore possible that through neofunctionalisation, the location and subsequent roles of β-ARs in fish have become different to humans (Froschauer *et al*, 2006). Therefore, in response to environmental β-blocker exposure, fish may show an unanticipated toxic reaction tha could not be predicted from mammalian studies.

1.6 Pharmaceutical Environmental Risk Assessments (ERAs)

The realisation that pharmaceuticals in the environment can cause sometimes severe toxicological effects has led to the enforcement of environmental risk assessments (ERAs). As stipulated by the committee for medicinal products for human use (CHMP) within the European medicines agency (EMEA), an ERA is required for all new marketing authorisation applications for a medicinal product through standardised procedure. Vitamins, electrolytes, amino acids, peptides, proteins, carbohydrates and lipids are exempted because they are unlikely to result in a significant risk to the environment.

According to the guideline on the environmental risk assessment of medicinal products for human use (EMEA/CHMP/SWP/4447/00 [2006]), the assessment procedure is a step-wise, phased procedure, consisting of two phases (see table 3). The first phase (Phase I) estimates the exposure of the environment to the pharmaceutical, the second phase (Phase II) assesses the fate and potential effects in the environment.

Table 3. The phased approach in the ERA (taken from EMEA/CHMP/SWP/4447/00).

Stage in regulatory evaluation	Stage in risk assessment	Objective	Method	Test/Data requirement
Phase I	Pre-screening	Estimation of exposure	Action limit	Consumption data, logK_{ow}; PEC
Phase II Tier A	Screening	Initial prediction of risk	Risk assessment	Base set aquatic toxicology and fate; PEC/PNEC
Phase II Tier B	Extended screening	Substance and compartment-specific refinement and risk assessment	Risk assessment	Extended data set on emission, fate and effects

In Phase I, the estimation of load into the environment is based on the drug substance, irrespective of its route of administration, pharmaceutical form, metabolism and excretion. Any substance with a log K_{ow} >4.5 is screened in Phase II regardless of its load into the aquatic environment as it has a high bioconcentration factor (BCF) and so is likely to exert an effect on aquatic organisms (Cleuvers, 2004).

The predicted environmental concentration (PEC) is calculated for a pharmaceutical based on its usage and assumes that the predicted amount used per year is evenly distributed over the year and throughout the geographic area, STW effluent is the main route of entry, there is no biodegradation and metabolites in the patient are not taken into account. The PEC in the surface water ($PEC_{surfacewater}$) is calculated using the following equation:

$$PEC_{surfacewater} = \frac{\text{Maximum daily dose consumed per inhabitant x Percentage of market use}}{\text{Amount of waste per inhabitant per day x dilution factor}}$$

If the $PEC_{surfacewater}$ exceeds $10ngL^{-1}$, the pharmaceutical is required for Phase II screening. PEC levels are usually over-estimated, which decreases the likelihood of

getting false-negative errors in the phase II assessments. Arguably, it is better to have more false-positive pharmaceuticals that require further testing than underestimate the pharmaceutical loads into the environment (Ferrari *et al*, 2004). Phase II Tier A assesses the fate and effects of a pharmaceutical in the environment according to test protocols issued by the European commission, Organisation for economic co-operation and development (OECD) or the International organisation for standardisation (ISO). A predicted no-effect concentration (PNEC), at which no adverse effect on the aquatic environment function is to be expected is calculated by dividing the lowest 50%-effect concentration from standard long-term toxicity tests using three aquatic test-species, one plant (algal growth inhibition test), one invertebrate (*Daphnia* reproduction test), and one fish vertebrate (fish early life stage test). In addition to standard toxicity tests, data on the physical-chemical structural properties, such as quantitative structure-activity relationships (QSARs), primary and secondary pharmacodynamics, toxicology, metabolism, excretion, degradability and persistence of the drug substance and/or its metabolites are also taken into account when calculating the PNEC (Cleuvers, 2004). A risk quotient PEC/PNEC is then calculated. If this ratio equals or exceeds 1, an ecological risk is suspected and further evaluation on the fate of the drug and/or its metabolites in the aquatic environment is conducted in Phase II Tier B. This ERA represents a marked improvement over previous risk assessments, which in the past have determined the PNEC of a pharmaceutical using acute toxicity tests only. Chronic toxicity tests are arguably more sensitive than acute toxicity tests and are reflective of actual environmental concentrations and loads (Webb, 2001).

There are, however, some reservations to this ERA approach. The first is that the ERA only examines individual pharmaceutical toxicity. This is not environmentally realistic as drugs in the environment are seldom found as single contaminants but instead occur as mixtures. There is therefore the potential for pharmaceuticals to behave synergistically (the presence of one pharmaceutical enhances the effects of another), antagonistically (the presence of one pharmaceutical inhibits the effects of another) or independently (the effects of a pharmaceutical are not affected by the presence of another) (Laenge *et al*, 2006). At least 50 pharmaceuticals have been detected in the aquatic environment and so

there is the potential for complex drug-interactions, which should be evaluated (albeit simplistically at first) in the ERA (Cleuvers *et al*, 2004). Secondly, pharmaceutical tests cover only a small set of laboratory organisms, which are not sensitive enough to unravel the adverse effects of pharmaceuticals. Furthermore, the physiological endpoints used do not necessarily address the pharmaceutical mechanism of action and therefore have limited use when trying to extrapolate to different scenarios (Forbes *et al*, 2006). Thirdly, the fish early life stage test examines for mortality over a 10-day period only. Considering that the effects of environmentally relevant concentrations of ethinyloestradiol have not been measured in any staged fish under a 10-day period suggests that its toxicity would probably not have been detected using the current ERA physiological endpoints (Sumpter, 2007).

1.7 Emergence and application of "omic" technologies to environmental monitoring

A biomarker can be defined as a "measurable biological response to a chemical or chemicals that gives a measure of exposure, and sometimes, also of toxic effect (Peakall, 1994). For example in current ERA assessments, the physiological responses of algae, daphnia or fish are used as biomarkers of exposure to individual pharmaceuticals. The emergence of "omic" techniques has considerably enhanced the potential of subtle, mechanism of action-revealing biomarker discovery in the field of environmental monitoring. The use of genomics, which is the "study of an organism's collection of genes in an attempt to describe the functions they perform, the products they create and their interaction with one another" (Kuska, 1998; Shugart & Theodorakis, 1998), provides considerable advantages over standard physiological biomarkers as it is sensitive, provides early detection of response and allows for comparison between species (Ankley *et al*, 2006; Hook *et al*, 2007). As a result "ecotoxicogenomics", which is defined as "the study of gene and protein expression in non-target organisms in response to environmental contaminant exposure", and combines genomics, proteomics and transcriptomics, has emerged (Snape *et al*, 2004). Several studies have examined the response of aquatic organisms to pharmaceutical exposure using an ecotoxicogenomic approach, including measuring changes in gene expression levels to both individual (Kohno *et al*, 2008) and pharmaceutical mixtures (Filby *et al*, 2007) and measuring

changes in steroidal levels, such as cytochrome P450 aromatase activity (Fossi *et al*, 2006; Hinfray *et al*, 2006; Lyssimachou *et al*, 2006) and plasma sex steroid levels (Mauder *et al*, 2007; Ankley *et al*, 2008); most of this work has examined for endocrine disruption upon exposure to EE_2. However, to date, none of these biomarkers have been adopted for further ERA use. One of the most recent "omic" techniques to emerge is metabolomics. Metabolomics is defined as the quantitative and comprehensive analysis of all metabolites in a biological sample (Nicholson & Wilson, 2003). Metabolites are bi-products of metabolism, which is itself the process of converting food energy into mechanical energy or heat. There are around 2,000 to 3,000 different metabolites in the human body, compared to an estimated 30,000 genes and 1,000,000 proteins (Schmidt, 2004). Of the estimated 3,000 metabolites, secondary metabolites and signaling molecules, researchers are particularly interested in the small, low-molecular weight compounds, such as lipids and sugars and amino acids, which serve as substances and products in various metabolic pathways. Metabolomics can therefore be used to investigate metabolic regulation in response to environmental stressors or stimuli (Nicholson *et al*, 2002). The metabolome is defined as "the total quantitative collection of metabolites present in a cell or organism which participate in metabolic reaction required for growth, maintenance and normal function" and represents the final "omic" level in a biological system. Therefore, unlike genomics, transcriptomics and proteomics, which may only indicate the potential for biochemical change, metabolomics reveals actual functional changes in a study organism (Nicholson & Wilson, 2003; Goodacre *et al*, 2004). Additionally, there are far fewer metabolites than genes or gene products to be studied and, unlike the other aforementioned omics', metabolomics does not require a sequenced genome for the study organism. Also, the two most widely used metabolomic techniques – mass spectrometry (MS) and nuclear magnetic resonance (NMR) – can be rapid and highly automated, lending themselves to effective high-throughput analysis (Ekman *et al*, 2008). Accordingly, albeit with some debate, metabolomics has been proposed as the "greatest omic" technology (Ryan & Robards, 2006). The use of metabolomics for biomarker development has been used extensively within the pharmaceutical industry as an effective preclinical screen in drug discovery and also in clinical and biomedical studies (Robertson, 2005). For example, metabolomics has been

used measures changes in metabolite profiles over time (Nicholson *et al*, 2002) and it has been proposed that by using metabonomics to measure changes in specific blood plasma metabolite over time as biomarkers to specific drug treatments, one could personalize drug therapy treatment (Clayton *et al*, 2006).

1.7.1 Use of metabolomics in environmental studies

Metabolomics has been used in several aquatic environmental toxicology studies measuring the response of organisms to natural environmental stressors including response of the common carp (*Cyprinius carpio*), Japanese medaka (*Oryzias latipes*) and killfish (*Austrofundulus limnaeus*) to hypoxia (Borger *et al*, 1998; Pinccetich *et al*, 2005; Prodrabsky *et al*, 2007) and juvenile rainbow trout (*Oncorhynchus mykiss*) to elevated water temperatures (Viant *et al*, 2003). Additionally, the response of aquatic organisms to diseases, such as red abalone (*Haliotis rufscens*) to the Rickettsiales-like prokaryote *Candidatus Xenohaliotis californiensis* (Rosenblum *et al*, 2005), have also been examined using metabolomics. Metabolomics has also been used to examine for the response of aquatic organisms to environmental pollutants including the red abalone and the common carp to copper (Viant *et al*, 2002 and De Boeck *et al*, 1997, respectively), Japanese medaka embryogenesis to trichloroethylene (Viant *et al*, 2005) and the fathead minnow to the fungicide vinclozolin (Ekman *et al*, 2007).

To date, only a handful of aquatic toxicity studies have used metabolomics to understand the subtle responses to pharmaceutical exposure. Samuelsson and colleagues (2006) and Ekman and colleagues (2008) both used metabolomics to examine for the effects of EE_2 on the rainbow trout and fathead minnow, respectively. Samuelsson and colleagues examined the metabolomic profiles of rainbow trout exposed to $10ngL^{-1}$ EE_2 and were able to measure the standard EE_2 biomarker vitellogenin, and its metabolites. Ekman and colleagues examined the metabolite profiles of male fathead minnows exposed to $10ngL^{-1}$ and $100ngL^{-1}$ EE_2 and suggested that there were striking similarities in the metabolite profiles between the exposed males and control females. Both studies demonstrated the proof-of-principle that metabolomics can be used to identify biomarkers of EE_2 exposure. However the effects of EE_2 are well understood and, in the instance of Samuelsson *et al*,

measuring vitellogenin has been achieved using far cheaper and simpler methods such as enzyme-linked immunosorbant assays (ELISAs). As such, further validation studies using pharmaceuticals that induce a subtle effect are required to fully appreciate the potential advantages metabolomics has to offer.

1.8 Aims and objectives

The aim of the PhD was to develop an intelligent testing approach to understand pharmaceutical toxicity in fish. Using the non-specific β-blocker propranolol and fathead minnow as the test pharmaceutical and test species, respectively, the first aim was to establish whether the fathead minnow possessed and expressed the β_3-AR in fish. A previous study had identified the β_1- and β_2-AR in the fathead minnow (Giltrow, 2008), and so by identifying the β_3-AR, one completes the picture in terms of understanding β-AR expression in the fathead minnow. This is particularly important for justifying an *in vivo* exposure assay to propranolol that in humans interacts with all three β-AR subtypes. Secondly, the use of metabolomics and Real Time β_3-AR expression studies were examined for their suitability in identifying sensitive, mechanism of action-revealing biomarkers of propranolol exposure, thereby evaluating their potential as suitable techniques for environmental monitoring studies.

As part of an EU-collaboration to conduct a risk assessment on β-blockers, propranolol was selected as the test-pharmaceutical for this study. Propranolol is the most widely used of all β-blockers and is the most highly lipophillic. Therefore, of all the β-blockers, it is most likely to induce a toxic effect in an aquatic organism. The fathead minnow (as described in more detail in Section 3.1.3) is a widely used test species for ecotoxicological studies and, as such, a robust pair-breeding *in vivo* assay has been developed to examine for effects on survival, reproduction, growth and behaviour. As part of the EU risk assessment of β-blcokers, the pair-breeding *in vivo* assay was used to examine the effects of atenolol on the fathead minnow and therefore, to allow for a direct comparison to be made between atenolol and propranolol, the same assay was adopted for this study.

CHAPTER 2: IDENTIFYING THE β_3-AR IN THE FATHEAD MINNOW

2.1 INTRODUCTION

Traditionally, aquatic-based ecotoxicology tests have investigated the response of an organism(s) to a particular compound(s) (e.g. heavy metal, xenobiotic) by examining for, and quantifying, anticipated physiological, biological and biochemical changes. The medical applications of pharmaceuticals are achieved by binding with specific drug targets (e.g. receptor) in a tissue(s) and triggering a cascade of desirable responses. By understanding the mechanism of action of a particular pharmaceutical in mammals, one can design an ecotoxicological experiment that looks at mechanism of action-related responses in a test-species. This approach has been applied in ecotoxicology tests; for example previous assessments of beta-blocker exposure to fish have investigated for cardiovascular changes in rainbow trout as the heart is one of the principal targets for beta-blockers in humans (Larsson *et al*, 2006; Owen *et al*, 2007). However, this mechanism of action approach has not necessarily been adopted for use prior to conducting an *in vivo* experiment. It can be assumed that for a drug to elicit a response in an aquatic organism, the organism in question must express the intended drug target, albeit in different tissues. Therefore before running an *in vivo* pharmaceutical exposure procedure, the intended drug-target should be first identified in the test-organism. From this, a test-species and mechanism-of-action-specific *in vivo* exposure procedure can be developed, which also has ethically beneficial ramifications.

Before running an *in vivo* fish exposure to the non-specific β-blocker propranolol, the practical aim was to identify whether the fathead minnow expressed the β_3-AR target for propranolol. Prior to this study Giltrow (2008) identified the β_1- and β_2-AR in the heart and liver tissues of the fathead minnow, respectively, which are the same tissue locations as in humans. Therefore, the fathead minnow was likely to possess the β_3-AR, which would justify conducting an *in vivo* study.

2.1.1 The β_3-adrenergic receptor structure

The β_3-AR is a single polypeptide chain of up to 366 amino acids with an extracellular amino (N) terminus and an intracellular carboxyl (C) terminus and resides embedded in the plasma membrane of cells. The membrane bound receptor is coupled to an

intracellular guanine nucleotide regulatory protein (G-protein) that, upon ligand binding to the receptor, mediates a response (Watson & Arkinstall, 1994). The β_3-AR shares a similar topological structure to all G-protein coupled receptors (GPCRs) and other serpentine receptors as it characteristically spans the lipid bilayer of the cell membrane in seven distinct α-helical trans-membrane domains (TMDs); see figure 7. Each TMD contains 18-30 hydrophobic amino acids (Lekowitz & Caron, 1988).

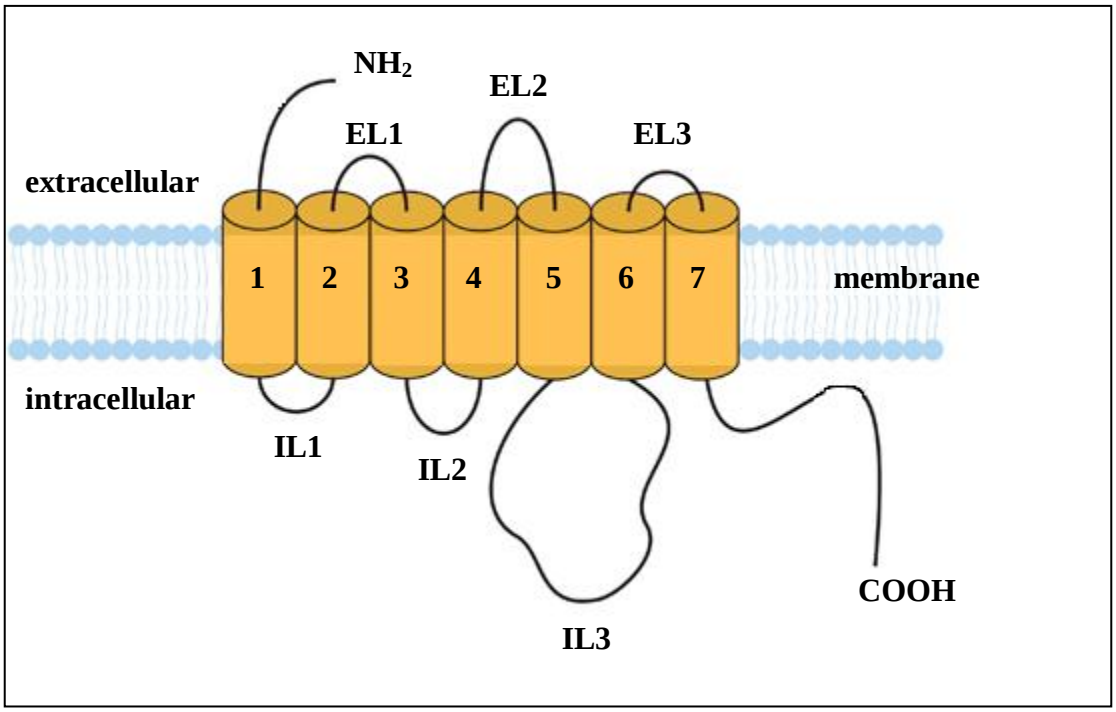


Figure 7. A schematic representation of a β-AR in a cell membrane showing the external N terminus (NH_2) and internal C terminus (COOH). IL = intracellular loop; EL = extracellular loop. Figure adapted from that documented by Blockaert & Pin (1999).

All GPCRs serve a similar function in that their role is to bind to a ligand and therefore there is considerable sequence homology between them. A series of "fingerprint" amino acid residues, or motifs, such as the TMDs, are responsible for the three-dimensional structure and functional properties of GPCRs and are conserved in 95-98% of the receptors (Schwartz, 1996).

In β_3-ARs, the ligand binding site is composed from an arrangement of four of the seven TMDs. Noradrenaline, for example, binds to aspartic acid in TMD 3 by the formation of

a salt bridge, two serine residues in TMD 5 and a serine residue in TMD 4 via hydrogen bonds and a phenylalanine in TMD 6 by van der Waals interactions. Although noradrenaline also binds to aspartic acid and tyrosine in TMDs 2 and 7, respectively, this is more important for signal transmission to the G-protein than for actual ligand binding (Strosberg & Pietri Rouxel, 1996). The arginine residue located at the intracellular pole of TMD 3 (seen as part of the 'DRY' motif) also plays a crucial role in the signal transduction process and is the only amino acid that is totally conserved across every GPCR (Schwartz in Foreman & Johansen, 1996).

The three-dimensional structure is established via a series of chain folding and bridging between amino acid residues, as shown in figure 8 The N-linked glycosylation process, although not directly important for ligand binding, is the principle posttranslational modification to proteins which, through recognition by specific proteins in the rough endoplasmic reticulum, ensures that the protein folds correctly (Schwartz, 1996). The site for N-glycosylation in the β_3-AR, and all GPCRs, is the asparagine residue in the NH_2 terminal region before TMD 1 (Blin *et al*, 1993). The disulfide bridge formed between the cysteine residues found in the second and third extracellular loops is common to all GPCRs and this effectively closes the gap between TMD 3 and TMD 4, which is generally considered to be the central column in the seven-helical bundle (Schwartz, 1996).

Additional motifs necessary for ligand interaction include the conserved proline residues located in TMDs 5, 6 and 7. They create weak points in the TMD helices and so it has been suggested that they enable the protein receptor wobble to allow ligands and G-protein subunits to associate and dissociate (Lodish *et al*, 1995). The interaction between the asparagine and arginine residues located in TMDs 2 and 3, respectively, are involved in intramolecular signal-transduction and subsequent G-protein signalling (Schwartz, 1996).

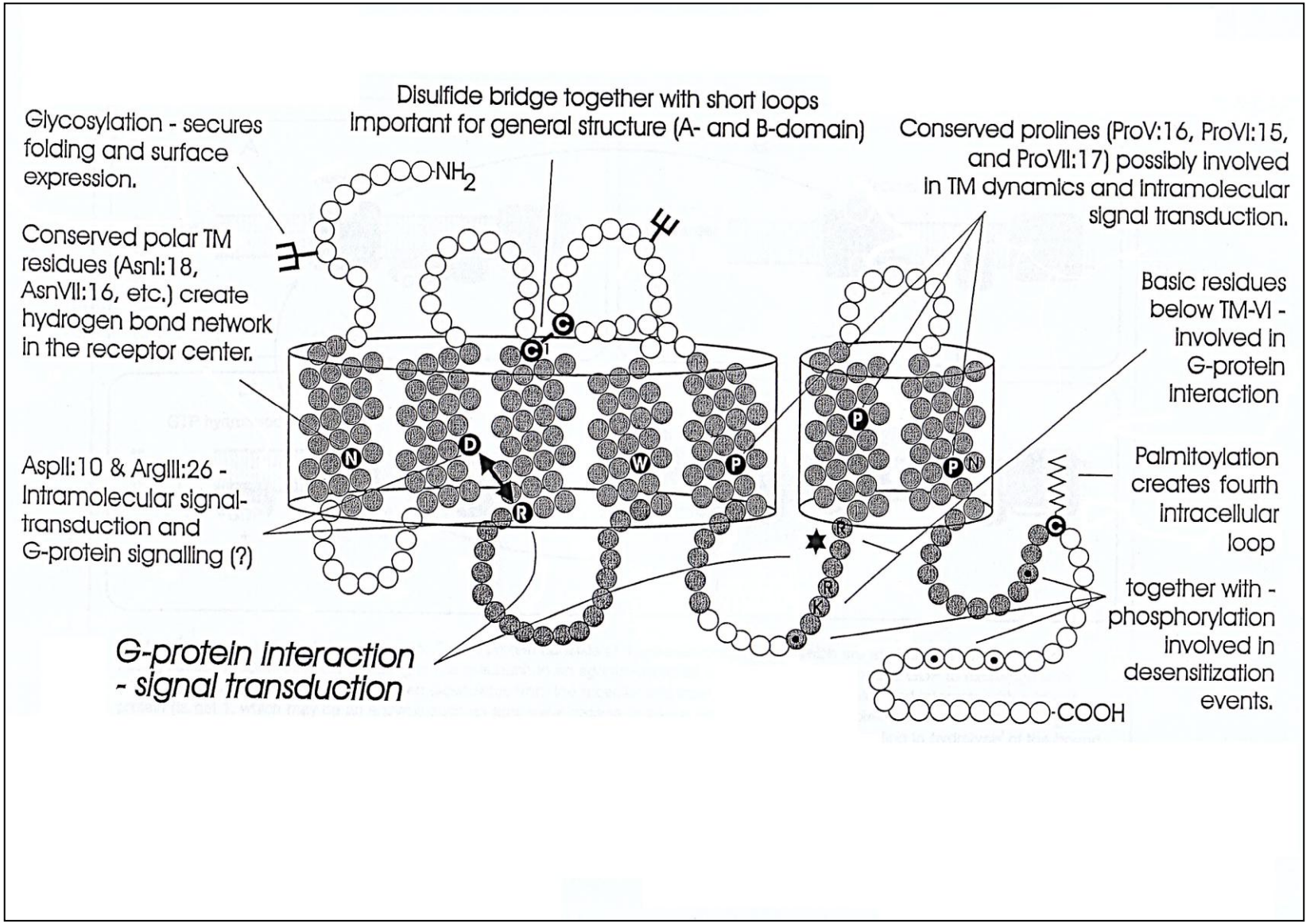


Figure 8. Structural characteristics of the rhodopsin-like GPCR family. Residues located in the transmembrane helices are shaded in light grey. The key fingerprint residues of each helix are highlighted in black: AsnI:18, CysIII:01, ArgIII:26, TrpIV:06, ProV:16, ProVI:15 and ProVII:17. Image taken from Schwartz (1996).

Two further post-translation events are involved with receptor desensitisation and subsequent regulation of G-protein signal transduction. The first is palmitoylation, which occurs at the cysteine residue found 15-20 amino acids C-terminally to the TMD 7, creating a fourth intracellular loop. Palmitoylation has been shown to mediate adenylyl cyclase stimulation by an agonist-bound receptor, possibly by promoting the insertion of several adjacent residues in the membrane, thus forming an additional intracellular loop which results in an active conformation for G-protein coupling (Strosberg & Pietri-Rouxel, 1996). The second is phosphorylation, whereby a phosphate group is added to amino acid in the presence of protein kinase A, which is regulated by cAMP (Lodish *et al*, 1995; Schwartz in Foreman & Johansen, 1996). Stimulation of GPCRs will therefore lead to phosphorylation of the serine and threonine residues in the 3rd intracellular cytoplasmic loop (between TMDs 4 and 5) and in the COOH terminal region.

The necessary motifs required for ligand binding, three-dimensional structure maintenance and G-protein signal transduction and desensitisation, therefore, should be present in a fathead minnow β_3-AR.

2.1.2 β-ARs in higher vertebrates

Beta-adrenergic receptors have been found in many species. Table 4 summarises the list of higher vertebrates β-ARs have been found in. Many of the sequences, especially in those species that are not commonly used as test models (e.g. the jaguar) have been computer-software generated from genomic sequence studies on the national centre for biotechnology information (NCBI) database (http://www.ncbi.nlm.gov/entrez/) and have not yet been confirmed. One must therefore be cautious when using these sequences for further analysis.

Table 4. Summary of β-ARs found in non-fish species. Sequences found using a blast search on the NCBI database.

Mammal	Latin name	β–AR subtype	Accession numbers
Brown rat	*Rattus norvegicus*	β_1, β_2, β_3	NM_012701; NM_012492; NM_03108
Cattle	*Bos taurus*	β_1, β_2, β_3	NM_194266; NM_174231; NM_174232
Chimpanzee	*Pan troglodytes*	β_2, β_3	XM_521608; Q28509; AAB53939.1
Cotton top tamarin	*Saguinus oedipus*	β_2, β_3	AY091938; AY091947
Dog	*Canis familiaris*	β_1, β_2, β_3	NM_001008713; NM_001003234; NM_001003377
Domestic cat	*Felius catus*	β_1, β_2, β_3	AF192344; NM_001009247; Q9TST4
Goat	*Capra hircus*	β_3	AAD26148
Golden hamster	*Mesocricetus auratus*	β_1	X03804
Gorilla	*Gorilla gorilla*	β_2, β_3	AY091936; AY091945
Grey short tailed possum	*Mododelphis domestica*	β_2	XM_001380743
Human	*Homo sapiens*	β_1, β_2, β_3	NM_000684; AAB82149; AAA35550
Jaguar	*Panthera onca*	β_2	AY011308
Mouse	*Mus musculus*	β_1, β_2, β_3	NM_007419; NM_007420; AAI32001
Pig	*Sus scrofa*	β_1, β_2, β_3	NM_00112307; NM_001128436; Q95252
Rhesus monkey	*Macaca mulatta*	β_1, β_2, β_3	X75540.1; NM_001042774; Q28524
Sheep	*Ovis aries*	β_1, β_2, β_3	AF072433; NM_001130154; NP_0011532929
Turkey	*Meleagris gallopavo*	β_1	U14958

2.1.3 β-ARs in fish

Table 5 details the β-ARs found in fish. However, only four β-ARs have actually been confirmed in the laboratory. The trout β_2- and β_3-ARs (highlighted in red) were identified by Nickerson *et al* in 2001 and 2003, respectively. The black bullhead β_2- and β_{3b}-ARs were identified by Dugan *et al* in 2008, however these sequences have not yet been

registered on the NCBI database. The remaining sequences were all predicted from NCBI computational analysis. Interestingly, two subtypes of the β_3-AR were identified in the trout. Both sequences were analogous to the human β_3-AR, however they were expressed in different tissues (β_{3a} predominantly in the heart and gill, whilst β_{3b} predominantly in the blood) and were only 84% identical, and so were considered to be two different subtypes.

Table 5. Summary of β-ARs found in fish. Rainbow trout sequences highlighted in red as they are the only sequences to be confirmed in the laboratory. The Atlantic salmon was the only fish species to have a predicted β_4-AR sequence and is highlighted in blue. Accession numbers collected from NCBI database, unless stated.

Fish species	Latin name	β-AR subtype	Accession numbers
Zebrafish	*Danio rerio*	β_1, β_2, β_{3bi}, β_{3bii}	NM_001128689; XP_700720; NW_001513985; NW_001513970
Rainbow trout	***Oncorhynchus mykiss***	**β_2, β_{3a}, β_{3b}**	**AAK94672.1; AY216465; AY216466**
Tiger puffer fish	*Takifugu rubripes*	β_2, β_3	**http://ensembl.fugu-sg.org** AAQ02695.1; CAG42650.1
Spotted green puffer fish	*Tetraodon nigroviridis*	β_2, β_3	**http://ensembl.fugu-sg.org** CAG10129.1; CAG12607.1
Three-spined Stickleback	*Gasterosteus aculeatus*	β_1	**http:pre.ensembl.org/Gaserosteus_aculeatus/Info/Index** IPR000507
Hagfish	*Myxine glutinosa*	β_2	CAA06539.1
Marine lamprey	*Petromyzon marinus*	β_1	EB084301
Black bullhead	***Ameiru melas***	**β_2, β_{3b}**	**Not registered**
Catfish	*Ictalurus punctatus*	β_2	AF127775
Atlantic salmon	***Salmo salar***	**β_{4C}**	**NM_001140454**

Interestingly a putative β_{4C}-AR was identified in the Atlantic salmon (highlighted in blue). It has been suggested that a β_4-AR is expressed in the human atrium (Molenaar *et al*, 1997) and that there is considerable sequence homology, and resultant receptor activity, between the β_3-AR and β_4-AR. Furthermore, it has been demonstrated that a β_3-

AR agonist initiated the same cardiostimulant effect observed in mice lacking a functional β_3-AR gene (β_3-AR knockout) as wild-type mice, thus supporting the notion that a β_4-AR is expressed in atrial tissues (Kaumann *et al*, 1997). Therefore it is possible for any species belonging to the vertebrate lineage to express a β_4-AR. The Atlantic salmon β_{4C}-AR was 90% and 97% similar to the trout β_{3a}- and β_{3b}-ARs, respectively, suggesting that either the receptor is a β_{4C}-AR, or that with such a close similarity score, a β_{3b}-AR. There are contradictory reports about the existence of a β_4-AR in humans, and it has been suggested that the β_4-AR is a β_1-AR derivative (Konkar *et al*, 2000). Therefore the Atlantic salmon β_4-AR could in fact be a β_1-AR, and therefore the trout β_{3b}-AR could also be a β_1-AR derivative (the β_1-AR has not yet been identified in the trout). There are still considerable gaps in knowledge regarding sequence information for all fish species.

The two zebrafish beta β_{3b}-AR sequences seem to be the most suitable starting templates for designing primers to find the β_3-AR in the fathead minnow as both fish species belong to the same Cyprinidae family and consequently sequence homology is likely to be highest between these fish. However, as demonstrated above, there are considerable difficulties involved with using sequences that firstly have not been confirmed and secondly may not have been labelled correctly. One must therefore use these sequences conservatively and with a degree of caution.

2.1.4 Aim

As a suitable first testing stage for an intelligent-testing protocol, the aim of the study was to establish whether the fathead minnow expresses the β_3-AR before starting a fathead minnow pair-breeding assay exposure to the non-specific β-blocker propranolol.

2.2 METHODOLOGY

2.2.1 Tissue acquisition

The fathead minnow (*Pimephales promelas*) and the zebrafish (*Danio rerio*) (figure 9 were both selected as test species for experimental procedures. Tissue acquisition, and subsequent genomic deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) isolation, from both species was thus conducted in the same manner.

Figure 9. Adult male fathead minnow (A) and adult male zebrafish (B). Images taken from www.mblaquaculture.com/content/organisms/pime and www.focusonnature.be/gallery/zebraifsh, respectively.

Prior to any dissection work, all work surfaces and equipment were cleaned with 100% industrial methylated spirit (IMS) (Fisher Scientific, Cat # M/4400/25) and RNase away (Molecular Bioproducts, Cat # 7000) to ensure complete removal of any potential RNA contamination. Additionally, all microcentrifuge tubes were autoclave sterilised to remove any contaminants. All fish were culled via anaesthetisation with $100mgL^{-1}$ Ethyl 3-aminobenzoate methanesulfonate salt (MS-222) (Sigma, Cat # A5040) buffered with $200mgL^{-1}$ $NaHCO_3$. Individual fish were checked for complete anaesthetisation by rolling them onto on side and observing no fin movement and balancing response. The

fish were subsequently blotted dry and killed by lobotomy. The caudal tail was firstly removed and blood collected in a heparinised micro capillary tube and emptied into a pre-weighed microcentrifuge tube. The microcentrifuge tube was subsequently centrifuged at 12,000 x g for 10 minutes at 4^{o}C and the upper blood plasma layer removed. The remaining whole red blood cell samples from each male within the same species were pooled together to increase the likelihood of extracting sufficient quantities of RNA and snap frozen in liquid nitrogen. The hearts, livers, brains, adipose tissues, gonads and gills were quickly dissected, transferred into individual clean microcentrifuge tubes and snap frozen in liquid nitrogen. All microcentrifuge tubes were subsequently stored at -80^{o}C to reduce RNA degradation. To minimise the risk of RNA contamination between different tissues belonging to the same fish and also individual fish, all dissecting equipment was wiped down with RNase away in between removing individual tissue samples.

2.2.2 Preparation for PCR and subsequent cloning work

For all molecular-based experimental work, including cloning, all procedures were conducted under strict sterile conditions. All work surfaces, including pipettes, were wiped down with 70% IMS and RNase away. Gloves were worn. All pipette tips and microcentrifuge tubes were autoclave sterilised prior to use.

The principal technique for rapid gene identification is polymerase chain reaction (PCR) (as described in section 2.2.8) and the starting material required is either genomic DNA or RNA.

2.2.3 Genomic DNA isolation from tissues

Prior to examining whether fathead minnow tissues expressed the β_3-AR, it was deemed more useful to determine whether the fathead minnow possessed the β_3-AR at the genomic level, thereby removing the variable of tissue choice and life-cycle stage. For example, it was possible to begin examining for β_3-AR expression in a tissue that does not express it or examine for expression in selected tissues from a particular aged fish, for example an adult, that no longer expressed the β_3-AR because the functioning purpose of it in the fathead minnow was during early fry development. Genomic DNA was therefore

extracted from both the fathead minnow and the zebrafish (which served as a positive control for the zebrafish-template designed primers) tissues. Genomic DNA was extracted directly from tissues using the 'DNeasy purification of total DNA from animal tissues' (QIAGEN; 69504). An individual tissue sample (up to 25mg) was removed from the -80^{o}C freezer and placed on ice to slowly thaw. 360µl of a lysis buffer* and 20µl proteinase K was added and subsequently mixed thoroughly by vortexing and then incubated at 56^{o}C for 3 hours to allow for complete lysis. Proteinase K is an endolytic proteinase that is used to digest proteins and rapidly inactivate nucleases that might otherwise degrade the DNA or RNA during purification. To ensure the genomic DNA was RNA-free, 4µl RNase A (100mgL^{-1}) was added and the sample was vortexed for 15 seconds. 200µl of a second lysis buffer (and 200µl ethanol (100%)) were added to the sample in successive fashion, with the sample mixed by vortexing after adding each reagent. The mixture was subsequently pipetted into a 2ml DNeasy mini spin column and centrifuged at 8,000 x g for 1 minute at room temperature. The genomic DNA selectively bound to the spin column membrane. The flow-through was thus discarded and the DNeasy mini spin column containing the membrane-bound genomic DNA was placed in a new 2ml microcentrifuge collection tube. The remaining contaminants and enzymes were removed by adding 500µl of two individual wash buffers; centrifuging the sample at 17,900 x g for 3 minutes at room temperature in between the addition of each buffer. The DNeasy spin column with contaminant-free genomic-bound DNA was then placed into another clean 2ml microcentrifuge tube and 200ul of an elution buffer was pipetted directly onto the spin-column membrane and incubated at room temperature for 1min. During the incubation period, the genomic DNA was released from the membrane. The spin-column was subsequently centrifuged at 17,900 x g for 1 minute at room temperature to elute the genomic DNA. The 200µl purified genomic DNA was consequently stored at -20^{o}C until required for a PCR reaction.

To measure the quantity and quality of isolated genomic DNA, 1.5µl of sample was analysed on a Nanodrop N-1000 spectrophotometer (Fisher Scientific, Loughborough) against a blank reading of 1.5 µl dH_2O water. This same procedure was used to examine isolated RNA, mRNA, cDNA and cloned cDNA.

* Note, unless stated, all buffers belonged to a particular kit(s) and due to trade secrecy it was not possible to ascertain buffer ingredients.

2.2.4 Direct mRNA isolation from tissues

Of the four types of RNA (messenger RNA, transfer RNA, ribosomal RNA and micro RNA), it is messenger RNA (mRNA) that carries the coding information obtained from DNA during transcription and which is used for translating information into proteins (Weaver, 2002) and therefore is considered most useful for examining receptor expression. It is possible to examine total RNA for receptor expression, however for optimal sensitivity it is recommended to use mRNA (Sambrook & Russell, 2006). Messenger RNA, however, makes up only 1-5% of total RNA and is therefore difficult to obtain in sufficient quantities. Moreover, it usually requires a two-step procedure to firstly isolate total RNA and secondly isolate mRNA, and so there is the potential for a poor yield of mRNA. Advances in scientific technology meant that it was possible to directly isolate mRNA from fish tissues using a GenElute direct mRNA miniprep kit (Sigma; Cat # DMN10). An individual tissue sample (up to 25mg) was removed from the -80^{o}C freezer and immediately placed on ice. Once thawed, 1ml of lysis solution containing proteinase K was added to the sample. The sample mix was subsequently homogenised until no visible tissue pieces were visible. The mix was then incubated at 65^{o}C for 10 minutes to allow for complete degradation of nucleases and other proteins. Subsequetly, the mixture was removed from the heat and, to facilitate mRNA bead-binding, 64µl of 5M NaCl was added to the digested tissue lysate. Prior to addition to the sample mix, the oligo dT beads were thoroughly vortexed to ensure complete resuspension in buffer and, following on from this, 25µl of the resuspended oligo dT bead mix was added to the sample mix. The mix was vortexed thoroughly and subsequently incubated at room temperature for 10 minutes. During this incubation the poly(A) tails of the mRNA hybridised with the oligo dT on the beads. The bead-mRNA complex was collected in the form of a pellet via centrifugation at 17,900 x g for 5 minutes at room temperature. The DNA and protein-containing supernatant was carefully removed and discarded, leaving behind the pellet. To get a more highly enriched mRNA preparation, the bound mRNA material was released from the beads into a fresh lysis solution and then rebound to the same beads. This incorporation of re-binding helped to remove any unwanted material that may have been pelleted. To achieve this, 0.5ml of lysis solution and 32µl of 5M sodium chloride solution was added to the pellet and

vortexed thoroughly to resuspend the pellet. The mixture was subsequently incubated at 65^{o}C for 5 minutes and then incubated at room temperature for 5 minutes. The supernatant was subsequently removed leaving behind a more highly enriched mRNA-bead complex. The pellet was subsequently washed in 350µl of a high salt wash solution. The pellet-wash solution mixture was transferred into a spin filter-collection tube and centrifuged at 17,900 x g for 2 minutes at room temperature. The flow-through was disregarded, whilst the spin filter that contained the mRNA-bead complex was washed a further 2 times in 350µl of a low salt wash solution, each time the flow through was disregarded. The spin filter was transferred into a fresh, sterile collection microcentrifuge tube and 50µl of pre-heated (65^{o}C) elution buffer was pipetted onto the filter surface, ensuring that contact was made between the elution buffer and the mRNA-bead complex. The mix was subsequently incubated at 65^{o}C for 2 minutes before centrifugation at 17,900 x g for 2 minutes at room temperature. The mRNA-containing flow-through was subsequently collected and stored at -80^{o}C.

2.2.5 Total RNA isolation from fathead minnow blood

Nickerson *et al* (2003) documented that the β_3-AR was expressed in rainbow trout (*Oncorhynchus mykiss*) whole blood cells. It was therefore important to examine whether the fathead minnow also expressed the β_3-AR in whole blood cells. It was not possible, however, to use the GenElute direct mRNA miniprep kit to directly extract mRNA from fathead minnow whole blood cells and an alternative quick-step method was not available. Therefore, total RNA was firstly extracted using the two-step Tri reagent for blood derivatives protocol (Sigma; Cat # T3809) and this was followed by the mRNA isolation procedure. The Tri reagent method is an improvement on the single step method for total RNA extraction reported by Chomczynski and Sacchi (1987). The whole red cell samples were thawed slowly on ice. Once thawed, 0.75ml of tri reagent, supplemented with 20µl of 5N acetic acid was added to the whole red plasma sample and mixed via shaking. To ensure complete dissociation of nuclear protein complexes, the sample was incubated at room temperature for 5 minutes. Following that, 0.2ml chloroform was added and the sample was shaken vigorously for 15 seconds and incubated for 3 minutes at room temperature. The mixture was subsequently centrifuged at 17,900 x g for 15

minutes at 4^{o}C. The centrifugation procedure separated the mixture into 3 layers; the lower red organic phase (containing protein), the interphase (containing DNA) and the colourless upper aqueous phase (containing RNA).

The colourless RNA layer was carefully transferred into a clean microcentrifuge tube via pipetting and 0.5ml isopropanol was added and then the sample mixed and incubated for 10 minutes at room temperature. The sample was then centrifuged at 17,900 x g for 8 minutes at room temperature, which produced a precipitated RNA pellet. The supernatant was removed and the pellet washed with 1ml 75% ethanol (prepared in-house from 99% ethanol; Fisher Scientific, Cat # A405P-4) via vortexing followed by centrifugation at 17,500 x g for 5 minutes at room temperature. The ethanol was subsequently removed and the pellet air-dried for 10mins. Finally, the pellet was solubilised in 20µl double-distilled water (dH_2O). The purified RNA sample was stored at -80^{o}C.

In addition to examining the RNA quantity and quality by Nanodrop, 1-2µg of RNA in 9µl of dH_2O with 2 µl of loading buffer was also examined on a 1.2% agarose gel for any signs of DNA contamination and/or RNA degradation. The gel was run for 45 minutes at 80volts against a 1Kb DNA ladder (Helena Bioscience, Cat # N3232) (see section 2.2.9 for further details).

2.2.6 Isolation of poly A^+ mRNA from total RNA

Following total RNA isolation, red blood cell mRNA was isolated using the Oligotex mini mRNA isolation kit (QIAGEN; Cat # 72022). This protocol works on the principle of binding the poly A^+ tails of the mRNA to oligo dT-coated polystyrene-latex particles, thus dissociating them from total RNA and finally eluting the captured mRNA. A total RNA sample (20µl) was first thawed slowly on ice as to minimise RNA degradation. The total RNA sample was pipetted into an RNase-free 1.5ml microcentrifuge tube and the volume was adjusted to 250µl with diethylpyrocarbonate (DEPC) water. 250µl of digestion buffer and 15µl of Oligotex suspension, both of which had been pre-heated to 37^{o}C, were pipetted into the microcentrifuge tube. The contents were thoroughly mixed by reverse pipetting. The mixture was subsequently incubated for 3 minutes at 70^{o}C in a

water bath. After removal from the water bath, the sample was stored for 10 minutes at 20^{o}C. The mixture was pelleted by centrifugation at 17,900 x g for 2 minutes at room temperature and the supernatant was removed. The pellet was re-suspended in 400µl of a wash buffer and transferred into a small spin column within a microcentrifuge tube. The sample was then centrifuged at 17,900 x g for 1 minute room temperature. The spin column was transferred to a new RNase-free 1.5ml microcentrifuge tube and a further 400µl of a second wash buffer was added to the column. The flow through was discarded. The column was again centrifuged at 17,900 x g for 1 minute at room temperature and the flow-through was again discarded. The spin column was again transferred to a new RNase-free 1.5ml microcentrifuge tube. 20µl of a hot elution buffer (heated to 70^{o}C) was added to the spin column and reverse pipetted to resuspend the poly A^{+} mRNA. The column was again centrifuged at 17, 900 x g for 1 minute at room temperature. The flow-through contained the poly A^{+} mRNA and was thus kept. To maximise yield of mRNA, a further 20µl of hot elution buffer was added to the spin column, centrifuged and mRNA collected. The mRNA samples were stored at -20^{o}C until required for synthesis of first-strand copy DNA (cDNA). At this stage, there was suitable mRNA from several fathead minnow tissues.

2.2.7 Generation of complementary DNA by reverse transcription

Complementary cDNA was created from the isolated mRNA using the SuperScript III First-Strand synthesis system for reverse transcription-PCR kit (Invitrogen, Cat # 18080-051). In a single 0.5ml microcentrifuge tube, 1µl of 50µM oligo$(dT)_{20}$ primer, 1µl of 10mM deoxyribonucleotide triphosphate (dNTP) mix and 8µl of mRNA (up to 5µg) sample were combined together, the mixture briefly centrifuged, and incubated at 65^{o}C for 5 minutes and then placed on ice for 1 minute. This step annealed the oligo$(dT)_{20}$ primer to the mRNA. Following on from that, 2µl of 10 x RT buffer, 4µl of 25mM $MgCl_2$, 2µl of 0.1M DTT, 1µl of RNaseOUT^{TM} (40 U/µl) and 1µl of SuperScript^{TM} III RT (200 U/µl) was added, mixed by reverse pipetting and then briefly centrifuged at 17,900 x g. The mixture was subsequently incubated at 50^{o}C for 50 minutes to create the cDNA product. The reaction was terminated by incubation at 85^{o}C for 5 minutes and the mixture was then chilled on ice for approximately 2mins. Residual RNA was removed by

adding 1µl of RNase H (2 U/µl) to the reaction and incubating it at 37°C for 20 minutes. The newly-created cDNA was stored at -20°C until required for a PCR reaction.

2.2.8 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is an *in vitro* technique which allows the amplification of a specific DNA sequence in a complex mixture where the ends of the sequence are known. Primers, which are designed to complement the DNA region of interest, bind to the DNA and are extended by the enzyme DNA polymerase in the presence of dNTPs. Under optimum conditions, this reaction synthesises a new complementary DNA strand. Over the course of 30-40 cycles, the quantity of desired DNA increases exponentially, as shown in figure 10 (Newton & Graham, 1994). The PCR is a robust, relatively cheap, sensitive and easy to use technique and, as such, has been utilised in many ecotoxicological studies examining the effects of pharmaceutical pollutants including the fathead minnow (Halm *et al*, 2002; Zerulla *et al*, 2002; Miracle *et al*, 2006).

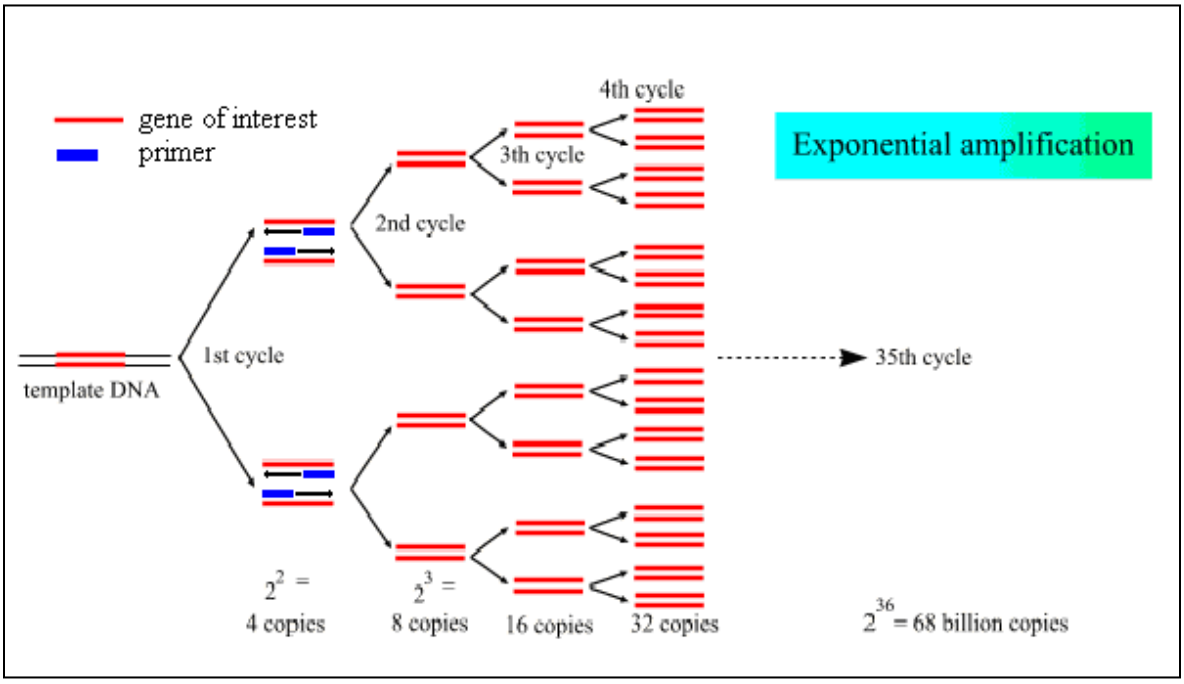


Figure 10. Overview of PCR reaction. Figure adapted from Vierstraete (1999).

2.2.8.1 Factors affecting PCR

There are several factors that can influence a PCR reaction and resultant success:

1. **Primers**

Primer designing

Template sequences

The only published data on putative fish β_3-ARs was that by Nickerson *et al* (2003) who identified two probable β_3-AR's in the rainbow trout (*Oncorhynchus mykiss*) as β_{3a}- and 3_b-AR. Additionally, using the NCBI database engine, two β_{3b}-AR sequences were found in the zebrafish. The zebrafish belongs to the same Cyprinidae family as the fathead minnow (Linnaeus, 1758) and so the likelihood of sequence conservation between the two species was higher than between the fathead minnow and the rainbow trout. However, the zebrafish β_3-AR sequences were computer-software generated and had not been supported by laboratory findings. Therefore, in addition to using the published primers by Nickerson *et al* (2003), the two zebrafish β_{3b}-AR sequences, which for the ease of designing primers for this project were named β_{3bi}-AR and β_{3bii}-AR, were used as templates for designing primers. Table 6 summarises the primer sets successfully used to isolate the β_3-adrenergic receptor in the fathead minnow.

Primer conditions

All primers were between 16 and 30 nucleotides long and were ordered from Sigma Genosys (http://orders.sigma-genosys.eu.com) and dissolved with Milliq water to a concentration of 100μM. Each primer had a guanine and cytosine (G and C) content of 40-60% and, in most cases, had a guanine or cytosine nucleotide at the 3' end. Guanine and cytosine bind to each other using 3 covalent bonds, which make a stronger link compared to the link between adenine and thymine, which only uses 2 covalent bonds. As such, a high GC content and a 3'end guanine or cytosine nucleotide strengthened the bond between DNA template and primer, thus assisting with the amplification process. DNA Taq polymerase has a melting point of 72^{o}C, therefore each primer pair was

designed to have a melting point between 58-68^{o}C; the higher the melting point, the more specific the binding between primer and DNA template and the less likelihood of a primer binding non-specifically. If the annealing temperature was too low, the primer could bind non-specifically to undesired regions of DNA producing unwanted PCR products. Each primer pair was designed to have a similar, if not the same, melting point; these were calculated using the oligo-calculator on the Sigma Genosys website (www.sigmaaldrich.com/life-science/custom-oligos.html). To improve the likelihood of isolating the desired sequence, nested primers were also used. These were primers designed to the same DNA template as the initial primers but were 'inside' of the first pair and therefore they were used in a second PCR reaction to further amplify the product obtained from the first PCR and minimize the profile of any non-specific products amplified in the first round.

In addition to optimizing the PCR temperature conditions, the risk of primers binding non-specifically, either to themselves producing primer dimmers or to other regions of DNA, was minimized by first examining the likelihood of that happening using programmes such as http://frodo.wi.mit.edu/ and www.basic.northwestern.edu/biotools/oligocalc.html. A primer was re-designed if it had the potential for binding non-specifically.

Table 6. Primer sets used to isolate the β_{3bi}- and β_{3bii}-ARs in the fathead minnow.

Primer name	**β_{3bi}-AR 5' - 3'**	**β_{3bii}-AR 5' - 3'**	**Annealing Temp (^{o}C)**
Round 1			
forward	GTAACCTCCTGGTCATCATTG	GCGTTGGTCATCTTCTTGACG	62
reverse	GTAGATGATAGGGTTGAGTCC	GCGTAGATGATTGGGTTCAG	
Nested round 2			
forward	CCTCCAGCTGCAGACTAC	CTCTCCTCCACACCACCAC	62
reverse	GCCTAACCAGTTTAAGAGACG	CGTAGCCCAGCCAGTTCAAC	
Nested round 3			
Forward	GCCAGCATAGAGACTCTATG	CGCTAGCATTGAGACATTGTG	64
reverse	GCGTAGATGATGTTCGCCAC	GCCAACAGAGACTGAAGATG	

2. DNA polymerase enzyme

Several DNA polymerase enzyme kits are available, all with varying levels of accuracy. Platinum Taq DNA polymerase high fidelity (Invitrogen, Cat # 11304-011) was used. This was an enzyme mixture composed of two DNA Taq polymerases which improved both the accuracy and efficiency of the PCR reaction.

3. Temperature

The entire PCR reaction relies upon several stages of temperature. Initially the temperature must be between 95^{o}C and 100^{o}C for the denaturation of template DNA, whilst from the second cycle onwards it is sufficient for the temperature to be between 92 and 95 ^{o}C. Denaturation temperatures are unlikely to be changed. The annealing temperature of the primer to the DNA strand is particularly crucial and specific to each primer. The optimum temperature for primer extension was 72^{o}C.

4. Number of cycles

As standard, the number of PCR cycles ranges from 25 – 35 cycles. As the number of cycles exceeds 30, the amount of DNA Taq polymerase enzyme in the reaction tube becomes limited. This in turn limits the efficiency of the PCR reaction. Figure 11 outlines a typical PCR thermal cyclic programme used for this project.

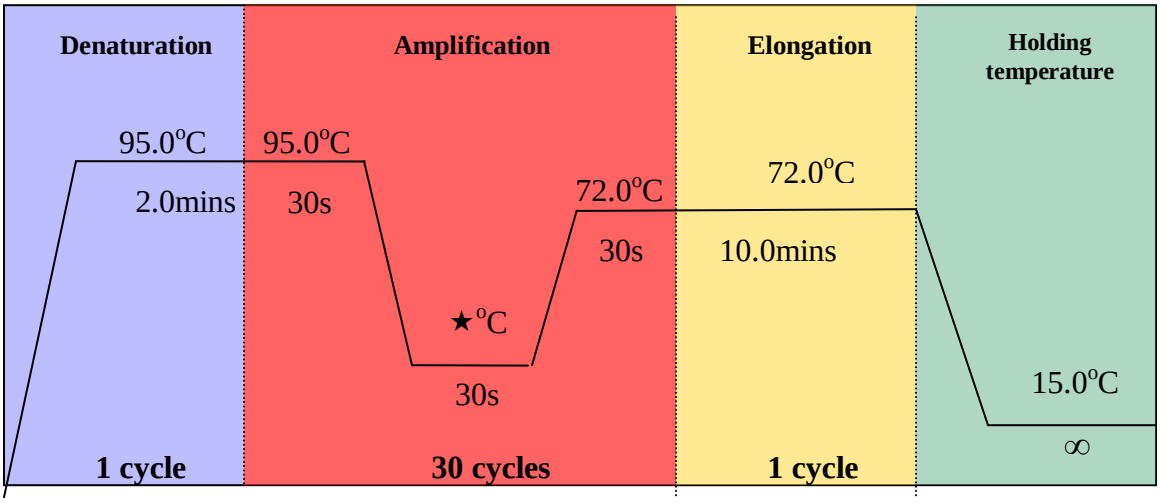


Figure 11. PCR protocol. ★ indicates temperature specific to GSP.

5. Quantities of additional PCR components

A specific buffer is used to complement the specific DNA Taq polymerase enzyme, which further improves the efficiency of the enzyme. Additionally, magnesium sulphate ($MgSO_4$) is required to form a soluble complex with dNTPs to allow them to be incorporated into the extending strand. Hence the concentration of $MgSO_4$ and dNTP, and their corresponding ratio, in the final reaction mix was of paramount importance and can dramatically affect the yield of PCR reactions; insufficient $MgSO_4$ results in a low yield of PCR product; too much $MgSO_4$ can produce high levels of non-specific products. Table 7 summarises quantities, and resultant final concentrations, of components used in the PCR first and nested rounds. The only difference between the first round PCR and a nested round was the quantity of added DNA template. Only 0.5µl of DNA template was added to the nested PCR reaction as the quantity of DNA was significantly higher than in the first round as it was the product of exponential amplification from the 1st PCR round.

Table 7. Quantities and respective final concentrations of components added to a standard first round and nested PCR reaction.

Component	**1st Round PCR**	**Final concentration**	**Nested rounds PCR**	**Final concentration**
10 x high fidelity PCR Buffer	2.5µl	1 x	2.5µl	1 x
10 mM dNTP mixture	0.5µl	0.2mM	0.5µl	0.2mM
50mM $MgSO_4$	1µl	2mM	1µl	2mM
Forward primer	0.5µl	0.4µM	0.5µl	0.4µM
Reverse primer	0.5µl	0.4µM	0.5µl	0.4µM
Platinum Taq high fidelity	0.25µl	1.0 unit	0.25µl	1.0 unit
Sample	2.5µl genomic DNA or cDNA		0.5µl PCR product from previous round	
Double distilled water	To 25µl	not applicable	To 25µl	not applicable

2.2.9 Gel electrophoresis

To visualise the PCR reaction, and determine whether a product had been generated, each PCR reaction mixture was run on an agarose gel via electrophoresis. The principle of gel electrophoresis, in brief, is that DNA formed during the PCR reaction is negatively charged. When added to an agarose gel matrix and exposed to an electrical current, DNA products migrate towards the positive anode; smaller products will move further and faster than large products, thereby separating DNA according to product size in a series of bands. A DNA ladder was used as a means of indicating the size of DNA bands. Visualisation was achieved by subsequent ultraviolet (UV) illumination of the gel (Sambrook & Russell, 2006). Expected correct band size was estimated at the primer-designing stage.

The gel was made by adding 1.2g of certified biological agarose (Bio-Rad, Cat # 163-2111) with 100ml, 1 x Tris borate ethylenediaminetetraacetic acid (EDTA) (TBE) buffer (see appendix 1) in a 250ml conical flask, producing a 1.2% gel. The mixture was heated in a microwave for approximately 2.5 minutes until boiling point. The conical flask was shaken and reheated in the microwave to ensure the agarose powder had fully dissolved in the buffer. Once cooled, 5µl of ethidium bromide (Sigma, E1510) was added to the flask and the solution mixed by swirling. Ethidium bromide stains DNA under U/V conditions. The gel was poured into a plastic mould that had been taped at either end with autoclave tape. The comb, used to create wells for the PCR product samples, was inserted 1 cm from the top edge. The gel was left to cool and solidify for 30 - 45 minutes. The gel-loading buffer was prepared by adding 750µl 40% Ficol (Fisher Scientific, Cat # 26873-85-8), 100µl 10 x TBE, 150µl dH_2O and 8µl 0.1% xylene cyanol (Sigma, Cat # 220-167-5). 2.5µl of gel-loading buffer was added to 20µl of sample. The gel-loading buffer gives both colour and weight to a sample, thus aiding in loading the sample into the gel and ensures the sample does not dissipate out of the well. Once each sample had been loaded into the gel, a 1Kb DNA ladder (Helena Bioscience, Cat # N3232) was loaded. The gel was then run for 45 minutes at 80 volts. The gel was then examined under UV and a photograph taken.

2.2.10 Cutting and gel-extraction of PCR product

Upon examination, if a band of the correct size was observed, it was excised from the gel under UV light using a clean, sharp scalpel (Swan-Morton, Sheffield) and transferred to a clean 2.0ml microcentrifuge tube. UV rapidly breaks-down DNA and so this process was conducted quickly. The gel slice was weighed and the DNA extracted from the gel according to the protocol from the MinElute gel extraction kit (Qiagen, Cat # 28604). Firstly, the gel slice was weighed and three volumes of digestion buffer were added to 1 volume of gel. The mixture was then incubated at $50^{o}C$ for 10 minutes to ensure the gel had been completely dissolved. One gel volume of isopropanol was added to the sample and mixed by inverting the tube several times. The mixture was then transferred to a MinElute column inside a 2ml collection tube, which was centrifuged at 17,900 x g for 1 minute at room temperature. The flow-through was discarded and 500µl of a wash buffer

was added to the spin column, which was centrifuged at 17,900 x g for 1 minute at room temperature. The flow through was again discarded and 750µl of a second wash buffer was added to the spin column, which was left for 3.5 minutes and centrifuged at 17,900 x g for 1 minute at room temperature. The flow through was disregarded and the spin column was centrifuged for an additional minute. The MinElute column was placed into a clean microcentrifuge tube. 10µl of sterile dH_2O was added to the spin column, which was centrifuged at 17,900 for 1 minute at room temperature. The eluate that contained the DNA was collected, quantified and stored at -20^{o}C to await either cloning or sequencing preparation.

2.2.11 Recombinant DNA cloning

One problem of using PCR is that a DNA band of the correct size may actually be comprised of several DNA bands, all of which have the same size. This is particularly problematic when examining for an unknown DNA sequence with primers that may not be specific to the sequence. Recombinant DNA cloning presents an additional method of separating and identifying all potential DNA sequences that may be found within a single DNA band of interest and subsequently amplifying the desired DNA product. The process of recombinant DNA cloning is divided into two principal stages. The first stage is ligation; the DNA product is inserted into a bacterial plasmid vector. The second stage is transformation, where the plasmid is inserted into a suitable bacterial host cell and subsequently grown on agar plates to amplify the desired DNA product (Watson *et al*, 1992).

2.2.11.1 Ligation

The DNA formed during a PCR reaction has single adenine residues formed at either end, which is referred to as a "sticky end" as it allows for insertion into a plasmid vector that have a uracil overhang. Only a single DNA product can be inserted into a plasmid vector, thus separating different DNA products of the same size. Ligation was achieved using the PCR cloning kit (Qiagen, Cat # 231222). 4µl of extracted DNA solution was added to 1µl pDrive cloning vector (50ng/µl) and 5µl 2 x ligation master mix. The solution was made

up to 10µl with distilled water and incubated for 2 hours at 15^{o}C in an incubator. During this incubation time, the adenine (5' end) and uracil (3' end) residues in the DNA product and cloning vector, respectively, hybridized together to produce a complete plasmid (see figure 12). In addition to the region where exogenous DNA fragments can be inserted, there is also, importantly, a drug-resistant gene present in the plasmid which confers resistance to the antibacterial compound ampicillin. The drug-resistance offers a screening process so that only cells with the inserted plasmid can colonise on ampicillin-containing agar and reduces that risk of bacterial contamination.

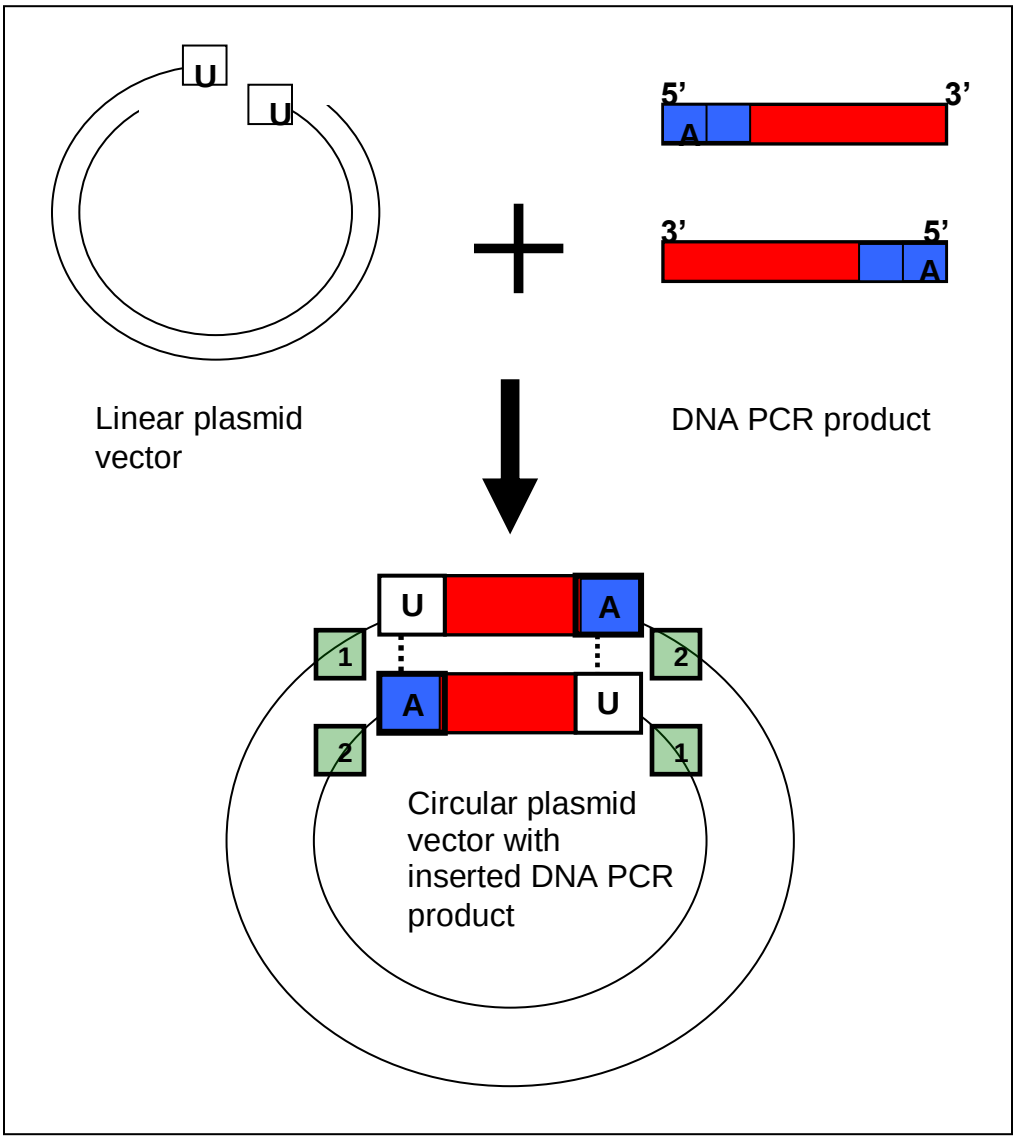


Figure 12. Schematic representation of ligation procedure with insertion of DNA PCR product. Green boxes 1 and 2 represent the promoter genes SP6 and M13R, respectively. A hydrogen bond was formed between the adenine and uracil nucleotide bases at either end of the PCR product.

2.2.11.2 Transformation

Immediately following the ligation incubation period, 2µl of the ligation mix was added to quickly thawed QIAGEN EZ Competent cells (Qiagen, Cat # 231222) and left on ice for 5 minutes. The cell mixture was then heat shocked for 30 seconds at 42 ºC and returned immediately to ice. 250µl of room temperature super optimum broth with catabolite repression (SOC) medium was added, briefly mixed by reverse pipetting and spread over 4 agar plates using a sterilised glass spreader. The agar plates were made of 100 ml agar (see appendix 2), 100µl ampicillin, 192µl Xgal and 50µl Isopropyl β-D-1-thiogalactopyranoside (IPTG). Ampicillin provided the means of preventing growth of cells without plasmids on the agar, Xgal expresses the β-galactosidase enzyme, which is encoded in the lacZ gene and was used to facilitate the differentiation between cells with and without an inserted DNA sequence within the plasmid vector (which appeared as white and blue colonies, respectively). The DNA insert within the plasmid breaks the LacZ gene and therefore the colour remains white, whilst those colonies without a DNA insert within the plasmid vector have an intact lacZ gene and so the colour remains blue. IPTG further induces the transcription of β-galactosidase and thus enhances the colour differentiation.

The plates were incubated overnight at 37 ºC. The following day, the plates were incubated at 4^{o}C to facilitate colour development (i.e. blue and white colonies) and white colonies were selected for PCR checking of DNA insert.

2.2.12 PCR-checking of colonies

White colonies were checked with SP6 (5’ AT TTA GGT GAC ACT ATA G 3’) and M13 reverse (M13 R) (5’ CAG GAA ACA GCT ATG ACC ATG 3’) primers in a PCR reaction. These are primers designed to amplify the region between both promoters, which was the exogenous DNA PCR product insert, as shown in figure 9. Up to 58 colonies were selected for PCR-checking and they were subsequently visualised on a 1% agarose gel to determine that DNA PCR products of the correct size had been inserted into the plasmid vectors. Those colonies that had the correctly sized DNA inserts were

selected and grown in individual 5ml cultures of Luria broth (L-broth) (see appendix 3) and 5µl ampicillin overnight at 37^{o}C to maximise the quantity of DNA insert.

2.2.13 Preparation of glycerol stocks

Once the cultures had grown overnight, 250µl of a culture mix was added to 250µl of 20 % glycerol in a 2ml microcentrifuge tube and subsequently stored at -80^{o}C. This provided a stock of cells that could be re-used repeatedly for future sequencing work.

2.2.14 Plasmid (mini-prep) preparation

The remaining culture mix was centrifuged at 2,000 x g at room temperature for 10 minutes, which produced a pelleted culture. The supernatant was disposed of into a previously-prepared multi-purpose disinfectant solution (Virkon; RMS, Cat # VIR-05) whilst the pelleted culture underwent plasmid preparation using the QIA prep spin miniprep kit (Qiagen, Cat # 27104). Firstly, 250µl of a lysis buffer containing RNase A was added and the cells re-suspended by repeat pipetting. 250µl of a second lysis buffer was then added, followed by 350µl of a suspension buffer. The tube was thoroughly mixed by inserting the tube upside down several times. The subsequent mixture was centrifuged at 17,900 x g for 10 minutes at room temperature, which produced a white pellet. The supernatant was then pipetted onto a QIA prep spin column and centrifuged for at 17,900 x g for 1 minute at room temperature. 500µl of a wash buffer was then added to the spin column and centrifuged. During these steps, the bacterial lysate adsorbed to the silica-gel QIA prep spin column membrane and all impurities were washed away in the buffer. The DNA was finally eluted in 50µl of elution buffer. The DNA quality and quantity was subsequently examined using the Nanodrop spectrophotometer. In addition 2µl loading buffer was added to 5µl of DNA sample and was run on a 1.2% agarose gel at 80V for 45 minutes and visually examined for correct product size.

2.2.15 Sequencing

Plasmid mini-prep DNA products of the correct size were diluted to a concentration of 600ng in 30ml of dH_2O. The diluted samples were then sent to the Sequencing Service,

Dundee University (www.dnaseq.co.uk) for direct sequencing analysis. Sequencing analysis ran from either the SP6 or M13R promoter region, or both as required. The results from the sequencing were sent back as a chromas plot indicating the nucleotide sequence of the DNA product, as shown in figure 13a. Once the nucleotide peaks had become large, sharp and well defined, the sequence was taken. Figure 13b shows a sequence that had been obtained from a PCR sample without cloning. It can be clearly observed that there was more than one nucleotide sequence; hence sequencing of cloned PCR products was the preferred method.

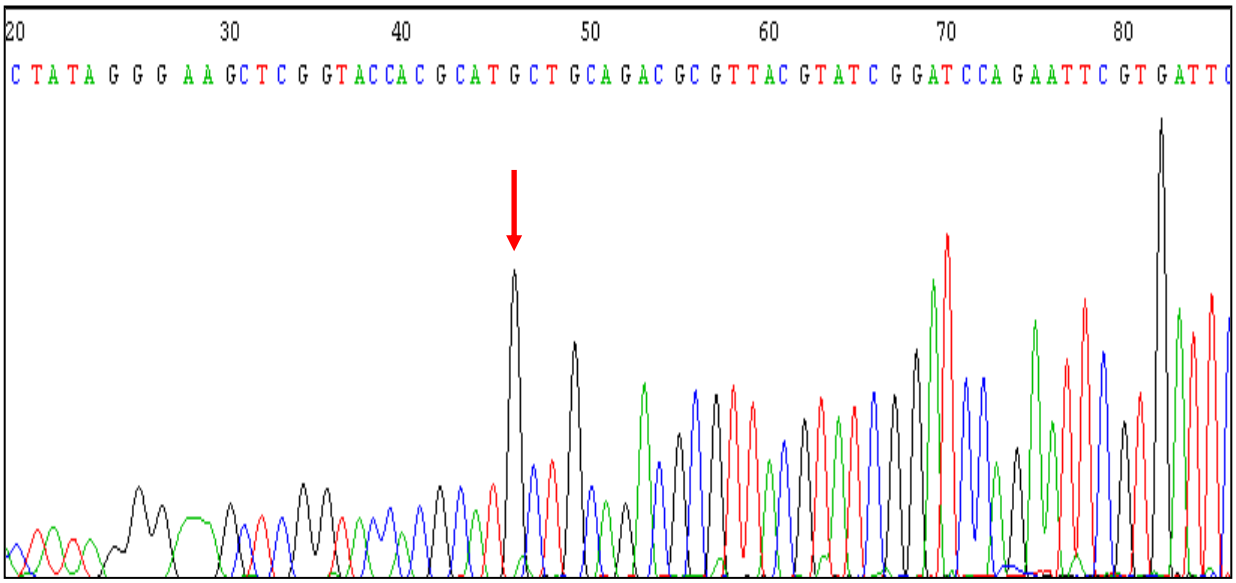


Figure 13a. Example of a chromas file indicating the nucleotide sequence. The red arrow indicates where the strength of sequence peaks was judged to be satisfactory.

