Pharmaceuticals in the Aquatic Environment: β-blockers as a case study

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by

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Abstract

The presence of many human pharmaceuticals in the aquatic environment is now a worldwide concern and yet little is known of the chronic effects that these bioactive substances may be having on aquatic organisms. This study used mammalian pharmacodynamics to predict the mode of action of the β-blocker, propranolol, on fish, in order to identify chronic effects in fathead minnows. β-blockers target β1- and β2-adrenergic receptors in humans and hence these receptors were characterised in the fathead minnow. It was found that fish possess β1- and β2-ARs that are structurally very similar to their mammalian counterparts. Further, the distributions of these two β-ARs in various organs of the fathead minnow were similar to those in mammals. Pair-breeding assays were conducted, in which fathead minnows were exposed to various concentrations of propranolol. To discover whether β-ARs had been up or down regulated by propranolol, molecular analysis was conducted on different tissues of the exposed fish using real-time polymerase-chain reactions (RT-PCR). Exposure of fathead minnows to propranolol caused acute toxicity at 10 mg/L. Propranolol caused a statistically significant decrease in reproduction at 1.0 mg/L, dose-related decreases in male weight, condition index and fatpad weight, and a dose-related increase in female GSI. Molecular analysis of β1- and β2-AR expression levels revealed a dose-related decrease in β2-AR expression in fathead liver and heart. LOEC and NOEC values were 0.1 mg/L and 0.01 mg/L, respectively. Propranolol plasma concentrations in fish exposed to water concentrations of 0.1 and 1.0 mg/L were greater than the human therapeutic concentration and hence these data very strongly support the fish plasma model proposed by Huggett et al. (2001).

Keywords- adrenergic receptor, β-blockers, fathead minnow, pharmaceuticals, propranolol

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Chapter 1 Introduction

1.1 Pharmaceuticals in the environment

In Britain alone, approximately 3,000 different human pharmaceutical substances are registered for use, of which a variety (e.g. painkillers, aspirin, paracetamol, the anti-epileptic carbamazepine, and antibiotics) sell an excess of 10 tonnes per year (ENDS Report, 2005; Thomas & Hilton, 2004). This may be because a number of illnesses require long term treatment, and so a lot of some particular drugs are used and may enter the environment. With regard to veterinary pharmaceuticals, a trend has developed towards more intensive farming and more reliance is being placed in pharmaceuticals, feed additives, hormones and potent pesticides (Di Guardo et al., 2001).

There are many routes of pharmaceutical entry into the aquatic environment, but the main route is recognised as being in effluent from sewage treatment works (STWs) (Andreozzi et al., 2003). Humans that are prescribed drugs excrete metabolites and often a percentage of the parent compound, in urine and faeces. Sewage is taken to STWs and although the majority of human pharmaceuticals are removed during treatment processes, complete degradation does not occur and so a small percentage of the pharmaceutical reaches the aquatic environment via effluents. In addition to this, a lot of pharmaceuticals are excreted as conjugates that are formed via phase II reactions within the body. Conjugation (e.g. the addition of a glucuronide or sulphate group to the active molecule) brings about an increase in molecular weight and polarity of a molecule, which also has the effect of making the parent molecule less toxic. However, during sewage treatment, the conjugate is often cleaved off, thus releasing the active moiety (Gros et al., 2007). Hence, a varying amount of a pharmaceutical can routinely end up in treated effluents, rivers, and lakes and more rarely in groundwater. Occurrence of pharmaceuticals, although less frequent, has also been detected in estuarine and coastal waters (Thomas & Hilton, 2004). Other routes of entry of pharmaceuticals into the aquatic environment can occur during the production of drugs, direct disposal of surplus household drugs that leach from landfill sites, runoff from irrigating crops with treated wastewater and leaching of compounds from the application of sludge onto agricultural land (Pedersen et al., 2005; Ternes, 1998). The fate of veterinary and human drugs is quite different from each other. Unlike human pharmaceuticals, veterinary pharmaceuticals don't have to pass through a STW prior to entering a river and hence they can enter the aquatic system directly from runoff or by leaching from manure (Chen et al., 2006; Ternes, 1998).

1.2 Concentrations of pharmaceuticals in the environment

Pharmaceutical compounds such as analgesics, antibiotics, anticonvulsive drugs, cancer drugs, lipid regulators, psychiatric drugs and even recreational drugs have been detected in a range of water bodies (Jones et al., 2004). However, when the design of pharmaceuticals is taken in to consideration, this is hardly surprising. The majority of drugs are designed to be generally resistant to hydrolysis and persistent, so that their chemical structures can be retained long enough for their therapeutic work to be done (Jones et al., 2002). For example, the ethinyl group on ethinyl estradiol makes this estrogen much more resistant to biodegradation and conjugation in the liver, so that it is tough enough to be taken orally but not degraded in the gut, so that enough of the drug can reach the blood and target organ. These factors unfortunately may enable pharmaceuticals to remain in the environment for a significant period of time. As well as having the above characteristics, some pharmaceuticals are also designed to ensure that they can be taken at relatively low doses, yet can have an extremely potent effect. However, it has to be remembered that there is often only a small percentage of people in the population taking such potent drugs at any one time, and hence these drugs are often found at low concentrations in the environment.

Concentrations of drugs in rivers are often dependent on the pharmacokinetic properties of the drug, i.e. what the body does to a pharmaceutical, which includes the distribution, metabolism and elimination of a pharmaceutical. The physical properties of a pharmaceutical can also give an indication of how long it may reside in the environment, where it partitions to, and how readily it can be degraded. For example, log K_{ow} provides a measure of the hydrophobicity of a chemical, i.e. its tendency to move from water (a polar liquid) into a non-polar liquid (i.e. fat) when the chemical is at thermodynamic equilibrium (Walker et al., 1997). Pharmaceuticals with a high K_{ow} have a propensity to adsorb on to organic particles and settle at the bottom of streams, or undergo biological uptake into fatty tissue of animals and plants (Brun et al., 2006). However, in addition to hydrophobicity partioning, independent mechanisms such as cation exchange, cation bridging at clay surfaces, surface complexation and hydrogen binding are also involved in determining the fate of a pharmaceutical compound in the aquatic environment (Calamari et al., 2003)

Kd values define the sorption constant of a pharmaceutical, which defines the partition of a compound between the sludge and the water phase, and pKa values help us understand how much the chemical will dissociate and to what extent, at environmental pH values. The half life of a drug in a stream is also affected by the physical dynamics of the environment. For example, the amount of natural stream flow will dictate the amount of

dilution and mixing of a drug, whilst the landscape characteristics such as drainage, soil and sediment type will affect partioning dynamics (Brun et al., 2006).

Tables 1.1 and 1.2 give a rough guide to which pharmaceuticals are known to be present in rivers and STW effluents at some of the highest concentrations around the world. It must be remembered that not all pharmaceuticals were tested for in each paper, and detection limits and methodology between laboratories are often different. For example, matrix effects are often encountered when determining the concentration of a pharmaceutical, which can lead to signal suppression or enhancement, possibly leading to erroneous results. There are different ways to reduce matrix effects (e.g. selective extraction, effective sample clean up after extraction, improvement of chromatographic separation, internal standard or dilution of samples extracts) but these can often be expensive, time consuming, lead to analyte losses or be unavailable (Gros et al., 2006). However, these Tables do show that a range of pharmaceuticals can be present in the environment in the low μg/L range, although many are in the high ng/L range.

Ibuprofen, an analgesic with anti-inflammatory properties, features quite frequently in these Tables. This is probably due to high over the counter sales of this pharmaceutical, and because it is poorly metabolised in humans, with 70 – 80 % of the parent compound and metabolites being excreted. In addition to this, ibuprofen is not readily degradable and has the potential to bioaccumulate (log K_{ow} is 3.5), hence it is not surprising that it is found in both effluents and rivers at relatively high concentrations for pharmaceuticals (Ashton et al., 2004; Thomas & Hilton, 2004).

Country	Pharmaceutical found at the highest concentration in each study in effluent (ng/L)			Reference
	Drug	**Type of drug**	**Range concentration (ng/L)**	
U.K.	Ibuprofen	Analgesic and anti-inflammatory	<20 - 27256	Ashton et al., 2004
U.K.			1800 - 3800	Hilton & Thomas 2003
EU countries			50 - 7110	Andreozzi et al., 2003
Canada			381 - 1191	Gagné et al., 2006
U.S.A.	Naproxen	Analgesic and anti-inflammatory	380	Yu et al., 2006
U.S.A.			81 - 106	Boyd et al., 2003
Canada	Salicylic acid (aspirin)	Analgesic, antipyretic and anti-inflammatory	bld - 35,000	Brun et al., 2006
Canada			1675 - 3522	Lajeunesse & Gagnon, 2007
Germany	Diatrizoate	X-ray contrast media	80 - 8700	Ternes, 2001
4 EU countries	Diclofenac	Non-steroidal anti-inflammatory	bld - 5450	Ferrari et al., 2003
Germany	Bezafibrate	Lipid regulator	<250 – 4600	Ternes 1998
U.S.A.	Meprobamate	Minor tranquilizer	1270	Vanderford & Snyder, 2006
India	Ciprofloxacin	Antibiotic	28,000,000-31,000,000	Larsson et al., 2007
Croatia	Acetaminophen (paracetomol)	Analgesic and antipyretic	blq - 5990	Gros et al., 2006
S. Korea	Iopromide	X-ray contrast media	1170 - 4030	Kim et al., 2007

Table 1.1 Pharmaceuticals found at the highest concentrations in effluent in different countries. It must be noted that these data by Larsson et al., (2007) are quite unique in that about 90 pharmaceutical companies were discharging their liquid wastes into just one STW. blq: below limit of quantification. bld: below limit of detection.

Country	Pharmaceutical found at the highest concentration in rivers (ng/L)			Reference
	Drug	**Type of drug**	**Range or actual concentration (ng/L)**	
Canada	Naproxen	Analgesic and anti-inflammatory	94 - 551	Metcalfe et al., 2003a
U.S.A.			bld - 68	Boyd et al., 2003
Spain	Atenolol	β-blockers	160 - 465	Gros et al., 2007
Croatia			bld - 250	Gros et al., 2006
Finland	Metroprolol		39 - 107	Vieno et al., 2007
U.K.	Erythromycin	Antibiotic	1000	Hilton & Thomas 2003
Germany	Bezafibrate	Lipid regulator	350 - 3100	Ternes 1998
Canada	Salicylic acid (aspirin)	Analgesic, antipyretic and anti-inflammatory	bld - 17,000	Brun et al., 2006
U.K.	Ibuprofen	Analgesic and anti-inflammatory	<20 - 5044	Ashton et al., 2004
Italy	Hydro-chlorothiazide	Diuretic	bld - 255.8	Calamari et al., 2003
Italy	Lincomycin	Antibiotic	80	Castiglioni et al., 2004
S. Korea	Iopromide	X-ray contrast media	20 - 361	Kim et al., 2007

Table 1.2. Pharmaceuticals found at the highest concentrations in rivers in different countries. bld: below limit of detection

1.3 The risk pharmaceuticals pose to non-target organisms

Many, and perhaps most, pharmaceuticals are probably present in the environment at concentrations too low to cause any effects on fauna and flora (Sumpter 2007). However, some pharmaceuticals pose a greater potential threat to the aquatic environment than others, as some will have a higher usage, others will be more potent at low levels, whilst some will be poorly degraded and have the ability to bioaccumulate in aquatic organisms. To highlight pharmaceuticals that may cause harm to aquatic organisms, risk characterisation ratios can be calculated.

$$\text{Risk characterisation ratio} = \frac{\text{Predicted environmental concentration (PEC)}}{\text{Predicted no-effect concentration (PNEC)}}$$

PEC is the predicted environmental concentration in surface waters calculated from a worst case scenario. This value is based on usage, physical properties, chemical and biological properties, STW effluent flows and surface water flows. PNEC is the predicted highest concentration considered unlikely to cause an effect, and is an estimate of the concentration at which no potential effects on aquatic organisms and ecosystems might occur. It is usually obtained using ecotoxicology data from the open literature and quantitative structure-activity relationships (QSAR). Substances with a ratio greater than 1 are deemed to be of 'potential concern' (Ashton et al., 2004). However, these ratios can only be as good as the data available to feed into the calculation. Table 1.3 shows two different drugs with very different PEC/PNEC ratios.

Drug	UK use (tonnes per year)	PEC µg/L	PNEC µg/L	PEC/PNEC
Propranolol	11.8	2.17	1.87	1.16
Ethinylestradiol	0.029	0.005	0.84	<0.01

Table 1.3. Summary of PEC and PNEC values and ratios for 2 pharmaceuticals found in the environment. Taken from Webb: Pharmaceuticals in the environment (2001)

Propranolol, a β-blocker, has a ratio >1 and therefore is shown to be of 'potential concern'. Studies show it has been found to affect medaka (*Oryzias latipes*) reproduction at concentrations as low as 0.005 mg/L, although these data have not been repeated since (Huggett et al., 2003a). However, from the calculations in Table 1.3a, ethinylestradiol has a ratio much less than 1, and therefore is deemed to be of no concern. Yet ethinylestradiol is found at environmental concentrations at < 1ng/L and has been found to be a most potent

endocrine disrupter, demonstrating an estrogenic potency over a thousand times greater than any xenobiotic mimic, and found to elicit a response in fish at concentrations as low as 0.1 - 0.5 ng/l (Purdom et al., 1994, Thorpe et al. 2003). The PNEC data from Webb (2001) seems to be the acute toxicity value and not the chronic PNEC, for if recalculated using a PNEC of 0.5 ng/L, the resulting risk characterisation ratio is 10, and hence shows ethinylestradiol to be of great concern, highlighting how this ratio relies heavily on accurate data.

The above example shows that those drugs of environmental concern are not necessarily those in high production, but can also be those with a high environmental persistence, that have a high potency or have effects on key biological functions such as reproduction (Fent et al., 2006). However, the vast majority of pharmaceuticals do not exhibit high acute ecotoxicity, in that they are not lethal to aquatic life at concentrations less than 1mg/L. More than 90 % of these compounds have EC50 values greater than 1mg/L, and only 1 % have EC50 values less than 0.01mg/L (Cunningham et al., 2004). Hence, most pharmaceuticals are not expected to cause lethal effects, as they are found at very low concentrations in the environment.

Despite this, there is a lack of knowledge about long term risks that the presence of a large variety of drugs may pose for non-target organisms, even though they may be found at low concentrations (Gros et al., 2006). However, many pharmaceuticals do not enter aquatic organisms easily, and even though a drug may be very potent, for the drug to cause any effect, it must first get into the organism (e.g. through gills of a fish), and reach the site of action without being metabolized. Once the drug is inside an organism, its distribution and consequently its effect will be dependant on factors such the K_{ow} of the drug. For example, if a drug is lipid soluble it would accumulate in the fat portion of an animal, and unless metabolized in times of stress or deprivation, it could be assumed that the drug would remain in the fat as an inactive compound. However, if the drug becomes partitioned into the blood and circulation, the plasma concentration could increase in the organism and come close to, or even exceed, the therapeutic level that is set in humans (Huggett et al., 2003). Various models have been developed to predict the internal concentration of a pharmaceutical based on the chemical properties of a pharmaceutical and likely concentration in the environment, although currently very little data are available to assess the accuracy of these models (e.g. Huggett et al., 2003).

Drugs can reach non-target animals through unexpected routes, and once inside an animal may act unexpectedly based on what is known of the drug, leading sometimes to unexpected outcomes. In 2004, diclofenac, an analgesic and nonsteroidal anti-inflammatory pharmaceutical, caused extremely high death rates in three species of vultures in India and Pakistan, resulting in the death of more than 95 % of the population of the oriental white backed vulture (*Gyps bengalensis*) (Fent et al., 2006; Oaks et al. 2004). By eating carcasses of cows which had been treated with the drug, the vultures ingested diclofenac and consequently died of renal failure, visceral gout and kidney malfunction from the accumulation of uric acid throughout the body cavity (Fent et al., 2006). This is a very important message, as we may be unable to predict all the different modes of action of a pharmaceutical, which can in turn sometimes lead to very serious consequences. Only time and further research will show whether the case of diclofenac killing vultures is a unique example, or is more common than realized currently.

Another way of evaluating the risk posed by a pharmaceutical is the acute: chronic ratio (ACR) of the drug. If the ACR of a drug is high, then the patient can tolerate a high dose without the drug causing significant side effects and therefore it could follow that if the ACR of a drug is high in humans, it is also likely to be high in other organisms. For example, pharmaceuticals that have low ACRs (e.g. lower than 10) are perhaps more likely to cause an acute effect in another organisms. There are very limited data available to assess whether or not this is true (Fent et al., 2006), although presumably the ACR for diclofenac is much lower for vultures than it is for cows, since vultures receive an acute dose from eating a small amount of carcass of a cow that had been treated with diclofenac, yet the cow did not die from the drug.

1.4 Different modes of action of pharmaceuticals

The study of pharmacodynamics describes the biochemical and physiological effects of a pharmaceutical on an animal, i.e. what a pharmaceutical does to an animal and how it does it. In order to alter an array of structures, functions and metabolic processes within the body of a patient, pharmaceuticals are designed to stimulate a response in humans and animals at low doses, and so their design is often engineered such that they have a very specific mode of action (Jones et al., 2004; Calamari et al., 2003). The modes of action of pharmaceuticals can be categorized into four recognized groupings, as shown in Figure 1.1.

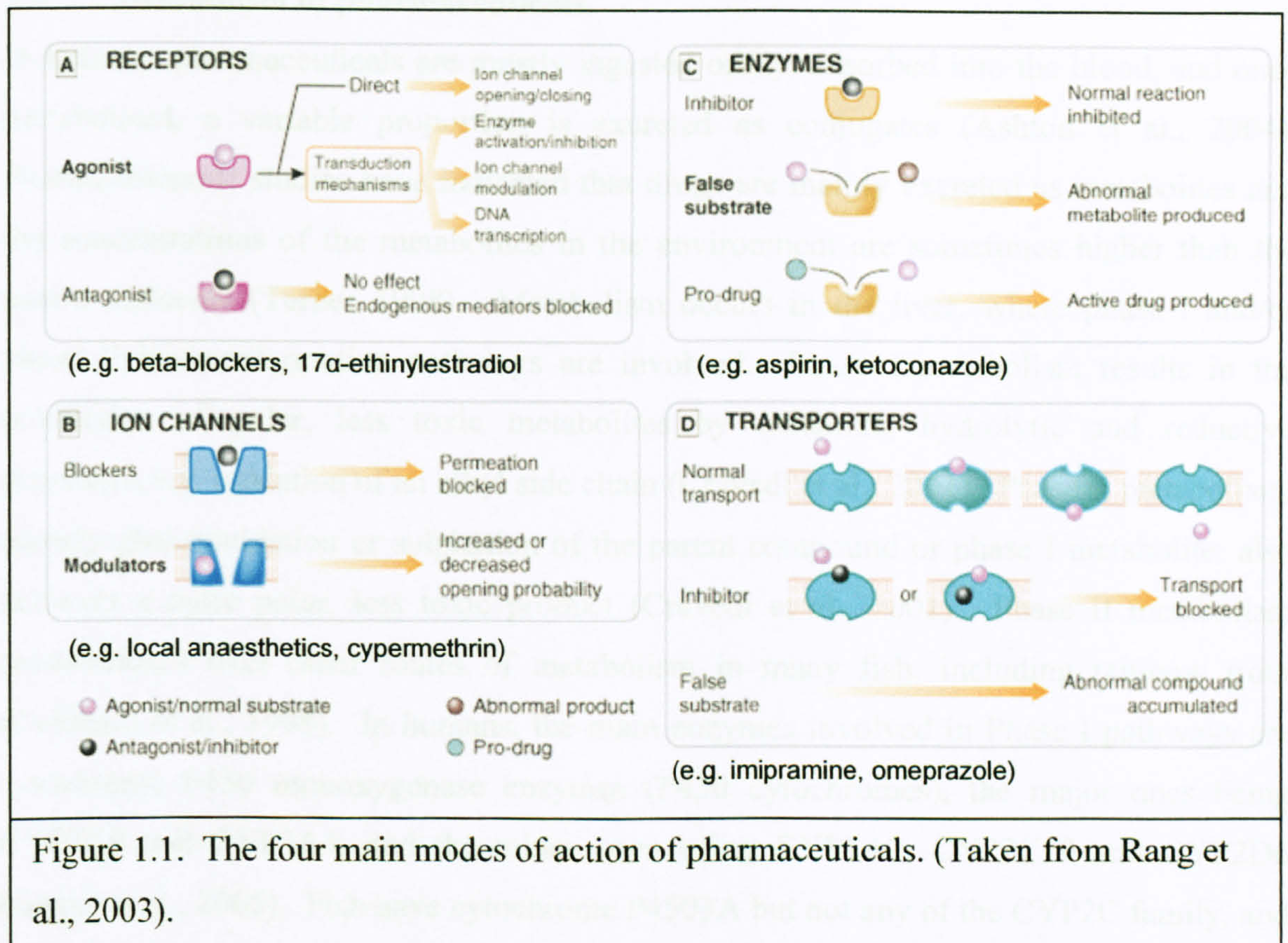


Figure 1.1. The four main modes of action of pharmaceuticals. (Taken from Rang et al., 2003).

Most receptors and enzymes found in humans have also been identified and characterized in fish and other vertebrates, showing that between mammalian and teleost systems there is a great deal of conservation. For example, sequence homologies for receptors and enzymes in fish compared to mammals range from 31 – 88% (Huggett et al., 2003). Therefore if a drug has a particular mode of action in humans, we would expect to see the equivalent mode of action in other organisms that also have the same receptor or enzyme. However, it is much less clear whether invertebrates exposed to a drug would respond with the same mode of action that occurs in humans. This is because currently there is not enough information available to suggest that the same biochemical and molecular machinery exists in these animals.

If it can be assumed that the same mode of action will occur in humans and other classes of vertebrates for a particular pharmaceutical, the next logical step might be to ask whether the effect of a pharmaceutical occurs at the same therapeutic dose, i.e. if the concentration of the pharmaceutical in the blood is the same for both humans and other vertebrates, will the drug elicit the same effect? Unfortunately little data are available to assess this theory, as very few ecotoxicology studies have included measurement of the plasma concentration of test drugs.

1.5 Metabolism of pharmaceuticals

In humans, pharmaceuticals are mostly ingested orally, adsorbed into the blood, and once metabolised, a variable proportion is excreted as conjugates (Ashton et al., 2004). Pharmacokinetic studies have indicated that drugs are mainly excreted as metabolites and the concentrations of the metabolites in the environment are sometimes higher than the parent molecule (Ternes 1998). Metabolism occurs in the liver, where phase I and/or phase II biotransformation pathways are involved. Phase I metabolism results in the production of polar, less toxic metabolites by oxidative, hydrolytic and reductive processes, e.g. oxidation of an alkyl side chain (Cravedi et al., 2001). Phase II metabolism, namely glucuronidation or sulphation of the parent compound or phase I metabolite, also achieves a more polar, less toxic product (Cravedi et al., 2001). Phase II metabolism predominates over other routes of metabolism in many fish, including rainbow trout (Coldham et al., 1998). In humans, the main enzymes involved in Phase I pathways are cytochrome P450 monoxygenase enzymes (P450 cytochromes), the major ones being CYP2C9 and CYP3A4, and the minor ones being CYP1A2, CYP2C19 and CYP2D6 (Gagné et al., 2006). Fish have cytochrome P4503A but not any of the CYP2C family, and so fish will metabolise drugs differently to humans in an attempt to eliminate them and prevent toxic accumulation. Since many pharmaceuticals require phase I oxidative metabolism to assist in their elimination, without equivalents of the human cytochromes, fish have the potential to bioaccumulate these drugs (Gagné et al., 2006).

1.6 Current legislation and testing of pharmaceuticals (guidelines)

In the U.S.A., the food and drug administration (FDA) have monitored pharmaceuticals in the environment since 1977 under the National Environmental Policy act of 1969. Regulation occurred via an environmental review process for new drug applications and since 1980 this has become more extensive (Jones et al., 2004). For new drug applications, the expected introductory concentration (EIC) of a drug into the environment over 5 years had to be calculated. If this concentration does not exceed 1 µg/L, then no further action need be taken. But if the EIC is greater than 1 µg/L, then a formal environmental risk assessment has to be carried out, which includes a tiered set of ecotoxicology tests on microbial, algal, invertebrate and fish species. However, if a drug had the ability to bio-accumulate, then chronic testing is also considered (Jones et al., 2004).

In Europe, up until recently, a less rigorous procedure was in place, and hence for the majority of medicinal products and their metabolites, an environmental risk assessment has never been carried out (Ternes 1998). The old system (EMEA/CHMP/96268/2005) looked

at PEC values; if these were less than 0.01 μg/L, then no further assessment was needed. If the PEC value was greater than 0.01 μg/L, then the PEC/PNEC ratio was calculated and phase 2 testing was conducted if the ratio was greater than 1 (Jones et al., 2004). However, there was no legal requirement for this to be done, and there were many criticisms of this system as no terrestrial component was considered, providing physico-chemical data such as rate of adsorption (Koc) or Pow or LC_{50} EC_{50} and NOEC were not mandatory, and there was an inclination towards acute rather than chronic effects (Jones et al., 2004; Ternes 1998).

However, the recent realisation that many pharmaceuticals are present in the environment has triggered a new proactive approach, and a proposal to include an environmental risk assessment in the registration procedure for medicinal products was issued (Escher et al., 2005). In 2000 the European Medicines Evaluation Agency (EMEA) issued guidelines to assess the environmental impact of veterinary medicines, and draft guidelines for human pharmaceuticals (CPMP/SWP/4447/00) (Escher et al., 2005). The approach is a step-wise, two phase procedure, as shown in Table 1.4.

Stage in regulatory evaluation	**Stage in risk assessment**	**Objective**	**Method**	**TEST /DATA REQUIREMENT**
Phase I	Pre-screening	Estimation of exposure	Action limit	Consumption data, log Kow
Phase II Tier A	Screening	Initial prediction of risk	Risk Assessment	Base set of aquatic toxicology and fate data
Phase II Tier B	Extended	Substance and compartment-specific refinement and risk assessment	Risk Assessment	Extended dataset on emission, fate and effects

Table 1.4. Summary of the EMEA guidelines in assessing the impacts of human pharmaceuticals in the environment (EMEA document: CPMP/SWP/4447/00)

The first phase estimates the concentration of a drug substance in the environment by calculating a $PEC_{SURFACEWATER}$ value. If the $PEC_{SURFACEWATER}$ value is equal or above 0.01µg/L, phase 2 Tier A is carried out. In some cases, if the drug substances may affect the reproduction of vertebrate or lower animals at concentrations lower than 0.01 µg/L, then a tailored risk assessment strategy is followed that addresses the specific mode of action of the drug, regardless of the PEC value obtained. Phase 2 Tier A assesses the fate and effects of a pharmaceutical in the environment using Organization for Economic Co-operation and Development (OECD) protocols on three aquatic species, one plant (algal growth inhibition test), one invertebrate (Daphnia reproduction test), and one vertebrate (fish early life stage test) (Sumpter 2007). If a potential risk is detected in Phase II Tier A, then Phase II Tier B is conducted into extended effects analysis.

The recent EMEA guidelines are a vast improvement on former guidelines, and data from Germany collected over the last ten years show that the EMEA method provides a good approximation of environmental concentrations (Castglioni et al., 2004). Nevertheless, it is unlikely that the guidelines would have highlighted the dangers posed to the environment from ethinylestradiol or diclofenac (Sumpter 2007). The very nature of pharmaceuticals is their specificity to target particular enzymes and receptors and their narrow scope of biological activity and potency. These properties mean that the use of standard acute ecotoxicology tests may not be suitable for assessing the risks caused by pharmaceutical compounds (Thomas & Hilton, 2004). Another criticism of the guidelines is that they only consider one pharmaceutical at a time and ignore the fact that some compounds can cause additive or even synergistic toxic effects when in the presence of other compounds (Nunes et al., 2005). For example, a number of different representatives of the same class of pharmaceutical may be present in the environment at the same time, for instance a number of different β-blockers are undoubtedly present in the environment simultaneously and these might have an additive effect. Then there is the issue of the simultaneous presence of quite different drugs, such as an estrogen and a progesterone, and the question here would be whether these would act synergistically or independently of each other.

1.7 β-blockers and their use in humans

β-blockers are designed to target β_1- and/or β2-adrenergic receptors (β_1-ARs, β_2-ARs) and currently there are at least 14 different β-blockers that are widely used. There are two types of β-blocker; selective β-antagonists, such as atenolol, which only block β_1-ARs and are prescribed most commonly for treating heart conditions such as angina, arrhythmia and heart attacks, and non-selective β-antagonists, such as propranolol, which block both β_1- and β_2-ARs. In addition to treating heart problems, non-selective β-antagonists are also prescribed to treat migraines, essential tremors, stress, and some, such as timolol, are used for the treatment of glaucoma (Lee et al., 2007). Appendix A shows the chemical structures of these compounds.

1.8 Environmental concentrations of β-blockers

Since the aquatic environment has been termed 'the ultimate sink' for natural and man-made chemicals, it is hardly surprising that β-blockers have been detected in the aquatic environment, with them and other pharmaceutical contaminants now present in surface waters throughout the world (Sumpter, 1998; Lindqvist et al., 2005). After patient administration, non-metabolised β-blockers and their metabolites are excreted by patients. Due to the incomplete removal of these biologically active compounds at sewage treatment plants, these drugs enter our rivers primarily via sewage treatment works effluent. Table 1.5 details the environmental concentrations of β-blockers that have been reported in STW wastewater and surface waters from different studies conducted around the world. Propranolol has also been detected in 41 % of estuarine water samples at a maximum concentration of 56 ng/L and a median concentration of 13 ng/L (Thomas & Hilton, 2004). It can be seem from Table 1.5 that β-blockers are frequently detected in both rivers and wastewater at concentrations normally in the ng/L to low μg/L range. The maximum concentration recorded in effluent was 950μg/L; however this was measured in samples obtained from India, where the particular wastewater originated primarily from about 90 bulk drug manufacturers (Larsson et al., 2007).

Compound	Wastewater concentration (ng/L)	Surface water concentration (ng/L)	Reference
Acebutolol	<10 – 130		Andreozzi et al., 2003
	80 - 230	<0.8 - 8	Vieno et al., 2006
	308		Lee et al., 2007
		3 – 14	Vieno et al., 2007
Atenolol		3.44 - 41.7	Calamari et al., 2003
		241	Calamari et al., 2003
		23	Castiglioni et al., 2004
		49.5 – 169.9	Zuccato et al., 2000
	360		Ternes et al., 2003
	80 - 440	<11.8 - 25	Vieno et al., 2006
	879	6 - 859	Vanderford & Snyder, 2006
	<10 – 1,150	<10 - 250	Gros et al., 2006
		17 -55	Vieno et al., 2007
	987		Lee et al., 2007
	160 - 465		Gros et al., 2007
Betaxolol	190	28	Ternes, 1998
	Not detected		Andreozzi et al., 2003
Bisoprolol	370	2,900	Ternes, 1998
	24		Lee et al., 2007
Carazolol	120	110	Ternes, 1998
Labetalol	81		Lee et al., 2007
Metoprolol	2,200	2,200	Ternes, 1998
	100 - 390		Andreozzi et al., 2003
	1,200		Huggett et al., 2003a

Table 1.5. Summary of reported levels of β-blockers in wastewater discharges and surface waters from studies conducted around the world.

Compound	Wastewater concentration (ng/L)	Surface water concentration (ng/L)	Reference
Metoprolol (continued)	1,700		Ternes et al., 2003
	910 – 1,070	<3.8 - 116	Vieno et al., 2006
	<34	<34	Gros et al., 2006
	800,000 – 950,000		Larsson et al., 2007
	244		Lee et al., 2007
		39 - 107	Vieno et al. ,2007
Nadolol	360		Huggett et al., 2003a
	60	Not detected	Ternes, 1998
	56		Lee et al., 2007
Oxprenolol	<10 - 50		Andreozzi et al.,2003
Propranolol	10 – 40		Ferrari et al., 2004
	290	590	Ternes, 1998
	130 - 180	<10 - 37	Hilton et al., 2003
	300	100	ENDS Report 2005
	16 - 284	<10 - 115	Ashton et al., 2004
	26 - 1900		Huggett et al., 2003a
	180		Ternes et al., 2003
	<16	<16	Gros et al., 2006
	30		Lee et al., 2007
	<2 - 63		Gros et al., 2007
Sotalol	1320		Ternes et al., 2003
	160 - 300	<3.9 - 52	Vieno et al., 2006
	<10 - 210	<10 - 70	Gros et al., 2006
		30 - 86	Vieno et al., 2007
	264		Lee et al., 2007
Timolol	70	10	Ternes, 1998
	<6		Lee et al., 2007

Table 1.5 continued. Summary of reported levels of β-blockers in wastewater discharges and surface waters from studies conducted around the world.

1.9 Effects of β-blockers on aquatic organisms

From acute EC_{50} data it would seem that atenolol is non-toxic to aquatic organisms, whilst metroprolol would be classified as toxic, and propranolol as very toxic (Cleuvers 2005). Atenolol and metroprolol have PEC/PNEC ratios of less than 1 and hence the environmental risk based on acute studies seems to be relatively low. However, if as stated by Webb (2001), propranolol has a PEC/PNEC ratio of 1.16, it is classed as being of environmental concern. Chronic data with respect to aquatic life and β-blockers are not found in abundance. Owen et al., (2007) summarises in vivo data of β-blocker activity in fish and chapter 3.1.3 reviews more specifically the biological effects that β-blockers have been found to have on plants, bacteria, invertebrates and fish. The chronic effects of β-blockers include inhibited plant growth, reduced bioluminescence and photosynthesis in plants and bacteria and reduced movement, growth and reproductive capability in invertebrates and fish. These chronic effects have been found to occur in the low to mid mg/L concentrations.

1.10 Mechanisms of action of β-blockers

In mammals, the 'fight or flight' syndrome, or a response to stress, is controlled by two hormones that are produced in the adrenal medulla found within the middle of the adrenal gland (Roberts, 1986). The two hormones, adrenaline and noradrenaline (also termed epinephrine and norepinephrine, respectively), are catecholamines that are synthesised in chromaffin cells from the amino acid tyrosine via the Blaschko pathway (Campbell, 1993; Reid et al., 1998). Catecholamines are charged compounds that contain a catechol moiety (see Figure 1.2), and amongst other things, their presence in the blood enables an immediate supply of energy to be released that allows for rapid contraction of major locomotor muscles (Lodish et al., 2000).

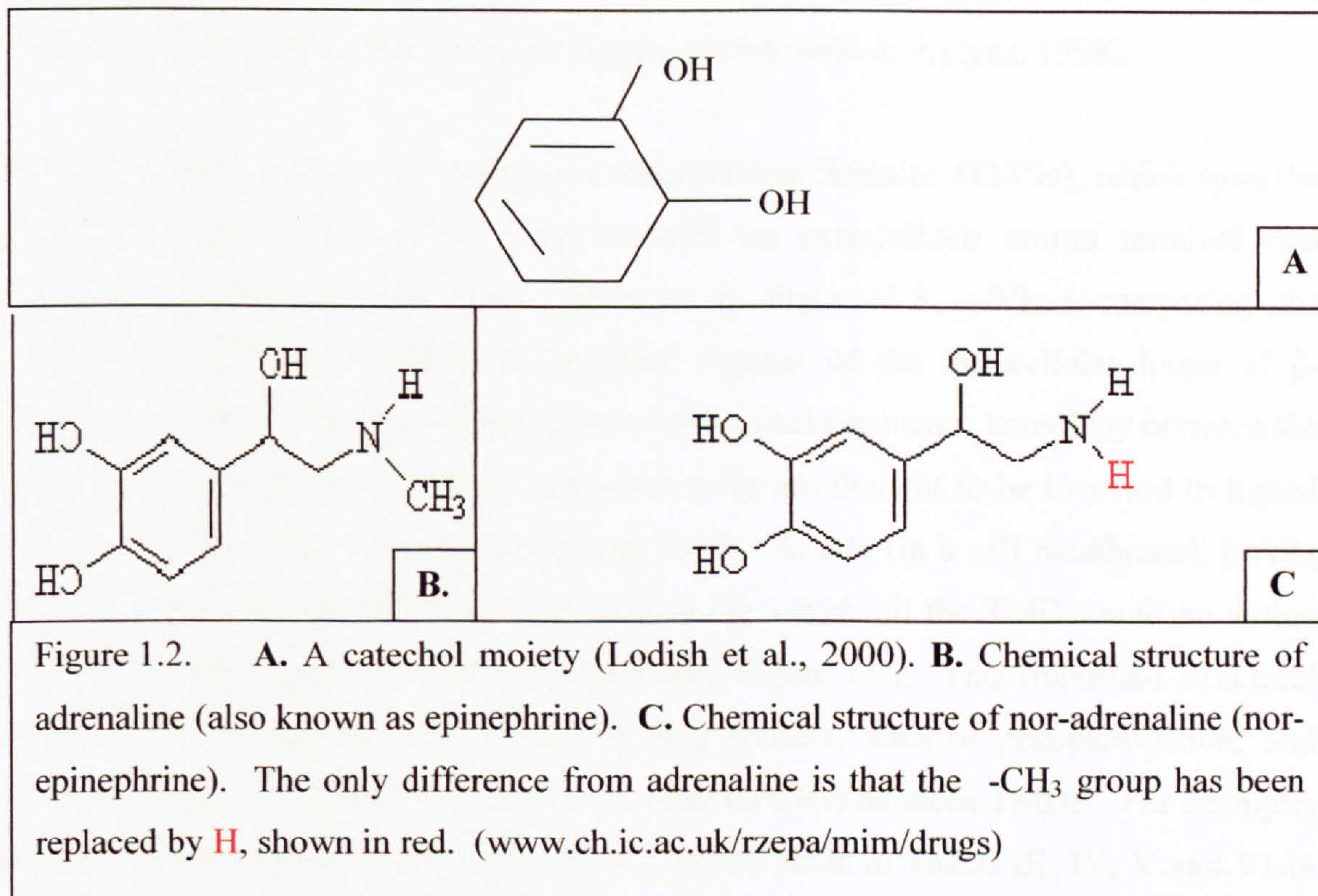


Figure 1.2. **A.** A catechol moiety (Lodish et al., 2000). **B.** Chemical structure of adrenaline (also known as epinephrine). **C.** Chemical structure of nor-adrenaline (nor-epinephrine). The only difference from adrenaline is that the $-CH_3$ group has been replaced by H, shown in red. (www.ch.ic.ac.uk/rzepa/mim/drugs)

Most hormones derived from amino acids are unable to pass through plasma membranes of their target cells and the same is true for catecholamines. Catecholamines bind to specific receptors, known as adrenergic receptors, that are located on the outer surface of particular cells. A receptor is a molecular structure within a cell, or cell membrane, that produces a particular physiological response in the cell after a specific ligand binds to it. G-protein coupled receptors (GPCRs) are membrane-bound serpentine receptors that make up one of the largest families of proteins in vertebrate species. Adrenergic receptors (ARs) belong to the large rhodopsin branch of GPCRs and are divided into two main 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 and relative affinity for adrenaline and noradrenaline. In humans, β_1-ARs are found in cardiac muscle and when circulating catecholamines bind to these receptors, an increase in heart rate and contractility is observed, which consequently increases blood supply to tissues. 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). β_3-ARs in mammals have a major role in the mediation of the adipose tissue thermogenic response (Nickerson et al., 2003). Liver and adipose tissues also possess β-ARs and the binding of catecholamines to these receptors causes the liberation of glucose and fatty acids to the blood within seconds (Lodish et al., 2000). β-ARs are also found on

leukocytes (white blood cells) and by neuro-immune communication they are responsible for many stress-related immunological changes (Jozefowski & Plytycz, 1998).

GPCRs are made up of seven α-helical transmembrane domains (TMDs), which span the membrane in an anti-clockwise manner, with an extracellular amino terminal and intracellular carboxyl terminus, as illustrated in Figure 1.3. When comparing the hydrophobic TMDs and membrane proximal regions of the intracellular loops of β-adrenergic receptors (β-ARs), a high degree of amino acid sequence homology between the different ARs can be found. These conserved areas are thought to be involved in ligand binding and G-protein interaction (Strosberg 1997). *In situ* (in a cell membrane), β-ARs have a complex three-dimensional (3D) structure in which all the TMDs, and the amino acids in the TMDS, are close to each other (see Figure 1.3). This important structural feature allows the formation of ligand binding pockets, sites of phosphorylation, and regions of protein interaction in β-ARs (and other GPCRs) between TMDS. For example, adrenaline has been predicted to interact with amino acids in TMDs III, IV, V and VI in the β_2-AR (Freddolino et al., 2004). Hence, the ligand-binding site is not a consecutive row of amino acids, but an assortment of residues spread throughout the receptor. The ligand binding site forms a pocket and develops hydrogen bonds, salt bridges and hydrophobic interactions with hydroxyl and methyl groups on the ligand (Freddolino et al., 2004). Other key residues form similar bonds to maintain the 3D structure of the receptor (Rezmann-Vitti et al., 2004).

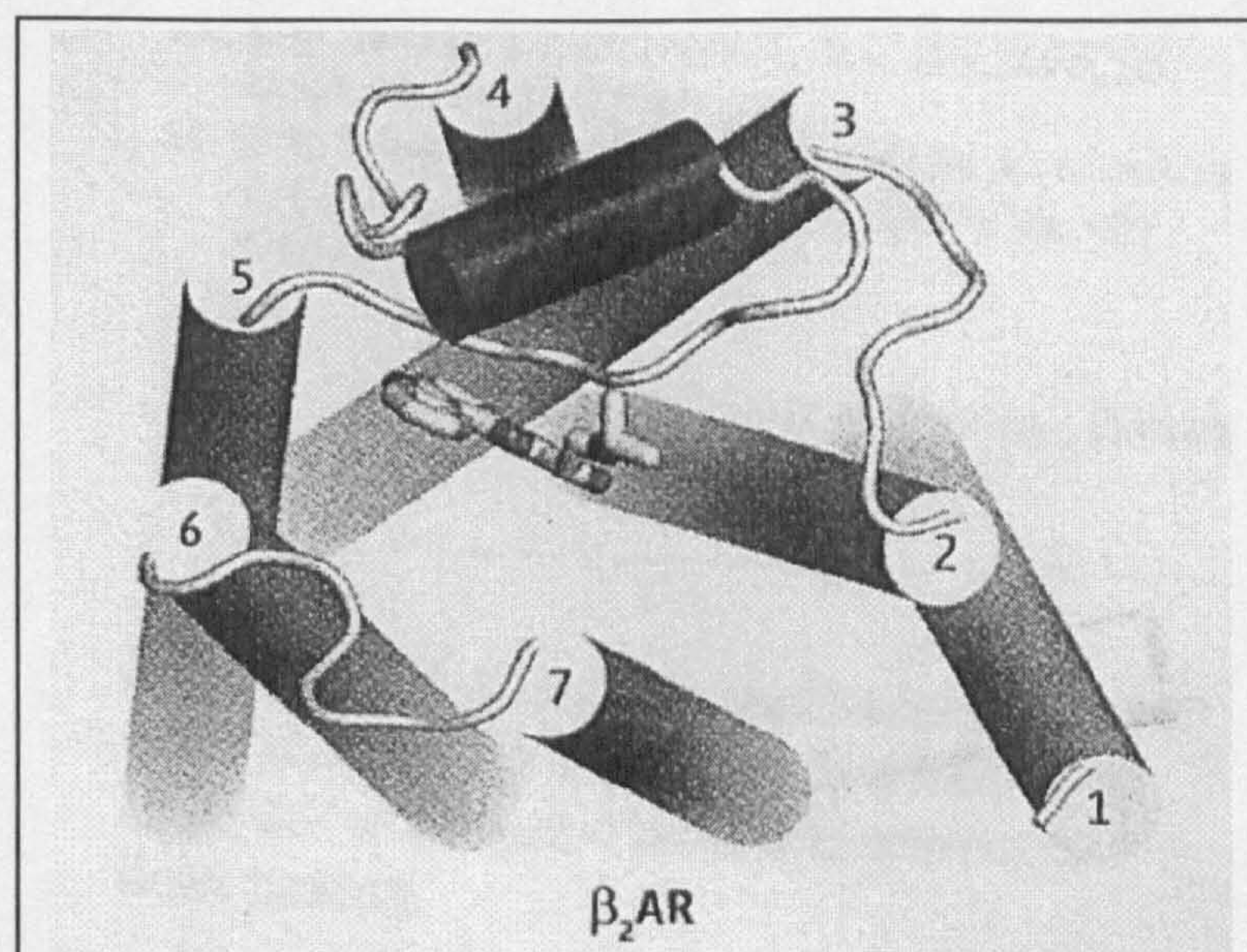


Figure 1.3. Three dimensional view of a β2-AR. The numbers 1 to 7 refer to each TMD. (Ranganathan, 2007). Note the ligand in the centre of the receptor.

The signalling pathway in ARs (and other GPCRs) is brought about by a series of biochemical changes within the cell, which result in a physiological change (see Figure 1.4). The classical signal transduction pathway involves intracellular heterotrimeric guanine proteins, (G-proteins) that are made up of 3 subunits; Gα, Gβ and Gγ, of which the Gα- subunit has three different subtypes: Gαq-, to stimulate phospholipase C; Gαs-, and Gαi- to stimulate or inhibit adenylyl cyclase, respectively (Fitzgerald et al., 1999). When an endogenous ligand such as adrenaline or noradrenaline binds to a β-AR, the transformation of GTP to GDP + Pí occurs, causing the stimulatory part of G-protein (Gαs) to dissociate from the Gβγ subunit, and interact with the enzyme adenylyl cyclase. The interaction triggers the synthesis of cAMP, by hydrolysis of ATP, and switches the receptor to an active state. However, the conversion of ATP also hydrolyses GTP in the Gαs- protein back to GDP, and hence the synthesis of cAMP only lasts for a few seconds. The receptor now returns to an inactive state and the stimulatory Gαs-protein moves back to the remaining Gβγ subunit.

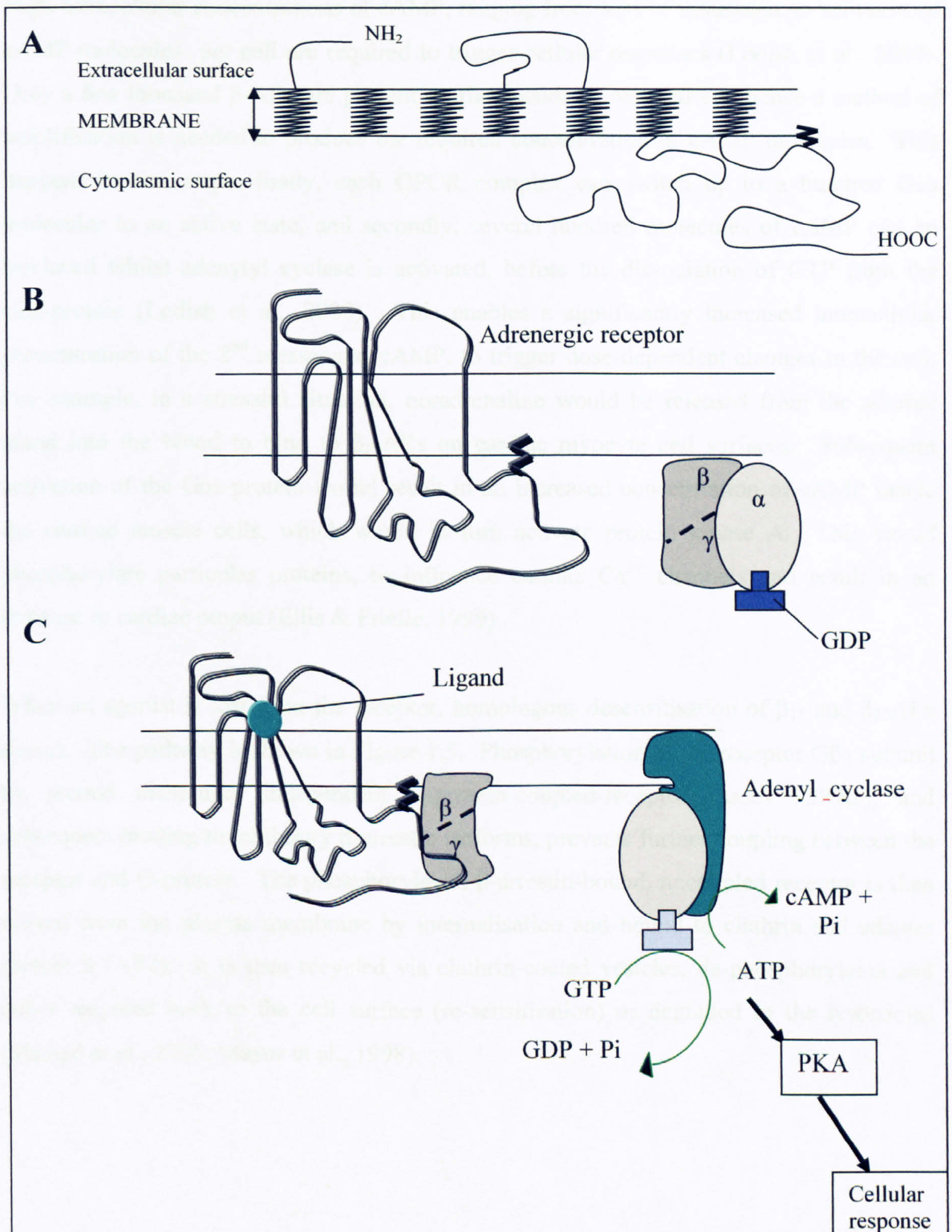


Figure 1.4. Schematic representation of the signal transduction pathway in an adrenergic receptor. (**A**) 2D diagram of a GPCR, based on the model for the rhodopsin receptor. (**B**) Adrenergic receptor in a basal state with GDP bound to Gα-s. (**C**) Upon a ligand binding to the receptor, GTP replaces GDP and Gα-s dissociates from the Gβ-γ complex and binds to adenylyl cyclase, resulting in the synthesis of cAMP. Consequently, intracellular concentrations of cAMP increase and activate proteins, such as protein kinase A (PKA), that induce a physiological response.

High intracellular concentrations of cAMP, ranging from tens of thousands, to millions, of cAMP molecules per cell are required to trigger cellular responses (Lodish et al., 2000). Only a few thousand β-ARs are present on the outside of one cell and hence a method of amplification is needed to produce the required concentration of cAMP molecules. This happens in two steps; firstly, each GPCR complex can switch up to a hundred Gαs molecules to an active state, and secondly, several hundred molecules of cAMP can be produced whilst adenylyl cyclase is activated, before the dissociation of GTP from the Gαs-protein (Lodish et al., 2000). This enables a significantly increased intracellular concentration of the 2nd messenger, cAMP, to trigger dose-dependent changes in the cell. For example, in a stressful situation, noradrenaline would be released from the adrenal gland into the blood to bind to β_1-ARs on cardiac myocyte cell surfaces. Subsequent activation of the Gαs-protein would result in an increased concentration of cAMP inside the cardiac muscle cells, which would in turn activate protein kinase A. This would phosphorylate particular proteins, to influence cardiac Ca^{2+} channels and result in an increase in cardiac output (Ellis & Frielle, 1999).