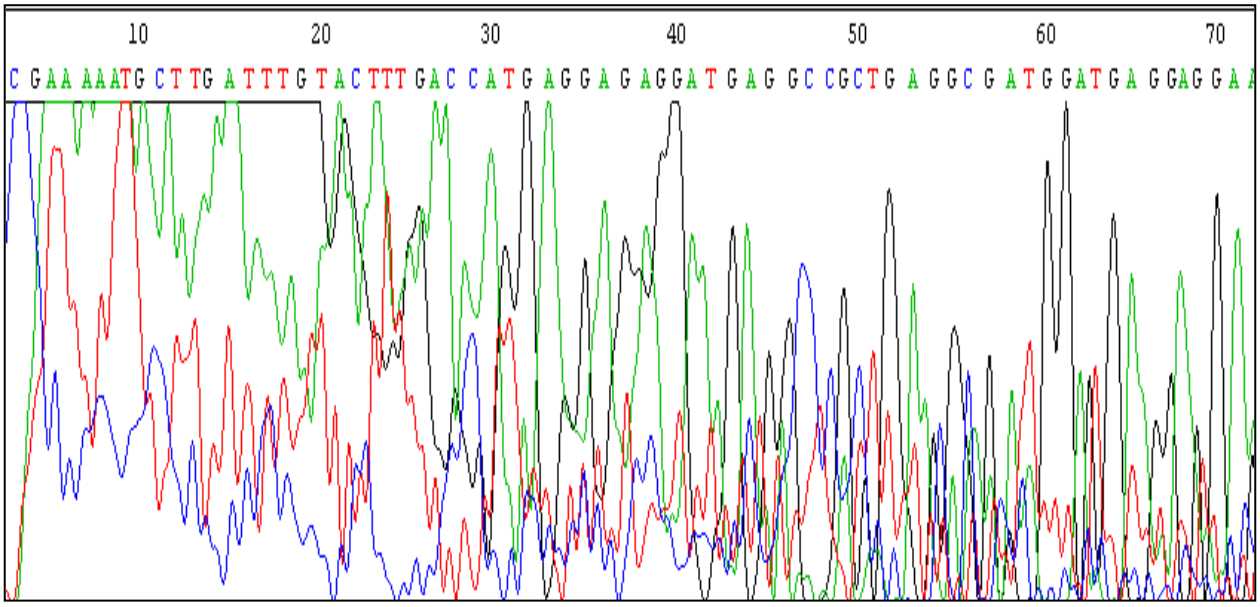


Figure 13b. Example of a chromas file taken from a PCR sample without cloning. The sequence is not clean and there are many overlaps suggesting that there is more than one sequence. This sequence would not have been used for further analysis.

2.2.16 Rapid amplification of cDNA ends (RACE) PCR

Once a fragment of the β_3-AR had been characterised, it was possible to identify the entire sequence using RACE PCR. Using gene specific primers (GSP) designed to the known sequence, RACE PCR was used to fill in both the 5' (5' RACE) and 3' (3' RACE) ends; figure 14 illustrates the principle of RACE PCR. The SMART RACE cDNA amplification kit (Clonetech, Cat # 634914) was used to amplify the β_3-AR 5' and 3' ends. For both types of RACE PCR, the starting mRNA template was firstly reverse transcribed into cDNA. The 3' end of an mRNA sequence has a string of adenine residues (referred to as a poly A^+ tail) and therefore during reverse transcription an oligo(dT) adapter primer (AP) with an enriched GC end was incorporated into the cDNA. A PCR was then run using the existing GSP and AP to amplify the specific β_3-AR product. Nested PCR rounds were then used to increase the specificity of the PCR.

The 5' end of an mRNA sequence does not have a distinguishable feature such as a poly A^+ tail and, therefore once the reverse transcriptase enzyme reached the 5'end of the mRNA sequence during reverse transcription, it added several cytosine residues, thereby

extending the cDNA sequence slightly. This extended 5' tail anchor served as a template for a 5' end-specific primer. Similarly to the 3' RACE, a PCR was then run using the GSP and 5'end-specific primer. A nested PCR round was used to increase specificity.

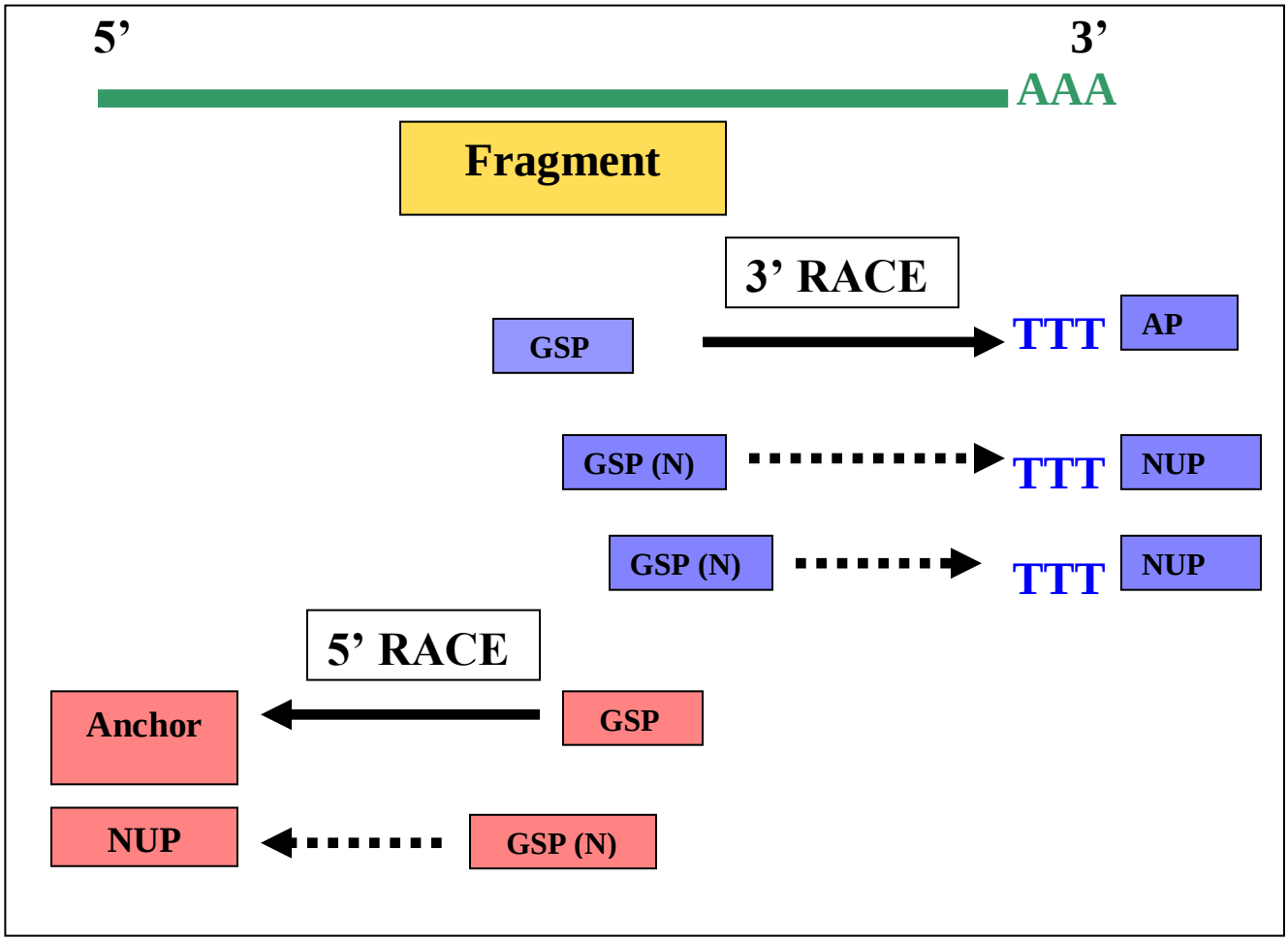


Figure 14. Rapid amplification of cDNA ends (RACE) schematic. GSP = gene specific primers; GSP (N) = nested gene specific primers; AP = adapter primer; NUP = nested universal primer. The yellow box represents the identified β-3-AR fragment; the green line represents the entire sequence from 5' to 3' end.

The GSPs and 5' and 3' primers used to amplify the β_3-AR sequence used are summarised in table 8.

Table 8. Summary of primers used for 3' and 5'RACE. Annealing temperatures specified in brackets.

		GSP	AP/Anchor primer
3'RACE	Round 1	TTCAATCGAGATCTGCTAACC (68°C)	CTAATACGACTCACTATAGGGC (70°C)
	Nested	CAGAGTTTCGTGCGGCCTT (68°C)	AAGCAGTGGTATCAACGCAGAGT (70°C)
5'RACE	Round 1	CGATGTCCATAATCTGCACG (68.9°C)	CTAATACGACTCACTATAGGGC (70°C)
	Nested	GGTCTGCAGATGGGAGGT (69.6°C)	AAGCAGTGGTATCAACGCAGAGT (70°C)

The PCR cycle protocol adopted for both RACE experiments is outlined in figure 15. The main stage of annealing and subsequent exponential amplification of PCR product was stage 3. The annealing temperatures naturally varied depending on which set of primers were used, as shown in table 8, above.

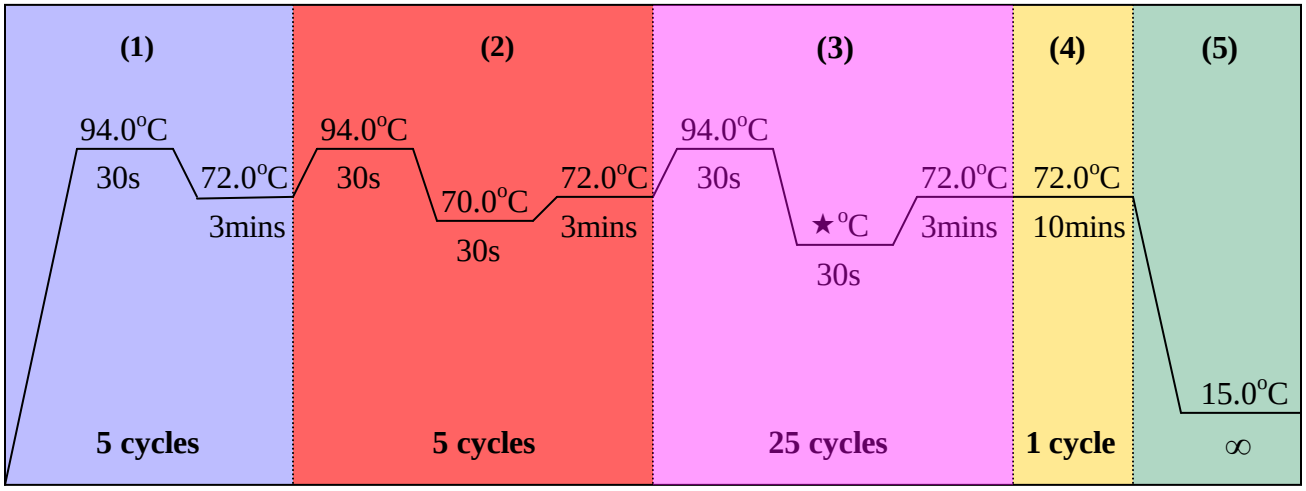


Figure 15. RACE PCR protocol. ★ indicates temperature specific to GSP. Annealing temp ranged from 68.0°C to 70.0°C depending on the GSP. Stage number indicated in brackets.

In addition to running the RACE PCR primers, additional primers were designed to confirm regions of the β_{3bi}-AR sequences, to resolve any discrepancies. In total, two sets of primers were used, as shown in table 9 and were run according to the PCR protocol outlined in figure 14, using the indicated annealing temperature.

Table 9. Summary of primers used for examining sequence discrepancies. Annealing temperatures specified in brackets.

	Forward **5' – 3'**	**Reverse** **5' – 3'**
Test 1	GCGACTACTCACACGACGCTGCTATCTGC (76^oC)	CATAGAGTCTCTATGCTGGCAGTGACGC (72^oC)
Test 2	GCGTCACTGCCAGCATAGAGACTCTATG (72^oC)	GCGTAATGATGTTCGCCACAAAGAAAG (73^oC)

2.2.17 Confirmation of β_{3bi}-AR by primer walking

To ensure that both RACE products and additional PCR products were from the same sequence and to confirm the β_{3bi}-AR, additional first round and nested primers were designed in the 5' and 3' untranslated regions (UTR), which are regions specific to the proposed β_3-AR. A PCR and nested PCR reaction was subsequently performed. The product from the nested round was gel extracted and purified, inserted into a plasmid vector, transformed into competent cells and grown on agar plates. Suitable colonies were PCR checked for the correct insert and a single colony was subsequently grown in L-broth and served as the template for the whole primer walking process, which was conducted by the Sequencing Service. Primer walking is a process whereby the entire sequence is characterised in the 5'3 and 3'5 direction using a series of overlapping primers. Unlike in a PCR, the primers are not used to produce a product but as probes to continue the sequencing process. Sequencing accuracy is limited to approximately 750 nucleotides. Therefore, with an anticipated sequence of approximately 2,100 nucleotides, additional primers (termed 'internal primers') designed by the Sequencing Service were used to enable the complete sequencing, and confirmation, of the β-3bi-AR product in both the 5'3 and 3'5 direction. Table 10 summarises the primers used.

Table 10. Summary of primers used to confirm sequence in both 5'3 and 3'5 directions. Annealing temperatures specified in brackets. The internal primers were those designed by and used by the Sequencing Service to enable complete sequencing of the β_{3bi}-AR product.

Primer	Sequence 5' – 3'
5'3	CACGCTGACTGAACCTCCTCC (68^{o}C)
5'3 nested	GCGGACTACTTCAACACGACGGC (73^{o}C)
3'5	CCGGGCTGAAGTGGATTGCTCCAGTAC (76^{o}C)
3'5 nested	CTCCATGTTACACTCCTTCATGAACAGTACC (70^{o}C)
Internal 1 (5'3)	ACCTCCCAGCTGCAGACC
Internal 2 (5'3)	TTCAATCGAGATCTGCTAACC
Internal 1 (3'5)	AAGGCCGCACGAAACTCTG
Internal 2 (3'5)	CGATGTCCATAATCTGCACG

2.2.18 Computational analysis

2.2.18.1 Sequence alignments

Nucleotide sequence products were analysed for both similarity to other β_3-AR sequences and for annotation. The nucleotide sequences were analysed in their sense (5'3') direction. In cases where the sequence product was in the anti-sense direction, the nucleotide sequence was reverse complemented using the website www.bioinformatics.vg/bioinformatics_tools/reversecomplement.shtml. The nucleotide sequences were subsequently run on the blastx programme on the NCBI database, where the protein database was searched using the translated nucleotide sequence. Regions of similarity between the query sequence (proposed fathead minnow β_{3bi}-AR) and all other amino acid sequences listed on the NCBI database. Those sequences that were significantly similar to the query sequence were listed in rank order of most similar to least similar. Alongside each sequence was an E value, which describes the number of likelihood of "hitting" the specific sequence by chance. The closer the E value was to zero, the more significant the match.

Sequences were aligned using the ClustalW (1.83) multiple sequence alignment database (www.ebi.ac.uk/Tools/clustalw/index.html) using default settings for pairwise alignment. The proposed fathead minnow β_3-AR nucleotide sequences were run against the zebrafish, trout and human β_3-ARs to examine for regions of similarity. The fathead minnow sequences were also aligned against other fish and human β-adrenergic receptors to examine for the possibility that the sequences may have been a β_1 or β_2-AR.

The accession numbers of selected sequences from the NCBI database, unless stated otherwise, used for ClustalW sequence alignment were as follows: For β_3-ARs, *Danio rerio*: NW_001513985; *Oncorhynchus mykiss* β_{3a}-AR: AY216465, *Oncorhynchus mykiss* β_{3b}-AR: AY216466, *Takifugu rubripe*: CAG42650.1, *Tetraodon nigoviridis*: CAG12607.1, *Homo sapiens*: X70811.

For β_1-ARs: *Danio rerio*: XP 685300, *Pimephales promelas* AR: characterised by Giltrow (PhD thesis 2008) and as yet does not have an NCBI accession number, *Tetraodon nigoviridis*: CAG12607.1, *Homo sapiens*: NP00675.1.

For β_2-ARs: *Danio rerio*: XP 700720, *Pimephles promelas* β_{2i}-AR and β_{2ii}-ARs: characterised by Giltrow (PhD thesis 2008) and as yet do not have NCBI accession numbers: *Oncorhynchus mykiss*: AAK94672.1, *Tetraodon nigroviridis*: CAG10129.1, *Takifugu rubripe*: AAQ02695.1, *Myxine glutinosa*: CAA06539.1, *Petromyzon marinus*: CAA06540.1, *Homo sapiens*; AAB 82149.

The nucleotide sequence products were subsequently translated into protein sequences using the ExPASy proteomics server of the Swiss Institute of Bioinformatics (www.expasy.ch/tools/dna.html). The nucleotide was translated into all 6 possible reading frames. Frame 1 was obtained from translating from the first nucleotide, frame 2 from the second and frame 3 from the third. Frames 4 to 6 were the same as frames 1 to 3 but in the reverse direction. The frame that produced the longest reading frame with no stop codons was selected. The protein sequences were also aligned against other β_3-, β_2- and β_1-AR protein sequences using ClustalW.

2.2.18.2 Hydropathy analysis

The seven hydrophobic transmembrane domains synonymous to GPCRs must be of a length ranging from 23 to 30 hydrophobic amino acids to span the hydrophobic cell membrane bilayer (Strosberg & Peitri-Rouxel, 1996). Accordingly, hydrophilic amino acids are commonly found outside of the bilayer. To examine for these seven hydrophobic amino acid chains, the ExPASy proteomics server of the Swiss Institute of Bioinformatics (www.expasy.ch/_tools/dna.html) was used and several programmes utilised. Using the Kyte-Doolittle hydropathy index (Kyte & Doolittle, 1982), which ranged from -4.5 (hydrophilic) to 4.5 (hydrophobic), each amino acid was allocated a hydropathy score. It is the hydrophobicity of the amino acid that determines its position in the final protein structure. Those regions of hydrophobic amino acids were subsequently allocated a TMD, and those hydrophilic positioned outside of the TMD. The software programmes used to identify the TMDs were as follows:

1. TMpred – Prediction of Transmembrane Regions and Orientation (Hofmann & Stoffel (1993). (www.ch.embnet.or/software/TMPRED_form.html)
2. TMHMM (2.0) Prediction of transmembrane helices in proteins, The Centre for Biological Sequence Analysis at the Technical University of Denmark, (Krogh *et al*, 2001). (www.cbs.dtu.dk/services/TMHMM-2.0/)
3. SOSUI Result Classification and Secondary Structure Prediction of Membrane Proteins (Hirokawa *et al*, 1998; Mitaku *et al*, 2002). (http://bp.nup.nagoya-u.ac.jp/sosui/cgi-bin/adv_sosui.cgi)
4. HMMTOP transmembrane topology prediction server (Tusnády & Simon, 2001). (www.enzim.hu/hmmtop/)
5. Dense Alignment Surface (DAS) Transmembrane Prediction server (Cserzo *et al*, 1997). (www.sbc.su.se/~miklos/DAS/tmdas.cgi)

The allocated hydrophobic amino acid chains varied between the different programmes. Therefore, additional criteria based on information about GPCR and β-AR biology was used to help with the decision process of allocating TMDs to particular hydrophobic amino acid regions.

2.2.18.3 Phosphorylation sites analysis

Potential phosphorylation residues of serine, threonine and tyrosine in the third intracellular loop and cytoplasmic tail were identified using NetPhose 2.0 (http://www.cbs.dtu.dk/services/NetPhose/) (Bloom *et al*, 1999).

2.2.18.4 Phylogenetic analysis

Phylogenetic trees are binary-rooted trees constructed from multiple sequence alignments, and are used to illustrate genetic distance and species relationships (Penny *et al*, 1992). For the purpose of this study, the β_3-, β_2- and β_1-AR amino acid sequences were aligned using a multiple cluster and topological algorithm alignment server using the GeneBee software programme (www.genebee.msu.su) (Brodsky *et al*, 1995). The results were presented in the form of a rectangular tree. Bootstrap values indicate the significance of relationship between the sequences, much like p values are used in statistical analysis, which is particularly important when examining the relevance of a particular phylogenetic tree (Thompson *et al*, 1994). Bootstrap values were calculated by re-sampling each alignment 100 times and expressing the similarity as a percentage.

2.2.19 Examining β_{3bi}-AR expression in fathead minnow tissues

Once the sequence had been classified as a β_{3bi}-AR, several tissues from both adult male and female fathead minnows were examined for β_{3bi}-AR expression via PCR. The GSP pair designed in the 3' end UTR, and consequently used for real-time PCR analysis (see section 5.2.4), were used to examine the heart, liver, brain, gill, red blood cell, adipose and gonad tissues for β_{3bi}-AR expression.

2.3 RESULTS

2.3.1 RNA extraction from blood

Unlike fathead minnow tissues, from which mRNA could be directly extracted, RNA was first extracted from fathead minnow whole blood cells and visually examined on a 1% agarose gel to give an indication of quality. Figure 16 shows an example of RNA inspection and demonstrates both a clean set of extracted RNA samples (16A), note the two distinct 28S and 18S bands and an unclean extracted RNA sample (16B). The 28S band should be approximately twice as strong as the 18S. There were no signs of DNA contamination. The blurring in figure 15B was indicative of RNA degradation.

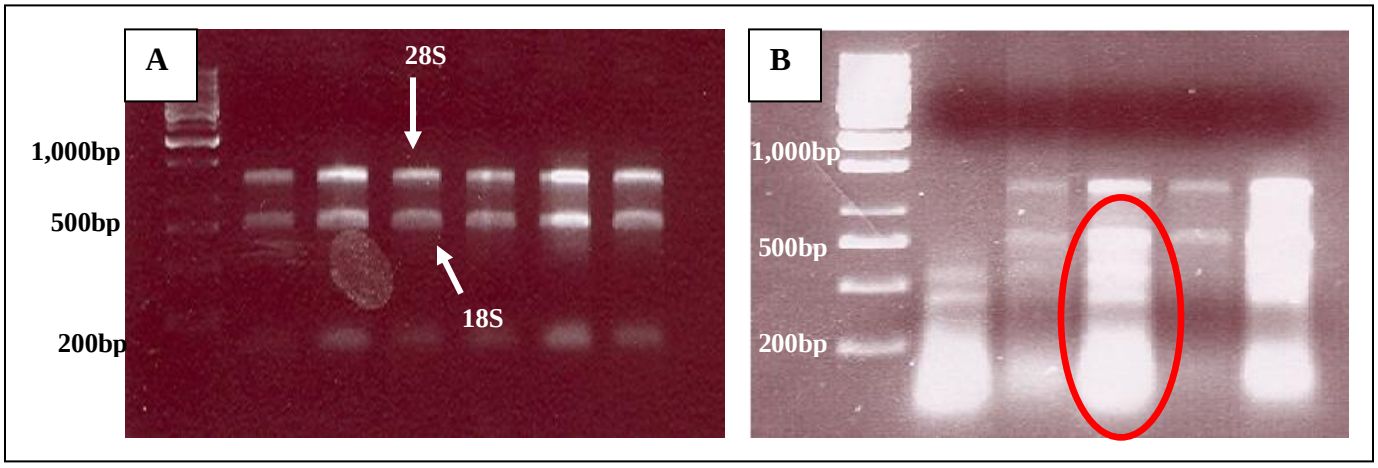


Figure 16. UV images of RNA samples run on a 1% agarose gel. Image A shows a cleanly extracted set of RNA samples. Note the clear S bands. Image B shows some blurring (as highlighted by the red circle), which is indicative of RNA degradation.

2.3.2 Identifying the zebrafish β_3-AR

The primers used to identify the fathead minnow β_{3bi}-AR were originally designed using the zebrafish β_3-AR nucleotide sequences. Therefore, prior to looking for the β_3-AR in the fathead minnow, zebrafish heart mRNA was examined for β_3-AR expression. The heart was chosen as Nickerson *et al* (2003) found the β_3-AR in the rainbow trout heart. Figure 17 shows a very strong band presence for both β_{3bi}-AR and β_{3bii}-AR. The band was of the correct size of approximately 800bp.

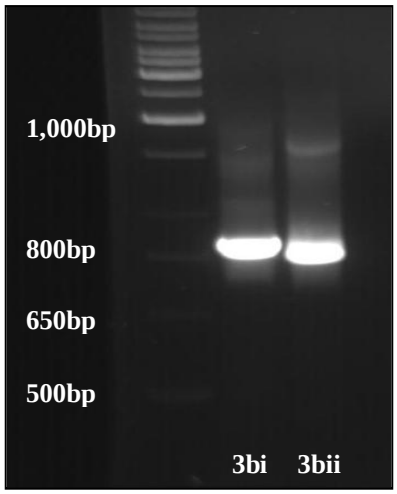


Figure 17. UV image of zebrafish heart mRNA β_{3bi}-AR and β_{3bii}-AR sequences. Bands were at approximately the correct size.

2.3.3 Identifying the fathead minnow β_{3bi}-AR

The fathead minnow genomic DNA was examined for the β_{3bi}-AR as a first screening to indicate that the fathead minnow possessed the β_{3bi}-AR before trying to find which fish tissues and life-stages the receptor was expressed in. Figure 18 shows the results from 3 nested genomic PCR rounds. Both β_{3bi}-AR subtypes were amplified during each PCR round. Product bands at the correct size were identified for both receptor subtypes in PCR nested rounds 2 (approximately 800bp) and nested 4 (approximately 600bp). There was, however a higher degree of non-specific binding in nested round 4. This was anticipated for each round since the primers were designed against a different fish species, albeit from the same family. There was no amplification in PCR nested round 3, which suggested that primers did not work. There was no relationship between strength of band and β_3-AR subtype as the β_{3bii}-AR band was stronger from nested round 2 but the β_{3bi}-AR band was stronger in nested round 4. Confirmation of sequence amplification was achieved via direct sequencing.

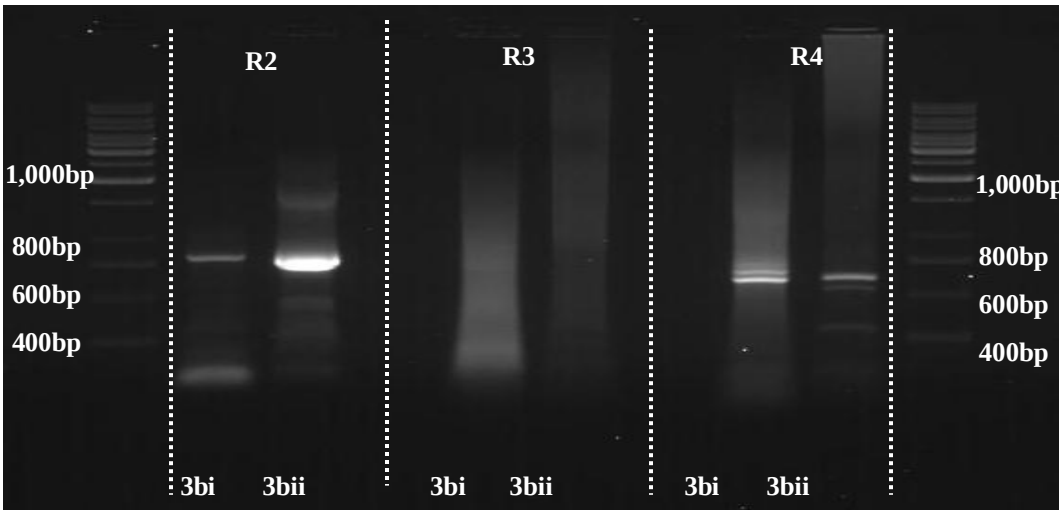


Figure 18. UV image showing genomic PCR nested rounds 2, 3 and 4. Clear product bands for both β_{3bi}- and β_{3bii}-AR at the correct sizes can be seen in rounds 2 and 4. There as a higher degree of non-specific binding in round 4.

Subsequently, heart mRNA from the fathead minnow was examined for β_3-AR expression. The nested round 3 primers were excluded as they did not produce a product in the genomic PCR investigation and thus the nested round 4 primers served as nested round 3 primers. The results from both β_3-AR subtype PCR rounds are shown in figure 19. The product bands are seen at the correct size for each corresponding PCR round. The nested round 3 products were cut out of the gel and subsequently gel extracted.

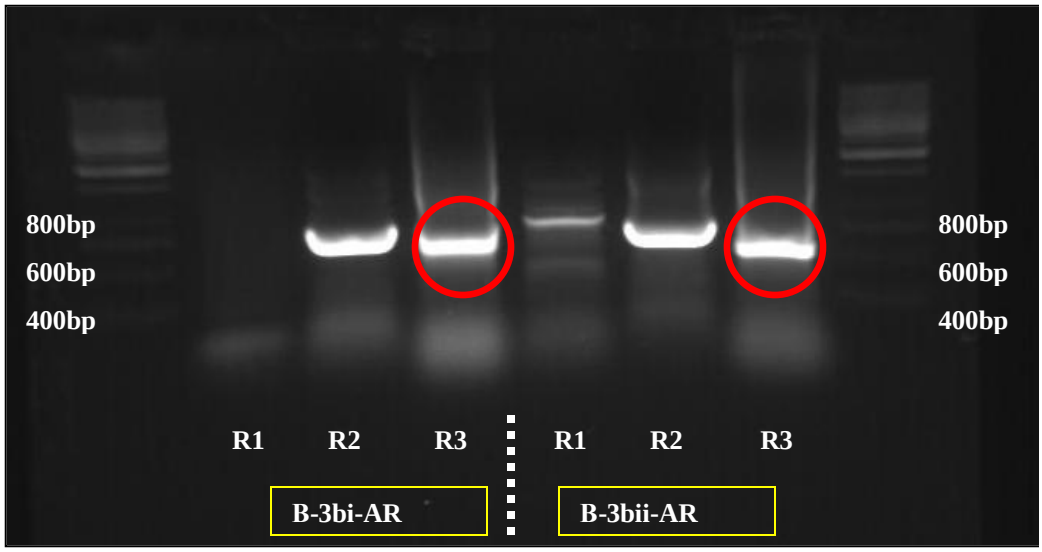


Figure 19. UV image of PCR rounds 1, 2 and 3 for identifying the β_{3bi}- and β_{3bii}-AR. Both nested rounds 2 and 3 have clear, clean product bands at approximately the correct size of 800 and 600bp, respectively. Both products from nested round 3 (highlighted in red circles) were cut out and subsequently gel extracted.

The gel extracted products were subsequently ligated into a cloning vector and transformed into competent cells. Selected colonies were PCR checked for the insert, as shown in figure 20. Each colony had the correct sized insert. The products were approximately 200 nucleotides larger than their original size as there was additional cloning vector sequence. Several colonies were selected, grown overnight in L-broth, mini-prepped and subsequently sent off for sequencing.

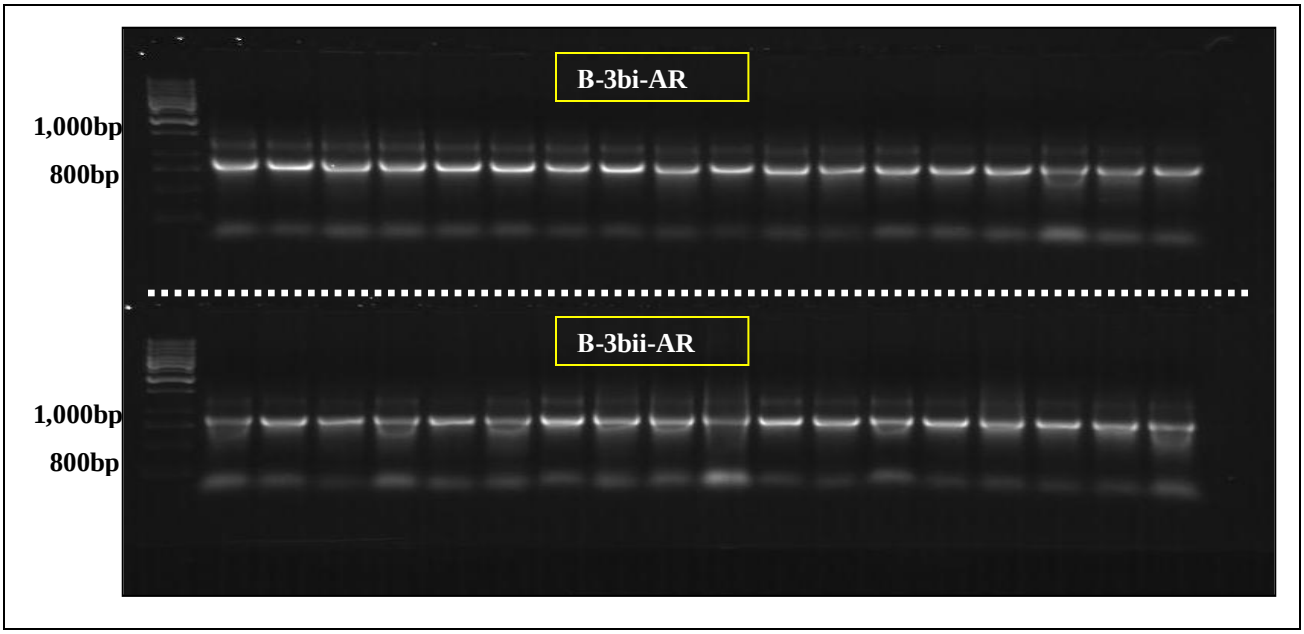


Figure 20. UV PCR-colony check image. Each colony had the same-sized product insert. The original product size was 600bp, however on the gel it is 800bp due to additional vector sequence. Several colonies were selected for sequencing analysis.

The results from the sequencing analysis confirmed that the male fathead minnow expressed both the β_{3bi}-AR and β_{3bii}-AR in the heart. Figure 21 shows that the fathead minnow β_{3bi}-AR and β_{3bii}-AR were 46% similar to each other, however when compared to their zebrafish counterparts were 92% (figure 22) and 76% (figure 23) identical, respectively.

```
3bi          CATCATTACAGTGGATCGGTATATTGCGATCACACGGCCACTGCGGCATAAAGTGCTTCT 60
3bii         CGCCATCGCCGTTGAGCGTTATGTTGCGGTCACAAGACCCCTCGAGCATCAGGTCCTCCT 60
             *  ***  * ** ** ****** ***** ******* ** **    *** * ** ** **

3bi          GAATAAGTGCAGAGCTCGAATTATTGTTTGCATTGTGTGGATAGTATCGGCTTGCATCTC 120
3bii         GGGCAAGCGCAGAGCCGTCTACATCGTATGCATAGTGTGGATGGTGTCGTCCCTGGTTTC 120
             *   *** *******       ** ** ***** ******** ** *** *     ****

3bi          CTTTATCCCCATAATGTACGGTTTTTGGCGTAGAGACCCTAATGATGATGTAGCAACTGC 180
3bii         CTTCGTGCCAATCATGCACCATTATTCTCATGCA------AATGCCACCGTTCCGGAAGA 174
             ***  * ** ** *** ** *** **  * *         ****     **  *    *

3bi          ATGTTATAGTGATACAAACTGCTGTGATTTTATAACAAACATGACCTATGCCATCATTTC 240
3bii         CTGTTACAATAACGAAGAGTGCTGTGATTTCCATACTAACGAGGTCTATGCCATCTTCTC 234
              ***** * * *   * * ***********     * ***  *  ********** * **

3bi          ATCAGTGGTTTCATTCTACATCCCACTCTTCATCATGATTTTTGTATATGCACGTGTGTT 300
3bii         CTCAGTGGTGTCATTCTATGTGCCCCTCGTGATTATGGTGTGTTTGTACGGGAAGGTGTT 294
              ******** ********  * ** *** * ** *** * * * * ** *     *****

3bi          TCTCATTGCCACACGACAGGTTCAGTTAATTGACAAGAGCCGTCTGAGGTTCCAAAACGA 360
3bii         TGCCATCGCGACCAGACAGCTGAAACTCATCGAAAAAGACAGACTGCGCTTTCCGAGCAC 354
             *** ** ** ***** * * * ** ** ** * * *** * ** * * *

3bi          GTACATGGGAAATCAGGTACAGCCACCGACCATTGGCAACAATAACTTGCCATCTATGTG 420
3bii         AGGCATGGAAGAT--------GCTAGTGA--------AAGTACAGTT-----TCTGCAAG 393
                ***** * **        ** *   *        **  * *  *     ***    *

3bi          TAATAACGTCGGAGGGATGACAGCAAAGAGGAAGAGTTCTCGAAGGCGACCTTCCAGATT 480
3bii         CAA-AACAGCTGGTGGTTG--------TAGGAGGAAGTCGACGAGGC----------ATT 434
              ** ***  * *  ** **         **** **  **    ****          ***

3bi          AACTGTAATCAAGGAGCACAAGGCCCTCAAGACACTGGGCATCATCATGGGCGTCTTTAC 540
3bii         A--TGTAA---AAGAACACAAGGCGCTCAAAACACTAGGGATTATTATGG---------- 479
             *  *****   ****************** ***** ** ***** ****

3bi          GCTGTGCTGGCTACCTTTCTTT 562
3bii         ----------------------

Similarity score = 46%
```

Figure 21. ClustalW alignment of the fathead minnow β_{3bi}-AR and β_{3bii}-AR sequences. Sequences shared a 46% similarity.

```
zf3b_i_-AR      TGCATCATTGCAGTGGATCGGTATATTGCGATCACACGGCCACTGCGGCATAAAGTGCTT 960
FHM3bi          --CATCATTACAGTGGATCGGTATATTGCGATCACACGGCCACTGCGGCATAAAGTGCTT 58
                  ******* **************************************************

zf3b_i_-AR      CTGAATAAGTGCAGAGCTCGAATTATTGTTTGCATTGTGTGGATAGTATCGGCTTGCATC 1020
FHM3bi          CTGAATAAGTGCAGAGCTCGAATTATTGTTTGCATTGTGTGGATAGTATCGGCTTGCATC 118
                ************************************************************

zf3b_i_-AR      TCCTTTATCCCCATAATGTACGGTTTTTGGCGTAGAGACCCTAATGATGATGTAGCAACT 1080
FHM3bi          TCCTTTATCCCCATAATGTACGGTTTTTGGCGTAGAGACCCTAATGATGATGTAGCAACT 178
                ************************************************************

zf3b_i_-AR      GCATGTTATAGTGATACAAACTGCTGTGATTTCATAACAAACATGACCTATGCCATCATT 1140
FHM3bi          GCATGTTATAGTGATACAAACTGCTGTGATTTTATAACAAACATGACCTATGCCATCATT 238
                ****************************** ************************** 

zf3b_i_-AR      TCATCAGTGGTTTCATTCTACATCCCACTCTTCATCATGATTTTTGTATATGCACGTGTG 1200
FHM3bi          TCATCAGTGGTTTCATTCTACATCCCACTCTTCATCATGATTTTTGTATATGCACGTGTG 298
                ************************************************************

zf3b_i_-AR      TTTCTCATTGCCACACGACAGGTTCAGTTAATTGACAAGAACCGTCTGAGGTTCCAAAAC 1260
FHM3bi          TTTCTCATTGCCACACGACAGGTTCAGTTAATTGACAAGAGCCGTCTGAGGTTCCAAAAC 358
                ************************************ ******************

zf3b_i_-AR      GAGTACATGGGAAATCAGGTACAGCCACCGACCATTGGCAACAATAACTTGCCATCTATG 1320
FHM3bi          GAGTACATGGGAAATCAGGTACAGCCACCGACCATTGGCAACAATAACTTGCCATCTATG 418
                ************************************************************

zf3b_i_-AR      TGTAATAACGTCGGAGGGATGACAGCAAAGAGGAAGAGTTCTCGAAGGCGACCTTCCAGA 1380
FHM3bi          TGTAATAACGTCGGAGGGATGACAGCAAAGAGGAAGAGTTCTCGAAGGCGACCTTCCAGA 478
                ************************************************************

zf3b_i_-AR      TTAACTGTAATCAAGGAGCACAAGGCCCTCAAGACACTGGGCATCATCATGGGCGTCTTT 1440
FHM3bi          TTAACTGTAATCAAGGAGCACAAGGCCCTCAAGACACTGGGCATCATCATGGGCGTCTTT 538
                ************************************************************

zf3b_i_-AR      ACGCTGTGCTGGCTGCCTTTCTTTGTGGCGAACATCATCTACGCGTTTGTACCACAAGAC 1500
FHM3bi          ACGCTGTGCTGGCTACCTTTCTTT---------------------------------- 562
                ************** *********

Similarity score = 92%
```

Figure 22. ClustalW alignment of fathead minnow (FHM) and zebrafish (zf) β_{3bi}-AR sequences. Sequences were 92% identical

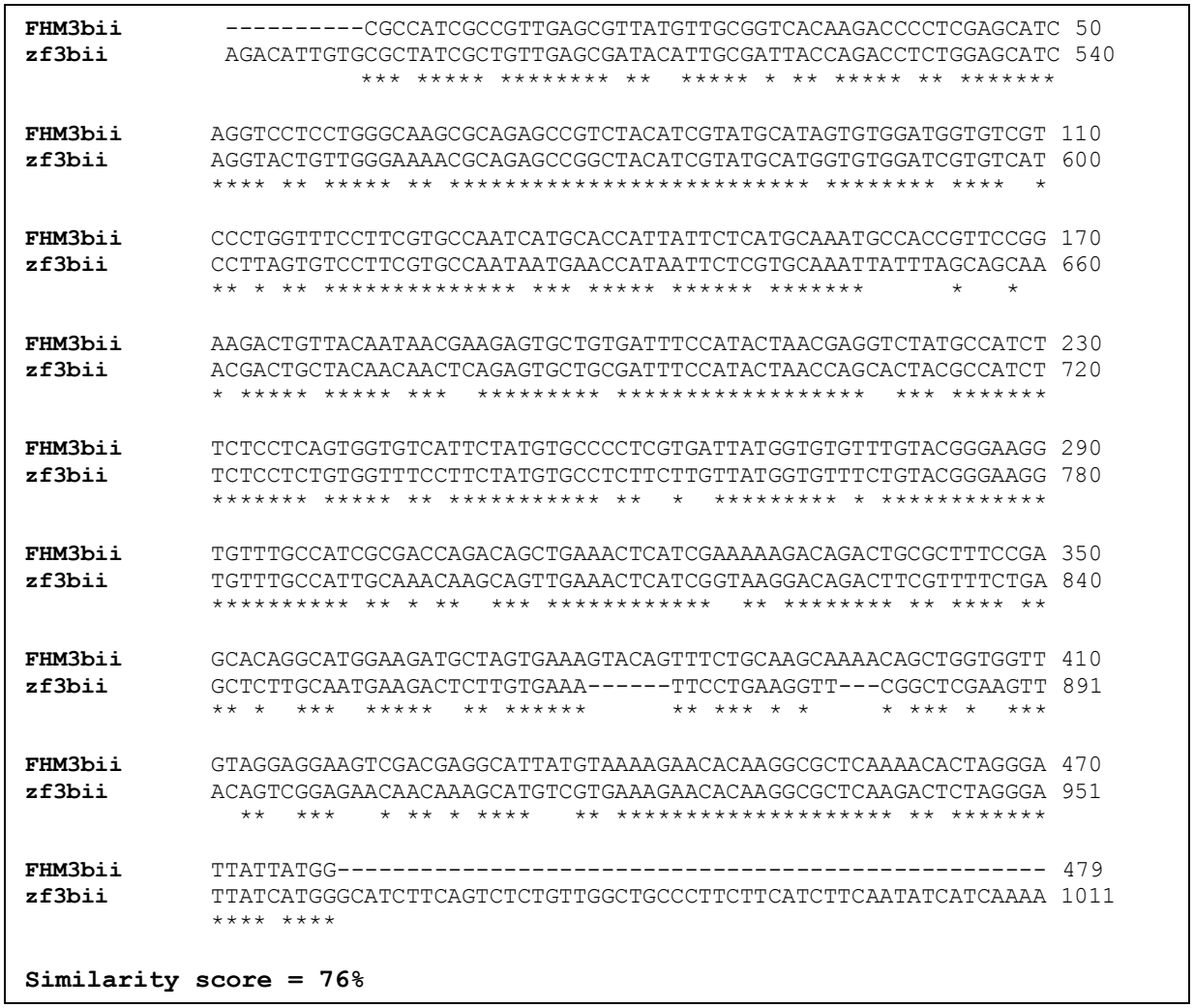


Figure 23. ClustalW alignment of fathead minnow (FHM) and zebrafish (zf) β_{3bii}-AR sequences. Sequences were 76% identical.

2.3.4 Identifying entire fathead minnow β_{3bi}-AR sequences using RACE PCR

The β_{3bi}-AR sequence was identified by piecing together several different fragments that were obtained from PCR, 5' RACE PCR and 3' RACE PCR. It was not possible to obtain the entire β_{3bii}-AR sequence beyond the first fragment. Figure 24 shows an outline of how the different PCR fragments fitted together. The 3'RACE was used to successfully identify the poly (A^+) tail, whilst the 5'RACE was able to identify up to 950 nucleotides in the 5'UTR. One clear observation made was that the overlap between the 1st fragment and 5'RACE fragment and also between the 1st fragment and 3'RACE fragment was not 100% similar. This was unusual as the 1st fragment was representative of the middle of

the sequence and covered the 2nd to the 6th trans-membrane regions; a section that is usually highly conserved and is partly responsible for the structure of the receptor. The test 1 and test 2 fragments were highly similar in sequence to the 5'RACE and 3'RACE products, but were also not 100% similar to the 1st PCR fragment. It could be possible that the 1st fragment had identified a $\beta_{3bi.1}$-AR, whilst the other fragments belonged to a $\beta_{3bi.2}$-AR.

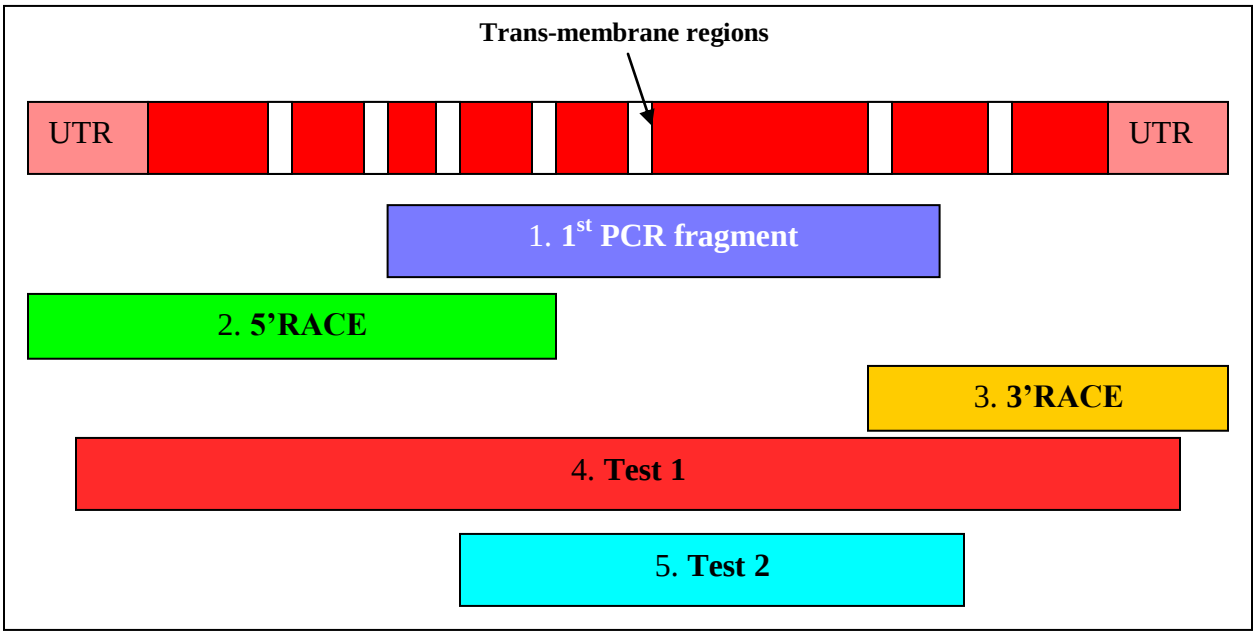


Figure 24. Outline schematic illustration of the five different fragments used to construct the entire molecular sequence of the β_{3bi}-AR.

2.3.5 Confirmation of sequence

The β_{3bi}-AR sequence was conservatively pieced together and two sets of primers were designed to the 5' and 3'UTR, the second pair was used as nested primers. The primers were then run against the fathead minnow heart tissue cDNA template in a PCR and the products were subsequently visualised on a 1.2% agarose gel, as shown in figure 25. The product from PCR round 1 was cleaner than round 2 and was therefore cut out, cloned and subsequently evaluated by PCR to identify suitable colonies.

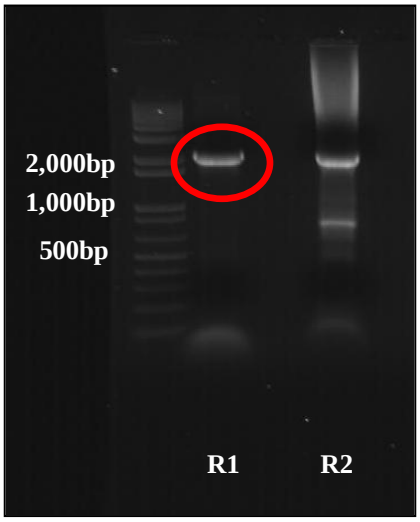


Figure 25. UV image of 1.2% agarose gel. R1 = round 1 and R2 = round 2. The red circled band was cut out of the gel for subsequent gel extraction.

A single colony of the correct sized was grown overnight and subsequently analysed by primer-walking sequencing. Figures 26 and 27 outline the complete β_{3bi}-AR nucleotide and amino acid sequences, respectively. The nucleotide sequence was 2,203 nucleotides long and the translated protein sequence was 434 amino acids long. Typically the transcription initiator sequence, commonly referred to as a TATAA box because of its nucleotide sequence, is found 25-35bp before the start codon. It was not possible to accurately identify the TATAA box in the fathead minnow β_{3bi}-AR sequence.

5’ GAATTCAGATTCACGCTGACTGAACCTCCTCCACTTCAGCTCAAACTCCACTTCAG
CGGACTACTTCAACACGACGGCTGCTTATCTGCCTTTTCTCTGCTGGTTTCAAGAGCTTTT
CTAAAAGTTAAAATATTTTTATTTCCCCCATTTCTCTCGGACCAGTTTTAACTCAGAATA
AAAGGGGCTTTTTGTTTTCTTTCCTCCTACATCTTCAGTTCAGATGTGTGGAACATGGCAT
ATATGTTTGCTTCAAACGCCACAGGACTGCTGGATATGCACTATTTTTCAGGATGACTGA
ATAATATCCCAAAAGGAATTGATGCTGTTGCTCAACATTTGGAGATATGGTGGTATAAA
ATGCAAGATTAAATACATCATCGTCATGGTATCTCAATCACTCGACTGAAGACCGAATA
GCACCTAGCTATGTCTCTTTGCTGCTCGTTTCTGACATACAGTTTGAAAAACCACAAACA
TTGATCTCTGAATACTGAAAC**ATG**GAATCTCTGACAAACAGCTCTCAAACAACCCCTGT
GCCAACAAGTGCACCTCTTCAAGGGTGGACATCATTTCAGCTGTTCATCATGATCATCAT
CATCGCGATTATCGTTGTGGGTAACCTTATGGTCATCATCGCCATAGCACGC**ACCTCCC**
AGCTGCAGACCACGACGAATATCTTCATTATGTCGTTGGCATGTGCTGACCTCATAATG
GGGGTGGTGGTGGTGCCACTGGCTGCCATGATTGTTGTAAAAGGGGAATGGACACTTGG
TGAGGTAC**CGTGCAGATTATGGACATCG**GTTGATGTTTTATGTGTCACAGCCAGTATA
GAGACCCTTTGCATCAACGCAGTGGATCGGTATATTGCGATCATGCGGCCACTGCGGTA
TAAAGTGCTCCTGAATAAGTGCAGGGCTCGAATCATCGTTTGTGTCGTGTGGCTGCTCTC
AGCTCTAATCTCCTTCGTCCCAATCATGAACGACTGGCATGCTGGGGCCGATACAGGAA
ACAAAAATGACACAGACAATTACAAAGACACCTGTGCTTTTGATACAAACATGGCCTTT
GCTATATTTTCATCAGGGATTTCGTTCTACATCCCACTACTCATCATGATTTTTGTCTATG
CACGCGTCTTCCTCGTCGCCACAAGACAGGTACAGCTCATTGGCAATAACCGTCTGAGG
TTCCAAAACGAGTGCATAGGAAATCAGGTCCATGGCAACAACAACTTGCCTTCGATGTG
CAATAACGTCGGTGGGATGACGGCAAGGCGGAAAAGTTCCCGGAGGCGACCGTCCAAA
TTAACCGCTGTGAAGGAGCACAAGGCCCTCAAGACGCTGGGCATCATCATGGGCATCTT
CACACTGTGCTGGCTGCCTTTCTTCGTGGCGAACATCATCAATGTT**TTCAATCGAGATC**
TGCTAACCATGTATGTTTTTCGATATTTAAACTGGTTAGGCTACATCAACTCCAGCCTCA
ATCCCATCATCTACTGCCGCAGCC**CAGAGTTTCGTGCGGCCTT**CAAGAACCTTCTCGG
CTGTCCCTGGGTATCCCCGCTGAGAATGAACTTCCTTTACAAGGAGCTTCGGACTCGATG
CACTTGCTTCCTGGGTTCCGCAGAGTCGGGAATGCCTGGATCGTTCGAGAAGCCCCCCA
CATCCCCAGGGGCACTGCCTGGGGAAGGGAGCAGCCAGAGCAGCTACAGGAGCGAGGA
GCCGTCGCCGGGACCACCGCACTCCAACGGACGCACGTTCTTCAGTGATTTCTCCGAGC
CGGAGACGGAGTTCTGTAACCTA**TAG**GAGCAGGATGGCTGAGAGTCTCTAGCATGGCTG
AAATGAAAGGACGATGAGGAAGAAGAAACAGGGAAAAGGACTGACTTACATCACAGTT
GGCGGGACTTTCCCAGCAGAGACCACAAGGGCCCAGCAAAAAACATCCATGAAAGCCA
TCAGGGGAGCCTGCTCCAAAACTGAAAGACAGAAGAGTGCAAAGATATAACCATAGAG
AGGCAAGCACTATGGGAACTTTCTCACAAACAGCTCGTCTATGGAAAGAACGACTGAAC
TGGTACTGTTCATGAAGGAGTGTAACATGGAGTGATCCGATACTGCTAACGTTCTATTAA
GTACTGGAGCAATCCACTTCAGCCCGGTTTGTTTTAGTTCGTTTGACAAAAAAAAAAAA
AAAAAAAAAAAAAAAA **3’**

Figure 26. Fathead minnow β_{3bi}-AR nucleotide sequence. The start (ATG) and stop (TGA) codons are highlighted in bold pink. The blue and red highlighted sequences represent the primers used to confirm the sequence in the 5’ and 3’ direction by the Sequencing service, respectively. The green and orange sequences represent the 5’ and 3’ UTR’s, respectively. The highlighted yellow sequences represent primers used to identify the entire β_3-AR sequence.

N' MESLTNSSQTTPVPTSAPLQGWTSFQLFIMIIIIAIIVVGNLMVIIAIARTSQLQTTTNIFI
MSLACADLIMGVVVVPLAAMIVVKGEWTLGEVPCRLWTSVDVLCVTASIETLCINAVDR
YIAIMRPLRYKVLLNKCRARIIVCVVWLLSALISFVPIMNDWHAGADTGNKNDTDNYKDT
CAFDTNMAFAIFSSGISFYIPLLIMIFVYARVFLVATRQVQLIGNNRLRFQNECIGNQVHGN
NNLPSMCNNVGGMTARRKSSRRRPSKLTAVKEHKALKTLGIIMGIFTLCWLPFFVANIINV
FNRDLLTMYVFRYLNWLGYINSSLNPIIYCRSPEFRAAFKNLLGCPWVSPLRMNFLYKELR
TRCTCFLGSAESGMPGSFEKPPTSPGALPGEGSSQSSYRSEEPSPGPPHSNGRTFFSDFSEPET
EFCNL **C'**

Figure 27. Fathead minnow β_{3bi}-AR amino acid sequence.

2.3.6 Characterising the β_{3bi}-adrenergic receptor

2.3.6.1 Hydropathy analysis

To identify the TMDs in the fathead minnow β_{3bi}-AR sequence, the ExPASy proteomics server of the Swiss Institute of Bioinformatics (www.expasy.ch/ tools/dna.html) was used and several hydropathy programmes were used. None, however, produced the same results. Therefore, additional information on GPCRs and β-adrenergic receptors, summarised in table 11, was used to compare the information produced by the hydropathy software programmes. For example it was used to examine whether a specific motif common to β-adrenergic receptors was located in the designated TMD or, because of incorrect hydropathy analysis, was outside of the TMD.

Table 11. Summary of motifs found specifically in GPCRs and β-adrenergic receptors, amended from that described by Giltrow (2008).

Motif	**Amino Acid sequence**	**Reference**
N-terminal region is extracellular, asparagine-linked and glycosylated	**NxS/T**	Emorine *et al* (1988)
Cysteine residues between the second and third extracellular loops. Required for disulfide bond formation and essential for ligand binding.	**C,C**	Foreman & Johansen (1996)
Motif occurring at the end of the TMD 3.	**DRY**	Devic *et al* (1997)
Residues required for α_s activation found in TMD3 (N) and TM 7 (Y).	**N, Y**	Strosberg & Peitri-Rouxel (1996)
Ligand binding residues found in TMD 2 (D) TMD 3 (D), TMD 5 (S, S) and TMD 6 (F)	**D, D, S,S, F**	Strosberg & Peitri-Rouxel (1996),
Tryptophan reside (W) found in the middle of TMD 4 and TM 6.	**W**	Devic *et al* (1997)
Cysteine residue is located 10-20 residues C-terminally to TM 7 and is palmitoylated	**C**	Foreman & Johansen (1996)
Proline residues located in TMD 5, TMD 6 and TMD 7. Facilitates interchange between different conformations of associated and dissociated receptor.	**P**	Foreman & Johansen (1996)
Arginine residue located in the second intracellular loop just below TMD 3. Involved in signal transduction process	**R**	Blin *et al* (1993)
Residues found in TMD 7 and are characteristic of the α-rhodopsin group.	**NSxxNPxxY**	Friedriksson *et al* (2003)
Found in β-1-AR receptors at the start of the COOH loop after TM 7.	**RSPDFRKA FKR**	Mason *et al* (1999)

Figure 28 summarises the results from the hydropathy analysis. The GPCR- and β-AR-specific motifs summarised in table 12 are also included. It was clear that most motifs

were found at their specified locations in the fathead minnow β_{3bi}-AR. The exception to this, however, was that the TMHMM and HMMTOP programmes did not produce a 7th TMD and therefore in these cases all motifs associated with the 7th TMD were not included. To evaluate which hydropathy analysis programme or combination of programmes was most suitable for allocating the TMD to the fathead minnow $\beta_3$bi-AR sequence, all the information was first summarised, as shown in tables 13 and 14.

Table 12. Summary of TM regions produced by all five different hydropathy programmes. Red highlighted figures represent those TMDs that were too small (18-30 amino acids) according to Strosberg & Peitri-Rouxel (1996).

TM region	TMpred	TMHMM	SOSUI	DAS	HMMTOP
1	22	22	23	23	22
2	26	23	23	25	26
3	19	23	23	20	**10**
4	19	20	23	20	20
5	24	23	24	24	27
6	22	23	23	20	22
7	20	**0**	23	18	**6**

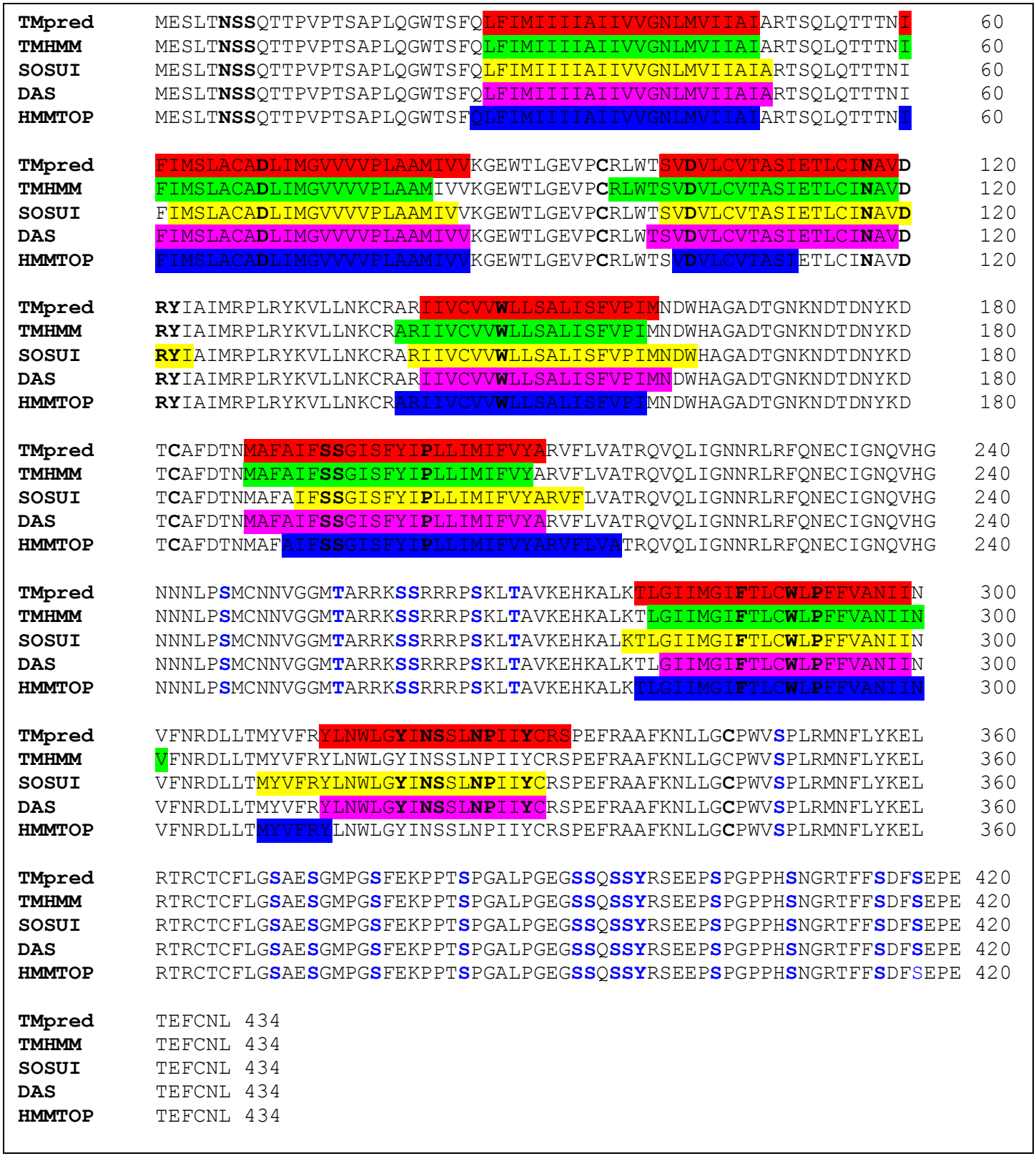


Figure 28. Results of the hydropathy analysis of the fathead minnow β_{3bi}-AR sequence. Different results were obtained from the available hydropathy programmes. The highlighted TM regions are TMpred = red, TMHMM = green, SOSIU = yellow, DAS = pink and HMMTOP = blue. Motifs highlighted in bold. Blue highlighted motifs indicate potential phosphorylation sites identified by NetPhos 2.0 (Bloom *et al*, 1999).

Table 13. A summary showing which motifs are associated with the TMDs according to the different hydropathy programmes.

Motif	TMpred	TMHMM	SOSUI	DAS	HMMTOP
Number of TMDs	7	6	7	7	7
NxS	Yes	Yes	Yes	Yes	Yes
C,C	Yes	Yes	Yes	Yes	Yes
DRY	Yes	Yes	No	Yes	Yes
N (TM 3), Y (TM 7)	Yes	Yes	Yes	Yes	Yes
D (TMD 2 & 3), S,S (TMD 5), F (TMD 6)	Yes	Yes	Yes	Yes	yes
W in the middle of TM 4	Yes	Yes	Yes	Yes	Yes
and TMD 6	Yes	Yes	Yes	Yes	Yes
C at COOH end of TMD 7	Yes	No	Yes	Yes	Yes
P in TMD 5,	Yes	Yes	Yes	Yes	Yes
6	Yes	Yes	Yes	Yes	Yes
and 7	Yes	No	Yes	Yes	No
R in 2nd intracellular loop	Yes	Yes	Yes	Yes	Yes
NSxxNPxxY	Yes	No	Yes	Yes	No
RSPDFRKAFKR	No	No	No	No	No

Tables 12 and 13 demonstrate that each topology programme highlighted different TMDs. As each topology programme used different criteria to allocate TMDs, it was important that only a single programme was chosen to select each TMD. Accordingly, TMHMM was not selected as it did not produce a TMD 7 and HMMTOP was excluded as it produced a TMD that was smaller than 18 nucleotides in length. The SOSUI programme did not produce a DRY motif immediately after TMD 3 (it was found within TMD 3) as so was not used, whilst TMHMM did not have a C motif at the COOH end of TMD 7. Both TMpred and DAS produced very similar results. Giltrow (2008) used TMpred to allocate TMDs in the fathead minnow β_1- and β_2-ARs and, accordingly, TMpred was used to assign TMDs to the fathead minnow β_{3bi}-AR.

Table 13 indicates that the amino acid sequence RSPDFRKAFKR was not found in the sequence, which suggested that the sequence was not a β_1-AR.

2.3.6.2 Comparison of the fathead minnow β_{3bi}-AR sequence with other β_3-ARs

The fully annotated proposed fathead minnow β_{3bi}-AR amino acid sequence was aligned with fish and human β_3-AR sequences and is shown in figure 29 and the similarity scores across the TMDs highlighted in table 14.

```
Tetraodon3      ------------------------------------------------------------
human3          ----MAPWPHENSSLAPWPDLPTLAPNTANTSGLPG-------VPWEAALAGALLALAVL 49
Fugu3           ------------------------------------------------------------
trout3a         MHEKSILPLCMSFLMKMDFNLSSIVLYAVNTTVTPFHNNTGPYHPAAGPWSLLLLVIIIM 60
trout3b         MHEKSILPLCMSFLMKMDFNLSSIVLYAVNTTVTPFHNNTGPYHPAAGPWSLLLLVIIIM 60
zebrafish3      ----------------------MTNNTQNVTQN----------EGFKS-LLFVMVIIIT 26
FHM3            --------------------MESLTNSSQTTPVPTSAP-----LQGWTSFQLFIMIIIIA 36

Tetraodon3      ---------IVAIAWTPRLQTMTNVFVTSLAAADLVMGLLVVPPAATLALTGHWPLGATG 51
human3          ATVGGNLLVIVAIAWTPRLQTMTNVFVTSLAAADLVMGLLVVPPAATLALTGHWPLGATG 109
Fugu3           ---------IVAIAWTPRLQTMTNVFVTSLAAADLVMGLLVVPPAATLALTGHWPLGATG 51
trout3a         TIMVGNLLVIIAIARTSQLQTITNIFIMSLGCADLIMGVLVVPLGATIVLTGNWQLSDTS 120
trout3b         TIVVGNLLVIIAIARTSQLQTMTNIFIMSLACADLIMGVLVVPLGATIVVTGNWQLSDMS 120
zebrafish3      ITIVGNLLVIIAIARTSQLQTTTNIFIMSLACADLIMGVLVVPLGATIVVTGQWKLNNTF 86
FHM3            IIVVGNLMVIIAIARTSQLQTTTNIFIMSLACADLIMGVVVVPLAAMIVVKGEWTLGEVP 96
                         *:*** *.:*** **:*  **..***:**::*** .* :.:.*.* *.

Tetraodon3      CELWTSVDVLCVTASIETLCALAVDRYLAVTNPLRYGALVTKRCARTAVVLVWVVSAAVS 111
human3          CELWTSVDVLCVTASIETLCALAVDRYLAVTNPLRYGALVTKRCARTAVVLVWVVSAAVS 169
Fugu3           CELWTSVDVLCVTASIETLCALAVDRYLAVTNPLRYGALVTKRCARTAVVLVWVVSAAVS 111
trout3a         CELWTSVDMLCVTASIETLCVIAVDRYIAITRPLRHKVLLNKWRARLIVCVVWIVSALIS 180
trout3b         CELWTSVDVLCVTASIETLCVIAVDRYIAITRPLRHKVLLNKWRARLIVCVVWIVSALIS 180
zebrafish3      CELWTSVDVLCVTASIETLCIIAVDRYIAITRPLRHKVLLNKCRARIIVCIVWIVSACIS 146
FHM3            CRLWTSVDVLCVTASIETLCINAVDRYIAIMRPLRYKVLLNKCRARIIVCVVWLLSALIS 156
                *.******:**********  *****:*: .***: .*:.*  **  * :**::** :*


Tetraodon3      FAPIMSQWWRVGADAE--AQRCHSNPRCCAFASNMPYVLLSSSVSFYLPLLVMLFVYARV 169
human3          FAPIMSQWWRVGADAE--AQRCHSNPRCCAFASNMPYVLLSSSVSFYLPLLVMLFVYARV 227
Fugu3           FAPIMSQWWRVGADAE--AQRCHSNPRCCAFASNMPYVLLSSSVSFYLPLLVMLFVYARV 169
trout3a         FVPIMNQYWRAEDDPE--AVLCYQDPACCDFLINRTFAIISSVVSFYIPLLIMIFVYAKV 238
trout3b         FVPIMNQLWRAEEDPE--AMVCYQDPACCDFMTNSTFAIISSVVSFYIPLLIMIFVYVKV 238
zebrafish3      FIPIMYGFWR-RDPNDDVATACYSDTNCCDFITNMTYAIISSVVSFYIPLFIMIFVYARV 205
FHM3            FVPIMNDWHAGADTGNKNDTDNYKDT--CAFDTNMAFAIFSSGISFYIPLLIMIFVYARV 214
                * ***          :      :.:.  * *  * .:.::** :***:**::*:***.:*

Tetraodon3      FVVATRQLRLLRGELGRFPPEESPPAPS------RSLAPAPVGTCAP-----PEGVPACG 218
human3          FVVATRQLRLLRGELGRFPPEESPPAPS------RSLAPAPVGTCAP-----PEGVPACG 276
Fugu3           FVVATRQLRLLRGELGRFPPEESPPAPS------RSLAPAPVGTCAP-----PEGVPACG 218
trout3a         FLIATRQVQLIDKIRLRFQSECPVPQTQQLSHHSNNILPLGYGGSCSRSSTSSARKRSSR 298
trout3b         FLIATRQVQLIDKTRLRFQSEWPAPTTTQLSNHSCNILPLGYGGSS--SCTSSPRKRSSK 296
zebrafish3      FLIATRQVQLIDKNRLRFQNEYMGNQVQPPTIGNNNLPSMCNNVGG-----MTAKRKSSR 260
FHM3            FLVATRQVQLIGNNRLRFQNECIGNQVH----GNNNLPSMCNNVGG-----MTARRKSSR 265
                *::****::*:     **  *              .: .   .         .    :.
```

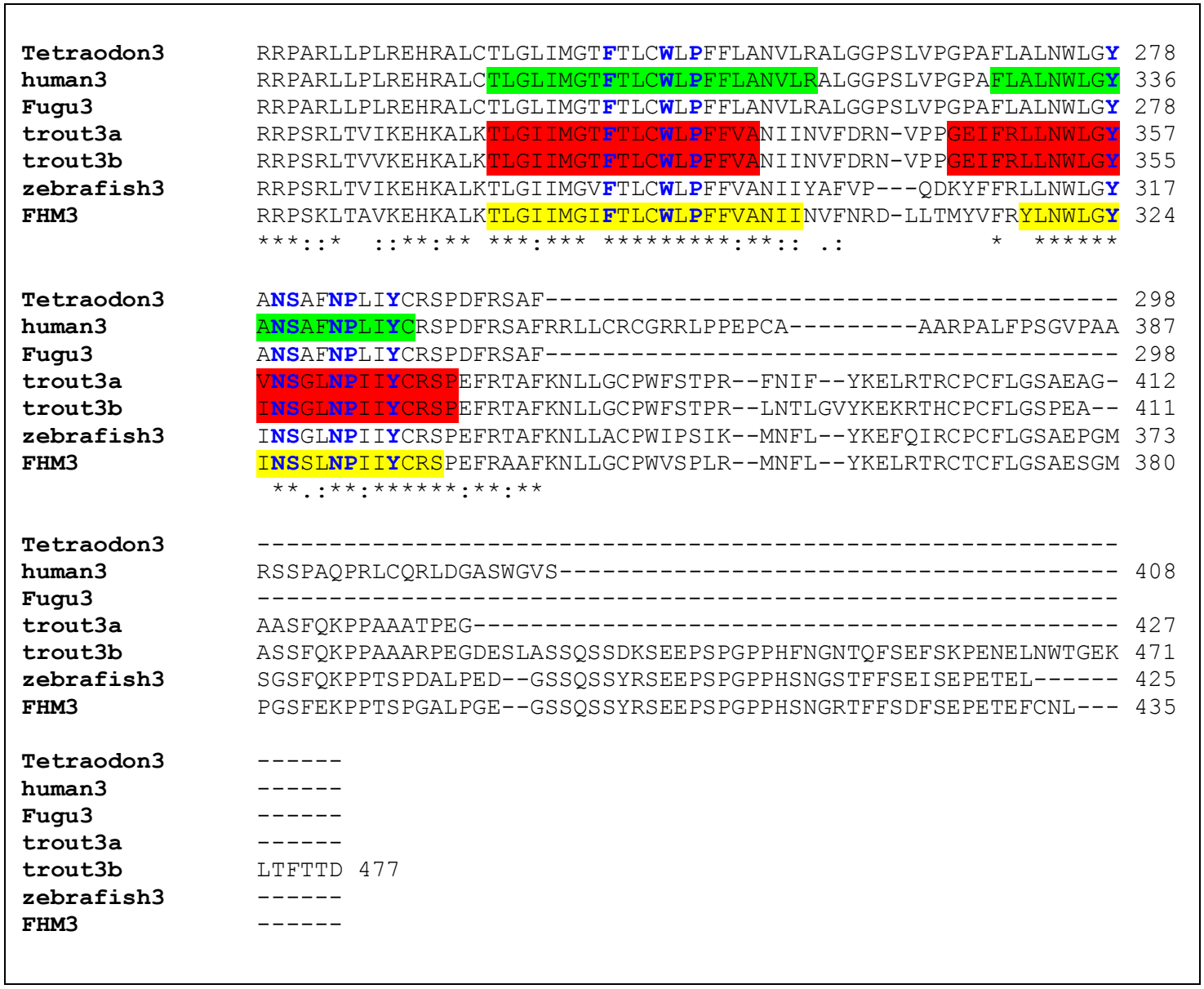


Figure 29. ClustalW aligned amino acid sequences of the fathead minnow (FHM), zebrafish (ZF), trout a and b, Tetraodon, fugu and human β_3-AR sequences. Calculated TMDs for fathead minnow highlighted in yellow, documented TMDs for trout β_{3a}- and β_{3b}-ARs highlighted in red and proposed TMDs for human β_3-AR highlighted in green. GPCR and β-adrenergic receptor motifs highlighted in blue font. Highlighted purple amino acid residue at alignment position 75 represents a potential tryptophan (W) replacement for arginine (R) in the human β_3-AR compared to other vertebrates (including the fathead minnow); only confirmed amino acid residues highlighted.