When an agonist is lost from the receptor, homologous desensitisation of β_1- and β_2-ARs occurs. The pathway is shown in Figure 1.5. Phosphorylation of the receptor Gβγ subunit by second messenger independent G-protein-coupled-receptor-kinases (GRKs), and subsequent binding to inhibitory β-arrestin isoforms, prevents further coupling between the receptor and G-protein. The phosphorylated, β-arrestin-bound, uncoupled receptor is then moved from the plasma membrane by internalisation and bound to clathrin and adapter protein 2 (AP2). It is then recycled via clathrin-coated vesicles, de-phosphorylated and either recycled back to the cell surface (re-sensitisation) or degraded in the lysosomes (Métayé et al., 2005; Mayor et al., 1998).

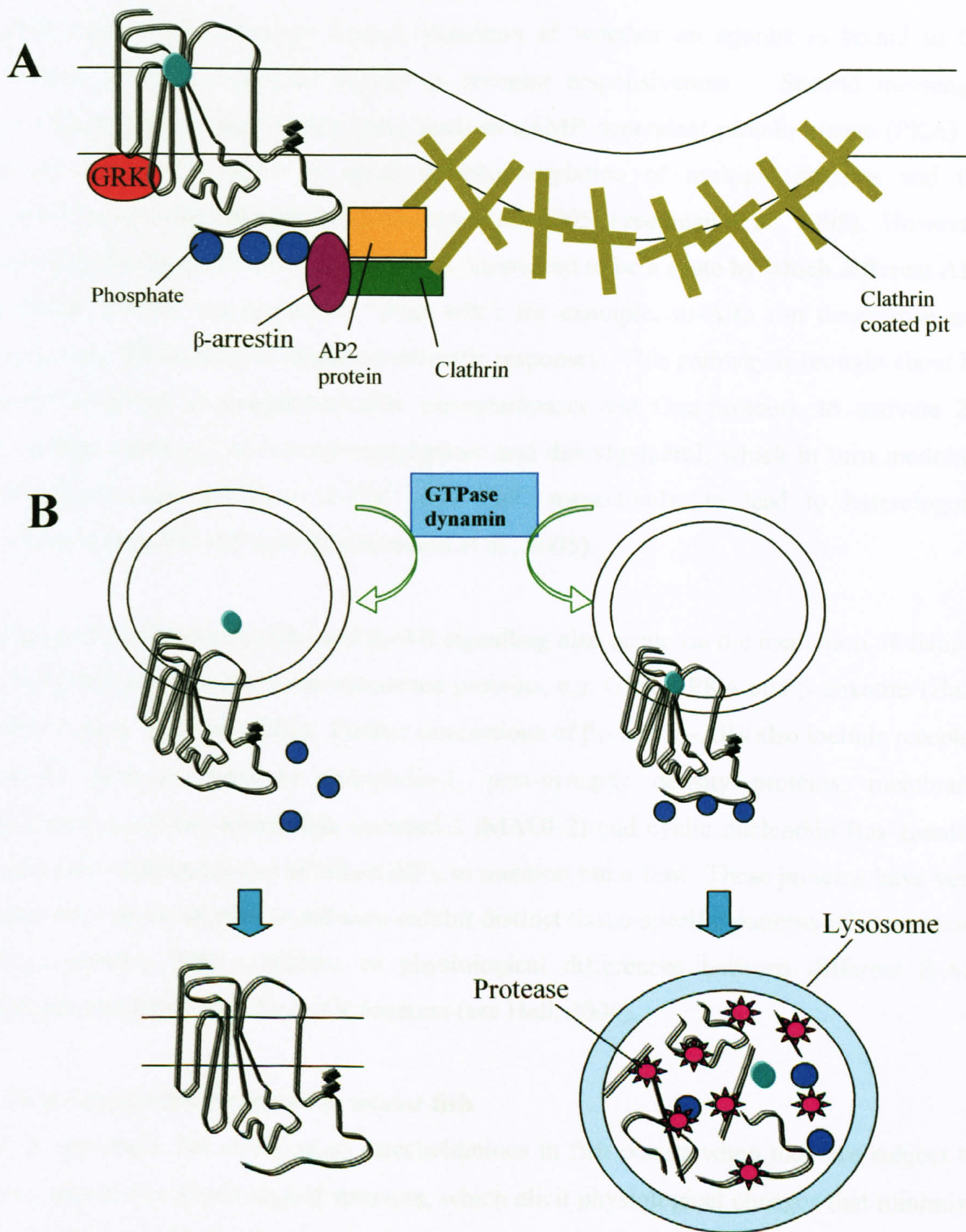


Figure 1.5. Summary of the endocytotic pathway, leading to recycling or degradation of ARs . **(A)** GRK binds to the Gs-βγ/receptor complex and phosphorylates the receptor, which promotes binding of β-arrestin. β-arrestin acts as an intermediary protein and binds the receptor complex to clathrin coated pits via clathrin and AP2 proteins. **(B)** In the presence of GTPase dynamin, endocytic vesicles are formed. The receptor complex then undergoes resensitization, where it is recycled back to the membrane surface, or it is trafficked to lysosomes to be degraded by proteases.

Heterologous desensitisation occurs regardless of whether an agonist is bound to the receptor, to cause a general decline in receptor responsiveness. Second messenger generating systems activate enzymes, such as cAMP dependent protein kinase (PKA) or protein kinase C (PKC), to result in phophorylation of multiple proteins and the desensitisation of β-AR activity (Guimond et al., 2005; Freedman et al., 1995). However, heterologous desensitisation has also been discovered to be a route by which different ARs modulate activity via molecular 'cross talk'; for example, α_1-ARs can desensitise and regulate β_2-ARs and affect cardiac contractile responses. This pathway is brought about by α_1-AR coupling to inositide-specific phospholipases via Gαq-proteins, to activate 2nd messenger pathways of inositol-triphosphate and diacylgylcerol, which in turn modulate intracellular concentrations of Ca^{2+} and PKC respectively, to lead to heterologous desensitisation of β-AR activity (Guimond et al., 2005).

Additional mechanisms of β_1- and β_2-AR signalling also occur via the mediation of distinct cytoplasmic proteins and trans-membrane proteins, e.g. GRKs, PKA and β-arrestins (Hall, 2004; Tsao & Zastrow, 2001). Further interactions of β_1- and β_2-ARs also include receptor specific proteins, such as endophilin-1, post-synaptic density proteins, membrane associated guanylate kinase-like inverted-2 (MAGI-2) and cyclic nucleotide Ras guanine nucleotide exchange factor (CNRasGEF), to mention but a few. These proteins have very particular functional effects and they exhibit distinct tissue-specific patterns of expression. These proteins help contribute to physiological differences between different β-AR subtypes and tissue specific β-AR function (see Hall, 2004).

1.11 β-Adrenergic receptors in teleost fish

As in mammals, the secretion of catecholamines in fish occurs when they are subject to environmental or physiological stressors, which elicit physiological changes that minimise the detrimental effects of stress on physiological function (Perry & Bernier, 1999). Such stressors can include air exposure, environmental hypoxia or hypercapnia, metabolic acidosis, exercise, handling, physical disturbance, anaemia, anaesthesia or hypotension. However, not all stressful situations in fish have been found to elicit a catecholamine response. For example, rainbow trout will not release catecholamines in response to mild or moderate environmental hypoxia, sustained aerobic exercise or to a 20-25 % reduction in blood pressure (Perry & Bernier, 1999).

Fish, like mammals, also possess α and β adrenergic receptors and the transduction pathway for fish β-ARs seems to resemble the one present in mammalian cells, whereby

the occupation of a GPCR receptor causes stimulation of adenylyl cyclase and the synthesis of cAMP (Fabbri et al., 1998). On activation of the afferent limb of the humoral adrenergic stress response (i.e. the secretion of catecholamines), the efferent limb (i.e. the physiological reactions elicited by the catecholamines) responds by modulating cardiovascular and respiratory function to maintain an adequate supply of oxygen to the blood and tissues, and mobilization of energy stores, such as glucose and lipids, to provide for the increased energy demands (Reid et al., 1998).

The storage of catecholamines is different in fish. Catecholamines are still produced by chromaffin cells, but in teleosts these are primarily located in the walls of the posterior cardinal vein in the region of the head kidney and in close association with lymphoid tissue of the kidneys, where they have been observed to exist singularly or in clusters (Perry & Bernier, 1999; Reid et al., 1998). However, it must be noted that in less evolutionary advanced fish, the location of chromaffin cells differs again.

Adrenaline and noradrenaline are stored in separate types of chromaffin cells; those storing adrenaline contain spherical or elongated granules (30 nm diam.), whilst chromaffin cells storing noradrenaline contain spherical, electron dense granules (200 nm diam.) (Reid et al., 1998). Levels of catecholamine storage also differ between different groups and species of fish, and one particular catecholamine can be stored at greater concentrations than another; for example, in teleosts, adrenaline is the predominant catecholamine that is stored (Reid et al., 1998). However, levels of catecholamine storage can also be affected by pollution, nutritive and/or physical stress, hormonal inference and anoxia (Reid et al., 1998).

In teleost fish, three subtypes of β-AR receptor exist (β_1, β_2 and β_3), as they do in mammals, with a further subdivision of β_3-AR into β_{3a} and β_{3b}. β_1-AR has been reported to be present on red blood cells of many fish species and is associated with the Na^+/H^+ antiporter to increase intracellular pH and allow greater affinity of haemoglobin for oxygen and consequently increased oxygen transport in the blood (Fabbri et al., 1998). In addition to this, β_1-AR is also reported to be present on the hepatic membrane of coho salmon, and on activation, increased cAMP levels activate triacylgycerol lipase, resulting in glycerol and free fatty acid release (Fabbri et al., 1998).

The most characterised β-AR receptor in fish is the β_2-AR. In the channel catfish, the primary β-AR receptor present on the membranes of the liver, head kidney leukocytes and

spleen leukocytes is the β_2-AR, whilst in rainbow trout, β_2-AR, a 409 amino acid protein, is highly expressed in the liver and red and white muscle, with lower expression levels in the gills, heart, kidney and spleen (Nickerson et al., 2001; Finkenbine et al., 2002). When catecholamines are released into the blood, the function of liver β_2-AR in rainbow trout and other species of teleosts has been found to be the mobilisation of hepatic glycogen (Nickerson et al., 2001).

Through phylogenetic relationships, it has been suggested that teleost β3-ARs are homologous to mammalian β3-ARs. However, their roles are completely different, for in mammals these receptors mediate the adipose tissue thermogenic response, a response that fish are unlikely to have (Nickerson et al., 2003). β3a-AR mRNA has been found to be highly expressed in the gill and heart of rainbow trout, whilst evidence of the presence of β3b-AR has been found on red blood cells (Nickerson et al., 2003). Strong evidence exists that the activation of β3b-AR by catecholamines, predominantly noradrenaline, results in an increase in the oxygen-binding affinity of red blood cells (Nickerson et al., 2003). The resulting rise in cAMP from the activation of the red blood cell β3b-AR, results in activation of protein kinase A that consequently activates the β-Na^+/H^+ exchanger by phosphorylation. H^+ then extrudes from the red blood cell in exchange from Na^+ and the increase in alkalisation/pH increases the affinity of haemoglobin for oxygen, and hence increases oxygen transport in the blood (Nickerson et al., 2003). Following adrenergic stimulation, the blood oxygen capacity was found to increase two-fold within minutes (Wang et al., 1999). The two β3-ARs in rainbow trout have been found to have an 84 % degree of sequence conservation, both at the nucleotide and amino-acid level, with β3a-AR encoding a protein of 429 amino acids and β_{3b} encoding one for 477 amino acids (Nickerson et al., 2003).

β-ARs have also been found on white blood cells (leukocytes) in fish (with phagocytic, adherent cells bearing more receptors than lymphocytes), and in the special sphincter in puffer fish (Ng et al., 1973; Jozefowski & Plytycz, 1998). However, β-AR subtypes have been found to be specific to species, tissues and the function of the tissues, but there is little explanation as to why the high diversity of adrenergic receptor systems exists in teleosts (Fabbri et al., 1998). The sensitivity of receptors also differs between fish species; for instance, adrenaline is 100 times more effective in stimulating carp liver pieces than noradrenaline is, whereas chinook salmon liver pieces responded equally well to both catecholamines (Fabbri et al., 1998). Temperature can also effect adrenergic sensitivity and density of β-ARs; for example, a heart trout acclimated at 8 °C is more sensitive to

adrenaline than one acclimated at 18 °C, due to the cardiac cells at 8 °C having a greater cell surface adrenoreceptor population (Keen et al., 1993).

Almost all fish tissue is potentially able to remove catecholamines from the blood via two deactivation enzymes; monoamine oxidase and catechol-o-methyl transferase (Fabbri et al., 1998). Once circulating catecholamines have been deactivated, the blood level of catecholamines returns to the resting concentration of between 1 to 10 nM, which is a 100-fold reduction from circulating catecholamine concentrations occuring during periods of acute stress (Fabbri et al., 1998).

1.12 Differences between β-ARs of different species

A lot of research and information has been gathered with regard to the structure and genetics of mammalian β-ARs, but few studies have dealt with β-ARs of non-mammalian species. The completion of the zebrafish (*Danio rerio*) and pufferfish (*Takifugu rubripes* and *Tetraodon nigroviridis*) genomes has allowed β-ARs to be identified in these species of fish, showing that β-ARs in fish and humans demonstrate a high sequence homology. In humans, the sequences of β_1- and β_2-ARs are 54% identical (71% when only TMDs are compared). However, the efficacy (the inherent ability of the receptor to produce a signal) of β_1-ARs in humans is six times lower than that of β_2-ARs in transducing a given receptor occupancy signal, yet the molecular basis for this difference is poorly understood (Birnbaumer et al., 1994; Frielle et al., 1987). It seems that changes in efficacy may occur as a consequence of relatively small molecular differences within a single species, let alone when data are extrapolated across species.

The specificity of β-ARs for adrenaline and noradrenaline, and other agonist and antagonists, is a feature that distinguishes each β-AR subtype. β-ARs in the goldfish head kidney share a similar affinity for adrenaline and noradrenaline to mammalian β-ARs (Jozefowski & Plytyczt, 1998). However, in rainbow trout (*Oncorhynchus mykiss*), the β_{3b}-AR shows a high affinity for propranolol and nadolol (non-selective β-AR antagonists), which is not consistent with mammalian β_3-ARs, which show low affinities for these compounds (Nickerson et al., 2003). Currently, not enough is known about the specificities of fish ARs to know whether they will be similar or different from their mammalian counterparts.

Physiological endpoints between humans and fish β-ARs also differ. For example, in mammals, β_1-, β_2- and β_3-ARs are all found to stimulate lipolysis in fat cells. However in

carp, β_1-ARs inhibit lipolysis, whereas β_2-ARs stimulate it (Vianen et al., 2004). Hence, the signal transduction of β-ARs may be similar between mammals and teleost fish, yet the physiological outcomes may be completely different. Small structural differences may account for some of the above differences, such as the lack of phosphorylation sites within G-protein binding domains and the finding of three potential phosphorylation sites in the carboxyl terminus in rainbow trout β_2-ARs (Nickerson et al., 2001). These sites play an essential role in desensitisation pathways in mammals, and the change or removal of them has unknown consequences.

1.13 Evolution of β-ARs

It is most likely that all ARs arose from a common gene, and despite 350 million years of evolution, overall ligand-binding character, order of potency and efficacy of agonists has been found to be highly conserved between zebrafish and human α_2- adrenergic receptors (α_2-AR) (Ruuskanen et al., 2005). However, gene duplication, in particular complete genome duplication in the bony fish lineage after the split from mammals (leading to some, but not all, families of fish being tetrapoloid), and amino-acid polymorphisms, may account for some of the differences between teleost and mammalian β-ARs (Yang-Feng et al., 1990). The abundance of trace-amine receptors in zebrafish (*Danio rerio*) compared to Japanese puffer fish (*Takifugu rubripes*) is an example of how protein evolution can proceed at a rapid rate (Gloriam et al., 2005). Hence, despite transduction pathways for fish β-ARs seeming to resemble, in all details, the ones present in mammalian cells (Fabbri et al. 1998), it has to be considered that the pharmacodynamic activities of β-blockers, and their metabolites, could induce effects that are totally different, or even if similar, more potent, than the original therapeutic actions that were intended for humans (Laville et al., 2004).

1.14 Aims and objectives

The general aim of my research was to improve the understanding of the consequences to fish of the presence of human pharmaceuticals in the aquatic environment. I chose to focus on β-blockers, mainly because it is well documented that these are present in the aquatic environment. If β-blockers do affect wild fish, they are most likely to do so via the AR system of the fish. Hence, basing my research on the fathead minnow (*Pimephales promelas*), because of its widespread use in ecotoxicology, I investigated several of the key issues that need to be resolved if any possible effects of β-blockers on fish are to be understood. Specifically, to demonstrate that fathead minnows are capable of responding to β-blockers in the expected manner, I characterised two of their β-ARs, namely β1- and

β2-AR. Then, as an aid in indicating what physiological processes these β-ARs regulate (and hence what effects β-blockers might cause), I determined the tissue location of these receptors. Finally, to begin the process of determining the possible effects of β-blockers on fish, I investigated whether the β-blocker propranolol affected reproduction.

Chapter 2 β1- and β2-adrenergic receptors in fathead minnow

2.1 Introduction

β-adrenergic receptors (β-ARs) have been found in many species of organism from mammals to plants. Whether their functions are the same in each organism it is not yet known. However, the completion and annotation of genome databases has enabled bioinformaticists to identify β-ARs in a range of organisms.

2.1.1 The genetic code

An organism develops by replicating information that is held within the nucleic acids of genes. Nucleic acids are found in all living cells and viruses and the two main types are deoxyribonucleic acid (DNA), which is found in the nucleus of cells, and ribonucleic acid (RNA), found mostly in the cytoplasm (Roberts, 1986). The building blocks of nucleic acids are known as nucleotides and consist of a 5-carbon sugar (deoxyribose in DNA and ribose in RNA), phosphoric acid and an organic base. There are four organic bases, (adenine (A), guanine (G), cytosine (C) and thymine (T), which is replaced by uracil (U) in RNA) and the organic base determines the name of the nucleotide. It is the sequence of the bases that carries the information which determines an organism's development.

DNA consists of two parallel polynucleotide chains and these are held together by pairs of bases that are linked by hydrogen bonds. The pyrimidine bases, T and C, have a single hexagonal ring and join to the larger purine bases, A and G, which have a hexagonal ring joined to a pentagonal ring. However, the only possible pairings are A to T via two hydrogen bonds, and C to G via three hydrogen bonds (see Figure 2.1). Other variations of bonding are not possible because either the bases repel each other, or the pattern of hydrogen donors and acceptors does not correspond.

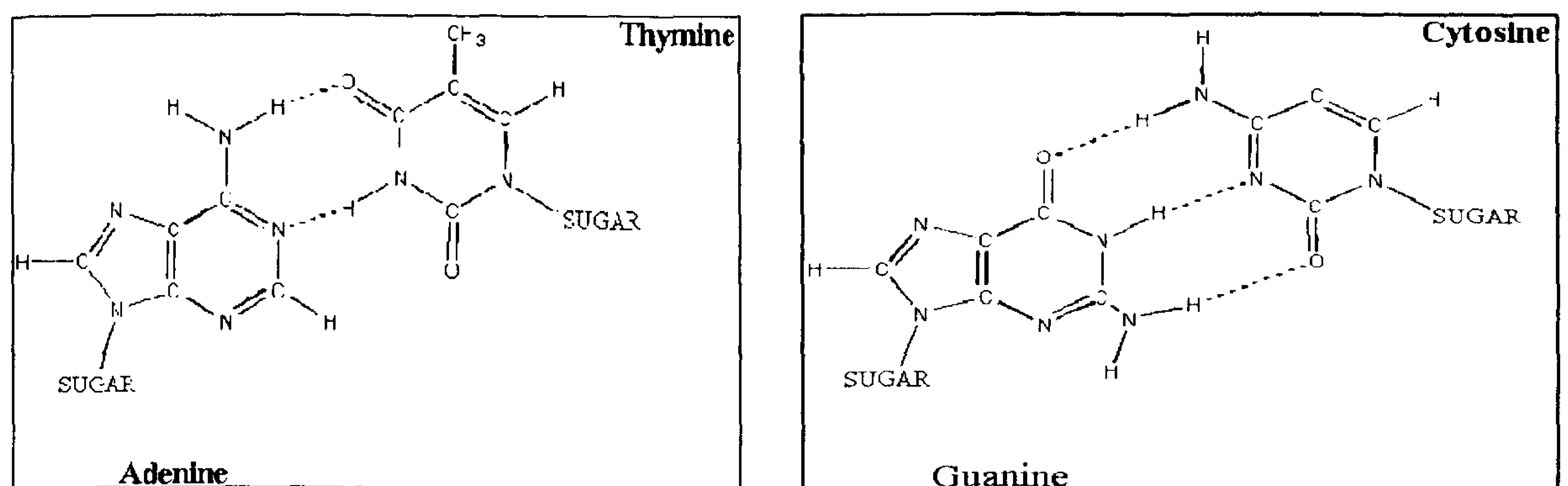


Figure 2.1. Chemical structure of nucleotide bases showing the hydrogen bonds between adenine and thymine, and between cytosine and guanine. (www.ncc.gmu.edu/dna/three.htm)

The two strands of polynucleotide chains run in opposite directions and are twisted to form a double helix. This model was proposed by James Watson and Francis Crick in 1953. The 5' and 3' ends of the polynucleotide strand are identified with regard to which carbon in the sugar is attached to the phosphate group, e.g. carbon 5 or 3 of the sugar, respectively. One strand runs in the 5' to 3' direction, this is known as sense strand, and the other complimentary strand runs in the 3' to 5' direction, and this is known as the anti-sense strand.

A triplet of bases, known as a codon, codes for a particular amino acid and is the basis for the genetic code. Table 2.1 details the 20 amino acids and the codons that are required to produce them in DNA (In RNA the same code applies except thymine is replaced by uracil). As shown, most amino acids can be coded for by more than one codon, e.g. leucine and serine. In order to make proteins, DNA instructs the cell which amino acids have to be put together in which order for the protein to be synthesised.

First Letter	Second Letter								Third Letter
	T		**C**		**A**		**G**		
T	**TTT**	Phenylalanine (Phe, F)	**TCT**	Serine (Ser, S)	**TAT**	Tyrosine (Tyr, Y)	**TGT**	Cysteine (Cys, C)	**T**
	TTC		**TCC**		**TAC**		**TGC**		**C**
	TTA	Leucine (Leu, L)	**TCA**	Serine (Ser, S)	**TAA**	Stop	**TGA**	Stop	**A**
	TTG		**TCG**		**TAG**		**TGG**	Tryptophan (Trp, W)	**G**
C	**CTT**	Leucine (Leu, L)	**CCT**	Proline (Pro, P)	**CAT**	Histidine (His, H)	**CGT**	Arginine (Arg, R)	**T**
	CTC		**CCC**		**CAC**		**CGC**		**C**
	CTA	Leucine (Leu, L)	**CCA**	Proline (Pro, P)	**CAA**	Glutamine (Gln, Q)	**CGA**	Arginine (Arg, R)	**A**
	CTG		**CCG**		**CAG**		**CGG**		**G**
A	**ATT**	Isoleucine (Ile, I)	**ACT**	Threonine (Thr, T)	**AAT**	Asparagine (Asn, N)	**AGT**	Serine (Ser, S)	**T**
	ATC		**ACC**		**AAC**		**AGC**		**C**
	ATA	Isoleucine (Ile, I)	**ACA**	Threonine (Thr, T)	**AAA**	Lysine (Lys, K)	**AGA**	Arginine (Arg, R)	**A**
	ATG	Methionine	**ACG**		**AAG**		**AGG**		**G**
G	**GTT**	Valine (Val, V)	**GCT**	Alanine (Ala, A)	**GAT**	Aspartic acid (Asp, D)	**GGT**	Glycine (Gly, G)	**T**
	GTC		**GCC**		**GAC**		**GGC**		**C**
	GTA	Valine (Val, V)	**GCA**	Alanine (Ala, A)	**GAA**	Glutamic acid (Glu, E)	**GGA**	Glycine (Gly, G)	**A**
	GTG		**GCG**		**GAG**		**GGG**		**G**

Table 2.1. A table depicting the amino acids specified by each codon of three nucleotides. The abbreviation of each amino acid with its coded letter are in brackets. The bold letters refer to nucleotides. ATG codes for the start codon, and TAA, TAG or TGA codes for the stop codon. Adapted from www.people.virginia.edu

2.1.2 Gene expression

Gene expression is the process whereby information encoded in a particular gene is decoded to generate a protein (Lodish et al., 2000). Proteins are synthesised in ribosomes which are found in the cytoplasm. In order to transfer the information stored in DNA from the nucleus across to the cytoplasm, another molecule, messenger RNA (mRNA), is made, which consists of only one strand of polynucleotides. In this process an enzyme, RNA polymerase II, splits the double stranded DNA apart at the appropriate point. This is the most important step in transcription, for it determines which genes are expressed and how much mRNA and subsequently proteins are produced (Lodish et al., 2000). The DNA sequence that specifies where RNA polymerase II starts this process is known as a

promoter, and transcription from the promoter is controlled by DNA binding proteins named transcription factors (Lodish et al., 2000).

The anti-sense strand (3' to 5') acts as a template for the formation of mRNA. Free RNA molecules, which can only be added to the 3' end of the polymer, align themselves to pair with their complimentary base, and in this way the mRNA strand elongates in the 5' to 3' direction. Once assembled, the mRNA, which has the same sequence as the sense strand of DNA, peels itself off from the template and the DNA strands re-join. This whole process is known as transcription. Immediately after transcription, a poly(A) tail is added to the cleaved 3' end of the mRNA, a process catalyzed by polyadenylate polymerase. The function of the poly (A) tail is to protect the mRNA molecule from exonucleases, for the export of mRNA from the nucleus, and for translation (www.en.wikipedia.org). Nearly all mRNAs contain the sequence AAUAAA, 10 – 35 nucleotides upstream from the poly (A) tail, which enables the poly(A) tail to be added.