Table 14. Percentage similarity scores of TMDs between the fathead minnow β_3-, trout β_{3a}- and β_{3b}- and human β_3-AR amino acid sequences.

	Fathead minnow β-3-AR							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**Whole sequence**
human β_3-AR	33	53	90	47	61	72	70	**40**
trout β_{3a}-AR	55	66	16	0	0	89	73	**57**
trout β_{3b}-AR	61	75	25	0	0	89	78	**61**
Tetraodon β_3-AR								**50**
Fugu β_3-AR								**50**

Overall, the proposed fathead minnow β_{3bi}-AR was 57% and 61% similar to the trout β_{3a}-AR and β_{3b}-AR sequences, respectively, 74% similar to the zebrafish β_{3bi}-AR, 50% similar to the Tetraodon and fugu β_3-ARs and 40% similar to the human β_3-AR. However, the location of the TMDs (excluding Tetraodon and fugu as they have not been documented) varied considerably between the sequences and therefore, the percentage similarity across the TMDs varied significantly. This was most obvious for the trout β_{3a}-AR and β_{3b}-AR TMDs 4 and 5, where there was no overlap between the sequences and the fathead minnow β_3-AR. The TMDs, documented by Nickerson *et al* (2003) were not long enough to span the cell membrane bilayer; for example TMDs 2 and 3 were only 12 amino acids long and, as such, may have been incorrectly identified. The location of the TMDs in the human β_3-AR, as proposed by Strosberg & Pietri-Rouxel (1996), were in line with the fathead minnow, and this was reflected in the percentage similarity scores. Several amino acid motifs specific to GPCRs and β-adrenergic receptors were found in all the sequences, although in the case of the trout β_{3a}-AR and β_{3b}-ARs, not necessarily in the correct locations, which was possibly due to the incorrect allocation of TMDs.

In July, 2008, the zebrafish β_{3bi}-AR sequence classified on the NCBI database, and used as the template for original primer designing, was re-classified as a β_1-AR sequence (NM_001128335). This, therefore, suggested that the fathead minnow β_{3bi}-AR sequence was potentially a β_1-AR. To examine this possibility, the pre-existing zebrafish β_1-AR amino acid sequence and the fathead minnow β_1-AR amino acid sequence characterised by Giltrow (2008) were compared to the newly classified zebrafish β_1-AR amino acid

sequence. Table 15 shows that the fathead minnow and current zebrafish β_1-AR were 91% identical whilst the proposed zebrafish β_1-AR was only 45% similar to both β_1-AR sequences. Moreover the amino acid motif sequence typically found in all β_1-ARs (see table 11) was absent from both the fathead minnow β_{3bi}-AR and the proposed zebrafish β_1-AR sequences, thus providing further evidence that the sequences were more likely to be β_3-ARs.

Table 15. Percentage alignment scores between the fathead minnow and current zebrafish β_1-AR, the fathead minnow β_{3bi}-AR and the newly suggested zebrafish $\beta_{1(?)}$-AR. Scores in red highlight similarity between fathead minnow and current zebrafish β_1-AR. Scores in blue highlight similarity between newly proposed zebrafish β_1-AR and the fathead minnow and current zebrafish β_1-AR.

	FHM β-1-AR	**zebrafish β 1-AR**	**zebrafish β-?-AR**
FHM β-1-AR		**91**	44
zebrafish β 1-AR	**91**		45
zebrafish β-?-AR	**44**	**45**	

Table 16 compares similarity of the fathead minnow β_{3bi}-AR with the fathead minnow β_1-AR and β_2-AR sequences at the amino acid level. The level of similarity between the fathead minnow β_3-AR and other characterised fathead minnow β-ARs ranged from 38% to 43% for the fathead minnow β-1-AR and fathead minnow β_2-ARs respectively. This suggests that the proposed fathead minnow is unlikely to be a β_1-AR or β_2-AR. At 435 amino acids long, the β_3-AR was also considerably longer than other β-AR sequences.

Table 16. Sequence lengths and similarity scores (%) between the proposed fathead minnow β_{3bi}-AR and the classified fathead minnow β_1-AR and β_2-ARs amino acid sequences.

	Amino acid sequence lengths	**Similarity with FHM β_{3bi}-AR**
FHM β_1-AR	402	43
FHM $\beta_{2.i}$-AR	378	38
FHM $\beta_{2.ii}$-AR	397	38
FHM β_{3bi}-AR	435	

2.3.6.3 Phylogenetic analysis

The results from the phylogenetic analysis (figure 30) both supports and adds further observations to those, made in tables 14 - 17. The fathead minnow β_{3bi}-AR was most similar to the zebrafish β_3-AR. However, it was then most similar to the human β_1-AR. The human β_3-AR was closely similar to the fugu and tetraodon. However, neither the fugu nor the tetraodon sequences have been confirmed and it could be possible that the human β_3-AR was used as a template to identify a similar sequence in the fugu and tetraodon genome using software analysis but that the identified receptor sequences actually function as different receptors. The three characterised fish β_3-ARs (trout and fathead minnow) are all more closely linked to the human β_1-AR than β_3-AR. The fathead minnow β_{3bi}-AR was distinctly different to other fish β_1- and β_2-ARs.

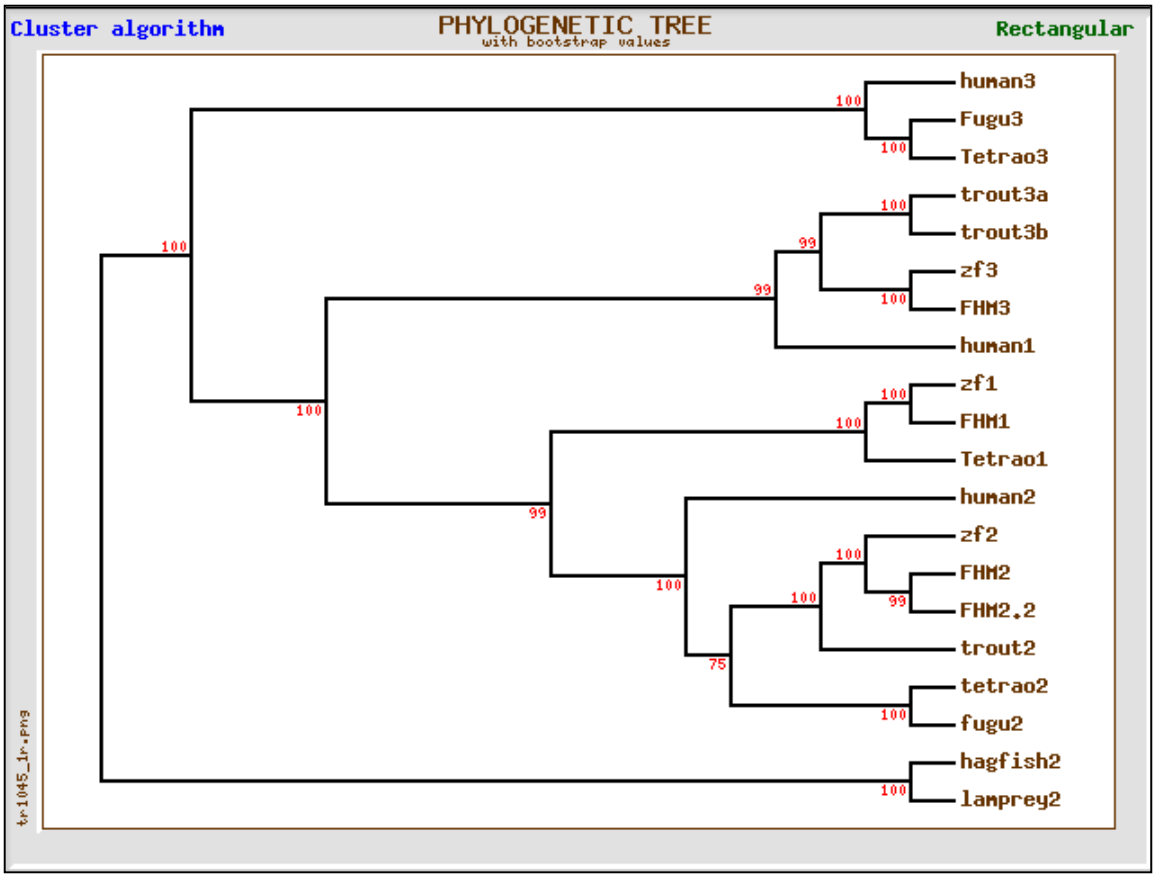


Figure 30. A phylogenetic tree for all fish and human β-adrenergic receptors; with bootstrap values.

2.3.7 β_{3bi}-AR expression in fathead minnow tissues

β_{3bi}-AR expression was visualised in several tissues via a 2.0% agarose gel (figure 31). β_{3bi}-AR was expressed in all tissues examined and expression levels appeared to be highest in the heart, gill and blood in both male and female fathead minnows suggesting the receptor role is the same in both sexes and maybe similar to that proposed by Nickerson *et al* (2003) in the trout.

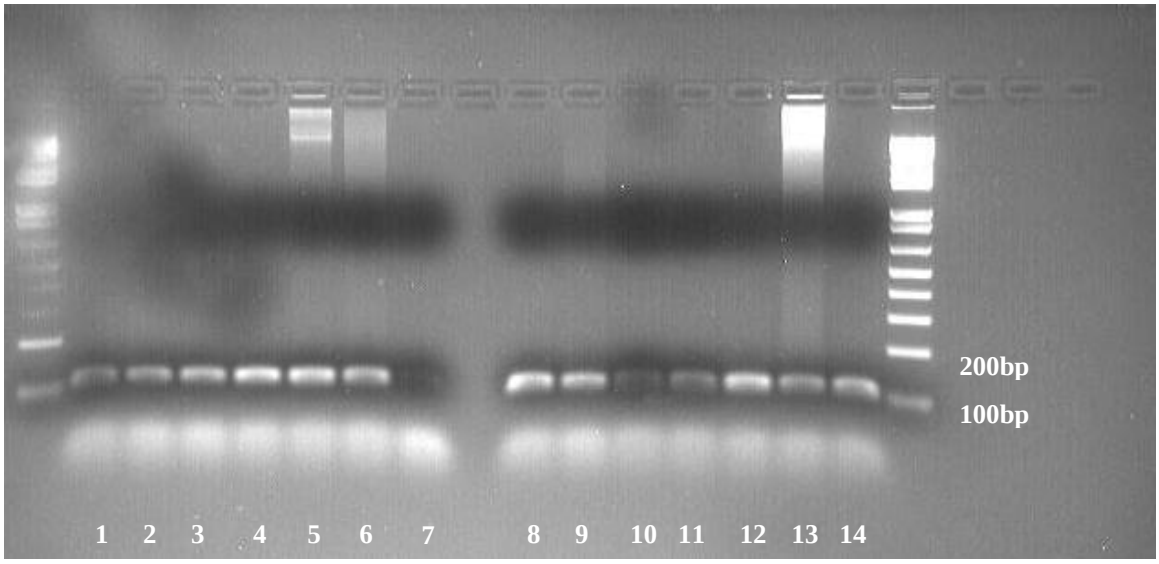


Figure 31. UV image of tissue expression of the β_{3bi}-AR in the fathead minnow. 1 – 6 = male liver, brain, gill, heart red blood cell, adipose and gonad; 7-13 = female heart, liver, brain, gill, red blood cell, adipose and gonad.

2.4 DISCUSSION

2.4.1 Expression of β_{3bi}-AR in the fathead minnow

Both adult male and female fathead minnows expressed the β_{3bi}-AR, as did the adult male zebrafish. The total length of the characterised fathead minnow β_{3bi}-AR sequences was 2,203 nucleotides and the amino acid sequence contained all the characteristic motifs at the specified locations that are associated with both GPCRs and β-ARs.

With the exception of the fathead minnow (present study), the only fish to have a fully characterised β_3-AR is the rainbow trout (Nickerson *et al*, 2003). The fathead minnow β_{3bi}-AR was 57% and 61% similar to the trout β_{3a}- and β_{3b}-AR. However there were some marked differences in similarity across the TMDs. The allocation of TMDs varied greatly between the fathead minnow β_{3bi}-AR and the trout β_3-AR sequences to the extent that there was minimal alignment between the respective TMD 3 sequences and no alignment similarity between TMDs 4 and 5. Nickerson *et al* used a gene conversion event database, GENECONV (version 1.7), to examine for TMDs but the length of some of the TMDs characterised in the trout were less than 18 amino acids in length and therefore, according to Lekowitz and Caron (1988), would not span a cell membrane. For example, TMDs 2 and 3 were only 12 amino acids long and of the seven TMDs, only TMDs 5 and 7 were long enough to span a membrane, which suggests that TMD allocation in the trout was possibly incorrect. The allocation of TMDs in the human β_3-AR (Strosberg & Peitri Rouxel, 1996), however, was very similar to those in the fathead minnow β_{3bi}-AR, thus supporting the findings from this study.

The fathead minnow expressed two different β_{3b}-AR subtypes, namely β_{3bi}-AR and β_{3bii}-AR. Both sequences were 46% identical at the nucleotide level and 53% identical at the amino acid level across the TMD 3 and TMD 4 region, which was unusually low as the TMD sequences are usually highly conserved across these functional domains. The trout β_{3a}- and β_{3b}-AR sequences were 84% identical across the entire sequence length; however it was not possible to identify the entire fathead minnow β_{3bii}-AR sequence and therefore examine the similarity between the complete β_{3b}-AR subtypes. In humans and higher vertebrates (all of which are diploid) there are three types of β-ARs, namely β_1-AR, β_2-

AR and β_3-AR. Teleost fish, however, have undergone additional gene duplication events and are tetraploid and therefore may potentially express many more β-AR subtypes (Froschauer *et al*, 2006); the fathead minnow could potentially express a β_{3ai} – and β_{3aii}-AR. As a result of these genomic duplication events in both diploid and tetraploid species, polymorphisms of genomic sequences can occur. Polymorphisms are variations in genomic sequences at an allele frequency of >1% in a single population and are often presented as single nucleotide substitutions. As such, polymorphisms may encode different amino acids (nonsynonymous polymorphisms) or may have no effect on the encoded amino acid (synonymous polymorphisms) (Small *et al*, 2003). A nonsynonymous polymorphism observed in the β_3-AR gene has been reported that results in a substitution of arginine for tryptophan at amino acid position 64, as shown in figure 29 (Clement *et al*, 1995). This residue is located either at the distal end of TMD 1 or within the first intracellular loop of the receptor. In higher vertebrates arginine is found at position 64, however in humans tryptophan has been found to replace arginine with an allele frequency of 8-10%, which suggests that there was a human-specific evolutionary pressure for dominance of the tryptophan residue. Both the fathead minnow and the trout β_3-AR sequences express arginine at alignment position 75, which corresponds to position 64 in the human β_3-AR. It is very unlikely, however, that both nonsynonymous and synonymous polymorphism events are responsible for the differences between the fathead minnow β_{3bi}- and β_{3bii}-AR sequences as the sequences are considerably different from one another. It is possible that a gene conversion event, which is the alteration of a sequence that does not affect its function, extending across the known sequence regions of both the β_{3bi}- and β_{3bii}-AR is responsible for the difference in sequence homology (Cantor & Smith, 1999; Betts & Russell, 2003).

Furthermore, results from the RACE PCR indicated that a β_{3bi}-AR sequence taken from approximately TMD 2 to TMD 5 was different to other PCR and RACE PCR products, including the confirmation primer-walking sequence. The sequence was also different to the β_{3bii}-AR sequence. Considering the occurrence of genomic duplication events and possible gene conversion events in teleost fish, this sequence may represent a second β_{3bi}-AR sequence.

2.4.2 Comparison with fathead minnow β_1- and β_2-AR sequences

The β_{3bi}-AR, consisting of 435 amino acids, was considerably longer than the fathead minnow β_1-AR and β_{2i}- and β_{2ii}-AR sequences which were 402, 378 and 397 amino acids long (Giltrow, 2008), respectively, which differs from the human β_1-, β_2- and β_3-ARs, where β_1 and β_2 are longer than β_3-AR (Skeberdis, 2004). It is difficult at this stage to postulate whether the differences in amino acid sequence lengths has any bearing on functional differences between the different β-AR subtypes. The fathead minnow β_{3bi}-AR was more similar to the β_1-AR (43% similar) than the β_2-ARs (38% similar to both subtypes) which goes someway to support previous suggestions that perhaps the β_3-AR is derived from a β_1-AR (Skeberdis, 2004).

Cluster algorithm analysis of aligned vertebrate β-AR amino acid sequences including the fathead minnow β-AR sequences produced a tree with three major groups corresponding to the three pharmacologically defined β-AR subtypes and placed the fathead minnow β_{3bi}-AR at the base of the β_3-AR group with strong statistical support (99%). All three groups of receptors were closely related according to the bootstrap values. The phylogenetic tree did support previous observations that the fathead minnow β_{3bi}-AR was more similar to the fathead minnow β_1-AR than the β_2-AR. Moreover, β_3-AR receptors in general were more similar to β_1-ARs than β_2-ARs, which further supports the theory that β_3-ARs may be the product of β_1-AR evolutionary divergence. However, since the fathead minnow β_1- and β_3-AR sequences were not closely similar, one can assume that they are different β-AR types. For absolute confirmation, kinetic ligand binding assays are required to examine the binding potential of both β-ARs to different catecholamines and to examine for different G-protein mediated responses to ligand binding.

2.4.3 Expression of the β_{3bi}-AR in fathead minnow tissues

The β_{3bi}-AR was found in all examined tissues, those being the heart, liver, brain, red blood cell, gonad, adipose tissue and gill from both adult male and female fathead minnows. Nickerson *et al* (2003) documented that the β_{3a}- and β_{3b}-ARs were found in the trout heart and gill and red blood cells, respectively, and suggested that the function of the β_{3b}-AR was to control the β-AR Na^+/H^+ exchanger (β-AR/β-NHE), which regulates

red blood cell intracellular pH, and in turn enhances the affinity of hemoglobin for oxygen, thus allowing for increased oxygen transport by the blood. Although the function of the β_{3bi}-AR in the fathead minnow heart and gill is not known, both tissues are arguably the most important for fish in regulating blood flow, in particular the gill with regards to binding oxygen to blood. As the β_{3bi}-AR appeared to be expressed most highly in the heart, gill and blood (although this was subjectively assessed and needs to be repeated), it is possible that the β_{3bi}-AR performs a similar role in the fathead minnow as in the trout. However, as it was expressed in all examined tissues, it is possible that the β_{3bi}-AR may perform a number of functions.

In humans and higher vertebrates, the β_3-AR is found predominantly in brown adipose tissue, although it has also been identified in the human brain, gallbladder, colon, internal mammary artery, bladder, mouse oocytes and the pig duodenum (Krief *et al*, 1993; Rodriguez *et al*, 1995 Bardou *et al*, 1998; Horinouchi & Koike, 1999; Takeda *et al*, 1999; Čikoš *et al*, 2005). Accordingly, the suggested principal role of the β_3-AR is to facilitate a thermogenic response, although researchers have postulated its role in other tissues, for example in brain development in juveniles and urine storage in adults. Unsurprisingly the β_{3bi}-AR was found in several tissues including the adipose tissue, albeit at apparently lower levels than the heart, blood and gill. According to Giltrow (2008), neither the fathead minnow β_1-AR nor the β_2-ARs were found in adipose tissue, providing further evidence that the β_{3bi}-AR belonged to the β_3-AR family, although its function in the fathead minnow is unknown since it is unlikely that a poikilothermic fish requires a thermogenic response. However, there is the possibility that the β_{3bi}-AR could mediate the mobilisation of energy reseves during fasting, migration or egg laying in fathead minnows via lipolysis. Ligand binding assays would need to be employed to examine the role of the β_{3bi}-AR in the fathead minnow.

There were sex-based differences in the expression patterns of the β_{3bi}-AR in the fathead minnow; however the technique of examining expression levels was, in comparison to RT-PCR, less sensitive and semi-quantitative. Therefore, before examining this anomaly,

a comprehensive, sensitive and accurate means of examining expression levels, such as RT-PCR, is required.

2.4.4 Limitations of the study

One of the main problems encountered was the interpretation of the outcomes from the different topology programmes used to predict the TMDs. The 5 different EXPasy programmes produced identified TMDs at different locations and, despite there being a level of agreement between the programmes on isolated TMDs, there was not a unanimous agreement between any two programmes for all seven TMDs. Furthermore, TMHMM and HMMTOP did not identify a TMD 7; this problem was also encountered when identifying the fathead minnow β_1- and β_2-ARs (Giltrow, 2008) suggesting that this may be an inherent problem with using these programmes. Using the information from each topology programme, along with the motif criteria, the TMDs were selected according to the TMpred programme.

The rainbow trout is the only fish to have a sequenced β_3-AR and therefore the use of all other sequences, including the zebrafish that was used as the template for primer designing, are based entirely on NCBI sequences produced by automated processing of existing genomic data and are therefore predicted sequences. This problem was highlighted further in the current study when the existing zebrafish β_{3bi}-AR sequence was changed from a predicted β_{3bi}-AR to a β_1-AR, which indicated that the fathead minnow sequence could be a β_1-AR. Upon further inspection, the sequence did not express the motif tag associated with all β_1-ARs (as suggested by Mason *et al*, 1999). Additionally, Giltrow (2008) identified the fathead minnow β_1-AR through PCR and cloning and the similarity between this sequence and the pre-existing zebrafish β_1-AR was 91%, whilst the similarity between the re-assigned zebrafish β_1-AR and the fathead minnow β_1-AR was 44%. Therefore, it is unlikely that that the zebrafish sequence is a β_1-AR but more likely a β_3-AR. This example demonstrates one of many problems when working with sequences that have not been confirmed in the laboratory.

2.4.5 Recommendations for future work

The tissue distribution of the β_{3bi}-AR supports findings from other studies that it is a β_3-AR subtype. However, for accurate confirmation, kinetic competitive ligand binding assays are required.

The fathead minnow, and certainly the zebrafish, represent a potentially suitable alternative model test species for pharmaceutical development. As the β_3-AR in humans is primarily located in adipose tissue and is directly involved in thermogenesis and lipolysis, activation of the β_3-AR is thought to be a possible treatment approach for obesity, type 2 diabetes, mellitus and frequent urination (Arner & Hoffstedt, 1999; Sawa & Harada, 2006). Therefore, in this context, it is extremely important that the β_3-AR identified in both the fathead minnow and zebrafish is confirmed as the use of either fish, most likely the zebrafish, as a test species may aid in anti-obesity drug development. Accordingly, it is also important to explore the number of β-AR subtypes in the fathead minnow to aid understanding from both an evolutionary, drug-development and environmental monitoring perspective.

2.4.6 Conclusion

The current study demonstrated that the fathead minnow expresses the β_3-AR in several fathead minnow tissues. Together with the previous study by Giltrow (2008), all three β-ARs have been identified in the fathead minnow, which provides suitable justification for a fathead minnow pair-breeding assay exposure to the non-specific β-blocker, propranolol. This study represents a successfully first stage of an intelligent test-protocol for examining pharmaceutical toxicity on fish.

CHAPTER 3: FATHEAD MINNOW EXPOSURE TO PROPRANOLOL

3.1 INTRODUCTION

The aim of the exposure experiment was to generate tissues suitable for metabolomic analysis and β_3-AR expression studies using real-time PCR. Accordingly, adult fathead minnows were exposed to several concentrations of the non-specific β-blocker propranolol, ranging from 0.001mgL^{-1} to 10mgL^{-1}, and tissues collected for metabolomic and β_{3bi}-AR expression analysis.

3.1.1 Pharmacokinetic properties of propranolol

Propranolol works by competitively inhibiting β-ARs, thus reducing the effects of adrenaline and noradrenaline, and is prescribed to patients suffering with angina and hypertension (high blood pressure), adrenal cushing's syndrome and corticotrophin-independent bilateral adrenal hyperplasia and to treat patients after a heart attack to prevent further attacks (Lacroix *et al*, 1997; UAB Health System, 2006; Fent *et al*, 2006). Upon consumption, propranolol undergoes extensive first stage hepatic metabolism by the cytochrome 2C19 and 2D6 P450 enzymes and only approximately 10% of the parent compound is excreted, most often in urine (Perkinson, 1996; Huggett *et al*, 2003a). Once excreted, propranolol enters wastewater treatment facilities. Ternes (1998) found that a German sewage treatment work (STW) removed 96% of propranolol, although this figure is likely to be lower in wet weather conditions. According to these calculations, 0.4% of the original propranolol product passes through the STWs into the aquatic environment. However, propranolol has been detected in the aquatic environment at concentrations ranging from <10ngL^{-1} to 2.2µgL^{-1} (Huggett *et al*, 2003a; Ashton *et al*, 2004), as shown in table 17. In the UK, Thomas & Hilton (2004) collected 45 STW water effluent samples and detected propranolol in every sample. These figures reflect the enormous usage of propranolol, especially across the developed Western countries.

Propranolol has an *n*-octanol-water partition coefficient (log K_{ow}) value of 3.67 and a half life of 16.8 days at 5.0^{o}C in winter indicating that it has the potential to accumulate in either biota or partition into sediments, thus prolonging the length of time it resides in the aquatic environment and therefore increase the likelihood of inducing an effect in resident biota. Like all drugs designed to be taken orally, propranolol is relatively

hydrophobic and it is therefore unlikely that propranolol will degrade via hydrolysis (Huggett *et al*, 2003b; Lee *et al*, 2007).

Table 17. Reported levels of the β-blocker propranolol in sewage discharges and surface waters.

Sewage concentration (ngL^{-1})	**Surface water concentration (ngL^{-1})**	**Location**	**Reference**
10-40		Germany	Ferrari *et al* (2004)
180	590	Germany	Ternes (1998); Ternes *et al* (2003)
16-284	<10-115	U.K	Thomas & Hilton (2004); Ashton *et al* (2004)
300	100	U.K.	ENDS (2005)
26-2200		U.S.A	Huggett *et al* (2003a)

3.1.2 Measured effects of propranolol on the aquatic environment

Several *in vivo* studies have examined the acute toxicity of propranolol to both invertebrates and vertebrates. Algae and plant inhibition of growth studies suggested the 50% effective concentration (EC_{50}) values were 0.73 and 0.8mgL^{-1} (48hr tests) (Huggett *et al*, 2002, Cleuvers, 2004), whilst the crustacean *Daphnia magna* appeared to be less sensitive to propranolol than algae and plants, having a recorded 48 hour EC_{50} for immobilisation of 7.7mgL^{-1}. However, contrary information from a 48-hour mortality tests suggested *Daphnia magna* had an observed 50% mortality (LC_{50}) at a concentration ranging from 1.66 – 2.75mgL^{-1} (Huggett *et al*, 2002; Ferrari *et al*, 2004). Regardless of these different observations, these measured EC_{50} and LC_{50} values do not reflect actual environmental concentrations. There is very little information that addresses the possible effects of long-term, chronic, exposure to propranolol. Early data suggested that over a chronic 10-day exposure to propranolol, *Dapnia magna* showed signs of reduced growth (lowest observed effect concentration (LOEC) = 0.44mgL^{-1}), fecundity (LOEC = 0.11mgL^{-1} and heart rate (0.055mgL^{-1}) (Dzialowski *et al*, 2006). Thus, there is the

potential for propranolol to have a cardiovascular effect on *Daphnia magna* in the environment, suggesting it may have the same target tissues in aquatic organisms as in humans.

The acute toxicity of propranolol has also been investigated in a handful of fish test species including the Japanese medaka (Huggett *et al*, 2003b), the zebrafish (Fraysse *et al*, 2006) and the rainbow trout (Larsson *et al*, 2006). Huggett and colleagues observed 50% mortality in 3 – 4 day old Japenese medaka exposed for 48hr to propranolol at a concentration of 24.3mgL^{-1}. Fraysse and colleagues (2006) examined the response of 4-day-old zebrafish larvae exposure to propranolol for 24, 48 and 80 hours. After 24 hours of exposure to propranolol, there was an observed decrease in the number of spontaneous tail movements (EC_{50} = 1.5mgL^{-1}), after 48 hours, there was a reduction in heart rate (EC_{50} = 1.5mgL^{-1}) and after 80 hours, no hatching was observed (32mgL^{-1}). Larsson and colleagues (2006), however, found no effect of exposure to propranolol in the rainbow trout at a concentration of 0.00709mgL^{-1}, which is not unusual considering the concentration was considerably lower than those that triggered a response in the Japanese medaka and zebrafish. Although this difference in response to propranolol could be due to species sensitivity, age sensitivity, it is thus most likely the result propranolol concentration levels; as propranolol concentrations increases, the magnitude of response increases. Similarly to the acute toxicity studies using invertebrates, the measured EC_{50} values do not reflect actual environmental concentrations.

The more environmentally realistic effects of chronic exposure to propranolol have also been examined in a handful of fish studies. The reproductive effects of chronic exposure of propranolol to fish have been detected in the Japanese medaka (Huggett *et al*, 2002). Following on from the acute exposure of 3 – 4 day old Japanese medaka to propranolol, Huggett and colleagues cultured the medaka in different concentrations of propranolol for 14 days and found that propranolol inhibited growth at a concentration of 0.5mgL^{-1} and reduced testosterone and increased oestradiol concentrations at a concentration of 0.001mgL^{-1}. Huggett and colleagues also exposed sexually-viable adult Japanese medaka pairs to propranolol concentrations for 4 weeks and observed a reduction in egg

production and hatchability at a concentration of 0.0005mgL^{-1}. However, there were several discrepancies regarding this data. Firstly, the data was no repeated, secondly, the exposures were not carried out in a flow-through system but rather in a semi-static system, indicating that exposure to propranolol could have been in acute "doses", thirdly, propranolol was dissolved in ethanol, which seemed unnecessary as propranolol readily dissolves in water at the assigned concentrations and so ethanol may have induced a toxic response. Finally, the reproductive and hatchability response of Japanese medaka to a chronic 4-week propranolol exposure was only observed at the lowest concentration. Accordingly, to date our understanding of fish response to chronic exposure to propranolol is limited.

As propranolol has the lowest log K_{ow} value of all beta blockers, it is the most lipophilic and therefore the most likely to bioaccumulate in fatty tissues. Several studies indicate that the sensitivity, and consequential response, of aquatic organisms to propranolol exposure is higher than other beta-blockers detected in the environment (Fent, 2006). Therefore, for the purpose of examining a measurable response of fish tissues, propranolol was the most suitable beta-blocker candidate.

3.1.3 Test organism: the fathead minnow (*Pimephales promelas*)

The fathead minnow (*Pimephales promelas*) along with the zebrafish (*Danio rerio*) and the Japanese medaka (*Oryias latipes*) is one of the most commonly used small fish test species used in ecotoxicological research.

The fathead minnow is a gonochoristic teleost species belonging to the family Cyprinidae, order Cypriniformes (Linnaeus, 1758). Etymology of the minnow's name derives from the shape of the head (*Pimephales* – "fathead") and it's colour in breeding males (*promelas* – "forward" and "black") (Mettee *et al*, 1996). The fathead minnow is a small freshwater fish; adults typically reach a maximum total length of 50mm and have a moderately compressed laterally-shaped body, a short blunt snout and a slightly subterminal snout (Rook, 1999; Yonkos *et al*, 2000). Sexually mature males have a striking appearance and are easily distinguished from the females – they are larger (3-5g

versus 2-3g respectively) and darker in colour with vertical bands on the body and head, they also have nuptial tubercules on the face and a prominent fatpad on top of the head (referred to as secondary sexual characteristics). In contrast females are olive/silver in colour and have a more pointed head (figure 32) (Yonkos *et al*, 2000).

Under optimal conditions the fathead minnow reaches sexual maturity at four to six months post-hatch. The sexually mature males select and prepare the nest site, which are typically located on the under-surface of submerged stones or branches. Females spawn every 2-16 days producing up to 10,000 eggs in the three month breeding season. The brooding males protect the eggs from predation and cannibalism and fan the egg masses with their caudal and dorsal fins to increase oxygen availability. The spongy dorsal fatpad is used for scrubbing the nest site to remove parasites. Eggs hatch within 4-5 days depending on temperature and begin feeding almost immediately (Harries *et al*, 2000; Yonkos *et al*, 2000; Jensen *et al*, 2001).

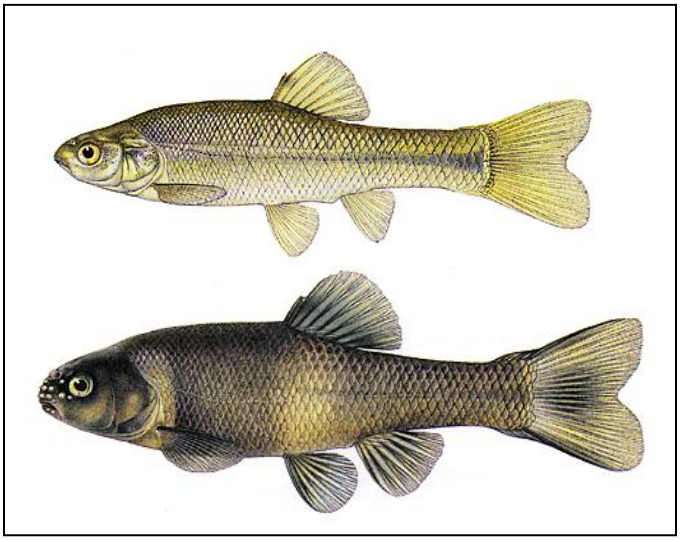


Figure 32. Female (top) and male (bottom) fathead minnow, *Pimephales promelas*. The male is illustrated in breeding condition. Illustrations taken from Yonkos *et al* (2000).

3.1.3.1 Role of the fathead minnow in ecotoxicological research

The fathead minnow has been, and still is, used as a model test species in routine regulatory toxicity tests by a multitude of agencies including the environmental

protection agency (EPA), the environment agency (EA) and the organisation for economic cooperation and development (OECD). These tests encompass short-term 48/96-hr lethality assays, complex partial and full life-cycle tests involving a battery of both apical (whole animal) and mechanistic/diagnostic endpoints (Harries *et al*, 2000). With developments in genomic, proteomic and histological techniques, the fathead minnow has become an established biomarker test species for a diverse number of contaminants including several pharmaceuticals; arguably the most extensively studied of which has been ethinyloestradiol (EE_2) (Harries *et al*, 1998; Harries *et al*, 2000; Halm *et al*, 2002; Zerulla *et al*, 2002; Leino *et al*, 2005; Ankley & Villeneuve, 2006; Miracle *et al*, 2006). The fathead minnow represents a potentially more advantageous test-species compared to an alternative OECD test species, the zebrafish, when examining the reproductive response to pharmaceutical exposure. Thorpe *et al* (2007) examined 200 different pair-breeding assays using the fathead minnow as the test species and found that egg production was more consistent and robust compared to the zebrafish, which according to Paull *et al* (2008) required higher levels of replication pairs when examining the reproductive response to EDCs.

To date, only a single study has examined the response of fathead minnows to β-blocker exposure. Winter and colleagues (2008) examined the impacts of chronic 21-day exposure of atenolol on both the reproductive output and subsequent embryo-larval development of fathead minnows. The results from the assay suggested that atenolol (concentrations ranging from $0.1mgL^{-1}$ to $10mgL^{-1}$) did not have an impact on the reproductive potential or embryo-larval survival, suggesting that it is less toxic than propranolol.

3.1.4 Aim of study

There were two aims for the study: to firstly examine the response of fathead minnows to chronic 21-day exposure to propranolol using the same pair-breeding assay and physiological endpoints originally described by Winter *et al* (2008); and to secondly acquire tissues suitable for NMR-metabolomic and Real Time PCR (RT-PCR) β_3-AR expression analysis for subtle biomarkers of propranolol exposure.

3.2 METHODOLOGY

3.2.1 Propranolol experimental design

The propranolol pair breeding assay adopted for this study was originally proposed by Harries *et al* (2000) and Ankley *et al* (2001) and subsequently adapted by Winter *et al* (2008) for examining the response of the fathead minnow to the β-blocker atenolol. In Brief, sexually viable adult fathead minnows aged 1 year post hatch (Thorpe *et al*, 2007), were housed in discrete male-female pairs and their reproductive potential assessed over two concurrent 21-day periods: one prior to propranolol exposure (referred to as the 'baseline period') and one during exposure to propranolol ('exposure period'). The nominal concentrations of propranolol were set at the environmentally relevant concentration of 0.001mgL^{-1} plus 0.01mgL^{-1}, 0.1mgL^{-1}, 1.0mgL^{-1} and 10.0mgL^{-1} and a dilution water control (DWC). The endpoints measured were survival, egg production, standard length, wet weight and condition index (CI), gonad weight and gonadosomatic index (GSI) and secondary sexual characteristics and also first generation (F1) survival.

The entire pair-breeding assay was repeated, which was in part due to experimental room size limitations, but it also presented both an ethical and statistical advantage. Ethically, if no physiological effect was observed in the first experiment, there would be no need to conduct the second experiment and unnecessarily expose adult fathead minnows to propranolol. Statistically, by repeating the study it was possible to examine how robust the first data set was and if there were no significant differences between the same treatment groups between the experiments, the data could be pooled to produce a larger and more statistically-powerful replicate number.

3.2.2 Preparation of experimental room

Prior to running the pair-breeding assay the walls and floors of the experiment room were washed with 'Pinefresh' detergent and subsequently washed with water. The header tanks were bleached and the bleach was left overnight to ensure any algae that may have accumulated in the system was killed. The glass tanks that were used to house the fish pairs were cleaned with Scale-Free and washed and rinsed in the tank washing machine

to ensure that any residual chemical or substance from a previous experiment was removed. The flow meters that were used to control the rate of header tank water entering the mixing chamber were also dismantled and cleaned, after which fresh PTFE tape was put around the screws; this was again to ensure any residual chemical or substance was removed.

3.2.3 Preparation of stock solutions and dosing of tanks and water quality

A continuous flow-through system was employed, ensuring a complete water change of dechlorinated tap water in each pair-breeding tank every 2.5hrs. Propranolol-hydrochloride (DL-Propranolol hydrochloride, *1–isopropylamino-3-(1-naphthyloxy) proprano-2-ol hydrochloride*) (CAS 318-98-9) (figure 33) was used as the source of propranolol. Propranolol hydrochloride is a racemic mixture that is 99% pure and was supplied from Astrazeneca, Brixham laboratories and because it is light sensitive was stored in a foil-wrapped beaker at 4^{o}C.

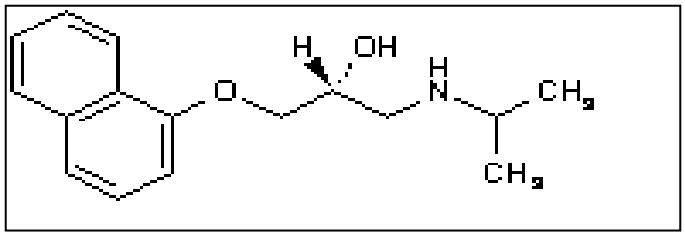


Figure 33. Chemical structure of propranolol hydrochloride.

Propranolol was readily dissolved in dilution water by vigorous shaking. Individual stocks of propranolol were prepared for each dosing concentration; table 18 shows the calculated volumes of propranolol used for each test concentration. To minimize photo-degradation of propranolol, each stock bottle was wrapped in foil. Figure 34 shows a close-up of the propranolol stock bottles and the peristaltic pump which was used to deliver each propranolol stock to their respective concentration tanks.

Table 18 Calculated volumes of propranolol required for each test concentration in 4L. 10mgL^{-1} concentration, highlighted in red, was only used for the first study and not the repeat.

Nominal concentration of Propranolol-HCl (mgL^{-1}) in fish tanks	Amount of propranolol-HCl added to each 4L bottle (g)
0.001	0.0016
0.01	0.016
0.1	0.16
1.0	1.6
10.0	**16.0**

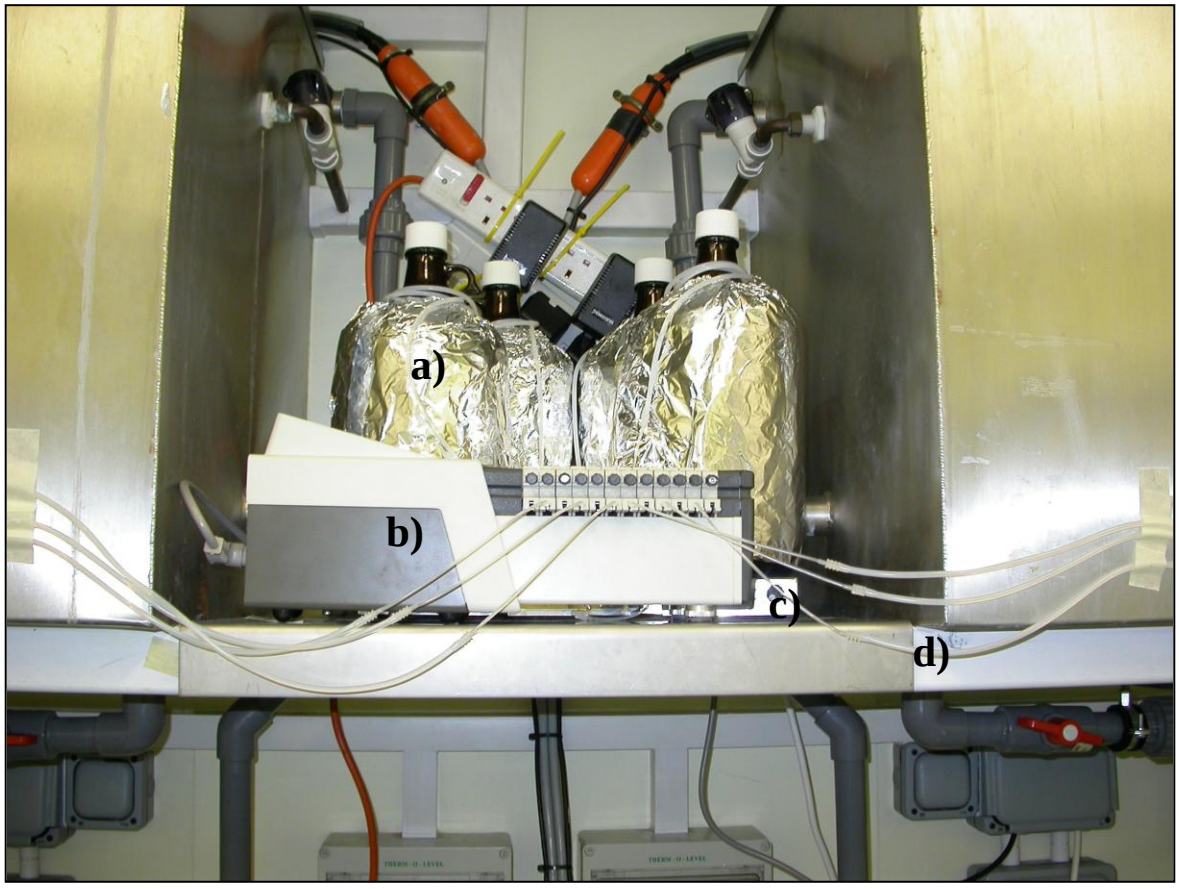


Figure 34. Close-up photograph of the **a)** the stock bottles wrapped in foil; **b)** the peristaltic pump; **c)** the magnetic stirrers used to ensure complete mixing of propranolol stocks and **d)** the tubing taking each propranolol stock to their respective concentration mixing vessel.

During the exposure period, all propranolol stocks were dosed at 1.0ml min^{-1}, using a Watson Marlow (Cornwall, UK) multi-channel peristaltic pump, into glass mixing vessels where they mixed with dilution header-tank water (400ml min^{-1}) before delivery into their respective test tanks at a flow rate of 80ml min^{-1}; see figure 35. All tubing within the system was medical grade silicon. Each stock was replenished every 2.78 days. Direct measurements of dilution water flow rates were made twice per week throughout the exposure period, with daily checks to ensure the correct operation of the dosing system. The flow rates of the concentrated propranolol stocks were checked twice per day.

Figure 35. A photograph of one side of the experimental room. **a)** represents the header tank; **b)** shows the mixing vessels from which water from the header tank and propranolol from the stock bottles mixed before flowing into the individual fish tanks (**c)**); **d)** indicates one of the flow meters; **e)** shows one of the clamps used to regulate water flow from the mixing vessel into each tank.

In the first experiment five test concentrations were used, however in the repeat experiment the four test concentrations used were 0.001mgL^{-1}, 0.01mgL^{-1}, 0.1mgL^{-1} and 1.0mgL^{-1} and also DWC.

3.2.4 Test apparatus

The tanks used for the adult pair-breeding study had a working volume of 10.5L and were constructed of glass. Screens were placed around the sides of all test vessels to prevent fish interacting with those in neighbouring tanks and to minimize disturbance due to operator movements. A spawning tile and tray (which consisted of a rectangular glass tray covered with a 0.5cm^2 stainless steel mesh (see figure 36) were placed in each vessel. The tile served as the nest-site for spawning whilst any eggs that fell of the tile were collected in the tray; the mesh allowed eggs to fall through into the tray, while preventing passage of the fish. Studies have suggested that this type of system of egg collection is highly robust and reduces the variability of egg numbers produced per couple per batch (Harries *et al*, 2000; Thorpe *et al*, 2007)

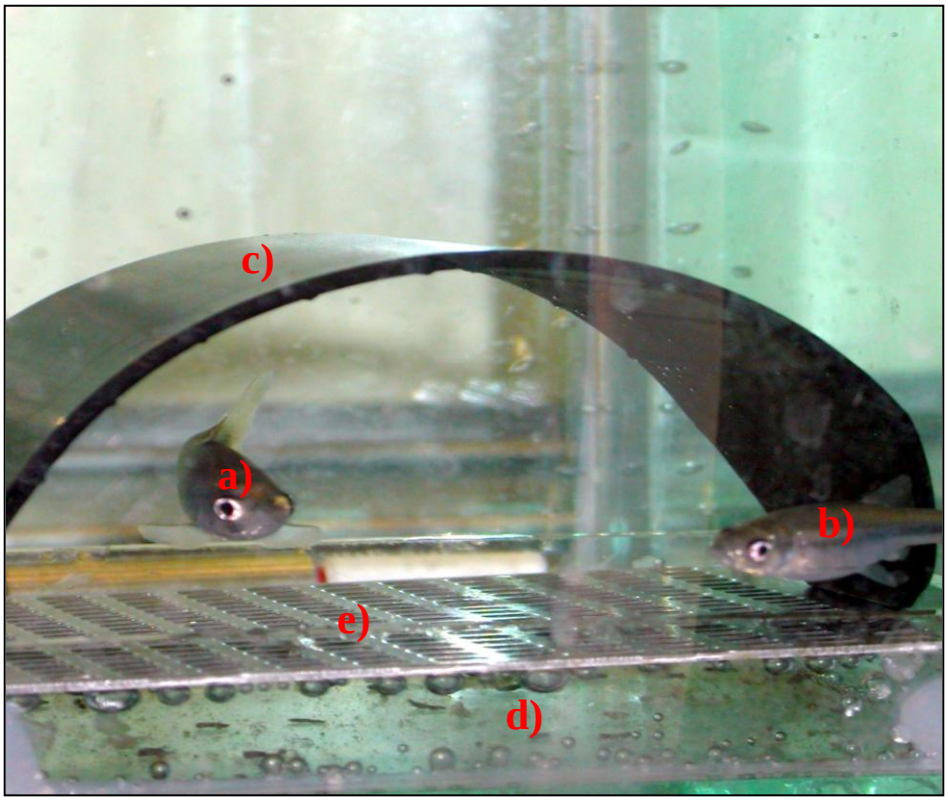


Figure 36. Spawning tank with **a)** male and **b)** female fathead minnow. **c)** shows the spawning tile; **d)** shows the glass tray and **e)** shows the metal mesh. Eggs were counted from **c)**, **d)** and **e)**.

3.2.5 Water, light and feeding parameters

Throughout the study the water temperature was maintained at 25 ± 1°C, dissolved oxygen (DO_2) was maintained above 75% saturation (equivalent to 6.2mgL^{-1}) and pH and water hardness were also recorded. Water from all exposure concentrations and DWC were tested for temperature and DO_2 on day 0 of initiation and recorded twice per week thereafter. Water hardness and pH were also tested for on day 0 and then on once per week thereafter. The photoperiod regime was set at 16 hours of light followed by 8 hours of darkness, with a 20 minute dawn: dusk transition.

The fish were fed twice a day with frozen adult brine shrimp (*Artemia salina*) during the week and once a day during the weekend. Fish flakes were also fed once a day. The sides and bottoms of all tanks were cleaned and the tanks siphoned to remove debris twice per week. All tanks were cleaned at the same times so that all received approximately equal levels of disturbance.

3.2.6 Test procedure

Figure 37 outlines a simplified timeline of the pair-breeding assay test procedure.

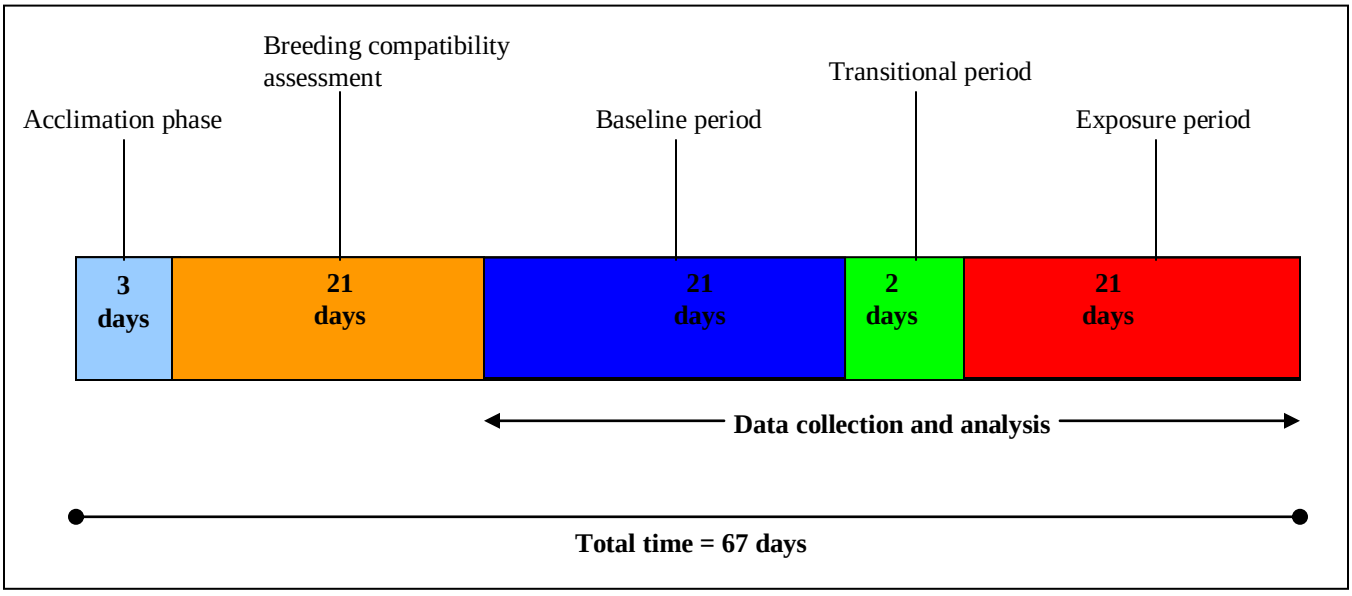


Figure 37. Test procedure schematic highlighting the acclimation phase, breeding compatibility assessment and baseline, transitional and exposure periods. Only data from the baseline and exposure period were analysed.

3.2.6.1 Acclimation and breeding compatibility assessment

To initiate the test, sexually mature male and female fish (1 year old) were selected at random and a pair of fish (1 male and 1 female) were placed into each test vessel tank, which were all receiving dilution water only. According to Thorpe *et al* (2007), fathead minnow pairs spawn at a 'normal' rate up to and including 16 months. There were 6 tanks per treatment, 4 were used to hold fish pairs (n = 4) and 2 were used for hatchability trials (see below), totaling 36 tanks.

The fish were then left to acclimate with minimal disturbance for 3 days. During this period, fish were only fed (water parameters were measured prior to introduction of the fish). After 3 days, the fish were left for a further 21 days to assess breeding compatibility. Breeding compatibility was assessed via determination of normal breeding behaviour, egg numbers, egg quality (egg viability and presence of abnormalities) and spawning ability (e.g. site where the female laid her eggs). This was achieved by examining the underside of each breeding tile once a day for egg production, as shown in figure 36. In the instance that eggs had been produced, the tile, mesh and glass base was removed from the tank and replaced with a clean set. The eggs were examined under the microscope to determine whether they were alive or not, dead eggs appear as opaque (as shown in figure 38). Egg numbers were counted, using a ticker counter, on the tile, mesh and base and recorded accordingly. The ratio of live: dead eggs was subsequently calculated. All eggs were subsequently killed via immersion in boiling hot water and then washed away. The tile, mesh and base was subsequently washed and dried.

To be deemed suitable for the pair-breeding assay, each fish pair must have spawned at least 4 times by the 21st day of the acclimation period; any fish that did not meet the breeding requirements were replaced by fish from the original batch. All fish movements were recorded. Any signs of distress and mortality were also recorded.

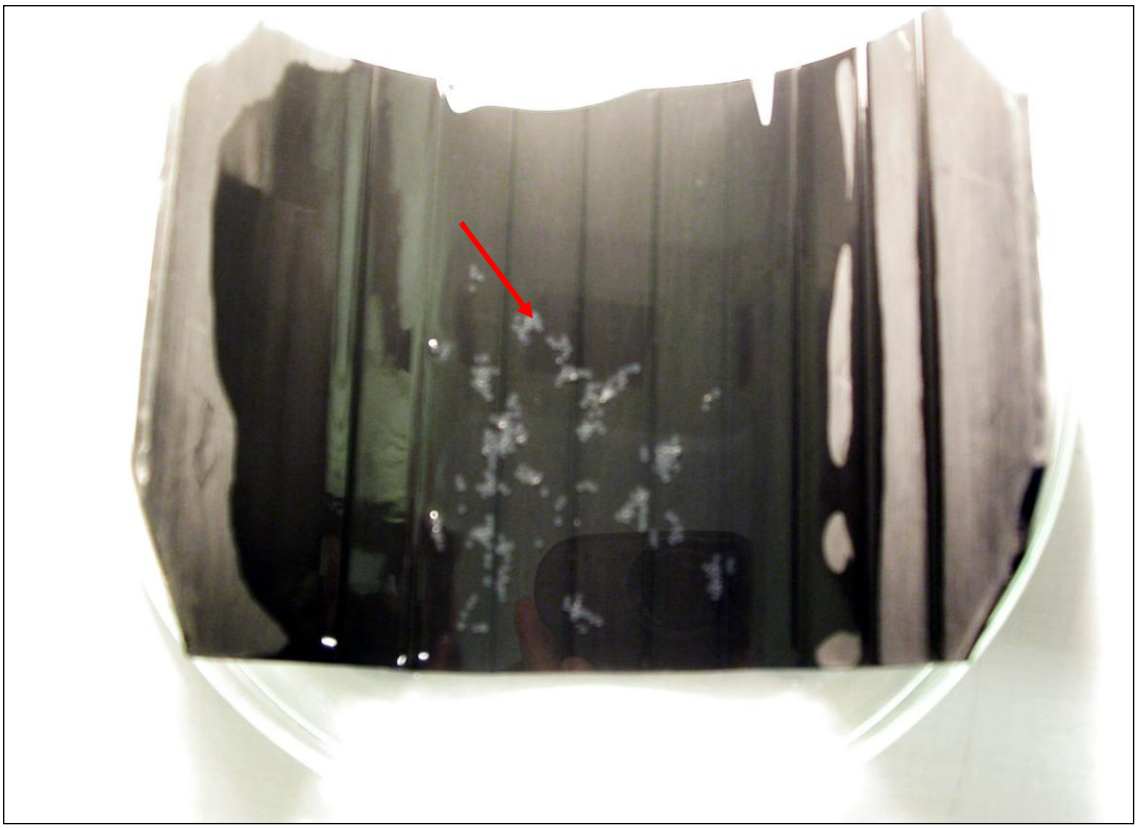


Figure 38. Close-up photograph of fathead minnow eggs laid on the underside of the tile, as highlighted by the red arrow.

3.2.6.2 Baseline period

After the initiation and acclimation period, the 21-day baseline reproduction period commenced. During this time, all tanks received dilution water only and breeding compatibility recorded. Each pair would thus serve as their own control. Each pair-breeding tank was examined once a day for egg production.

3.2.6.3 Transitional period

Following on directly from the baseline period a transitional period was undertaken where propranolol was introduced into the test vessels at the relevant concentrations. To ensure that the test vessels had reached their nominal concentrations prior to reproductive assessment the transitional period lasted for two days, during which the fish were left with minimal disturbance.

3.2.6.4 Exposure period

The exposure period lasted for a further 21 days, during which the assessment of reproductive performance under conditions of propranolol exposure were monitored in the same breeding pairs.

Water samples were collected from 3 randomly selected test vessels from each test concentration, including DWC, for offsite analysis of propranolol on exposure days 0, 4, 7, 14 and 21. Water samples (20ml) were collected in 40ml amber glass vials (Sword Scientific, Cat # 51-500), which had been previously washed twice with methanol and once with ethanol and dried, and stored at -20^{o}C until transportation to AstraZeneca Brixham Environmental Laboratory (Devon, UK) on dry ice for subsequent analysis.

3.2.7 Water chemistry analysis

Water samples were analysed by liquid chromatography for analyte separation with 0.1% ammonia in water and 0.1% ammonia in methanol over an elution gradient. Electrospray ionization-tadem mass spectrometry (ESI-MS/MS) was then used to quantify the analytes, which was carried out in the positive ionization mode (+) ESI. For increased selectivity, MS/MS with selected reaction monitoring (SRM) was used for detection of protonated propranolol ($[M + H]^+$, 260.2 Da) and Xcalibur^{TM} software was used for data acquisition and processing.

3.2.8 Hatchability trials

In addition to examining the reproductive potential of fathead minnow pairs exposed to propranolol, batches of eggs were taken from each fish pair throughout the study to examine the effects of propranolol on egg hatchability and survivability. For each hatchability trial, 50 eggs were selected at random from the tile and transferred into an incubation cup; this was made of a wire mesh that allowed for the constant movement of water across the eggs. The oxygen supply was left to bubble rapidly into the hatchability tanks to ensure this movement of water. In some cases there were less than 50 viable eggs or there were not enough eggs laid on the tile, therefore hatchability trials were

performed on smaller batch sizes taken from the stainless steel mesh and tray, in order of preference, with appropriate adjustment of statistical analysis.

Observations were made each day to assess the number of dead eggs (which were subsequently removed) and to score for abnormalities of stage development. Figure 39 shows the scoring process as developed by Tamsin Runnalls (2005). Hatching success (%) was also recorded. Eggs were incubated on the hatching apparatus until all eggs had hatched or at least two days elapsed since the final hatching.

Hatchability trials were undertaken during both the baseline and exposure period and 2 hatchability trials were conducted for each pair during both periods. If the hatching success from both trials differed by more than 30% an additional trial was undertaken. Following the assessment of hatchability, all larvae were sacrificed with an overdose of tricane methane sulhponate (MS222) anaesthetic, adjusted to pH 7.5.

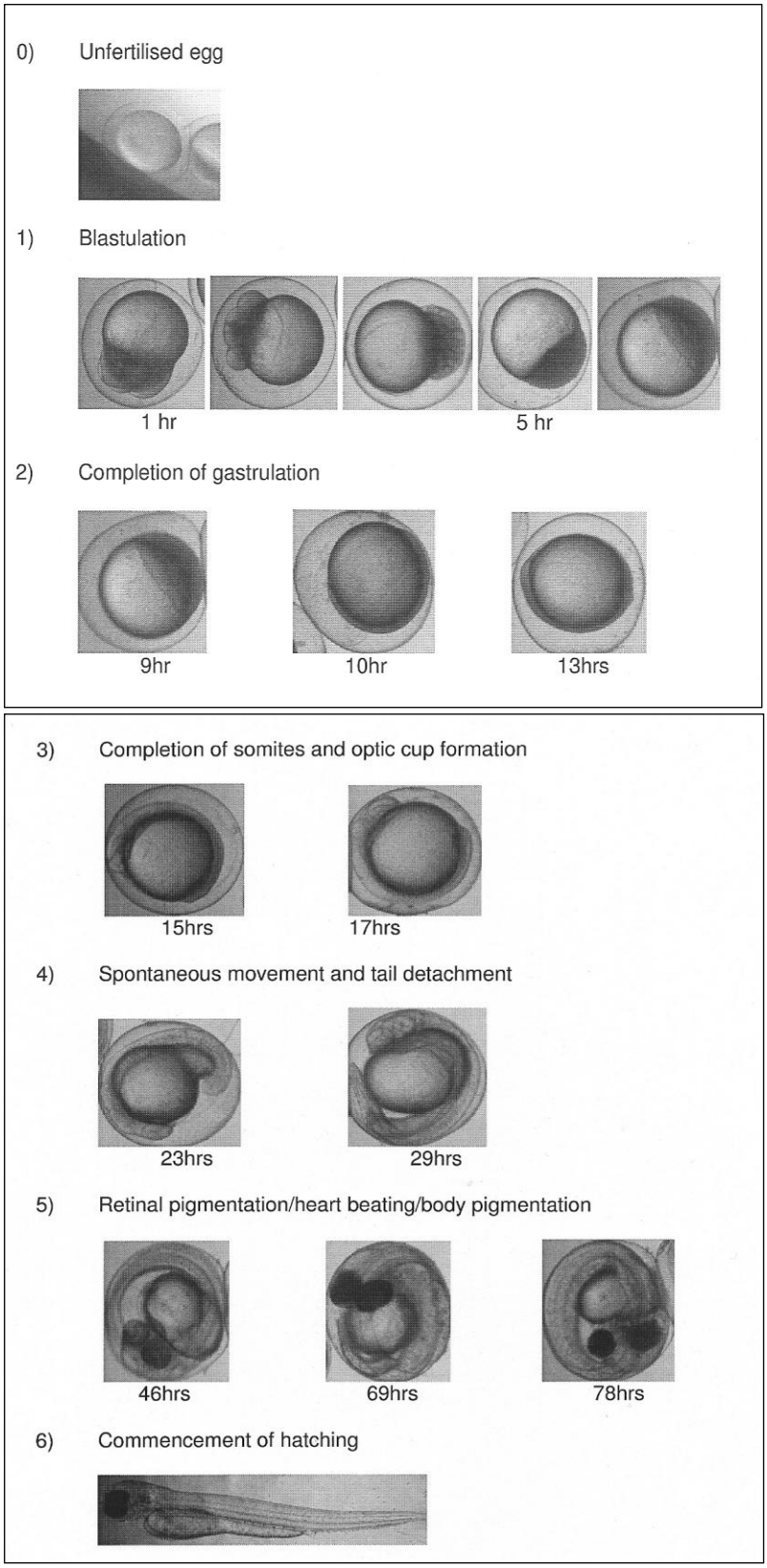


Figure 39. Photographs of the different stages of fathead minnow egg development (taken from the PhD thesis of Tamsin Runnalls, 2005).

3.2.9 Sampling Fish

On day 21 of the exposure period, adult fish were terminated by an overdose of pH-adjusted MS222 anaesthetic. For propranolol analysis, blood was collected via a tail cut (through the caudal peduncle) into 75µl heparinised capillary tubes and then decanted into Eppendorf tubes and kept on ice. Due to volume size limitations, blood plasma from female fish were pooled together. The blood samples were subsequently spun in a centrifuge at 700 x g for 5 minutes to separate whole red blood cells and plasma, and the blood plasma was carefully collected. The plasma samples were subsequently stored at -20^{o}C before transportation on dry ice to AstraZeneca Brixham Environmental Laboratory for subsequent analysis.

After blood collection, all fish were promptly measured for wet weight (g) and standard fork length (mm) and the CI was subsequently calculated (figure 38), which expressed the weight of the fish in relation to length. The liver was immediately extracted from each male fish for metabolomic analysis, promptly weighed and then snap frozen in liquid nitrogen before being stored at -80^{o}C. The gonad was subsequently extracted from each fish and weighed to determine the GSI (figure 40), which expressed the size of the gonad in relation to body weight. The female gonads, liver, heart and brain were subsequently snap-frozen in liquid nitrogen for Real Time PCR β_3-AR expression analysis.

$CI = \frac{\text{wet body weight (g)}}{\text{wet body weight (g)}} \times 100$	$GSI = \frac{\text{gonad weight (g)}}{\text{fork length (mm)}} \times 100$

Figure 40. Fish condition index (CI) and gonadosomatic index (GSI) calculations.

The fatpad from each male was subsequently weighed and the number of tubercles, and their prominence, assessed using the criteria outlined in table 19. Females were also examined for the presence of a fatpad and tubercles, which is indicative of androgenic or anti-oestrogenic effects.

Table 19. Fathead minnow tubercle scoring system (devised by Smith, 1978) used to assess tubercle prominence of male fathead minnows after exposure to propranolol for 21 days.

Score	Description
0	No visible sign of tubercles
+	Tubercles visible as white disks, not protruding above body surface
++	Tubercles project above body surface
+++	Tubercles are prominent, but not sharp
++++	Tubercles are prominent and sharp

3.2.10 Plasma chemistry analysis

All samples and standards were firstly prepared by protein precipitation (PPT) where 50µL of plasma was added to acetonitrile (200µL) (Fisher Scientific, Cat # 14952-0010) containing metoprolol internal standard ($0.25 \mu g L^{-1}$) in a filtration vial (0.45µm). After vortexing, the filtrate was transferred to a vial and diluted with 100µL 30:70 acetonitrile/water. Propranolol concentrations were then measured using online solid-phase extraction/liquid chromatography (SPE/LC).

3.2.11 The mammalian-fish plasma model

To quantitatively use mammalian pharmacology data in ecotoxicology, a conceptual model has been developed using the human No Observed Effect Concentration (NOEC) based on the therapeutic plasma concentrations and predicted environmental concentrations to calculate a Plasma Exposure Ratio (PER) (Huggett *et al*, 2003b). The PER indicates the likelihood for a fish to bioaccumulate a particular pharmaceutical across the gills and therefore indicates the potential for a pharmacological response.

A predicted fish stead state plasma concentration ($F_{SS}PC$) was obtained for propranolol at each treatment concentration by multiplying the treatment concentration value by the blood: water partition coefficient (logP), which was derived from the ratio of the compound in a water solvent and a hydrophobic solvent such as octanol the $\log K_{ow}$ value ($\log K_{ow} = 3.48$; logP = 45.7509) as logP is calculated from the ratio of solubility in water and in the hydrophobic solvent octanol (Leo *et al*, 1971). Propranolol PER was then

calculated by dividing the human therapeutic plasma NOEC (H_TPC) (as derived by Wong *et al*, 1979) by the measured $F_{SS}PC$. A PER <1 indicates that the $F_{SS}PC$ exceeds the H_TPC and so there is a high likelihood for a pharmacological response in a fish; a PER>1 indicates that the $F_{SS}PC$ is less than the H_TPC and so a pharmacological response in a fish is unlikely.

The aim was to examine how applicable the mammalian-fish plasma model was for predicting the potential pharmacological effects of propranolol.

3.2.12 Statistical Analysis

To compare water chemistry and blood plasma results from both exposures, a Mann-Whitney rank sum test was used, which compared the mean propranolol concentrations from each designated concentration treatment group between experiments 1 and 2 and examined for significant difference between the experiments. The Mann-Whitney rank sum test is the non-parametric alternative to the commonly used t-test.

To test the null hypothesis that there were no statistically significant differences between hatchability success between the baseline and exposure periods within each propranolol treatment group, the Mann-Whitney rank sum test was again adopted. To test the second null hypothesis that there was no significant difference in hatchability success between the different propranolol treatment groups, a Kruskal-Wallis One Way Analysis of Variance on Ranks (Kruskal-Wallis ANOVA) was performed. An ANOVA is a suitable alternative to running several t-tests and compares more than two data sets (Fowler *et al*, 1998). However, as the hatchability data was not normally distributed, a Kruskal-Wallis ANOVA was performed.

A parametric ANOVA was used to test the null hypothesis that there was no difference in egg production between the treatment groups. If a difference was found, a Holm-Sidak post-hoc test was used to identify which treatment groups were statistically significantly different to each other. Table 20 summarises the statistical tests used on the data collected on day 21 of the propranolol exposure.

Table 20. A summary of the null hypothesis for each parameter assessed, together with the statistical test that was applied, the post-hoc test used to accept or reject each null hypothesis. N/A indicates that there was not a statistical significant difference. Table amended from that used by Giltrow (2008).

Data set	Null hypothesis	Test used	Post-hoc test used
Wet Weight	There are no differences in wet weight between treatment groups.	Parametric ANOVA	Holm-Sidak
Fork length	There are no differences in fork length between treatment groups	Parametric ANOVA	N/A
Fat pad weight	There are no differences in fat pad weight between treatment groups	Non-parametric Kruskal-Wallis One Way Analysis of Variance on Ranks	Dunn's post hoc test
Tubercle number	There are no differences in tubercle number between treatment groups.	Non-parametric Kruskal-Wallis One Way Analysis of Variance on Ranks	Dunn's post hoc test
Tubercle prominence	There are no differences in tubercle prominence between treatment groups	Non-parametric Kruskal-Wallis One Way Analysis of Variance on Ranks	N/A
Male gonad somatic index (GSI)	There are no differences in the GSI between treatment groups.	Parametric ANOVA	Holm-Sidak
Female gonad somatic index (GSI)	There are no differences in the GSI between treatment groups.	Parametric ANOVA	Holm-Sidak
Male Condition index (CI)	There are no differences in the CI between treatment groups.	Parametric ANOVA	Holm-Sidak
Female Condition index (CI)	There are no differences in the CI between treatment groups.	Parametric ANOVA	N/A

3.3 RESULTS

3.3.1 Water Parameters

Figures 41 to 44 show the results for measured water temperature (^{o}C), dissolved oxygen (%), pH and water hardness ($CaCO_3$ mgL^{-1}). Water parameters for both pair-breeding assays (experimental tanks) and hatchability trials (progeny tanks) were routinely measured. For all figures the pink, filled green and orange boxes represent data collected in the baseline, transition and exposure periods, respectively. In some instances the water temperature dropped below the set limits. This was partly due to spells of hot weather which inadvertently activated the in-house chillers which in turn cooled the air temperature in the experiment rooms and as a result cooled the water temperature in the experimental tanks. Air bubbles also sometimes occurred in the in-flow tubes between the mixing chamber and an individual tank, thus preventing the flow of pre-heated water. Similarly to bubbles forming in water in-flow tubes, the pinching of oxygen inflow tubes occasionally prevented the flow of oxygen into individual tanks, which as a result caused levels of dissolved oxygen to drop below the set limit. However, these problems with tubing were quickly rectified and the set levels of both water temperature and DO_2 were restored within 48 and 24hrs, respectively, and so the likelihood of the differences in these water parameters inducing a measurable response in the fathead minnow pairs was negligible.