Once the mRNA is in the cytoplasm, it attaches itself to the surface of a ribosome, which provides a suitable surface for the attachment of mRNA and protein assembly. The protein is formed via transfer RNA (tRNA), which reads the information from the mRNA and translates the message into amino acids. tRNAs are clover-leaf shaped and have three unpaired bases projecting from them; these line up to the appropriate complimentary bases in the mRNA. Attached to the other end of the tRNA is the correct amino acid which codes for the codon sequence of the unpaired bases. When the tRNAs are lined up, peptide bonds form between adjacent amino acids, after which the polypeptide chain breaks away from the tRNA and a protein is formed.

A third kind of RNA, not described so far, is ribosomal RNA (rRNA). This type of RNA is synthesised in the nucleolus by RNA polymerase I and makes up the central part of the ribosome. Recently a fourth kind of RNA has also been discovered. This type of RNA, known as micro RNAs, are short lengths of RNA that control gene transcription through silencing genes (Groβhans & Filipowicz, 2008).

Not all nucleotides code for amino acids. At the start and end of mRNA there are two untranslated regions (UTR). Within the 5' UTR there is a component known as the DNA promoter region, otherwise known as the initiation site, or TATA box, (5' Y-Y-A^{+1}-N-T/A-Y-Y-Y 3'), where Y codes for C or T, which is found 25 – 30 bases from the start codon. The TATA sequence acts as a promoter to position RNA polymerase II for

transcription initiation (Lodish et al., 2000). Another nucleotide sequence in the 5' UTR includes the ribosomal binding site (RBS; also known as the Kozak sequence in eukaryotes). The RBS is the region where the ribosome binds to an mRNA to begin the translation of the mRNA into a protein (Oiveira et al., 2004). This series of purine rich bases (A and G) are located roughly 3 – 14 bases from the beginning of the gene, and their consensus sequence is AGGAG. However, the RBS sequence is highly degenerative, and may have a great deal of variation with respect to its location and its sequence.

Other specific nucleotide sequences include start and stop codons (see Table 2.1 for their coding). Most start codons encode for the amino acid methionine, but stop codons are known as nonsense codons. This is because they cannot be translated because they do not code for an amino acid, instead their purpose is to act as a signal to release the protein from the ribosome during translation.

2.1.3 Topology

Topology refers to the number of times a membrane protein spans the membrane, and the orientation of these membrane-spanning segments, all of which can be determined from a receptor sequence. For example, GPCRs and other serpentine receptors characteristically span the lipid bilayer of the cell membrane in seven α-helical trans-membrane domains (TMDs) (see Figure 2.2). Each TMD contains 20 to 25 hydrophobic amino acids and each particular TMD shows significant sequence homology to other corresponding TMDs within the GPCR gene family, e.g. The first TMD is similar in all classes GPCRs. The amino acid terminus of β-ARs is extracellular and the carboxy terminus is intracellular, whilst the 3rd cytoplasmic loop is characteristically longer than the 1st and 2nd (Strosberg, 1997; Frielle et al., 1987).

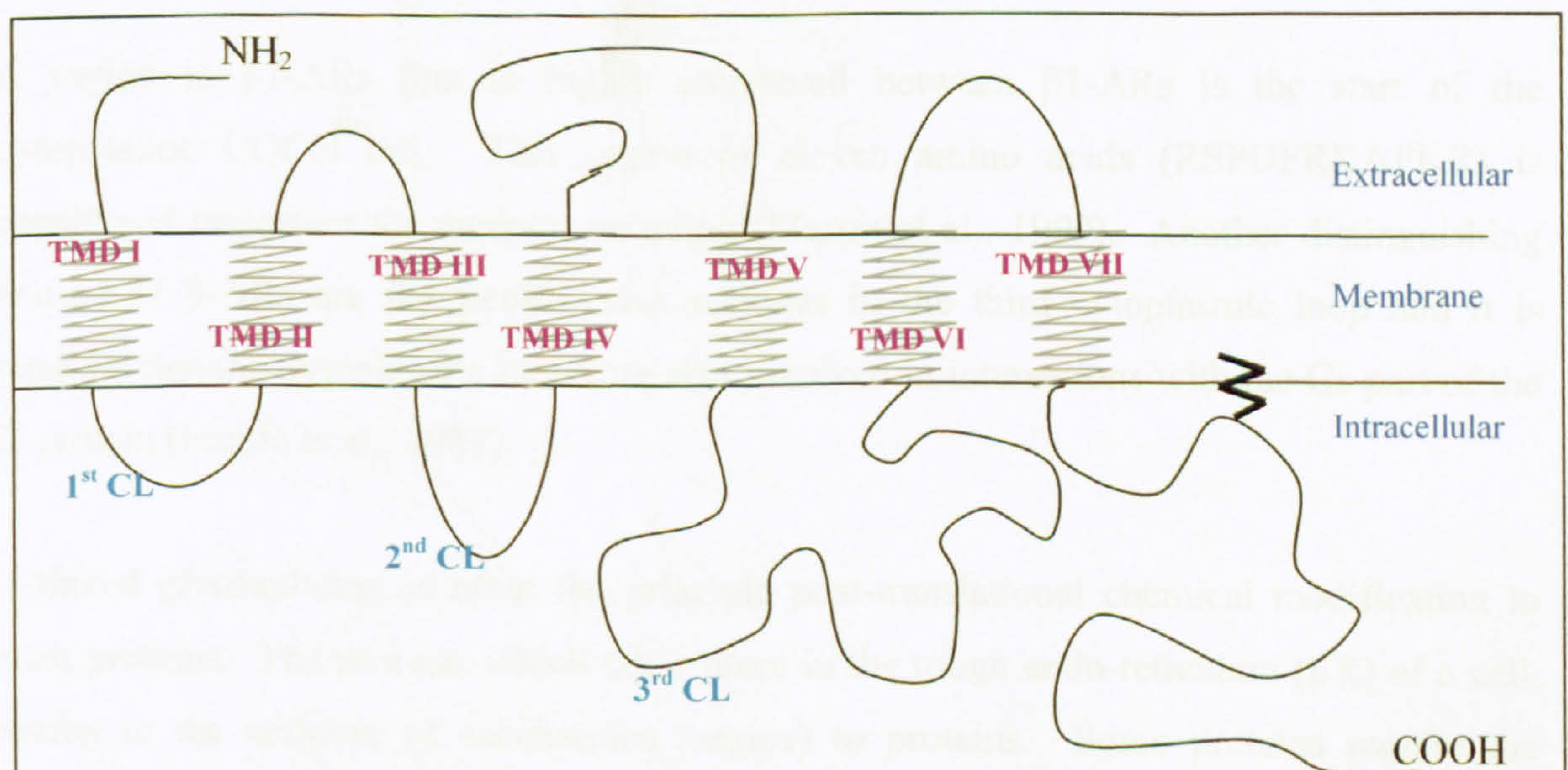


Figure 2.2. Diagrammatic representation of a β-AR in a cell membrane, showing the cytoplasmic loops (CL) and the trans-membrane domains (TMD).

2.1.4 GPCR motifs

In a similar way that start and stop codons are specific nucleotide sequences that are found in all genes, GPCRs also have conserved short nucleotide sequences that have specific functions and these sequences can be used to identify, characterise and distinguish GPCRs from each other and other genes. These motifs are often sequences that ensure the three dimensional (3D) structure and functional properties of GPCRs are conserved and some molecular sequences are preserved because of the ligand. Adrenergic ligands are small positively charged amines and in human β1-, β2- and β3-ARs, two asparagine residues are found in analogous positions and these are thought to act as counterions in order to maintain electric neutrality (Devic et al., 1997).

Because of the (3D) structure of GPCRs, the ligands bind with amino acid residues dotted throughout the molecular sequence. At least four of the seven TMDs are essential for ligand binding (Strosberg, 1997; Libert et al., 1989). For instance, in human β2-ARs, adrenaline binds with aspartic acid in TMD III by the formation of a salt bridge (seen as part of the 'DRY' motif), three serine residues in TMD V and asparagine in TMD VI via hydrogen bonds, isoleucine in TMD IV, and with both valine in TMD III and phenylanine in TMD VI by van der Waals interactions with the ligand (Freddolino et al., 2004; Cao et al., 1998). These residues (especially the serine residues in TMD V) are especially important for binding ligands, and the efficacy with which the ligand binds may be due to the ligand's orientation in the ligand binding pocket which affects the different affinities (strengths) between the ligand and the receptor. Subtle changes in the conformation of these bonds consequently provide weaker or stronger interactions between receptors and different ligands (Freddolino et al., 2004).

A region in β1-ARs that is highly conserved between β1-ARs is the start of the cytoplasmic COOH tail. This region of eleven amino acids (RSPDFRKAFKR) is considered important for receptor coupling (Mason et al., 1999). Another distinguishing feature of β-ARs are the **proline** rich residues in the third cytoplasmic loop and it is possible that the cytoplasmic loops are also involved in interactions with the Gs part of the G-protein (Freille et al., 1987).

N-linked glycosylation is often the principle post-translational chemical modification to most proteins. The process, which takes place in the rough endo-reticulum (ER) of a cell, results in the addition of saccharides (sugars) to proteins. Some proteins require the addition of an oligosaccharide in order to fold properly and to maintain stability (Lodish et

al., 2000). One agreed site for N-linked glycosylation in GPCRs is the **asparagine** residue in the NH_2 terminal region before TMD I (Cao et al., 1998; Frielle et al., 1987).

Phosphorylation is the process whereby a phosphate group is added to an amino acid in the presence of a protein kinase receptor, and this process is often crucial in regulating the activity of many cellular responses to an external signal (Lodish et al., 2000). There are consensus cAMP-dependent kinase phosphorylation sites in GPCRs which include **serine** and **threonine** residues in the 3rd cytoplasmic loop (between TMD V and TMD VI) and the COOH terminal region (after TMD VII), which may mediate receptor desensitization through phosphorylation (Machida et al., 1990; Emorine et al., 1989).

Disulfide bonds form covalent bonds between sulfhydril groups on two cysteine residues in the same or different polypeptide chains to help stabilize the tertiary and quaternary structure of many proteins (Lodish et al., 2000). A lot of GPCRs have conserved **cysteine** residues. These are believed to create a disulphide bridge between the loops to help keep the structural integrity of the protein (Cao et al., 1998).

2.1.5 β-Adrenergic receptors in mammals

ARs are found in many species and a blast search (www.ncbi.nlm.nih.gov) reveals that they have been characterised in many mammals. Table 2.2 shows the species for which β-ARs have been identified.

Mammal	**Latin name**	**β–AR subtype**
Human	*Homo sapiens*	β_1, β_2, β_3
Domestic cat	*Felius catus*	β_1, β_2, β_3
Rhesus monkey	*Macaca mulatt*	β_1, β_2, β_3
Dog	*Canis familiaris*	β_1, β_2, β_3
Brown rat	*Rattus norvegicus*	β_1, β_2, β_3
Gorilla	*Gorilla gorilla*	β_2, β_3
Chimpanzee	*Pan troglodytes*	β_2, β_3
Domesticated cattle	*Bos taurus*	β_1, β_2, β_3
Mouse	*Mus musculus*	β_1, β_2, β_3
Cotton top tamarin	*Saguinus oedipus*	β_2, β_3

Table 2.2. A list of mammals that have had β-ARs identified in their genome.

In the mammals named above, all three β-ARs have been characterised in most animals. However, just because the β1-AR has not been identified in the gorilla, chimpanzee or tamarin, does not mean that these animals don't have these receptors. It is more likely that they are present, but have not been annotated or characterised.

2.1.6 β-Adrenergic receptors in fish

Table 2.3 details the β-ARs found in fish. One noticeable difference between the fish and the mammalian data are that fish appear to have two subtypes of the β3-AR (β3a- and β3b-AR), whereas mammals only have one subtype. The division of the fish β3-AR was initiated by Nickerson et al. (2003). In this paper it is stated that the two novel β-ARs that had been discovered were both homologs of the mammalian β3-AR (52.0 and 52.8 % homology, respectively). However, due to the clearly different expression patterns of these two genes, and the 84% identity between them, they were considered two subtypes, namely β3a- and β3b-AR.

Fish species	Latin name	β-AR subtype	Source
Zebra fish	(*Danio rerio*)	β1, β2, β3, β3b	www.ncbi.nlm.nih.gov
Rainbow trout	(*Oncorhynchus mykiss*)	β2, β3a, β3b	Nickerson et al., 2001;2003
Tiger puffer fish	(*Takifugu rubripes*)	β1, β2	www.Ensembl.org
Spotted green puffer fish	(*Tetraodon nigroviridis*)	β1, β2	www.genoscope.cns.fr
Stickleback	Gasterosteus aculeatus	β1, β3	**www.ensembl.org/**
Hagfish	(*Myxine glutinosa*)	β	www.ncbi.nlm.nih.gov
Marine lamprey	(*Petromyzon marinus*)	β	www.ncbi.nlm.nih.gov
Catfish	(*Ictalurus punctatus*)	β1 (fragment), β2	www.ncbi.nlm.nih.gov

Table 2.3. A list of the β-ARs reported in various species of fish.

These data in Table 2.3 were found from a variety of sources and sometimes initial results could not be taken at face value. For instance, with respect to annotation of the stickleback β-ARs, a search under 'beta adrenergic receptor' of the stickleback genome at

www.ensemble.org provides eight matches and identifies each sequence with an annotation. However, on comparison of these matched sequences to β-ARs in zebrafish, sequence identity analysis suggests that some of the annotations are incorrect, due to the extremely low overall sequence similarity and lack of identity in TMD regions. Analysis suggest and that only one of the annotated β1-ARs has its identity predicted correctly, and that another of the named β1-ARs is in fact a β3-AR, as detailed in Table 2.4.

β-AR as assigned by ENSEMBL	ENSEMBL gene ID	Subtype of zebrafish β-AR to which each ENSEMBL gene was compared	% Identity	Proposed β-AR subtype
β1-AR	ENSGACG00000006578	β1-AR	76	**β1-AR**
	ENSGACG00000005437	β1-AR	2	-
	ENSGACG00000007811	β1-AR	2	-
	ENSGACG00000011022	β1-AR	48	-
	ENSGACG00000011022	β2-AR	45	-
	ENSGACG00000011022	β3-AR (XM_681440)	65	**β3-AR**
β2-AR	ENSGACG00000011022	β3b-AR (XM_696013)	49	-
β3-AR	ENSGACG00000006598	β3-AR	6	-
	ENSGACG00000015178	β3-AR	3	-

Table 2.4. A comparison of putative stickleback β-ARs as annotated by ENSEMBL, compared to authentic zebrafish β-ARs. The % identity is the result of the pair wise comparison between the Ensemble stickleback gene and a particular zebrafish gene.

Zebrafish is another species that required some analysis to fully understand the β-AR sequences given in the annotation. From the NCBI database, two β2-ARs have been annotated for zebrafish, one has been placed on chromosome 14 (Accession number XM_689100) and appears to begin half way through the first TMD, as shown in Figure 2.3, and the second sequence has not yet been placed on a chromosome (Accession number XM_695628).

```
XM_689100           ------------------------------------------------------------M 1
XM_695628           MEGDNTLITENTSLYMNISAGLNASSPVSLSVSEYSDAEVVLISILIGILVLVIVFGNAL 60
                                                                               :

XM_689100           VITAISRFQRLQNVTNCFITSLACADLVMGLVVIPFCALYMVGDTWHFGHFLCDFWTATD 61
XM_695628           VISAIVRFQRLQTVTNYFISSLACADLVMGLMVVPFGACYILLNTWHFGNFFCEFWTATD 120
                    **:** ******.*** **:***********:*:** * *:: :*****:*:*:******

XM_689100           VLCVTASIETLCVIAMDRYIAITSPFRYQSLLTKNRARFIVLMVWVIAGLVSYLPIHMEW 121
XM_695628           VLCVTASIETLCVIALDRYIAIMWPLRYQSMLTKRKACGMVIGVWAVAALISFLPIHMEW 180
                    ***************:******  *:****:***.:*  :*: **.:*.*:*:*******

XM_689100           WISTDNETLCCYNNTYCCEFDITYSYAIVSSIISFYIPLVIMVFVYSRVFQEARKQLKKI 181
XM_695628           WVSEDPEALSCLEEPTCCDFNTNAAYAVPSSIISFYIPLVIMAFVYSRVFQEARRQLQKI 240
                    *:* * *:*.* ::. **:*: . :**: *************.***********:**:**

XM_689100           NKTEGRFHAQKSNSRNQDVANNHSEMRSTKKAKFYLKEHKALKTLGIIMGVFTLCWLPFF 241
XM_695628           DRIEGRIRTQSLSTQEGNEIKN-------RRTKFCMKDHKALKTLGIIMGTFTMCWLPFF 293
                    :: ***:::*. .::: :  :*       :::** :*:************.**:******

XM_689100           VLNVIP---KGSVDIWTFRILNWIGYANSAFNPLIYCRSPDFRYAFKEILCLNRSRYPNV 298
XM_695628           VLNVVAAIWKMDNIMLPFRILNWIGYANSAFNPLIYCRSPEFRCAFQEILCWRPSHLPST 353
                    ****:.   * .  : .***********************:** **:****  .*: *..

XM_689100           RPNNGYIYNAHSWQSENREQSKGSSGDSDHAEGNLAKEECLSADKTDSNGNCSKAQMRVL 358
XM_695628           RSKKGYLYSGHSWKVHTKTTRPREP--SPACETEIG-AECLTAGTKNKNGNYNKTVTSI- 409
                    *.::**:*..***: ..:  ..     *  .* ::.  ***:*...:.***.*:    :
```

Figure 2.3. Clustal alignment of probable zebrafish β2-ARs (Accession no's XM_689100, XM_695628): TMDs highlighted in yellow in XM_689628, were predicted using the programme www.ch.embnet.org/software/TMPRED with a window size of 19.

On further investigation, the upstream data for XM_689100 in the NCBI website can be found by looking at contig 14, which is accessed through NW_633576. However, 37 nucleotides upstream from the 5' end XM_689100 in the contig information, 10 unknown nucleotides (NNNNNNNNNN) occur. The translation of the extra sequence information obtained from contig 14 shows that it very closely resembles the protein sequence found in the zebrafish β2-AR, accession number: XM_695628 up to the point of the unknown nucleotides (XXXX), see Figure 2.4.

```
Extended XM_689100 HAV-KSQF-YCENK-CSALKN-ETERSRIHDCIKVFMGILVDNLYLKKVLSLI-TH-NHH
XM_695628          ------------------MEG---DNTLITENTSLYMNISAG-LNASSPVSLS-------
                     :    :          ::.   :.: * :  .::*.* .. *  .. :**

Extended XM_689100 IW-KKNIRV-VIQSSDHKECFIS-ICFILSKFYQEIPAKMF-SV-MWCD-ICQPRSTVIM
XM_695628          ----------VSEYSDAEVVLIS-------------------------------------
                   :     : : * : ** :  :** : :::  :   :   ::  : :    :      :::

Extended XM_689100 SSTLQHVITRIKQE-T-CFTVVCKTQYNPVKCRVSSVL-SITHTKNS-ICFMCT-NYTAN
XM_695628          ------------------------------------------------------------
                      :  ::  :       : ::       :   :  ::  :       : ::

Extended XM_689100 IIIXXXXSILVLIIVFGNVMVITAISRFQRLQNVTNCFITSLACADLVMGLVVIPFCALY
XM_695628          ILIG----ILVLVIVFGNALVISAIVRFQRLQTVTNYFISSLACADLVMGLMVVPFGACY
                   *:*     ****:*****.:**:** ******.*** **:***********:*:** * *

Extended XM_689100 MVGDTWHFGHFLCDFWTATDVLCVTASIETLCVIAMDRYIAITSPFRYQSLLTKNRARFI
XM_695628          ILLNTWHFGNFFCEFWTATDVLCVTASIETLCVIALDRYIAIMWPLRYQSMLTKRKACGM
                   :: :*****:*:*:*********************:******  *:****:***.:*  :

Extended XM_689100 VLMVWVIAGLVSYLPIHME
XM_695628          VIGVWAVAAL---------
                   *: **.:*.*:  : : :
```

Figure 2.4. Alignment of the translation of the extended XM_689100, using contig 14, and XM_695628. The extension of contig. 14 is shown in purple, and unknown nucleotides have been represented by X. The gold highlighted amino acids show the very close resemblance of the extended part to XM_695628

Upstream of the unknown nucleotides (X) in the extended section, homology is lacking between the two sequences. This may be because there are more than 10 nucleotides that are unknown in this region. However, when different frames of translation are used and compared to XM_695628, no homology is found.

Hence, XM_696100 could be a truncated protein. If the methionine (**ATG**) start codon highlighted in blue, in Figure 2.5, is the actual start codon, then the initiator element sequence (5' Y-Y-A^{+1}-N-T/A-Y-Y-Y 3') expressed at CCATTCTT, can be found 25-30 bp from the start codon. This cannot be found if the alternative methionine start codon (ATG) is used. However, in the truncated form the ribosomal-binding site (RBS) (ACCAUGG) cannot be found 7-13 bp before the start codon, but it can also not be found up to 600bp upstream of the alternative methionine start codon (ATG) either. Hence, because the RBS cannot be found upstream regardless of whether the receptor sequence is extended or not, may mean that this receptor is not actually expressed and is a pseudo gene.

```
AAACTAAGAAACTGAGCGATCACGCATACATGACTGCATAAAAGTGTTTATGGGTATTCTTGTAGATAATCT
TTACTTAAAAAAAGTTTTGTCTCTCATCTAAACACACTGAAATCATCACATTTGGTGAAAAAAAAACATCAG
AGTTTGAGTCATTCAGTCTAGTGATCACAAAGAGTGTTTCATTTCTTAAATCTGTTTCATTTTGTCCAAATT
TTACCAAGAAATTCCAGCAAAAATGTTTTGAAGTGTTTAAATGTGGTGTGACTAAATATGCCAACCACGTTC
CACCGTAATCATGTCTTCCACTTTACAACACGTTATAACTCGAATTAAACAAGAATGAACATGATGTTTTAC
AGTGGTGTGTAAGACACAGTACAACCCAGTAAAATGTAGGGTATCATCTGTCCTGTAATCAATCACACACAC
AAAAAACTCATAGATTTGTTTTATGTGCACATGAAATTATACTGCAAATATCATAATTGNNNNNNNNNNGTC
CATTCTTGTCTTGATCATCGTCTTTGGCAATGTGATGGTGATTACAGCCATTAGCCGCTTCCAGCGTCTTCA
GAACGTCACTAACTGCTTCATTACATCACTTGCGTGCGCTGATCTGGTCATGGGATTGGTGGTGATTCCCTT
CTGTGCCCTTTA
```

Figure 2.5. The 5'UTR of β2-AR zebrafish accession number XM_689100 as obtained from contig 14 is shown in purple. The 5' end of XM_689100 is highlighted in black. N shows a region of unknown nucleotides. ATG is the start codon before the extension. CCATTCTT is the initiator element sequence. ATG is the alternative start codon in the extended area.

Because gene XM_695628 (protein accession number XP_700720) has 7 TMDs, an initiator element sequence found 22 bp from the start codon, an RBS, as is found in the human and fathead minnow β2-AR sequences, start and stop codons, and the 'AAUAAA' motif 10 to 35 nucleotides upstream from the poly (A) tail, as shown in Figure 2.6, this gene was used to represent the zebrafish β2-AR.

```
tgagcacactttgtgagagcagaatactttagagaacaacatctcaaagggattcgttttg
gatatctcacgggagtccatgaaaagtggatttaagttggacaactcacaccctcttctg
atttagtcaaagctcatggagggagacaatacgctgatcacggagaacacctccctgtat
                 M   E   G   D   N   T   L   I   T   E   N   T   S   L   Y
atgaacatttcagctgggctaaacgcttcctcaccagtgtctctctctgtctcagagtac
M   N   I   S   A   G   L   N   A   S   S   P   V   S   L   S   V   S   E   Y
agcgatgcagaggtggtcttaatcagcatcttaataggcattctggttctagtcattgtc
S   D   A   E   V   V   L   I   S   I   L   I   G   I   L   V   L   V   I   V
tttggtaatgcgctggtcatcagtgccattgtacgattccagcgcttgcagactgtcaca
F   G   N   A   L   V   I   S   A   I   V   R   F   Q   R   L   Q   T   V   T
aactacttcatcagctccctggcgtgtgctgatctagtcatgggtcttatggtggtgccc
N   Y   F   I   S   S   L   A   C   A   D   L   V   M   G   L   M   V   V   P
ttcggcgcatgctacattctcctcaatacatggcactttggaaacttcttttgcgagttt
F   G   A   C   Y   I   L   L   N   T   W   H   F   G   N   F   F   C   E   F
tggacggctacggatgtattgtgtgtgaccgctagcattgagacattgtgtgtgattgct
W   T   A   T   D   V   L   C   V   T   A   S   I   E   T   L   C   V   I   A
ctggaccggtacatagccatcatgtggcctctgcgttatcagtctatgcttacaaagcgg
L   D   R   Y   I   A   I   M   W   P   L   R   Y   Q   S   M   L   T   K   R
aaggcctgcgggatggtcataggggtgtgggcagtggccgcccttatctcgttcctaccc
K   A   C   G   M   V   I   G   V   W   A   V   A   A   L   I   S   F   L   P
atccacatggagtggtgggtgtcagaggatcctgaggcgctgagctgcttggaagaaccc
I   H   M   E   W   W   V   S   E   D   P   E   A   L   S   C   L   E   E   P
acctgctgcgacttcaacaccaacgctgcgtacgccgtgccctcctccattatctccttc
T   C   C   D   F   N   T   N   A   A   Y   A   V   P   S   S   I   I   S   F
tacatcccgctggtcatcatggcgttcgtgtacagccgggtcttccaagaggcccgtagg
Y   I   P   L   V   I   M   A   F   V   Y   S   R   V   F   Q   E   A   R   R
cagcttcagaaaatagaccgcattgagggcgaatacgaacacagagcttaagcacccag
Q   L   Q   K   I   D   R   I   E   G   R   I   R   T   Q   S   L   S   T   Q
gagggaaatgagataaagaacaggaggaccaagttctgcatgaaggaccacaaagctctg
E   G   N   E   I   K   N   R   R   T   K   F   C   M   K   D   H   K   A   L
aagaccttggggatcatcatgggaaccttcaccatgtgttggctgccatttttgtgctt
K   T   L   G   I   I   M   G   T   F   T   M   C   W   L   P   F   F   V   L
aatgtggtggcagccatctggaagatggacaacatcatgttgcccttcaggatcttaaat
N   V   V   A   A   I   W   K   M   D   N   I   M   L   P   F   R   I   L   N
tggattggctacgccaactctgccttcaacccattaatctactgcaggagtcctgagttt
W   I   G   Y   A   N   S   A   F   N   P   L   I   Y   C   R   S   P   E   F
agatgtgcttttcaggaaatcctgtgctggagaccctctcatctaccttccacaaggagt
R   C   A   F   Q   E   I   L   C   W   R   P   S   H   L   P   S   T   R   S
aaaaagggatatctctacagtggtcacagctggaaggttcacacaaagaccaccagacca
K   K   G   Y   L   Y   S   G   H   S   W   K   V   H   T   K   T   T   R   P
cgagagccctcgccggcttgtgaaacagaaattggtgctgaatgtttgactgcaggcacc
R   E   P   S   P   A   C   E   T   E   I   G   A   E   C   L   T   A   G   T
aaaaacaaaaatgggaattataataaaactgtgacgtccatttagggcactaaatatatc
K   N   K   N   G   N   Y   N   K   T   V   T   S   I   -   G   T   K   Y   I
cggttgaaactgtttttgtctgtgacaaaacatccatttttagatatttaagcctttgaac
R   L   K   L   F   L   S   V   T   K   H   P   F   -
caagatggtttgcctatatttagacaatatttaattatgaaacttcagaacaaacggaaa
tgaagtactgctttgggacaaattgtgtgcatgaaatccaggacagatttggaaaggcaa
gacagtcagaaagtctacctctggattacatttactacctcgagacacttttatgtctc
aaaaaacctgcattgatatttcatttgtaatttatgaataaatgtcccaacttacaaccg
gaatatgtggattatagatcagaaggagcagtgtcttat
```

Figure 2.6. The translated sequence of XM_695628 (protein accession number XP_700720). Orange nucleotides represent the UTR, **atg** and **tag** are the start and stop codons respectively, **tcacaccc** is the initiator element sequence, the RBS sequence is **catgg,** the 3' UTR motif is **aataaa** found 10-35 nucleotides from poly(A) tail, TMDs are highlighted in yellow and the positions of the TMDs were predicted using the programme TMPRED.

These analyses carried out here of the putative (provisionally named) β-ARs of both the stickleback and the zebrafish demonstrate the considerable difficulties associated with firstly identifying genes that definitely code for a functional β-AR, secondly, the naming of any β-AR correctly (i.e. is the sequence a β1-, β2- or β3-AR?), and thirdly, comparing the sequences of β-ARs. The latter issue includes comparisons of β-AR sequences within a single species (e.g. how similar are β1-, β2- and β3-ARs in a given species?), and between species (e.g. how similar are the β1-ARs of different species?). It is apparent that unless the β-AR genes can be identified and named correctly, any sequence comparisons can be misleading, if not incorrect.

2.1.7 Aims

To characterise the sequences of the β1-AR and β2-AR in the fathead minnow. This will be carried out by first isolating a short sequence from each receptor using polymerase chain reaction (PCR), and then the remaining sequence in each direction, will be found using 3' and 5' rapid amplification of cDNA ends polymerase chain reaction (RACE PCR).

2.2 Materials and Methods

2.2.1 Tissue acquisition

Fathead minnow (*Pimephales promelas)* and rainbow trout, (*Oncorhynchus mykiss*), as shown in Figure 2.7, were both used in experimental procedures and tissue samples from both species were collected in the same way.

Figure 2.7. (**A**) Image of a fathead minnow (*Pimephales promelas*) taken from www.duluthstreams.org/understanding/fatheadminnow.html.

(B) Image of a juvenile rainbow trout (*Oncorhynchus mykiss)* taken from www.nps.gov/isro/NR_Profile_Internal/NR_stills/fish_imag/index1.htm

All bench surfaces and equipment were wiped down with RNase away (Molecular Bio Products) and the microtubules were autoclaved before use to remove any enzymes or other sources of contamination. The fish were anaesthetised in methanesulfonate salt (MS-222) (Sigma, Dorset) until the fish rolled onto one side and no fin movement was observed. Each fish was killed by lobotomy, and the relevant tissues such as the heart, liver, brain and gonads were extracted from each fish by dissection. Each organ was placed in a microtubule and immediately snap frozen in liquid nitrogen to be later stored in a – 80°C freezer.

2.2.2 RNA isolation

Before any work began, all work surface, and instruments were wiped down with RNase away. A tissue sample was obtained from the –80°C freezer and immediately put on ice. The sample was weighed, and 1 ml of TriReagent (Sigma, Dorset) was added per 50 to 100 mg of tissue. The tissue sample was homogenised (initially with only 200 μl of TriReagent in a 1.5 ml eppendorf tube, after which the remaining 800 μl was added), and then left to stand at room temperature for 5 minutes. 0.2 ml of chloroform (Sigma, Dorset), per ml of TriReagent, was added to the tube, which was shaken for 15 seconds, at which stage the sample looked like a milkshake, as shown in Figure 2.8. The tube was left to stand at room temperature for 10 minutes, after which the sample was spun in a centrifuge (Gilson, Anachem, Bedfordshire) at 12,000*g* for 15 minutes at 4°C.

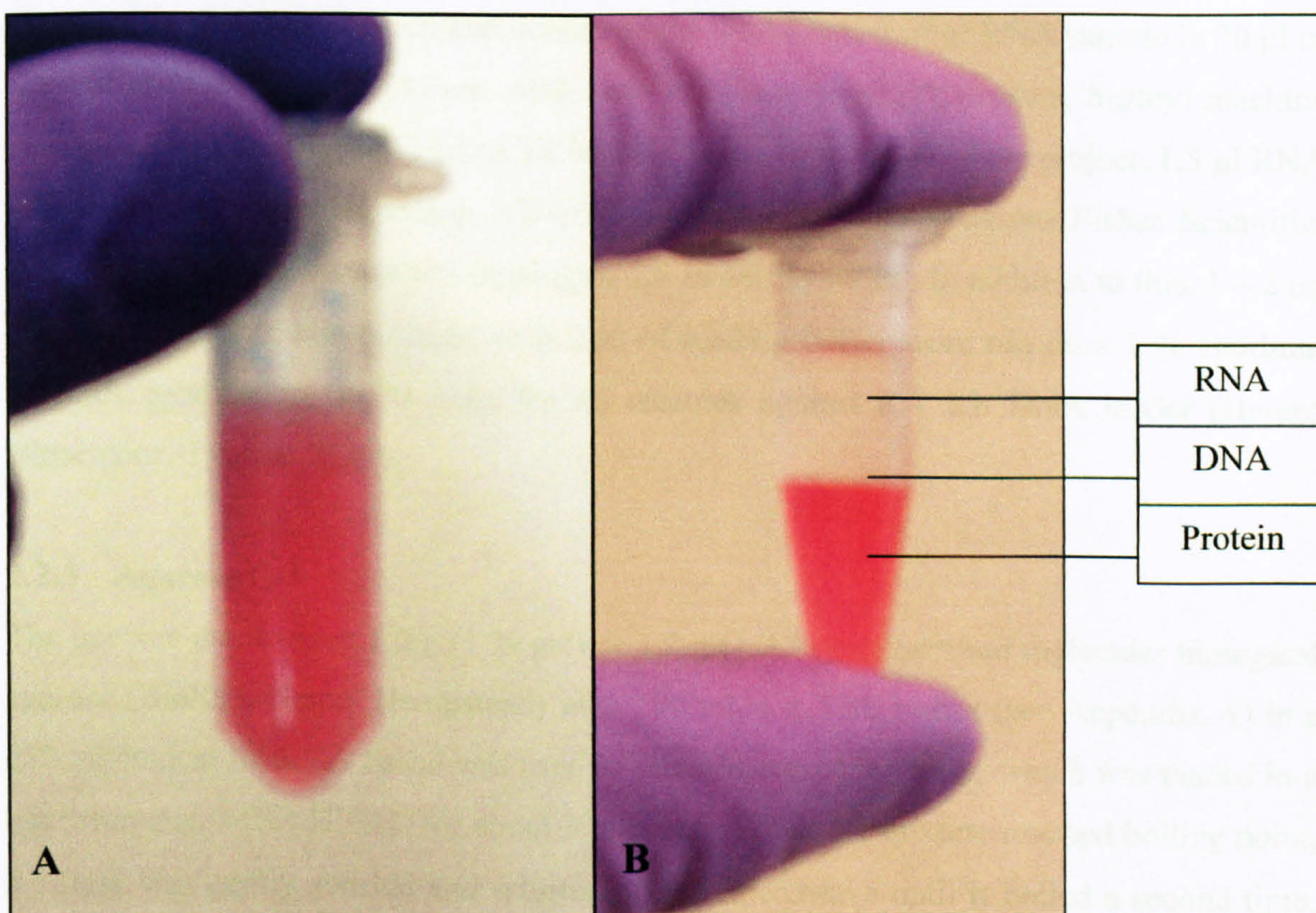


Figure 2.8. (**A**) An eppendorf tube containing TriReagent, chloroform and homogenised tissue. Its appearance is similar to a milkshake. (**B**) Appearance of tube (A) after centrifugation. RNA, DNA and protein have become separated into three distinct layers.

The RNA supernatant was very carefully removed, using a pipette, and placed in a clean eppendorf tube. To ensure no DNA contamination occurred, chloroform was added again to the RNA supernatant and the previous steps were repeated. Following this, 0.5 ml of Isopropranol (Sigma, Dorset) per ml of TriReagent used was added to the RNA supernatant and the sample was mixed and left to stand for 6 minutes. The sample then

underwent further centrifugation at 12,000g, for 10 minutes at 4°C, after which a pellet formed at the base of the tube. The supernatant was removed and 1ml of 75 % ethanol for 1 ml TriReagent was added, and the sample vortexed until the pellet came detached from the base of the tube. The sample was then centrifuged at 7,500g for 5 minutes at 4°C, the supernatant was removed with a pipette, and the remaining pellet was left to air dry for 5 to 10 minutes or until it became clear around the edges. Depending on the size of the pellet, 30 to 400 μl of Milliq water was added to the pellet before it was then placed in a 55 °C water bath (Grant, Chelmsford) for 10 minutes. If the pellet had not dissolved, further small amounts of Milliq water were added to re-suspend the pellet, taking care not to over-dilute the sample.

To measure the concentration and quality of the total RNA, 1 μl of RNA sample in 70 μl of TE buffer (see Appendix A) was analysed on a GeneQuant (Pharmacia, Surrey) machine against a blank reading of 71 μl of TE buffer. In the later stages of the project, 1.5 μl RNA was quantified on a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Loughborough) against a blank reading of 1.5 μl Milliq water. In addition to this, 1 – 2 μg of RNA in 9 μl of water mixed with 2 μl of loading buffer were run on a 1 % ethidium bromide agarose gel at 80 volts for 45 minutes against a 1 Kb DNA ladder (Helena Bioscience, Tyne & Wear).

2.2.3 Agarose Gel

The gel was made using 1.0 g (1 % gel) or 1.2 g (1.2 % gel) certified molecular biological agarose (BioRad, Hemel Hempstead) with 100 ml, 1 x TBE buffer (see Appendix A) in a 250 ml conical flask. A tissue was used to plug the top of the flask, which was placed in a microwave at 'MED HIGH' for about 3 minutes. When the mixture reached boiling point, the flask was gently swirled and returned to the microwave until it boiled a second time. The flask was removed from the microwave and constantly swirled under cold running water to cool the contents. 5 μl of ethidium bromide (Sigma, Dorset) was added to the flask and the solution mixed by swirling. The gel was poured into a plastic mould that had been taped at either end with autoclave tape, and the comb inserted 1 cm from the top edge. The gel was left to set for at least 30 minutes before the samples were loaded.

2.2.4 Messenger RNA (mRNA) isolation

Four types of RNA exist, messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA) and micro RNAs, and it is only mRNA that contains the coded information obtained from DNA during transcription (Lodish et al., 2004). Since mRNA only constitutes 1 – 5 % of total RNA, a significant reduction of sample occurs when mRNA is isolated. In addition to this, isolation of mRNA is an extra step which can result in a further loss of sample. However, if maximum sensitivity is required, then mRNA is the preferred choice of material (Bustin, 2000).

mRNA was isolated from 50 to 100 μg total RNA in 200 to 500 μl of water using a Nucleotrap nucleic acid purification mini kit (BD Sciences, USA) using the protocol supplied with the kit. The maximum mRNA yield using this mini kit is 10 μg. To calculate the concentration of mRNA, 1 μl of mRNA in 70 μl of TE buffer was analysed on a GeneQuant machine against a blank reading of 71 μl of TE buffer. In the later stages of the project, mRNA was isolated using Genelute mRNA miniprep kit (Sigma, Dorset) and 1.5 μl mRNA was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Loughborough) against a blank reading of 1.5 μl Milliq water. The Genelute kit required 5 to 500 μg total RNA in 250μl water to give a yield of 2- 5 % of the starting material.

2.2.5 Reverse transcription

Complementary DNA (cDNA) was obtained using Superscript III reagents and protocol (Invitrogen, Paisley). 1 ng to 5 μg of total RNA or mRNA was required together with 1 μl 10mM deoxynucleoside triphosphate (dNTP) mix, 1 μl Oligo (dT), and enough DEPC-treated water to make the total volume up to 10 μl. These ingredients were mixed and placed in a heated block (PCR machine, Omnigene, Cambridge) for 5 minutes at 65 °C to denature the RNA. The tube was placed on ice for at least a minute, after which 2 μl 10 x RT buffer, 4 μl 25mM magnesium chloride, 2 μl 0.1M DTT, and 1 μl RNase OUT were added. The tube was mixed and returned to the incubator for 2 minutes at 42 °C and 1 μl Superscript III RT was added, and the mixture was left on the incubator block for a further 50 minutes. During this time, reverse transcription took place, whereby adenine is replaced by thymine, and vice versa, and cytosine is replaced by guanine, and vice versa. To terminate the reaction, the incubator temperature was changed to 70 °C for 15 minutes, after which the tube was left to cool on ice for 1 minute. To complete the process, any remaining RNA is destroyed by the addition of 1 μl of *E. coli* RNase H 2U/ μl and the tube incubated at 37 °C for 20 minutes. This ensures only the newly made cDNA remains.

2.2.6 Primers

All primers were oligonucleotide primers, i.e. short single strand pieces of DNA between 20 to 30 nucleotides long, and were ordered from Sigma Genosys (http://orders.sigma-genosys.eu.com) and dissolved with Milliq water to 100 μM. Primer pairs were designed to have the same melting points; these were calculated using the oligo-calculator on the Sigma Genosys website. Taq polymerase has a melting temperature of 72 °C and therefore primers were designed to have melting points between 60 – 68 °C. Primers were also designed to have a guanine and cytosine (GC) content of between 40 – 60%. Guanine and cytosine bind to each other using 3 covalent bonds, which make the link stronger than between adenine and thymine, which link using only 2 covalent bonds. Because of this, primers were designed to end in a C or a G so that the enzyme, taq polymerase, could easily bind and start the amplification process. Degenerate primers were also designed and used when the sequence was unknown and an educated guess meant that the sequence could be either one of two different nucleotides.

In order to ensure that the primers did not bind to themselves or other primers and consequently reduce the yield of the reaction, secondary structures of the primers were investigated. Secondary structures are produced through inter- and intra-molecular interactions and include structures such as hairpins, self dimers and cross dimers. The possibilities of these forming can be identified using programmes such as http://frodo.wi.mit.edu/ and www.basic.northwestern.edu/biotools/oligocalc.html. If a primer design had a secondary structure potential that was stronger than a weak association, or if it produced a primer dimer, then it was re-designed.

2.2.7 Polymerase Chain Reaction (PCR)

PCR allows specific regions of DNA to be amplified. The enzyme, DNA polymerase, extends primers, designed to be complementary to particular parts of a DNA sequence, onto a single DNA template in the presence of dNTPs. AmpliTaq Gold (Applied Biosystems, Warrington) was used in all PCR reactions. Under optimum conditions, this results in the synthesis, in the 5' to 3' direction, of new complimentary DNA strands (Newton & Graham, 1997). Figure 2.9 details how this process works. Amplification of the target region occurs exponentially after cycle 3 of the PCR. Single adenine residues form at the end of each PCR product and these are utilised by the Uracil overhang during ligation of the PCR product into a vector.

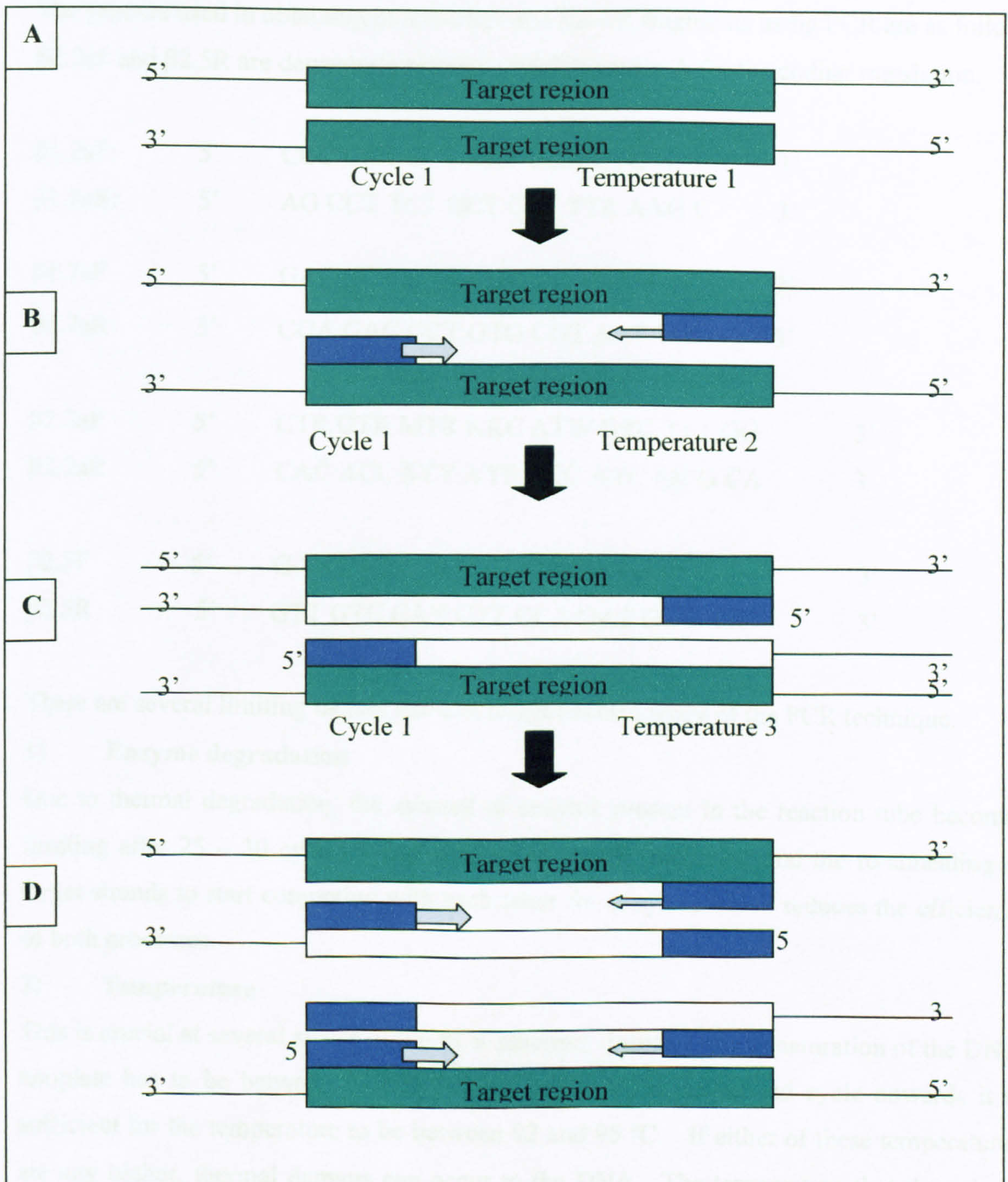


Figure 2.9. Diagrammatic explanation showing how a specific region of a DNA strand undergoes amplification in a PCR reaction. **A.** Firstly, the double-stranded DNA is denatured by thermal denaturation. **B.** Once the DNA has cooled, the primers anneal to the target regions at a temperature specific to the primers **C.** DNA polymerase is then used to extend the primers in the presence of dNTPs and buffer, and the target strands re-anneal. **D.** In second and subsequent PCR cycles, the three processes above are repeated.

The primers used in obtaining putative β1- and β2-AR fragments using PCR are as follows. β2.2aF and β2.5R are degenerate primers. See Appendix A for the coding translation.

β1.2aF:	5'	CCC CAT CCT AAT GCA CTG G	3'
β1.2aR:	5'	AG CCT TCT GCT CTT TTA AAG C	3'
β1.7aF	5'	GAC TCT AAA CGC GCC ACG	3'
β1.7aR	5'	CGA GAC CCT GTG CGT AAT TG	3'
β2.2aF	5'	CTR GTK MTR KKC ATW GTC TTT GG	3'
β2.2aR	5'	CAC ACC SYY AYS ACC AYC MCG CA	3'
β2.5F	5'	G TAC GTC GCC ATC ATG TGG	3'
β2.5R	5'	GTT GTC CAY CTT CCA GAT GGY YR	3'

There are several limiting factors that can affect the efficiency of the PCR technique.

1) Enzyme degradation

Due to thermal degradation, the amount of enzyme present in the reaction tube becomes limiting after 25 – 30 cycles. This causes the primer annealing and the re-annealing of target strands to start competing with each other for enzyme, which reduces the efficiency of both processes.

2) Temperature

This is crucial at several stages of the PCR reaction. Initially, the denaturation of the DNA template has to be between 95 and 100 °C, whilst from the second cycle onwards it is sufficient for the temperature to be between 92 and 95 °C. If either of these temperatures are any higher, thermal damage can occur to the DNA. The temperature that the primer anneals to the DNA strand is also crucial and is specific to each primer, whilst the optimum temperature for primer extension is 72 °C.

3) Number of cycles

If the number of cycles is increased to over 30, it is usual to see an increase in the number of unwanted artefactual products.

4) Quantities of other components

Magnesium chloride ($MgCl_2$) forms soluble complexes with dNTPs to allow them to be incorporated into the extending strand, and also stimulates the polymerase activity of the enzyme. Hence the concentration of $MgCl_2$ in the final reaction mix can radically affect

the specificity and yield of PCR reactions, whereby insufficient $MgCl_2$ results in a low yield or too much $MgCl_2$ can produce an accumulation of non-specific products.

Table 2.5 details the quantities of components added for first and second (with nested primers) rounds of PCR.

Component	**1st Round PCR**	**2nd Round PCR (With nested primers)**
Double distilled water	35.75 µl	37.25
5 µl GeneAmp 10 x B II Buffer	5 µl	5 µl
25 mM Magnesium chloride	5 µl	5 µl
10 mM dNTP mix	1 µl	1 µl
Forward primer	0.5 µl	0.5 µl
Reverse primer	0.5 µl	0.5 µl
AmpliTaq Gold	0.25 µl	0.25 µl
Sample	2 µl RNA or mRNA	0.5 µl PCR product

Table 2.5 Table detailing the amounts of the various components used in each PCR reaction.

Nested primers are used in a second round of PCR and are primers that amplify a smaller amount of more specific DNA from the previously amplified segment obtained in the first round of PCR. Nested primers were used to increase the specificity of the PCR. A gradient PCR (Icycler, BioRad, Hemel Hempstead, see Figure 2.10) was used, for which the annealing temperature for most reactions was between 50 to 64 °C.