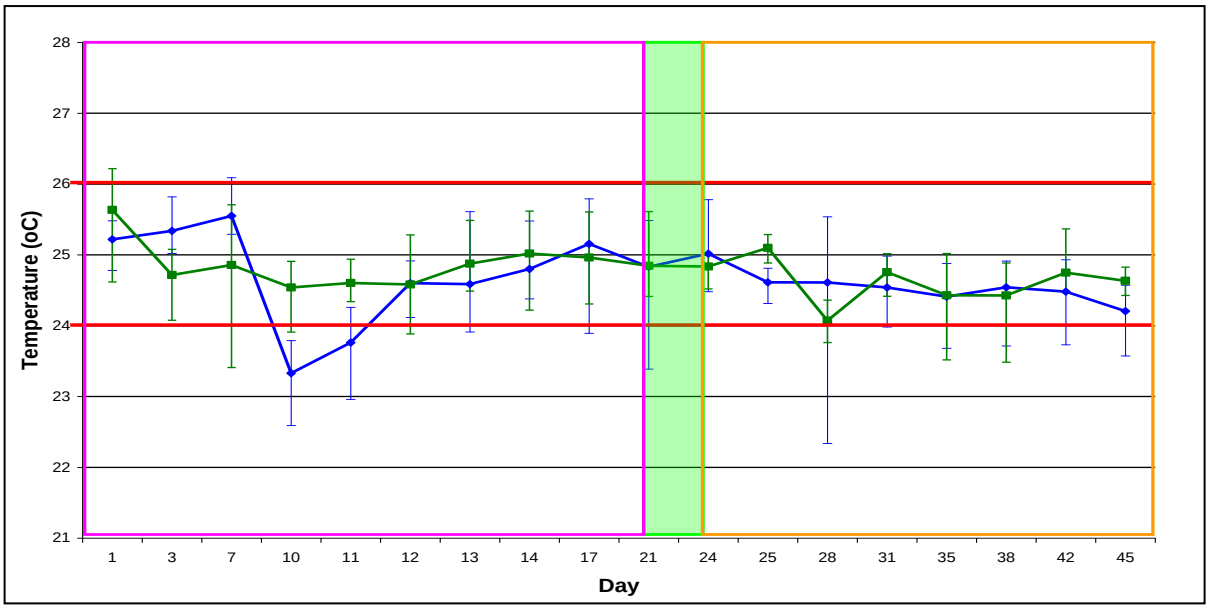


Figure 41. Water temperature in experiments 1 (blue) and 2 (green). Red lines represent temperature limits of 25°C ± 1°C. Error bars represent maximum and minimum recorded temperatures.

With the exception of days 10 and 11 where the temperature in experiment 1 dropped below 24°C as the in-house chillers were activated, water temperature both between experiments and experimental periods (e.g. baseline and exposure period) did not vary considerably.

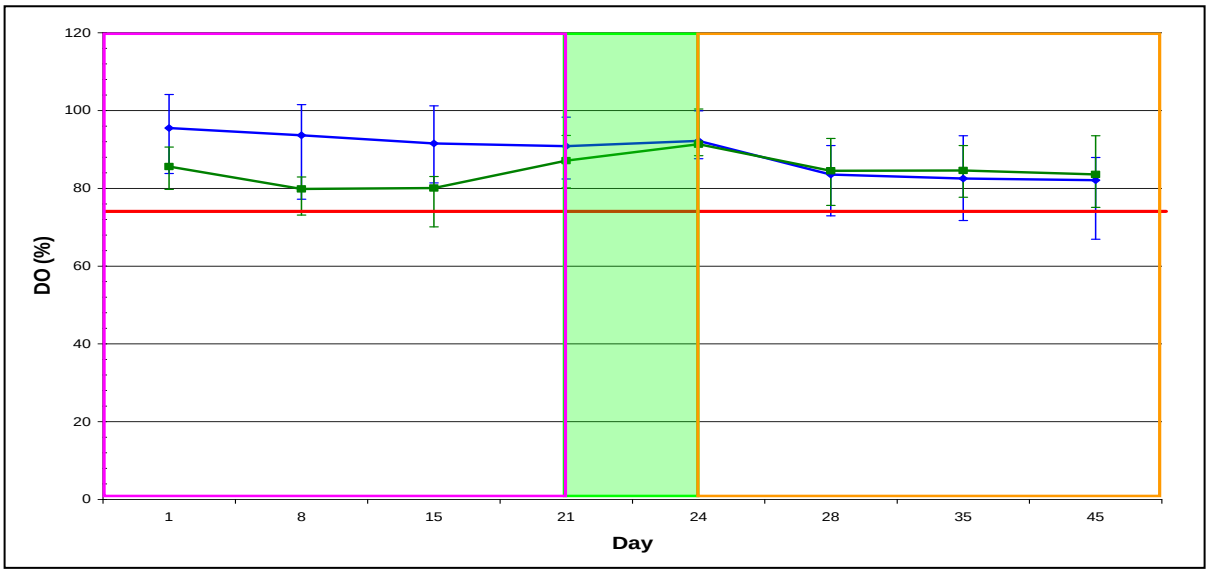


Figure 42. DO_2 in experiments 1 (blue) and 2 (green). Red line represents the limit below which oxygen levels should not fall. Error bars represent maximum and minimum recorded DO levels.

The levels of DO_2 did not vary significantly between both experiments and exposure periods. There were isolated instances where air inflows to individual tanks failed, however there was no relationship between propranolol exposure concentration and failure incidence of oxygen inflow.

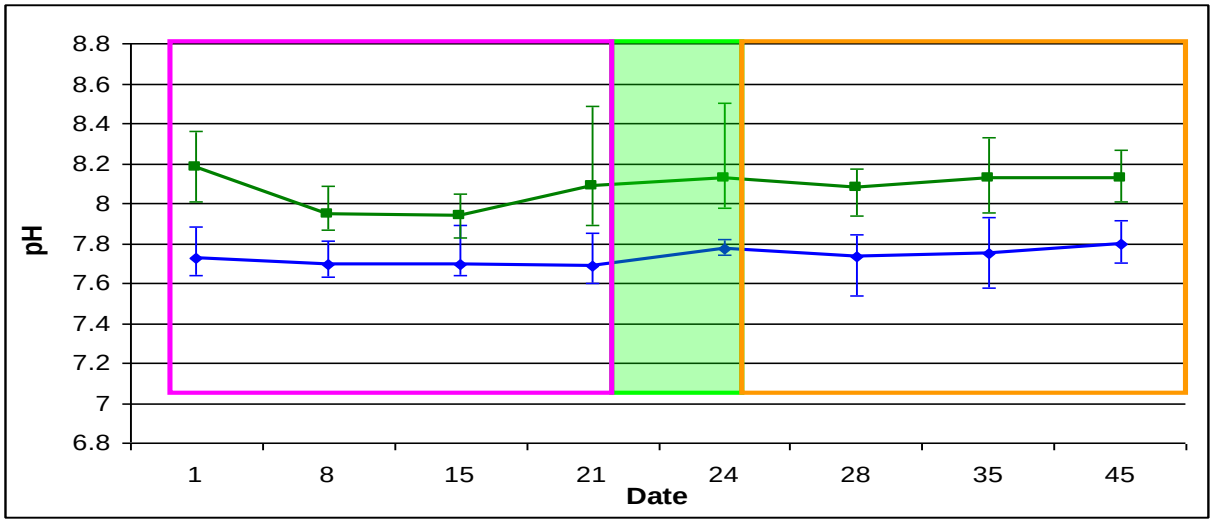


Figure 43. pH levels in experiments 1 (blue) and 2 (green). Error bars represent maximum and minimum recorded pH levels.

Although there was a notable difference between measured pH levels from exposure 1 and exposure 2, all treatment tanks were measured in the range of 7.7 to 8.2 and so were all considered to be neutral. There was no relationship between pH levels and fish response to propranolol. One can observe that in both exposures pH levels increased during the propranolol exposure period (with the exception of day in exposure 2). This was most notable in the progeny tanks, as all progeny tanks held the maximum number of steel meshes. Steel is comprised from iron, which is alkalising. Therefore it is possible that the presence of steel over a 42-day period (including the baseline period) increased the pH, albeit minimally.

The water hardness levels from both experiments ranged from 110 to 140mgL^{-1} $CaCO_3$ over both exposure periods. Water hardness levels were higher than those specified by Winter *et al* (2008), however this was due to location as $CaCO_3$ levels in water from the Thames catchment are generally higher than those from the Dorset catchment (where

Winter *et al* (2008) conducted the pair-breeding assay using the non-specific β-blocker atenolol).

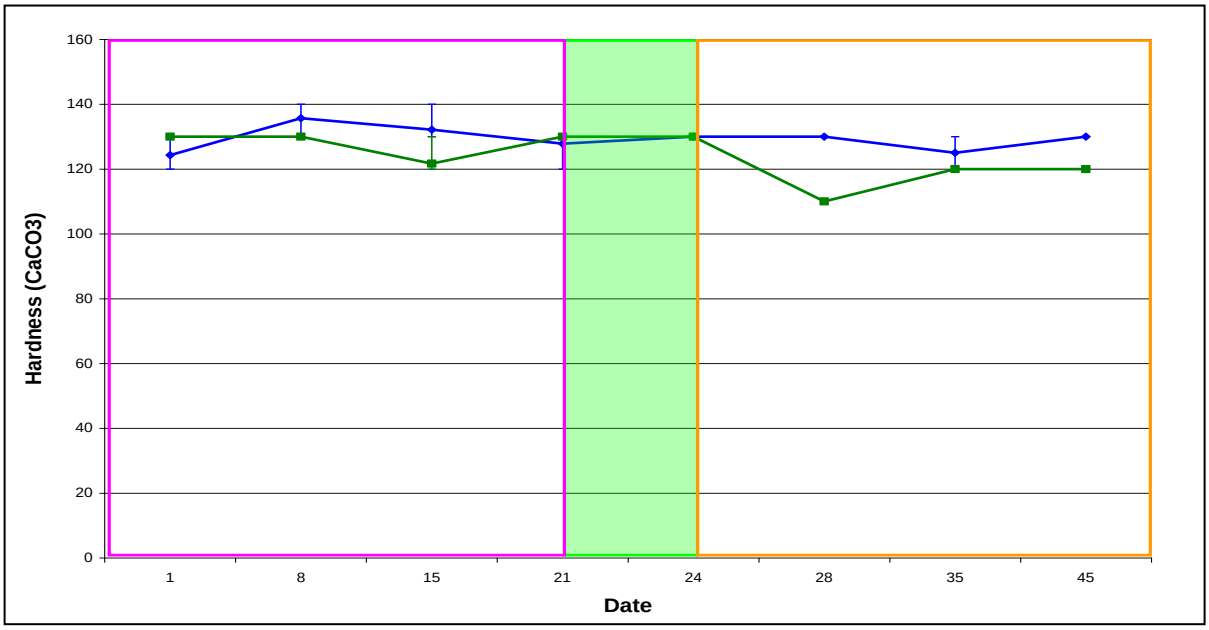


Figure 44. Levels of $CaCO_3$ in experiments 1 (blue) and 2 (green). Error bars represent maximum and minimum recorded levels.

3.3.2 Exposure concentrations

The mean propranolol levels for each concentration sampled on days 0, 4, 7, 14 and 21 of the exposure period are shown in tables 21 and 22. In total, 4 tanks out of 6 from each concentration were randomly selected and sampled. In experiment 2, there was a high level of mortality in the 1.0mgL^{-1} treatment group and by day 13 all fish from this exposure had either died or were culled for ethical reasons. Therefore, water samples were taken both on day 13 and 14; the prepared 1mgL^{-1} stock ran out after day 14. In experiment 1, all fish had died within 48 hours of receiving 10mgL^{-1} propranolol and therefore, there was only a single water sample available.

Table 21. Summary of water chemistry results from propranolol experiment 1.

Nominal Concentration (mg/L)	Mean propranolol concentration of water samples					Average result (mgL^{-1}). % of nominal concentration in brackets
	0	4	7	14	21	
DWC	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	**<0.0001 (100%)**
0.001	0.00014	0.000927	0.00063	0.000733	0.001567	**0.00084 (84%)**
0.01	0.005867	0.003933	0.004	0.004933	0.009333	**0.0053 (53%)**
0.1	0.092	0.083333	0.101	0.116667	0.128333	**0.098 (98%)**
1.0	1.1	1.133333	1.0366667	1.166667	2.133333	**1.16 (116%)**
10	3.4	-	-	-	-	**3.4 (34%)**

Table 22. Summary of water chemistry results from propranolol experiment 2.

Nominal Concentration (mg/L)	Mean propranolol concentration of water samples						Average result (mgL^{-1}). % of nominal concentration in brackets
	0	4	7	13	14	21	
DWC	-	<0.0001	<0.0001	-	<0.0001	<0.0001	**<0.0001 (100%)**
0.001	0.00074	0.0011	0.002467	-	0.000957	0.001233	**0.0013 (130%)**
0.01	0.006767	0.008867	0.010567	-	0.006633	0.007033	**0.0080 (80%)**
0.1	0.038	0.065	0.065	-	0.113333	0.106667	**0.078 (78%)**
1.0	0.476667	1.043333	0.983333	1.28	1.06	-	**0.97 (97%)**

Water chemistry results from both propranolol experiments indicate that fish were exposed to propranolol and that the drug was absent from the dilution water control tanks indicating there was no contamination. With the exception of the $10mgL^{-1}$ tanks in experiment 1, the level of propranolol in each concentration was within the expected concentration range. Solubility issues and/or an unexpected uptake rate into fish were the

most likely causes of such a low concentration of propranolol (34%) compared to the nominal concentration in the 10mgL^{-1} tanks. In all other treatments, concentrations ranged from 53 to 130% of the nominal concentration and, more importantly, there was no overlap of measured propranolol concentrations between treatments; there was at least a factor of x 6 between concentrations. Statistical analysis of the water chemistry results indicated that there were no significant differences in the mean levels of propranolol between the same concentrations from both experiments and so it was possible to pool the two sets of data together.

3.3.3 Blood plasma results

To ensure an accurate measurement could be taken, blood plasma from female fish from each treatment had to be pooled as there was very little plasma available for analysis. Blood plasma from individual male fish, however, could be analysed. The results, in table 23, highlight that there was less than 0.00025mgL^{-1} of propranolol in the dilution water control fish plasma, which was the threshold of the mass spectrometry technique used to analyse the samples. Thus this can be interpreted as there was no propranolol in the fish plasma from the dilution water control treatment. As water propranolol concentration increased, the level of propranolol in blood plasma increased accordingly. It was evident that once the concentration of propranolol exceeded 0.1mgL^{-1}, propranolol bioaccumulated in the plasma at a concentration that exceeded the environmental water concentration (exceeding 100%); this was also noted in female fathead minnows at the 0.01mgL^{-1} treatment group.

Table 23. Mean concentration of propranolol in male and female blood plasma. Figures in bold represent where plasma concentrations of propranolol exceeded water propranolol concentrations.

Nominal Concentration (mg/L)	Actual mean water concentrations from both exposures (mg/L)	Mean propranolol concentration in plasma (mgL^{-1})		Plasma concentration as percentage of mean water concentration (%)	
		Male	Female	Male	Female
DWC	<0.0001	<0.00025	<0.00025	<0.00025	<0.00025
0.001	0.00107	0.000555	0.000690	51.87	64.5
0.01	0.00665	0.00605	**0.00855**	90.98	**128.57**
0.1	0.088	**0.34**	**0.21**	**386.36**	**238.64**
1.0	1.065	**15.00**	**5.75**	**1408.45**	**539.91**

3.3.3.1 Mammalian-fish plasma model

Although the predicted $F_{SS}PC$ at $1mgL^{-1}$ was considerably higher than the mean measured $F_{SS}PC$, the predicted and measured $F_{SS}PC$ values at each concentration, as shown in table 24, were all within one order of magnitude of each other and there was no overlap in predicted levels. This suggests the model was relatively accurate. The calculated PER values were less than 1 at both the $0.1mgL^{-1}$ and $1.0mgL^{-1}$ treatment concentrations, thus predicting a pharmacological response to propranolol.

Table 24. Comparison of mean measured $F_{SS}PC$ values based on measured plasma concentrations against predicted $F_{SS}PC$ values based on the mammalian-fish plasma model by Huggett *et al* (2003b). $F_{SS}PC$ = fish stead state plasma concentration; H_TPC = human therapeutic plasma (as stipulated by Wong *et al*, 1979) NOEC; PER = Plasma Exposure Ratio.

Nominal Concentration (mg/L)	Actual mean water propranolol concentration (mg/L)	Mean measured $F_{SS}PC$ of propranolol (mg/L)		Predicted $F_{SS}PC$ of propranolol (mg/L)	H_TPC (mg/L)	PER	
		Male	Female			Male	Female
DWC[a]	<0.0001	<0.00025	<0.00025	<0.00458	0.146	>584	>584
0.001	0.00107	0.000555	0.000690	0.0595		263.06	211.59
0.01	0.00665	0.00605	**0.00855**	0.3660		24.13	17.08
0.1	0.088	**0.34**	**0.21**	3.5686		**0.43**	**0.70**
1.0	1.065	**15.00**	**5.75**	44.3784		**0.0097**	**0.025**

3.3.4 Physiological response to propranolol exposure

3.3.4.1 Fish mortality

By original design, there were four pairs of fish per treatment concentration and there was a sixth propranolol concentration of $10mgL^{-1}$. However, during the transition period in experiment 1 (when the drug was turned on and the fish were only observed and fed) all but 2 of the female fish died. The two remaining female fish showed signs of distress and an inability to orientate themselves in the tank and therefore, for ethical reasons, they were culled and their tissues collected. This suggested that at $10mgL^{-1}$, propranolol was acutely toxic to the fathead minnows. By day 5 of propranolol exposure in experiment 1, two of the males from the $1mgL^{-1}$ treatment group started to show similar signs of distress as the fish from the $10mgL^{-1}$ group and so they were culled and tissues collected. This suggested that propranolol at $1mgL^{-1}$ maybe chronically toxic to fathead minnows.

In experiment 2, the $10mgL^{-1}$ treatment was not repeated. It was noted that by day 2 the fish from the $1mgL^{-1}$ group had a reduced appetite. By day 4, two of the males were showing the same signs of distress as the two males from the $1mgL^{-1}$ treatment from experiment 1. Therefore, these males were culled and their tissues were collected. The pairing females began showing signs of distress on day 5 and so were also culled and their tissues collected. The remaining fish from the $1mgL^{-1}$ group, though showing of reduced appetite, showed no further signs of distress. However, on day 11, one male was found dead outside the tank, which was unusual as there had been no signs of distress between day 5 and day 11. The one remaining pair of fish from this treatment were culled as they started to show signs of distress. Thus, there was no cumulative egg production data from the $1mgL^{-1}$ treatment group from experiment 2 after day 11.

A further four female fish died during both experiments as they had become “egg bound”. This is where an egg becomes stuck in the female ovaries and hence the females can no longer spawn and thus swell. These incidences of egg-bound females were not related to propranolol exposure. A summary of the number of fish pairs used to collect both egg production and fish condition data is summarised in table 25.

Table 25. Summary of fish pairs used for data collection.

	Fish pairs used for egg production data				
Concentration (mg/L)	**Number of fish pairs used in experiment 1**	**Number of fish pairs used in experiment 2**	**Total number pairs**	**Total number of fish for tissue collection**	
				Male	**Female**
DWC	3	5	**8**	**9**	**8**
0.001	4	3	**7**	**8**	**8**
0.01	4	4	**8**	**8**	**8**
0.1	4	3	**7**	**8**	**7**
1	3	3 for 11 days	**6 (for 11 days)**	**4**	**6**
10	0	n/a	**0**	**0**	**0**

Since there was no egg production data collected from the $10mgL^{-1}$ group and the $10mgL^{-1}$ exposure was not repeated for experiment 2, data analysis was only conducted on treatment groups DWC to $1.0mgL^{-1}$.

3.3.4.2 Egg production

The total number of eggs produced during the exposure period was less than during the baseline period for all treatments including dilution water control, as shown in figure 45. By the time the exposure period had started each fathead minnow pair had been together for at least 6 weeks, inclusive of acclimation period, and so the spawning rate naturally slowed down. It was not possible to statistically compare cumulative egg production data between propranolol treatments as different numbers of fish pairs were used to collect the data, therefore cumulative egg production data was compared between the same treatment groups between the baseline and exposure periods. There was a statistically significant difference ($p<0.05$) in cumulative egg production between the baseline and exposure period from the $1mgL^{-1}$ treatment group.

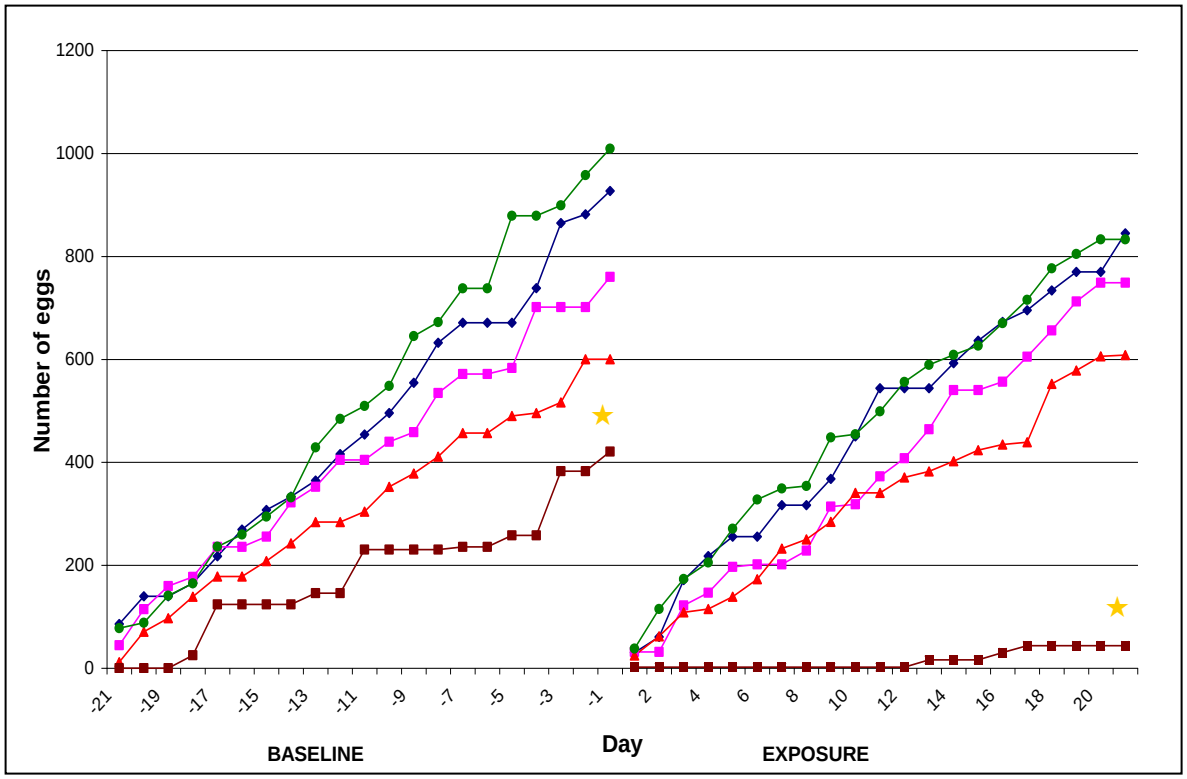


Figure 45. Pooled cumulative egg production data from each treatment group during the 21-day baseline period and 21-day exposure period. ♦ = DWC; ■ = 0.001mgL^{-1}; ◆ = 0.01mgL^{-1}; ● = 0.1mgL^{-1}; ■ = 1.0mgL^{-1}. ★ denotes statistical significant difference ($p<0.05$) between baseline and exposure periods.

3.3.4.3 Hatchability success

There was no apparent relationship between propranolol concentration and hatching success, as shown in figure 46, suggesting that propranolol did not have an affect on hatching success and initial survivability.

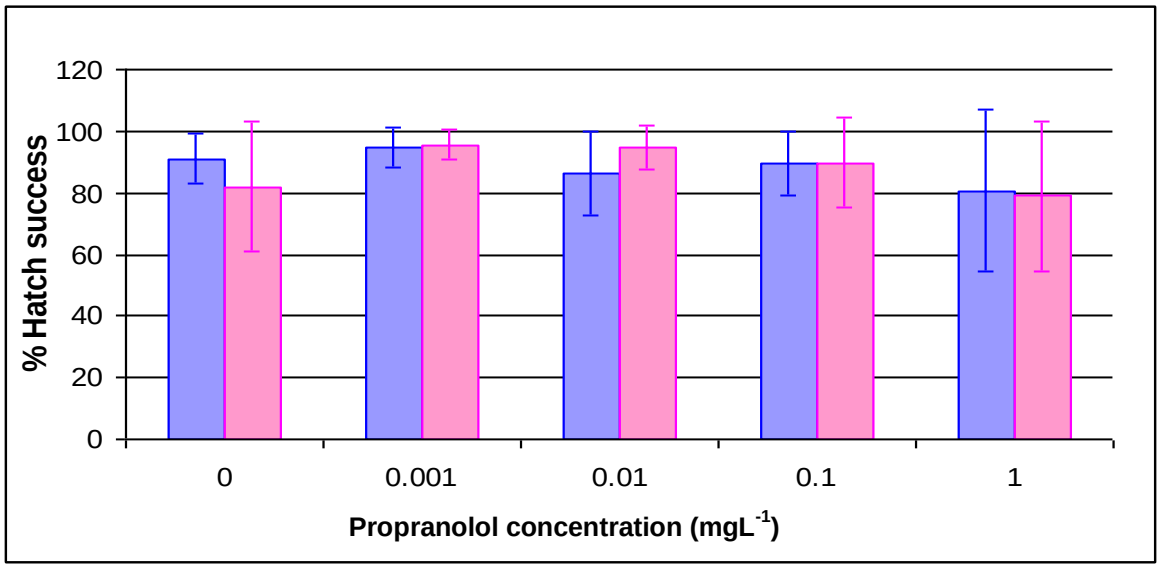


Figure 46. Mean hatching success from hatchability trials from all propranolol treatment groups. Error bars represent standard error of mean hatchability success. The blue and pinks bars represent baseline and exposure hatching success, respectivelyThere was no significant difference ($p<0.05$) between the DWC and propranolol treatment groups.

3.3.4.4 Fish condition endpoint

Figures 47 to 52 show fish weight, fork length, male fatpad weight, male tubercle number and prominence, fish GSI and condition. Wet weight ranged from 2.43 to 5.68 and from 0.81 to 2.49g in male (blue bars) and female (pink bars) fish, respectively. Male weight showed a dose-dependent decrease in weight as propranolol concentration increased and there was a statistically significant difference ($p<0.05$) in weight between the DWC and 1.0mgL^{-1} treatment groups. Female wet weight, however, did not vary considerably and there was no relationship between propranolol concentration and weight.

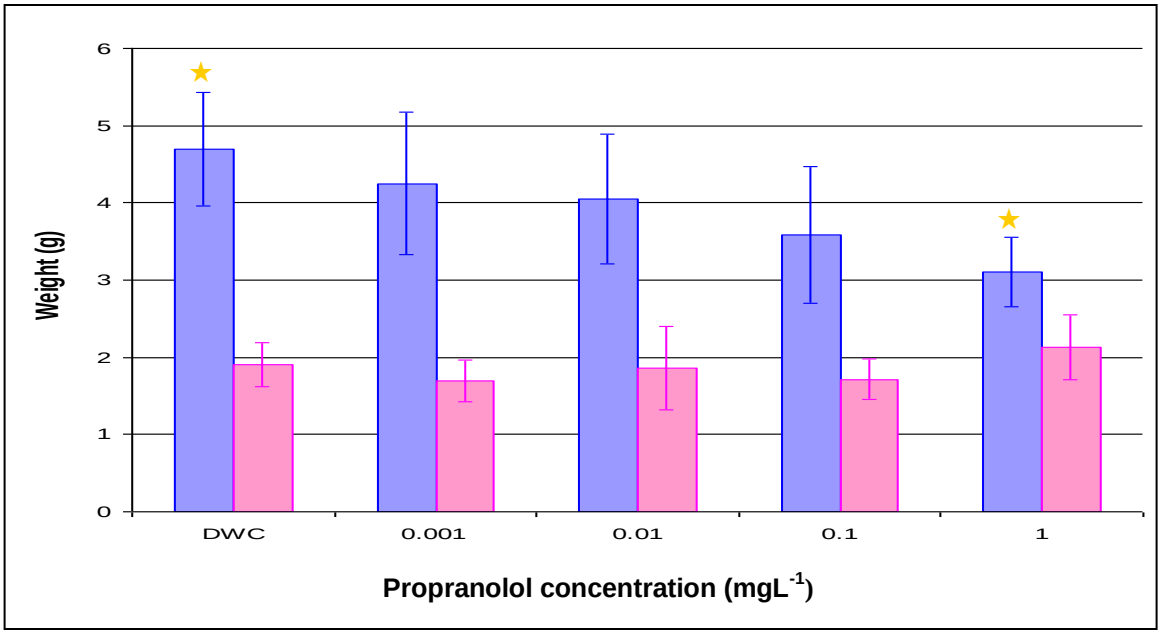


Figure 47. Wet weight of male (blue) and female (pink) fathead minnows sampled on day 21 of propranolol exposure. ★ denotes statistical significant difference ($p<0.05$) between DWC and 1.0mgL^{-1} treatment groups. Error bars represent standard error of mean weight.

Fork lengths ranged from 52 to 71 mm and 42 to 65 mm in male (blue bars) and female (pink bars) fathead minnows, respectively. There was no significant relationship between fork length and propranolol concentration for both male and female fish; there was a slight trend between fork length decrease in male fathead minnows and increase in propranolol concentration, however this was not statistically significant.

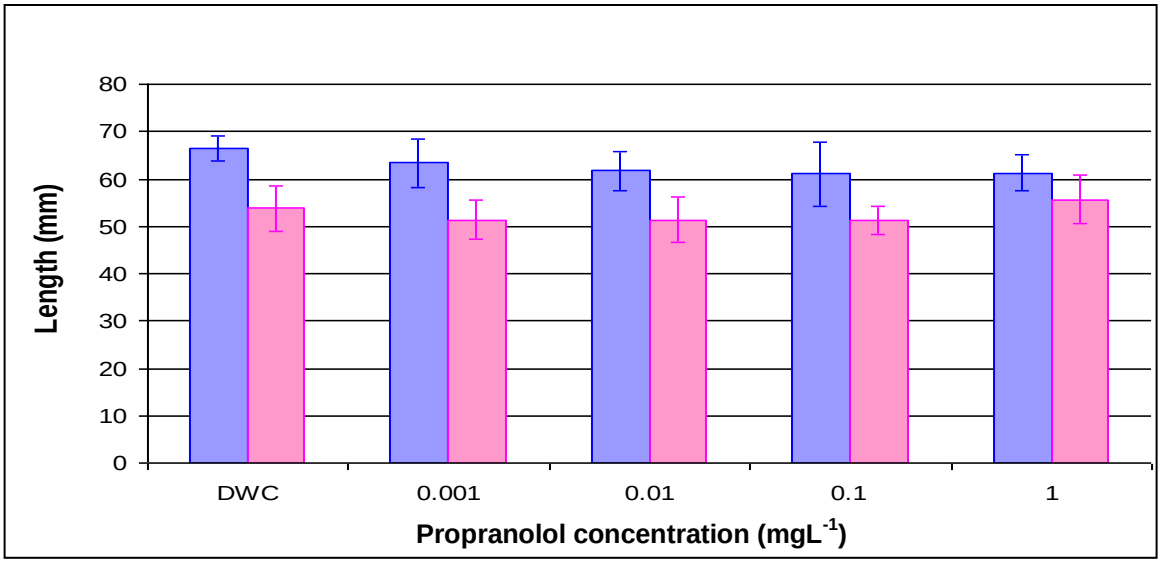


Figure 48. Fork length of male (blue) and female (pink) fathead minnows sampled on day 21 of propranolol exposure. Error bars represent standard error of mean length. There was no significant difference ($p<0.05$) between the DWC and propranolol treatment groups.

Fatpad index from 0.09 to 0.48g across all treatment groups and, once the propranolol concentration exceeded 0.01mgL^{-1}, there was an inverse relationship between fatpad index and propranolol concentration; with a statistical significant difference ($p<0.05$) being observed between 0.01 and 1.0mgL^{-1}.

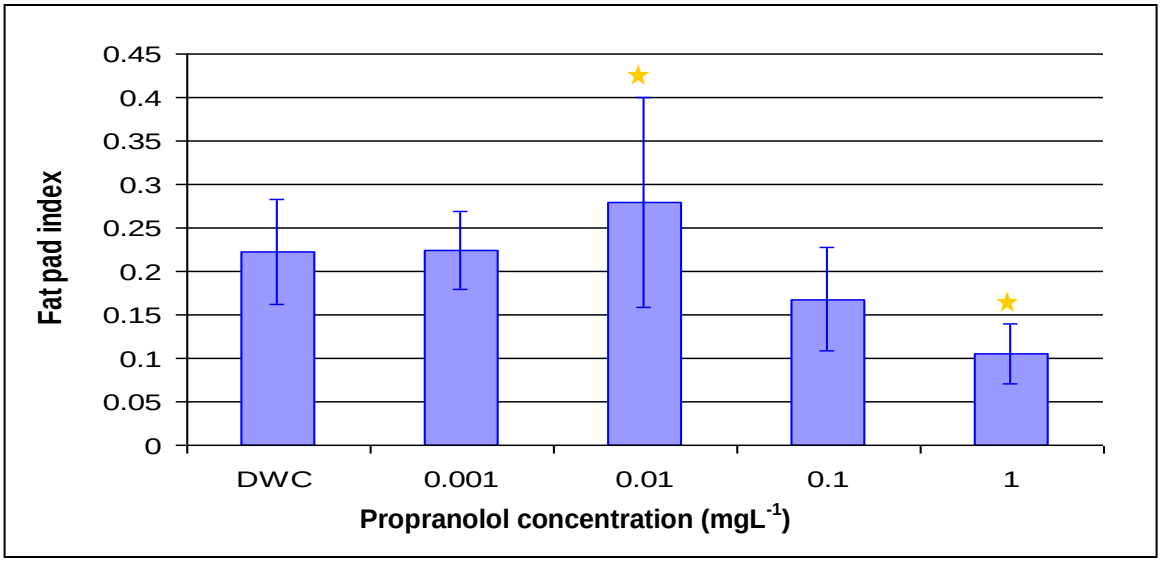


Figure 49. Fatpad index of male fathead minnows sampled on day 21 of propranolol exposure. Error bars represent standard error of mean fatpad index. ★ denotes statistical significant difference between treatment groups. There was no significant difference ($p<0.05$) between the DWC and propranolol treatment groups.

Figure 50, below, shows tubercle number (bar) and prominence (line) assessed using the qualitative scheme devised by Smith (1978). Tubercle numbers ranged from 12 to 16 and prominence score from 1 to 4. There was no relationship between propranolol concentration and tubercle number or prominence. No secondary sexual characteristics commonly associated with males were observed in any of the females.

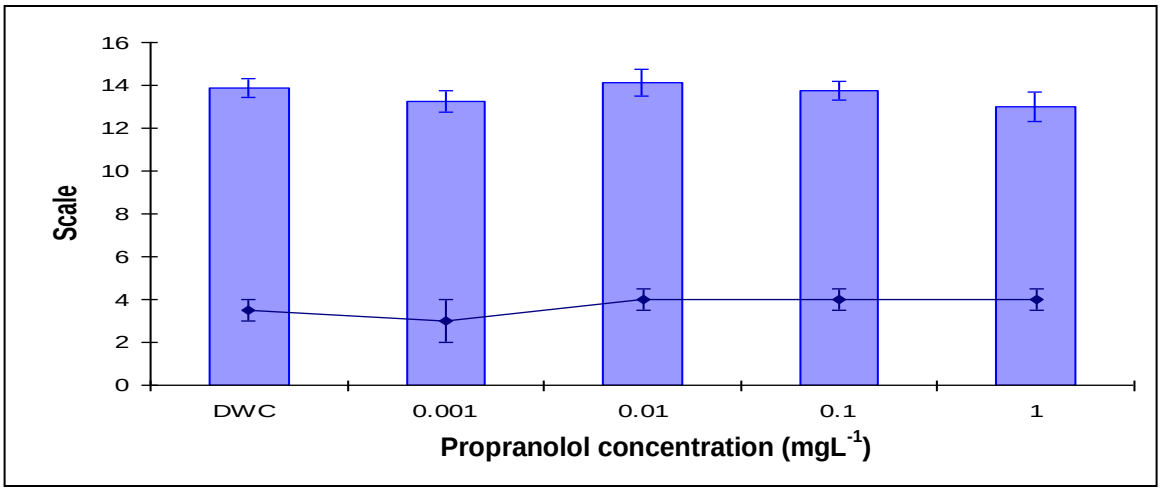


Figure 50. Tubercle number (bar) and prominence (line) of male fathead minnows sampled on day 21 of propranolol exposure. Error bars represent standard error. There was no significant difference ($p<0.05$) between the DWC and propranolol treatment groups.

Gonad somatic index expresses gonad weight as a proportion of body weight and therefore serves as an additional indicator of reproductive status. Gonad weights in males (blue bars) ranged from 0.01 to 0.074 g and GSI ranged from 0.22 to 1.39 % and in females (pink bars) ranged from 0.01 to 0.357 g and GSI ranged from 1.32 to 17.14 %. In males there was an increase in GSI as propranolol increased from DWC to 0.01mgL^{-1}, with a statistically significant difference observed between the two treatment groups, however this trend did not continue for the two higher propranolol concentration treatment groups. Female GSI exhibited a dose-related response, as propranolol concentration increased, GSI increased. Statistical significant differences ($p<0.05$) were noted between DWC and 0.1 mg/L, DWC and 1.0 mg/L, and between 0.001 and 1.0 mg/L.

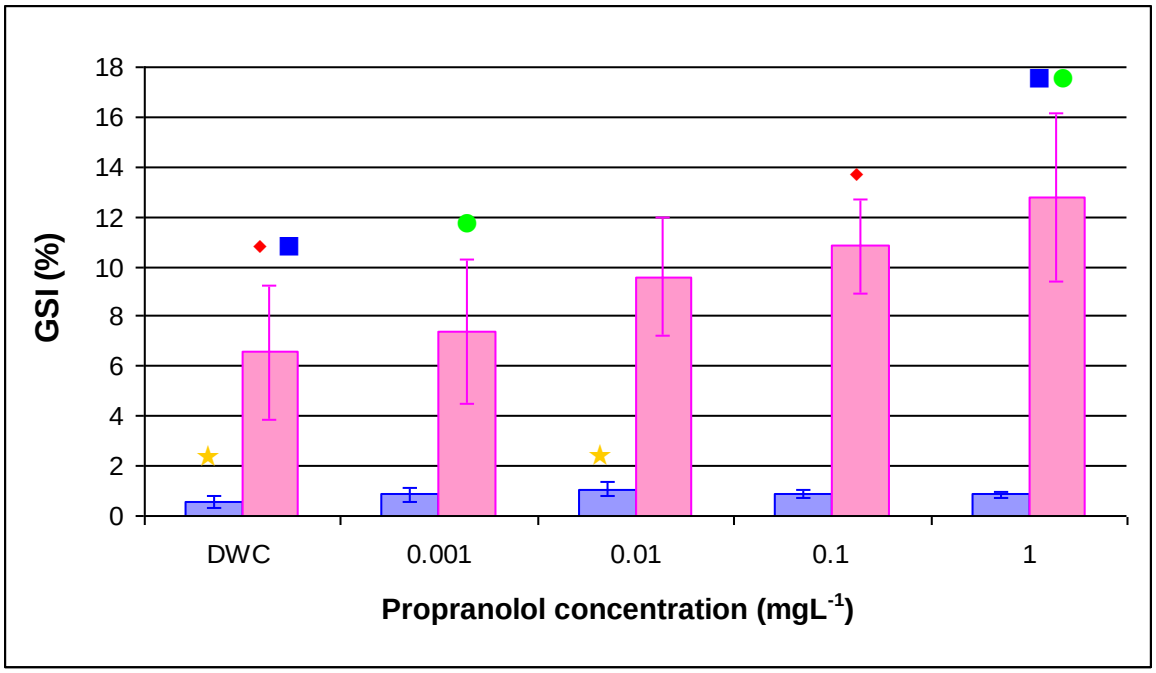


Figure 51. Male (blue bar) and female (pink bar) GSI. Error bars represent standard error of mean GSI. ★denotes statistical significant difference between DWC and 0.01mgL^{-1} in male GSI; ◆ denotes statistical significant difference between DWC and 0.1mgL^{-1} in female GSI; ■ denotes statistical significant difference between DWC and 1.0mgL^{-1} in female GSI; ● denotes statistical significant difference ($p<0.05$) between 0.001mgL^{-1} and 1.0mgL^{-1} in female GSI.

The condition index provides a useful overall assessment of general fish condition. CI ranged from 1.21 to 1.99 and 1.00 to 1.58 in male and female fish, respectively. Similarly to male fatpad index, male GSI showed an inverse relationship between propranolol concentration and index score once propranolol concentration exceeds 0.01mgL^{-1} and a statistical significant difference ($p<0.05$) was found between 0.001mgL^{-1} and 1.0mgL^{-1} and also between 0.01mgL^{-1} and 1.0mgL^{-1}, indicating male fathead minnows from the highest dose group had the poorest condition. There was no treatment-related effect on female CI.

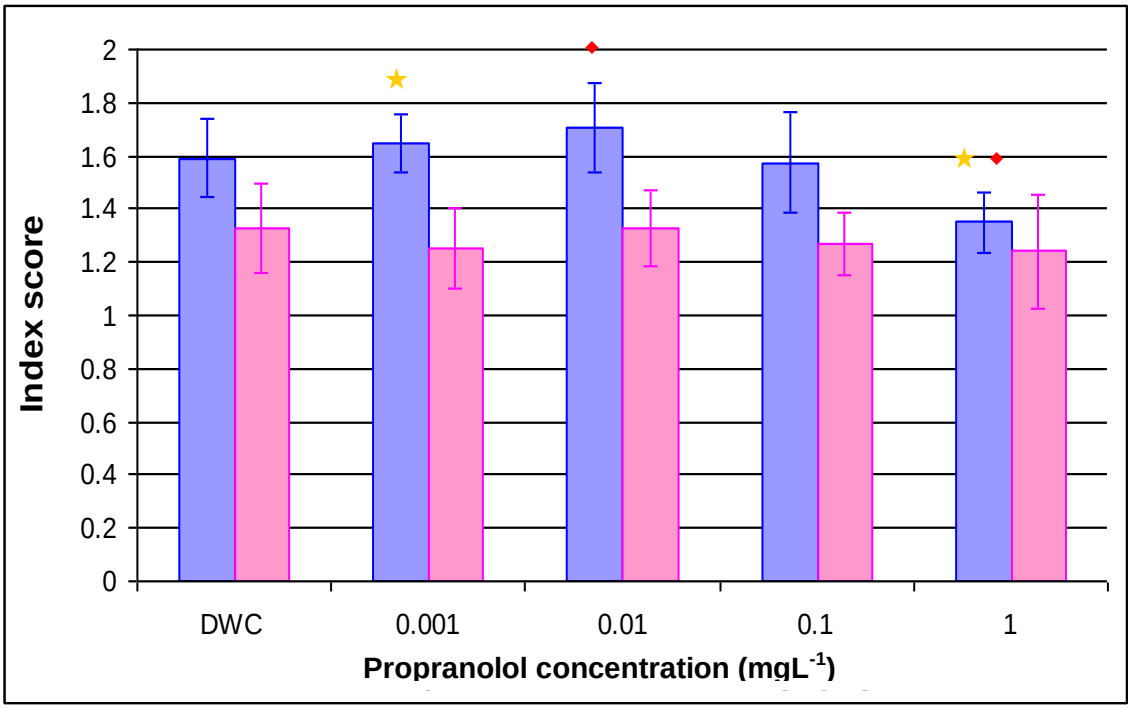


Figure 52. Male (blue bar) and female (pink bar) CI. Error bars represent standard error of mean CI. ★denotes statistical significant difference between 0.001mgL^{-1} and 1.0mgL^{-1} in male CI; ◆ denotes statistical significant difference ($p<0.05$) between 0.01mgL^{-1} and 1.0mgL^{-1} in male CI. There was no significant difference ($p<0.05$) between the DWC and propranolol treatment groups.

3.3.5 Conclusive physiological LOEC and NOEC from propranolol study

Table 26 shows the LOEC and NOEC (non-observed effect concentration) for all physiological endpoints measured during the propranolol pair-breeding assay. There must have been a significant difference between a specified concentration and the DWC for the concentration to be deemed an LOEC. Propranolol concentrations expressed as their measured nominal concentration.

Table 26. LOEC and NOEC for physiological endpoints measured during propranolol pair-breeding.

Endpoint	**LOEC nominal concentration (mg/L)**		**NOEC nominal concentration (mg/L)**	
	Male	Female	Male	Female
Survival	1.0	10.0	0.1	1.0
Egg production	-	1.0	-	0.1
Hatchability success	>1.0	>1.0	>1.0	>1.0
Wet weight	1.0	> 1.0	0.1	>1.0
Fork length	>1.0	>1.0	>1.0	>1.0
Fatpad index	>1.0	-	>1.0	-
Number of tubercles	>1.0	-	>1.0	-
Prominence of tubercles	>1.0	-	>1.0	-
GSI	>1.0	0.1	>1.0	0.01
Condition index	>1.0	>1.0	>1.0	>1.0

3.4 DISCUSSION

3.4.1 Response of fathead minnow pairs to propranolol exposure

The toxicity data obtained from the 21-day chronic exposure to propranolol study reported acute mortality for both sexes at $10mgL^{-1}$ and also male mortality at $1.0mgL^{-1}$ after 11 days. To date, this is the first study to have reported acute toxicity of any β-blocker on the fathead minnow and as such the observed affect of propranolol was unanticipated. Since the remit of the study was to examine for the chronic impacts of propranolol on reproduction in the fathead minnow, it was decided not to repeat the $10mgL^{-1}$ treatment.

Prior to euthanisation, the fish in the $10mgl^{-1}$ and $1.0mgL^{-1}$ treatments group (nominal concentration of 3.4 and $0.97mgL^{-1}$, respectively) were observed to be very disorientated and swimming on one side or nosing the bottom of the tanks, which suggested that propranolol, or possibly a metabolite derivative, may have been acting as a central nervous system (CNS) toxin. As propranolol is lipophilic, it can move across the blood-brain barrier in organisms with relative ease (Olesen *et al*, 1978). Propranolol has been found in human brain tissue at concentrations ten to twenty six times higher than in plasma, compared to atenolol which has a brain: plasma ratio of 0.2 (Cruickshank & Neil-Dwyer, 1985). Therefore, it was possible that fathead minnows exposed to $10mgL^{-1}$ and $1.0mgL^{-1}$ propranolol bioaccumulated the drug within the CNS, which induced the observed toxicological responses. The observed symptoms in the fish also mirrored those described upon fish exposure to ammonia (Arillo *et al*, 1981; Ronan *et al*, 2007). It has been well documented that drug-induced liver disease such as cirrhosis can lead to the production and accumulation of ammonia in humans, albeit the drug in question is most often alcohol (Zimmerman, 1995). The liver is the principal site of propranolol metabolism and studies have indicated that there was a non-linear relationship between dose and pharmacokinetic bioavailability of propranolol in human and rat livers and that the metabolic activity of propranolol in liver microsomes was higher than predicted from the plasma concentrations (Masubuchi *et al*, 1994; Kiriyama *et al*, 2007). The plasma concentrations of propranolol from fathead minnows exposed to $10mgL^{-1}$ and $1mgL^{-1}$ exceeded the human therapeutic plasma concentration and therefore this combination of

propranolol overdose and elevated propranolol activity in the liver microsomes may have induced liver failure, resulting in hyperammonemia and thus caused the observed severe neurological dysfunction symptoms (Wong *et al*, 1979; Ronan *et al*, 2007). Overdoses of propranolol on the CNS in humans have induced similar symptoms to those observed in the fathead minnows including convulsions, drowsiness and in severe cases, coma (Frishman *et al*, 1979; Tynan *et al*, 1981; Das & Ferries, 1988). Male fathead minnows are larger than females and therefore there is an increased likelihood of rapid bioaccumulation of propranolol, which explains why males showed symptoms of propranolol toxicity earlier than females.

Further propranolol-induced effects included a decrease in male wet weight (LOEC = 0.97mgL^{-1}; nominal concentration = 1.0mgL^{-1}) and a decrease in GSI in females (LOEC = 0.078mgL^{-1}; nominal concentration = 0.1mgL^{-1}). It is difficult to determine whether propranolol toxicity was directly responsible for the loss of weight in the males from the 1.0mgL^{-1} treatment group as the fish were not weighed prior to the study. However, in addition to showing symptoms of CNS toxicity and reduction in egg production, food accumulated in the bottom of the tanks suggesting the fish were not feeding. Egg production was severely impaired at the 10mgL^{-1} and 1mgL^{-1} treatment concentrations. However, because of the observed mortality at both concentrations, it is less likely that propranolol behaved as a reproductive toxin but rather as an acute toxin.

3.4.2 Comparison with other aquatic β-blocker studies

This toxicity data reported from this study provides a stark contrast to directly comparable data examining atenolol toxicity (Winter *et al*, 2008), where there was at least a 10-fold difference in the magnitude of response. Fathead minnows were able to tolerate high concentrations of atenolol (LOEC^{survival} = >10mgL^{-1}). The most likely reason for this difference in observation was that atenolol has a much lower log K_{ow} value than propranolol and is therefore less likely to readily bioaccumulate. Plasma concentrations of atenolol in relation to nominal water concentrations ranged from 1.8-12.2%, whereas plasma concentrations of propranolol reached up to 1550% of the nominal water concentration.

When Huggett *et al* (2002) exposed Japanese medaka (*Oryzas latipes*) to propranolol for a chronic 28-day period, egg production and egg viability were found to be significantly lower at 0.0005mgL^{-1} and 0.0001mgL^{-1}, indicating propranolol was acting as a reproductive toxin. The data from this study, however, indicated that propranolol inhibited reproduction at 1.0 mgL^{-1}, which is a 1000 to 2000-fold higher concentration. This immediately suggests that the Japanese medaka is a potentially more sensitive species to propranolol exposure. However upon further inspection, there was not a dose-response to propranolol exposure in the medaka, which was unusual considering the magnitude of difference between the control and aforementioned treatment groups. Secondly, the LOEC^{survival} values for the fathead minnow were 1.0mgL^{-1} and 10mgL^{-1} for males and females, respectively, which was much more sensitive than the recorded LC_{50} of 24.3mgL^{-1} for the medaka, indicating that the fathead minnow was more acutely sensitive to propranolol. Thirdly, the work conducted in this study was repeated and the same result trends were recorded whilst the results from the medaka exposure have not been repeated.

The pair-breeding assay primarily examined for mortality and a reproductive response to propranolol exposure. Arguably these are the most important endpoints in terms of species long-term survivability and, considering propranolol had been found to both inhibit reproduction and induce mortality in other fish species (Huggett *et al*, 2002), were particular relevant for this study.

3.4.3 Plasma concentrations of propranolol in the fathead minnow

It can be assumed that for a drug to elicit a pharmacological response in a human (target) system, the concentration of the drug must be sufficient enough to bind with the target site (e.g. receptor) in adequate numbers to trigger a measurable response. This desired concentration is most accurately reflected in the therapeutic plasma concentration. Therefore, it has been hypothesised that, assuming the species in question expresses the target site, the same concentration of drug would be required to affect another species. The mammalian-fish plasma model, proposed by Huggett *et al* (2003b) makes this

assumption and compares the predicted (or measured) plasma concentration, based on the physiochemical property of a drug, with the measured human therapeutic plasma concentration in order to predict whether the drug may induce a pharmacological effect in a different species.

The predicted $F_{SS}PC$ was within one order of magnitude to the measured $F_{SS}PC$ and, as such, was relatively accurate in predicting the plasma concentration of propranolol. In the case of propranolol inducing a pharmacological effect on the fathead minnow, the mammalian-fish plasma model was relatively accurate. The model predicted that at both $0.1mgL^{-1}$ and $1.0mgL^{-1}$ propranolol would induce a pharmacological effect. The plasma concentrations at these nominal water concentrations exceeded the water concentration dose demonstrating that propranolol was able to bioaccumulate because of its lipophilic nature. As a result female GSI was significantly lower in the $0.1mgL^{-1}$ group and male mortality was observed in the $1.0mgL^{-1}$ group. These data support the mammalian-fish plasma model and demonstrate that the read-across information from humans to fish is plausible, allowing for existing human pharmaco and toxicodynamic data coupled with the physicochemical property of a pharmaceutical to be used as a suitable starting point to predict the potential effects on organisms in the environment.

In summary, the data suggests that propranolol was not acting as a reproductive toxin but as an acute toxin. Measured environmental concentrations of propranolol ranged from $<0.000012 - 0.000255mgL^{-1}$ (approximately 1000-fold lower than the lowest pharmacological concentration in the current study) and so it is extremely unlikely that propranolol will elicit a significant toxicological effect to fathead minnows in the aquatic environment. The data from the current study supports the use of the mammalian-fish plasma model and recommends this approach for future use. Ultimately, exposure to propranolol elicited both a strong acute-toxic and lesser physiological response in the fathead minnow. As such, the study provided tissues that were suitable for both subtle biomarker analysis using metabolomics and RT-PCR, as well as robust physiological endpoints for which any results from the biomarker analysis could be related to.

CHAPTER 4: IDENTIFYING A BIOMARKER OF PROPRANOLOL EXPOSURE USING ^{1}H NMR METABOLOMICS

4.1 INTRODUCTION

Any analytical technique used for metabolomics must be quantitatively interpretable and relatively unbiased in its interpretation. Additionally, it is most desirable that the results are easily reproducible and robust and that the analytical or technical variation introduced during data collection is less than the typical biological variation in the "normal" population of interest (Keun, 2006). With this in mind, there are two principal collection techniques used in metabolomics: mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy.

4.1.1 Mass spectrometry (MS)

In brief, a mass spectrometer measures the characteristics of individual molecules by converting them into their constituent ions and manipulating them in external electric and magnetic fields. Individual ions (which represent different metabolites) are separated according to their mass-to-charge ratio and presented as signal peaks (Scoog *et al*, 1998). Because the signals are dispersed by the ion mass-to-charge ratio, considerable information is provided on the elemental composition of the metabolites. For example, the Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) has a <1 part per million mass accuracy, which allows for only one or a few elemental composition(s) to be assigned to each peak, thus aiding metabolite identification (Marshall *et al*, 1998). Furthermore, mass spectrometry is a sensitive method for detecting metabolites. Mass spectrometry is often combined with different initial sample separation techniques: the most commonly used are gas chromatography (GC), high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE).

However, compared to NMR based metabolomics, the use of mass spectrometry is still relatively new and, as such, the studies are not as reproducible and the protocols are not as well developed as for NMR metabolomics. For these reasons, NMR was adopted for the current study.

4.1.2 Nuclear magnetic resonance spectroscopy (NMR)

High resolution NMR spectroscopy is a quantitative technique that can measure hundreds of compounds (Viant, 2003). The principle of NMR is based on the behaviour of atoms placed in a strong electromagnetic field. The nuclei of some atoms possess a "spin" property. The "spinning" of these nuclei generates a magnetic moment along the axis of the spin, so that they act like tiny magnetic bars. Under normal conditions, the magnetic moments in a collection of nuclei in a metabolite sample will be randomly orientated (no net spin). However, when an external magnetic field (B_o) is applied, they can adopt different orientations with different energy levels (Engelke, 2007). Superconducting NMR magnets provide this strong, homogenous magnetic field. There are different types of NMR machines to interact with particular nuclei such as ^{1}H, ^{13}C and ^{31}P, which interact with hydrogen, carbon and phosphorus nuclei, respectively. ^{1}H-NMR, also referred to as proton-NMR, is used to examine samples for all metabolites (it is non-targeted as all metabolites have hydrogen atoms), whereas ^{13}C and ^{31}P are used more specifically to examine samples that have carbon and phosphorus atoms, respectively (Skoog *et al*, 1998).

On exposure to B_o in a proton-NMR, ^{1}H nuclei can only adopt two orientations, either aligned with (parallel) or against (anti-parallel) the direction of the external magnetic field. In a given sample, a slight majority of ^{1}H nuclei point in the direction of the magnetic field, termed bulk magnetisation or M_o. This net magnetisation will be aligned with the applied B_o, which is defined as the z-direction (M_z). In this equilibrium state, the net magnetisation vector does not produce a detectable signal. To acquire an NMR signal, a short radio-frequency radiation pulse is applied to the sample, as shown in figure 53. This causes the M_z to rotate 90^o (hence it is called a 90^o pulse) from the z-axis to the x,y plane. The resulting signal, called the free induction decay (FID) is subsequently detected, processed and then presented as a ^{1}H-NMR spectrum. An NMR spectrum provides a characteristic "fingerprint" of the proton-containing metabolites (Engelke, 2007).

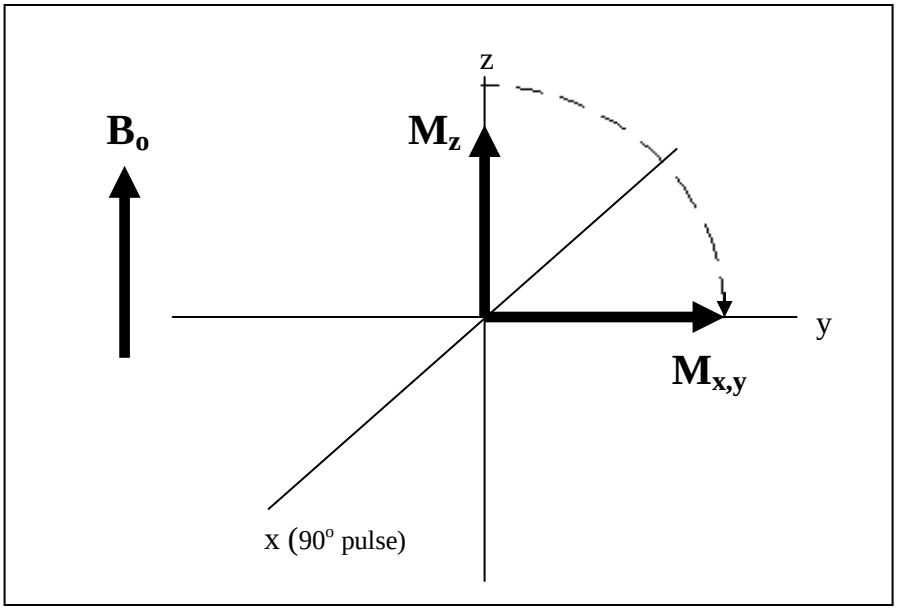


Figure 53. A short radio-frequent radiation rotates M_z to $M_{x,y}$. The resulting signal is detected and presented on a ^1H NMR spectrum.

One can differentiate between different metabolites according to their structure and chemical environment. For example, as shown in figure 54, the CH_3 moiety has three hydrogen atoms, whilst the COH moiety only has one. The hydrogen atoms belonging to the CH_3 moiety generate a stronger electron shield than the single hydrogen belonging to the COH moiety. Electron shielding is where the electrons within the hydrogen atoms shield the effect of B_o. Additionally, the oxygen atom will withdraw electron density away from the hydrogen atom and so the hydrogen atom will experience a higher level of B_o than the three hydrogen atoms. This effect is called the 'chemical shift' and causes nuclei with different chemical environments to resonate at different frequencies, which therefore provides structural information and enables the user to differentiate between metabolites when examining the produced NMR spectrum (Harris, 1986; Engelke, 2007).

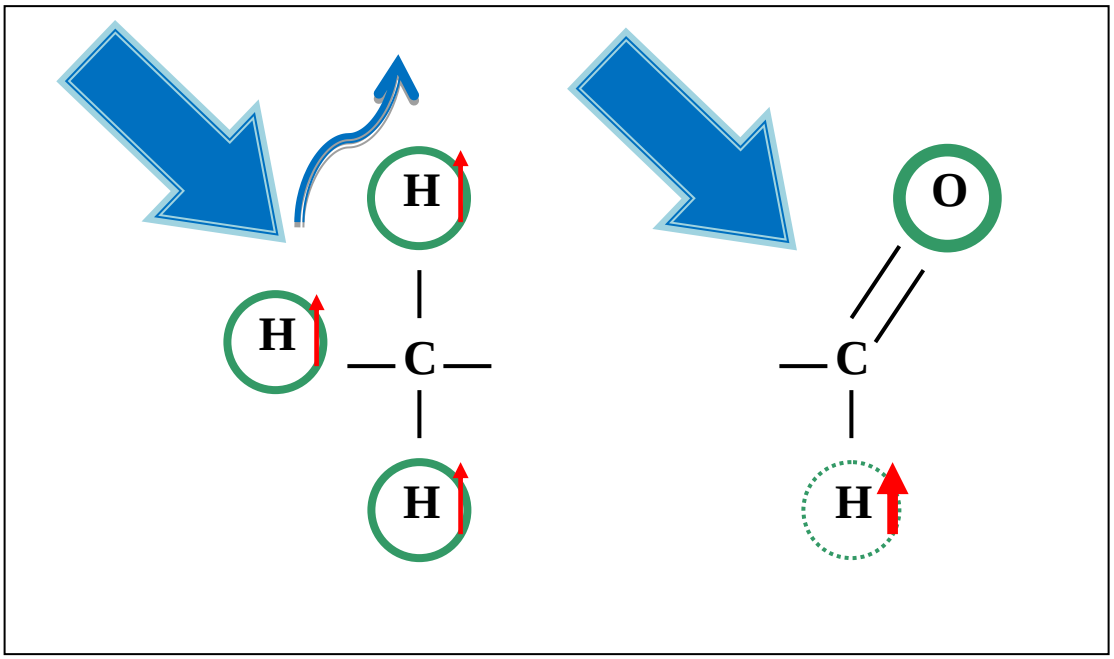


Figure 54. A simplistic schematic representation of differentiating metabolites according to their structure. The CH_3 molecule has considerable electron shielding (green circles around each H atom) from the external magnetic field (bold blue arrow) and therefore there is minimal energy change (red arrows) and loss of excitation energy (small blue arrow). The oxygen atom belonging to the COH molecule withdraws electrons away from the H atom and therefore upon excitation frequency, the hydrogen atom will experience a higher energy change (denoted by a bold red arrow).

There are two principal types of ^{1}H-NMR spectroscopy: One dimensional (1D) and two-dimensional (2D). Whilst 1D-NMR data presents the spectrum as a measure of frequency (x-axis) against intensity (y-axis), 2D-NMR data presents the data along the x and y (frequency) axes and z (intensity) axis, which provides additional information and resolution to solve overlap and problems in metabolite identification (Engelke, 2007).

4.2 The use of NMR-metabolomics in pharmaceutical-based aquatic ecotoxicity studies

To date, only two studies have examined the response of a fish, or any aquatic organism for that matter, to pharmaceutical exposure using metabolomics; both studies used ^{1}H-NMR spectroscopy and EE_2 (the principal component of the contraceptive pill) as the NMR technique and test drug, respectively. Samuelsson and colleagues (2006) exposed

rainbow trout (*Oncorhynchus mykiss*) to several concentrations of EE_2 and observed elevated levels of vitellogenin in fish plasma in the highest treatment group (10ng/L), which was supported by enzyme-linked immunosorbant assay (ELISA) results. Vitellogenin (VTG) is a protein produced in the livers of female teleost fish and EE_2-exposed male fish (Tyler & Routledge, 1998). The use of VTG as a biomarker of EE_2 exposure is well characterised and routinely used. The study by Samuelsson and colleagues, therefore, demonstrated a proof of principle that a well-known protein, and associated metabolites, can be identified using metabolomics. More recently, Ekman and colleagues (2008) examined the liver metabolite profiles of adult fathead minnows exposed to EE_2 and found that the metabolite profile of exposed male livers showed striking similarities to female livers. Furthermore, closer inspection of the exposed male liver metabolite spectra revealed that the most influential metabolites in feminization originated from increases in the amino acids glutamate and alanine. Alanine is one of the most abundant amino acids in a protein and it, along with glutamate, has been identified as a key amino acid in vitellogenin (Miles-Richardson *et al*, 1999) and so it is unlikely that glutamine and alanine were biomarkers of EE_2 exposure but are indicative of vitellogenin production.

4.3 Aim

With the exception of EE_2, metabolomics has not been used to understand the potential toxic mechanism of action and subsequent effects of pharmaceuticals on aquatic organisms. Following on from the pair-breeding assay, in which physiological endpoints were used, the aim of this study was to examine the effect of the non-specific β-blocker propranolol on the metabolite profiles of adult male fathead minnow livers and to determine whether it was possible to identify a robust and applicable biomarker of exposure.

4.2 METHODOLOGY

4.2.1 General protocol for NMR-based metabolomics

The general protocol for a NMR-based metabolomics experiment is tissue extraction, metabolite extraction, NMR data collection and finally data analysis using multivariate pattern recognition techniques and univariate statistics; and this process was applied to study the metabolic profiles of several fathead minnow (*Pimephales promelas*) tissues.

4.2.2 Assessing the use of ^{1}H-NMR-metabolomics to examine fathead minnow tissues

Prior to running fish exposure studies, it was important to firstly examine whether it was viable to use ^{1}H-NMR metabolomics to examine fathead minnow tissues (excluding urine); for example one would argue that if it was not possible to discriminate between different biological tissue samples using ^{1}H-NMR, the technique may not be suitable for this type of ecotoxicological study. Therefore the aim of the preliminary study was to analyse a range of fathead minnow tissues and assess whether ^{1}H-NMR metabolomics could differentiate between them.

Ten stock adult (20 months old) male fathead minnows were culled via an overdose of MS222 anaesthetic at 40-100 ppm in dilution water, adjusted to pH 7.5 with 1M NaOH (Fisher Scientific, Cat # J/7620/15) to obtain complete anaesthesia (Kreiberg, 2000). The heart, brain, liver and adipose tissues were rapidly extracted and frozen.

In any metabolomic study it is extremely important that the biological material should be as homogenous as possible, minimizing the level of analytical variation introduced at the sampling level, and ideally leaving the biological variation as the only inherited variation (Keun, 2006). Therefore the tissues were immediately snap-frozen in liquid nitrogen to prevent any metabolic degradation. The samples were subsequently stored at -80^{o}C until starting the extraction procedure.

4.2.3 Metabolite extraction protocol

The metabolite extraction protocol used in this study was based on the methanol/chloroform/water method adapted by Wu *et al* (2007) from that first described by Bligh and Dyer (1959) and considered to be the best in terms of reproducibility and metabolite recovery. Tissues were weighed (mg wet tissue) and the following solvent quantities determined:

Solvent quantities for polar layer: 8µL/mg (wet tissue) HPLC-grade 100% methanol (MeOH) (Fisher Scientific, Cat # A456-1); 2.5µL/mg 100% deioinised water.

Solvent quantities for non-polar layer: 8µL/mg pesticide-grade 100% chloroform ($CHCl_3$) (Fisher Scientific, Cat # C/4920/08); 4.0µL/mg 100% deionised water.

The heart tissues were considerably smaller than the other tissues and so had to be pooled together at a ratio of 3 tissues to form 1 sample. Tissue preparation was conducted in batches of eight and it was particularly important that tissue selection was randomized (i.e. in a given batch there were a mixture of tissue types).

All solvents were pre-chilled to 4^{o} C and kept on ice. Firstly methanol and deionised water were pipetted into individually labeled Precellys vials, which contained ceramic beads, according to their calculated volumes and kept on ice. Each frozen tissue was added to its' respective Precellys vial, containing the methanol-water mix and subsequently homogenised by the Precellys-24 bead-based tissue homogeniser at 6400 rpm for 2 cycles of 10 seconds, separated by a 5 second gap. Each mixture was then transferred to a 1.8ml glass vial using a glass Pasteur pipette. Calculated volumes of $CHCl_3$ and deionised water were added to their respective glass vials via a Hamilton syringe ($CHCl_3$ will cause leaching of plastics if transferred via a plastic pipette). Each solvent-tissue mixture was subsequently vortexed for 30 seconds before centrifugation at 3000rpm for 10 minutes at 4^{o}C. At this point, the mixture was bi-phasic (2 layers: polar and non-polar).

The upper, polar, layer was carefully removed using a Hamilton syringe so as not to extract any interphase layer (containing unwanted proteins and lipoproteins) and transferred into a clean 1.8ml glass vial. All vials were subsequently dried at room temperature using a Thermo Electron RVT4104 Refrigeration Vapor Trap speed vacuum concentrator. The dried pellets were subsequently stored at -80^{o}C.

4.2.4 1D ^{1}H and 2D ^{1}H J-resolved NMR spectroscopy

The dried polar extracts were re-suspended in 640µL sodium phosphate buffer in 90% H_2O and 10% D_2O (100mM, pH 7.0) containing sodium 3-trimethylsilyl-1-2,2,3,3-d_4-propionate (TMSP), which served as an internal chemical shift standard. D_2O served as the template signal for locking the magnetic radiofrequency onto a sample. Each glass vial was vortexed for 10 seconds to ensure the pellet was displaced before centrifugation at 12,000rpm for 10 minutes at room temperature. 600µL of the re-suspended sample was subsequently transferred to a clean NMR tube. All NMR spectra were measured at 500.18 MHz using an advanced DRX-500 spectrometer (Bruker, Coventry, UK) equipped with a cryoprobe. One-dimensional ^{1}H NMR spectra were collected at a temperature of 300 K using excitation sculpting to suppress the water resonance (Hwang & Shaka, 1995). Spectra were obtained using a 8.4-µs (60^{o}) pulse, 6-kHz spectral width and 2.5 second relaxation delay with 80 transients collected per sample, requiring a total acquisition time of 6.5 minutes. Datasets were zero-filled to 32k points and exponential line-broadenings of 0.5Hz applied prior to Fourier transformation. All spectra were phased, manually baseline corrected using a quadratic function and calibrated (TMSP at 0.0ppm) using TopSpin (version 1.3, Bruker).

A limitation of using 1D ^{1}H-NMR is spectral congestion which limits the number of metabolites that can be uniquely identified and quantified. For this reason 2D ^{1}H-predicted J-resolved (JRES) NMR spectra were also collected. A JRES spectrum separates the chemical shift and spin-spin coupling data onto different axis (termed F2 and F1, respectively) thereby increasing proton signal dispersion and producing a less congested spectrum, thus aiding in biomarker identification (Viant, 2003). Spectra were obtained using a 90^{o} pulse; 16 transients per increment for 16 increments were collected

into 16k data points, with spectral widths of 6kHz in F2 and 50Hz in F1. A 4.0 second relaxation delay was employed, requiring a total acquisition time of 25 minutes per sample. Datasets were zero-filled in F1 and both dimensions multiplied by sine-bell window functions prior to Fourier transformation. JRES spectra were tilted by 45^{o}, symmetrized about F1 and then calibrated, all using TopSpin (Bruker). Data were finally exported as the 1D skyline projections (along F2) of the JRES spectra (termed pJRES) (Parsons *et al*, 2009).

4.2.5 Spectral pre-processing

Both 1D and 2D pJRES ^{1}H NMR data were converted to an appropriate format for multivariate analysis using custom-written *ProMetab* software in Matlab (version 7.1; The MathsWorks, Natick, MA, USA) (Viant, 2003). A single NMR data set can contain thousands of data points, which is too large to efficiently analyse and so a 'binning technique' was used. In this approach, the predictors within a window of a defined width in ppm were replaced by a single value corresponding to the sum of the intensities in the window. This meant that tens of thousands of data points were conveniently reduced into approximately two thousand and yet the overall character of each spectrum was not affected (Purohit *et al*, 2004). For this study, each spectrum was segmented into 0.005 ppm (2.5Hz) chemical shift 'bins' between 0.6-10.0ppm with residual water bins excluded from all spectra. The total area of each spectrum (referred to as total spectrum area; TSA) was normalized to one which allowed for comparisons to be made between different spectra despite differences in original sample size and metabolite quantity. Additionally, bins containing pH sensitive peaks were compressed into a single bin. The binned data describing each spectrum were then compiled into a matrix, with each column containing binned NMR data and each row representing individual samples. The data was finally generalized log-transformed which tended to equalize the weightings of smaller and larger peaks. Spectral data were mean centered before multivariate statistical analysis (Viant, 2003; Purohit *et al*, 2004; Parsons *et al*, 2009).

4.2.6 Processing of data using multivariate statistical analysis

To analyse both the 1D and 2D pJRES ^{1}H NMR data, principal component analysis (PCA) was employed using PLS_Toolbox (version 3.53, Eigenvector Research, Manson, WA, USA). PCA is termed a "non-supervised" technique because no *a priori* knowledge (i.e. class) of the samples is required; it effectively reduces a great number of correlated variables into a smaller number of uncorrelated variables, called principal components (Nicholson *et al*, 2002). The first principal component explains the greatest variability in the data; the second principal component is orthogonal to the first component and is second best at explaining the variability of the data and so on. Each sample was plotted on a coordinate space, called a 'scores plot', and samples of a similar metabolic nature clustered together. A corresponding 'loadings plot' was then used to assess which variables were most responsible for the positioning/clustering of a sample/s. A PCA model can be used to quickly visualize which samples in a data set are similar or dissimilar to each other and which variables, ultimately corresponding to biomarkers, were the route causes of any separation (Keun, 2006).

Similarly to PCA, partial least squares discriminate analysis (PLSDA) is a multivariate analytical technique used to classify samples. PLSDA is performed to enhance the potential separation between groups of samples by rotating PCA components such that maximum separation between sample groups is achieved. Unlike PCA, PLSDA is a 'supervised' technique, whereby sample information (i.e. which treatment group/tissue type each sample belongs to) is input into the algorithm, thus enhancing the likelihood of achieving separation of sample groups. To evaluate a PLSDA model, a root mean square error of cross-validation (RMSECV) prediction value for each sample group ranging from zero to one is given. A value closer to zero indicates that a sample has been accurately assigned to a modeled group, whereas a value closer to one indicates that that a sample has been inaccurately assigned to a modeled group (Westerhuis *et al*, 2008).

4.2.7 Metabolic analysis of liver tissues extracted from propranolol exposed fish

One limitation of NMR is that tissues must exceed 30mg wet weight to be effectively analysed using both 1D and 2D pJRES spectrometry; for the preliminary assessment of

metabolomics as a suitable tool for analysis using the fathead minnow the hearts and brains were pooled together. Therefore the liver tissue was selected for NMR analysis; additionally the liver is also the primary site of propranolol metabolism (Huggett *et al*, 2003). The protocols for tissue preparation and metabolite extraction, 1-D ^{1}H and 2-D ^{1}H pJRES NMR spectroscopy and both spectral pre- and post-processing (e.g. PCA) used to assess the suitability of ^{1}H-NMR metabolomics to examine fathead minnow tissues were adopted for this study. Additionally relative standard deviation analysis was adopted to assess the quality of the data (Parsons *et al* 2009).

4.2.8 Data processing – calculating Relative Standard Deviation (RSD)

Relative Standard Deviation (RSD), also referred to as the coefficient of variance (CV), is a statistic that describes the level of variation within and between data sets. This is vital in metabolomics as one is ultimately trying to identify which variables are changing significantly under different conditions and without this understanding data can be, at worst, falsely interpreted (Keun *et al*, 2002; Teahan *et al*, 2006).