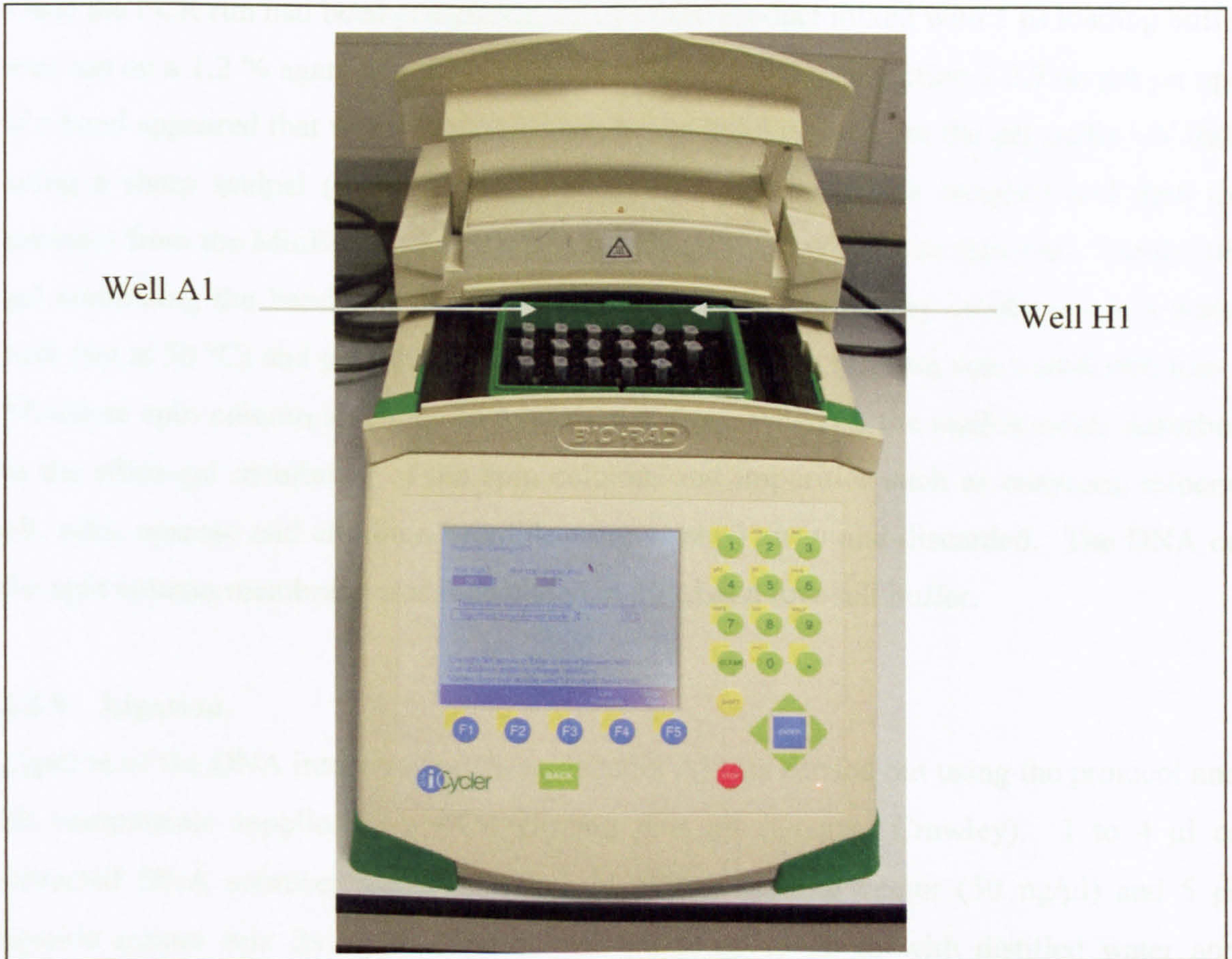


Figure 2.10. An Icycler made by BioRad, Hemel Hempstead. In a temperature gradient PCR, wells A1 to A12 (top left to bottom left) function at the highest temperature in the gradient. Wells H1 to H12 are set to the lowest temperature in the gradient (top right to bottom right).

The PCR programme used for most primers pairs is set out in Figure 2.11.

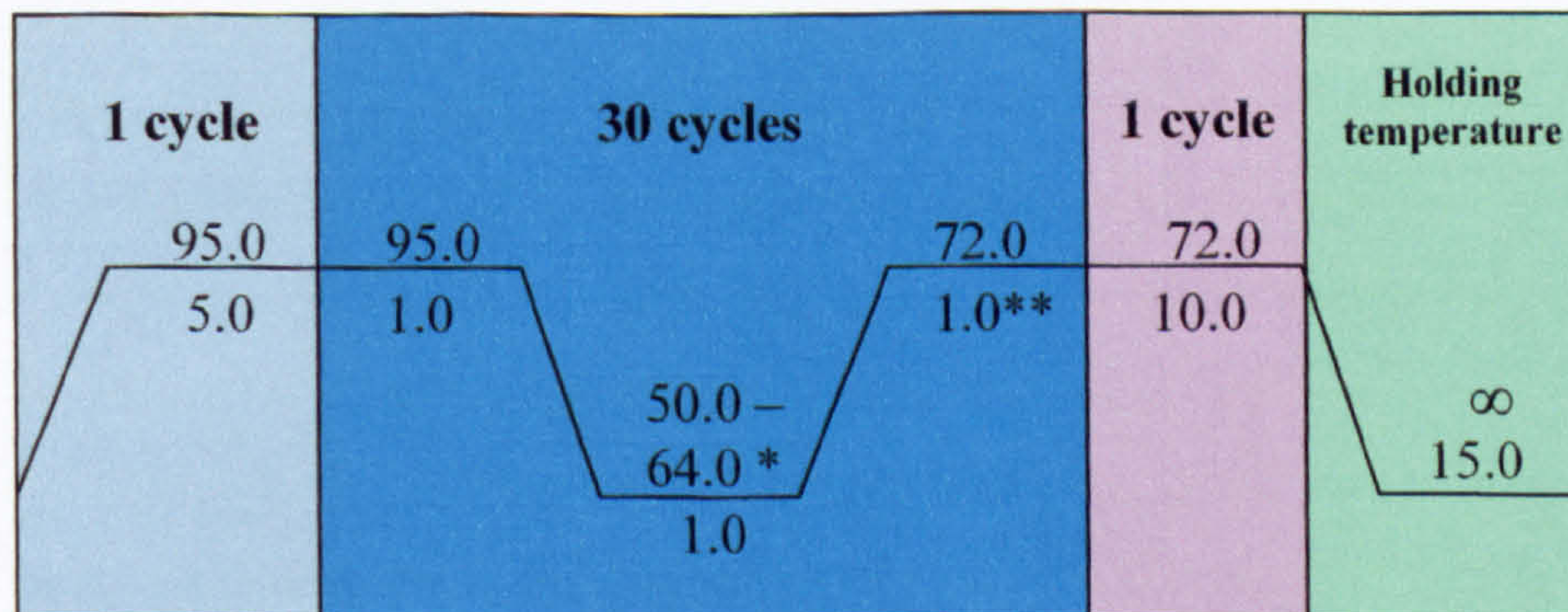


Figure 2.11. Diagram detailing the PCR program used. The number above the line represents the temperature (°C) and the number below represents the time (minutes). * This is the annealing temperature, which is specific to each set of primers, ** this is the extension time, during which the DNA is synthesised within the target region. Both these parameters can be altered to improve the efficiency of the PCR. As a rule of thumb, extension occurs at about 1 Kb per minute.

2.2.8 Extraction of DNA from agarose gel

Once the PCR run had been completed, 25 μl of the product mixed with 5 μl loading buffer was run on a 1.2 % agarose gel for 45 minutes at 80 Volts (see section 2.2.3 on gel set up). If a band appeared that was the correct size, it was quickly cut from the gel under UV light using a sharp scalpel (Swan-Morton, Sheffield). The band was weighed and then the protocol from the MinElute gel extraction kit (Qiagen, Crawley) was followed. Firstly, the gel containing the band was dissolved in a pH-sensitive buffer by incubation in a water bath (set at 50 °C) and periodic vortexing. The now yellow solution was transferred into a MinElute spin column and spun in a mini-centrifuge, whereon the nucleic acids adsorbed to the silica-gel membrane of the spin column, and impurities such as enzymes, mineral oil, salts, agarose and ethidium bromide were washed away and discarded. The DNA on the spin column membrane was then eluted in 10 μl of a low-salt buffer.

2.2.9 Ligation

Ligation of the DNA into a vector (See appendix A) was carried out using the protocol and kit components supplied in a PCR cloning plus kit (Qiagen, Crawley). 1 to 4 μl of extracted DNA solution was added to 1 μl pDrive cloning vector (50 ng/μl) and 5 μl ligation master mix 2x. The solution was made up to 10 μl with distilled water and incubated for 15 °C for 2 hours in an incubator. During incubation, under optimal conditions provided by the master mix, the linear vector, which has a Uracil (U) overhang at each 3' end, hybridises to the A overhang of each PCR product (see Figure 2.12).

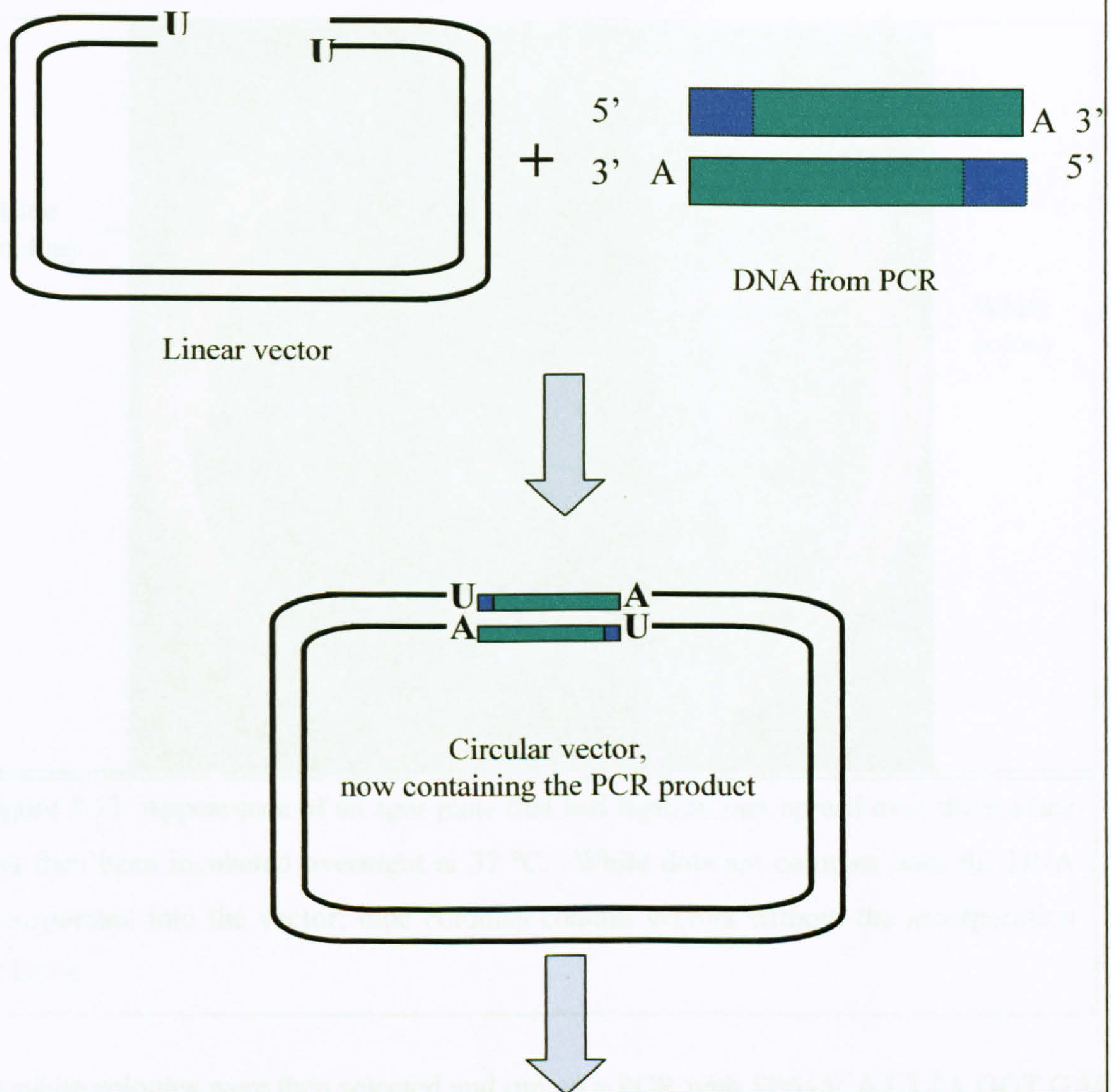


Figure 2.12. Diagrammatic explanation of the orientation of the PCR product in the vector.

After incubation, 2 µl of the ligation-reaction mix was added to QIAGEN EZ Competent Cells (Qiagen, Crawley) and left on ice for 5 minutes. The cell mixture was then heat shocked for 30 seconds at 42 °C and returned immediately to the ice. 250 µl of room temperature SOC medium was added, and after mixing, the solution was spread over 4 agar plates using a sterilised glass spreader. The agar plates were made up of 100 ml agar (see appendix A), 100 µl ampicillin, 160 µl Xgal and 50 µl IPTG. Once the cells had sunk into the agar, the plates were turned upside down and left overnight at 37 °C.

The next day, the plates were left in the fridge for 30 minutes to let the colour develop. The vectors that had DNA incorporated into the plasmid appeared white, whilst those that did not have incorporated DNA appeared blue (see Figure 2.13).

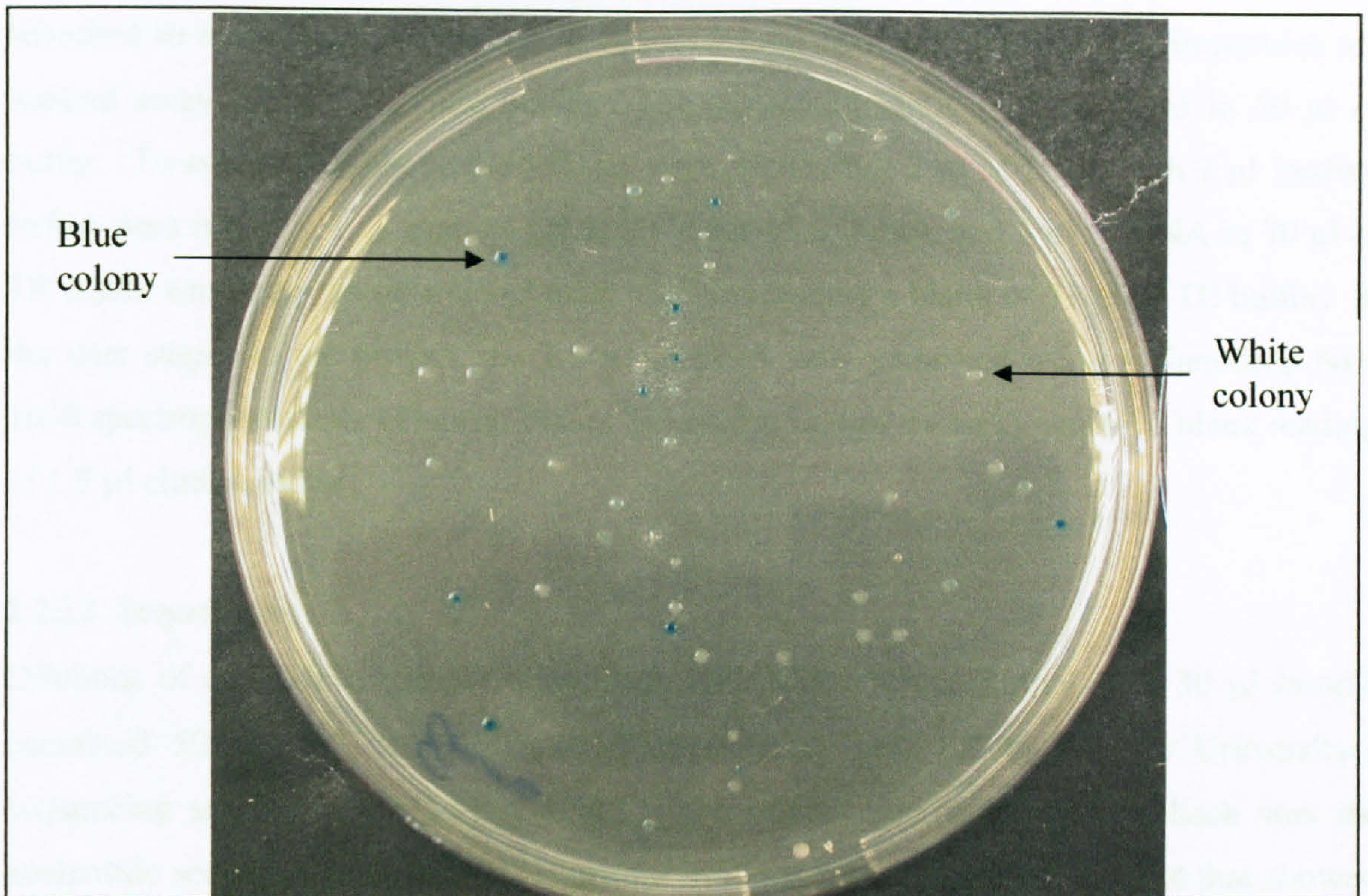


Figure 2.13. Appearance of an agar plate that had ligation mix spread over the surface and then been incubated overnight at 37 °C. White dots are colonies with the DNA incorporated into the vector; blue colonies contain vectors without the incorporation of DNA

The white colonies were then selected and run on a PCR with SP6 (5' AT TTA GGT GAC ACT ATA G 3') and M13 reverse (5' CAG GAA ACA GCT ATG ACC ATG 3') primers. The PCR products were then run on a 1 % agarose gel to ensure that a DNA fragment of the expected size had been inserted into the plasmids. The successful colonies were picked from the agar plates and grown up in a culture of 5 ml L broth (see Appendix A) and 5 μl ampicillin overnight in a 37 °C shaker.

2.2.10 Preparation of glycerol stocks

250 μl of 20 % glycerol and 250 μl of a colony were mixed in a 1.5 ml eppendorf tube and stored at –80 °C. These stocks can be used to grow up the same colony repeatedly, by streaking a loop from the stock over an agar plate and growing colonies overnight at 37 °C.

2.2.11 Plasmid (mini-prep)preparation

The culture tubes were spun at 2,500 rpm at room temperature for 10 minutes. The supernatant was poured away and any excess liquid was blotted out onto tissue. Using the protocol set out in the QIA prep spin miniprep kit (Qiagen, Crawley), a buffer was added

and the cells re-suspended by repeat pipetting. After several steps, the bacterial lysates are adsorbed to a silica-gel membrane in the QIA prep spin column, and any impurities are washed away using a washing buffer. The remaining DNA is then eluted in 50 µl of buffer. To ensure that above procedures were successful, 2 µl of DNA with 2 µl loading buffer were run on a 1 % agarose gel at 80 V for 45 minutes, and 1 µl of DNA in 70 µl of TE buffer was analysed on a GeneQuant machine against a blank of 71 µl of TE buffer. In the later stages of the project, the 1.5 µl of DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Loughborough) against a blank reading of 1.5 µl elution buffer.

2.2.12 Sequencing

Dilutions of each DNA sample were made with Milliq water so that each 30 µl sample contained 500 ng of DNA. These dilutions were sent off to Dundee University's sequencing service (www.dnaseq.co.uk). The information that was sent back was the nucleotide sequence from the M13R primer in the vector, and a chromas plot that showed the quality of the sequence. Figures 2.14 and 2.15 are examples of chromas plots.

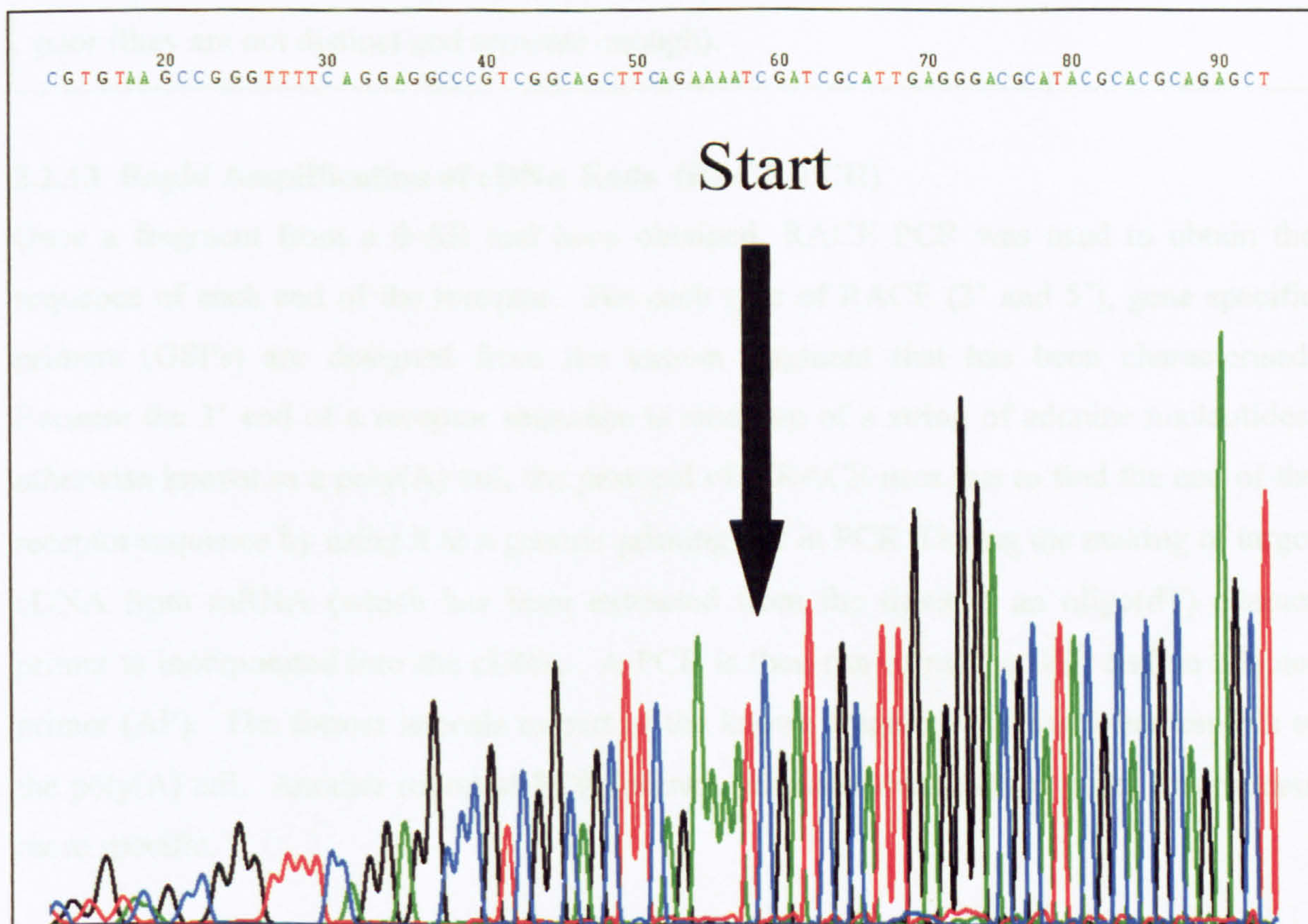


Figure 2.14. This shows peaks from a chromas plot and at which point the information would be collected from the sequence. This point was determined by myself. Once the peaks become sharp and well defined, the quality of these data is good enough to start obtaining the sequence.

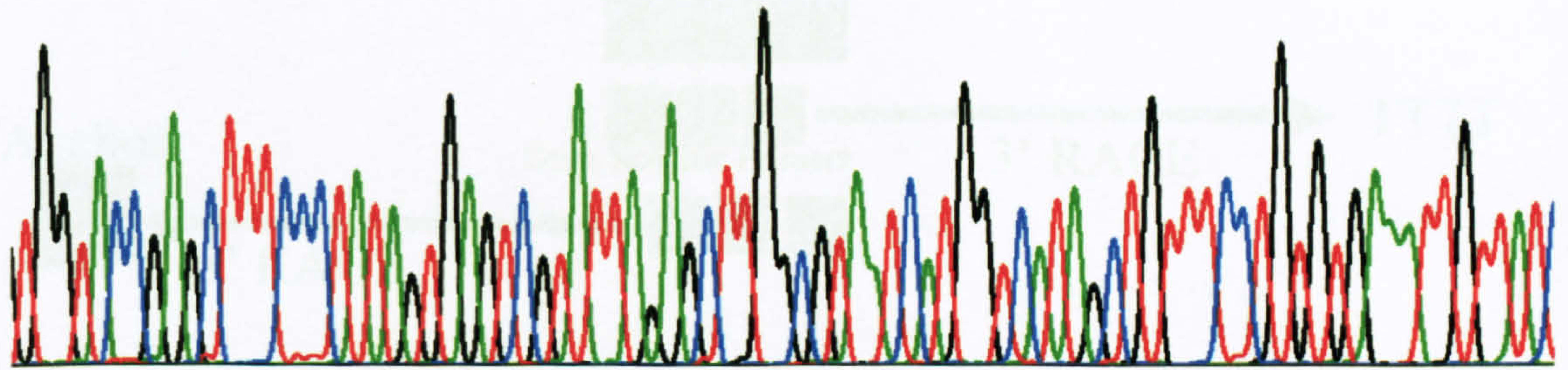


Figure 2.15. Peaks from a chromas plot. These peaks are poorly defined and in places are overlapping each other. The amino acids translated from plots such as these would not have been used for sequencing characterisation, as the quality of the peaks is too poor (they are not distinct and separate enough).

2.2.13 Rapid Amplification of cDNA Ends (RACE PCR)

Once a fragment from a β-AR had been obtained, RACE PCR was used to obtain the sequence of each end of the receptor. For each type of RACE (3' and 5'), gene specific primers (GSPs) are designed from the known fragment that has been characterised. Because the 3' end of a receptor sequence is made up of a string of adenine nucleotides, otherwise known as a poly(A) tail, the protocol of 3'RACE uses this to find the end of the receptor sequence by using it as a generic priming site in PCR During the making of target cDNA from mRNA (which has been extracted from the tissues), an oligo(dT) adapter primer is incorporated into the cDNA. A PCR is then run using the GSP and an adapter primer (AP). The former anneals to part of the known fragment and the latter anneals to the poly(A) tail. Another round of PCR follows, with a nested GSP, to make this process more specific.

The 5' end of the receptor does not have a poly(A) tail or an equivalent marker at the 5' end of the receptor. So in the 5' RACE protocol, an anchor is inserted at the 5' end of the receptor during the synthesis of cDNA from mRNA. After this process is complete, 5'RACE works in a similar manner to 3'RACE. GSPs are designed from the known

fragment and these are run with primers that detect the inserted anchor during two rounds of PCR. In this way the receptor is sequenced in 2 parts, i.e. from the known fragment to the 3' end and from the known fragment to the 5' end of the receptor, as diagrammatically illustrated in Figure 2.16.