To date, RSD has been predominantly used to establishing the stability of metabolite peaks and consequently used as an indicator of the strength of the results (Keun *et al*, 2002; Teahan *et al*, 2006; Parsons *et al*, 2009). In this study, however, RSD analysis was used to examine the variability of tissue metabolite profiles within and between treatment groups and to assess the quality of the data.

During exposure to propranolol, a number of male fish from all concentrations, including DWC, were culled prior to the 21-day termination date. One reason for this was that males in the highest concentration of 1.0mgL^{-1} showed signs of distress and, to abide with strict ethical guidelines, were culled. Another reason was that some female fish became “egg-bound”, which is where eggs get caught and therefore the female fish can no longer spawn yet they continue to produce eggs and so the females swell and become distressed and eventually die. There was no relationship between propranolol concentration and incidence of egg-bound females. In these cases both the females and males were culled as the pair-breeding assay could not continue. In addition to the liver

tissues collected on day 21 of the propranolol exposure, the dissected liver tissues from pre-21-day culled fish also underwent the metabolite extraction protocol. Therefore, by measuring the level of variation of liver metabolite profiles with and without the pre-21-day culled tissues, RSD analysis was used to determine whether the pre-21-day extracted livers could be included in the data set. RSD analysis was conducted on both 1D and 2D pJRES data and also used to compare the liver metabolite profile variation between the different treatment groups. It should be noted that, unlike data prepared for PCA, data used for RSD analysis was not log transformed.

The RSD protocol was adopted from that described by Parsons *et al* (2009). Before the data could be analysed using RSD, bins containing solely "noise" were removed from each NMR spectra. Noise refers to "signals" that are below a pre-defined signal-to-noise limit. They often give an artificially high amount of variation thereby heavily distorting the RSD distribution. To filter out this noise, the intensity of each bin in both the 1D and 2D pJRES ^{1}H NMR data was examined. Each spectrum was divided into 32 sections and the standard deviation of signal intensity for each section calculated. The noise threshold for the spectrum was then estimated as 3 times the smallest standard deviation of the 32 sections. If the bin intensity was less than the corresponding noise threshold for each spectrum in the data set, the bin was deemed as "noise" and was removed. Only bins that had a signal greater than the noise threshold were included for RSD analysis. Finally, RSD was calculated for each bin (point) in the filtered spectrum as shown in figure 55.

$$\textbf{RSD} = \frac{\text{standard deviation of signal intensity}}{\text{mean signal intensity}} \times 100 = \%$$

Figure 55. RSD calculation (according to Parsons *et al*, 2009).

The distributions of the RSD were then compared using the Wilcoxon rank sum test for comparing two distributions (i.e. pre- and post-21-day exposure data) and the Krustal-Wallis test for multiple distributions (i.e. comparing treatment groups) to establish if the RSD distributions differed significantly at $p<0.05$ (using the Matlab statistics toolbox).

4.2.9 Identification of biomarker metabolites using univariate statistics followed by False Discovery Rate (FDR)

Both 1D and 2D pJRES ^{1}H NMR data were analysed by one-way Analysis of Variance (ANOVA) using the Statistics Toolbox within Matlab (with treatment group as the factor; $p = 0.05$). To ensure a conservative assessment of significant bins was made, false discovery rate (FDR) was subsequently applied to the ANOVA output-data. Unlike the more commonly used Bonferroni technique that controls for the chance of any false positives (the proportion of inaccurately rejected null hypotheses), FDR controls for the expected proportion of false positives among a threshold, which is determined by the size of the observed p-value distribution, and hence is adaptive to the amount of signal in a data set (Nichols and Hayasaka, 2003). Therefore for a large and complex data set such as NMR data, the Bonferroni technique, which uses a fixed estimated error rate, is too conservative and prohibitive whilst FDR offers a more powerful statistical tool (Benjamini & Hochberg, 1995; Garcia, 2003). Through the use of ANOVA followed by FDR, bins whose intensities changed significantly between treatment groups were identified.

Bins that were identified as "significantly different" by ANOVA and FDR were then identified as peaks on non-generalized log-transformed and non-normalized 1D ^{1}H NMR spectra and quantified (measuring area under each peak, representing intensity levels) using Chenomx NMR Suite and associated libraries (version 4.5; Chenomx Inc, Edmonton, Canada). Intensity levels were divided by the TSA for each spectrum to ensure comparisons were accurately made between different spectra and treatment groups. Finally, average levels of metabolites were compared between different treatment groups as percentages (Ekman *et al*, 2007).

4.2.10 Examining metabolic variation in fathead minnow livers

4.2.10.1 Experimental design

Metabolomic analysis of adult fathead minnow livers from the propranolol study revealed considerable variation between fish within individual treatment groups. Therefore the aim of the experiment was to address potential sources of this variation.

During the propranolol exposure, fish were not starved prior to culling and so on the day of termination, it took several hours to cull all the fish and so it was possible that fish would have fed for different lengths of time depending on when they were culled. The liver is a principal site of food metabolism in the teleost body and, as such, a variation in diet and feeding regimes has a considerable influence on the metabolic activity of the liver (Cowley & Sargent, 1979; Sánchez-Muros *et al*, 1998; Pérez-Jiménez *et al*, 2007 Enes *et al*, 2008). It is therefore likely that unregulated feeding in the propranolol study could have been a source of intra-fish variation.

Another source of variation could be the result of "tank effects". Each tank held only a single pair of fish, which in terms of metabolomic analysis (examining males only) effectively means a tank only held a single fish. It was also likely that tank position within the room could be a source of variation. For example fish in the tank closest to the door may have experienced more stress than fish in tanks further away. In order to ensure that the room position had no bearing on the results for different propranolol exposures, the tank positions were randomized.

Therefore, the aim of the experiment was two-fold: the first was to assess the effect of feeding on the metabolic profile of adult male fathead minnow livers and the second was to examine whether tank effects have any effect on the metabolic profile. This was achieved by setting up four tanks (working volume of 20L) each of which received dilution water (400ml min^{-1}) only. Mature adult males, aged 26 months, were distributed into each tank (n = 16) and were fed twice per day for 6 days; on the 6^{th} day, fish were terminated by an overdose of MS222 anaesthetic, measured for wet weight (g) and

standard length (mm), and livers extracted for metabolomic analysis. Two of the tanks were fasted for 24 hours prior to termination whilst two of the tanks were fed for all 6 days. Thus it was possible to examine for both metabolite differences of fish livers between feeding treatments and also between replicate tanks ("tank effects").

MS222 anaesthetic does not degrade rapidly in dilution water (Yuwiler & Samuel, 1974) and so it is common practice in ecotoxicological studies to prepare a "stock" of MS222 and use the same stock throughout the culling procedure for entire fish experiments. However, MS222 is taken up via the gills and so as fish number increases, MS222 concentration decreases due to removal, thereby increasing the time taken for fish anaesthetisation and consequently the stress a fish is likely to endure increases. Previous studies have suggested that stress can have a considerable effect on the metabolic activity of tissues (Morales *et al*, 1990; Kolanczyk *et al*, 2003) and so in the propranolol study, where a batch stock of MS222 was prepared, individual fish could have experienced varying levels of stress due to the time taken for complete anaesthetisation and so this could have been a source of variation. With this in mind, individual MS222 stocks were prepared for the feeding study. A concentrated stock of MS222 was prepared and then aliquoted into individual beakers for each fish; dilution water was then added to each beaker to achieve a concentration of $0.5gL^{-1}$ (pH adjusted to 7.5). By preparing individual stocks of MS222, each fish was exposed to the same concentration of anaesthetic and therefore time taken for complete anaesthetisation was comparable between fish and as such stress endured by each fish should also be comparable.

Metabolite extraction, 1D ^{1}H NMR spectroscopy, spectral pre- and post-processing of data, RSD calculation and biomarker analysis protocols were followed as previously described.

4.2.11 Analysis of data using Linear Discriminate Analysis (LDA)

After analyzing the data using PCA, the "supervised" multivariate technique Fisher's LDA was applied to principal components 1 and 3 using Matlab version 7.1. LDA examines for linear combinations of variables so that the ratio of inter-group variation to

intra-group variation is maximized, thereby maximizing the separation of "decision regions" (or treatment groups) in the scores plot (Hines *et al*, 2007). Similarly to PLSDA, LDA was a "supervised" technique as the software was given information as to which treatment group each sample belonged to, unlike the "non-supervised" PCA technique, where no additional information of the data set was given.

4.3 RESULTS

4.3.1 Preliminary metabolomic assessment

Prior to assessing the effects of propranolol on the metabolic profile of male fathead minnow livers, ^{1}H NMR metabolomics was employed to examine different fathead minnow tissues. As different tissues have very different metabolic profiles, this was a proof-of-principle assessment to determine whether metabolomics could be used to differentiate different tissues. As shown in figure 56, it was possible to separate fathead minnow according to tissue type on a PCA scores plots using 2D pJRES ^{1}H NMR. This suggested that ^{1}H NMR metabolomics could potentially be a viable technique for assessing the effects of propranolol exposure on the fathead minnow, where the metabolomic nature of a single tissue may vary according to the concentration of propranolol exposure. Fathead minnow adopise tissues had the greatest metabolic variation, as can be seen by the large ellipse and an outlying sample, whilst all other tissues fitted within the designated two standard deviation of the mean ellipses.

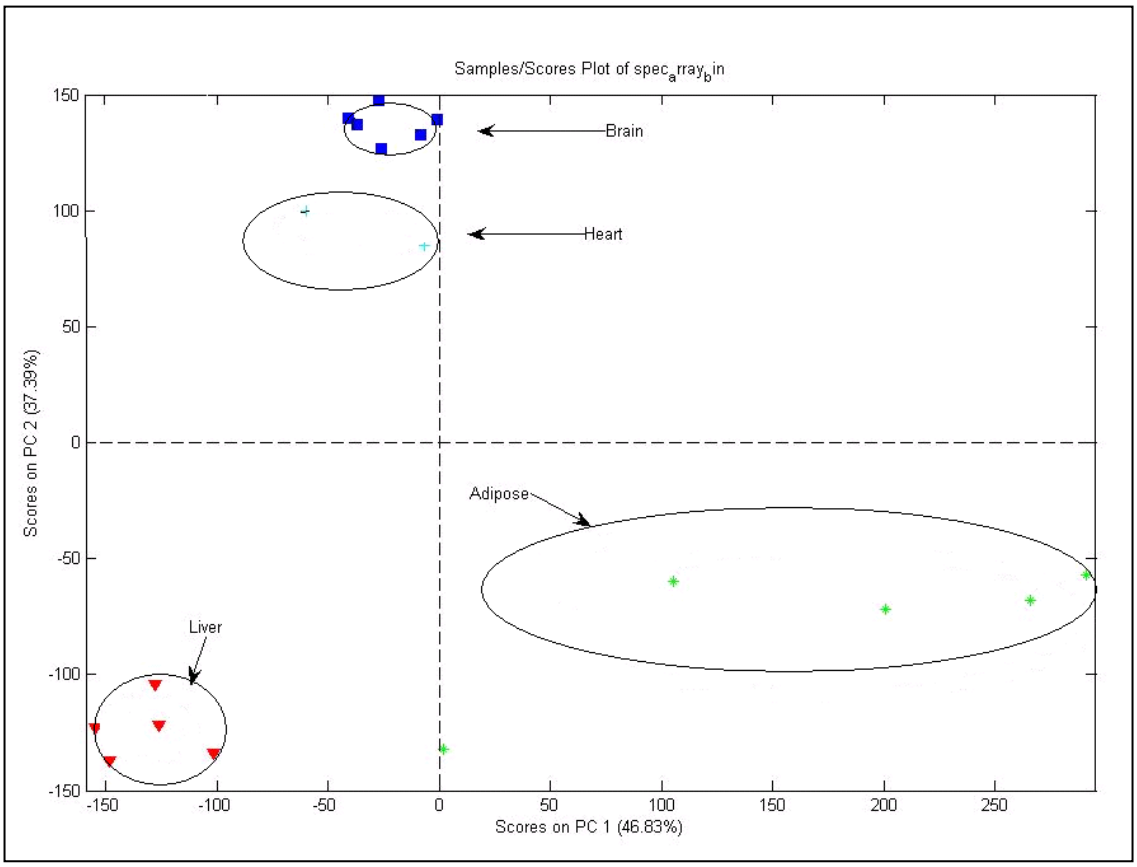


Figure 56. PCA scores plot of ^{1}H NMR metabolomics data showing adipose, brain, heart and liver plots. Ellipses drawn at two standard deviations (SD).

4.3.2 Propranolol exposure metabolomic results

4.3.2.1 Liver tissues used for metabolomic analysis

As discussed in chapter 3 section 3.4.1, 10mgL^{-1} propranolol was acutely toxic to male fathead minnows and therefore no liver data from this treatment group was available for analysis. Additionally several male liver tissues were extracted before the end of the 21-day exposure period (termed pre 21-day liver data) from several treatment groups. Livers were collected from these fish and prepared for metabolomic analysis. The minimum amount of liver for a ^{1}H NMR is 30mg and profiles produced by tissues under this size had a significant effect on the overall treatment metabolomic profiles and for this reason all metabolomic profiles from tissues under 30mg were excluded from both 1D and 2D pJRES data analysis. Table 27 summarises the number of suitable tissues used for metabolomic analysis.

Table 27. Summary of number of male liver tissues extracted for metabolomic analysis. Tissues that were below 30mg were excluded from analysis.

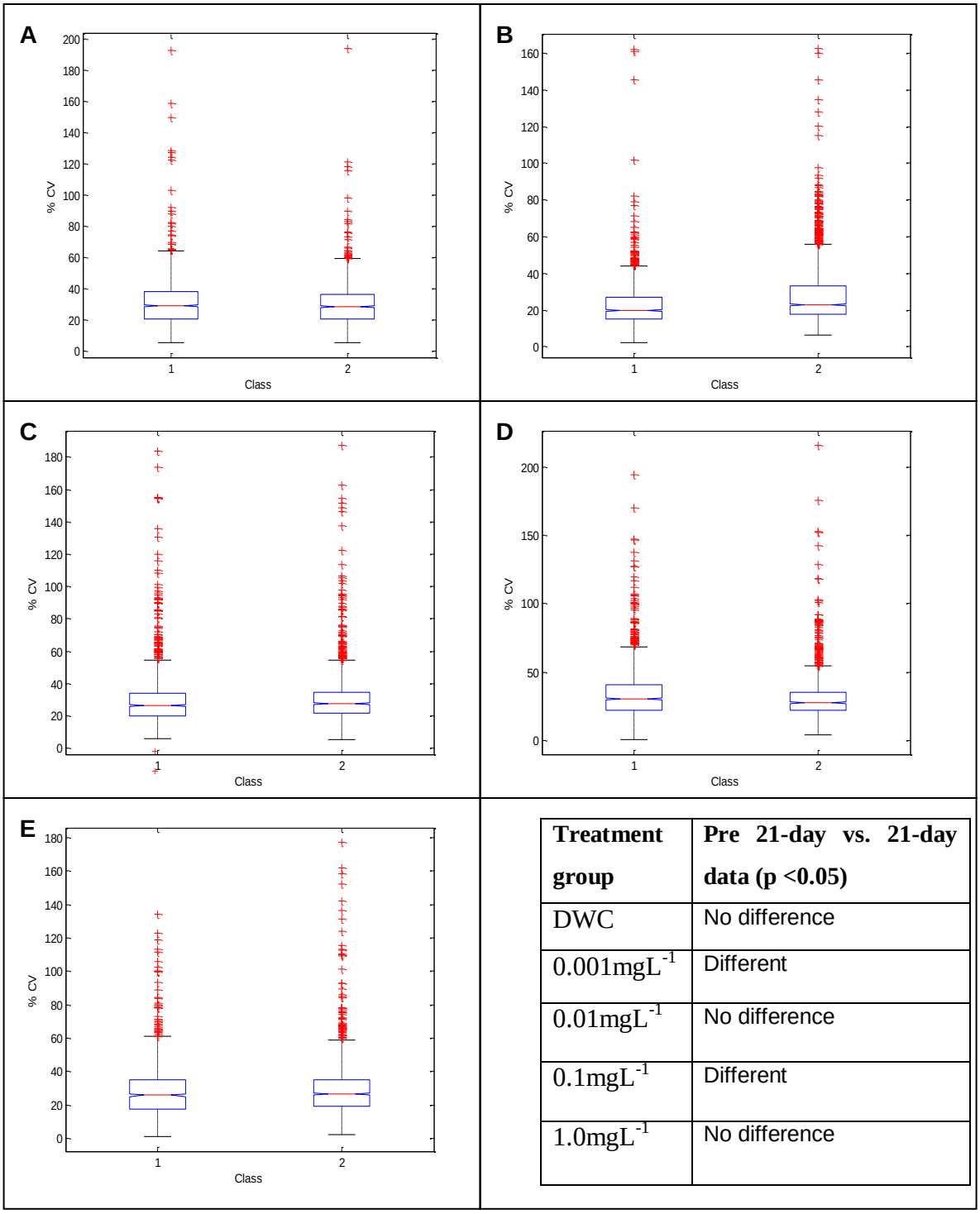
Propranolol treatment group (mgL^{-1})	Number of tissues used for metabolite extraction
DWC	8
0.001	8
0.01	8
0.1	6
1.0	4

4.3.2.2 Relative standard deviation

During both propranolol exposures, several male fish were culled (and their livers extracted) during the 21-day exposure period either because they were showing signs of distress (such as in the 1mgL^{-1} treatment group) or because the females had become "egg-bound", which is when an egg becomes trapped in the ovary duct and prevents the females from spawning; in these instances, the females were culled. Therefore, male fish were exposed to propranolol for different lengths of time, which may be a potential

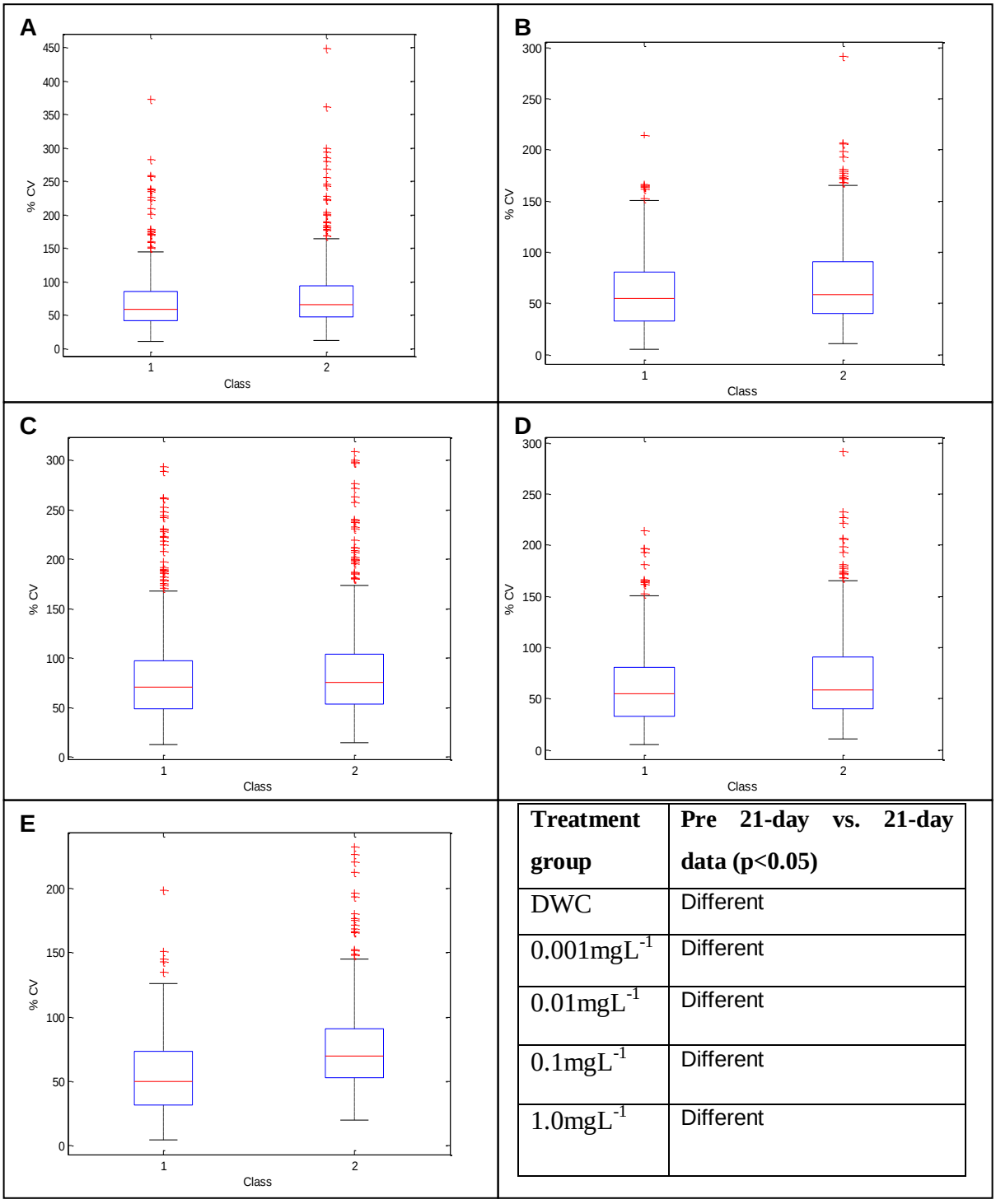
source of metabolic variation within each treatment group. RSD was therefore used to examine the level of metabolic variation in each treatment group with and without the pre-21 liver data. Figures 57 and 58 show several box plots of median variation taken from 1D and 2D pJRES ^{1}H NMR data with associated 10^{th}, 25^{th}, 75^{th} and 90^{th} percentile ranges, respectively. Each red cross represents the level of variation between all male livers within the same treatment group at a single outlying bin. There were over 2000 bins per liver spectrum and therefore, although it appears that a significant quantity of data points are outside the 90^{th} percentile ranges, the box plots do accurately represent the data sets. The level of variation was significantly higher ($p<0.05$) in the $0.001mgL^{-1}$ and $0.1mgL^{-1}$ treatment groups from the 1D data and significantly higher in all treatment groups from the 2D pJRES data. Therefore, the pre-21 liver metabolic data was excluded from further analysis. Table 28 summarises the number of tissues used for further metabolomic analysis at each propranolol concentration.

One noticeable difference between the 1D and 2D pJRES data was that the level of median variation was considerably higher in the 2D pJRES data (median RSD variation ranged from 47.7-70.5%) than in the 1D data (median RSD variation ranged from 19.6-30.2%). This is further highlighted in figure 59A and B. The variation in the $0.001mgL^{-1}$ treatment group was significantly lower than the DWC and $0.1mgL^{-1}$ group in the 1D data ($p<0.05$), whereas in the 2D p JRES data the DWC and $0.01mgL^{-1}$ treatment groups had a significantly higher level of variation ($p<0.05$) compared to the other treatment groups. However, there was no relationship between propranolol concentration and observed level of variation between liver metabolite profiles. This data supports the findings of Parsons *et al* (2009), who suggested that because of the increased level of resolution and accuracy, 2D pJRES data has a higher level of variation.



Treatment group	Pre 21-day vs. 21-day data (p <0.05)
DWC	No difference
0.001mgL^{-1}	Different
0.01mgL^{-1}	No difference
0.1mgL^{-1}	Different
1.0mgL^{-1}	No difference

Figure 57. RSD box plots taken from 1D male liver data from all propranolol treatment groups. A = DWC; B = 0.001mgL^{-1}; C = 0.01mgL^{-1}; D = 0.1mgL^{-1}; E = 1.0mgL^{-1}. For each figure class 1 represents 21-day data and class 2 represents pre 21-day and 21-day data. Table summarises significant statistical difference ($p<0.05$) between data sets.



Treatment group	Pre 21-day vs. 21-day data (p<0.05)
DWC	Different
0.001mgL^{-1}	Different
0.01mgL^{-1}	Different
0.1mgL^{-1}	Different
1.0mgL^{-1}	Different

Figure 58. RSD box plots taken from 2D pJRES male liver data from all propranolol treatment groups. A = DWC; B = 0.001mgL^{-1}; C = 0.01mgL^{-1}; D = 0.1mgL^{-1}; E = 1.0mgL^{-1}. For each figure class 1 represents 21-day data and class 2 represents pre 21-day data. Table summarises significant statistical difference ($p<0.05$) between data sets.

Table 28. Summary of number of male liver tissues used for metabolomic analysis. Tissues smaller than 30mg and also tissues extracted during the 21-day propranolol exposure were excluded from further analysis.

Propranolol treatment group (mgL^{-1})	Number of tissues used for multivariate and univariate statistical analysis
DWC	7
0.001	6
0.01	7
0.1	4
1.0	3

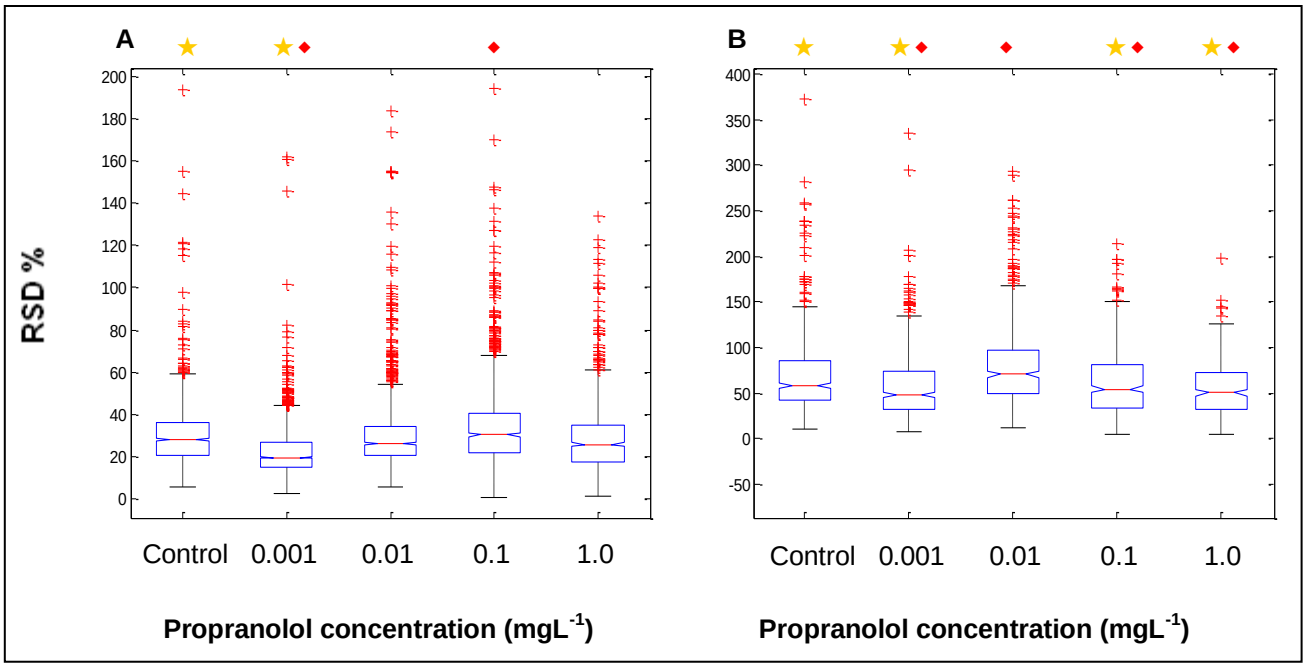


Figure 59. RSD box plots taken from 1D (A) and 2D pJRES (B) male liver data from all propranolol treatment groups. ★denotes statistical significant difference ($p<0.05$) between treatment groups exclusive for data set only. ◆ denotes statistical significant difference ($p<0.05$) between treatment groups exclusive for data set only.

To explore whether the observed level of metabolic variation in the DWC fathead minnow livers was inherent to fish species, RSD data from the control liver 1D data was compared with that from other fish test species (courtesy of Parsons *et al*, 2009) as shown

in figure 60. The three-spined stickleback (*Gasterosteus aculeatus*) and flounder (*Platichthys flesus)* lab samples were taken from fish bred under laboratory conditions, whereas the flounder Alde samples were taken from flounder living in the River Alde, Suffolk. The observed levels of metabolomic variation within each of these test species was similar to those observed in the fathead minnow. The median RSD values were 28.8% for the fathead minnow, 26.8% for the three-spined stickleback, 27.3% for the flounder (from the laboratory) and 30.1% for the flounder taken from the river Alde.

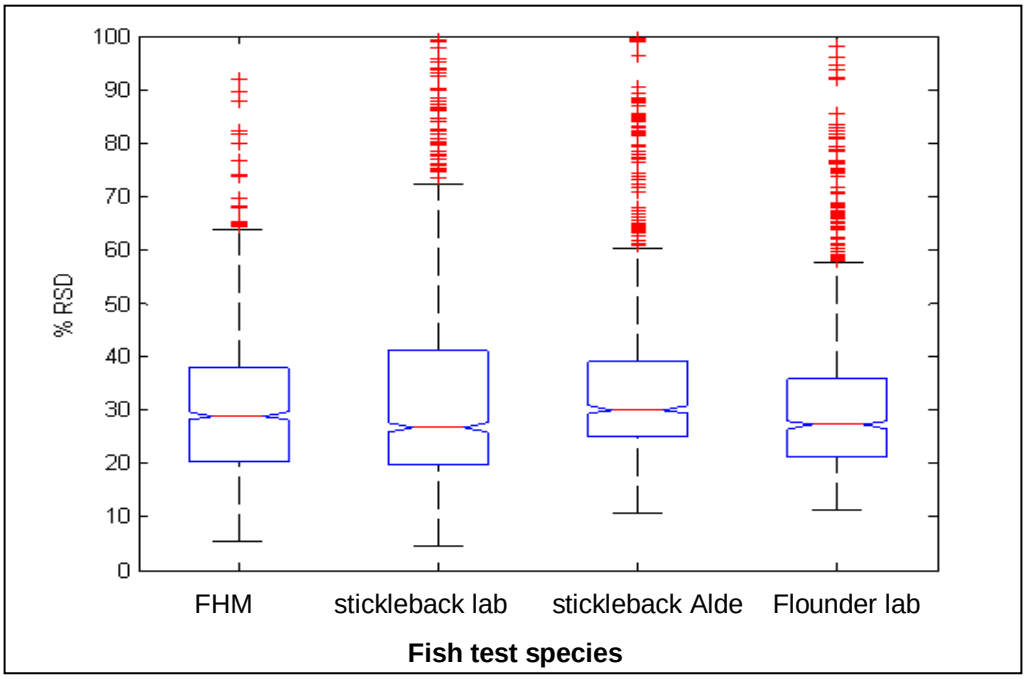


Figure 60. RSD box plots comparing control liver 1D data between different fish test species. Data taken from Parsons *et al* (2009).

4.3.2.3 Multivariate analysis

4.3.2.3.1 Principal component analysis

PCA was used to group each propranolol data set according to the concentration of the two metabolites that showed the greatest variation in concentration between all liver tissues (termed principal components 1 and 2). However, the level of variation within each treatment group for both 1D (figure 61) and 2D pJRES (figure 62) data was such that it was not possible to separate the data according to propranolol treatment

concentration, which was in stark contrast to the clear grouping of tissues achieved in the preliminary study (figure 56).

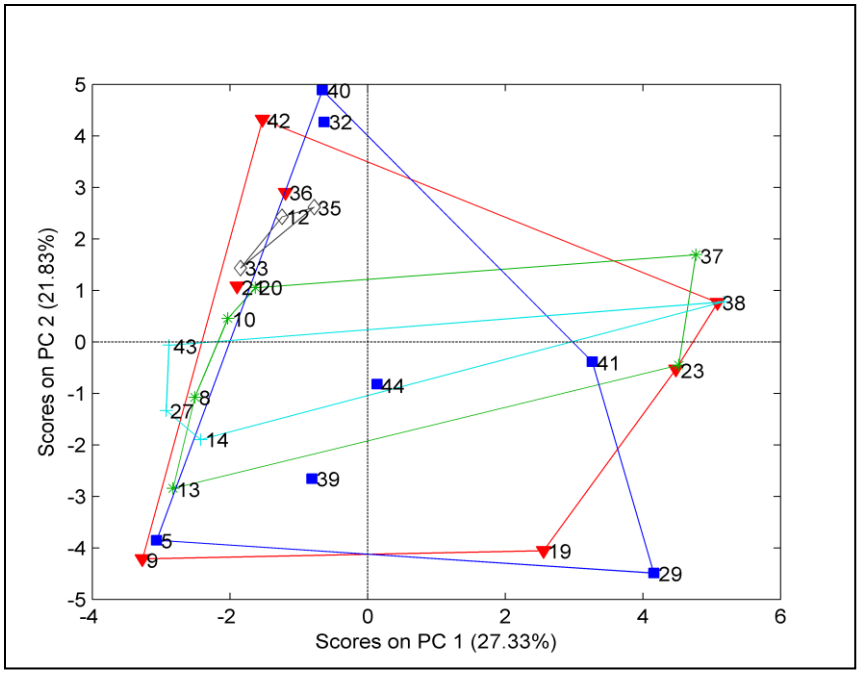


Figure 61. PCA scores plot of PC1 vs. PC2 for 1D NMR fathead minnow liver data grouped according to classes. ▲ = control group; * = 0.001mg L^{-1}; ■ = 0.01mg L^{-1}; + = 0.1mg L^{-1}; ◊ = 1.0mg L^{-1}.

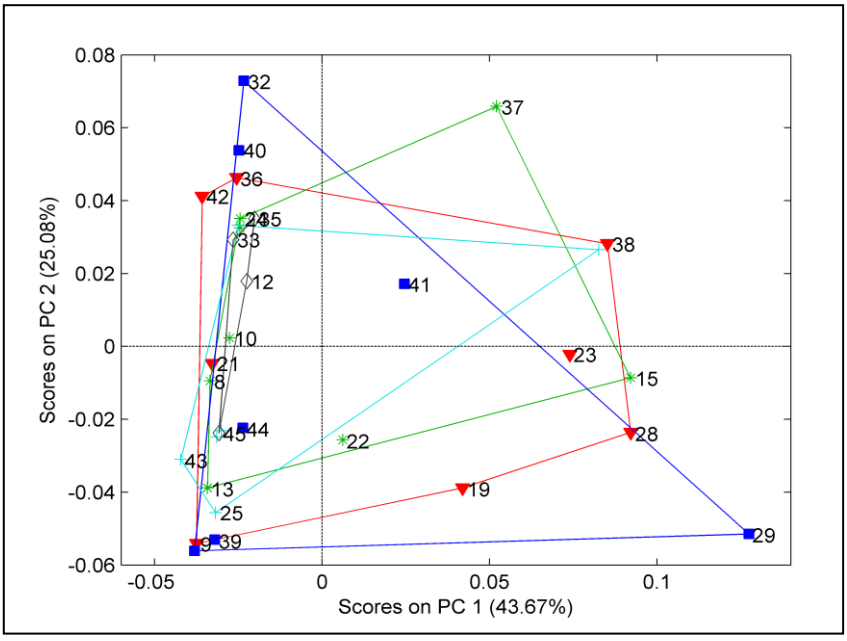


Figure 62. PCA scores plot of PC1 vs. PC2 for 2D pJRES NMR fathead minnow liver data grouped according to classes. ▲ = control group; * = 0.001mg L^{-1}; ■ = 0.01mg L^{-1}; + = 0.1mg L^{-1}; ◊ = 1.0mg L^{-1}.

Figures 63 and 64 are examples of PCA plots that included both metabolic data from samples less than 30mg in weight and also metabolomic data taken from the two surviving females in the 10mgL^{-1} group (propranolol experiment 1). There was a clearer separation of the 1.0mgL^{-1} and 10mgL^{-1} groups. It was not clear whether this was as result of propranolol exposure, tissue size or, for the 10mgL^{-1} group, sex. Figure 64, also shows a pink highlighted male liver spectrum taken from a dying fish in the 1.0mgL^{-1} treatment group. Note the metabolomic profile of this tissue was markedly different to other profiles, including those from the 1.0mgL^{-1} group. Also note that figure 64 represents a PC1 vs. PC3 plot, indicating that there were at least 3 factors that influenced the variability in between tissues, besides the propranolol treatment, which further supports the variation-based observations made in figures 57-60.

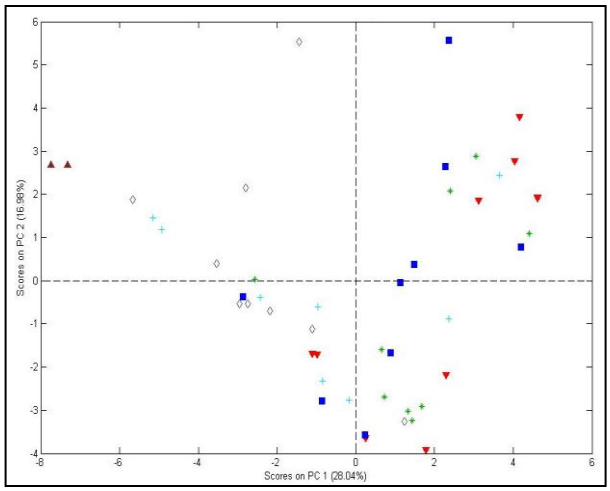


Figure 63. PCA scores plot of PC1 vs. PC2 for 2D pJRES NMR fathead minnow liver data grouped according to classes. ▲ = male control group; * = male 0.001mg L^{-1}; ■ = male 0.01mg L^{-1}; + = male 0.1mg L^{-1}; ◊ = maleb1.0mg L^{-1}. ▲= 10mgL^{-1} female liver data.

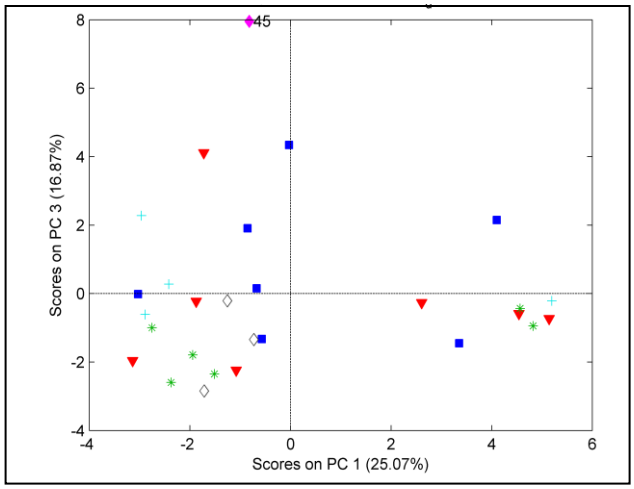
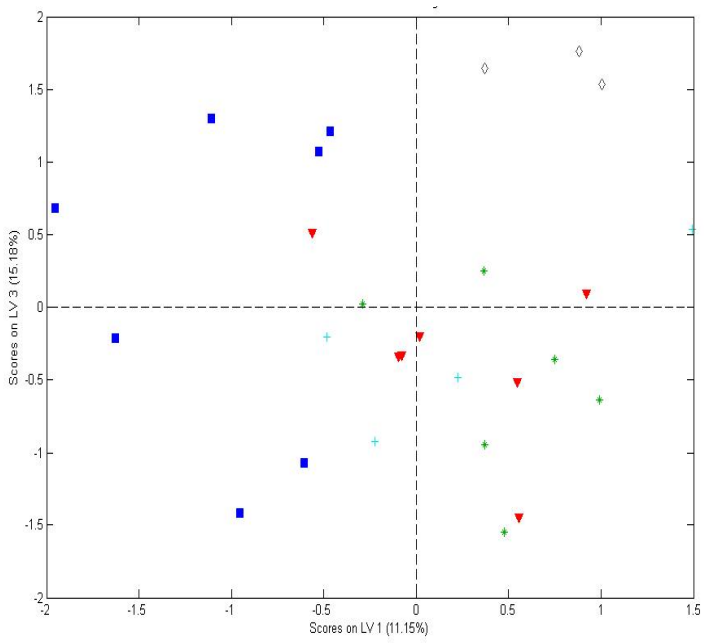


Figure 64. PCA scores plot of PC1 vs. PC3 for 2D pJRES NMR fathead minnow liver data grouped according to classes. ▲ = male control group; * = male 0.001mg L^{-1}; ■ = male 0.01mg L^{-1}; + = male 0.1mg L^{-1}; ◊ = male 1.0mg L^{-1}. Highlighted pink diamond (♦) represents male fish that was dying.

4.3.2.3.2 Partial least squares discriminate analysis

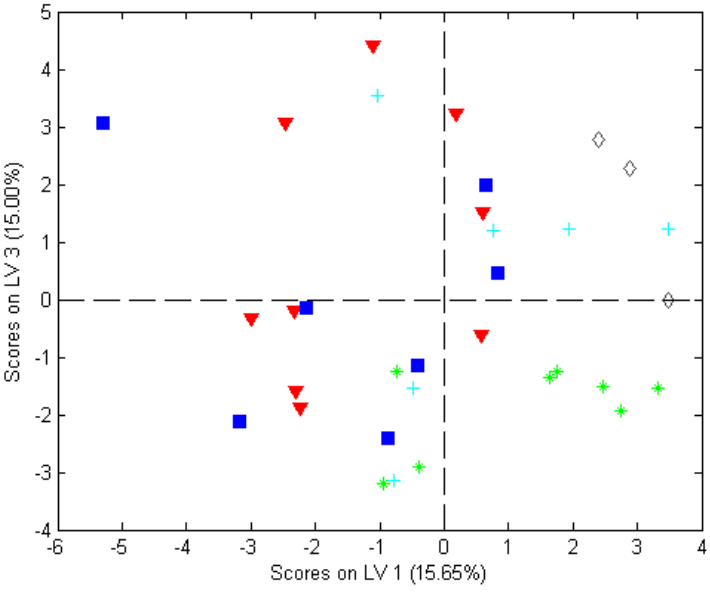
Although there appears to be a clearer separation of treatment groups, as shown in figures 65 and 66, the associated root mean square error of cross-validation (RMSECV), which is

the likelihood of the algorithm incorrectly placing a data point on the PLSDA plot for an individual treatment, ranged from 0.23 (1.0mgL^{-1} data points) to 0.68 (0.001mgL^{-1} data points) for the 1D data and 0.28 (DWC data points) to 0.70 (0.01mgL^{-1} data points). A statistically satisfactory RMSECV value should be <0.01.



Propranolol treatment group mgL^{-1}	RMSECV
DWC	0.36
0.001	0.37
0.01	0.68
0.1	0.53
1.0	0.23

Figure 65. PLSDA plot of LV1 vs. LV3 with associated RMSECV for 1D male fathead minnow liver data. ▲ = control group; * = 0.001mg L^{-1}; ■ = 0.01mg L^{-1}; + = 0.1mg L^{-1}; ◊ = 1.0mg L^{-1}.



Propranolol treatment group mgL^{-1}	RMSECV
DWC	0.28
0.001	0.49
0.01	0.51
0.1	0.70
1.0	0.55

Figure 66. PLSDA plot of LV1 vs. LV3 with associated RMSECV for 2D p JRES male fathead minnow liver data. ▲ = control group; * = 0.001mg L^{-1}; ■ = 0.01mg L^{-1}; + = 0.1mg L^{-1}; ◊ = 1.0mg L^{-1}.

4.3.2.4 Identifying a biomarker of propranolol exposure using One-way ANOVA and FDR

It was not possible to examine for a biomarker of propranolol exposure using multivariate statistics. Therefore, one-way ANOVA was used to examine for a difference in concentration at each 'binned data' between different liver metabolic profiles from each propranolol treatment concentration. To ensure a conservative assessment of significant bins was made, FDR was subsequently applied to the ANOVA output-data. Figure 67 shows the 1D data plot of all the fathead minnow liver spectra with highlighted bins that were significantly different to each other in terms of intensity/concentration between the different propranolol treatment groups. In total, 129 bins were significantly different, 17 of which were identified as metabolites. The Chenomix software used to identify the metabolites is not a comprehensive library and so it is possible that a number of the 112 bins that did not correspond to a metabolite were in fact representative of metabolites and some were "noise", which would suggest that the noise-threshold was not set high enough. However, since the Chenomix suite is not complete, it is not possible to identify between the two possibilities.

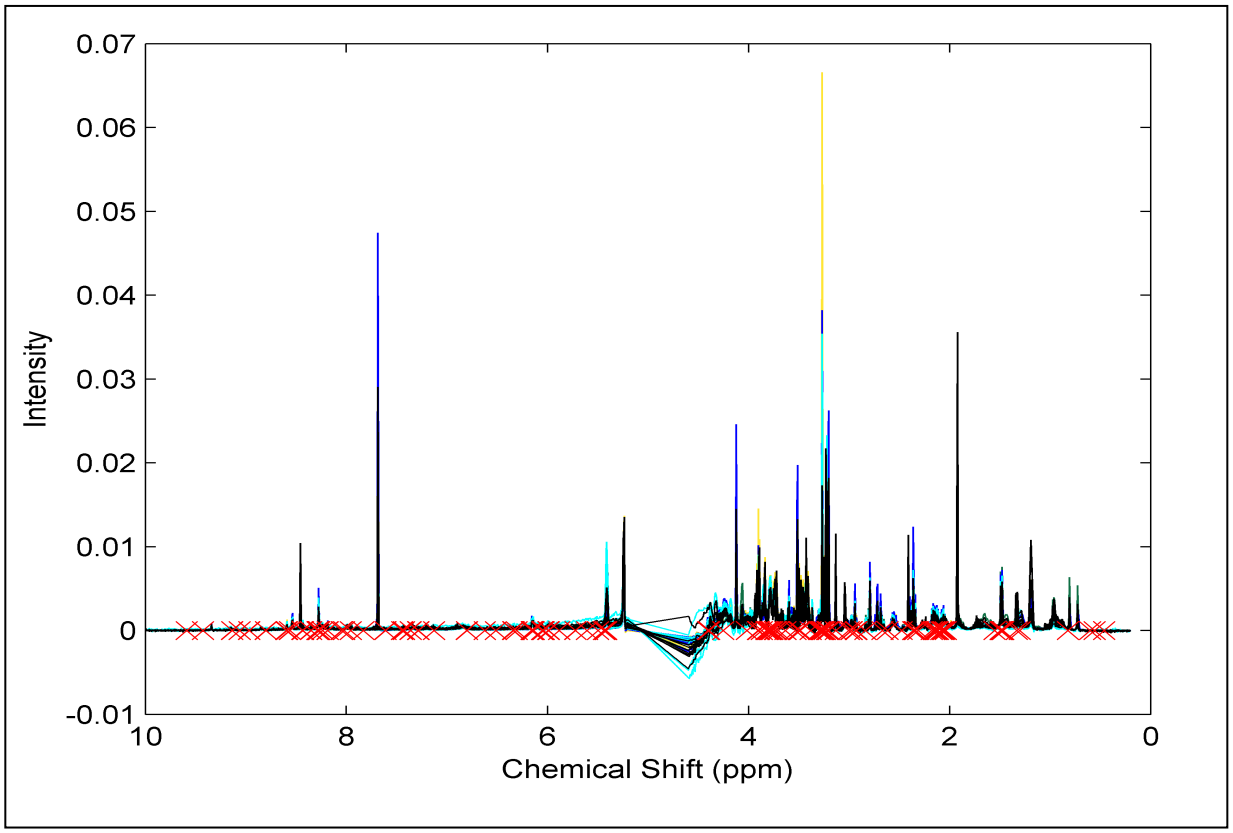


Figure 67. Plot of fathead minnow liver spectrum with highlighted (red crosses) bins of statistical significant difference. Yellow = control; green = 0.001mg L^{-1}; blue = 0.01mg L^{-1}; cyan = 0.1mg L^{-1}; black = 1.0mg L^{-1}.

Using Chenomx^{TM} software, each fathead minnow male liver spectrum was analysed and metabolites that significantly differed between each treatment group were identified as glutamate, glucose and nicotinamide adenine dinucleotide (NAD+), as shown in figure 68. Relative concentrations were calculated by measuring the area under each peak. To compare metabolite levels between fish livers between treatment groups, metabolite concentrations were normalized to the total spectrum area for each sample. The calculated concentrations indicated that there was an inverse dose relationship between propranolol concentration and glutamate levels (figure 69); as concentration increases, glutamate levels decreased. Glucose levels (figure 70) decreased as concentration increased from DWC to 0.01mgL^{-1}, reaching a trough of 60.5% of DWC concentration, before increasing as propranolol levels increased. There was not a dose-dependent relationship between NAD+ levels and propranolol concentration (figure 71), though all NAD+ levels were lower in all propranolol exposed fish compared to control fish.

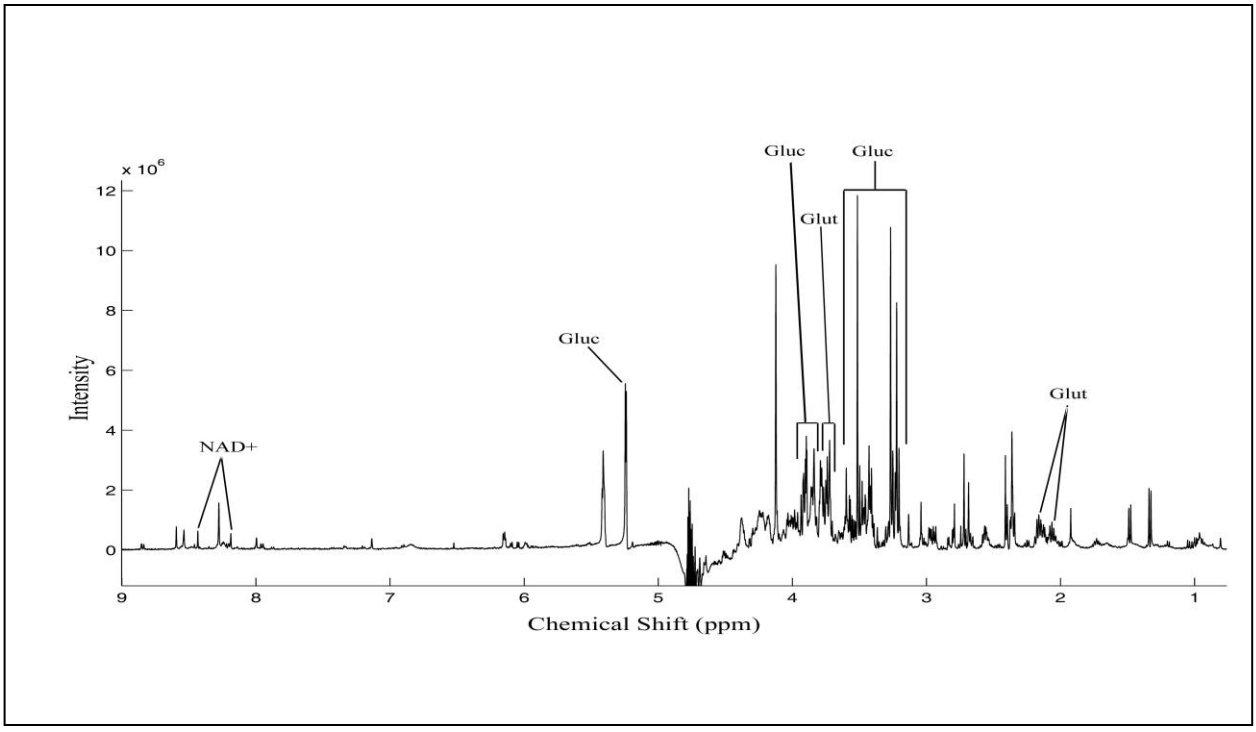


Figure 68. One-dimensional ^{1}H-NMR spectrum of a control fathead minnow liver sample analysed using ChenomX^{TM} for assigning metabolite resonances. Glut = glutamate; Gluc = glucose; NAD+ = NAD+.

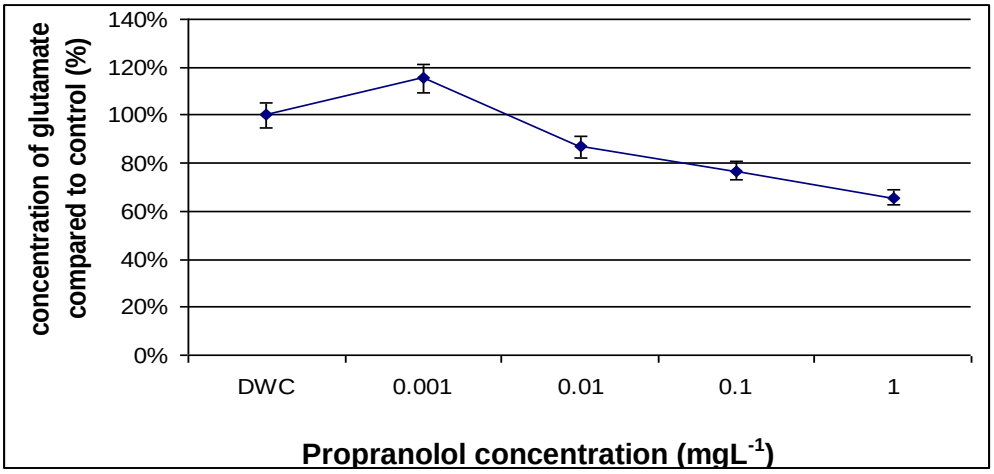


Figure 69. Concentrations of glutamate from propranolol-exposed livers as a percentage of measured glutamate levels in DWC livers.

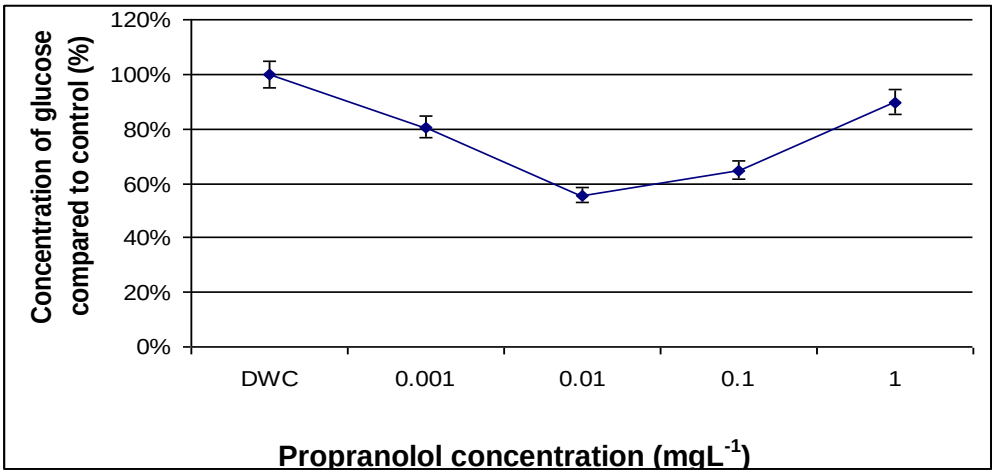


Figure 70. Concentrations of glucose from propranolol-exposed livers as a percentage of measured glucose levels in DWC livers.

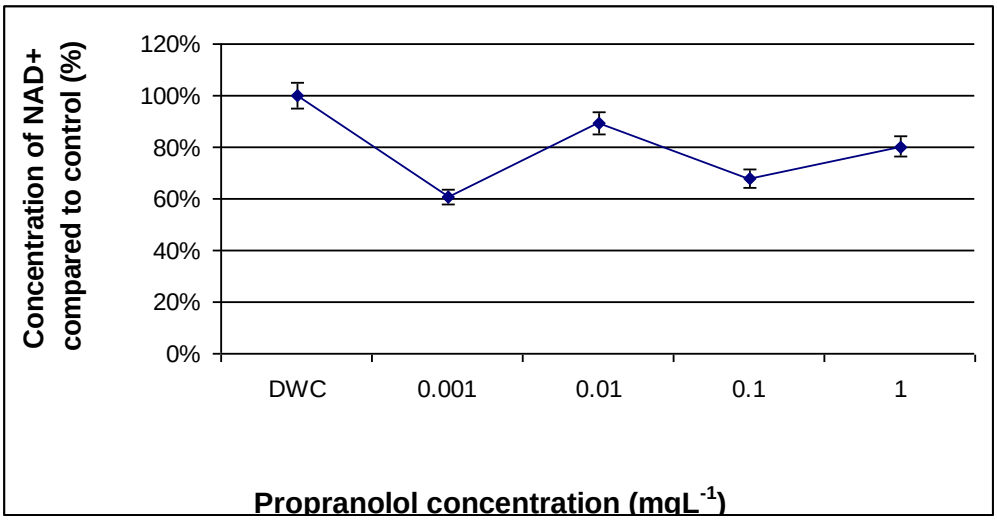


Figure 71. Concentrations of NAD+ from propranolol-exposed livers as a percentage of measured NAD+ levels in DWC livers.

An interesting point to note is that, following on from examining the metabolomic profile of the liver taken from the dying male fish from the 1.0mgL^{-1} propranolol treatment group on a PCA scores plot, the metabolic spectrum of this fish was markedly different from other fish from the same treatment group, as shown in figure 72. Levels of choline, lactate, formate and succinate were distinctly higher in the dying fish compared to other 1.0mgL^{-1} fish.

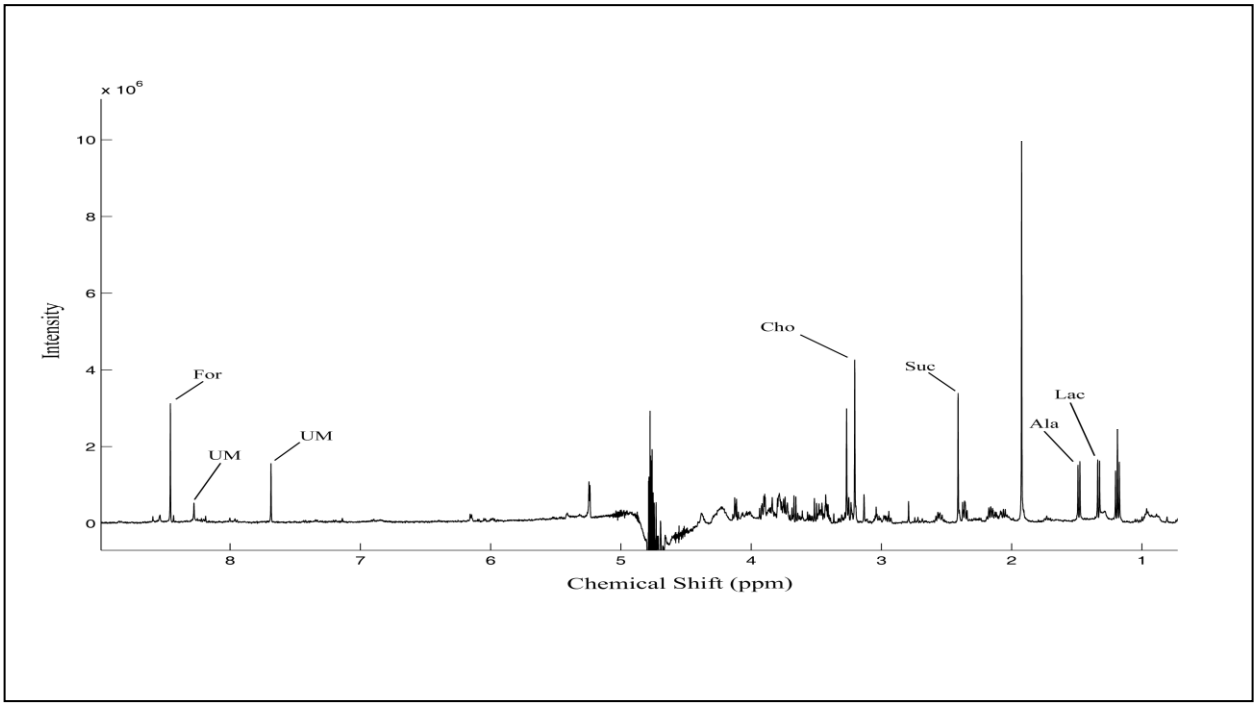


Figure 72. One-dimensional ^{1}H-NMR spectrum of fathead minnow liver sample 45, which was culled early because of signs of distress. Ace = acetone; Cho = choline; Eth = ethanol; For = formate; Lac = lactateSuc = succinate; UM = unidentified metabolite.

4.3.3 Examining sources of fathead minnow metabolic liver variation

4.3.3.1 Summary of liver tissues used for metabolomic analysis

Due to the cost of running the ^{1}H NMR and based on the results from the propranolol exposure, it was deemed unnecessary to collect 2D pJRES data, thus only 1D data was collected from male livers. Fish in all four tanks were sexually mature adult males and during the 6-day experiment several males died. Many of the dead males showed signs of bruising and hemorrhaging, suggesting that the more dominant and aggressive males may have killed these less dominant smaller fish. During the 6-day period all four tanks were treated identically and so it was unlikely that there was an alternative source for the observed mortality. Table 29 summarises the total number of liver tissues used from each experimental tank.

Table 29. Summary of number of male liver tissues used for metabolomic analysis.

Propranolol treatment group (mgL^{-1})	**Number of tissues used for analysis**
Starved tank 1	9
Starved tank 2	9
Fed tank 1	10
Fed tank 2	9

4.3.3.2 Relative Standard Variation

The level of variation did not significantly improve in the fasted tanks, as shown in figure 73; on the contrary, compared to the level of variation observed in the fed tank 1, the level of variation was considerably higher.

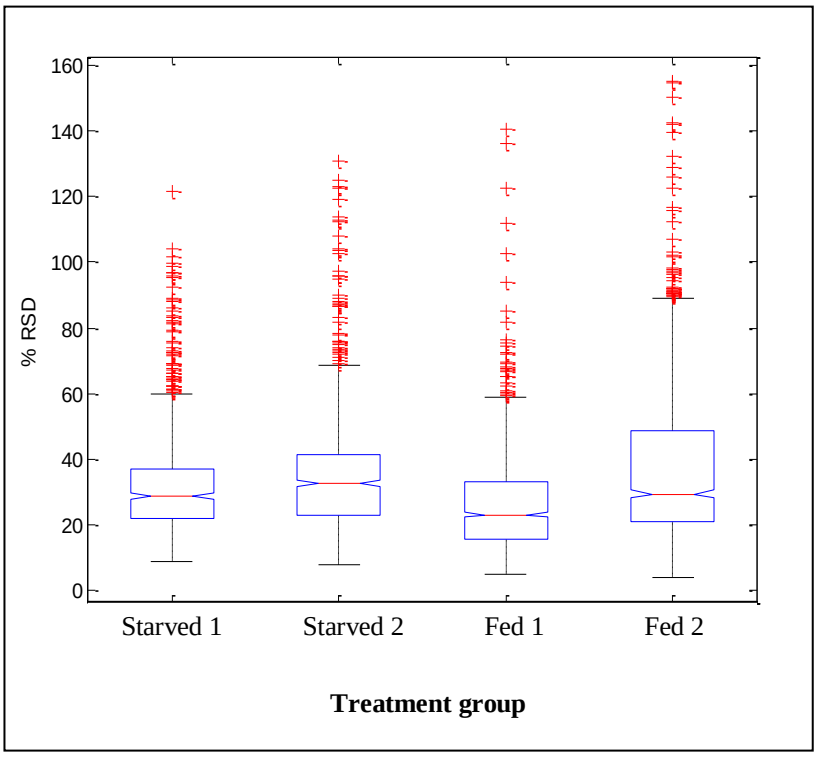


Figure 73. RSD box plots comparing male liver metabolomic variation between starved and fed fish up to and including day of culling.

4.3.3.3 Multivariate statistical analysis using PCA and LDA

The PCA scores plot, as shown in figure 74, illustrated a degree of separation on the PC3 scale between the two fasted 24 hrs prior to culling (blue and red circles) and the two fed for the entire experiment (green and cyan circles) treatment groups. The degree of separation was more apparent on the LDA plot as shown in figure 75. Similarly to figure 56, outlying liver samples from the constructed ellipses were observed from both the starved and fed fish. The elipses represent one standard deviation of the mean and so at least two thirds of all samples will fit within the ellipses. Accordingly, samples that do not fit within the ellipse demonstrate the the considerable level of variation between liver tissues within treatment groups.

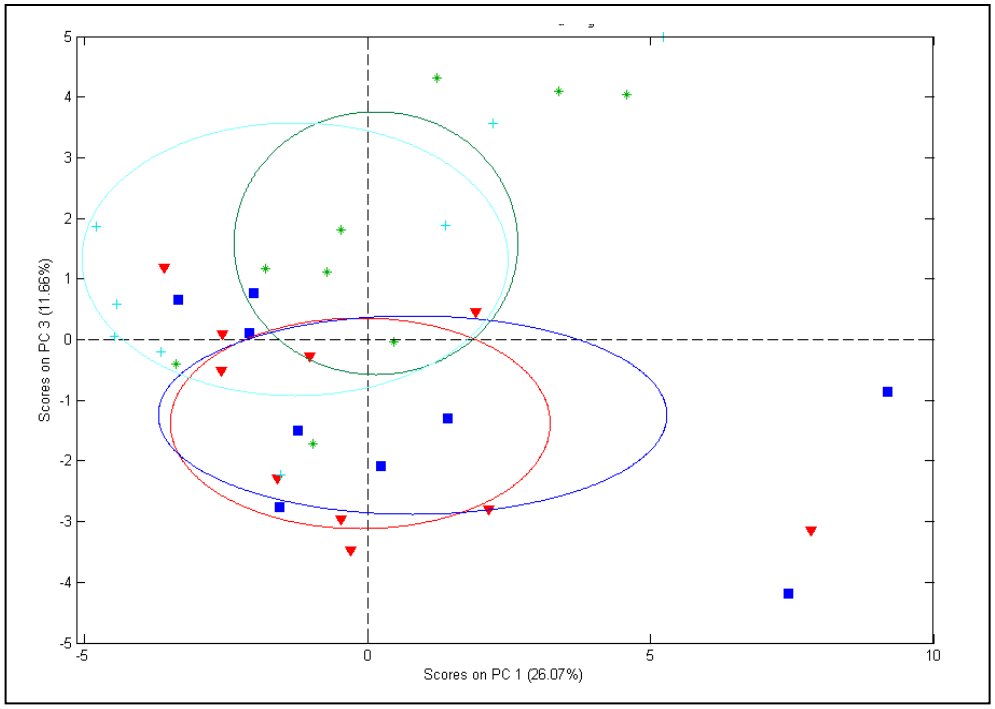


Figure 74. PCA scores plot of PC1 vs. PC3 for 1D NMR fathead minnow liver data grouped according to classes. ▲ = starved group 1; ■ = starved group 2 * = fed group 1; + = fed group 2.Ellipses represent one standard deviation of the mean.

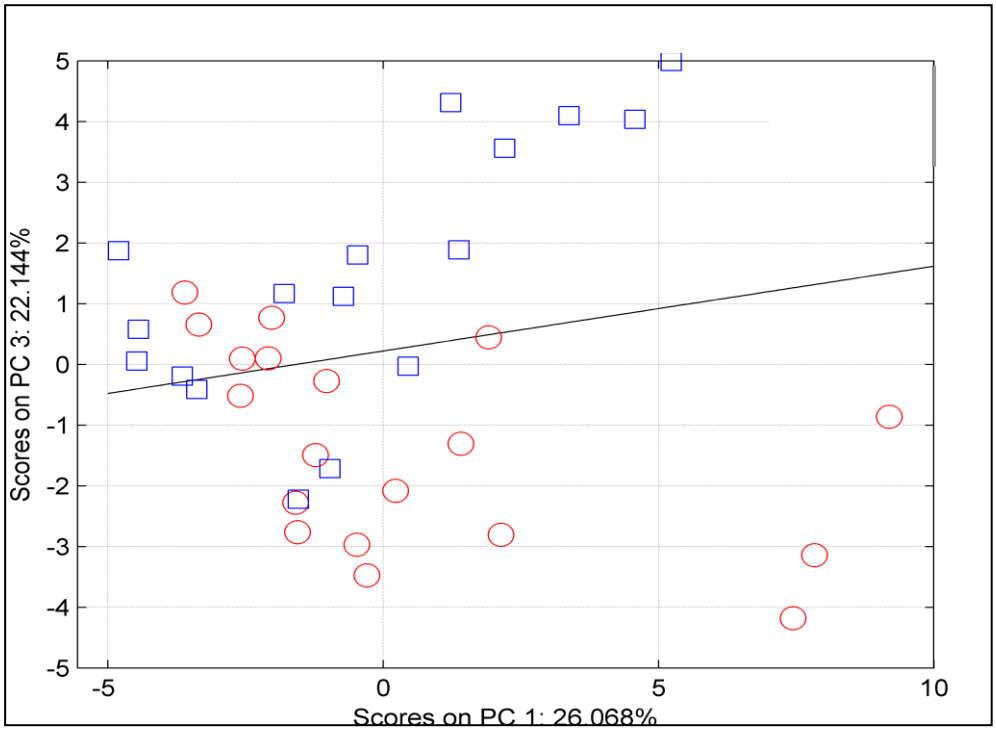


Figure 75. LDA scores plot of PC1 vs. PC3 for 1D fathead minnow liver data grouped according to classes. ○= fasted 24hrs prior to culling; □= fed for entire experiment.

4.3.3.4 Identifying a biomarker of feeding using One-way ANOVA and FDR

Using One-way ANOVA followed by FDR correction, 53 bins were found to differ significantly between the fasted for 24hrs and fed treatment groups, as shown in figure 76. Of the bins, 35 were deemed "noise" and 18 as identifiable metabolites.

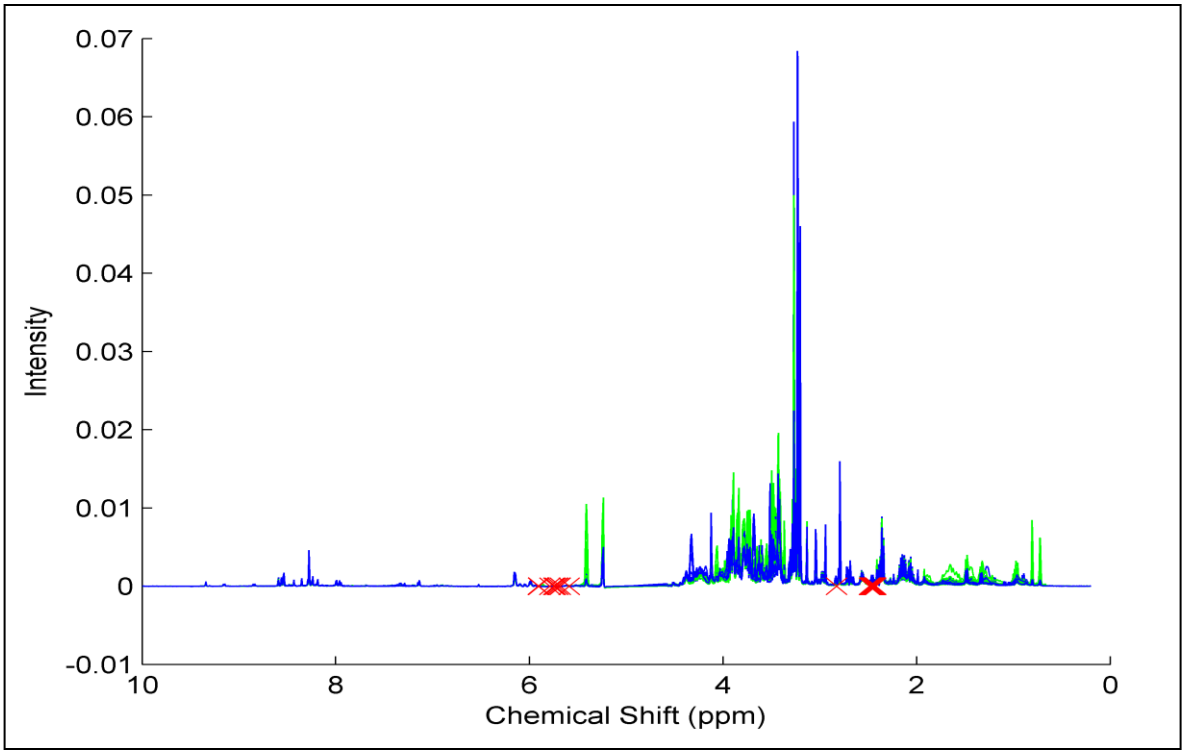


Figure 76. Plot of fathead minnow liver NMR spectra with highlighted (red crosses) bins of statistical significant difference ($p<0.05$). Blue = starved fish livers; green = fed fish liver.

As there was separation between treatment groups on the PCA and LDA along the PC3 scale, it was possible to transpose the highlighted bins of significance onto the PC3 loadings plot (figure 77) to examine whether the metabolites identified were markers of fasting or feeding. The bins highlighted by the red circle correspond to peaks arising from metabolites, whereas the other significant bins correspond to noise. The highlighted peaks were negative on the PC3 loadings plot which, when compared to the PCA and LDA scores plots, represented the starved fish liver metabolite profiles. Therefore, the metabolites that significantly differed between the two treatments were biomarkers of starved fish.

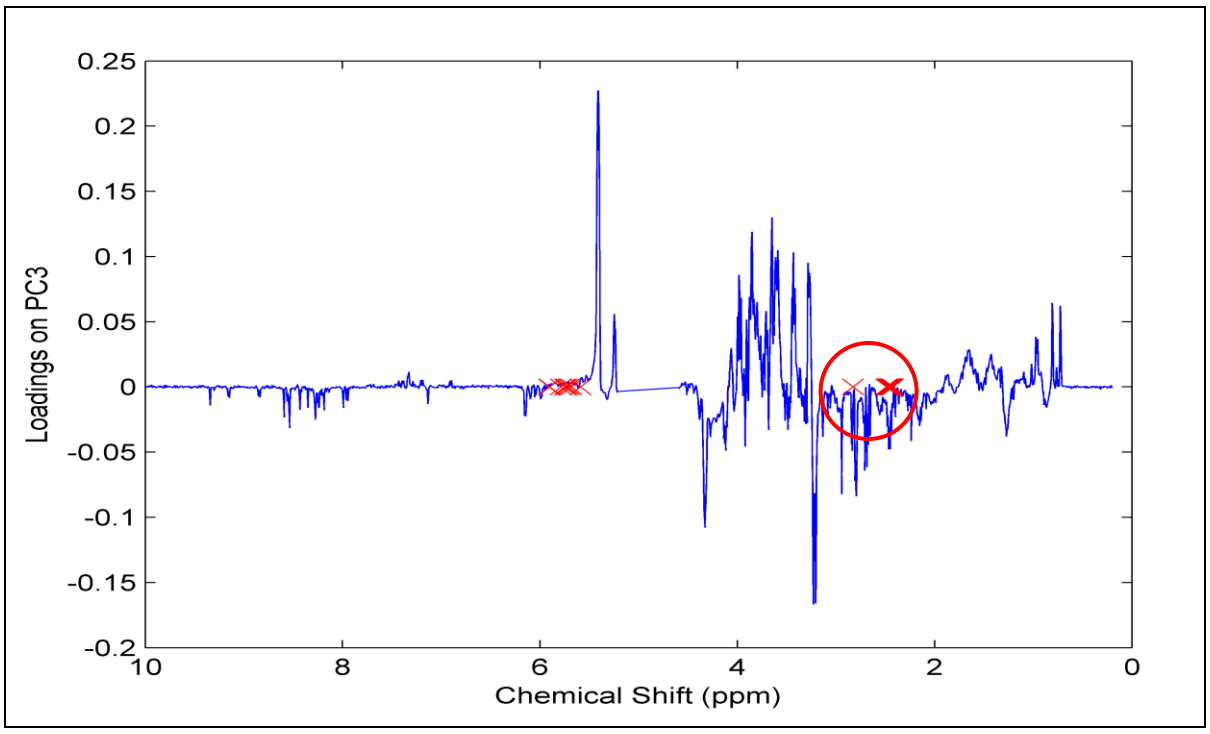


Figure 77. PC3 loadings plot with highlighted bins of significant difference ($p<0.05$) between the two metabolite profiles. The peaks arising from metabolites that were significantly different between treatments are highlighted by the red circle.

Analysis of the liver spectra using Chenomx^{TM} software was used to reveal which metabolites were found at significantly higher levels in starved fish (figure 78). Table 30 indicates that glutamine levels in starved livers were 153% of those levels in fed male fathead minnow livers.

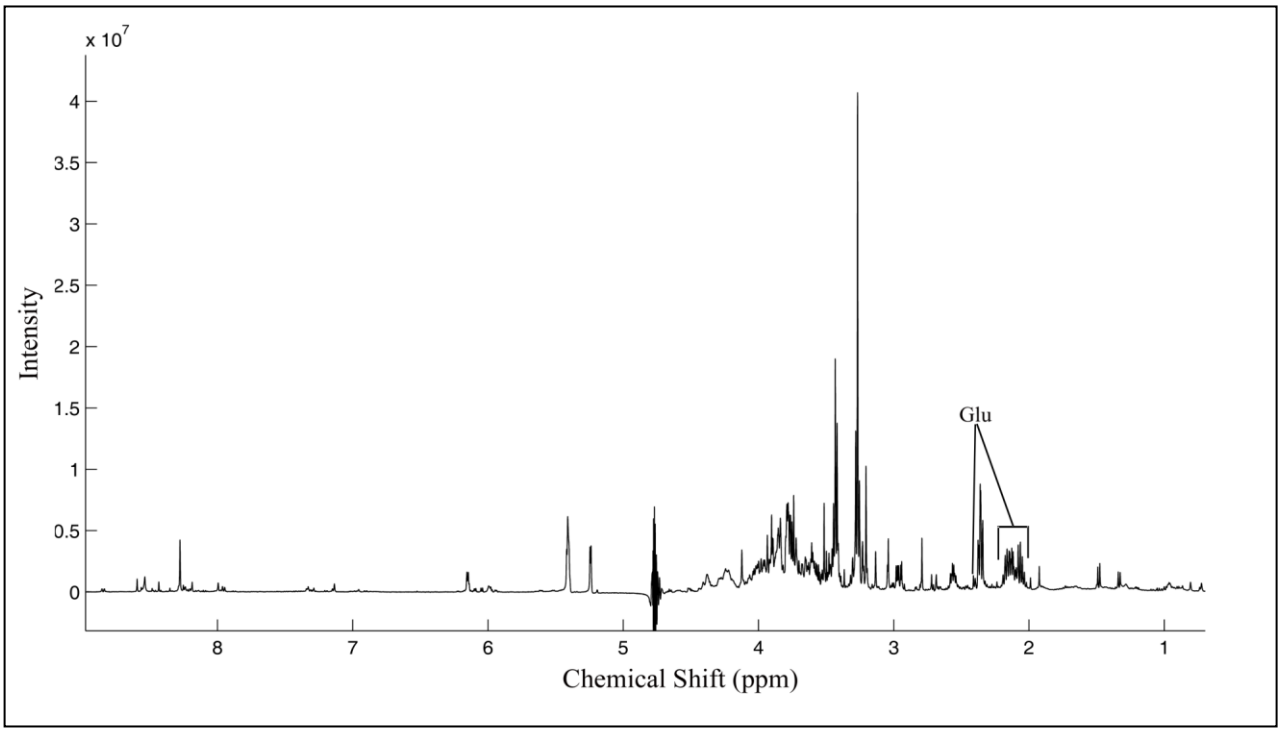


Figure 78. One-dimensional ^{1}H-NMR spectrum of a liver sample taken from a male fathead minnow starevd for 24hrs prior to culling. Glu = glutamine.

Table 30. Comparison of glutamine levels between fed fish livers and starved livers.

Metabolite	**Fed fish livers up to and including day of culling**	**Starved fish livers 24hrs prior to culling**
Glutamine	100%	153%

4.4 DISCUSSION

4.4.1 Proof of principle

The results from the preliminary study showed that ^{1}H-NMR metabolomics was capable of examining and differentiating four different tissues from fathead minnow. Although this does not in itself provide new knowledge, it demonstrated the proof of principle that metabolomics could potentially be used in aquatic toxicity studies to examine the metabolic response of tissues to exposure to pharmaceuticals and other contaminants. The use of ^{1}H NMR metabolomics to examine the metabolic response of fathead minnow livers to exposure to propranolol, however, was limited by metabolic variation between tissues from the same treatment groups (including control liver tissues).

4.4.2 Addressing metabolic variation in fathead minnow livers

Propranolol exerted an acute toxicological effect on male fathead minnows exposed to a concentration of 1.0mgL^{-1} and as the liver was the principal site of propranolol metabolism, it was anticipated that a metabolic response would be detected using ^{1}H and 2D pJRES NMR metabolomics (Masabuchi *et al*, 1994). However, the significant level of metabolic variation between liver tissues within the same treatment group was such that it was not possible to group them according to propranolol treatment concentration using both non-supervised PCA and supervised PLSDA. Therefore, it was not possible to identify a metabolic change in liver activity as a response to exposure to propranolol using multivariate analysis. One essential requirement of a metabolomic experiment is that the analytical variation introduced post-collection is less than the typical biological variation in the 'normal' population of interest (Keun, 2006). Therefore to address the source of variation and determine whether it was 'normal' or possibly introduced, RSD analysis was employed. Traditionally, RSD is used to examine the stability of metabolite peaks and consequently used as an indicator of the strength of results. In the current study, RSD was used to examine liver metabolomic variation levels within the same treatment group, according to the protocol described by Parsons *et al* (2009). The median RSD value for control male fathead minnow liver tissues was 28.8% and 55.6% for 1D and 2D p JRES data, respectively. Technical variation introduced by ^{1}H NMR repeats

have been recorded at 3.07% and 12.5% for 1D and 2D pJRES data, respectively, indicating the variation observed in the fathead minnow tissues was most likely reflective of 'normal' biological population variation, as opposed to 'introduced' variation (Parsons *et al*, 2009). Furthermore, the median RSD levels in control stickleback, laboratory-sampled flounder and wild flounder livers were closely comparable to that observed in the fathead minnow, suggesting further that this level of variation may be natural in teleost liver populations. There was no relationship between RSD liver variation and propranolol dose, indicating that any metabolic effect of propranolol on the livers did not override the natural variation in liver activity.

4.4.2.1 Sources of metabolic variation

4.4.2.1.1 Natural variation

There are several potential reasons for the observed variation in the fathead minnow liver tissues. First and foremost, it could simply be natural. The metabolic phenotype can be described by the simple genetic relationship relating phenotype (observable physical or biochemical characteristics of an organism) to the variables that control it accordingly (German *et al*, 2003):

Phenotype = (genotype + environment) + (genotype x environment)

Considering the level of genetic variation between individuals and the sum of all external variables, there is the potential for considerable phenotypic variation within a single population. One method of minimizing metabolic phenotypic variation, which has been adopted for pharmacological studies, is to use inbred test species, which minimises genetic variation. For example, a typical median RSD level in the rat plasma (*Ratus norvegicus*) is 8.00% (Parsons *et al*, 2009), which is much lower than the median RSD levels in fathead minnow livers. The environmental relevance of using an inbred fish test species for an ecotoxicological experiment is questionable. However, the use of inbred fish may serve as a good starting point to understand toxicant mechanism of action before expanding upon the work with genetically diverse fish.

4.4.2.1.1 Introduced variation: technical variation

The two-step tissue extraction protocol of methanol:chloroform:water optimized by Wu *et al* (2008) was unlikely to have contributed towards the observed level of variation as the recorded median RSD for technical replications (i.e. examining the same sample twice) was only 4.62%, as stated by Parsons *et al* (2009), although a technical replication median RSD was not recorded for the current study.

4.4.2.1.2 Introduced variation: handling and anaesthetisation stress

There are a handful of potential external sources of variation that may be introduced in all aquatic toxicology studies, including stress. In the propranolol exposure study, fish were culled by an overdose of MS222 and subsequent brain dislocation. It is possible that during the culling procedure, the fish may have experienced high levels of stress. Past studies have documented that handling and MS222 anaesthetisation stress can affect primary liver activity and biotransformation rates in the Arctic charr (*Salvelinus alpinus*) and rainbow trout (*Oncorhynchus mykiss*) (Morales *et al*, 1990 and Kolanczyk *et al*, 2003, respectively), resulting in decreased cytochrome P450 levels, which can ultimately transform the liver metabolic profile. However, Hemre & Krogdahl (1996) suggested that handling and anaesthetisation stress had a minimal effect on secondary changes in carbohydrate metabolism in the Atlantic salmon (*Salmo salar*) and so possibly the magnitude and effect of stress may be species-specific. It was possible that different fish experienced different levels of stress and this variation in stress level may have had an effect on the overall variation in liver metabolite profiles.

Regulated MS222 anaesthetisation for individual fish, used in the fish feeding experiment, did not improve the median level of RSD in each tank and so MS222 anaesthetisation was also an unlikely source of introduced variation in the propranolol study.

4.4.2.1.3 Introduced variation: Diet – feeding and fasting

Another external source of variation could be feeding; in the propranolol exposure study, the fathead minnows were fed up to and including the day of culling; food was added

each morning. There were in total 40 fish (males and females) culled on both sampling days. Therefore, fish were culled at different times during each sampling day and so fish culled late on in the day may have had time to feed more than those culled earlier on. Diet has a huge influence on the hepatic metabolome (Griffin *et al*, 2007), and so it was possible that unregulated feeding may have contributed to the variation observed in the liver tissues.

Fasting the fish 24 hours prior to culling did not reduce (improve) variation levels between liver metabolic profiles. The median RSD was indeed higher in livers taken from the 'starved' tanks than in livers taken from the 'fed' tanks. At the start of the 6 day feeding trial, 16 adult male fathead minnows were introduced into each tank and were fed for either 5 ('fasted') or 6 days. It was very difficult to quantify the volume of food each fish consumed over this period as, naturally, fish hierarchy was established and thus some fish were likely to have consumed more food than others. It was apparent that there was an aggressive hierarchy in each tank as several smaller males died and showed signs of abdominal bruising, which is indicative of fighting between fish. When sexually mature, the male fathead minnow is a very territorial freshwater species (Cole and Smith, 1987). For the purpose of this study only adult male livers were suitable for ^{1}H NMR metabolomic analysis because of their large size and so this hierarchal behaviour was anticipated. It has been suggested that to attempt to counter-balance this aggressive territorial behaviour, female fish should be introduced in sufficient numbers. However, previous studies have suggested that introducing several females into male tanks does not alter male fathead minnow behaviour (Pyron & Beitinger, 1989). As such, this observed hierarchal behaviour could have influenced feeding rates by individual fish in all tanks and possibly liver metabolomic profiles within the same tank and thus increase metabolic variation. Regardless, one can conclude that feeding up to and including the day of culling in the propranolol study was unlikely to be the principal source of introduced variation in the propranolol study.

4.4.2.1.4 Introduced variation: Tank effects

It is possible that fish in different tanks can respond differently to exposure to the same concentration of a toxicant. For example, in the propranolol study each male fish was housed in an individual tank (6 tanks each with a different propranolol concentration) and so the fish in the tanks closest to the door may have experienced more stress than fish in tanks further away. However, the PCA scores plot from the feeding experiment suggested that there was little or no difference in liver metabolic profiles between both repeat tanks from the feeding and fasting treatments, suggesting there was no major tank effect. Therefore, different tank environments probably had a minimal contribution towards the observed variation between individual liver metabolic profiles within the same propranolol treatment groups.

4.4.3 Identifying Biomarkers

4.4.3.1 Propranolol exposure

Univariate analysis was employed to identify a biomarker of propranolol exposure in male fathead minnow livers. Using one-way ANOVA ($p<0.005$) followed by FDR correction on the binned data, 17 bins that contained peaks from metabolites were identified as varying significantly between propranolol treatment groups; these metabolites were subsequently identified as glucose, nicotinamide adenine dinucleotide (NAD^+) and glutamate. There was an observed decrease in all three metabolites upon exposure to propranolol, although glutamate was the only metabolite to show a dose-dependent decrease. Glutamate is used in the tricarboxylic acid cycle and is therefore linked to energy production. There was an inverse dose-dependent response that as propranolol treatment concentration increased, glutamate levels decreased. Propranolol reduces energy metabolism and subsequent oxygen consumption in humans (Krag *et al*, 2006) and so it is possible that this same effect was observed in propranolol-exposed fathead minnow. There is contradictory information regarding the effects of propranolol on glucose levels in humans and higher vertebrates. Hunt *et al* (2002) examined the effects of propranolol on glucose clearance and premature fatigue during exercise in the rat (*Rattus norvegicus*). They suggested that propranolol inhibited the epinephrine-

mediated pathway of insulin-stimulated glucose uptake during muscle contraction and, as a result, plasma glucose levels were elevated. Allison *et al* (1969), however, found that propranolol slightly lowered plasma glucose levels and had no significant effect on insulin levels in humans and so the effect of propranolol on the insulin-stimulated glucose uptake is still unclear. There is little information which addresses the direct effects of propranolol on the metabolic profile of liver tissues. Epinephrine regulates glycogenolysis in muscle and liver tissues, which is the conversion of glucagon to glucose (McMurry, 1988). In the liver, glucose-6-phosphate is produced and is subsequently de-phosphorylated into glucose, which then diffuses into the bloodstream for uptake by other tissue cells (Berg & Tymoczko, 2002). It was not possible to differentiate between glucose and glucose-6-phosphate using the Chenomix software and, considering the rate at which glucose diffuses into the bloodstream, it is more likely that glucose-6-phosphate was detected. It was evident at all concentrations that fish were showing signs of stress and, as such, there would have been an increase in epinephrine levels (Bonga, 1997) and an increase in glyogenolysis. However, propranolol may have inhibited this pathway at the lower propranolol concentrations ($0.001 mgL^{-1}$ to $0.1 mgL^{-1}$) and as a result the glycogenolysis rate was lowered and so there was a decrease in liver glucose-6-phosphate levels. Once blood plasma glucose levels fall below a threshold (hypoglycemia) in humans and higher vertebrates, glucagon is produced to stimulate the conversion of stored glycogen into glucose in the liver (Kieffer & Habener, 2000). Therefore, it is possible that glucose levels in fathead minnows exposed to $1 mgL^{-1}$ propranolol fell below the threshold, and in response glucagon induced the production of glucose-6-phosphate in the liver and by day 21 of the exposure period, glucose levels were almost restored to the same levels as measured in the DWC fish livers. However, if this did happen, one would have expected a corresponding decrease in glycogen levels in the $1 mgL^{-1}$ fish livers, which was not detected. Metabolomics takes a "snap-shot" of tissue metabolic activity and so it is difficult to examine a metabolic response over time, as hypothesised. Secondly, as it was not possible to measure glucose levels in blood plasma because of limited sample sizes, it was not possible to examine whether glucose-6-phosphate levels corresponded with glucose levels.

Due to the considerable variation observed between livers, it is possible that this response may have occurred but was not detected using ^{1}H NMR metabolomics. NAD^{+} is a coenzyme involved in many redox reactions during metabolism. In higher vertebrates, NAD^{+} has been identified as an important cofactor of cytochrome P450 propranolol metabolism (Vu and Abramson, 1980) and so it is possible that upon exposure to propranolol, hepatic cytochrome P450 activity levels increased in the fathead minnow and there was a resultant decrease in NAD^{+} levels. There was not a dose-related response between NAD^{+} and propranolol concentration; this coupled with the fact that NAD^{+} levels were not dramatically lower than the control levels suggests that perhaps what has been observed is an example of individual variation or that because NAD^{+} is involved in many critically important cellular pathways, levels are maintained around a set point.

A single liver tissue from a dying male from the 1.0 mgL^{1} propranolol treatment group was also analysed using univariate one-way ANOVA and FDR. The metabolomic profile from this single male liver suggested that there were several metabolites found at higher levels compared to DWC livers. Elevated levels of lactate, formate and succinate, which are all characteristic metabolites of liver failure, were observed (Oka & Sakai, 1969; Thompson, 1986; Sewell *et al*, 1997; Riordan & Williams, 2002). Elevated levels of choline were also observed. Choline is a constituent of cell membranes and so if there was a breakdown in the cell membranes, choline may have been released. This data supports the theory described in 3.4.1, that liver failure was potentially the cause of death observed in fathead minnows exposed to pharmacologically-relevant levels of propranolol and that the identified biomarkers were representative of dying.

Two female tissues analysed from the $10mgl^{-1}$ treatment group, where acute toxicity was observed in both male and female fish, also demonstrated a significant different metabolite profile and were grouped accordingly. However, it was not possible to determine whether the differences were due to propranolol exposure, phenotype-related metabolic profile differences (Hines *et al*, 2007; Ekman *et al*, 2008) or because the tissues were smaller than 30mg and thus giving potential errors in NMR analysis.

Of the three identified "biomarkers" of propranolol exposure (excluding those indicative of dying), glutamate is perhaps the most suitable. Unlike glucose and NAD^+, glutamate levels showed a dose-dependent response, with minimal variation and therefore may have served as an indicator of changes to energy production/metabolism. However, one cannot be sure that glutamate production (either decreased or increased) is specific to exposure to propranolol and therefore the use of glutamate as a biomarker must be validated against other pharmaceuticals. Additionally, considering the level of metabolic variation observed between liver tissues, the significance of glutamate as a biomarker remains questionable and also requires further validation. The use of univariate analysis has been frequently adopted to identify biomarkers of xenobiotic/contaminant exposure in aquatic organisms and therefore presents a suitable method of analysis, however the relevance and reliability of these identified biomarkers is still uncertain.

4.4.3.2 Feeding/fasting

Despite the levels of variation within each tank, it was possible to separate and group the liver metabolic profiles according to their feeding treatment using multivariate PCA and LDA. Combining this with univariate one-way ANOVA followed by FDR ($p<0.05$), glutamine was identified as a metabolite that varied significantly in concentration between livers from fish fed up to and including the day of culling and livers from fish starved 24 hours prior to culling; glutamine was therefore a potential biomarker of starving. Glutamine is a non-essential amino acid produced from muscles during gluconeogenesis, where glucose is generated from non-carbohydrate carbon substrates such as lactate, glycerol and glucogenic amino acids, during which nitrogen is converted to glutamine in the liver (Jungas *et al*, 1992). In higher vertebrates and teleosts, glutamine is an important substrate for red muscles and is an important fuel source when metabolism is increased and dietary sources of non-carbohydrate carbon substrates are low (Chamerlin *et al*, 1991; Balzola & Boggio-Bertinet, 1996). Gluconeogensis typically occurs during periods of fasting, starvation or intense exercise (Nelson & Cox, 2000) and therefore it is likely that gluconeogensis activity in the livers of fish fasted for 24 hours increased which resulted in the increased production of glutamine. Surprisingly, ^{1}H-NMR metabolomic analysis of the livers was not able to identify a decrease in glycogen levels

which has been observed in fasting rainbow trout (*Oncorhynchus mykiss*) and European sea bass (*Dicentrchus labras*) (Pottinger *et al*, 2003 and Pérez-Jiménez *et al*, 2007, respectively). However, the analytical techniques were used specifically for the determination of glucose-derivatives.

The results from the feeding study demonstrated that there was not necessarily a direct relationship between RSD variation and the ability for unsupervised multivariate techniques to extract a biomarker of response. The median RSD levels were very comparable between livers from the feeding and propranolol exposure and yet it was possible to identify a biomarker of response using uni- and multivariate analysis from the feeding exposure whereas for the propranolol exposure, only univariate analysis was used. The trend in response to a 'treatment' (i.e. the magnitude in change of specific metabolites) in addition to the level of metabolic variation is a crucial factor as to the likelihood of successful multivariate analytical interpretation (separation of 'treatment' groups and identification of a biomarker) of data.

4.4.4 Recommendations for future work

Despite the considerable variation observed in both studies, it was possible to extract and identify potentially subtle biomarkers of propranolol exposure and fasting using 1D and 2D pJRES ^{1}H-NMR metabolomics. The use of RSD analysis for examining variation provided a clearer insight for understanding the multivariate analysis data. Additionally, RSD provided a valuable tool to determine whether data could be included for analysis. The use of RSD should be continued in this fashion for further aquatic toxicology based studies where genetic and phenotypic variation could potentially be very high and, with additional studies, it could possibly be used to determine benchmarks of variation.

Efforts to minimise the level of variation could include using a larger fish test-species, such as the rainbow trout, and conduct time-dependent metabolomic analysis (i.e. sampling blood plasma every 24 hours) both before and during pharmaceutical exposure; each fish would thus serve as its own control. This approach is often used in human-based pharmacological metabolomic studies as effectively each patient serves as their

own control, thus dramatically reducing variation levels (Clayton *et al*, 2006). There are however, ethical implications for such a study. The use of in-bred fish also presents problems in the interpretation and relevance to the environmental situation.

Considering the physiological impact of propranolol on the adult male fathead minnows, it was surprising that it was not possible to extract a robust and sensitive biomarker of propranolol exposure. One principal limitation of using NMR is that it detects in the range of 50-100 metabolites (Skoog *et al*, 1998), whereas a fish metabolome contains probably more than 2000 metabolites, and so NMR is only able to examine 2.5-5% of all metabolites. As such, there is a high likelihood that the metabolic response of a particular tissue/sample may not be detected by NMR. Of the metabolites that NMR can detect, it is possible that NMR is too sensitive and so subtle changes in metabolites are masked by the large variations between individual tissues.

Another potential limitation of this study was that only polar metabolites were extracted in the methanol stage. Assuming propranolol has a similar mode of action in fish as it does in humans and higher vertebrates, one may assume that propranolol interacts with the β_3-AR in the fathead minnow, which is located in the adipose tissue, as well as other tissues. The β_3-AR plays a key role in lipolysis and in response to interacting with propranolol, there may have been measurable changes in triglyceride and fatty acid concentrations (Rühling *et al*, 1980). It was not possible to measure these metabolites, and thus examine a potential direct mode-of-action of propranolol as they are non-polar and as such were extracted in the chloroform stage of metabolite extraction. Considering the level of variation observed in both metabolomic studies, it is difficult to postulate at this stage whether any significant changes would have been observed using multivariate analysis on non-polar metabolites. Regardless, it is important in the future to examine both polar and non-polar metabolites from samples to gain a better understanding of metabolic responses to pharmaceutical exposure.

Perhaps the most realistic and long-term advantageous tactic for expanding our knowledge would be to firstly examine and understand the potential routes and causes of

natural biological variation and explore the possibility of using biofluids such as plasma, which is not as heavily influenced by sudden metabolic changes brought on by stressor factors (Polkowska *et al*, 2004). Ekman *et al* (2007) examined fathead minnow urine and plasma, however because of the small volumes analysed, the length of NMR analysis was considerably longer than for analyzing liver tissues and the subsequent measured RSD levels were 52.9% and 62.6%, respectively. This therefore provides an additional motive for using larger fish test-species.

In conclusion, when considering firstly the limitations of NMR and secondly the possibly high inherent variation between tissues from a small fish test-species, ^{1}H NMR metabolomics certainly offers promise for future studies in examining for a response of a fish test-species to an environmental stressor. This study has not only provided further proof-of-principle that it is possible to identify a biomarker of pharmaceutical exposure using ^{1}H NMR and subsequent univariate statistical analysis, but it has (perhaps more importantly) also addressed current problems and made valuable recommendations for improvements to the use of metabolomics in ecotoxicological studies.

CHAPTER 5: EXAMINING CHANGES IN FATHEAD MINNOW β_{3bi}-AR EXPRESSION IN RESPONSE TO PROPRANOLOL EXPOSURE

5.1 INTRODUCTION

The polymerase chain reaction (PCR) allows the operator to amplify and subsequently identify a particular gene of interest from a complex sample and is widely used in molecular biological investigations (Lodish *et al*, 2005). Furthermore, the PCR is a robust, relatively cheap, sensitive technique and has therefore been utilised in a wide range of studies including those of an aquatic toxicology nature (Halm *et al*, 2002). The result from a PCR experiment, which is visualised on an agarose gel under U/V conditions illustrates whether the sample in question possesses and potentially expresses the gene of interest. There are, however, limitations to using a standard PCR reaction. For example, the whole process of extracting RNA (for reverse transcription PCR), reverse transcribing it to cDNA, running the PCR, collecting the samples and subsequently visualising them on an agarose gel is time consuming and cannot be achieved through an automated process. Secondly, results are entirely based on discriminating different sized products on an agarose gel, which can be very subjective and imprecise. Finally, and arguably most importantly, a standard PCR does not provide a quantitative measure of gene expression; it only tells the operator whether or not it is expressed. Therefore it is not possible to examine whether a particular gene is up- or down-regulated under different conditions.

Real-time PCR (RT-PCR), also referred to as quantitative real time PCR (Q-PCR) is a technique based on PCR, which is used to detect, amplify and simultaneously quantify a target DNA molecule (Nolan *et al*, 2006). In brief, a fluorescent probe is added to a standard PCR reaction which binds with newly formed double-stranded DNA and, upon binding, emits a fluorescence signal which is detected and analysed. The greater the fluorescent signal, the greater the concentration of DNA and therefore the greater the starting expression level of the gene of interest. In addition to providing a reliable quantitative account of expression levels, real-time PCR also allows the user to measure the number of molecules during each exponential amplification stage, which represents a significant time-efficient advantage over a standard reverse transcription PCR (VanGuilder *et al*, 2008).

As such, RT-PCR represents a useful tool to identify gene expression changes in tissues exposed to a range of environmental conditions that may perhaps result in the elucidation of a potential biomarker(s) (Alberti *et al*, 2005).

5.1.1 Types of RT-PCR

There are two types of RT-PCR, the first is relative RT-PCR and the second is absolute RT-PCR. Both techniques allow for gene expression quantification using a small amount of RNA template and are equally sensitive. The difference between the techniques is how the measurements are quantified. Relative RT-PCR determines the changes in RNA levels of a gene and expresses it relative to the levels of an internal control "housekeeping" gene RNA. The theory behind this is that expression levels of housekeeping genes do not vary significantly between the same tissues taken from different treatment groups. Therefore, it is possible to directly compare the levels of the gene in question from different treatment groups with the internal housekeeping gene control (Thellin *et al*, 1999). As such, relative quantification does not require standards with known concentrations. A difficulty with using relative RT-PCR can arise if the treatment variable interacts with the housekeeping gene. For example Runnalls (2005) found that the pharmaceutical clofibric acid interacted with the housekeeping gene β-actin in the fathead minnow and therefore β-actin was not a suitable internal control.

Absolute RT-PCR uses an amplicon of the gene of interest as the control external standard. Once the number of molecules in the defined standard has been measured, a range of standard concentrations (for example 10^1 to 10^7 molecules) are run on each assay plate and their fluorescent levels measured, creating a standard curve. The standard curve is used as an indicator of RT-PCR efficiency. Using the standard curve, it is possible to calculate the number of molecules of a sample per µl. Absolute RT-PCR represents a more efficient and robust technique of quantifying expression levels as the standard RNA used to compare expression levels is the same as the gene RNA of interest and preparation of samples has been uniform. Hence, the standard and sample strands simultaneously undergo reverse transcription and amplification under the same conditions

at the same time, with the same primers, and therefore the experimental variables are the same, thus reducing the potential for errors in quantification.

5.1.2 Use of mRNA in RT-PCR

Traditionally, most RT-PCR studies use total RNA (tRNA) as the starting template as the isolation of mRNA from tRNA requires an additional step and can therefore result in the loss of starting material. However, for any RT-PCR, it is the mRNA that contains all the coded information stored in DNA and, thus, it is the mRNA expression level which is actually being measured (Lodish *et al*, 2005). Therefore, when measuring the expression of a potentially poorly expressed receptor in a tissue it is advantageous to use mRNA as the starting template as this will reduce potential background "noise", which is imperative when using an extremely sensitive technique.

5.1.3 Use of RT-PCR in aquatic ecotoxicological studies

Like most techniques, the use of RT-PCR in aquatic ecotoxicological studies has, to date, been primarily used to examine the responses of aquatic organisms upon exposure to endocrine disrupting chemicals (EDCs) including EE_2, testosterone, oestradiol (E_2), polycylic aromatic hydrocarbons (PAHs) and dioxin pesticides (Hoffman & Oris, 2006). Accordingly, investigations have primarily focused on examining the responses of genes known to play a key role in reproduction, growth and development on exposure to EDCs. Kohno *et al* (2008), for example, observed differences in the expression of gonadal oestrogen receptors ESR1 and ESR2 belonging to wild juvenile American alligators caught in different Florida wetlands. Male juvenile alligators taken from a renowned pesticide-contaminated lake exhibited higher levels of testicular ESR1 and ESR2 mRNA than juvenile male alligators from different lakes, suggesting exposure to such contaminants up-regulated oestrogen receptor expression. Furthermore, male alligators demonstrated the same ESR1: ESR2 ratio as females from a reference site. Using RT-PCR, this study began to address the underlying basis for the abnormalities that has been observed in testicular and ovarian biology in alligators from pesticide-contaminated wetlands, which up until then had not been possible. The up-regulation of P450 aromatase mRNA expression in the liver and brain in response to environmental EDCs

have also been examined in the Atlantic salmon (Lyssimachou *et al*, 2006) as has the up-regulation of vitellogenin mRNA expression in the so-iuy mullet (*Mugil soiuy*) (An *et al*, 2006). Both absolute RT-PCR (Lyssimachou *et al*, 2006; Kohno *et al*, 2008) and relative RT-PCR (An *et al*, 2006) techniques were used, indicating that there is not a routinely prefered RT-PCR technique.

Pharmaceuticals often exert their effect by up- or down-regulating mRNA expression and, as such, there have been a handful of laboratory studies that have examined for up- or down-regulation of gene mRNA expression in aquatic organisms exposed to pharmaceuticals. Lange *et al* (2008), for example, exposed roach to concentrations of EE_2 and observed that effects on gonadal development were associated with alterations in expression of estrogen receptor ESR 1 and aromatase CYP19a1b genes and demonstrated that both aromatase and estrogen receptors were associated with sexual differentiation in roach, and alteration in their expression can signal for disruptions in sexual development.

5.1.4 Tissue selection for RT-PCR analysis

The current study was part of a larger EU-collaborative study conducting a risk assessment on propranolol and one of the designated endpoints was the effect on β_1- and β_2-AR expression. As such, selected tissues for RT-PCR analysis had to express all three β-ARs. For this reason, the heart, liver, brain and gonad were selected. Although propranolol is a non-specific beta-blocker, the heart is the major target organ and therefore the heart was selected for RT-PCR analysis. Additionally, propranolol is a highly extracted drug in the liver (up to 95% removal) and so there is likely to be changes in β-AR expression in the liver (Ludden, 1991). In humans and higher vertebrates, β-ARs are found predominantly in smooth muscles around contracting hollow organs (i.e. heart and lungs) and their expression levels in skeletal muscle is, in comparison, minimal. Therefore, to examine for the same distribution pattern in the fathead minnow, the brains were also examined. Finally, Huggett *et al* (2002) demonstrated that propranolol exhibited an inhibitory effect upon the reproductive potential of Japanese medaka (*Oryzias latipes*) at concentrations as low as $0.5 \mu g L^{-1}$. It was possible that propranolol may have interacted with and either up- or down-regulated β-AR mRNA expression in

the gonads, which may subsequently have induced the observed inhibitory reproductive effect. To examine this theory, β-AR expression was measured in fathead minnow gonads.

The male liver and heart tissues were originally selected for metabolomic analysis. Therefore, female heart, liver, brain and gonad tissues were chosen for RT-PCR β-AR expression analysis.

5.1.5 Aim

The aim of the study was to examine the effects of propranolol exposure on β_3-AR expression in selected female fathead minnow tissues using absolute RT-PCR, thereby providing a better understanding of the mode of action of propranolol.

5.2 METHODOLOGY

5.2.1 Tissue acquisition

Female fathead minnow tissues were analysed for β_3-AR expression upon exposure to propranolol. Therefore female fathead minnow liver tisssues were used for the RT-PCR work. All tissue and subsequent mRNA extraction were acquired as described in sections 2.2.1 and 2.2.4, respectively. The female fathead minnow hearts were too small for accurate and reliable mRNA extraction from individual tissues and therefore all the hearts from a single propranolol concentration were pooled together. The liver, brain and gonads were analysed individually.

5.2.2 Exploring β-actin as a housekeeping gene for relative RT-PCR

β-actin is a highly conserved protein in vertebrates including fish. Accordingly, it has previously been used in fathead minnow tissue RT-PCR studies as a housekeeping reference gene (Runnalls, 2005). Although it was not suitable for the study conducted by Runnalls (2005), it was examined whether β-actin could be used as a suitable housekeeping gene for this study as a different test substance was used. Several fathead minnow tissues from both male and female fathead minnows were examined by PCR for β-actin expression. All cDNA levels were diluted so that each PCR reaction contained the same quantity of starting mRNA material. The PCR reactions were finally visualised on a 2% agarose gel under U/V conditions, as shown in figure 79.

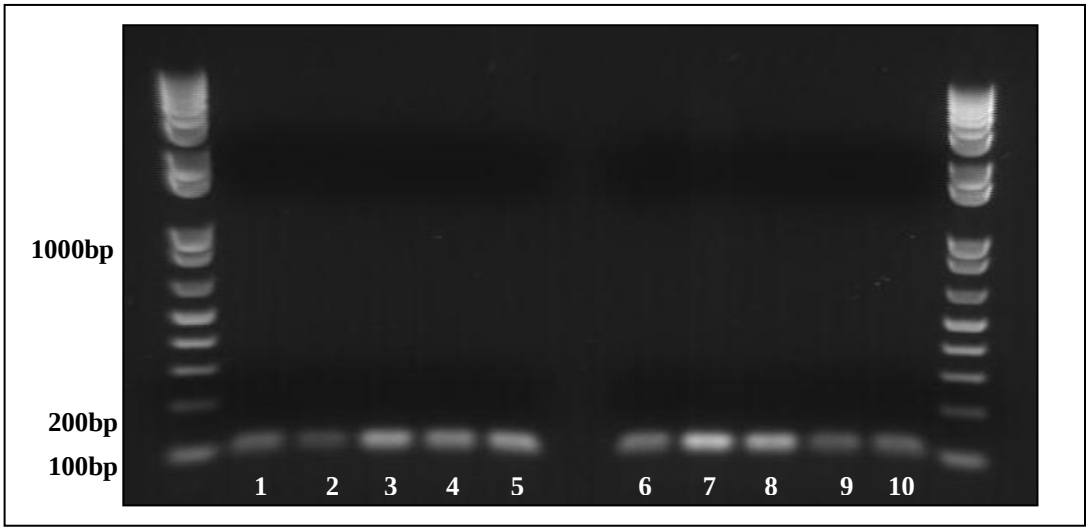


Figure 79. UV photograph of a 2% agarose gel examining for beta-actin expression. 1 = male heart; 2 = male liver; 3 = male brain; 4 = male gill; 5 = male blood; 6 = female heart; 7 = female liver; 8 = female brain; 9 =female gill; 10 = female blood. β-actin was expressed in every tissue at the correct size of 100bp.

Although β-actin was expressed in every tissue, it appeared to be expressed at different levels, which could present a problem when used for analysing and comparing different tissue expression of the β_{3bi}-AR. For this reason, and taking into account the advantages of using absolute RT-PCR, it was decided not to use relative RT-PCR but instead use absolute RT-PCR.

5.2.3 Absolute RT-PCR overview

Absolute RT-PCR was used to quantitatively examine the levels of β_{3bi}-AR expression in female fathead minnow tissues upon exposure to propranolol using the QuantiTect SYBR Green RT-PCR kit (Qiagen; Cat # 204243). During the assay, mRNA was firstly reverse transcribed to cDNA. Subsequently, during the primer annealing stage, DNA was formed at an exponential rate. During this step the SYBR green fluorescent dye bound to the newly-formed double-stranded DNA molecules and emitted a fluorescent signal. The Real Time PCR machine measured the level of green fluorescent signal and indicated the quantity of starting material. In terms of β_{3bi}-AR expression: the higher the expression of β_{3bi}-AR, the more DNA template formed and the more green fluorescence emitted.

Effectively the SYBR green dye will bind to any DNA and therefore care was taken to remove any contaminant genomic DNA from the mRNA samples. Additionally, care had to be taken to ensure that non-specific primer annealing did not occur, as this would produce non-specific secondary DNA products that the SYBR green dye would have bound to, which would have thus produced a incorrectly high result.

5.2.4 RT-PCR primer designing

To minimise the potential for non-specific primer binding, primers were designed to the 3' untranslated region (UTR). For a high efficiency RT-PCR, the amplicon (target DNA region that will be amplified) should be as short as possible, however it must be longer than 75 nucleotides so that it can be distinguished from primer dimers. The amplicon must not have any secondary structures and the guanine: cytosine (G: C) content should be between 40 and 60%.

Both primers should be between 18 and 30 nucleotides in length (the longer the primer the less likelihood of non-specific binding) and have similar annealing temperatures of ideally 60^{o}C. Each primer should also have a G: C content of between 50 and 60% and have no more than two guanine or cytosine nucleotides in the last five bases, yet end with either a guanine or cytosine nucleotide. Additionally, both primers should be significantly different from each other as to minimise the likelihood for primer dimers occurring.

The Primer Express software, supplied by Applied Biosystems, was used to both locate a suitable amplicon region and find primers that met the outlined criteria. Figure 80 shows the entire β_{3bi}-AR sequence with highlighted 3'UTR, selected primers and anticipated amplicon region and table 31 gives details on the primers and amplicon region. The primers were manufactured by Applied Biosystems as the RT-PCR machine was calibrated to work optimally with these primers and also because the software used to generate the primers was developed by Applied Biosystems.

5’

TGCTTGTGGATCGTTGTCGCTTACAGTCCTCTAGTCCTCGCCATC**atg**acactgcatgctgccgaaacag
aatcaaatgaccaggccaatgcaaggacaccttggtcttggatcaacatggcttagctaatttcatcaggattcgttcacatcccac
tactcatcatgattttgtctatgcacgcgtcttcctcgtcgcacaagacaggtacagctcattgcataccgtctaggttccaaacgag
tgcataggaaatcaggtccatggcacaacaacttgcttcgatgtgcaataacgtcggtgggatgacggcaaggcggaaaagttt
cccggaggcgaccgtccaaattaaccgctgtgaaggagcacaaggccctcaagacgctgggcatcatcatgggcatcttcaca
ctgtgctggctgcctttcttcgtggcgaacatcatcaatgttttcaatcgagatctgctaaccatgtatgtttttcgatatttaaactggtt
aggctacatcaactccagcctcaatcccatcatctactgccgcagcccagagtttcgtgcggccttcaagaaccttctcggctgtc
cctgggtatccccgctgagaatgaacttcctttacaaggagcttcggactcgatgcacttgcttcctgggttccgcagagtcggga
atgcctggatcgttcgagaagccccccacatccccaggggcactgcctggggaagggagcagccagagcagctacaggagc
gaggagccgtcgccgggaccaccgcactccaacggacgcacgttcttcagtgatttctccgagccggagacggagttctgtaa
cctataggagcaggatggc**tga**gagtctctagcatggctgaaatgaaaggacgatgaggaagaagaaacagggaaaaggac
tgacttacatcacagttggcgggactttcccagcagagaccacaag**ggcccagcaaaaaacatcc***atgaaagccatcaggg*
*gagcctgctccaaaactgaaagacagaagagtgcaaagatataaccata***gagaggcaagcactatgggaa**ctttctcac
aaacagctcgtctatggaaagaacgactgaactggtactgttcatgaaggagtgtaacatggagtgatccgatactgctaacgttc
tattaagtactggagcaatccacttcagcccgg 3’

Figure 80. β_3-AR nucleotide sequence. The 3’ UTR is highlighted by the yellow region. The two primers are shown in bold red and the amplicon region in italics. The start and stop codon are shown by **atg** and **tga**, respectively.

Table 31. Summary of primer and amplicon nucleotide sequences.

Primer	**Sequence**	**Length (bp)**	**Melting temp**	**GC (%)**	**Conc (nM)**
Forward RT	GGCCCAGCAAAAAACATCC	19	59	53	50
Reverse RT	TTCCCATAGTGCTTGCCTCTC	21	58	52	50
Amplicon		**105**	**79**	**47**	

The primers were run in a PCR using control female fathead minnow liver cDNA to examine firstly whether the primers amplified the desired amplicon region, and secondly to examine for any non-specific amplified products. The PCR products were visually analysed on a 2% agarose gel under UV light, as shown in figure 81. The amplicon was distinguishable from the primer dimer. There was no indication of non-specific binding.

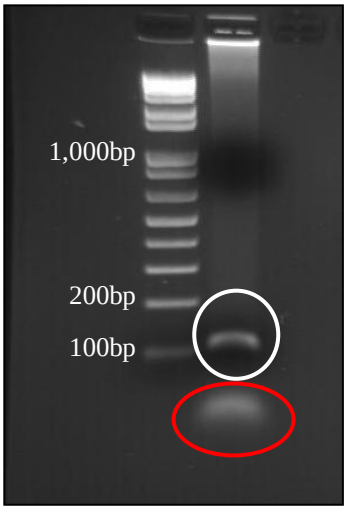


Figure 81. UV photograph of PCR product run on a 2% agarose gel. The white circle highlights the amplicon product, which is the correct size of 105 nucleotides; as compared with the 1Kb DNA ladder. The amplicon was distinguishable from the primer dimer, highlighted by the red circle.

The product was gel extracted and cloned, and the amplicon sequence was confirmed as shown in figure 82. In addition to sequence confirmation, the cloned amplicon was also used to generate the external standard. Thus it was important to determine the insert direction, which was found to be anti-sense and sense for clones 1 and 2, respectively. There was a 100% match between the sequence results and the original sequence.

>colony 1
tcccatagtgcttgcctctc*tatggttatatctttgcactcttctgtctttcagttttggagcaggctcccctgatggctttcat***ggatgttttttgctggg gcc**

>colony 2
ggcccagcaaaaaacatcc*atgaaagccatcaggggagcctgctccaaaactgaaagacagaagagtgcaaagatataaccata***gaga ggcaagcactatgggaa**

Figure 82. Results from sequencing of amplicon product. Red highlights the primers and the italics highlights the amplicon.

5.2.5 Preparation of external standard

The external standard was made from clone 1 with the amplicon inserted in the anti-sense direction. The standard was amplified in a PCR reaction using SP6 (5' ATT TAG GTG ACA CTA TAG 3') and M13R (5' CAG GAA ACA GCA TGA CCA TG 3') primers, which also amplified the T7 promoter region, as shown in figure 83.

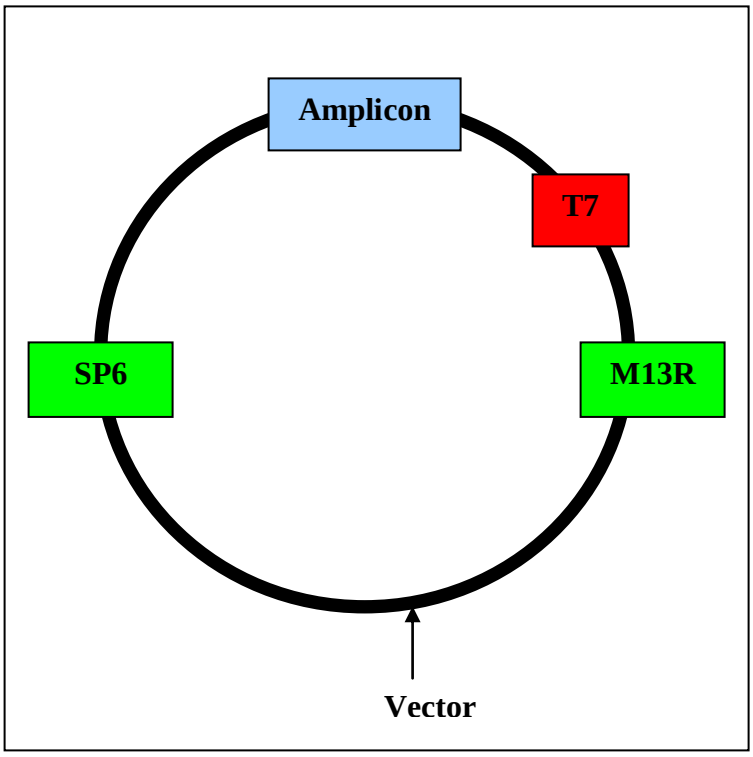


Figure 83. Diagram of pDrive cloning vector that contains the amplicon. Using SP6 and M13R primers, the vector was essentially cut in half so that the transcript and T7 promoter were specifically amplified.

The PCR products were run on a 2% agarose gel and DNA extracted, as detailed in section 2.2.9. The DNA sample was then cleaned using the QIAquick PCR purification kit (Qiagen; Cat # 28104). In brief, 50µl of lysis wash buffer was added to the 10µl of gel extracted PCR product and mixed by reverse pipetting. The mixture was then transferred onto a spin column in a 2ml collection tube and centrifuged at 17,900 x g for 1 minute at room temperature. The DNA product absorbed to the membrane whilst enzyme

contaminants passed through. The flow-through was discarded and 750µl of a second wash buffer, containing ethanol, was added to the spin column and subsequently centrifuged at 17,900 x g for 1 minute at room temperature. The flow-through was discarded and the spin column was centrifuged again at 17,900 x g for 1 minute at room temperature to remove any residual ethanol. The spin column containing the absorbed DNA was transferred into a clean collection tube and 50µl of elution buffer was added to the spin column and left for 1min before centrifugation at 17,900 x g for 1 minute at room temperature. The absorbed DNA was released at subsequently collected in the flow through. The DNA was quantified on the NanoDrop-1000 Spectrophotometer (as detailed in section 2.2.3) and run on a 2% agarose gel for 45 minutes at 80 volts and subsequently visually examined under U/V conditions (see figure 84).

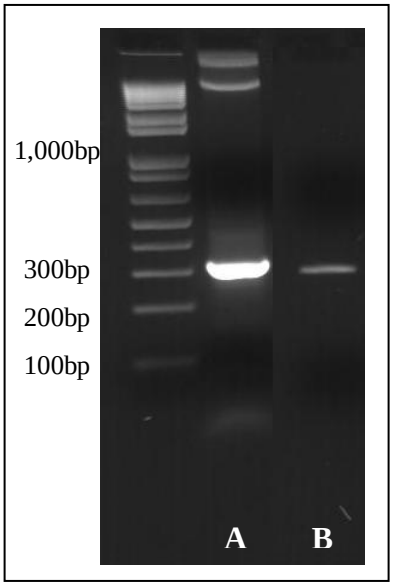


Figure 84. Comparing non-purified (A) and purified (B) gel extracted external standard product. Note there are no additional bands in B. The Amplicon is larger than in figure 79 as it contains additional vector sequence.

The cleaned DNA then underwent in-vitro transcription, creating RNA (to be used as the starting template for RT-PCR work) from DNA using the Riboprobe in vitro transcription systems (Promega; Cat # P1460). As the amplicon was inserted in the anti-sense direction, T7ase was used. 20µl of transcription-optimised buffer, 10µl 100mM DTT,

2.5µl Recombinant RNasin ribonuclease inhibitor, 20µl of rNTP, 2.22µl of T7ase and 43µl of nucleasefree water was added to 2µl of DNA sample and incubated at 37^{o}C for 2 hours. The newly made RNA was subsequently treated with 4µl DNAse I enzyme (Invitrogen; Cat # 18047019), to remove any contaminant DNA, and incubated at 37^{o}C for 15 minutes. The sample was then cleaned to remove any salts and enzymes and precipitated to concentrate the RNA by adding 100µl T.E buffer, 10µl 4M lithium chloride and 300µl 100% ethanol to the sample and storing it at -20^{o}C for 1 hour. The sample was subsequently centrifuged at 17,900 x g for 10 minutes at 4^{o}C. This produced a concentrated RNA pellet. The supernatant was removed and discarded. To remove any residual enzyme contaminants, 500µl of 75% ethanol was added to the precipitate and the sample centrifuged at 17,900 x g for 10 minutes at 4^{o}C. The supernatant was removed and the pellet left to air dry for approximately 5 minutes. The pellet was subsequently reconstituted in 50µl of dH_2O. The RNA sample was quantified on the NanoDrop-1000 spectrophotometer and visually examined on a polyacrylamide gel.

5.2.6 Examining RNA using a polyacrylamide silver staining procedure

The problem with examining RNA on an agarose gel (which is usually used to visualise DNA) is that RNA forms extensive secondary structures via intra-molecule base pairing, which prevents it from migrating strictly according to nucleotide sequence size. One commonly used alternative method of visually examining RNA is to firstly treat the RNA with formaldehyde, which removes the secondary RNA structures and subsequently run the RNA on a formaldehyde agarose gel, which provides a better resolution. However, the problem with this is, firstly, formaldehyde is presents a health and safety hazard (when considering the large volumes) and, secondly the results are not always robust and reproducible (Southern, 1975).

Therefore, a silver staining polyacrylamide gel technique modified from that first developed by Switzer *et al* (1979) was adopted for this study to visually inspect the RNA, as shown in figure 85. A polyacrylamide: urea gel was made from 4ml 10 x TBE buffer (see appendix 1), 19.2g urea (Fisher Scientific, Cat # BPE 169-212), 10ml 40% polyacrylamide gel (National Diagnostics, Cat # EC-850) and made up to 40ml with

dH_2O. Glass plates were firstly cleaned with acetonitrile (Sigma, Cat # 34967), which is a powerful solvent that dissolves protein and nucleic matter. Between each plate, two strips of 4mm thick waterproof sheeting were laid at either end and the plates held together with bulldog clips. 20ml of the polyacrylamide: urea gel was aliquoted into a sterile falcon tube (Fisher Scientific, Cat # 661347) and 125µl 10% ammonium persulfate (APS) (Fisher Scientific, Cat # 7727-54-0) and 16µl tetramethylethylenediamine (TEMED) (Fisher Scientific, Cat # 110-18-9 was added to induce polymerisation of the gel. The gel was then pipetted into a gap between the plates and, through capillary action, dispersed between the plates. A comb was subsequently added to the top of the plates and the gel was left to set. One litre of 0.5 x TBE buffer was prepared and added to the positive and negative gel electrophoresis reservoirs. Once the gel had set, the comb was gently removed and each well rinsed with dH_2O to remove any bubbles. The gel-containing plates were then clamped to the gel electrophoresis stand and buffer topped up so that there was contact between both the positive and negative buffer reserviors and the gel. An aluminium plate was placed across the plates to ensure that there was even distribution of electrical current across the gel. The electrophoresis set-up was connected to the anode and cathode on the power pack and the gel was pre-run at 700 volts.

1µl of sample RNA was diluted in 50µl RNA buffer (8ml 100-% formamide (Fisher Scientific, Cat # 75-12-7)), 20µl 0.5M EDTA (annex), 0.5ml 1% Bromophenol Blue (Fisher Scientific, Cat # 62625-28-9), 0.2ml 2.5% xylene cyanol (Sigma, Cat # 220-167-5) and 1.6ml dH_2O to produce a working concentration of 0.06pmol. Two replicate RNA samples were examined. 1µl of DNA ladder was also added to 20µl RNA buffer. The RNA and DNA ladder samples were subsequently denatured at 85^oC for 2 minutes.

Urea dissociates in a gel and can therefore sit at the bottom of a loading well preventing the migration of a loaded RNA sample. Therefore prior to loading the RNA samples, each well was washed with TBE buffer. Each sample (including the DNA ladder) was loaded onto the gel at three different volumes: 2.5µl, 5µl and finally 10µl. The gel was run for 3 hours at 700 volts. During this time the first wash buffer (see table 32) was prepared.

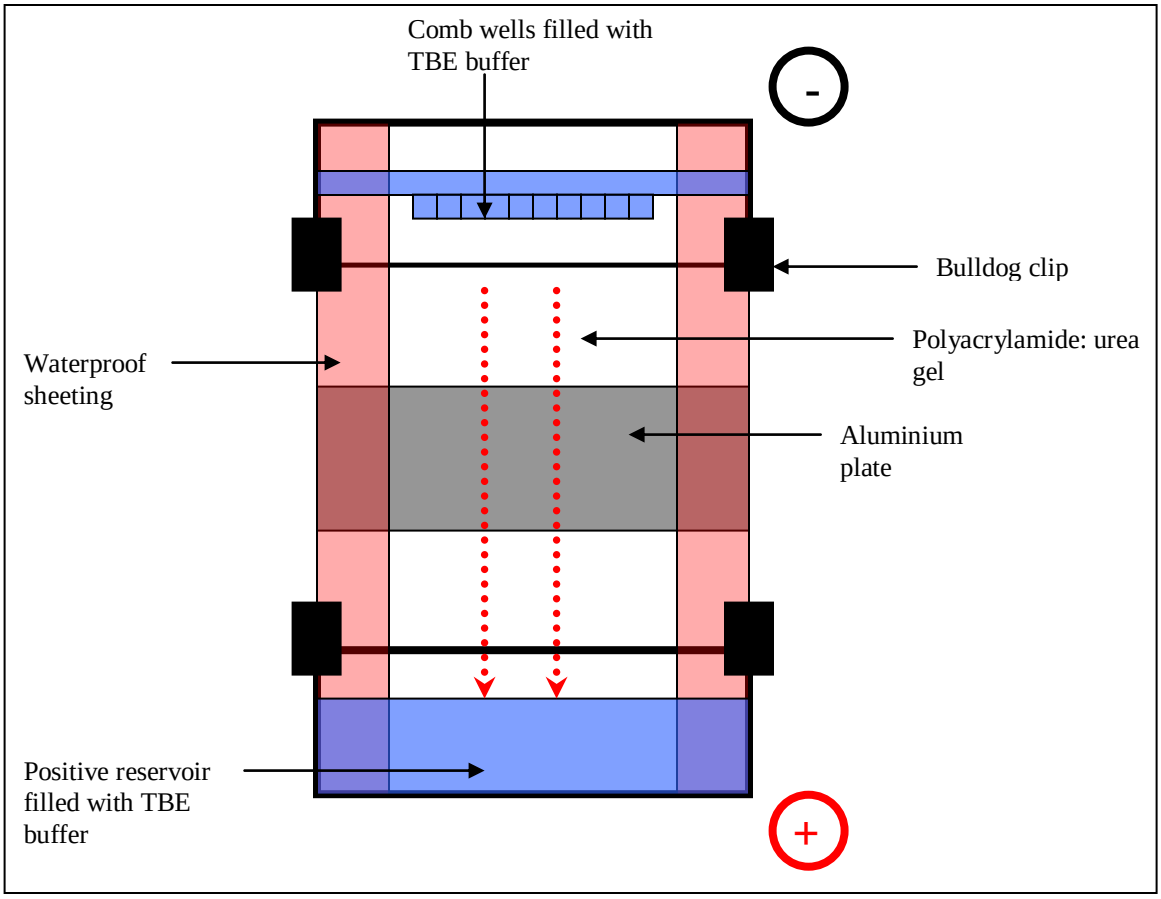


Figure 85. Schematic illustration of polyacrylamide: urea gel set-up. The red arrow indicates the movement of RNA down the gel from the negative cathode to the positive anode.

After 3 hours, the top plate was gently removed and the polyacrylamide gel carefully transferred into an acetonitrile-washed glass dish. The gel was subsequently washed in several consecutive reagents, as shown in table 32.

Table 32. Wash protocol for silver staining of polyacrylamide gel. The total volume for each wash stage was 500ml. Fisher Scientific supplied all reagents: 95% methanol (Cat # A456-1), 99.9% acetic acid (Cat # A/0360/25), 95% ethanol (Cat # E/0500/17), silver nitrate (Cat # S/1240/48), sodium carbonate 0.5M (Cat # J/7520/17), 10% formaldehyde (Cat # F/1451/25).

Wash stage	Wash Buffer	Time	Reason
1	40% methanol (v/v) 10% acetic acid (v/v)	30 minutes	Wash off urea and fix RNA
2	10% ethanol (v/v) 5% acetic acid (v/v)	15 minutes	Wash off urea
3	10% ethanol (v/v) 5 % acetic acid (v/v)	15 minutes	Wash
4	dH_2O	Quick wash	Remove alcohol
5	12mM silver nitrate (v/v) (Cat # S/1240/48)	30 minutes	Stain RNA
6	dH_2O	Quick wash	Removal residual silver
7	0.28M Sodium carbonate (v/v) 0.0185% formaldehyde (v/v)	Quick wash	Remove silver from surface of gel
8	0.28M Sodium carbonate (v/v) 0.0185% formaldehyde (v/v)	Quick wash	Develop RNA
9	5% acetic acid (v/v)	15 minutes	Fix RNA and stop reaction with sodium carbonate

After the final wash, the gel was scanned, as shown in figure 86. The RNA from each of the three loading concentrations was very clean and of the correct size. There was no DNA contamination and so it was decided that the RNA was suitable as an external standard for RT-PCR work. The RNA external standard was then separated into 3µl aliquots and immediately stored at -80^{o}C to prevent any RNA degradation. A single aliquot was used for each RT-PCR assay.

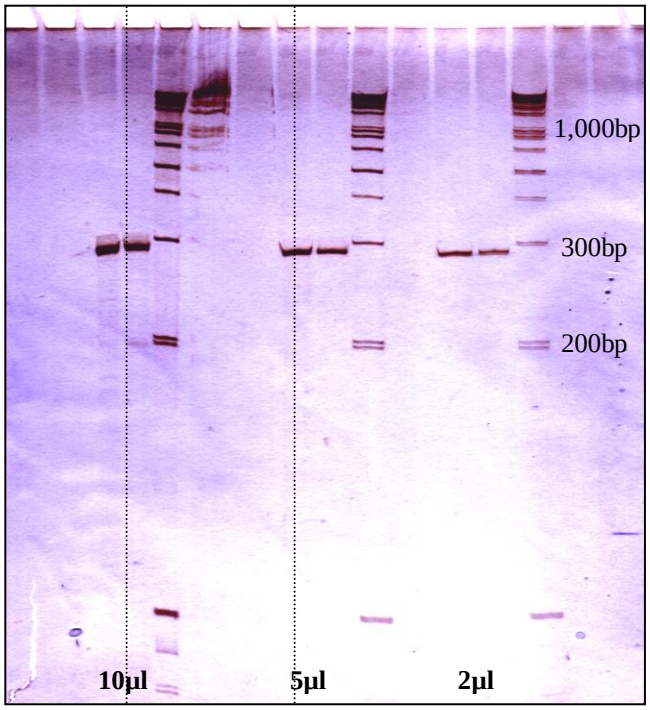


Figure 86. Scanned image from silver staining of polyacrylamide gel. The bands are of the correct size (~280 nucleotides) and were consistent across the different loading concentrations. There was no DNA contamination.

5.2.7 RT-PCR assay

As the first stage of running the RT-PCR assay was to use a serial dilution of RNA standard and examine the efficiency of the assay, the number of molecules was calculated using the equation shown in figure 87.

$$\frac{\text{Xg/µl RNA}}{[\text{transcript length} \times 340] \times 6.022 \times 10^{23}} = \text{Y molecules/µl RNA}$$

Figure 87. Equation used to determine the number of RNA molecules per µl of sample in the external standard. Xg/µl refers to the concentration of RNA and transcript length is the inserted product from, and including, the SP6 to M13R promoters. There are 6.022×10^{23} molecules per mole of RNA.

Prior to examining the RNA samples, the RT-PCR assay conditions were optimised using the external standard.

5.2.7.1 Optimising conditions using the external standard

All surfaces, pipettes, microcentrifuge tube holders and the centrifuge were cleaned with 75% IMS and wiped down with RNase way to remove any sources of DNA or RNA contamination. All components were thawed slowly on ice. The external standards were made by a serial dilution so that the working concentrations ranged from 10^7 to 10^2 molecules. The stock standard of 10^9 molecules was made by adding 2.4µl of sample to 1ml of dH_2O. The sample was mixed thoroughly by reverse pipetting and flicking, before being placed on ice for at least 5 minutes. The first standard of 10^7 molecules was then made by adding 1µl of the stock standard to 100µl dH_2O and subsequently mixing the sample thoroughly and placing on ice for 5 minutes. The subsequent serial dilutions were each made by adding 10µl of previous sample to 100µl dH_2O, creating a 10 x serial dilution each time. Each standard was thoroughly mixed and placed on ice for 5 minutes.

The external standards were run on every single plate, including those with RNA samples as they provided an indication of RT-PCR efficiency of RT-PCR.

RT-PCR assays were made in 96-well plates. The master mix was made by adding the following components (final concentration in brackets); Quantitect SYBR green enzyme (1x), forward primer (0.5µM), reverse primer (0.5µM) (as described in section 5.2.4), RT-PCR mix (0.5µl per reaction), RNase-free water (make up to 20µl per well). The master mix was mixed thoroughly by reverse pipetting and briefly centrifuged at 17,900 x g at room temperature before adding 20µl of mix into each well. This was not conducted on ice as some methods suggest that making the master mix on ice can create primer-dimers. Triplicate 5µl samples of RNA standard or sterile water (also referred to as the non-template control or NTC) were added to the designated well and a clear cover was stuck down onto the plate. The plate was then transferred to the RT-PCR machine on ice, centrifuged briefly at 13,000 x g at 4^oC before being loaded onto the RT-PCR machine.

Figure 88 shows the original RT-PCR protocol used. The annealing temperature was set at 55°C.

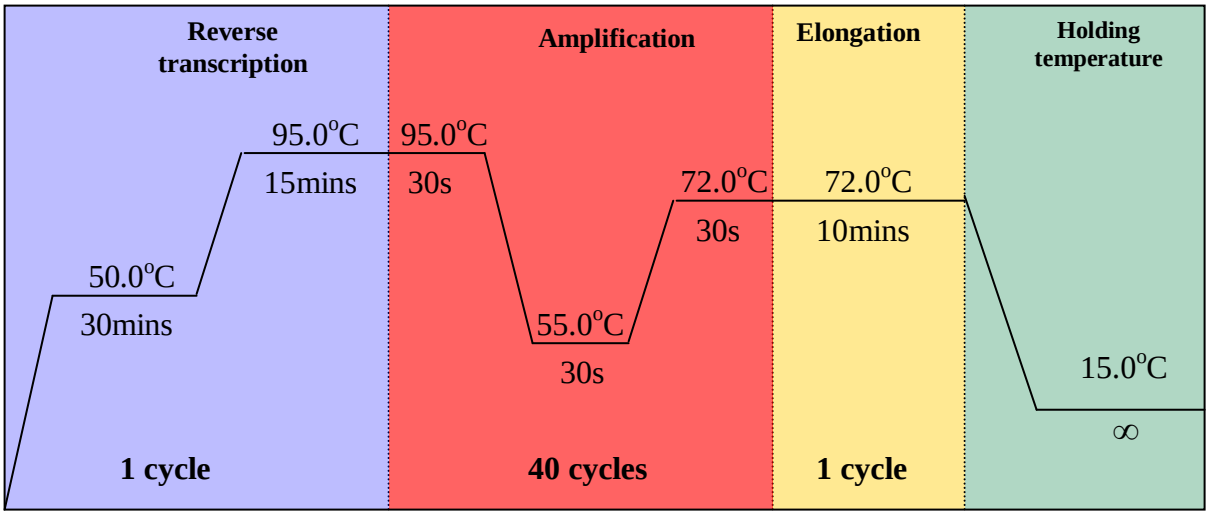


Figure 88. RT-PCR protocol for external standard.

5.2.7.2 Interpretation of results

The NTC was firstly examined for any amplification. Any fluorescence seen in the NTC suggested that there was DNA contamination and so the RT-PCR assay would need to be repeated. Secondly, the wells were examined for any primer dimer formation. It was important to remove the potential for primer-dimers as these would produce an incorrectly high expression value. The efficiency of the RT-PCR assay was calculated from the serial dilution standard curve gradient and calculated according to figure 89. RT-PCR efficiency must be greater than 90% for each plate. All RT-PCR assay work was analysed using SDS 2.1 (Applied Biosystems), this had been previously configured to the RT-PCR machine.

1. $$\frac{1}{-(\text{slope})} = a$$

2. $$[(10\ x\text{^}y^{a}) - 1] \times 100 = \text{Efficiency (\%)}$$

Figure 89. Equation used to determine efficiency of RT-PCR assay.

Figures 90, 91 and 92 show the standard curve plot, amplification plot and dissociation curve, respectively, from the first RT-PCR assay. The calculated RT-PCR efficiency was 90% and was therefore suitable for analysis. The amplification plot compares the number of cycles against fluorescence. As cycle number increases, fluorescence increases. The red C_T line represents the cycle threshold, which is defined as the number of cycles required for the fluorescent signal to exceed background noise. Ct levels are inversely proportional to the amount of target nucleic acid in the sample. Once a sample exceeds the cycle threshold, it can be accurately measured (this is more applicable when running unknown samples as in the case of examining standards, the amount of RNA is already known).

The red arrow highlights that there was fluorescence detected in one of the NTC wells, indicating DNA contamination, and so the assay was repeated.

Figure 90. Standard plot for external standard for RT-PCR. Slope and calculated efficiency are also presented. Each triplicate value is shown. There was slight variation at 10^4 molecule concentration.

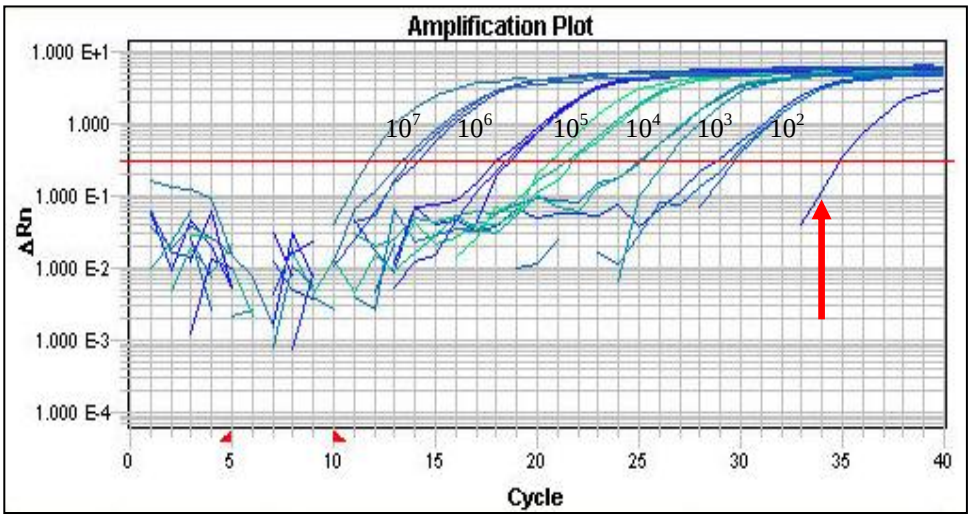


Figure 91. Amplification plot for external standard RT-PCR. Each standard plot molecule concentration is highlighted. The red arrow highlights amplification in the NTC, suggesting contamination. The red line is the automatically set C_T threshold line. ΔRn is calculated from subtracting the signal baseline from the initial stages of PCR from the real time data and it represents the magnitude of the signal.

As SYBR green indescriminately amplifies any double-stranded DNA product, the dissociation curve, figure 91, is used to show what products were amplified. The large peak represents the desired amplicon. However, a primer-dimer (circled in yellow) has also been formed. In addition, the smaller peak (circled in red) shows the NTC amplification, supporting the amplification plot observation and further suggesting that there was DNA contamination in that single well.

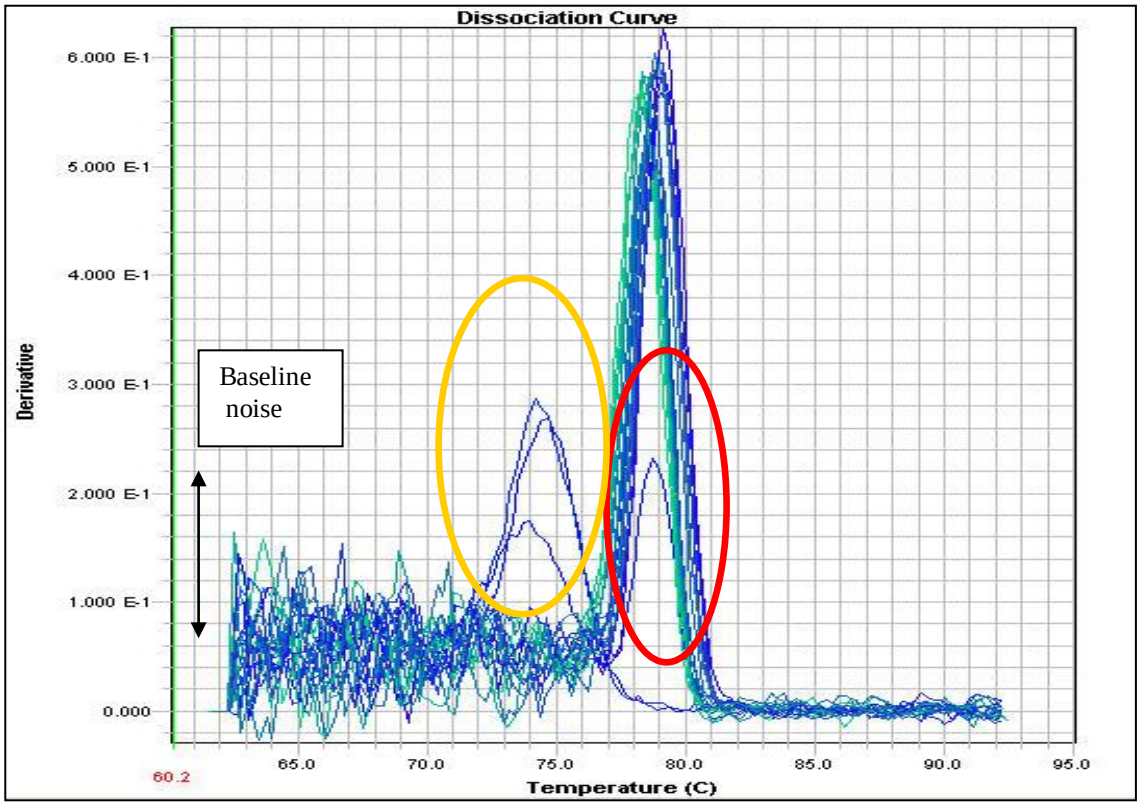


Figure 92. Dissociation curve for external standard RT-PCR. Large peak represents the desired amplicon. Red highlighted circle peak represents NTC DNA contamination, yellow highlighted circle peak represents primer dimer. Double-ended arrow represents the level of baseline noise.

A second RT-PCR assay was set up to explore minimising primer-dimer formation. Primer concentrations were diluted 1:1 (1µl primer to 1µl dH_2O), 1:4, 1:9 and 1:19. Each primer concentration was examined at all serial dilution concentrations. Figure 93A to D shows the dissociation curve results from the 1:1, 1:4, 1:9 and 1:19 primer dilutions, respectively. The 1:19 and 1:9 dilutions produce far weaker amplicon peaks. The very small peaks are not DNA contamination of NTC but are the 10^2 external standard peaks and 10^2 to 10^4 external standard peaks in figures C and D, respectively. The primer set had been diluted too much for the RT-PCR to work optimally, and this was reflected in the low RT-PCR efficiency values. Although the 1:1 primer dissociation curve (A) had a stronger amplicon peak, the level of baseline noise was higher compared to the 1:4 primer dissociation curve (B) and the 1:4 RT-PCR had a higher efficiency value of 94.3% compared to 92.9%. Therefore, a primer dilution of 1:4 was selected as the optimal concentration.