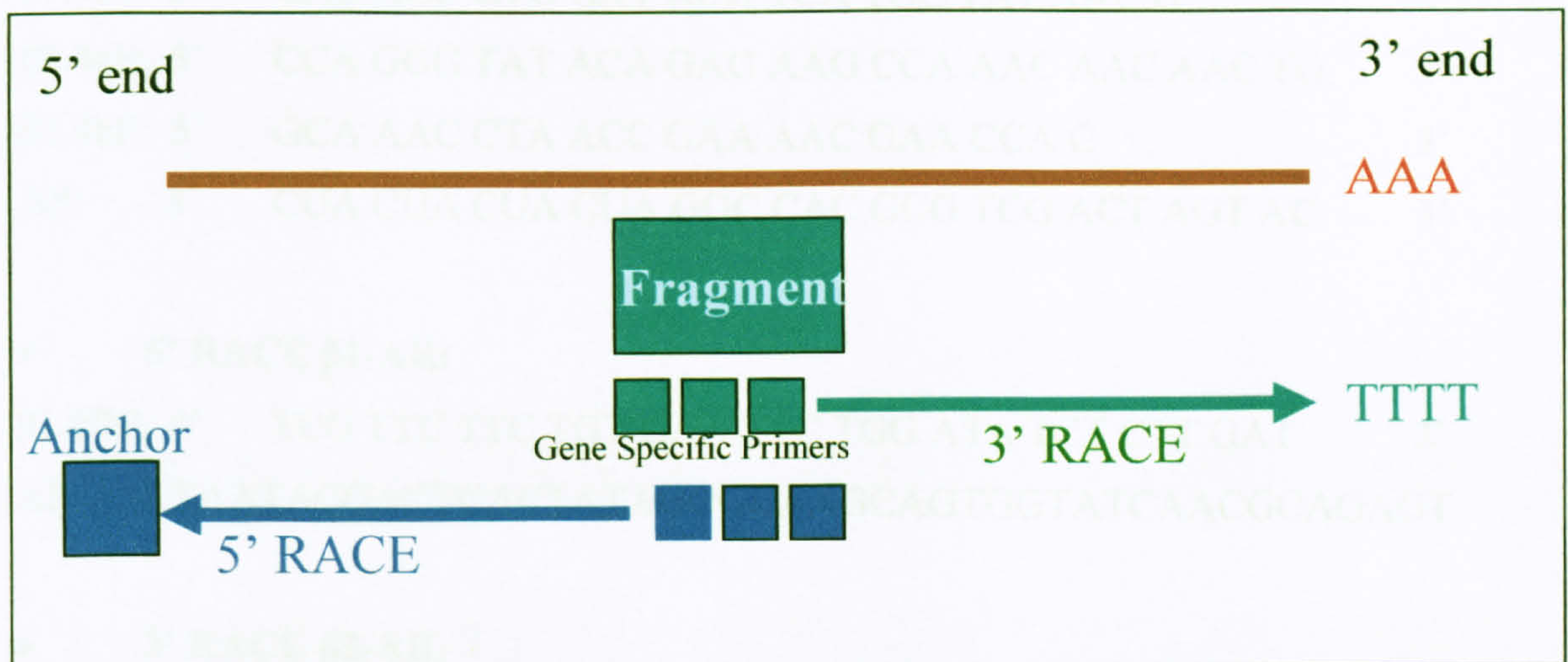


Figure 2.16. Diagrammatic explanation of 3' and 5' RACE PCR. The red line represents the whole length of a receptor with a poly(A) tail.

Two different methods were used to complete RACE PCR. The first was the RACE system from Invitrogen (Paisley), and the second was from Clontech (California, USA). When using both kits, the PCR was broken down into 3 stages to make it more specific, as shown in Figure 2.17. The annealing temperature for the first 5 rounds of PCR were carried out at minus 3°C of the GSP melting temperature, followed by 5 cycles of an intermediate annealing temperature, followed by 20 cycles of an annealing that was minus 3°C of the AP melting temperature.

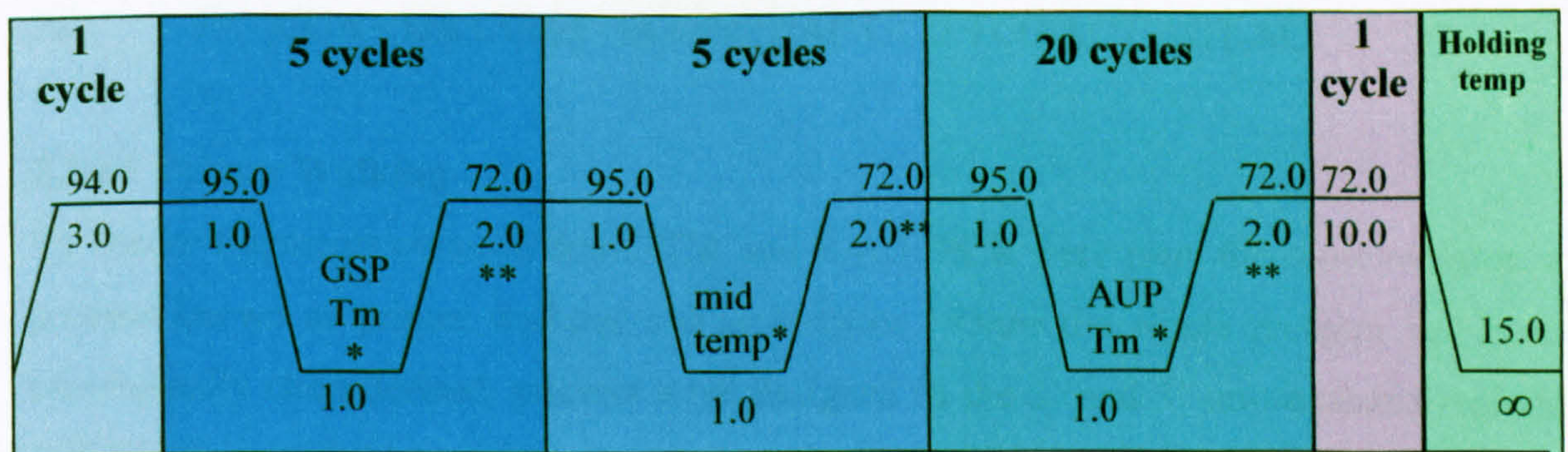


Figure 2.17. Diagrammatic representation of the PCR programme used in 3' RACE PCR. * denotes annealing temperature (°C). ** denotes extension time (minutes).

The GSPs used in 3' and 5' RACE PCR to successfully complete the sequence characterisation for the β1- and β2-ARs are as follows (U represents any nucleotide)

- **3' RACE β1-AR**:

β1.4F: 5' GAC ACC GTG GAT ACA TCA TGC TAT AAC G 3'
β1.4aF: 5' CCA GGG TAT ACA GAG AAG CCA AAC AAC AAC TG 3'
β1.4bF: 5' GCA AAC CTA ACC GAA AAC GAA CCA C 3'
AP: 5' CUA CUA CUA CUA GGC CAC GCG TCG ACT AGT AC 3'

- **5' RACE β1-AR:**

β1.6RR 5' TCG TTC TTC TGT GAA TTC TGG ATA TCT CTT GAT 3'
AP: CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT

- **3' RACE β2-AR**:

β2.7F 5' CAG GAC GGG AAC GAG ACG AAG AAC 3'
β2.7aF 5' G ACC ACA AAG CTC TGA AGA CCT TGG G 3'
β2.7bF 5' AAC ATT CAC CCT CTG CTG GCT GC 3'
AP: 5' CUA CUA CUA CUA GGC CAC GCG TCG ACT AGT AC 3'

- **5' RACE β2-AR**:

B2.8RR 5' CAC TTC GGA AAC TTC TTC TGC GAG TTT TG 3'
β2.8R 5' TAC ATC CTC CTC AAC ACG TGG CAC TTC 3'
β2.8aR 5' CAG ACG GGC ACC AAC TAC TTC ATC AG 3'
β2.8bR 5' GTC ATC AGC GCC ATT GTA CGA TTT CAA C 3'
AP: 5' CUA CUA CUA CUA GGC CAC GCG TCG ACT AGT AC 3'

2.2.14 Primer Walking

To ensure fragments obtained from PCR and RACE PCR were from the same receptor, a process known as primer walking was undertaken. Once the whole receptor had been provisionally characterised, primers were designed in the 3' and 5' un-translated regions (UTRs) to amplify the whole receptor using proof reading taq (Pwo SuperYield DNA polymerase, Roche, Sussex). Because proof reading taq does not add an overhang adenine to the end of the PCR product (i.e. it produces blunt-ended PCR product), one has to be added in order for the product to be inserted into a vector. This was carried out by adding A-addition master mix (Qiagen, Crawley) to the PCR product and incubating for 30 minutes at 37 °C. The PCR product was cloned and inserted into a plasmid. This sample

was then used through out the whole primer walking process. When a sequence that consists of roughly 1250 amino acids is sent for sequencing, only the first 350 amino acids can be reliably characterised, after which the sequence quality degenerates. To overcome this, a primer is designed at the start of the sequence, then an internal primer is designed after approximately 350bp and another is designed after approximately a further 350bp, and so on. This process is repeated until the end of the receptor has been reached, and was carried out in both the sense and the anti-sense directions, (see Figure 2.18).

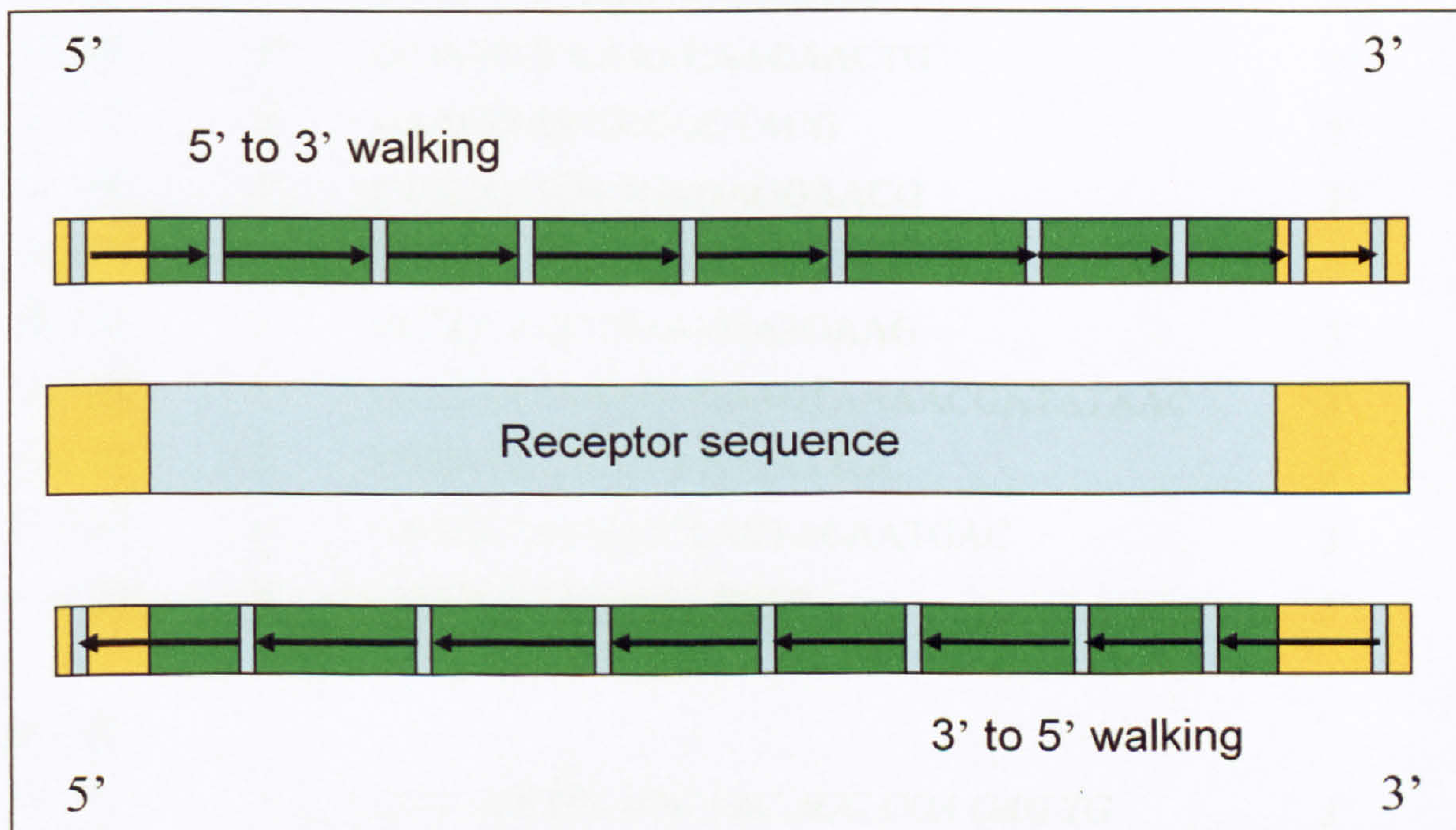
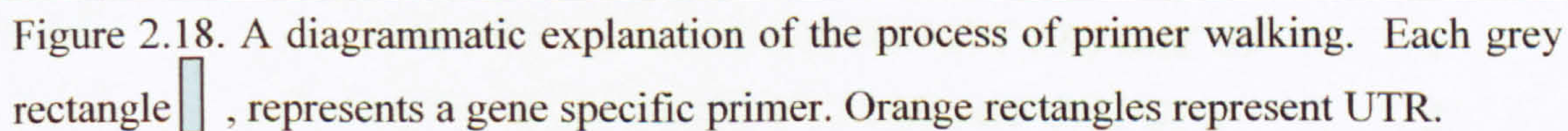


Figure 2.18. A diagrammatic explanation of the process of primer walking. Each grey rectangle , represents a gene specific primer. Orange rectangles represent UTR.

The primers used to obtain and verify the complete sequences (*in italics*) and also those used in primer walking, were as follows:

β1-AR:

β1.10R	*5'*	*CGT TTC AGA CAA ATT CGA AAA TAT TTC C*	*3'*
β1.11aF	*5'*	*GAG AGC GCG GAT GGA AG*	*3'*
β1.12F	5'	GGCTCCAGACGCTCACC	3'
β1.13F	5'	GCCTTTTAACGAAAGCACG	3'
β1.14F	5'	GAGAAGCCAAACAACAACTG	3'
β1.15F	5'	GAACTGGCTGGGGTACG	3'
β1.16F	5'	CTGCAGTCTAGAGAGGAACG	3'
β1.12R	5'	CTGCTCCATTGAGTTCACAAAG	3'
β1.13R	5'	GCACCACGTTAACGATGAAG	3'
β1.14R	5'	GACTATTAAAGGGATGTAAAACGATATAAC	3'
β1.15R	5'	GCGATGTACCTGTCAATTGC	3'
β1.16R	5'	CAGTATATTCCCCACTACAATGAC	3'
β1.11aR	5'	CTTCCATCCGCGCTCTC	3'

β2-AR:

β2.9F	*5'*	*CGA CAT TTA GTC TAC AGC CGA GAG TG*	*3'*
β2.9R	*5'*	*GTC TGT GAC AAA ACA TGG TTT TTA GAT GT*	*3'*
β2.9aF	*5'*	*CAT GAC CAG GTG ATC AAG AGT CGA GTG*	*3'*
β2.9aR	*5'*	*CCC TGG AGC ACT TGA GAG ATT TCT GC*	*3'*
β2.10R	5'	GCA GTA GAT GAG GGG ATT G	3'
β2.11F	5'	TCA ACG CTT GCA GAC G	3'
β2.12R	5'	CT TGG GGA TCA TCA TGG	3'
β2.13R	5'	CG CTA TCA GTC GAT GCT TAC	3'
β2.14F	5'	CCA CAA AGC TCT GAA GAC G	3'
β2.15R:	5'	CTC AAC ACG TGG CAC TTC	3'
β2.16F	5'	CATCGTCTCCTTCTACATCCC	3'

2.2.15 Sources of established β-AR sequences

Nucleotide and protein sequences of different β-ARs from different species were obtained from the websites detailed in Table 2.6.

Source	Website
NCBI	http://www.ncbi.nlm.nih.gov/
Wellcome trust Sanger Institute	http://www.sanger.ac.uk/
Ensembl Genome Browser	http://www.ensembl.org/index.html
Genoscope	http://www.cns.fr/

Table 2.6. A list of the databases and their websites that were used to obtain sequence data.

The accession numbers of selected sequences were from the NCBI website unless otherwise stated, and are as follows: For β1-ARs, *Homo sapiens*; NP 00675.1, *Danio rerio*; XP 685300, *Tetraodon nigroviridis*; CAG 12607.1, *Takifugu rubripes*; from www.ensembl.org: SINFRUG00000121458, *Gammarus aculeatus*; www.ensembl.org/, ENSGACG00000006578

For β2-ARs: *Homo sapiens*; AAB 82149, *Danio rerio*; XP_700720, *Oncorhynchus mykiss*; AAK94672.1, *Tetraodon nigroviridis*; CAG10129.1, *Takifugu rubripe*; AAQ02695.1, *Myxine glutinosa* (hagfish); CAA06539.1, *Petromyzon marinus* (Marine Lamprey); CAA06540.1, *Ictalurus puncatatus* (Catfish), (nucleotide only) AF127775.

Sequences were aligned using CLUSTAL W (1.83) multiple sequence alignment (www.ebi.ac.uk/Tools/clustalw/index.html) using default settings for pairwise alignment. To find the reverse complement of a sequence, the website www.bioinformatics.vg/bioinformatics_tools/reversecomplement.shtml was used. Translation of a nucleotide sequence to a protein sequence was carried out by ExPASy, a proteomics server of the Swiss Institute of Bioinformatics (www.expasy.ch/tools/dna.html). This website translates the nucleotide sequence into 6 possible reading frames. Frame 1 was obtained by translating the sequence from the first input nucleotide, frame 2 and 3 are from the second and third nucleotides, respectively. Frames 4 to 6 are the same as frames 1 to 3, but are the translations in the reverse direction.

To try to annotate an unknown nucleotide sequence, a blastx programme was run at the website www.ncbi.nlm.nih.gov/blast/Blast.cgi to find regions of local similarity between sequences of the input fragment and listed NCBI proteins. This programme translates the

nucleotide sequence into 6 frames and searches its large database against each of them. Against each result the programme denotes an E number, which describes the number of hits one can 'expect' to see just by chance when searching a database of a particular size. The closer the E value is to zero, the more significant the match is.

2.2.16 Hydropathy analysis

GPCRs are made up of seven hydrophobic trans-membrane domains (TMDs), as shown in Figure 2.19. Each TMD needs a row of 18-20 hydrophobic amino acids to be able to cross the very hydrophobic bilipid layer of a cell membrane, which means that hydrophilic amino acids are found primarily outside of the membrane.

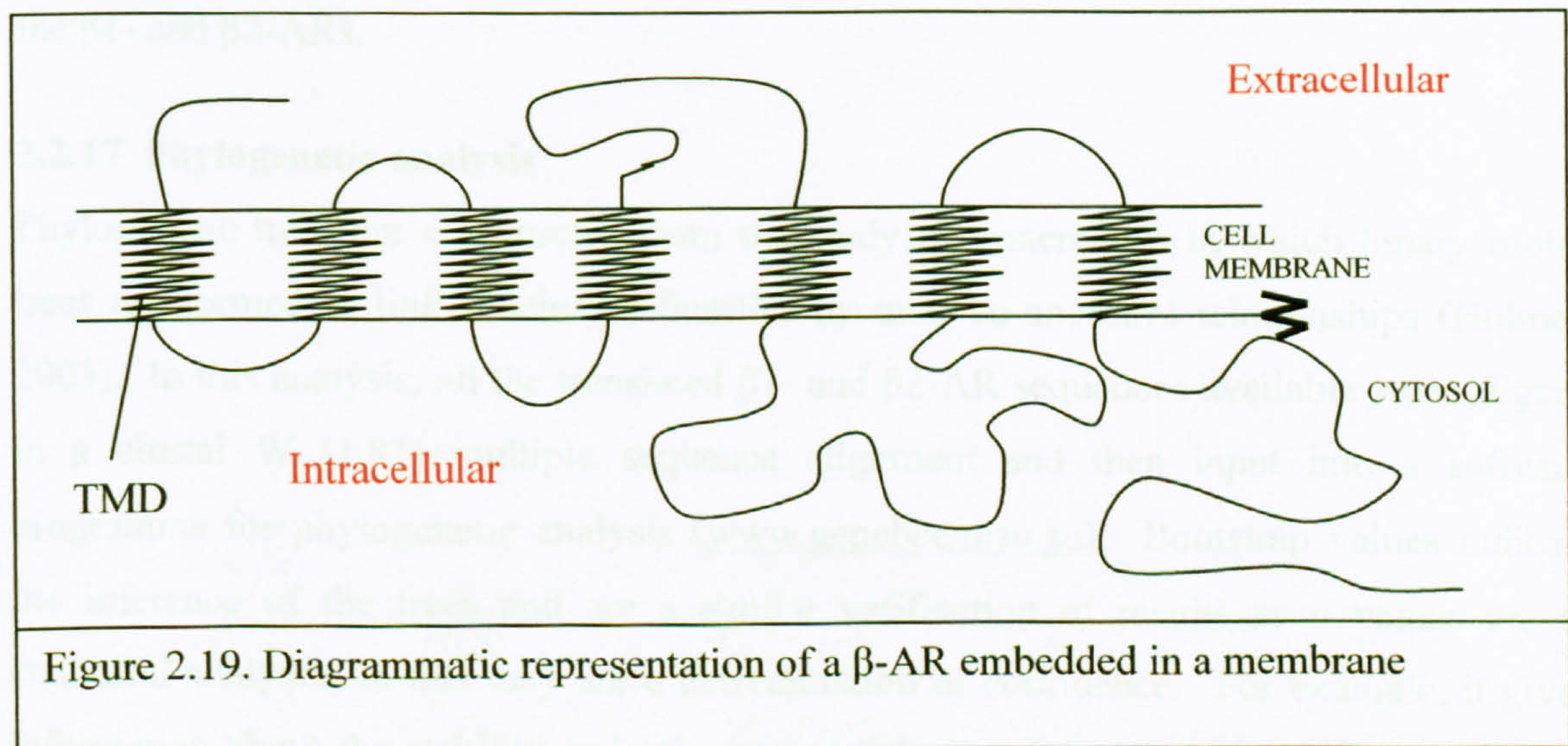


Figure 2.19. Diagrammatic representation of a β-AR embedded in a membrane

From sequence information put into a software programme, the TMD topology can be predicted. In these programmes each amino acid is designated a hydropathy score from the Kyte-Doolittle Hydropathy index from between -4.5 and 4.5. A score of 4.5 is the most hydrophobic and a score of -4.5 is the most hydrophilic. The hydrophobicity of the amino acids determines where the amino acid will be located in the final structure of the protein (Kyte & Doolittle, 1982). Once the hydropathy score has been assigned, a window size is set. A window size is the number of amino acids whose hydrophobicity scores will be averaged and assigned to the middle amino acid in the window. When looking for surface regions in a globular protein, a window size of 9 is found to give the best results. When looking at GPCRs, a window size of 19 is best. TMDs are identified by peaks with scores greater than 1.8 using a window size of 19.

The software programmes used were:

www.ch.embnet.org/software/TMPRED_form.html,

ConPred_all (http://bioinfo.si.hirosaki-u.ac.jp/~ConPred2/),

http://bp.nuap.nagoya-u.ac.jp/sosui/cgi-bin/adv_sosui.cgi,
www.cbs.dtu.dk/services/TMHMM-2.0/, and
www.sbc.su.se/~miklos/DAS/tmdas.cgi

TMD topology prediction calculations vary between programmes and criteria were established to help decide which programme was the most accurate. As discussed in section 2.1, some nucleotides appear in set motifs in certain parts of a GPCR and the literature states that they appear in certain TMDs. By using this information, the best fit topology programme could be determined. The programme that fitted the TMDs to allow the motifs to appear within or around certain TMDs was used to determine the TMDs for the β1- and β2-ARs.

2.2.17 Phylogenetic analysis

Phylogenetic trees are constructed from the study of systematics in which binary rooted trees are formed to link species of families by their co-ancestral relationships (Holmes, 2003). In this analysis, all the translated β1- and β2-AR sequences available were aligned in a clustal W (1.83) multiple sequence alignment and then input into a software programme for phylogenetic analysis (www.genebee.msu.su). Bootstrap values indicate the inference of the trees and are a similar verification of results as p values are to statistical analyses, in that they are a determination of confidence. For example, it gives information about the stability and robustness of the tree topology (the branching order), and it helps assess whether the sequence data are adequate to validate the topology (Berry & Gascuel, 1996).

2.3 Results

Each procedure was carried out as described in the Methods and Materials section. Initially, to gain experience in molecular techniques, a fragment was cloned and sequenced from the already characterised β2-AR using the liver of a rainbow trout (*Oncorhynchus mykiss*). Once this procedure had been successfully carried out, liver, and sometimes heart, tissues from female fathead minnow (*Pimephales promelas*) were used to characterise the unknown β1- and β2-AR sequences of this species.

2.3.1 RNA extraction

RNA was isolated and run on a 1% agarose gel to give an indication of quality. Good quality intact RNA should appear as two distinct bands (28S and 18S), and the 28S band should be approximately twice as intense as the 18S band, as shown in Figure 2.20. If any DNA contamination is present, it can be seen on a gel under a UV light as this DNA sample remains in the well into which the sample was pipetted, because it is a larger, heavier molecule, and so does not migrate into the gel.