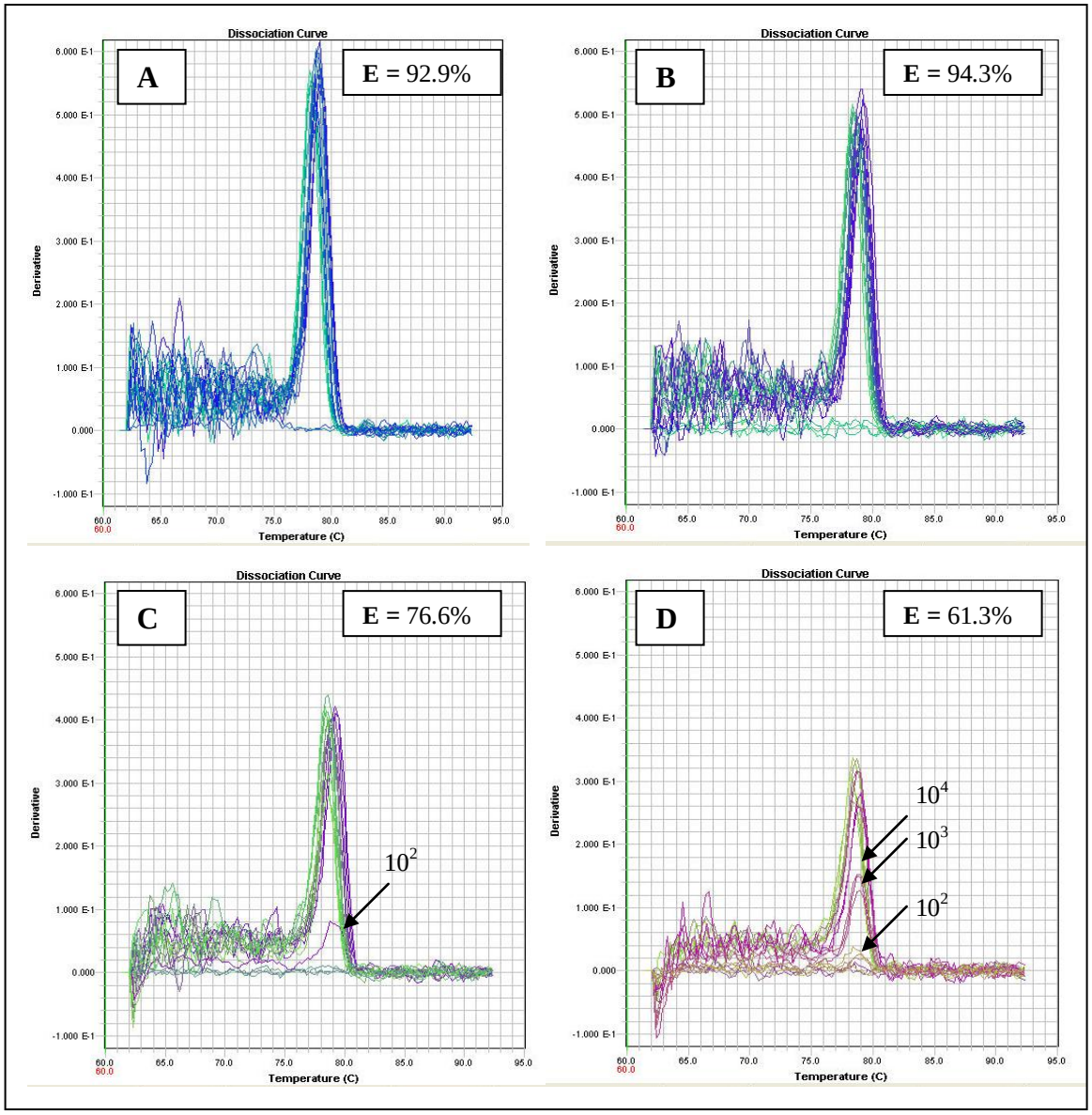


Figure 93. Dissociation curves for 1:1 (A), 1:4 (B), 1:9 (C) and 1:19 (D) primer dilutions. As dilution of primer increases, strength of amplicon peak decreases. The small peaks in figures C and D represent the lower external standard concentrations (as labelled). E values represent efficiency of each RT-PCR assay.

To try and minimise baseline noise, the length of each step in the amplification cycle was reduced from 30 seconds to 15 seconds. Figure 94 shows that there was no improvement in level of baseline noise when the length of amplification cycles was lowered, however the efficiency of RT-PCR was below the acceptable threshold of 90%. Thus, the length of each step in the amplification cycle was maintained at 30 seconds.

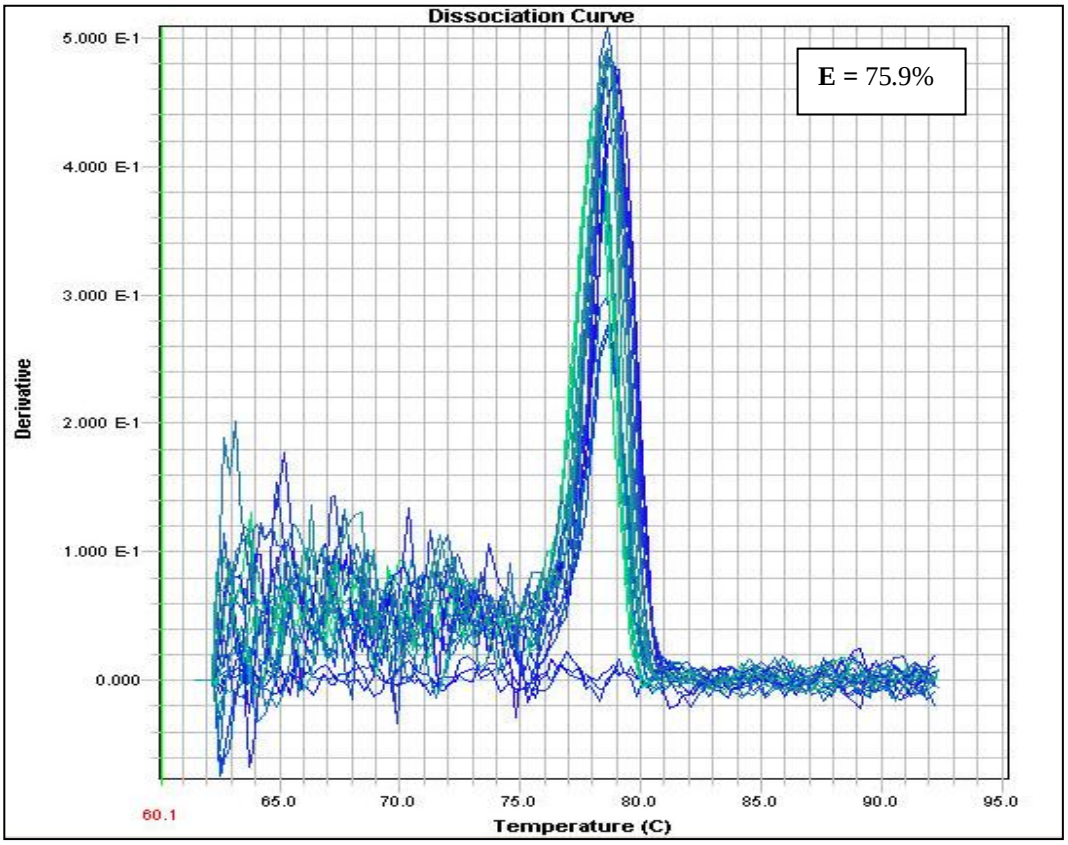


Figure 94. Dissociation for RT-PCR assay when length of each amplification cycle was reduced to 15 seconds. There was no improvement in the level of baseline noise. E value represents efficiency of the RT-PCR assay.

The annealing temperature of the primers was also examined as a potential cause of baseline noise. The annealing temperature was originally set at 55^{o}C, as stipulated by the Applied Biosystems manufacturer. However, the RT-PCR machine works optimally at an annealing temperature of 60^{o}C. Therefore two RT-PCR assays were run, one with an annealing temperature of 57^{o}C (figure 95A) and one with an annealing temperature of 60^{o}C (figure 95B). The level of baseline noise was marginally improved (~0.5 E-1 and 1.0 E-1 for 57^{o}C and 60^{o}C, respectively); however the efficiency of both RT-PCR assays fell below the ideal threshold of 90%. Therefore, the annealing temperature was maintained at 55^{o}C.

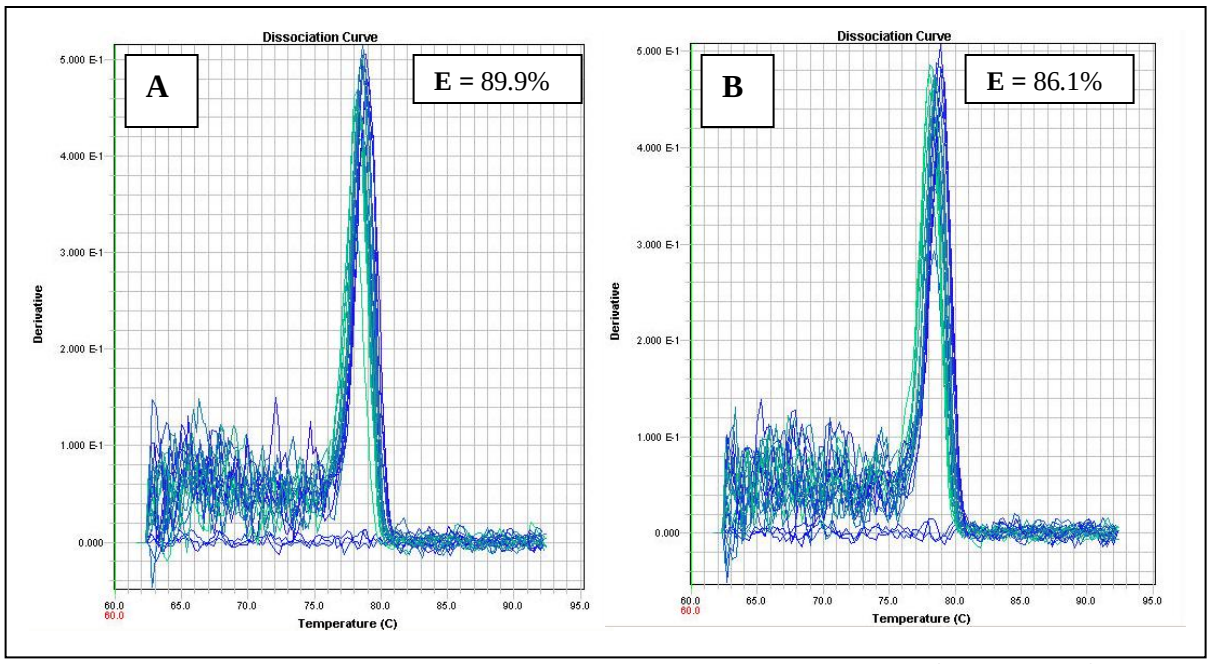
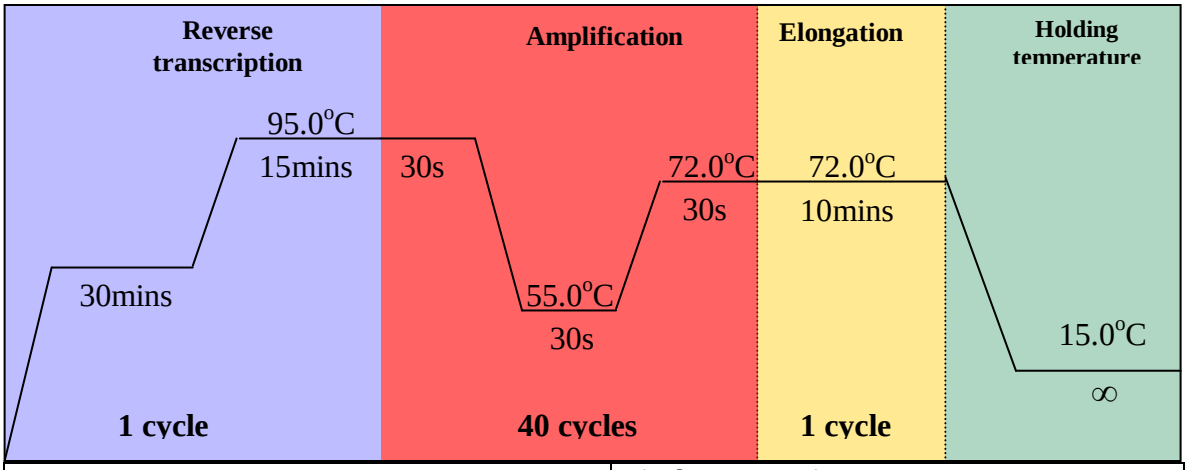


Figure 95. Dissociation curves for RT-PCR assay with an annealing temperature of 57°C (A) and 60°C (B). The baseline noise was marginally improved in both RT-PCR assays compared to the 55°C dissociation curve. However, the efficiency (E) of both assays fell below the threshold of 90%.

The RT-PCR assay conditions selected to examine β_{3bi}-AR expression in female fathead minnows exposed to several concentrations of propranolol is summarised in figure 96.



Component	Final concentration
Quantitect SYBR green enzyme	1 x
Forward primer	0.1µM
Reverse primer	0.1µM
RT-PCR mix	0.5µl per reaction
RNase-free water	Up to 20µl per well

Figure 96. Summary of RT-PCR protocol and component concentration used to examine β_{3bi}-AR expression in female fathead minnow tissues exposed to propranolol.

5.2.8 Preliminary examination of tissue profiles

Prior to examining β_{3bi}-AR expression of all propranolol-exposed tissues, it was firstly important to examine a handful of tissues to make sure there were no previously unforeseen problems. Therefore a small number of tissues were examined at 1, 3, 5 and 10ng/µl to determine which concentration was optimal. It was found that the expression of the β_{3bi}-AR in several tissues at the 1, 3 and 5ng/µl concentration did not fit within the external standard range. Therefore, the RT-PCR assay was repeated using external standards that ranged from 10^1 to 10^5 molecules. Figure 97 shows the standard plot taken from the latter experiment. The majority of the tissue β_3-AR concentrations fitted within this external standard range, the exception to this was from some tissues at the 1ng/µl concentration. It was decided to use the tissue concentration of 5ng/µl as, firstly, β_3-AR expression fitted within the external standard range at this concentration and, secondly, a previous study that examined β_1-AR and β_2-AR expression in the same female fathead minnow samples used a tissue concentration of 5ng/µl (Giltrow, 2008). Therefore, by

using this concentration it was possible to directly compare different β-AR expression levels.

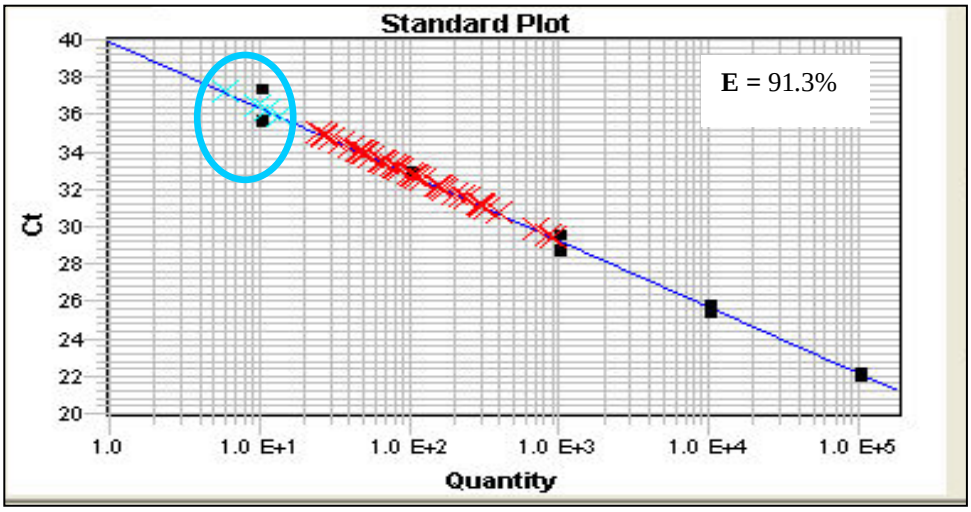


Figure 97. Standard plot for examining β_{3bi}-AR expression in several tissues. External standard concentration ranged from 10^1 to 10^5 molecules. All β_{3bi}-AR expression profiles fitted within standard range (red), with the exception of two tissues highlighted in cyan. Each tissue was analysed in triplicate and is therefore represented by three crosses.

5.2.9 Examining internal standard

In addition to running an external standard to assess the efficiency of each RT-PCR, an internal standard was also used. By analysing the same tissue sample on every plate, it is possible to get a measure of the inter-assay variation which is attributable to assays run on different plates at different times, and using different batches. The internal standard selected for the β_1-AR and β_2-AR expression study (Giltrow, 2008) was a liver sample at a concentration of 5ng/µl. Therefore, the same liver sample was examined for suitability as the internal standard in the current study. Figure 98 shows the standard plot from the RT-PCR assay and demonstrates that it was suitable for use as the internal standard.

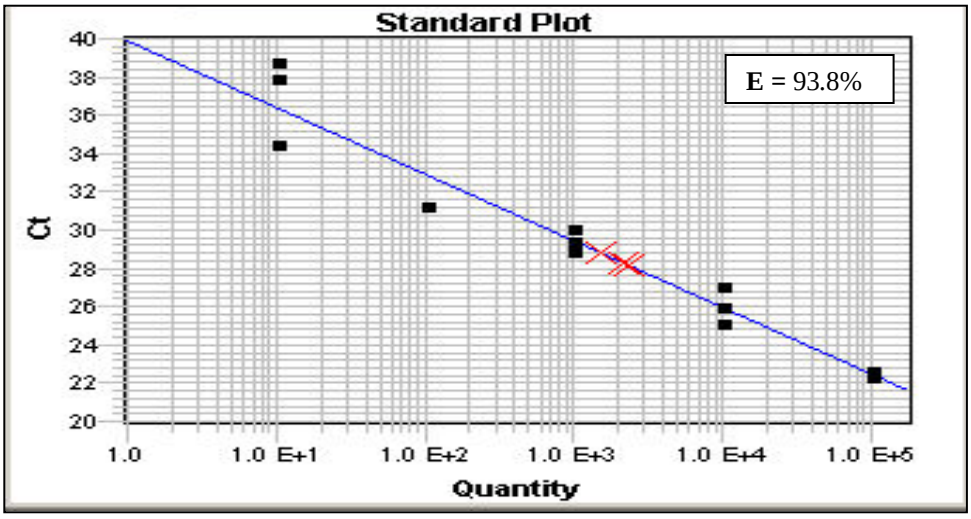


Figure 98. Standard plot taken from RT-PCR assay exploring suitability of liver internal standard at 5ng/µl. The β_{3bi}-AR expression levels fitted within the external standard range of 10^1 to 10^5 molecules. The efficiency of the RT-CPR assay was also above 90%.

5.2.10 Statistical analysis

To compare β_3-AR expression levels between different treatment groups and between control tissues, a Kruskal-Wallis One-Way ANOVA was performed. This is a non-parametric alternative to the commonly used ANOVA.

5.3 RESULTS

5.3.1 Examining external standards

The aim of the study was to examine β_3-AR expression changes in the female fathead minnow brain, liver, heart and gonad tissues exposed to several concentrations of propranolol. It was not possible to examine every tissue on the same RT-PCR assay plate. Therefore it was important that the efficiency of each RT-PCR assay was comparable between each tissue assay plate. To examine the efficiency of each absolute RT-PCR assay, a series of external standards (prepared from a cloned amplicon) were run on every plate. External standard concentrations ranged from 10^1 to 10^5 molecules per µl and each concentration was run in triplicate. RT-PCR efficiency for each assay was calculated from the gradient of the slope on the standard plot, where molecule quantity was plotted against Ct value. There was no significant difference ($p<0.05$) between the Ct values at each external standard concentration between each assay plate, as shown in figure 99, which suggested that amplification efficiency across all assay plates was directly comparable.

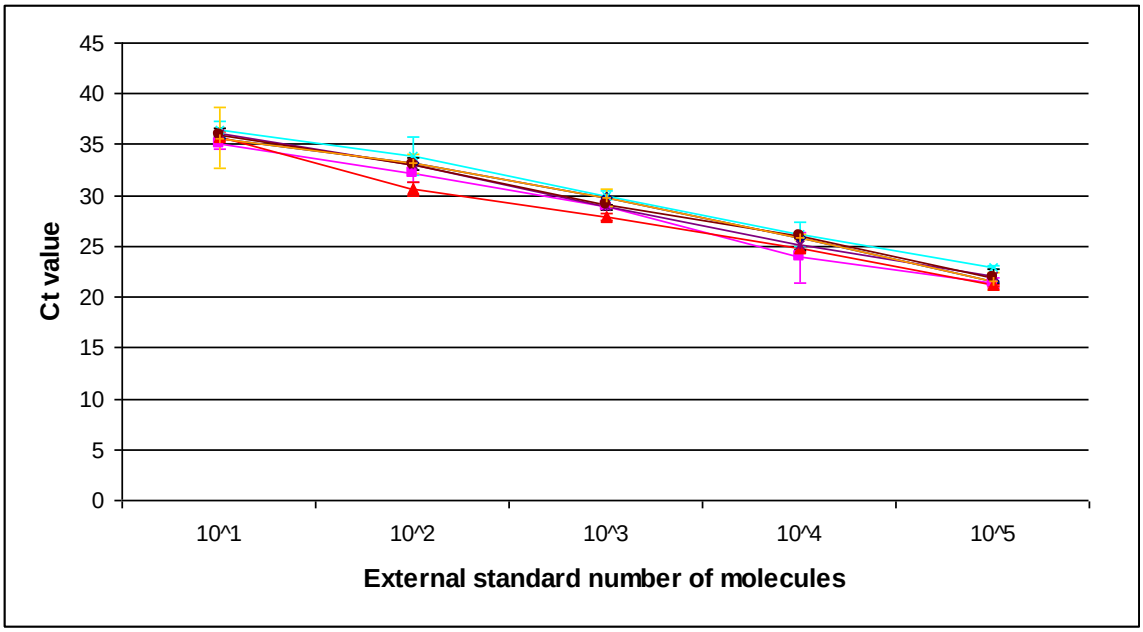


Figure 99. External standard plot of measured C_t value at each designated concentration. ♦ =plate 1; ■ = plate 2; ▲= plate 3; x = plate 4; ♦ = plate 5; + = plate 6; + = plate 7. There was no significant difference between Ct values at each concentration between the different assay plates ($p<0.05$).

5.3.2 Examining inter-assay variation

The inter-assay variation was assessed using a standard liver sample at 5ng/µl, which was included on all plates. As can be seen from figure 100, the mean values obtained ranged from 2600 molecules/µl (plate 4) to 5500 molecules/µl (plate 2), giving a 2.1-fold difference. The levels of internal standard in plates 1, 3 and 4 were significantly lower than plates 2, 5, 6 and 7 ($p<0.05$). The RT-PCR was repeated for plates 1, 3 and 4, however there was only a marginal increase in molecule numbers.

Two RT-PCR plates were used to analyse each tissue from both propranolol exposures (with the exception of the heart). Therefore to accurately compare changes in β_{3bi}-AR expression within tissues, a 2.1-fold difference in molecule numbers must exist between the DWC group and propranolol treatment groups. Similarly, to determine differences in β_{3bi}-AR expression between tissues, a 2.1-fold difference in molecule numbers must be present.

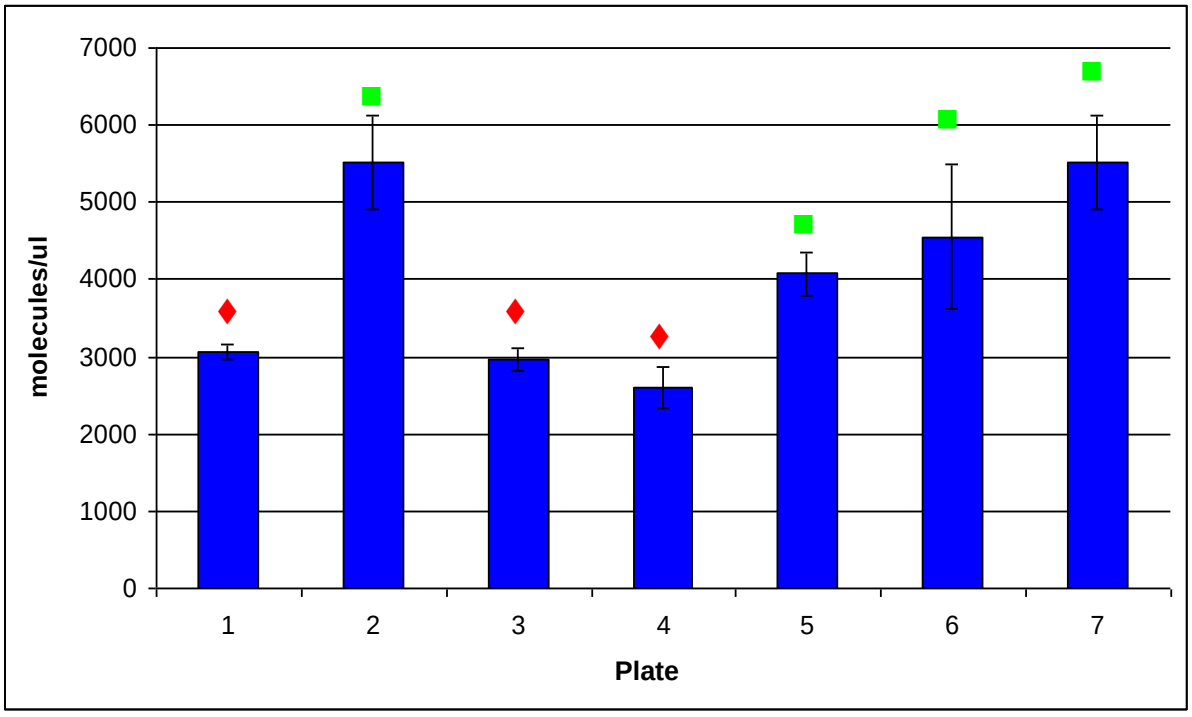


Figure 100. Internal control results for each plate in the β_{3bi}-AR RT-PCR assay. Each symbol denotes no significant difference, for example all plates that have a ■ are not different to each other. The brain samples were run on plates 1 and 2, the liver samples on plates 3 and 4, the gonad samples on plates 5 and 6 and the heart samples on plate 7.

5.3.3 β_{3bi}-AR expression in response to propranolol exposure

Figures 101 to 104 show the fold-changes in β_{3bi}-AR expression at all propranolol concentrations, compared to DWC levels, in the brain, liver, gonad and heart tissues, respectively. There was no significant ($p<0.05$) difference in β_{3bi}-AR expression between propranolol treatment groups and DWC up to and including 0.1mgL^{-1} in the brain (figure 101). However, there was a significant decrease ($p<0.05$) in β_{3bi}-AR expression between 1mgL^{-1} and DWC, with an expression fold-difference of -2.2.

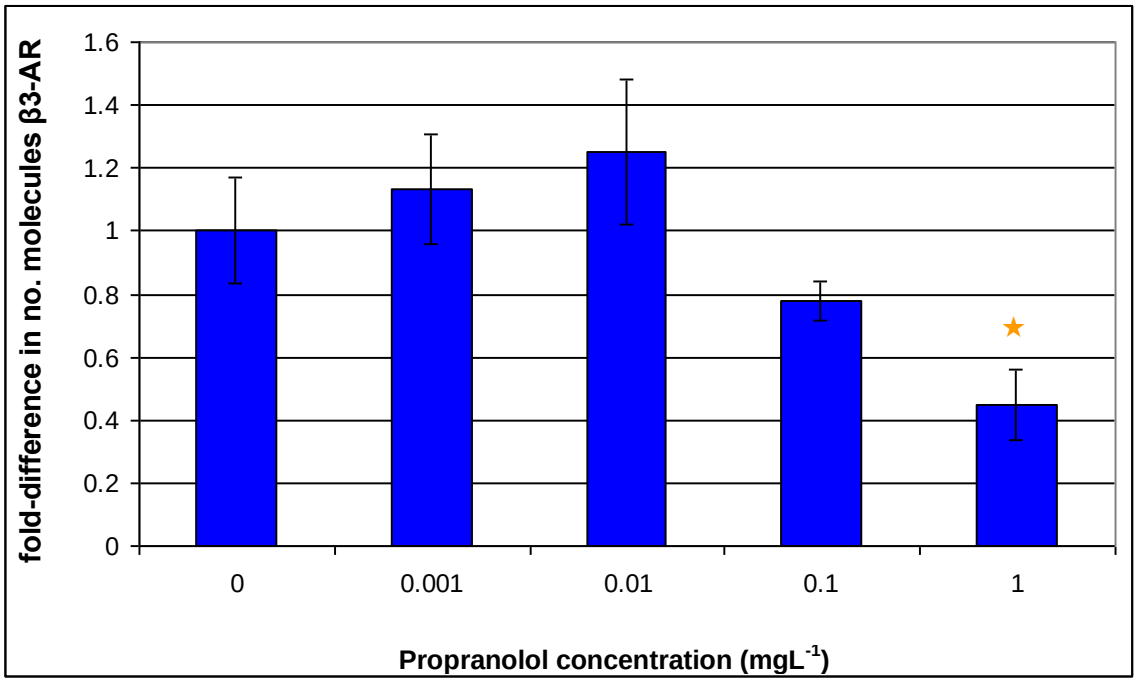


Figure 101. β_{3bi}-AR expression in female fathead minnow brain tissues upon exposure to propranolol. Symbols denote a statistically significant difference (p0.05 and fold-difference ≥ 2.1) between treatment and DWC groups.

Similarly in the liver, a significant decrease ($p<0.05$) in β_{3bi}-AR expression was observed between the 1mgL^{-1} and DWC treatment groups, with an expression fold-difference of -3.2 (figure 102).

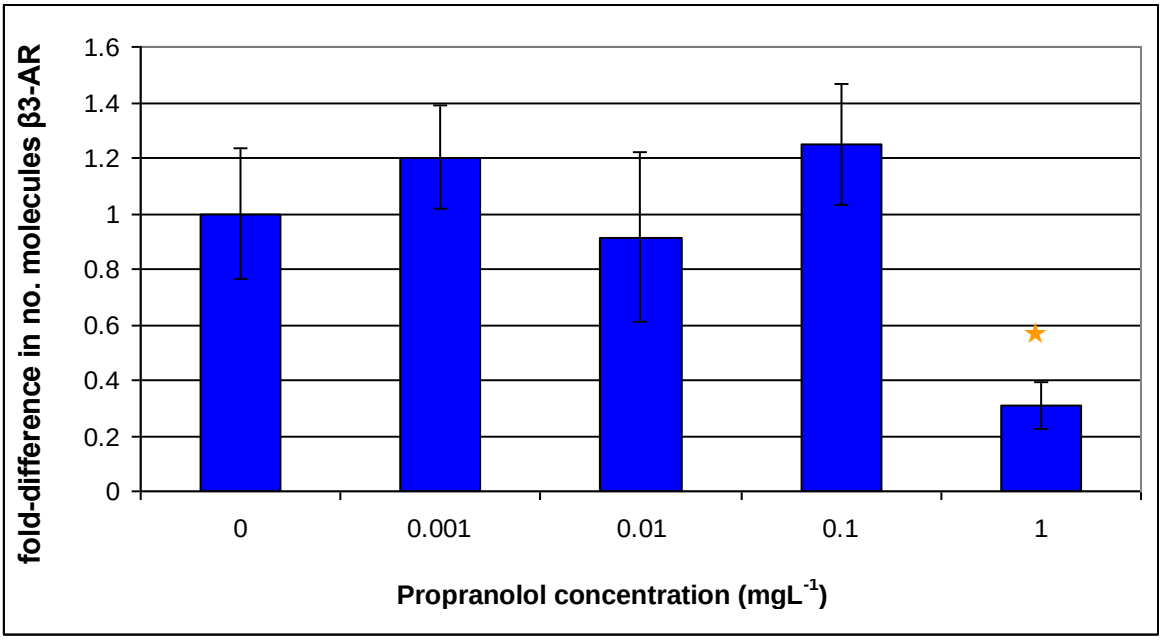


Figure 102. β_{3bi}-AR expression in female fathead minnow liver tissues upon exposure to propranolol. Symbols denote a statistically significant difference (p0.05 and fold-difference ≥ 2.1) between treatment and DWC groups.

Although β_{3bi}-AR expression in the gonad was 2.1-fold lower in the 0.1mgL^{-1} treatment group compared to the DWC group, it was not statistically significantly lower ($p<0.05$) because of considerable variation in β_{3bi}-AR expression in the DWC group (figure 103). There was no significant effect of propranolol on β_{3bi}-AR expression at any concentration, compared to DWC levels.

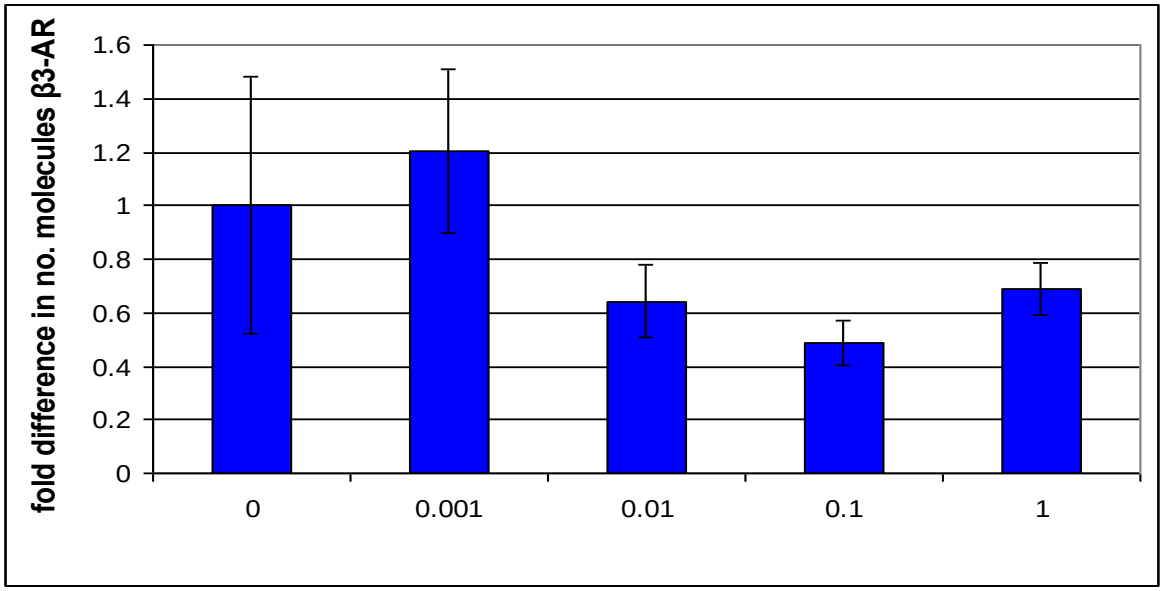


Figure 103. β_{3bi}-AR expression in female fathead minnow gonad tissues upon exposure to propranolol. There was no significant difference ($p<0.05$) in β_{3bi}-AR expression between propranolol treatment groups and DWC.

β_{3bi}-AR expression in the heart demonstrated a biphasic expression response. A shown in figure 104, as propranolol concentration increased from DWC to 0.mgL^{-1}, there was a significant increase ($p<0.05$) in expression with a noted +2.4, +3.1 and +2.6-fold difference in β_{3bi}-AR expression at 0.001mgL^{-1}, 0.01mgL^{-1} and 0.1mgL^{-1}, respectively, compared to DWC β_{3bi}-AR expression. However, at 1.0mgL^{-1}, there was a marked decrease in β_{3bi}-AR expression compared to the other propranolol treatment groups and the expression levels were almost the same as those in the DWC group.

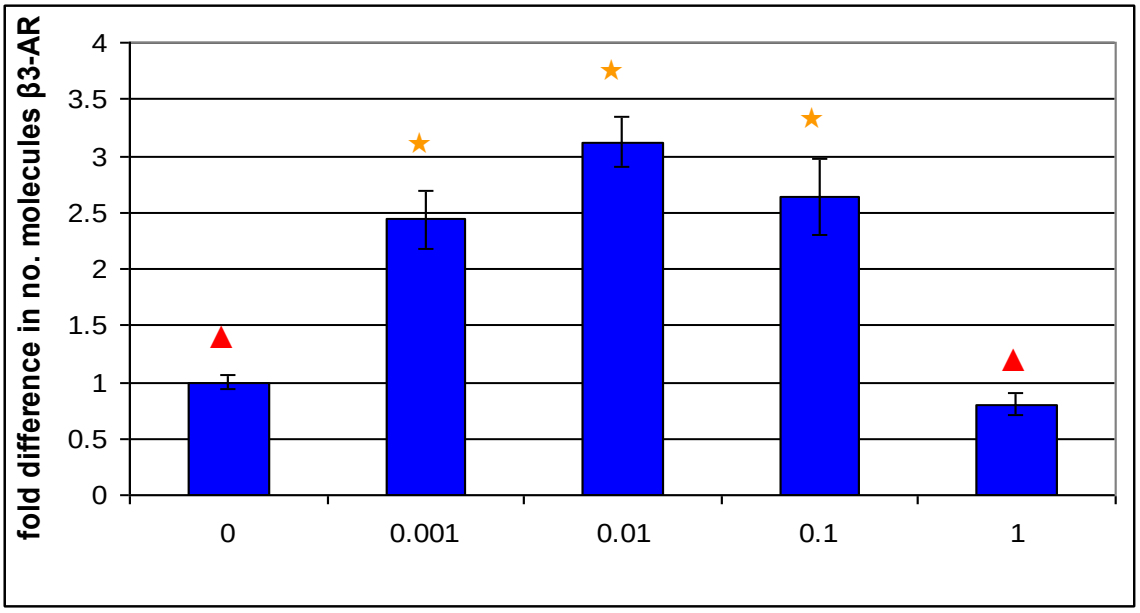


Figure 104. β_{3bi}-AR expression in female fathead minnow heart tissues upon exposure to propranolol. Different symbols denote significant difference ($p<0.05$) between treatment groups.

Table 33 summarises the fold-induction of the β_{3bi}-AR at each propranolol concentration for each tissue and highlights those that were both significantly different ($p<0.05$) to, and had a fold-induction difference of greater than 2.1 (± 2.1) to, DWC tissue β_{3bi}-AR expression levels. Propranolol induced a down-regulation of the β_{3bi}-AR at $1mgL^{-1}$ in both the brain and liver and yet had no significant ($p<0.05$) effect on β_{3bi}-AR expression in the gonad. Whilst in the heart, exposure to lower concentrations of propranolol ($1.001mgL^{-1}$ to $0.1mgL^{-1}$) exerted a significant ($p<0.05$) up-regulation of the β_{3bi}-AR. Although there was a marked decrease in β_{3bi}-AR expression at $1mgL^{-1}$, it was not significantly lower than that in the DWC heart.

Table 33. Summary of fold-induction difference of the β_{3bi}-AR at each propranolol concentration for each tissue. Expression figures highlighted in red represent those that were both significantly different to β_{3bi}-AR expression levels in, and were >2.1-fold different to, the respective DWC tissues.

	PROPRANOLOL CONCENTRATION (mgL^{-1})				
TISSUE	**DWC**	**0.001**	**0.01**	**0.1**	**1**
brain	1	+1.1	+1.3	-1.3	**-2.2**
liver	1	+1.2	-1.1	+1.2	**-3.2**
gonad	1	+1.2	-1.5	-2.0	-1.4
heart	1	**+2.4**	**+3.1**	**+2.6**	-1.2

5.3.4 β_{3bi}-AR expression in control fathead minnow tissues

β_{3bi}-AR expression levels were compared between control female fathead minnow tissues, as shown in figure 105 and summarised in table 34. The β_{3bi}-AR was expressed significantly highest ($p<0.05$) in the heart, at levels of 9800 molecules/µL, which corresponded to a +12.4, +2.5 and +11.7-fold (all of which exceeded 2.1-fold) difference to the brain, liver and gonad tissues respectively. The liver expressed the β_{3bi}-AR at the second-highest levels of 3940 molecules/µL, and this was +5 and 4.7-fold, and significantly ($p<0.05$), higher than in the brain and gonad, respectively. Finally, the brain and gonad expressed the β_{3bi}-AR at considerably lower levels of 840 and 790 molecules/µL, respectively. As there was only a 1.1-fold difference (<2.1-fold) in β_{3bi}-AR expression between the tissues, and as there was not a significant difference ($p<0.05$) in the number of β_{3bi}-AR molecules, it was not possible to distinguish them in terms of preferential β_{3bi}-AR expression.

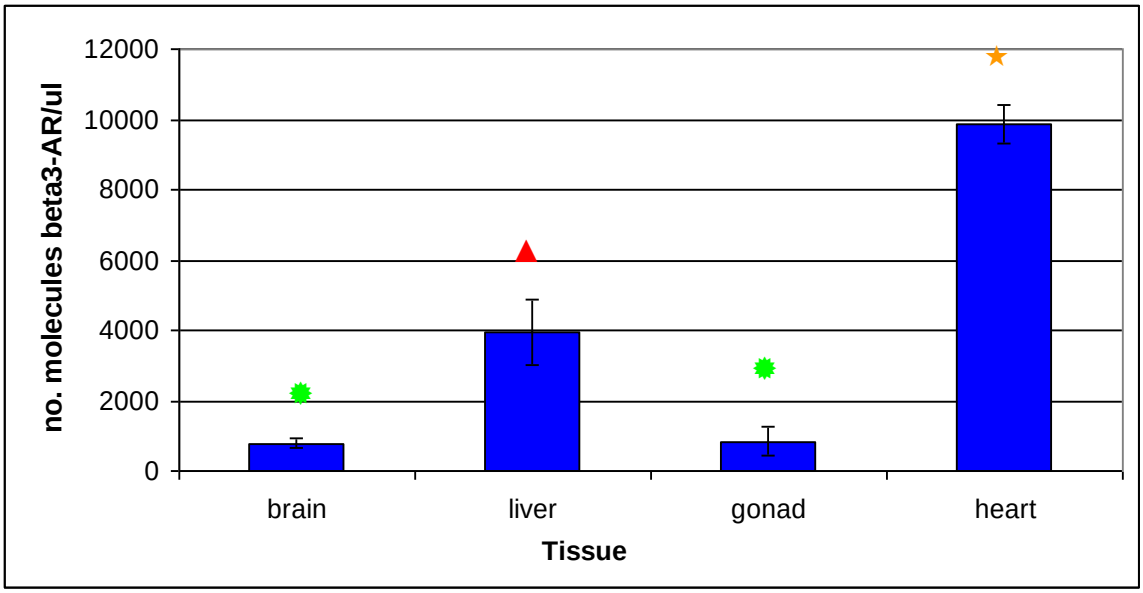


Figure 105. Comparison of β_{3bi}-AR expression between examined DWC female fathead minnow tissues. Different symbols denote statistically similar values.

Table 34. Summary of β_{3bi}-AR expression fold-difference between female fathead minnow control tissues. Figures highlighted in red represent both a significant difference ($p<0.05$) between tissues and a fold-difference >2.1.

	brain	**liver**	**gonad**	**heart**
brain		**5**	1.1	**12.4**
liver	**5**		**4.7**	**2.5**
gonad	1.1	**4.7**		**11.7**
heart	**12.4**	**2.5**	**11.7**	

5.4 DISCUSSION

Analysis of the brain, liver, gonad and heart tissues taken from female fathead minnows exposed to propranolol showed that expression levels of β_{3bi}-AR varied both between tissues and within the same tissues exposed to differing concentrations of propranolol.

5.4.1 β_{3bi}-AR expression in response to propranolol exposure – stress response

As described in chapter 2, fathead minnows exposed to propranolol showed signs of elevated stress, especially at the highest concentration of $1.0mgL^{-1}$. The stress response in teleost fish is very similar to that of terrestrial vertebrates and involve the production of the catecholamines adrenaline and noradrenaline from the brain-sympathetic-chromaffin cell axis (equivalent to the brain-sympathetic-adrenal medulla) and the production of corticosteroid hormones such as cortisol (Bonga, 1997). Cortisol increases blood pressure and blood sugar and reduces the immune response (Weber, 1998). In fish, toxic stressors and pollutants have been found to directly increase catecholamine and cortisol production. This is mainly related to the fact that fish are exposed to aquatic pollutants via the extensive respiratory surface of the gills. The high bioavailability of many chemicals (including pharmaceuticals) in water is an additional factor (Bonga, 1997).

In higher vertebrates (Spronsen *et al*, 1993) and the black bullhead fish (Dugan *et al*, 2008), the β_{3bi}-AR gene contains glucocorticoid-responsive element (GRE) sites and therefore glucocorticoids, such as cortisol, are able to modulate β_{3bi}-AR density as well as β_{3bi}-AR mRNA levels; in response to stress β_{3bi}-AR mRNA expression increases. During the pair-breeding assay, fish from all treatment groups were likely to be stressed under the laboratory conditions and therefore β_{3bi}-AR mRNA expression may have been elevated at all concentrations. Propranolol is a non-specific β-blocking pharmaceutical that binds to all β-AR subtypes and inhibits the response to adrenaline, noradrenaline and cortisol (Fent *et al*, 2006). Therefore, β_{3bi}-AR activity in those fish receiving non-toxic doses of propranolol ($0.001mgL^{-1}$ to $0.1mgL^{-1}$) may have had an inhibited response to elevated cortisol and catecholamine levels, compared to that in control fish. Although propranolol has a weaker binding affinity for β_{3bi}-AR than adrenaline and noradrenaline (Strosberg & Pietri-Rouxel, 1996), there may have been an overall net "muffling" effect

of propranolol on β_3-AR response to cortisol and catecholamine interaction. Therefore, to compensate for the reduced β_{3bi}-AR activity, β_{3bi}-AR mRNA expression may have been increased further in propranolol-exposed fish. It can be clearly seen in the heart that β_{3bi}-AR expression was significantly higher ($p<0.05$) in all propranolol treatment groups, with the exception of the $1mgL^{-1}$ treatment group (which may be due to a toxic desensitisation response as described in section 5.4.2), compared to DWC. There was also an increase in β_{3bi}-AR mRNA expression at $0.001mgL^{-1}$ in the brain, liver and gonad tissues, compared to DWC levels, however they were not significantly ($p<0.05$) higher and they were not necessarily higher at each propranolol concentration. One possible reason for this is that β_{3bi}-AR mRNA expression in the heart was 2.5 to 12 times higher than in the brain/gonad and liver, respectively, and so one is probably more likely to see a visible response to stress in the heart than in the respective tissues.

5.4.2 β_{3bi}-AR expression in response to propranolol exposure – desensitisation response

There was a significant ($p<0.05$) decrease in β_{3bi}-AR mRNA levels at $1mgL^{-1}$ in the brain and liver, compared to DWC levels and also the heart compared to the other propranolol treatment groups. It was unlikely that this response was directly related to the effects of increased stress levels as one would anticipate an increase in β_{3bi}-AR mRNA expression compared to DWC levels. The response observed here is more likely due to propranolol toxicity. At $1mgL^{-1}$, male and female fathead minnows showed signs of acute toxicity (mortality) and therefore it was possible that propranolol was acting as a toxin. Under such circumstances, catechlolamine and cortisol levels were likely to be elevated to such an extent that, considering the β_{3bi}-AR was a weaker binding affinity for propranolol than catecholamines and glucocorticoids, the net response would be the over-activation of the β_{3bi}-AR.

An important regulatory feature of G-protein signalling systems is that they are not constant but exhibit a memory of prior signalling and so the high activation of a receptor leads to a reduced ability to be stimulated in the future, termed desensitisation, and this leads to the down-regulation of the target receptor (Hausdorff *et al*, 1990).

Desensitisation prevents over-stimulation of a receptor and thus serves to "safeguard" against over-stimulation of a cellular signal, which could potentially be harmful (Gainetdinov *et al*, 2004). As such, following activation by an exogenous agonist ligand, most GPCRs undergo regulation by a cascade of events that promote receptor densitisation and endocytosis. Following endocytosis, receptors are recycled to the plasma membrane, retained in an intracellular compartment or targeted for degradation (Martini & Whistler, 2007).

There are two principal types of desensitisation; the first is homologous desensitisation, which is activation-dependent regulation and is commonly presented in the form of receptor sequestration. Sequestration is the rapid (within seconds to minutes of agonist interaction) movement of receptors from the cell surface by internalisation. Internalised receptors can be either recycled to the cell surface (resensitisation) or targeted for degradation in lysosomes (Gainetdinov *et al*, 2004). Prolonged stimulation generally leads to profound receptor loss from the cell surface. After long-term exposure, the net cellular expression of a receptor becomes decreased, termed down-regulated (Liggett *et al*, 1993; Bohm *et al*, 1997). An additional type of heterologous desensitisation is through the activation of GPCR protein kinases (GRKs), which discriminate between inactive and agonist-activated receptor states and, via phosphorylation, down-regulate the activated receptors. Once phosphorylated by a GRK, the activated receptor is bound by a member of another protein family, called the arrestins. Arrestins recognise both GRK phosphorylation sites on the receptor and the active conformation of the receptor and prohibit further G-protein activation by preventing the receptor from exchanging GTP to GDP on the G-protein α-subunit. GRKs, effectively promote receptor internalisation by virtue of helping to recruit arrestins to the activated receptor (Gainetdinov *et al*, 2004).

The second type is heterologous desensitisation, which is receptor activation-independent regulation of receptors and regulates the G-proteins directly or by altering the signalling efficiency of downstream effectors (Gainetdinov *et al*, 2004). The most common mechanism for heterologous desensitisation is the feedback regulation of receptors by the second-messenger-regulated kinases they activate. For example, upon stimulation, β-ARs

activate several protein kinases including the β-AR kinase (βARK) and the cAMP-dependent kinase protein kinase A (PKA). βARK and PKA in turn phosphorylate the β-ARs which alters the responsiveness of the receptor and they become less able to mount a response to subsequent active agonist interactions (Liggett *et al*, 1993; Gainetdinov *et al*, 2004).

For both types of desensitisation to occur, the G-protein receptor complex must possess specific amino acid residues that serve as sites for phosphorylation. For example, in β-ARs the sites of βARK phosphorylation reside in serine and threonine residues in the C-terminal portion, whilst the two consecutive serine residues in the third intracellular loop are critically important for PKA mediated desensitization (Clark *et al*, 1989; Liggett *et al*, 1993; Gainetdinov *et al*, 2004). The human β_3-AR lacks most of the structural determinants that, in the β_1- and β_2-AR, allow for agonist-induced receptor desensitisation (Nantel *et al*, 1993). In contrast to the 11 serines and threonines in the distal portion of the cytoplasmic tail of the β_2-AR, there are only three in the same region of the β_3-AR. In addition the β_3-AR lacks the consensus sequences in the third intracellular loop for PKA-mediated phosphorylation. Furthermore, the β_3-AR lacks the critical 10-amino acid sequence motif in the cytoplasmic tail, which has been implicated as playing a role in β_2-AR sequestration (Hausdorff *et al*, 1990). Finally, down-regulation response in the β_2-AR is mediated via PKA phosphorylation sites, which the human β_3-AR lacks (Valiquette *et al*, 1990; Liggett *et al*, 1993). As such, the human β_3-AR would appear to be resistant to receptor desensitisation.

The fathead minnow β_{3bi}-AR however, has 16 serine and threonine residues in the distal portion of the cytoplasmic tail and also expresses the consecutive serine residues in the third intracellular loop (at positions S^{263} and S^{264}) and therefore possesses the necessary sites for receptor desensitisation via PKA and βARK phosphorylation and sequestration. Despite fish and humans evolving from the same vertebrate lineage, they are separated by 450 million years of evolution and unlike humans which are diploid, fish have undergone tetraploidisation events, which in turn have led to functional divergence (Ruuskanen *et al*, 2005; Froschauer *et al*, 2006). Therefore, despite being most similar to the human β_3-AR

of all the β-ARs, the fathead minnow β_{3bi}-AR has evolved to express the necessary motifs for agonist-desensitisation.

A handful of studies have suggested that the β_3-AR in adipocytes in humans and rats is poorly coupled to adenyl cyclase and is able to mediate receptor desensitisation through a cAMP-independent manner via other protein kinases (Jockers *et al*, 1998; Ikezu *et al*, 1999). Considering the fathead minnow β_{3bi}-AR does express the essential amino acid residues for phosphorylation, it is unlikely that desensitisation occurs via cAMP-independent mechanisms, however further studies are required to examine this phenomenon of multiple potential desensitisation mechanisms further. Regardless, it was possible that upon prolonged exposure to elevated toxic levels of propranolol and elevated levels of cortisol and adrenaline/noradrenaline, β_3-AR homologous and heterologous desensitisation events led to receptor down-regulation.

As described in section 3.4.1, the two likely causes of mortality observed in fathead minnows exposed to $1mgL^{-1}$ propranolol were liver failure and/or CNS toxicity. As the brain and liver were the likely sites of propranolol toxicity, β_{3bi}-AR expression in these tissues may have been more sensitive to propranolol toxicity and subsequent over-stimulation and desensitisation events than β_{3bi}-ARs located in the heart and gonad.

5.4.3 β_{3bi}-AR expression levels in female fathead minnow tissues

The β_{3bi}-AR was most highly expressed in the heart, followed by the liver and then the brain and gonad tissues taken from un-treated, control fish. There was a 2.5-fold difference in β_{3bi}-AR expression levels between the heart and the liver and a 12-fold difference between the heart and the gonad and brain. This study supports the observation made by Nickerson *et al* (2003) that the β_3-AR subtypes were most highly expressed in rainbow trout hearts, blood and the gills. It was not possible to examine for β_{3bi}-AR expression in the blood as blood plasma was analysed for propranolol levels and the gills did not express the β_1 or β_2-AR (Giltrow, 2008) and were therefore not included for RT-PCR analysis. Nickerson *et al* (2003) proposed that the β_3-AR controlled red blood cell intracellular pH via Na^+/H^+ exchanger activity, which subsequently enhanced the affinity

of haemoglobin for oxygen and it is possible that it performs the same role in the fathead minnow. However, the rainbow trout did not express the β_3-AR subtypes in any other tissue, whilst the β_{3bi}-AR was found in all examined tissues, albeit at different concentrations. This suggests that the β_{3bi}-AR could serve more than one function in the fathead minnow, much like it does in humans and higher terrestrial vertebrates (Manara & Bianchetti, 1990; Krief *et al*, 1993; Skeberdis, 2004; Rozec *et al*, 2005).

5.4.4 Comparison of tissue distribution and response to propranolol between β_1-, β_2- and β_{3bi}-AR in the fathead minnow

Giltrow (2008) found that there was a relationship between the distribution of the β_1- and β_2-ARs in the fathead minnow and humans and higher vertebrates. The study found that the β_1-AR was found at higher concentrations higher in the heart compared to the β_2-AR, whilst the β_2-AR was expressed approximatelty 100-fold higher than the β_1-AR in the liver. This observed preference of tissue location is the same in the fathead minnow as in humans. Interestingly, this would suggest that the β_{3bi}-AR should be preferentially located in the adipose tissue. It was not possible to quantitatively examine β_{3bi}-AR expression in the adipose tissue. However results from chapter 2, supported by the expression levels in this study indicate that the β_{3bi}-AR is located in many tissues and preferentially located in the heart, gill and red blood cells (though it needs to be confirmed by RT-PCR for the latter two tissues). Nickerson *et al* (2003) suggested that the β_3-AR in the rainbow trout is preferentially located in the red blood cell and that its role is two regulate pH levels in, and facilitate oxygen binding to, haemoglobin, however this is still to be confirmed. Of all the β-ARs, the β_3-AR is the least well understood and further studies are still required to examine β_3-AR location in both higher vertebrates and fish.

Interestingly, Giltrow (2008) suggested that at $1mgL^{-1}$, propranolol induced a desensitisation response in the β_2-AR in the liver, which supports the observation made in this study. It also adds further support that as the liver is the prinicipal site of propranolol metabolism, β-AR expression in this tissue is the most sensitive to ligand-binding and thus desensitisation in response to over-activity. The β_1-AR was poorly expressed in the

liver compared to the β_2-and β_{3bi}-AR and so was less likely to show a desensitisation response.

5.4.5 Recommendations for future work

The RT-PCR assay used in the current study provided an insight into potential stress and toxic mechanisms of propranolol exposure to female fathead minnow tissues and how this may have induced a change in β_{3bi}-AR expression in all relevant tissues. Accordingly, it is recommended that RT-PCR is used in future studies to examine the response of a particular aquatic test organism to pharmaceutical exposure. However, conclusions made from this study were based on assumptions about corresponding protein, catecholamine and glucocorticoid levels. Therefore, it is important in future studies that one captures as much information as possible relating to either a known or unknown toxic mechanism of a particular pharmaceutical. For example, in this study, the toxic effects of propranolol could not have been predicted from the literature, especially since most of it was based on the responses of humans and terrestrial vertebrates. It is quite possible that other pharmaceuticals may also exert a toxic or stressful effect on aquatic organisms and so it is important that in addition to measuring expression levels of pharmaceutical-interacting receptors, one also measures the receptor protein, catecholamine and glucocorticoid levels so that a more complete picture as to the likely causes of response are understood.

Additionally, variation of gene expression both between and within the same tissues can sometimes be considerable. Whitehead & Crawford (2005) examined the expression of 192 genes in humans and found that 48% of them were differentially expressed among individuals within the same tissues. Therefore, when examining gene expression in tissues, one must interpret the data with an element of caution and, ideally, repeat the assays both in terms of technical and tissue replication to allow for a more robust and confident interpretation.

CHAPTER 6: GENERAL DISCUSSION

6.1 Pharmaceuticals in the aquatic environment: potential threat to environmental and human health

Pharmaceuticals represent important and indispensable elements in modern society. It has been well established that after consumption and elimination from the human body, pharmaceuticals and their metabolites are not completely removed during conventional biological treatment and thus enter the aquatic environment via wastewater treatment plants. Accordingly, more than 50 pharmaceuticals have been detected, albeit at low concentrations, in waterways throughout Europe, the America's, Asia and Australasia. Since the majority of drugs are designed to affect specific protein targets at relatively low doses, pharmaceuticals represent a potential environmental hazard, even at such low concentrations (Williams, 2006). For example, the analgesic diclofenac fed to livestock in India and Pakistan caused a dramatic decline in populations of vulture as a result of acute renal failure (Oaks *et al*, 2004). Further aquatic-based examples include the synthetic oestrogen in the human contraceptive pill, 17 α-ethinyloestradiol (EE_2), which induced fish feminisation downstream of sewage treatment works (Tyler & Routledge, 1998). To date, over 24 different pharmaceuticals have been found to elicit a toxicological effect on aquatic wildlife at environmentally relevant concentrations (see section 1.4).

As well as representing a potential threat to aquatic organisms, pharmaceuticals also represent a threat to human health via environmental exposure. Drinking water is taken from the same waterways that treated wastewater is discharged into and despite recent advancement in drinking water purification treatment technologies; several pharmaceutical products resist removal from water, because of their physicochemical nature, and have therefore been found to pass directly into drinking water (Dorne *et al*, 2007). Accordingly, pharmaceuticals have been detected in both the U.S and Europe in drinking and tap water (Loraine & Pettigrove, 2006; Donn *et al*, 2008) and it has been estimated that at least 41 million American homes receive pharmaceutical-contaminated drinking water (Donn *et al*, 2008).

Pharmaceuticals are designed to interact with protein targets in humans and so exposure to pharmaceuticals via drinking water is likely to induce an effect. Several studies have examined the impacts of endocrine disrupting, hormonally active chemicals on human infertility, testicular cancer, semen quality and subsequent male reproduction in adult males and breast cancer in adult females, and have suggested that at the detected low concentrations in drinking water, there is a weak association between exposure to endocrine disrupting drugs and endocrine-disruptive response (Saradha & Mathur, 2006; Foster *et al*, 2008; Garner *et al*, 2008; Phillips & Tanphaichitr, 2008). Johnson *et al* (2008) specifically examined the risk cytotoxic chemotherapy drugs pose to human health in the U.K and concluded that it was minimal to the adult population due to high removal rates during drinking water purification

The embryonic *in utero* stage is a particularly sensitive period and so the exposure of the pregnant mother, or more specifically her foetus, to pharmaceuticals may pose a greater threat than to the adult human population. Collier (2007) suggested that over a 9-month gestation period, approximately 13% of a minimum dose of the contraceptive pill will be ingested via contaminated drinking water in the U.S and 5% of the minimum dose of diazepam, which is known to cause floppy infant syndrome at the latter stags of pregnancy, will also be ingested. Currently, there is not enough to data to understand the ramifications these low level pharmaceuticals have on the developing foetus.

The drinking water directive (DWD) (Regulation 1882/2003/EC), which is supported by the European Union, has 48 parameters outlined within it; none however address pharmaceuticals. Recently, a cohort of pharmaceutical companies conducted a risk assessment on 26 pharmaceutical products based on their measured environmental concentrations using worldwide environmental monitoring data and used a similar ratio method to the current environmental risk assessment of medicinal products for human use guideline (EMEA/CHMP/SWP/4447/00 (2006)). By comparing the ratio between the measured concentration and the predicted no-effect concentration (PNEC) for children, a risk indicator was produced. None of the 26 pharmaceuticals examined posed a significant risk to human health. However, like the environmental risk assessment

guideline, there are several issues that require further assessment (Dorne *et al*, 2007). Regardless, there is currently no regulator guidance on pharmaceuticals in the environment and no safety limits set for pharmaceuticals in drinking water.

6.2 Conclusions from study

6.2.1 Propranolol toxicity on fish

One of the biggest contributions from this study was identifying that the β-blocking pharmaceutical propranolol poses a potential threat to teleost fish via environmental exposure. On exposure to $10mgL^{-1}$, female and male fathead minnows showed signs of acute toxicity with 100% mortality being recorded in the latter sex over a 24 hour period and at $1mgL^{-1}$, there was an observed 25% and 80% mortality, respectively. At these nominal water concentrations, propranolol was measured in fish blood plasma at a level that exceeded the human therapeutic concentration (Wong *et al*, 1979) and so it is possible that at therapeutic dose, propranolol exerted an unanticipated toxic effect via two main mechanisms (which may have either acted independently or alongside of each other). Fish in the $10mgL^{-1}$ and $1mgL^{-1}$ showed signs of disorientation, which suggested that propranolol may have been acting as a central nervous system (CNS) toxin. As propranolol is highly lipophilic, it can move across the blood-brain barrier relatively easily (Vu & Beckwith, 2007); propranolol has been found in human brain tissue at concentrations ten to twenty six times higher than in plasma which, when compared to another β-blocker atenolol that has a brain: plasma ratio of 0.2, is considerably high (Cruickshank & Neil-Dwyer, 1985). This may also help to explain why propranolol was found to exert a toxicological effect, whilst a previous study by Winter *et al* (2008) found that atenolol had no effect upon fathead minnow survivability at the same test concentrations. Propranolol may have also been acting as a liver toxin. The liver is the principal site of propranolol metabolism and the observed physiological symptoms may have been the result of increased ammonia levels, which is produced as a result of drug-induced liver disease (Arillo *et al*, 1981; Zimmerman, 1995).

Although it was not possible to accurately identify a robust metabolomic biomarker of propranolol exposure in fathead minnow livers using ^{1}H NMR metabolomics, there was a dose-dependent relationship between propranolol and glutamate levels and glutamate levels were significantly ($p<0.05$) lower in the $1mgL^{-1}$ livers than in the control livers. Glutamate is reflective of the energy status of a particular tissue/organism and although it was not possible to directly relate glutamate levels to potential propranolol-induced liver failure, it is reasonable to expect energy levels to be significantly lower in a tissue that is the site of drug toxicity. Furthermore, a brief examination of the metabolomic profile of a fish liver taken from a dying male from the $1mgL^{-1}$ treatment group revealed significantly ($p<0.05$) elevated levels of lactate, formate, succinate and choline, all of which are potentially representative of liver failure. Therefore the data from the metabolomics study further suggested that propranolol exerted its toxicity on the liver. This does not necessarily mean that propranolol did not exert toxicity on the CNS; it was not possible to analyse brain tissues using ^{1}H NMR metabolomics because of tissue size limitations. In response to exposure to $1mgL^{-1}$ propranolol, the β_3-AR was found to be significantly lower in the brain and liver, which was most likely due to desensitisation in response to continuously elevated levels of corticosteroids and catecholamines as a result of increased stress which was the consequence of propranolol toxicity. This further supports the notion that the brain and liver were both sites of propranolol toxicity as β_{3bi}-AR expression in these tissues may have been more sensitive to propranolol toxicity and subsequent over-stimulation and desensitisation events than β_{3bi}-ARs located in other tissues that were not sites of propranolol toxicity.

The current study demonstrated that through bioaccumulation, propranolol acted as both a CNS- and hepatic-toxin. It is unlikely that propranolol represents an acute threat to the aquatic environment as the concentrations at which it acted as a toxin were approximately 1000-fold higher than what has been measured in the environment. Nevertheless, it has been demonstrated that, due to its physical and chemical characteristics, propranolol has the potential to bioaccumulate in fish tissues and may therefore pose as a threat through chronic environmental exposure at environmentally relevant concentrations.

One of the most interesting results from the study was that the β_{3bi}-AR was found to be expressed at significantly higher levels than in other examined tissues. There was also a statistically significant response to propranolol at environmentally relevant concentrations ($0.001mgL^{-1}$ to $0.01mgL^{-1}$); as propranolol concentration increased, β_{3bi}-AR expression increased. This was the only significant expression response to propranolol at environmentally relevant concentrations and suggests that fish exposed to these concentrations in the aquatic environment may experience inhibited β_{3bi}-AR activity, which may have further ramifications at the physiological level. The function of the β_{3bi}-AR, however, has not been identified and so it is important to establish its function, so that the potential effects of propranolol in the aquatic environment are mopre comprehensively understood. This finding also gives further support as to developing *in vivo* assays that have specific pharmaceutical mode-of-action endpoints. In this study, cardio-specific physiological endpoints perhaps could have been adopted, such as heart rate and blood pressure. Larsson *et al* (2006) examined heart rate in the rainbow trout and found that at $1.5mgL^{-1}$, there was a significant decrease in heart rate. The fathead minnow, however, is considerably smaller than a rainbow trout and it would be extremely challenging to include this cardio-specific endpoints. Bearing in mind the tissue and sample size limitations of the metabolomics study and difficulty in obtaining large enough tissue samples for molecular analysis, perhaps the most advantageous step would be to use larger fish test species, such as the rainbow trout.

6.2.2 Use of current techniques in environmental risk assessments of pharmaceutical toxicity

The current pharmaceutical environmental risk assessment for pharmaceuticals (EMEA/CHMP/SWP/4447/00 (2006)) uses a step-wise, phased procedure. The first pre-screening phase examines the likelihood of a drug bioaccumulating in the environment and also determines the predicted environmental concentration (PEC). The second phase uses standard chronic-toxicity endpoints of immobilisation in the invertebrate *Daphnia magna*, inhibition of growth in algae and mortality in early life stage fish to calculate a predicted no-effect concentration and the third and final phase evaluates the fate of the drug and/or its metabolites in the aquatic environment. Although the ERA represents a

marked improvement over pervious risk assessments, it examines for a limited general toxicity response to predict the effects of low-level pharmaceuticals that have specific modes of action and are therefore likely to induce protein-target-specific responses that may not be detected using generalised endpoints. For example, using the current ERA risk assessment, the environmental effects of EE_2 would not have been predicted (Sumpter, 2007). This suggests that prospective testing could be made more powerful by including targeted test strategies, i.e. selection of species, tests and endpoints by transferring known information on pharmaceutical properties from mammalian studies.

The aim of the current study, therefore, was to develop an intelligent testing strategy that both examined pharmaceuticals based on known properties elucidated from mammalian pharmacology studies and also for subtle, perhaps more realistic, responses to pharmaceutical exposure (as opposed to just physiological responses). With this in mind, a proposed intelligent targeted testing strategy was developed (as shown in table 35).

Table 35. Proposed intelligent targeted testing protocol for assessment of pharmaceuticals in the aquatic environment

Phase	Description
I	Establishment of pharmaceutical protein-receptor target
II	*In vivo* exposure
III	Metabolomic analysis for subtle response to pharmaceutical exposure
IV	Expression analysis of pharmaceutical protein-target receptor upon exposure to pharmaceutical

Prior to conducting an *in vivo* exposure, the first phase established whether the test-species (fathead minnow) expressed the specific pharmaceutical protein-target, which in this study were the β-AR subtypes. It was assumed that as propranolol, like all pharmaceuticals, is found at low concentrations in the aquatic environment, any measurable adverse effects in non-target species are most likely to occur as a consequence of specific drug-target interactions rather than via an unspecific mode of action, such as narcosis. Thus evolutionary, well conserved targets in a given species are associated with an increased risk (Gunnarsson *et al*, 2008). Along with another study, the

β_1- and β_{2i}- and β_{2ii}- (Giltrow *et al*, 2008) and β_{3bi}-ARs were identified in the fathead minnow, which suggested that propranolol had the potential to bind and induce a response in its target receptors in the fathead minnow. As discussed in 6.2.1, an unanticipated toxic response to propranolol was observed in the fathead minnow and it was not possible to determine whether this was mediated via β-AR interactions. Nevertheless, phase I represents a good starting point for understanding potential pharmaceutical toxicity in an aquatic test-species, however the results from this study suggest that even if the test-species in question does not express the pharmaceutical protein-target receptor, an *in vivo* exposure should still be implemented and as such, phase II should not be dependent on phase I.

A pair-breeding assay was implemented for phase II, whereby pairs of fathead minnows were examined for their reproductive potential over two concurrent 21-day periods: the first prior to- and the second upon exposure to propranolol. The advantages of employing this assay were firstly, it examined for both acute and reproductive toxicity, which are arguably most important with regards to propagation and survivability of a species and secondly, each fish pair served as their own control, which thus allowed for a statistically robust comparison of breeding ability before and during pharmaceutical exposure. As discussed in section 6.2.1, results from the pair-breeding assay suggested that propranolol acted as a toxin at concentrations >1mgL^{-1} and the most likely causes of toxicity were liver failure and CNS toxicity. Although one could not confidently identify the cause of mortality at this stage; by working with smaller tanks and individual fish pairs and also by nature of each pair serving as its own control, it was easier to observe and understand the physiological responses of fish to propranolol, which may not have been possible if tank sizes and fish-group sizes were larger and more complex.

Phase III used metabolomics in an attempt to identify a robust and reliable biomarker of pharmaceutical exposure. Metabolomics is the quantitative and comprehensive analysis of all metabolites in a biological sample. Metabolites are bi-products of metabolism and the metabolome represents the final "omic" level in a biological system. Therefore, unlike genomics and transcriptomics, metabolomics reveals 'actual' functional changes in

a study organism, as opposed to potential changes (Nicholson *et al*, 2002; Nicholson & Wilson, 2003; Goodacre *et al*, 2004). Since the liver is the principal site of propranolol metabolism and considering the physiological impact of propranolol on the fathead minnow, it was anticipated that a significant response would be observed in propranolol-exposed fish livers. As mentioned in section 6.2.1, ^{1}H NMR metabolomic analysis of male fathead minnow livers provided more information as to the potential toxic actions of propranolol on the liver, however there were several difficulties encountered during metabolomic analysis (as mentioned in section 6.2.3) which still need to be addressed if it is to be reliably used as a diagnostic tool in future environmental-based pharmaceutical toxicity studies.

The fourth and final phase of the intelligent-testing protocol was to examine the response of the target receptor to pharmaceutical exposure using the highly sensitive real time polymerase chain reaction (RT-PCR). In response to exposure to 1mgL^{-1} propranolol, there was a significant decrease in β_{3bi}-AR expression in the liver and brain, which could possibly be the result of focused propranolol toxicity on these tissues and resultant desensitisation events in response to elevated levels of cortisol and catecholamines. By using RT-PCR, it is possible to examine expression level changes of a particular target receptor in response to pharmaceutical exposure and, in the context of this study, helps to build a more complete picture of pharmaceutical toxicity. However, as discussed in section 6.2.3, there are several limitations of interpreting expression study results that must be addressed for it to be more efficiently implemented in future pharmaceutical ecotoxicity studies.

In conclusion, the proposed testing strategy adopted in this study represents several advantages over the current ERA. First and foremost, it is a bespoke study that addresses individual pharmaceuticals according to their individual properties and mode of actions; secondly it is not limited by examining solely for physiological responses but analyses pharmaceutical interactions at the molecular (predictive toxicity), metabolomic (subtle measurable toxicity) and physiological (observable toxicity) level. Measuring the response of the fathead minnow to propranolol served as a good case study as there was

an unanticipated response to the drug which could not have been predicted from mammalian pharmacological data. It also revealed the limitations of the current study and allowed for recommendations to be made both to the proposed testing strategy and the current ERA testing strategy for examining pharmaceutical toxicity in the future, some of which may share similar characteristics with propranolol (such as its lipophilic nature and affinity to bioaccumulate).

6.2.3 Limitations of study

6.2.3.1 Variation

Considerable variation was observed at the molecular and, in particular, the metabolomic level. With regards to metabolomics, the principal problem was that inherent metabolomic variation between individual fish livers both within and between treatment groups was such that it was not possible to accurately and confidently identify a metabolomic biomarker of propranolol exposure. The measured variation was not significantly higher than other fish test-species and was therefore most likely indicative of "natural variation". Additionally the source of variation was not feeding, tank effects or MS222 anaesthetisation and was therefore unlikely to have been introduced. In human and higher vertebrate pharmacology studies, time-dependent studies (termed metabonomics) are used so that each test-organism serves as its own control, or genetically inbred animals are used to thus minimise variation. However, in ecotoxicological studies using small test-species (such as the fathead minnow), it is not particularly easy to conduct time-dependent studies (especially with regards to ethical guidelines) and the use of inbred fish strains has no environmental relevance.

Similarly with the RT-PCR study, there was considerable variation in β_{3bi}-AR expression both between the same tissues and between internal standard controls between plates (where there should be little variation as it is the same sample on every plate). In circumstances where β_{3bi}-AR expression levels were different between plates, the assay was repeated, however due to cost and time constraints it was not possible to fully understand the reasons for variations in internal standard expression between plates. Gene

expression levels can vary considerably between individuals (Whitehead & Crawford, 2005) and this is something that must be considered when conducting, and more importantly concluding from, RT-PCR expression studies.

6.2.3.2 Test species

The fathead minnow, *Pimephales promelas*, was the selected test species as it is a model test species used by a multitude of agencies including the Environmental Protection Agency (EPA), the Environment Agency (EA) and the Organisation for Economic Cooperation and Development (OECD). With developments in genomic, proteomic and histological techniques, the fathead minnow has become an established biomarker test species, with several physiological and biochemical endpoints, for a diverse number of contaminants including several pharmaceuticals; arguably the most extensively studied of which has been EE_2 (Harries *et al*, 1998; Harries *et al*, 2000; Halm *et al*, 2002; Zerulla *et al*, 2002; Leino *et al*, 2005; Ankley & Villeneuve, 2006; Miracle *et al*, 2006).

However, one major limitation of the fathead minnow is its size. The fathead minnow reaches a maximum length of 50mm and 1g in weight. Therefore, for metabolomic-based studies, the only viable tissue for analysis is the liver. For the purpose of this study, the liver was an ideal tissue because of its involvement in propranolol metabolism and being a potential site of propranolol toxicity. However, it was not possible to use ^{1}H NMR to analyse other tissues such as the brain, which would have been particularly useful to examine as it was the other proposed site of propranolol toxicity. Only liver tissues from male fathead minnows were suitable for analysis as female livers fell below the tissue threshold size. Furthermore, several liver tissues from males were excluded as they were too small.

6.3 Recommendations for future work

Although one cannot be entirely certain that propranolol did not induce toxicity via β-AR binding as only a handful of tissues were examined for β-AR expression changes, it appears that propranolol did not induce a toxicological response through interactions with the β-AR subtypes. Therefore it is perhaps essential that a robust *in vivo* assay is adopted

for pharmaceutical screening, regardless as to whether the aquatic organism possesses the drug target, such as the pair-breeding assay used in this study. The pair-breeding assay represented a robust, repeatable assay that examined for mortality and reproductive endpoints, which when considering the ramifications on the population level are arguably the most important. However, ideally a bespoke *in vivo* assay is required that will address both population-level endpoints and pharmaceutical-specific physiological endpoints; it is important to use mammalian pharmacology data to address the latter.

An alternative *in vivo* pharmaceutical examination is to use an embryo-larval bioassay, which addresses both lethal and sublethal effects and can be tailored for a specific drug mode of action. The problems with a standard adult toxicity assay include genetic variation, differences in the condition of individual animals, the missing knowledge about possible interaction effects between individuals and especially with micropathogens and also statistical problems such as sample sizes and pseudoreplication. Multi-factorial embryo tests on ecologically relevant test species can solve many of these issues (Wedekind *et al*, 2007). The use of in-bred embryos to minimise genetic variation may prove to be potentially hugely advantageous with regards to subsequent metabolomic analysis. For example Fraysse *et al* (2006) examined zebrafish (*Danio rerio*) embryo-larval development upon exposure to propranolol by measuring heart rate, spontaneous movement and hatching rate, thus collecting drug-specific and population-reflective data. This type of bioassay represents both ethical and financial advantages over the pair-breeding assay used in the present study; however it is difficult to collect suitable material for further metabolomic or genomic studies and so perhaps serves as an addition, rather than alternative, assay.

The use of larger in-bred fish to minimise genetic and metabolic variation presents several advantages. Firstly, it could be possible to conduct time-dependent studies, where each fish would serve as its own control, thus minimising variation further. Secondly, tissue size would not be a limiting factor and so it could be possible to extract samples for both metabolomic and genetic analysis from the same tissue, thus allowing for a direct

comparison to be made between gene expression and metabolic changes within an individual fish.

Although this study addressed pharmaceutical toxicity in the aquatic environment more comprehensively than current ERA assessments, informed assumptions were still made to piece together the overall picture of understanding propranolol toxicity. For example, it has been assumed that an increase/decrease in β_{3bi}-AR mRNA expression is directly related to β_{3bi}-AR activity, which may not necessarily have been the case. Likewise, it was not clearly evident whether the metabolic response to propranolol was linked to β_{3bi}-AR activity and changes in β_{3bi}-AR expression. When addressing pharmaceutical toxicity, it is hugely important to build a complete picture of understanding and, as such, future studies should also include proteomic and transcriptomic studies to confidently link together genomic and metabolomic responses. Additionally, laboratory-based studies should routinely measure cortisol and catecholamine levels to understand and potentially differentiate between a toxicological response directly related to the test pharmaceutical, and a stress response related to laboratory test-conditions and exposure to a pharmaceutical.

With regards to environmental relevance, the issue of mixture-toxicity should be addressed long-term. Pharmaceuticals are seldom found as single substances in the aquatic environment, but more commonly as complex mixtures. In mixtures, pharmaceuticals can either behave as *concentration addition* (toxicity exerted is greater than as individual substances) or *independent action* (toxicity exerted by each substance is independent of others, i.e. no greater toxicity) (Cleuvers, 2004; Brian *et al*, 2007; Johnson *et al*, 2008). Accordingly, both the proposed intelligent testing strategy and ERA require further modifications to allow for the examination of mixture-toxicity; this however may be location-specific, which in turn presents problems such as environmental (i.e. seasonal) and global change (Brian, 2008 *et al*; Wingfield, 2008) effects.

Pharmaceutical toxicity is a multi-faceted problem and, as such, requires an integrated scientific approach of understanding from the manufacturing, distributing and consumption of pharmaceuticals, to sewage treatment works, and finally to interactions with environmental receptors in exposed animals (Cotsier *et al*, 2007). As such, a comprehensive integrated programme of research is required to enable use to act efficiently to reduce the impact of pharmaceuticals in the aquatic environment.

REFERENCES

Aherne, G.W., & Briggs, R. (1989). The relevance of the presence of certain synthetic stroids in the aquatic environment. *Journal of Pharmaceutical Pharmacology*. **41**. 735-736.

Alberti, M., Kausch, U., Haindl, S., Leibiger, R., Budczies, J., Seifert, M. & Hock, B. (2005). Gene expression patterns – a tool for bioanalysis. *International Journal of Environmental Analysis and Chemistry*. **85(9-11)**. 589-608.

Alberts, B. (1994). Molecular biology of the cell. *Garland Science*. 44-1294.

Allison, S.P., Chamberlain, M.J., Miller, J.E., Ferguson, R., Gillet, A.P., Bermand, B.V. & Saunders, R.A. (1969). Effects of propranolol on blood sugar, insulin and free fatty acids. *Diabetologia*. **5(5)**. 339-342.

An, L., Hu, J., Zhang, Z. & Yang, M. (2006). Quantitative real-time RT-PCR for determination of vitellogenin mRNA in so-iuy mullet (*Mugil soiuy*). *Analytical and Bioanalytical Chemistry*. **386**. 1995-2001.

Andreozzi, R., Marotta, R. & Nicklas, P. (2003). Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. *Chemosphere*. **50**. 1319–1330.

Ankley, G.T., Jensen, K.M., Kahl, M.D., Korte, J.J. & Makynen, E.A. (2001). Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry*. **20**. 1276-1290.

Ankley, G.T. & Villeneuve, D.L. (2006). The fathead minnow in aquatic toxicology: past, present and future. *Aquatic Toxicology*. **78**. 91-102.

Ankley, G.T., Daston, G.P., Degitz, S.J., Denslow, N.D., Hoke, R.A., Kennedy, S.W., Miracle, A.L., Perkins, E.J., Snape, J., Tillett, D.E., Tyler, C.R. & Versteeg, D. (2006). Toxicogenomics in regulatory ecotoxicology. *Environmental Science and Technology*. **40**. 4055-4065.

Ankley, G.T., Miller, D.H., Jensen, K.M., Villeneuve, D.L. & Martinović. (2008). Relationship of plasma sex steroid concentration in female fathead minnows to reproductive success and population status. *Aquatic Toxicology*. **88**. 69-74.

Arch, J.R.S. (1989). The brown adipocyte β-adrenoceptor. *Proceedings of the Nutrition Society*. **48**. 215-223.

Arillo, A., Margiocco, C., Melodia, F., Mensi, P. & Schenone, G. (1981). Ammonia toxicity mechanisms in fish: studies on rainbow trout (*Salmo gairdneri*). *Ecotoxicology and Environmental Safety*. **5(3)**. 316-328.

Arner, P. & Hoffstedt, J. (1999). Adrenoceptor genes in human obesity. *Journal of Internal Medicine*. **245**. 667-672.

Ashton, D., Hilton, M. & Thomas, K.V. (2004). Investigating the environmental transport of human pharmaceuticals to streams in the United Kingdom. *Science of the Total Environment.* **333**. 167-184.

Ayscough, N.J., Fawell, J., Fanklin, G. & Young, W. (2000). Review of human pharmaceuticals in the environment. R&D technical report P390. *Environment Agency Dissemination Centre.* 1-105.

Balzola, F.A. & Boggio-Bertinet, D. (1996). The metabolic role of glutamine. *Minerva Gastroenterol Dietol*. **42**. 17-26.

Barceló, D. & Petrovic, M. (2007). Challenge and achievements of LC-MS in environmental analysis: 25 years on. *Trends in Analytical Chemistry*. **26(1)**. 2-11.

Bardou, M., Dousset, B., Deneux-Tharaux, C., Smadja, C., Naline, E., Chaput, J.C., Naveau, S., Manara, L., Croci, T. & Advenier (1998). In vitro inhibition of human colonic motility with SR 59119A and SR59104A: evidence of β_3-adrenoceptor-mediated effect. *European Journal of Pharmacology*. **353**. 281-287.

Becker, W.M., Kleinsmith, L.J., Hardin, J. & Bertonia, G.P. (2008). *Benjamin Cummings.*

Benjamini, Y. & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B*. **57**. 289-300.

Bensaid, M., Kaghad, M., Rodriguez, M., le Fur, G. & Caput, D. (1993). The rat β_3-adrenergic receptor gene contains an intron. *Federation of European Biochemical Societies Letters*. **318(3)**. 223-226.

Berg, , J.M. & Tymoczk, S. (2002). Biochemistry (5^{th} edition). *W.H. Freeman and Company*. 32-37.

Betts, M.J. & Russell, R.B. (2003). Amino acid properties and consequences of substitutions. ***In*** *Bioinformatics for Geneticists*. Barnes, M.R. & Gray, I.C. *Wiley*.

Bligh, E.G. & Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physics*. **37**. 911-917.

Blin, N., Camoin, L., Maigret, B. & Strosberg, A.D. (1993). Structural and conformational features determining selective signal transduction in the β3-adrenergic receptor. *Molecular Pharmacology*. **44**. 1094-1104.

Blockaert, J. & Pin, J.P. (1999). Molecular tinkering of G-protein coupled receptors: an evolutionary success. *European Molecular Biology Organisation*. **18**. 1723-1728.

Bloom, N., Gammeltoft, S. & Brunak, S. (1999). Sequence- and sequence-structure-based prediction of eukaryotic protein phosphorylation sites. *Journal of Molecular Biology*. **294**. 1351-1362.

Bohm, S.K., Grady, E.F. & Bunnett, .W. (1997). Regulatory mechanisms that modulate singalling by G-protein coupled receptors. *Biochemistry Journal*. **322**. 1-18.

Bonga, W.S.E. (1997). The stress response in fish. *Physiological Reviews*. **77(3)**. 591-625.

Borger, R., De Boeck, G., Van Audekerke, J., Dommisse, R., Blust, R., Van der Linden, A. (1998). Recovery of the energy metabolism after a hypoxic challenge at different temperature conditions: a ^{31}P-nuclear magnetic resonance spectroscopy study with common carp. *Comparative Biochemistry and Physiology Part A*. **120**. 143-150.

Bound, J.P. & Voulvoulis, N. (2004). Pharmaceuticals in the aquatic environment – a comparison of risk assessment strategies. *Chemosphere*. **56(11)**. 1143-1155.

Brian, J.V., Harris, C.A., Scholze, M., Kortenkamp, A., Booy, P., Lamoree, M., Pojana, G., Jonkers, N., Marcomini, A. & Sumpter, J.P. (2007). Evidence of estrogenic mixture effects on the reproductive performance of fish. *Environmental Science & Technology*. **41**. 337-344.

Brian, J.V., Harris, C.A., Runnalls, T., Fantinati, A., Pojana, G., Marcomini, A., Booy, P., Lamoree, Kortenkamp, A. & Sumpter, J.P. (2008). Evidence of temperature-dependent effects on the oestrogenic response of fish: implication with regard to climate change. *Science of the Total Environment*. **397**. 72-81.

Brodsky, L.I., Ivanov V.V., Kalai dzidis Ya.L., Leontovich A.M., Nikolaev V.K., Feranchuk S.I. & Drachev V.A. (1995) GeneBee-NET:Internet-based server for analyzing biopolymers structure. *Biochemistry*. **60(8)**. 923-928.

Brun, G.L., Bernier, M., Losier, R., Doe, K., Jackman, P. & Lee, H.B. (2006). Pharmaceutically active compounds in Atlantic Canadian sewage treatment plant effluents and receiving waters, and potential for environmental effects as measured by acute and chronic aquatic toxicity. *Environmental Toxicology and Chemistry*. **25(8)**. 2163-2176.

Calamari, D., Zuccato, E., Castiglioni, S., Bagnati, R. & Fanelli, R. (2003) Strategic survey of therapeutic drugs in the rivers Po and Lambro in northern Italy, *Environmental Science and Technology*. **37**. 1241–1248.

Chamberlin, M.E., Glemet, H.C. & Ballantyne, J.S. (1991). Glutamine metabolism in a holostean (*Amia calva*) and teleost fish (*Salvelinus namaycush*). *American Journal of Physiology Regulatory-Integrative and Comparative Phsyiology*. **260(1)**. 159-166.

Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*. **162(1)**. 156-159.

Christensen, F.M. (1998). Pharmaceuticals in the environment – a human risk? *Regulatory Toxicology and Pharmacology*. **28**. 212-221.

Čikoš, S., Veselá, J., Il'Ková, G., Rehák, P., Czikková, S. & Koppel, J. (2005). Expression of beta adrenergic receptors in mouse oocytes and preimplantation embryos. *Molecular Reproduction and Development*. **71**. 145-153.

Clark, R.B., Friedman, J., Dixon, R.A. & Strader, C.D. (1989). Identification of a specific site required for rapid heterologous desensitization of the beta-adrenergic receptor by cAMP-dependend protein kinase. *Molecular Pharmacology*. **36(3)**. 343-348.

Clayton, T., Lindon, J., Cloarec, O., Antti, H., Charuel, C., Hanton, G., Provost, J., Le, N.J., Baker, D., Walley, R., Everett, J. & Nicholson, J. (2006). Pharmaco-metabonomic phenotyping and personalized drug treatment. *Nature*. **440**. 1073-1077.

Clement, K., Vaisse, C., Manning, B.S.J., Basdevant, A. & Guy-Grand, B. (1995). Genetic variation in the beta-3-adrenergic receptor and an increased capacity to gain weight in patients with morbid obesity. *The New England Journal of Medicine*. **333**. 352-354.

Cleuvers, M. (2004). Initial risk assessment for three β-blockers found in the aquatic environment. *Chemosphere*. **59(2)**. 199-205.

Coe, T.S., Hamilton, P.B., Hodgson, D., Paull, G.C., Stevens, J.R., Sumner, K. & Tyler, C.R. (2008). An environmental oestrogen alters reproductive hierarchies, disrupting sexual selection in group-spawning fish. *Environmental Science and Technology*. **42**. 5020-5025.

Cole, K.S., & Smith, R.J.F. (1987). Male courting behaviour in the fathead minnow *Pimephales promelas*. *Environmental Biology of Fish*. **18**. 235-239.

Collier, A.C. (2007). Pharmaceutical contaminants in potable water: potential concerns for pregnant women and children. *EcoHealth*. **4**. 164-171.

Collins, S., Daniel, K.W., Rohlfs, E.M., Ramkmar, V., Taylor, I.L. & Gettys, T.W. (1994). Impaired expression and functional activity of the beta-3- and beta 1 adrenergic receptors in adipose tissue of congenitally obese (C57BL/6J ob/ob) mice. *Molecular Endocrinology*. **8**. 518-527.

Cotsier, C., Lin, L., Roig, B. & Touraud, E. (2007). Integrated approach to the problem of pharmaceutical products in the environment: an overview. *Analytical & Bioanalytical Chemistry*. **387**. 1163-1166.

Cowley, C.B. & Sargent, J.R. (1979). Nutrition. **In** W.S. Hoar, D.J. Randall & J.R. Brett (Ed.) *Fish Physiology volume VIII Bioenergetics and growth* (pp 1-58) *Academic Press Inc.*

Cruickshank, J.M. & Neil-Dwyer, G. (1985). Beta-blocker brain concentrations in man. *European Journal of Clinical Pharmacology*. **28**. 21-23.

Cserzo, M., Wallin, E., Simon, I., Von Heijne, G. & Elofsson, A. (1997). Prediction of transmembrane alpha-helices in procariotic membrane proteins: the dense alignment surface method. *Protein Engineering*. **10(6)**. 673-676.

Das, G. & Ferris, J.C. (1988). Generalised convulsions in a patient receiving ultrashort-acting beta-blocker infusion. *Drug Intelligence & Clinical Pharmacy*. **22**. 484-485.

Daughton, C.G. & Ternes, T.A. (1999). Pharmaceuticals and personal care products in the environment. *Environmental Health Perspectives*. **107(6)**. 906-941.

De Boeck, G., Borger, R., Van der Linden, A. & Blust, R. (1997). Effects of sublethal copper exposure on muscle energy metabolism of common carp, measured by ^{31}P-nuclear magnetic resonance spectroscopy. *Environmental Toxicology and Chemistry*. **16(4)**. 676-684.

Devic, E., Paquereau, L., Steinberg, R., Caput, D & Audigier, Y. (1997). Early expression of a β_1-adrenergic receptor and catecholamines in *Xenopus* oocytes and embryos. *Federation of European Biochemical Societies*. **417**. 184-190.

Donn, J., Mendoza, M. & Pritchard, J. (2008). Pharmaceuticals lurking in U.S. drinking water. *Association Press*. Published 10.03.2008.

Dorne, J.L.C.M., Ragas, A.M.J., Frampton, G.K., Spurgeon, D.S. & Lewis, D.F. (2007). Trends in human risk assessment of pharmaceuticals. *Analytical and Bioanalytical Chemistry*. **387**. 1167-1172.

Dugan, S.G., Chen, X., Nickerson, J.G., Montpetit, C.J. & Moon, T.W. (2008). Regulation of the black bullhead hepatic β-adrenoceptors. *Comparative Biochemistry and Physiology Part B*. **149**. 265-274.

Dzialowski, E.M., Turner, P.K. & Brooks, B.W. (2006). Physiological and reproductive effects of beta adrenergic receptor antagonists in *Daphnia magna*. *Archives of Environmental Contamination and Toxicology*. **50(4)**. 503-510.

ENDS, 2005 Report E. Chasing environmental risk assessment for pharmaceuticals. *ENDS Report 365*. 29-32. www.endsreport.com

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A. (2008). Growth performance and metabolic utilization of diets with native and waxy maize starch by gilthead sea bream (*Sparus aurata*) juveniles. *Aquaculture*. **274**. 101-108.

Ernst & Young for European Company Data (2003). Available from www.bioindustry.org/bigtreport/downloads/appendices_glossary/pdf. [Accessed 17 July 2008].

Ekman, D.R., Teng, Q., Jensen, K.M., Martinovic, D., Villeneuve, D.L., Ankley, G.T. & Collette, T.W. (2007). NMR analysis of male fathead minnow urinary metabolites: A potential approach for studying the impacts of chemical exposures. *Aquatic Toxicology*. **85**. 104-112.

Ekman, D.R., Teng, Q., Villeneuve, D.L., Kahl, M.D., Jensen, K.M., Durhan, E.J., Ankley, G.T. & Collette, T.W. (2008). Investigating compensation and recovery of fathead minnow (*Pimephales promelas*) exposed to 17 α-ethinyloestradiol with metabolite profiling. *Environmental Science and Technology*. **42**. 4188-4194.

Engelke, U. (2007). NMR Spectroscopy of body fluids: a metabolomics approach to inborn errors of metabolism. PhD Thesis, University Nijmegen. 17-190.

European Medicines Agency; Committee for medicinal products for human use. (2006). Guideline on the environmental risk assessment of medicinal products for human use. EMEA/CHMP/SWP/4447/00.

Fent, K., Weston, A.A. & Caminada, D. (2006). Ecotoxicology of human pharmaceuticals. *Aquatic Toxicology*. **76**. 122-159.

Ferrari, B., Mons, R., Vollat, B., Fraysse, B., Paxéus, N., Lo Giudice, R., Pollio, A., Carric, J. (2004). Environmental risk assessment of six human pharmaceuticals: are the current environmental risk assessment procedures sufficient for the protection of the aquatic environment? *Environmental Toxicology and Chemistry*. **23(5)**. 1344-1354.

Filby, A., Santos, E.D., Thorpe, K.L., Maack, G. & Tyler, C.R. (2007). Gene expression profiling for understanding chemical causation of biological effects for complex mixtures: A case study on oestrogens. *Environmental Science and Technology*. **41**. 8187-8194.

Finkenbine, S.S., Gettys, T.W. & Burnett, K.G. (2002). Beta-adrenergic receptors on leukocytes of the channel catfish, *Ictalurus punctatus*. *Comparative Biochemistry and Physiology Part C*. **131**. 27-37.

Folmar, L.C., Denslow, N.D., Rao, V., Chow, M., Crain, D.A., Enblom, J., Marcino, J. & Guillette, L.J. (1996). Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. *Environmental Health Perspectives*. **104**. 1096-1101.

Forbes, S., Clements, J., Dawson, E., Bamford, S., Webb, T., Dogan, A., Flanagan, A., Teague, J., Wooster, R., Futreal, P.A. & Stratton, M.R. (2006). Genetics and genomics: COSMIC 2005. *British Journal of Cancer*. **94**. 318-322.

Schwartz, T.W. (1996). Molecular structure of G-protein-coupled receptors. **In** J.C. Foreman & F. Johansen *Textbook of Receptor Pharmacology* (pp. 65-84). CRC Press.

Fossi, M.C., Casini, S. & Marsili, L. (2006). Endocrine disrupters in Mediterranean Top Marine Predators. *Environmental Science and Pollution*. **13(3)**. 204-207.

Foster, W.G., Neal, M.S., Han, M.S. & Dominguez, M.M. (2008). Environmental contaminants and human infertility: hypothesis or cause for concern. *Journal of Toxicology and Environmental Health Part B*. **11**. 162-176.

Fowler, J., Cohen, L. & Jarvis, P. (1998). Practical statistics for field biology 2nd edition. *John Wiley & Sons*. 74-185.

Fraysse, B., Mons, R. & Garric, J. (2006). Development of a zebrafish 4-day embryo-larval bioassay to assess toxicity of chemicals. *Ecotoxicology and Environmental Safety*. **63**. 253-267.

Fredriksson, R., Lagerstrom, M.C., Lundin, L.G., Schiöth, H.B. (2003). The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Molecular Pharmacology*. **63**. 1256-1272.

Frishman, W., Jacob, H. & Eisenberg, E. (1979). Clinical pharmacology of the new beta-adrenergic blocking drugs. Part 8. Self poisoning with beta-adrenergic blocking agents: recognition and management. *American Heart Journal*. **98**. 798-811.

Froschauer, A., Braasch, I & Volff, J.N. (2006). Fish genomes, comparative genomics and vertebrate evolution. *Current Genomics*. **7(1)**. 1-15.

Gainetdinov, PR., Premont, R.T., Bohn, L.M., Lefkowitz, R.J. & Caron, M.G. (2004). Desensitisation of g protein-coupled receptors and neuronal functions. *Annual Review of Neuroscience*. **7**. 107-144.

García, L.V. (2003). Controlling the false discovery rate in ecological research. *TRENDS in Ecology and Evolution*. **18(11)**. 554-555.

Garner, M., Turner, M.C., Ghadirian, P., Krewski, D. & Wade, M. (2008). Testicular cancer and hormonally active agents. *Journal of Toxicology and Environmental Health Part B*. **11**. 260-275.

German, J.B., Roberts, M.A. & Watkins, S.M. (2003). Personal metabolomics as a next generation nutritional assessment. *Journal of Nutrition*. **133**. 4260-4266.

Goodacre, R., Vaidyanathan, S., Dunn, W.B., Harrigan, G.G. & Kell, D.B. (2004). Metabolomics by numbers: acquiring and understanding global metabolite data. *Trends in Biotechnology*. **22**. 245-252.

Griffin, J.L., Nicholls, A.W., Daykin, C.A., Heald, S., Keun, H.C., Schuppe-Koistinen, I., Griffiths, J.R., Cheng, L.L., Rocco-Serra, P., Rubtsov, D. & Robertson, D. (2007). Standard reporting requirements for biological samples in metabolomics experiments: mammalian/in vivo experiments. *Metabolomics*. **3(3)**. 179-188.

Gross-Sorokin, M.Y., Roast, S.D. & Brighty, G.C. (2006). Assessment of feminization of male fish in English rivers by the environment agency of England and Wales. *Environmental Health Perspectives*. **114**. 147-151.

Gros, M., Petrović, M. & Barceló (2006). Multi-residue analytical methods using LC-tandem MS for the determination of pharmaceuticals in environmental and wastewater samples: a review. *Analytical and Bioanalytical Chemistry*. **386(4)**. 941-952.

Gros, M., Pizzolato, T.M., Petrović, de Alda, M.J.L. & Barceló (2008). Trace level determination of β-blockers in waste waters by highliy selective molcular imprinted polymers extraction followed by liquid chromatography-quadrupole-linear ion trap mass spectrometry. *Journal of Chromatography A*. **1189**. 374-384.

Gunnarsson, L., Jauhianen, A., Kristiansson, E., Nerman, O. & Larsson, D.G.J. (2008). Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. *Environmental Science and Technology*. **42(15)**. 5807-5813.

Halm, S., Pounds, N., Maddix, S., Rand-Weaver, M., Sumpter, J.P., Hutchinson, T.H. & Tyles, C.R. (2002). Exposure to exogenous 17β-oestradiol disrupts P450aromB mRNA expression in the brain and gonad of adult fathead minnows (*Pimephales promelas*). *Aquatic Toxicology*. **60**. 285-299.

Harries, J.E., Sheahan, D.A., Jobling, S., Matthiessen, P., Neall, P., Sumpter, J.P., Taylor, T. & Zaman, N. (1997). Ostrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environmental Toxicology and Chemistry*. **16**. 534-542.

Harries, J.E., Runnalls, T., Hill, E., Harris, C., Maddix, S., Sumpter, J.P. & Tyler, C.R. (2000). Development of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (*Pimephales promelas*). *Environmental Science and Technology*. **34**. 3003-3011.

Harris, R.K. (1986). Nuclear magnetic resonance spectroscopy. A physiochemical view. *Longman Scientific & Technical*. 1-42.

Hathaway, C.B. & Epple, A. (1989). Catecholamine release by catecholamines in the eels does not require the presence of brain or anterior spinal cord. *Journal of Experimental Zoology*. **249**. 338-342.

Hausdorff, W.P., Caron, M.G. & Lefkowitz, R.J. (1990). Turning off the signal: desensitisation of β-adrenergic receptor function. *The Federation of American Societies for Experimental Biology Journal*. **4**. 2881-2889.

Heberer, T. (2002). Occurrence, fate and removal of pharmaceutical residues in the aquatic environment: an extended review of recent research data. *Toxicological Letters*. **131 (1/2)** 377-382.

Hemre, G.I. & Krogdahl, A. (1996). Effect of handling and fish size on secondary changes in carbohydrate metabolism in Atlantic salmon, *Salmo salar* L. *Aquaculture Nutrition*. **2(4)**. 249-252.

Henschel, K.P., Wenzel, A., Dietrich, M. & Fliedner, A. (1997). Environmental hazard assessment of pharmaceuticals. *Regulatory Toxicology and Pharmacology*. **25**. 220-225.

Hernando, M.D., Petrovic, M., Fernández-Alba, A.R. & Barceló. (2004). Analysis by liquid chromatograph-electrospray ionization tandem mass spectrometry and acute toxicity evaluation for β-blockers and lipid-regulating agents in wastewater samples. *Journal of Chromatography A*. **1046**. 133-140.

Hines, A., Yeung, W.H., Craft, J., Brown, M., Kennedy, J., Bignell, J., Stentiford, G.D. & Viant, M.R. (2007). Comparison of histological, genetic, metabolomics, and lipid-based methods for sex determination in marine mussels. *Analytical Biochemistry*. **369**. 175-186.

Hinfray, N., Porcher, J.M. & Brion, F. (2006). Inhibition of rainbow trout (*Oncorhynchus mykiss*) P450 aromatase activites in brain and ovarian microsomes by varies environmental substances. *Comparative Biochemistry and Physiology, Part C*. **144**. 252-262.

Hirokawa, T., Boon-Chieng, S. & Mitaku, S. (1998). SOSUI: classification and secondary structure prediction system for membrane proteins. *Bioinformatics*. **14**. 378-379.

Hirsch, R., Ternes, T., Haberer, K. & Kratz, K.L. (1999). Occurrence of antibiotics in the aquatic environment. *Science of the Total Environment*. **225**. 109-118.

Hoffman, .L. & Oris, J.T. (2006). Altered gene expression: a mechanism for reproductive toxicity in zebrafish exposed to benzo[*a*]pyrene. *Aquatic toxicology*. **78**. 332-340.

Hofmann, K. & Stoffel, W. (1993). TMbase – a database of membrane spanning proteins segments. *Biological Chemistry Hoppe-Seyler*. **374**. 166.

Hook, S.E., Skillman, A.D., Small, J.A. & Schultz, I.R. (2007). Temporal changes in rainbow trout exposed to ethinyloestradiol. *Comparative Biochemistry and Physiology, Part C*. **145**. 73-85.

Horinouchi, T. & Koike, K. (1999). Characterisation of atypical β-adrenoceptors in the guinea pig duodenum. *European Journal of Pharmacology*. **376**. 61-66.

Huggett, D.B., Brooks, B.W., Peterson, B., Foran, C.M. & Schlenk, D. (2002). Toxicity of select beta adrenergic receptor-blocking pharmaceuticals (β-blockers) on aquatic organisms. *Archives of Environmental Contamination and Toxicology*. **43**. 229-235

Huggett, D.B., Khan, I.A., Foran, C.M. & Schlenk, D. (2003a). Determination of beta-adrenegic receptor blocking pharmaceuticals in United States wastewater effluent. *Environmental Pollution*. **121**. 199-205.

Huggett, D.B., Cook, J.C., Ericson, J.E. & Williams, R.T. (2003b). A theoretical model for utilizing mammalian pharmacology and safety data to prioritize potential impacts of human pharmaceuticals to fish. *Human and Ecological Risk Assessment*. **9**. 1789-1799.

Huggett, D.B., Benson, W.H., Chipman, K., Cook, J.C., Gray, L.E., Kinter, L.B., Meyerhoff, R.D. & Trudeau, V.L. (2006). Role of mammalian data in determining pharmaceutical responses in aquatic species. **In** R.T. Williams (Ed.) *Human Pharmaceuticals: assessing the impacts on aquatic ecosystems*. (pp 149-182). Society for Environmental Toxicology and Chemistry Press.

Hunt, D.G., Ding, Z. & Ivy, J.L. (2002). Propranolol prevents epinephrine from limiting insulin-stimulated muscle glucose uptake during contraction. *Journal of Applied Physiology*. **93**. 697-704.

Hwang, T.L. & Shaka, A.J.J. (1995). Excitation sculpting using arbitrary waveforms and pulsed field gradients. *Journal of Magnetic Resonance A*. **112**. 275-279.

Ikezu, T., Yasuhara, S., Granneman, J.G., Kraemer, F.B., Okamoto, T., Tompkins, R.G. & Martyn, J.A.J. (1999). A unique mechanism of desensitisation to lipolysis mediated by β_3-adrenoceptor in rats with thermal injury. *American Journal of Physiology: Endocrinology and Metabolism*. **277**. 316-324.

Jensen, K.M., Korte, J.J., Kahl, M.D., Pasha, M.S. & Ankley, G.T. (2001). Aspects of basic reproductive biology and endocrinology in the fathead minnow (*Pimephales promelas*) *Comparative Biochemistry and Physiology Part C*. **128**. 127-141.

Jessica, D., Robert, M., Frédéric, S., Arnaud, B., Delphine, L., Jean-Pierre, T. & Patrick, K. (2007). Do sewage treatment plant discharge substantially impair fish reproduction in polluted rivers? *Science of the Total Environment*. **372**. 497-514.

Jobling, S., Sheahan, D., Osborne, J.A., Mathiessen, P. & Sumpter, J.P. (1996). Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to oestrogenic alkylphenolic chemicals. *Environmental Toxicology and Chemistry*. **15**. 194-202.

Jobling, S., Nolan, M., Tyler, C.R., Brighty, G. & Sumpter, J.P. (1998). Widespread sexual disruption in wild fish. *Environmental Science and Technology*. **32**. 2498-2506.

Jobling, S., Beresford, N., Nolan, M., Rodgers-Gray, Brighty, G.C., Sumpter, J.P. & Tyler, C.R. (2002). Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. *Biological Reproduction*. **66**. 272-281.

Jockers, R., Issad, T., Zilberfarb, V., de Coppet, P., Marullo, S & Strosberg, A.D. (1998). Desensitisation of the β-adrenergic response in human brown adipocytes. *Endocrinology*. **139(6)**. 2676-2684.

Johnson, A.C., Williams, R.J. & Johnson, A.C. (1999). Comment on 'identification of oestrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. *Environmental Science and Technology*. **33(2)**. 369-370.

Johnson, A.C., Jürgens, M., Williams, R.J., Kümmerer, K., Kortenkamp, A. & Sumpter, J.P. (2008). Do cytotoxic chemotherapy drugs discharged into rivers pose a risk to the environment and human health? An overview and UK case study. *Journal of Hydrology*. **348(1-2)**. 167-175.

Jørgensen, S.E. & Halling-Sørensen, B. (2000). Drugs in the environment: Editorial. *Chemosphere*. **40**. 691-699.

Jozefowski, S.J. & Plytycz, B. (1998). Characterisation of β-adrenergic receptors in fish and amphibian lymphoid organs. *Developmental and Comparative Immunology*. **22**. 587-603.

Jungas, R.L., Halperin, M.L. & Brosnan, J.T. (1992). Quantitative analysis of amino acid oxidation and regional exchange of amino acids in health subjects. *Clinical Science*. **59**. 173-181.

Kaumann, A.J., Preitner, F., Sarsero, D., Molenaar, P., Revelli, J.P. & Giacobino, J.P. (1997). (-)-CGP 12177 causes cardiostimulation and binds to cardiac putative β_4-adrenoceptors in both wild-type and β_3-adrenoceptor knockout mice. *Molecular Pharmacology*. **53(4)**. 670-675.

Keun, H., Ebbels, T., Antti, H., Bollard, M., Beckonert, G., Schlotterbeck, G., Senn, H., Niederhauser, U., Holmes, E. & Lindon, J. (2002). Analytical reproducibility in 1 NMR-based metabonomic urinalysis. *Chemical Research in Toxicology*. **15**. 1380-1386

Keun, H.C. (2006). Metabonomic modeling of drug toxicity. *Pharmacology and Therapeutics*. **109**. 92-106.

Kieffer, T.J. & Habener, J.F. (2000). The glucagon-like peptides. *Endocrine Reviews*. **20(6)**. 876-913.

Kimball, J.K. & King, M.W (2008). The medical biochemistry page: second messengers. http://biochemistryquestions.wordpress.com/2008. Updated 17/02/2009.

Kiriyama, A., Honbo, A. & Iga, K. (2007). Analysis of hepatic metabolism affecting pharmacokinetics of propranolol in humans. *International Journal of Pharmaceutics*. **349 (1-2)**. 53-60.

Kohno, S., Bermudez, D.S, Katsu, Y. Igucki, T. & Guillette Jr, L.J. (2008). Gene expression patterns in juvenile American alligators (*Alligator mississippiensis*) exposed to environmental contaminants. *Aquatic Toxicology*. **88**. 95-101.

Kolanczyk, R.C., Fitzsimmons, P.N., McKim, J.M., Erickson, R.J. & Schmieder, P.K. (2003). Effects of anaesthesia (tricaine methanesulfonate, MS222) on liver biotransformation in rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology*. **64**. 177-184.

Konkar, A.A., Zhu, Z. & Granneman, J.G. (2000). Aryloxypropamolamine and catecholamine ligand interactions with the β_1-adrenergic receptor: evidence for interaction with distinct conformations of β_1-adrenergic receptors. *Journal of Pharmacology and Experimental Therapeutics*. **294(3)**. 923-932.

Krag, A., Simonsen, L., Henriksen, .H., Ottesen, L. & Bendtsen, F. (2006). Effect of meal and propranolol on whole body splanchnic oxygen consumption in patients with cirrhosis. *American Journal of Physiology – Gastrointestinal and Liver Physiology*. **291**. G8-G15.

Kreiberg, H. (2000). Stress and anaesthesia. In: Ostrander, G.K. The laboratory Fish. *Academic Press, London*. 503-513.

Krief, S., Lönnqvist, F., Raimbault, S., Baude, B., Van Spronsen, A., Arer, P., Strosberg, A.D., Ricquier, D. & Emorine, L. (1993). Tissue distribution of β3-adrenergic receptor mRNA in man. *The American Society for Clinical Investigation*. **91**. 344-349.

Krogh, A., Larsson, B., Von Heijne, G. & Sonnhammer, E.L.L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *Journal of Molecular Biology*. **305(3)**. 567-580.

Kuska, B. (1998). Beer, bethesda and biology: how "genomics" came into being. *Journal of the National Cancer Institute*. **90**. 564-565.

Kümmerer, K., Al-Ahmed, A. & Mersch-Sundermann, V. (2000). Biodegradation of some antibiotics, elimination of the genotoxicity and affection of wastewater bacteria in a simple test. *Chemosphere*. **40**. 701-710.

Kümmerer, K., Ericson, J., Hannah, B., Johnson, A., Sedlak, D.L. & Weston, J.J. (2006) Environmental fate and transport of human pharmaceuticals. **In** R.T. Williams (Ed.) *Human Pharmaceuticals: assessing the impacts on aquatic ecosystems*. (pp 111-148). Society for Environmental Toxicology and Chemistry Press.

Kyte, J. & Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology*. **157(1)**. 105-132.

Lacroix, A., Tremblay, J., Rousseau, G., Bouvier, M. & Hamet, P. (1997). Propranolol therapy for ectopic β-adrenergic receptors in adrenal cushing's syndrome. *The New England Journal of Medicine*. **337(20)**. 1429-1434.

Laenge, R., Steger-Hartmann, T. & Schweinfurth, H. (2006). The environmnetal risk assessment of human pharmaceuticals in the overall EU regulatory affairs process. *Regulatory Toxicology and Chemistry*. **45(3)**. 223-228.

Lange, A., Katsu, Y., Ichikawa, R. Paull, G.C., Chidgey, L.L., Coe, T.S., Iguchi, T. & Tyler, C.R. (2008). Altered sexual development in roach (*Rutilus rutilus*) exposed to environmental concentrations of the pharmaceutical 17α-ethinylestradiol and associated expression dynamics of aromatases and estrogen receptors. *Toxicological Sciences*. **106(1)**. 113-123.

Larsson, D.G.J, Fredriksson, S., Sandblom, E., Paxeus, N. & Axelsson, M. (2006). Is heart rate in fish a sensitive indicator to evaluate effects of β-blockers in surface water? *Environmental Toxicology and Pharmacology*. **22**. 338-340.

Larsson, D.G.J, de Pedro, C., Paxeus, N. (2007). Effluent from drug manufacturers contains extremely high levels of pharmaceuticals. *Journal of Hazardous Materials*. **148(3)**. 751-755.

Laville, N., Ait-Aissa, S., Gomez, E., Casellas, C. & Porcher, J.M. (2004). Effects of human pharmaceuticals on cytotoxicity, EROD activity and ROS production in fish hepatocytes. *Toxicology*. **196**. 41-55.

Lee, H.B., Sarafin, K. & Peart, T.E. (2007). Determination of β-blockers and β_2-agonists in sewage by solid-phase extraction and liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A* **1148**. 158-167.

Lefkowitz, R.J. & Caron, M.G. (1988). Adrenergic receptors: models for the study of receptors coupled to guanine nucleotide regulatory proteins. *The Journal of Biological Chemistry*. **263(11)**. 4993-4996.

Leino, R.L., Jensen, K.M., & Ankley, G.T. (2005). Gonadal histology and characteristic histopathology associated with endocrine disruption in the fathead minnow (Pimephales promelas). *Environmental Toxicology and Pharmacology*. **19**. 85-98.

Leo, A., Hansch, C. & Elkins, D. (1971). Partition coefficients and their uses. *Chemical Reviews*. **71(6)**. 525-616.

Liggett, S.B., Freedman, N.J., Schwinn, D.A. & Lefkowitz, R.J. (1993). Structural basis for receptor subtype-specific regulation revealed by a chimeric β_3/β_2-adrenergic receptor. *Proceedings of the National Academy of Sciences*. **90**. 3665-3669.

Lilius, H., Hastbacka, T. & Isomaa, B. (1995). A comparison of the toxicity of 30 reference chemicals to *Daphnia magna* and *Daphnia pulex*. *Environmental Toxicology and Chemistry*. **14**. 2085-2088.

Linnaeus, C. (1758). Tomus I. Systema naturae per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. *Editio desima, reformata, Holmiae*. **1-4**. 1-824.

Lodish, H., Baltimore, D., Berk, A., Zipursky, S.L., Matsudaira, P. & Darnell, J. (2000). Molecular cell biology. 5th Edition. *Scientific American Books*. 530-549.

Loraine, G.A. & Petigrove, M.E. (2006). Seasonal variations in concentrations of pharmaceuticals and personal care products in drinking water and reclaimed wastewater in southern California. *Environmental Science & Technology*. **40**. 687-695.

Ludden, T.M. (1991). Nonlinear pharmacokinetics: clinical applications. *Clinical Pharmacokinetis*. **20**. 429-446.

Lyssimachou, A., Jenssen, B.M. & Arukwe, A. (2006). Brain cytochrome P450 aromatase gene isoforms and activity levels in Atlantic salmon after waterborne exposure to nominal environmental concentrations of the pharmaceutical ethinyloestradiol and antioulant tributyltin. *Toxicological Sciences*. **91(1)**. 82-92.

Machida, C.A., Runzow, J.R., Searles, RP., Van Tol, H., Tester, B., Neve, K.A., Teal, P., Nipper, V. & Civelli, O. (1990). Molecular cloning and expression of the rat β_1-adrenergic receptor gene. *The Journal of Biological Chemistry*. **265(22)**. 12960-12965.

Manara, L. & Bianchetti, A. (1990). Further heterogenerity of the β-adrenoceptor. The phenylethanolaminotetralines: new selective agonists for atypical β-adrenoceptors. *Trends in Pharmacological Science*. **11(6)**. 229-230.

Marshall, A.G., Hendrickson, C.L. & Jackson, G.S. (1998). Fourier transform ion cyclotron resonance spectrometry: a primer. *Mass Spectrometry Reviews*.**17**. 1-34.

Martini, L. Whistler, J.L. (2007). The role of mu opioid receptor desensitization and endocytosis in morphine tolerance and dependence. *Current Opinions in Neurobiology*. **17(5)**. 556-564.

Mason, D.A., Moore, J.D., Green, S.A. & Liggetrt, S.B. (1999). A gain-of-function polymorphism in a G-protein coupling domain of the human β_1-adrenergic receptor. *Journal of Biological Chemistry*. **274**. 12670-12674.

Masubuchi, Y., Hosokawa, S. Horie, T., Suzuki, T., Ohmori, S., Kitada, M. & Narimatsu, S. (1994). Cytochrome P450 isoenzymes involved in propranolol metabolism in human liver microsomes. The role of CYP1A2 as N-desisopropylase. *American Society for Pharmacology and Experimental Therapeutics*. **22(6)**. 909-915.

Mauder, R.J., Matthiessen, P., Sumpter, J.P. & Pottinger, T.G. (2007). Rapid bioconcentration of steroids in the plasma of three-spined stickleback *Gasterosteus aculeatus* exposed to waterborne testosterone and 17β-oestradiol. *Journal of Fish Biology*. **70**. 678-690.

Mettee, M.F., O'Neil, P.E. & Pierson, J.M. (1996). Fishes of Alabama and the mobile basin. *Oxmoor House publishers*. 128-319.

Miles-Richardson, S.R., Kramer, V.J., Fitzgerald, S.D., Render, J.A., Yamini, B., Barbee, S.J. & Giesy, J.P. (1999). Effects of waterborne exposure of 17β-estradiol on secondary sexual characteristics and gonads of fathead minnows (*Pimephales promelas*). *Aquatic Toxicology*. **47**. 129-145.

Miracle, A., Ankley, G. & Lattier, D. (2006). Expression of two vitellogenin genes (*v*g1 ad *v*g3) in fathead minnow (*Pimephales promelas*) liver in response to exposure to steroidal estrogens and androgens. *Ecotoxicology and Environmental Safety*. **63**. 337-342.

Mitaku, S., Hirokawa, T. & Tsuji, T. (2002). Amphiphilicity index of polar amino acids as an aid in the characterization of amino acid preference at membrane-water interfaces. *Bioinformatics*. **18**. 608-616.

Molenaar, P., Sarsero, D. & Kaumann, A.J. (1997). Proposal for the interaction of non-conventional partial agonists and catecholamin lamines with the "putative β_4-adrenoceptor" in mammalian heart. *Clinical and Experimental Pharmacology and Physiology*. **24(9-10)**. 647-656.

Morales, A.E., Garćia-Rejón, L. & De La Higuera, M. (1990). Influence of handling and/or anaesthetisation on stress response in rainbow trout. Effects on liver primary metabolism. *Comparative Biochemistry and Physiology Part A: Physiology*. **95(1)**. 87-93.

Muzzin, P., Revelli, J, P., Kuhne, F., Gocaynes, J.D., McCombie, W.R., Venter, J.C., Giacobino, J.P. & Fraser, C.M. (1991). An adipose tissue-specific β-adrenergic receptor. *The Journal of Biological Chemistry*. **266(35)**. 24053-24058.

Nantel, F., Bonin, H., Emorine, L.J., Zilberfarb, V., Strosberg, A.D., Bouvier, M. & Marullo, S. (1993). The human beta 3-adrenergic receptor is resistant to short term agonist-promoted desensitisation. *Molecular Pharmacology*. **43(3)**. 548-555.

Nelson, D.L. & Cox, M.M. (2000). Lehringer Principles of Biochemistry. *Worth Publishers*. 724-725.

New drinking water directive on the quality of water intended for human consumption. (2003). European Council. Regulation 1882/2003/EC.

Newton, C.R. & Graham, A. (1994). PCR: introduction to biotechniques. *βIOS Scientific Publishers*. 1-133.

Nichols, T. & Hayasaki, S. (2003). Controlling the familywise error rate in functional neuroimaging: a comparative review. *Statistical Methods in Medical Research*. **12(5)**. 419-446

Nicholson, J.K., Connelly, J., London, J.C. & Holmes, E. (2002). Metabonomics: a platform for studying drug toxicity and gene function. *Nature Reviews*. **1**. 153-161.
Nicholson, J.K. & Wilson, I.D. (2003). Understanding 'global' systems biology: Metabonomics and the continuum of metabolism. *Nature Reviews*. **2**. 668-678.

Nickerson, J.G., Dugan, S.G., Drouin, G. & Moon, T.W. (2001). A putative β_2-adrenoceptor from the rainbow trout (*Oncorhynchus mykiss*). *European Journal of Biochemistry*. **268**. 6465-6472.

Nickerson J.G., Dugan, S.G., Drouin, G., Perry, S.F. & Moon, T.W. (2003). Activity of the unique β-adrenergic Na^+/H^+ exchanger in trout erythrocytes is controlled by a novel β_3-AR subtype. *American Journal of Physiological Regulation and Integrative Comparative Physiology*. **285**. R526-R535.

Nikolaou, A., Meric, S. & Fatta, D. (2007). Occurrence patterns of pharmaceuticals in water and wastewater environments. *Analytical and Bioanalytical Chemistry*. **387**. 1225-1234.

Nilsson, G.E. (1983). Autonomic nerve function in the vertebrates. Zoophysiology. Volume 13. *Springer-Verlag*. 121-354.

Nolan, T., Hands, R.E. & Bustin, S.A. (2006). Quantification of mRNA using real-time RT-PCR. *Nature Protocols*. **1**. 1559-1582.

Oaks, L.J., Gilbert, M., Vriani, M.Z., Watson, R., Meteyer, C.U., Rideout, B.A, Shivaprasad, H.L., Ahmed, S., Chauhry, M.J.I., Arshad, M., Mahmood, S., Ali, A. & Khan, A.A. (2004). Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature*. **427**. 630-633.

Ohno, S. (1970). Evolution of gene duplication. *Springer Verlag, New York*. 32-146.

Oka, H. & Sakai, T. (1969). Thyroid function in liver cirrhosis and effect of thyroxine on collagen metabolism in experimentally induced cirrhotic liver of rats. *Journal of Gastroenterology*. **4(2).** 95-96.

Olesen, J., Hourard, K. & Hertz, M. (1978). Isoproterenol and propranolol: ability to cross the blood-brain barrier and effects on cerebral circulation in man. *Stroke*. **9**. 344-349.

Owen, S.F., Giltrow, E., Huggett, D.B., Hutchinson, T.H., Saye, J., Winter, M.J. & Sumpter, J.P. (2007). Comparative physiology, pharmacology and toxicology of β-blockers: mammals versus fish. *Aquatic Toxicology*. **82**. 145-162.

Panter, G.H., Thompson, R.S., Beresford, N. & Sumpter, J.P. (1999). Transformation of a non-oestrogenic steroid metabolite to an oestrogenically active substance by minimal bacterial activity. *Chemosphere*. **38**. 3579-3596.

Parsons, H.M., Ekman, .R., Collette, T.W. & Viant, M.R. (2009). Spectral relative standard deviation: a practical benchmark in metabolomics. *Analyst*. **134**. 478-485

Paull, G.C., Van Look, K.J.W., Santos, E.M., Filby, A.L., Gray, D.M., Nash, J.P. & Tyler, C.R. (2008). Variability in measures of reproductive success in laboratory-kept colonies of zebrafish and implications for studies addressing population-level effects of environmental chemicals. *Aquatic Toxicology*. **87**. 115-126.

Peakall, D.B. (1994). The role of biomarkers in environmental assessment (1) Introduction. *Ecotoxicology*. **3(3)**. 157-160.

Peng, X., Yu, Y., Tang, C., Tan, J., Huang, Q. & Wang, Z. (2008). Occurrence of steroid oestrogens, endocrine-disrupting phenols and acid pharmaceutical residues in urban riverine water of the Pearl River Delta, South China. *Science of the Total Environment*. **397**. 158-166.

Penny, D., Hendy, M.D. & Steel, M.A. (1992). Progress with methods for constructing evolutionary trees. *Trends in Ecology and Evolution*. **7**. 73-79.

Pérez-Jiménez, A., Guedes, M.J., Morales, A.E. & Oliva-Teles, A. (2007). Metabolic responses to short starvation and refeeding in *Dicentrarchus labrax*. Effect of dietary composition. *Aquaculture*. **265**. 325-335.

Perkinson, A. (1996). In Klassen, C.D. (Ed). Biotransformation of xenobiotics, Casarett & Doull's toxicology, 5th edition. *McGraw-Hill Inc*. pp114-186.

Phillips, K.P. & Tanphaichitr, N. (2008). Human exposure to endocrine disrupters and semen quality. *Journal of Toxicology and Environmental Health Part B.* **11**. 188-220.

Pinccetich, C.A., Viant, M.R., Hinton, D.E. & Tjeerdema, R.S. (2005). Metabolic changes in Japanese medaka (*Oryzias latipes*) during embryogenesis and hypoxia as determined by in vivo ^{31}P NMR. *Comparative Biochemistry and Physiology Part C*. **140**. 103-113.

Polkowska, Z., Koslowska, K. & Namieśnik, J. (2004). Bioogical fluids as a source of information on the exposure of man to environmental chemical agents. *Critical Reviews in Analytical Chemistry*. **34**. 105-119.

Pottinger, T.G., Rand-Weaver, M. & Sumpter, J.P. (2003). Overwinter fasting and re-feeding in rainbow trout: plasma growth hormone and cortisol levels in relation to energy metabolism. *Comparative Biochemistry and Physiology Part B*. **136**. 403-417.

Prodrabsky, J.E., Lopez, J.P., Fan, T.W.M., Higashi, R. & Somero, G.N. (2007). Extreme anoxia tolerance in embryos of the annual killfish *Austrofundulus limnaeus*: insights from a metabolomics analysis. *The Journal of Experimental Biology*. **210** 2253-2266.

Purdom, C.E., Hardiman, P.A., Bye, V.A., Eno, N.C., Tyler, C.R. & Sumpter, J.P. (1994). Oestrogenic effects of effluents from sewage treatment works. *Chemistry and Ecology*. **8**. 275-285

Purohit, P.V., Rocke, D.M., Viant, M.R. & Woodruff, D.L. (2004). Discrimination models using variance-stabilizing transformation of metabolomic NMR data. *Journal of Integrative Biology*. **8(2)**. 118-130.

Pyron, M. & Beitinger, T.L. (1989). Behaviour of male fathead minnows in the presence of an additional male or female fathead minnow. *Texas Journal of Science*. **41**. 151-154.

Quinn, B., Gagne, F. & Blaise, C. (2008). An investigation into the acute and chronic toxicity of eleven pharmaceuticals (and their solvents) found in wastewater effluent on the cnidarian, *Hydra attenuata*. *Science of the Total Environment*. **389(2-3)**. 306-314.

Randall, D.J. & Perry, S.F. (1992). Fish Physiology. Volume 12. *Academic Press, Inc.* 256-289.

Rang, H.P., Dale, M.M., Ritter, J.M. & Flower, R. (2003). Pharmacology. *Churchill Livingstone.* 3-34.

Rathner, M. & Sonneborn, M. (1979). Biologically active oestrogens in potable water and effluents. *Forum Stadte-Hygiene*. **30**. 45-49.

Rautureau, Y., Toumaniantz, G., Serpillon, S., Jourdon, P., Trochu, J.N. & Gauthier, C. (2002). Beta 3-adrenoceptor in rat aorta: molecular and biochemical characterisation and signaling pathway. *British Journal of Pharmacology*. **137**. 153-161.

Reemtsma, T., Weiss, S., Mueller, J., Petrović, M., Gonzalez, S., Barcelo, D., Ventura, F. & Knepper, T.P. (2006). Polar pollutants entry into the water cycle by municipal wastewater: a European perspective. *Environmental Science and Technology*. **40**. 5451-5458.

Reid, S.D., Bernier, N.J. & Perry, S.F. (1998). The adrenergic stress response in fish: control and release of catecholamine storage and release. *Comparative Biochemistry and Physiology*. **120C**. 1-27.

Riordan, S. & Williams, R. (2002). Blood lactate and outcome of paracetamol-induced acute liver failure. *The Lancet*. **360(9332)**. 573-574.

Roberts, P.H., Thomas, K.V. (2006). The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment. *Science of the Total Environment*. **356**. 143-153.

Robertson, D.G. (2005). Metabonomics in Toxicology. *Toxicological Sciences*. **85(2)**. 809-822.

Robidoux, J., Kumar, N., Daniel, K.W., Moukdar, F., Cyr, M. & Medvedev, A.V. (2006). Maximal beta3-adrenergic regulation of lipolysis involves Scr and epidermal growth factor receptor-dependent ERK ½ activation. *Journal of Biological Chemistry*. **281**. 37794-37802.

Rodriguez, M., Carillon, C., Coquerel, A., Le Fur, G., Ferrara, P., Caput, D. & Shire, D. (1995). Evidence for the presence of β3-adrenergic receptor mRNA in the human brain. *Molecular Brain Research*. **29**. 369-375.

Rodriquez-Cuenca, S., Pujol, E., Justo, R., Frontera, M., Oliver, J., Gianotti, M. & Roca, P. (2002). Sex-dependent thermogenesis, differences in mitochondrial morphology and function, and adrenergic response in brown adipose tissue. *Journal of Biological Chemistry*. **277**. 42958-42963.

Ronan, P.J., Gaikowski, M.P., Hamilton, S.J., Buhl, K.J. & Summers, C.H. (2007). Ammonia causes decreased brain monoamines in fathead minnows (*Pimephales promelas*). *Brain Research*. **1147**. 184-191.

Rook, E.S.J. (1999). *Pimephales promelas*, fathead minnow. Available from www.rook.org/earl/bwca/nature/fish/pimephalespo.html. [Accessed 14/08/06].

Rosenblum, E.S., Viant, M.R., Braid, B.M., Moore, J.D., Friedman, C.S. & Tjeerdema, R.S. (2005). Characterising the metabolic actions of natural stresses in the California red abalone, *Haliotis rufescens* using ^{1}H NMR metabolomics. *Metabolomics*. **1(2)**. 199-209.

Rozec, B., Serpillon, S., Toumaniantz, G., Sèze, C., Rautureau, Y., Baron, O., Noireaud, J. & Gauthier, C. (2005). Characterisation of beta$_3$-adrenoceptors in human internal mammary artery and putative involvement in coronary artery bypass management. *Journal of American College of Cardiology*. **46(2)**. 351-359.

Rühling, K., Schauer, I. & Thielmann, K. (1980). Intraindividual variability of plasma cholesterol and triglycerides and the effect of propranolol treatment. *Artery*. **8(2)**. 140-145.

Runnalls, T. (2005). Pharmaceuticals in the environment: the effects of clofibric acid on fish. *PhD Thesis*. Brunel University.

Ruuskanen, J.O., Laurila, J., Xhaard, H., Rantanen, V.V., Vuoriluoto, K., Wurster, S., Marjamäki, A., Vainio, M., Johnson, M.S. & Scheinin, M. (2005). Conserved structural, pharmacological and functional properties among the three human and five zebrafish α_2-adrenoceptors. *British Journal of Pharmacology*. **144**. 165-177.

Ryan, D. & Robards, K. (2006). Metabolomics: the greatest omics of them all? *Analytical Chemisry*. **78**. 7954-7958.

Sacher, F., Lange, F.T., Brauch, H. & Blankenhorn, I. (2001). Pharmaceuticals in groundwaters. Analytical methods and results of a monitoring program in Baden-Württemberg, Germany. *Journal of Chromatography A*. **938**. 199-210.

Sambrook, J. & Russell, D.W. (2006). The condensed protocols from molecular cloning: A laboratory manual. *Cold Spring Harbour Laboratory Press*. 269-356.

Samuelsson, L.M., Förlin, L., Karlsson, G., Adolfsson-Erici, M. & Larsson, J.D.G. (2006). Using NMR metabolomics to identify responses of an environmental estrogen in blood plasma of fish. *Aquatic Toxicology*. **78(4)**. 341-349.

Sánchez-Muros, M., García-Rejón, L., García-Salguero, L., de la Higuera, M. & Lupiánez, J. A. (1998). Long-term nutritional effects on the primary liver and kidney metabolism in rainbow trout. Adaptive response to starvation and a high protein, carbohydrate-free diet on glutamate dehydrogenase and alanine aminotransferase kinetics. *The International Journal of Biochemistry & Cell Biology*. **30**. 55-63.

Sanyal, A.K., Royd, D., Chowdhury, B. & Banerjee, A.B. (1993). Ibuprofen, a unique anti-inflammatory compound with antifungal activity against dermatophytes. *Letters in Applied Microbiology*. **17**. 109-111.

Saradha, B., Mathur, P.P. (2006). Effect of environmental contaminants on male reproduction. *Environmental Toxicology and Pharmacology*. **21**. 34-41.

Sawa, M. & Harada, H. (2006). Recent developments in the design of orally bioavailable β_3-adrenergic receptor agonists. *Current Medicinal Chemistry*. **13**. 25-37.

Schiöth, H.B. & Fredriksson, R.F. (2005). The GRAFS classification system of G-protein coupled receptors in comparative perspective. *General and Comparative Endocrinology.* **142**. 94-101.

Schmidt, C.W. (2004).Metabolomics: What's happening downstream of DNA. *Environmental Health Perspectives*. **112(7)**. A411-A415.

Schweinfurth, H., Lange, R. & Gunzel, P. (1996). Environmental fate and ecological effects of steroidal oestrogens. *IBC Conference Proceedings of "Oestrogenic chemicals in the Environment", London. 9-10 May 1996*.

Scoog, D.A., Holler, F.J. & Nieman, T.A. (1998). Principles of Instrumental analysis. 5th Edition. *Saunders College Publishing*. 498-532.

Scott, A.P., Katsiadaki, I., Witthames, P.R., Hyllans, K., Davies, I.M., McIntosh, A.D. & Thains, J. (2006). Vitellogenin in the blood of male cod (*Gadus morhua*): A sign of oestrogenic endocrine disruption in the open sea? *Marine Environmental Research*. **61**. 149-170.

Scott, A.P., Sanders, M., Stentiford, G.D., Reese, R.A. & Katsiadaki, I. (2007). Evidence for oestrogenic endocrine disruption in offshore flatfish, the dab (*Limanda limanda*). *Marine Environmental Research*. **64(2)**. 128-148.

Sewell, A.C., Herwig, J., Böhles, H.J. & Sperl, W. (1997). Mitochondrial respiratory chain succinate-cytochrome-c oxidoreductase deficiency and hepatic cirrhosis. *Journal of Inherited Metabolic Disease*. **20(6)**. 837-838.

Shugart, L. & Theodorakis, C. (1998). New trends in biological monitoring: application of biomarkers to genetic toxicology. *Biotherapy*. **11**. 119-127.

Skeberdis, V.A. (2004). Structure and function of β_3-adrenergic receptors. *Medicina (Kaunas)*. **40(5)**.407-412.

Skoog, D.A., Holler, F.J. & Nieman, T.A. (1998). Principles of instrumental analysis 5th Edition: Nuclear magnetic resonance spectroscopy. *Saunders College Publishing*. 445-490.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology*. **98(3)**. 503-517.

Small, K.M., McGraw, D.W. & Liggett, S.B. (2003). Pharmacology and physiology of human adrenergic receptor polymorphisms. *Annual Review of Pharmacology and Toxicology*. **43**. 381-411.

Smith, R.J.F. (1978). Effects of 17 c-methyltestosterone on the dorsal pad and tubercles of fathead minnows (*Pimephales promelas*). *Canadian Journal of Zoology*. **52**. 1031-1038.

Snape, J.R., Maund, S.J., Pickford, D.B. & Hutchinson, T.H. (2004). Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. *Aquatic Toxicology*. **67(2)**. 143-154.

Soeder, K.J., Snedden, S.K., Cao, W., la Rocca, G.J., Daniel, K.W. & Luttrell, L.M. (1999). The beta3-adrenergic receptor activates mitogen-activated protein kinase in adipoxytes through a Gi-dependent mechanism. *Journal of Biological Chemistry*. **274**. 12017-12022.

Spronsen, A.V., Nahmias, C., Krief, S., Briend-Sutren, M-M., Strosberg, A.D. & Emorine, L.J. (1993). The promotor and intron/exon structure of the human and mouse β3-adrenergic-receptor genes. *European Journal of Biochemistry*. **213**. 1117-1124.

Strosberg, A.D. (1993). Structure, function and regulation of adrenergic receptors. *Protein Science*. **12**. 1198-1209.

Strosberg, A.D. & Pietri-Rouxel, F. (1996). Function and regulation of the β_3-adrenoceptor. *Trends in Pharmacological Sciences*. **17(10)**. 373-381.

Strosberg, A.D., & Nahmias, C. (2007). G-protein-coupled receptor signaling through protein network. *Biochemistry Society Transactions*. **35**. 23-27.

Stuer-Lauridsen, F., Birkved, M., Hansen, L.P., Lutzhøft, H. & Halling-Sørensen, B. (2000). Environmental risk assessment of human pharmaceuticals in Denmark after normal therapeutic use. *Chemosphere*. **40**. 783-793.

Sumpter, J.P. (2007). Environmental effects of human pharmaceuticals. *Drug Information Journal*. **41**. 143-147.

Switzer, R.C., Merril, C.R., Shifrin, S. (1979). A highly sensitive silver stain for detecting proteins and peptides and polyacrylamide gels. *Analytical Biochemistry*. **98**. 231-237.

Takeda, M., Obara, K., Mizusawa, T., Tomita, Y., Arai, K., Tsutsui, T., Hatano, A., Takahashi, K. & Nomura, S. (1999). Evidence for β_3-adrenoceptor subtypes in relacation of the human urinary bladder detrusor: analysis by molecular biological and pharmaceological methods. *The Journal of Pharmacology and Experimental Therapeutics*. **288(3)**. 1367-1373.

Ternes, T.A. (1998). Occurrence of drugs in German sewage treatment plants and rivers. *Water Research*. **32(11)**. 3245-3260.

Ternes, T.A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R.D. & Servos, M. (1999). Behaviour and occurrence of oestrogens in municipal sewage treatment plants – I. Investigations in Germany, Canada and Brazil. *The Science of the Total Environment*. **225**. 81-90.

Ternes, T.A., Jos, A. & Siegrist, H. (2004). Scrutinising pharmaceutical and personal care products in wastewater treatment. *Environmental Science and Technology*. **38**. 393-399.

Terzić, S., Senta, I., Ahel, M., Gros, M., Petrović, Barcelo, D., Müller, J., Knepper, Martí, Ventura, F., Jovančić, P. & Jabučar, D. (2008). Occurrence and fate of emerging wastewater contaminants in Western Balkan Region. *Science of the Total Environment*. **399**. 66-77.

Teahan, O., Gamble, S., Holmes, E., Waxman, J., Nicholson, J., Bevan, C. & Keun, H. (2006). Impact of analytical bias in metabonomic studies of human blood serum and plasma. *Analytical Chemistry*. **78**. 4307-4318.

Thellin, O., Zorzi, W., Lakaye, B., De Borman, B., Coumans, B., Hennen, G., Grisar, T., Igout, A. & Heinen, E. (1999). Housekeeping genes as internal standards: use and limits. *Journal of Biotechnology*. **75**. 291-295.

Thomas, K.V. & Hilton, M.J. (2004). The occurrence of selected human pharmaceutical compounds in UK estuaries. *Marine Pollution Bulletin*. **49**. 436-444.

Thompson, J.D., Higgins D.G. & Gibson T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*. **22**. 4673-4680.

Thompson, R.P. (1986). Measuring the damage – ethanol and the liver. *Gut*. **27(7)**. 751-755.

Thorpe, K.L., Benstead, R., Hutchinson, T.H. & Tyler, C.R. (2007). An optimised experimental test procedure for measuring chemical effects on reproduction in the fathead minnow, *Pimephales promelas*. *Aquatic Toxicology*. **81**. 90-98

Tusnády, G.E. & Simon, I. (2001). The HMMTOP transmembrane topology prediction server. *Bioinformatics*. **17**. 849-850.

Tyler, C.R. & Routledge, E.J. (1998). Natural and anthropogenic environmental oestrogens: the scientific basis for risk assessment. Oestrogenic effects in fish in English rives with evidence of their causation. *Pure and Applied Chemistry*. **70(9)**. 1795-1804.

Tynan, F., Fischer, D. & Ibels, L. (1981). Self poisoning with propranolol. *The Medical Journal of Australia*. **1**. 82-83.

UAB Health System. (2006). Beta blocking agents. Available from www.health.uab.edu/show.asp [Accessed 17.03.06].

Valiquette, M., Bonin, H., Hnatowich, M., Caron, M.G., Lefkowitz, R.J. & Bouvier, M. (1990). Involvement of tyrosine residues located in the carboxyl tail of the human β_2-adrenergic receptor

in agonist-induced down-regulation of the receptor. *Proceedings of the National Academy of Sciences*. **87**. 5089-5093.

Viant, M.R., Walton, J.H., TenBrook, P.L. & Tjeerdema, R.S. (2002). Sublethal actions of copper in abalone (*Haliotis rufescens*) as characterised by in vivo P NMR. *Aquatic Toxicology*. **57**. 139-151.

Viant, M.R. (2003). Improved methods for the acquisition and interpretation of NMR metabolomic data. *Biochemical and Biophysical Research Communications*. **310**. 943-948.

Viant, M.R., Werner, I., Rosenblum, E.S., Gantner, A.S., Tjeerdema, R.S. & Johnson, M.L. (2003). Correlation between heat-shock protein induction and reduced metabolic condition in juvenile rainbow trout (*Oncorhynchus mykiss*) chronically exposed to elevated temperature. *Fish Physiology and Biochemistry*. **29**. 159-171.

Viant, M.R., Bundy, J.G., Pincetich, C.A., Ropp, J.S. & Tjeerdema, R.S. (2005). NMR-derived developmental metabolic trajectories: an approach for visualizing the toxic actions of trichloroethylene during embryogenesis. *Metabolomics*. **1(2)**. 149-158.

Vieno, N.M., Tuhkanen, T. & Kronberg, L. (2006). Seasonal variation in the occurrence of pharmaceuticals in effluents from a sewage treatment plant and in the recipient water. *Environmental Science and Technology*. **39(21)**. 8220-8226.

Vieno, Heli Härkki, H., Tuhkanen, T & Kronberg, L. (2007). Occurrence of pharmaceuticals in river water and their elimination in a pilot-scale drinking water treatment plant. *Environmental Science and Technology*. **41(14)**. 5077-5084.

Vierstraete, A. (1999). Polymerase chain reaction principles. http://users.ugent.be/~avierstr/. Updated 05/01/2004.

Vu, V.T. & Abramson, F.P. (1980). The pathways of propranolol metabolism in dog and rat liver 10,000g supernatant fractions. *Drug Metabolism and Disposition*. **8(5)**. 300-304.

Warne, T., Serrano-Vega, M.J., Baker, J.G., Moukhametzianov, R., Edwards, P.C., Henderseon, R., Leslie, A.G.W., Tate, C.G. & Schertler, G.F.X. (2008). Structure of a β_1-adrenergic G-protein-coupled receptor. *Nature*. **454**. 486-491

Watson, S. & Arkinstall, S. (1994). The G-protein linked receptor factsbook. Adrenaline and noradrenaline. *Academic Press*. 32-54.

Watson, J.D., Gilman, M., Witkowski, J. & Zoller, M. (1993). Recombinant DNA 2nd Edition. *Scientific American Books*. 79-127.

Watson, J., Lowe, A., Silver, K., Yang, Y., Bodkin, N.L. & Hansen, B.C. (1997). The beta-3-adrenergic receptor in the obesity and diabetes prone rhesus monkey is very similar to human and contains arginine at codon 64. *Gene*. **188**. 207-213.

Weaver, R.F. (2002). Molecular Biology 2nd Edition. *McGraw-Hill Higher Education Inc*. 261-747.

Webb, S.F. (2001). A data-based perspective on the environmental risk assessment of human pharmaceuticals I – Collation of available ecotoxicity data. In Kümmerer, K. *Pharmaceuticals in the Environment – Sources, Fate, Effects and Risk*. Springer-Verlag, Berlin, Germany. pp 175-201.

Weber, C.E. (1998). Cortisol's purpose. *Medical Hypotheses*. **51**. 289-292.

Wedekind, C., Siebenthal, B. & Gibgold, R. (2007). The weaker points of fish acute toxicity tests and how tests on embryos can solve some issues. *Environmental Pollution*. **148**. 385-389.

Westerhuis, J.A., van Velzen, E.J.J., Hoefsloot, H.C.L. & Smilde, A.K. (2008). Discriminant Q^2 (DQ^2) for improved discrimination in PLSDA models. *Metabolomics*. **4(4)**. 293-296.

Whitehead, A., & Crawford, D.L. (2005). Variation in tissue-specific gene expression among natural populations. *Genome Biology*. **6(2)**. R13.3.

Williams, R.T. (2006).Human health pharmaceuticals in the environment – an introduction. **In** R.T. Williams (Ed.) *Human pharmaceuticals: assessing the impacts on aquatic ecosystems* (pp 1-46). *Society for Environmental Toxicology and Chemistry*. 7-298.

Williams, R.T., Hugget, D.B. & Sager, N.B. (2006). Human drug development and registration, including an example of how environmental safety is evaluated. **In** R.T. Williams (Ed.) *Human pharmaceuticals: assessing the impacts on aquatic ecosystems* (pp 47-70). *Society for Environmental Toxicology and Chemistry*. 7-298.

Wingfield, J.C., (2008). Comparative endocrinology, environment and global change. *General and Comparative Endocrinology*. **157**. 207-216.

Winter, M.J., Lillicrap, A.D., Caunter, J.E., Schaffner, C., Alder, A.C., Ramil, M., Ternes, T.A., Giltrow, E., Sumpter, J.P. Hutchinson, T.H. (2008). Defining the chronic impacts of atenolol on embryo-larval development and reproduction in the fathead minnow (Pimephales promelas). *Aquatic Toxicology*. **86(3)**. 361-369.

Wong, L., Nation, R.L., Chiou, W.L. & Mehta, P.K. (1979). Plasma concentrations of propranolol and 4-hydroxypropranolol during chronic oral propranolol therapy. *British Journal of Clinical Pharmacology*. **8**. 163-167.

Wu, H., Southam, A.D., Hines, A. & Viant, M.R. (2007). High-throughput tissue extraction protocol for NMR- and MS-based metabolomics. *Analytical Biochemistry*. **372**. 204-212.

Yonkos, L.T., Fisher, D.J., Reimschussel, R. & Kane, A.S. (2000). Atlas of fathead minnow normal histology. *An online publication of the University of Maryland Aquatic Pathology Center*. Available from http://aquaticpath.umd.edu/fhm. [Accessed: 14/08/06].

Yuwiler, A. & Samuel, D. (1974). Ethyl-m-aminobenzoate (MS-222) anaesthesia in the newt – effect of D_2O, pH and time of day. *Cellular and Molecular Life Sciences*. **30(9)**. 1058-1060.

Zerulla, M., Länge, R., Steger-Hartmann, T. Panter, G., Hutchinson, T. & Dietrich, D.R. (2002). Morphological sex reversal upon short-term exposure to endocrine modulators in juvenile fathead minnow (*Pimephales promelas*). *Toxicology Letters*. **131**. 51-63.

Zimmerman, H.J. (1995). Drug induced liver disease. *Clinics in Liver Disease*. **4(1)**. 73-96.

APPENDIX

APPENDIX 1: 10 x TRIS BORATE EDTA BUFFER REAGENTS

Add the following components to a 1L flask:

109g Tris Base
7.3g EDTA
55g Boric acid

- Make up to 1L with dH_2O.
- Autoclave for sterilisation and to ensure complete dissolution of components

To make 1 x TBE, dilute 10 x TBE ten-fold in dH_2O.

APPENDIX 2: AGAR REAGENTS

Add the following components to a 1L flask:

10g tryptone
5g yeast extract
10g NaCl
15g Bacto agar

- Make up to 1L with deionised dH_2O
- Autoclave for sterilisation and to ensure complete dissolution of components

APPENDIX 3: LURIA BROTH (L-BROTH) REAGENTS

Add the following components to a 1L flask:

10g tryptone
5g yeast extract
10g NaCl

- Make up to 1L with deionised dH_2O
- Autoclave for sterilisation and to ensure complete dissolution of components