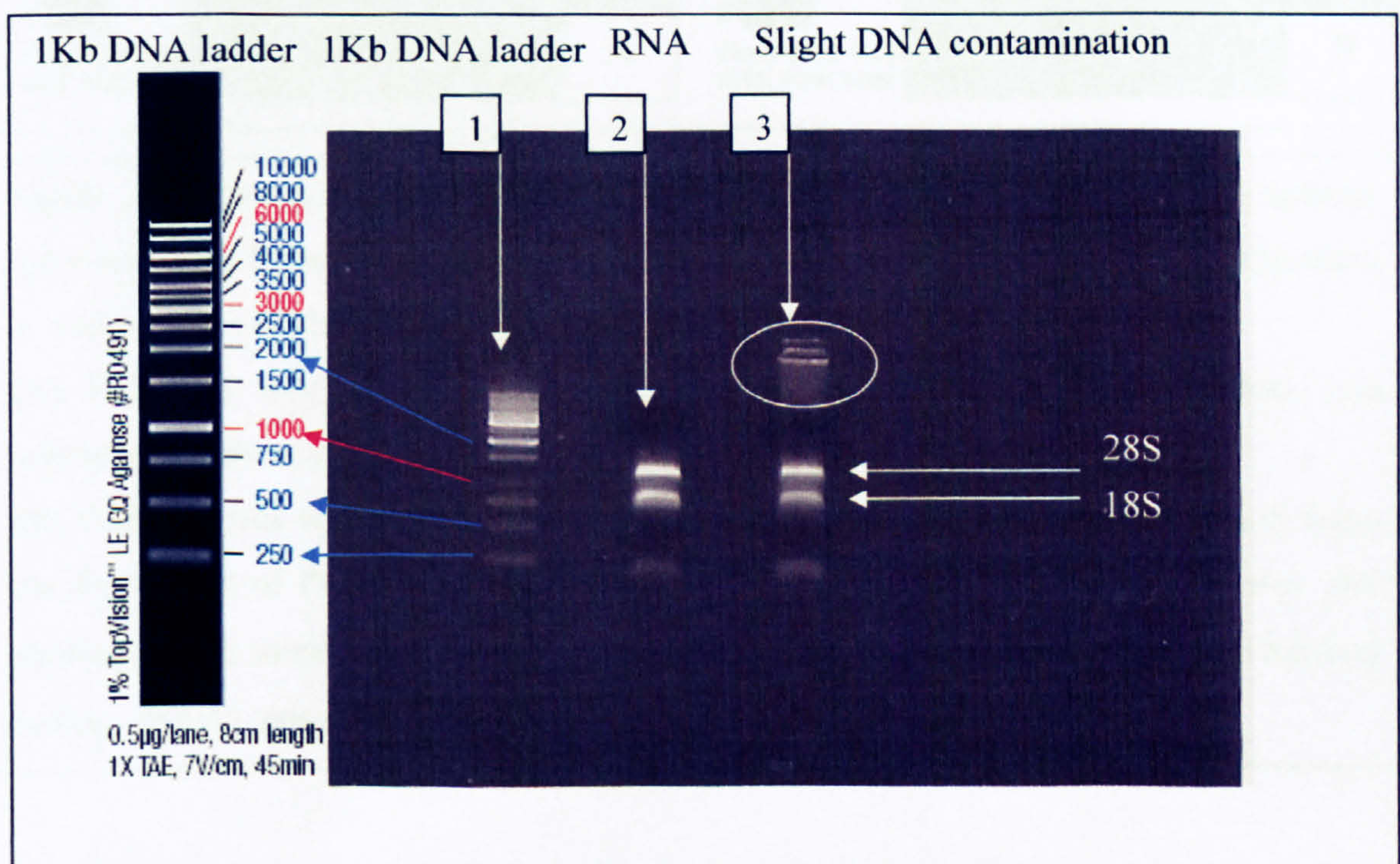


Figure 2.20. A photograph taken under UV light of RNA samples run on a 1 % agarose gel. Lane 1 is a 1Kb DNA marker. Lanes 2 and 3 are RNA samples. Both show good quality intact RNA as the 28S band is approximately twice the intensity of the 18S band. However, the RNA sample in lane 3 shows slight DNA contamination.

The 260/280 ratio of RNA read on a spectrophotometer also gives an indication of quality; a ratio greater than 1.8 shows that the RNA is of a good quality.

2.3.2 Characterisation of β2-AR in trout

As a practise exercise, a fragment of trout β2-AR was isolated from the liver. The sequence had been characterised by Nickerson et al. (2001), and so primers were designed to isolate a fragment that was about 450bp in length. It was discovered that the β2-AR could only be detected in PCR when mRNA (not total RNA) was used, and that nested primers increased this signal (see Figure 2.21).

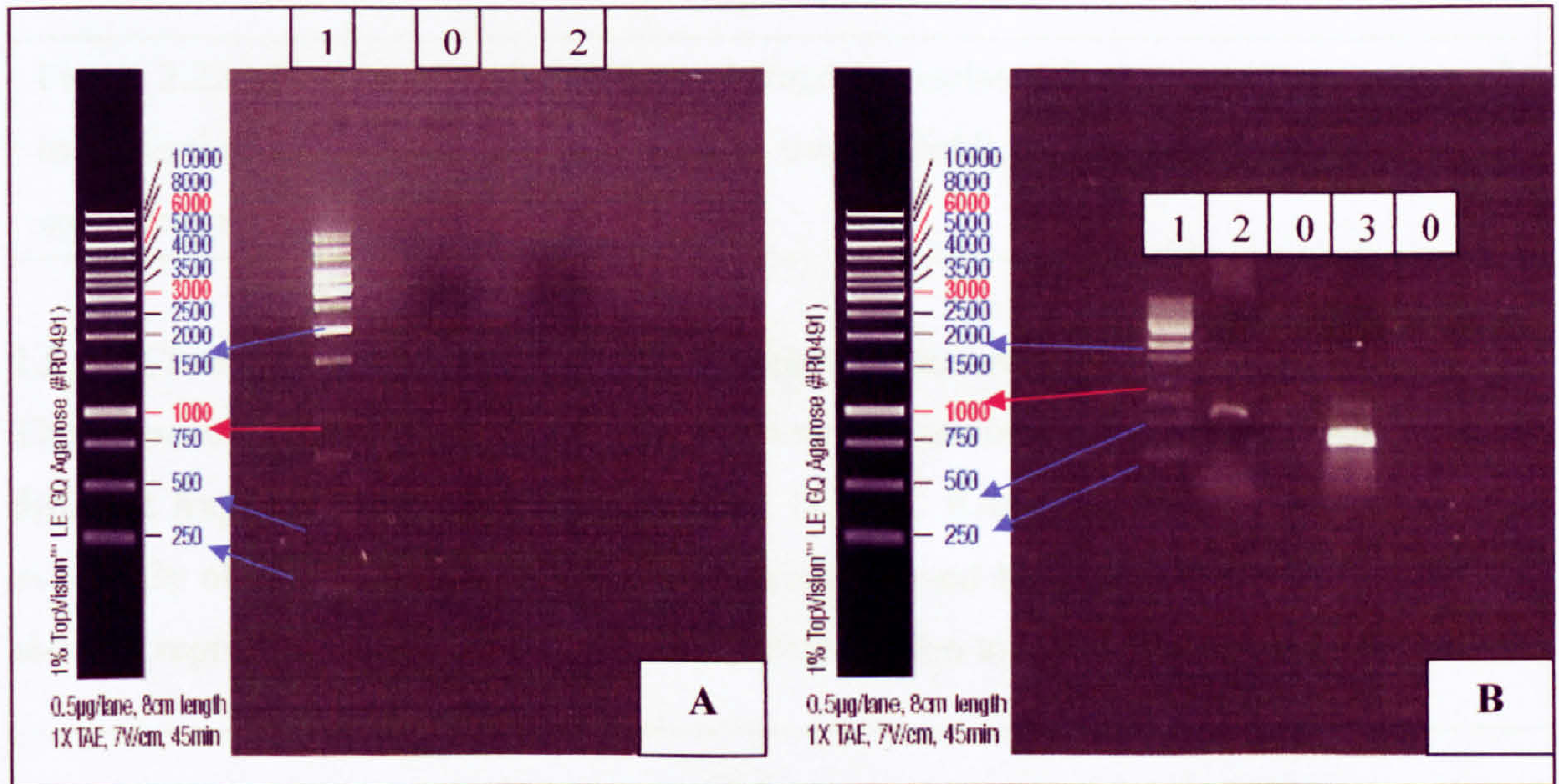


Figure 2.21. Photographs taken under UV light of PCR products run on a 1.2 % agarose gel where trout liver RNA was used in the PCR reaction. 1kb DNA markers are in lanes 1, and negative control samples are in lanes 0.

(A) PCR run was with total RNA in lane 2 and shows the PCR reaction was unsuccessful because no bands are visible.

(B) PCR run was with mRNA. Lane 2 shows the DNA product (as a faint band) from the first round of PCR, lane 3 is from the 2nd round of PCR with nested primers, and shows a much more intense band. The bands in lanes 2 and 3 show that this method (using mRNA) was sensitive enough to amplify a fragment of DNA.

The DNA was extracted from the agarose gel, cloned and sent for sequencing. The sequencing results showed that the fragment was 100% homologous to a part of the published sequence of trout β_2-AR, as shown in Figure 2.22. This demonstrated that the methodologies were sensitive and specific enough to detect β2-ARs in liver tissue.

Fragment	VMCIVFGNVLVITAIVRFQRLQTVTNMFITSLACADL
β2-AR trout	VMCIVFGNVLVITAIVRFQRLQTVTNMFITSLACADL
Fragment	VMGLLVVPFGACYILLNTWHFGSFLCEFWTAADVLCV
β2-AR trout	VMGLLVVPFGACYILLNTWHFGSFLCEFWTAADVLCV
Fragment	TASIETLCVIALDRYLAITSPLRYPSLLTKRKACVVVVTV
β2-AR trout	TASIETLCVIALDRYLAITSPLRYPSLLTKRKACVVVVTV

Figure 2.22. Results from the sequenced fragment isolated from trout liver, compared to a section of previously characterised trout β2-AR. The amino acids in both sequences are identical.

2.3.3 Characterisation of the β1-AR in fathead minnow

The sequence for the β1-AR in the fathead minnow was obtained by piecing together seven different fragments that were obtained from PCR, 3' RACE PCR and 5' RACE PCR to eventually obtain an entire sequence that was confirmed by primer walking. Figure 2.23 shows a representation of these fragments in comparison to a complete receptor sequence.

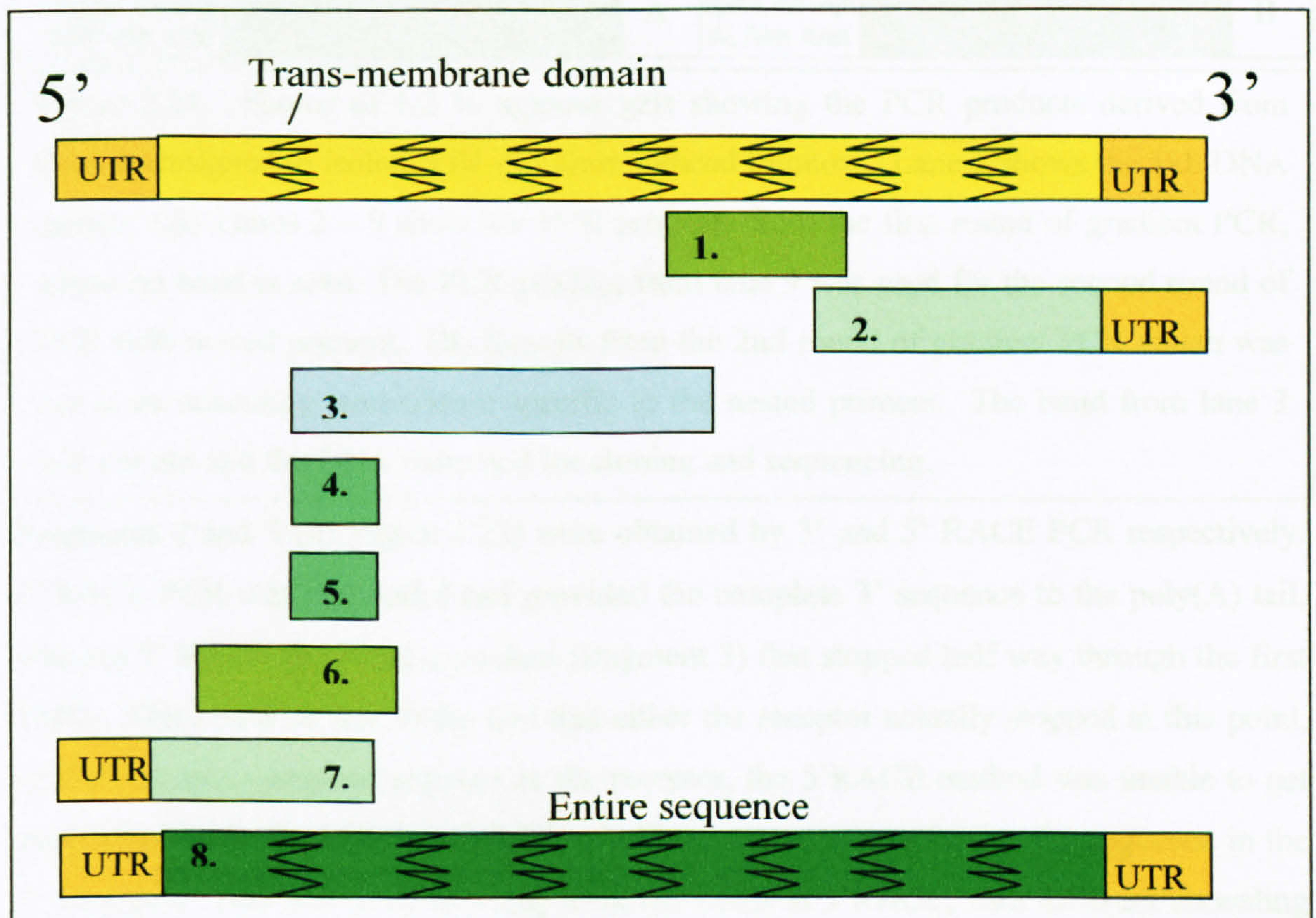


Figure 2.23. A diagrammatic representation of the 7 different fragments that were obtained to complete the entire molecular sequence of the β1-AR of fathead minnow. Fragment 8 was the amplification of the whole receptor sequence. UTR: Un-translated region.

Fragment 1 was obtained from 2 rounds of gradient PCR (see Figure 2.24). Gradient PCR was ideal for this task as it meant a range of temperatures could be used. This was extremely beneficial, as the primers only amplified DNA at certain temperatures and a difference of 4°C could determine whether the result was positive or negative (i.e. whether the desired fragment was or was not amplified).

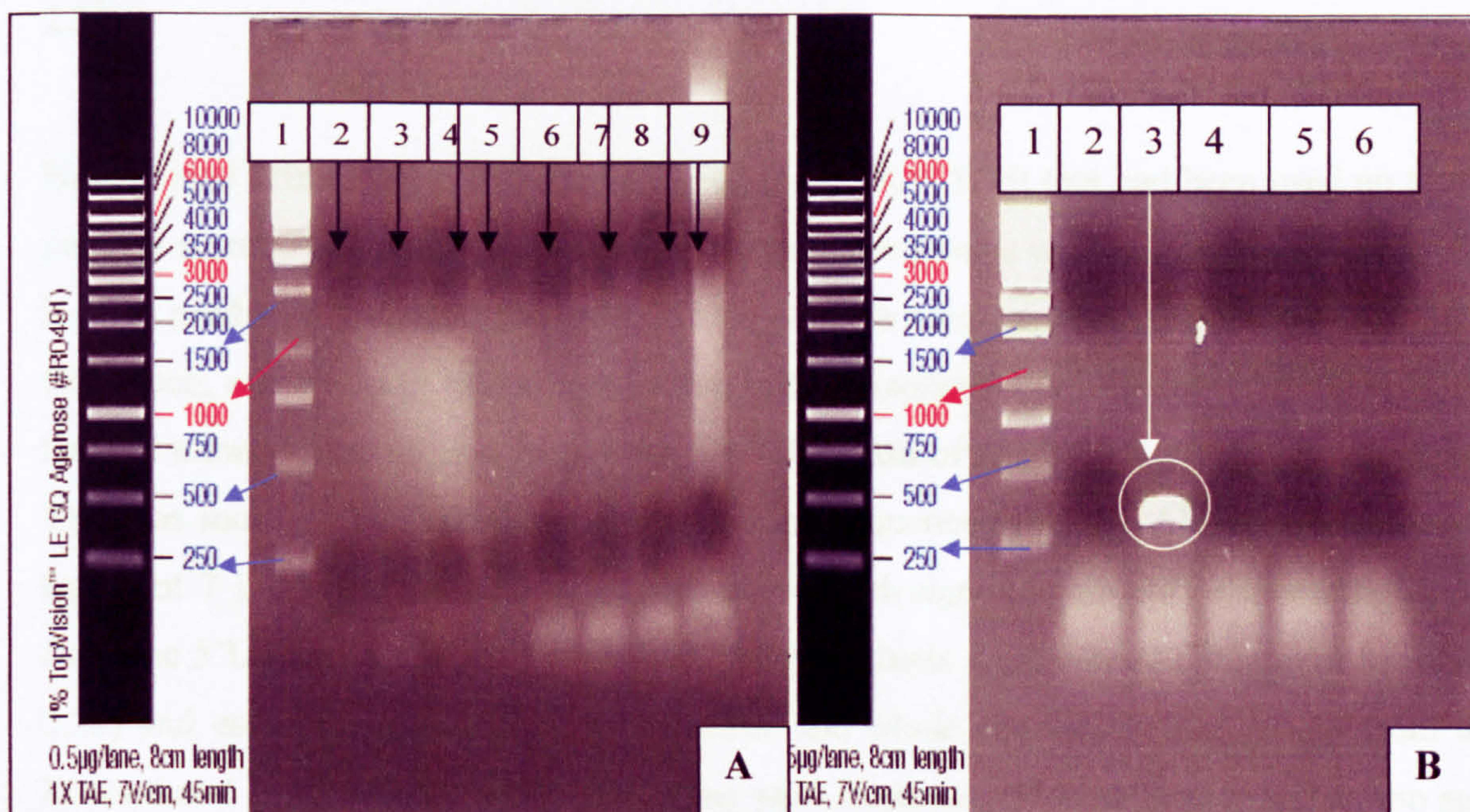


Figure 2.24. Photos of 1.2 % agarose gels showing the PCR products derived from primers designed to isolate a β1-AR from fathead minnow. Lane 1 shows the 1kb DNA marker. (**A**) Lanes 2 – 9 show the PCR products from the first round of gradient PCR, where no band is seen. The PCR product from lane 9 was used for the second round of PCR with nested primers. (**B**) Results from the 2nd round of gradient PCR which was run at an annealing temperature specific to the nested primers. The band from lane 3 was cut out and the DNA extracted for cloning and sequencing.

Fragments 2 and 3 (in Figure 2.23) were obtained by 3' and 5' RACE PCR respectively. 3' RACE PCR was successful and provided the complete 3' sequence to the poly(A) tail, whereas 5' RACE produced a product (fragment 3) that stopped half way through the first TMD. This could be due to the fact that either the receptor actually stopped at this point, or that due to a complex structure in the receptor, the 5'RACE method was unable to get past this point in the sequence. Repeated efforts were made to further the sequence in the 5' direction. This was done by using different GSPs in 5'RACE, with different annealing temperatures and extension times, new kits (to ensure the enzymes were working properly), open-ended PCR, freshly isolated RNA (to ensure there had been no degradation of the RNA sample during storage) and use of heart tissue instead of liver tissue, since β1-ARs (rather than other β-ARs) are predominant in human hearts. It was thought that β1-AR may be expressed at greater levels in the heart compared to the liver. All attempts at

extending the sequence in the 5' direction either gave no result or produced the same truncated product as fragment 3, as shown by fragments 4 and 5. Assuming that the receptor didn't finish at this point, PCR primers were designed to amplify a short fragment that would move us past this particular point. This strategy was successful, and a further short fragment of the β1-AR was obtained, which is represented by fragment 6 in Figure 2.23.

Having read different reviews on the Invitrogen 5' RACE kit that had been used up to this point, it seemed that many people had encountered problems with this particular kit, in that it often produced truncated products. These reviews recommended the 5' RACE kit from Clonetech, and so using this kit, and fresh mRNA isolated from the heart and liver of the fathead minnow, the sequencing of the 5'UTR region of the β1-AR was again attempted. This was successful and produced the remaining sequence of the β1-AR, as represented by fragment 7 in Figure 2.30. Lastly, primers were designed to amplify the whole β1-AR from the 5'UTR to the 3'UTR, of which three products were cloned (Fragment 8, Figure 2.23) and used in primer walking to confirm the whole sequence. The results from the forward and reverse walking for each clone were identical. The nucleotide and amino acid sequences for the β1-AR are set out below in Figure 2.25.

```
5'acgcgggggggctggagacGAGAGCGCGGATGGAAGCGCTCCACACAGGACCTGAAGTTC 60
TGAATGAGCGCGCTTCATTTCTCCACACCATGGGAGACGGTTTACCGTCTGTAAACTACA 120
GCAATGACTCTAAACGCACCCCGGATAACTTATCAGAACAGTGGCTCGTGGGCATGGGAA 180
TCATCATGGGTCTGGTAGTAATTGTCATTGTAGTGGGGAATATACTGGTTATAGTCGCCA 240
TAGCGCGGAATCAGAGGCTCCAGACGCTCACCAATGTTTTCATAGTGTCTCTGGCGTGCG 300
CAGACCTTATCATGGGGTTACTGGTGGTGCCATTTGGCGCAGACTTGGAGGTCAGAGGAT 360
CTTGGATGTATGGATCGTTCTTCTGTGAATTCTGGATATCTCTTGATGTACTTTGCGTCA 420
CGGCGAGCATCGAGACCCTGTGCGTAATTGCAATTGACAGGTACATCGCCATCACCTCTC 480
CATTTCGCTATCAAAGCCTTTTAACGAAAGCACGAGCCAAGGTGGTGGTGTGTGCAGTTT 540
GGGCTATATCAGCTCTTGTGTCATTTCCACCCATCCTAATGCACTGGTCCCGGGACACCG 600
TGGATACATCATGCTATAACGAACCCGAGTGCTGTGACTTCATCACCAACCGTGAATATG 660
CCATCTCATCCTCCGTTATATCGTTTTACATCCCTTTAATAGTCATGATATTCGTCTATG 720
CCAGGGTATACAGAGAAGCCAAACAACAACTGAAAAAAATTAACAAATGTGAGGGAAGAT 780
TCTACAACAATGGTACTAATTGCAAACCTAACCGAAAACGAACCACCAAGATCCTGGCTT 840
TAAAAGAGCAGAAGGCGTTGAAAACGTTGGGAATAATCATGGGAACATTCACTCTCTGCT 900
GGCTGCCGTTCTTCATCGTTAACGTGGTGCGGGTGTTTGGCAAAGAGGTGGTGAAAAAGG 960
AACTCTTCGTATTTTTGAACTGGCTGGGGTACGTCAACTCCGCCTTTAACCCCATCATAT 1020
ACTGTCGGAGTCCCGACTTTAGGAAAGCCTTTAAGAGGCTGTTGTGTTGTCCGAGGCAGG 1080
CGGACCGCAGGTTGCACGTGAGCTCGTGCGATCTGTCGCGCTGCACCGGGGGCTTTGTGA 1140
ACTCAATGGAGCAGAGCATGCTCGGGACCTGGTCGGACTGTAACGGCACGGACAGCCGCG 1200
ACTGCAGTCTAGAGAGGAACGGAAGGGTGTCCCATTCAGAGTCTCAGCTGTAAAAGTACA 1260
TCTGCGAATATCAAGTCAATATTGCACGAGCTGTTTGTTCGATTGGAAATCAGGTTATAG 1320
CCTACTGGTGCGCTAGTGATATGTGAGTGTGATTGTTTCAGTGAACGTTTCAGACAAATT 1380
CGAAAATATTTCCacaaaaaaaagaaaaaaaa    1412  (3' end)
```

A

```
MEALHTGPEVLNERASFLHTMGDGLPSVNYSNDSKRTPDNLSEQWLVGMGIIMGLVVIVI 60

VVGNILVIVAIARNQRLQTLTNVFIVSLACADLIMGLLVVPFGADLEVRGSWMYGSFFCE 120

FWISLDVLCVTASIETLCVIAIDRYIAITSPFRYQSLLTKARAKVVVCAVWAISALVSFP 180

PILMHWSRDTVDTSCYNEPECCDFITNREYAISSSVISFYIPLIVMIFVYARVYREAKQQ 240

LKKINKCEGRFYNNGTNCKPNRKRTTKILALKEQKALKTLGIIMGTFTLCWLPFFIVNVV 300

RVFGKEVVKKELFVFLNWLGYVNSAFNPIIYCRSPDFRKAFKRLLCCPRQADRRLHVSSC 360

DLSRCTGGFVNSMEQSMLGTWSDCNGTDSRDCSLERNGRVSH 402
```

B

Figure 2.25. The nucleotide sequence (A) and amino acid sequence (B) of the β1-AR in the fathead minnow. Orange letters show the UTRs. Orange letters that are not capitals have not been confirmed by primer walking as the primers made to sequence the whole receptor did not include these nucleotides. Nucleotides in blue represent the start (ATG) and stop (TAA) codons. Nucleotides highlighted in green show the 3' UTR motif. The ribosomal binding site is highlighted in yellow

2.3.4 Characterisation of the β2-AR in the fathead minnow

The same approach used to characterise the β1-AR was used to obtain the molecular sequence for the β2-AR in the fathead minnow. Only 5 fragments were needed to gather the whole receptor sequence, as shown in Figure 2.26.

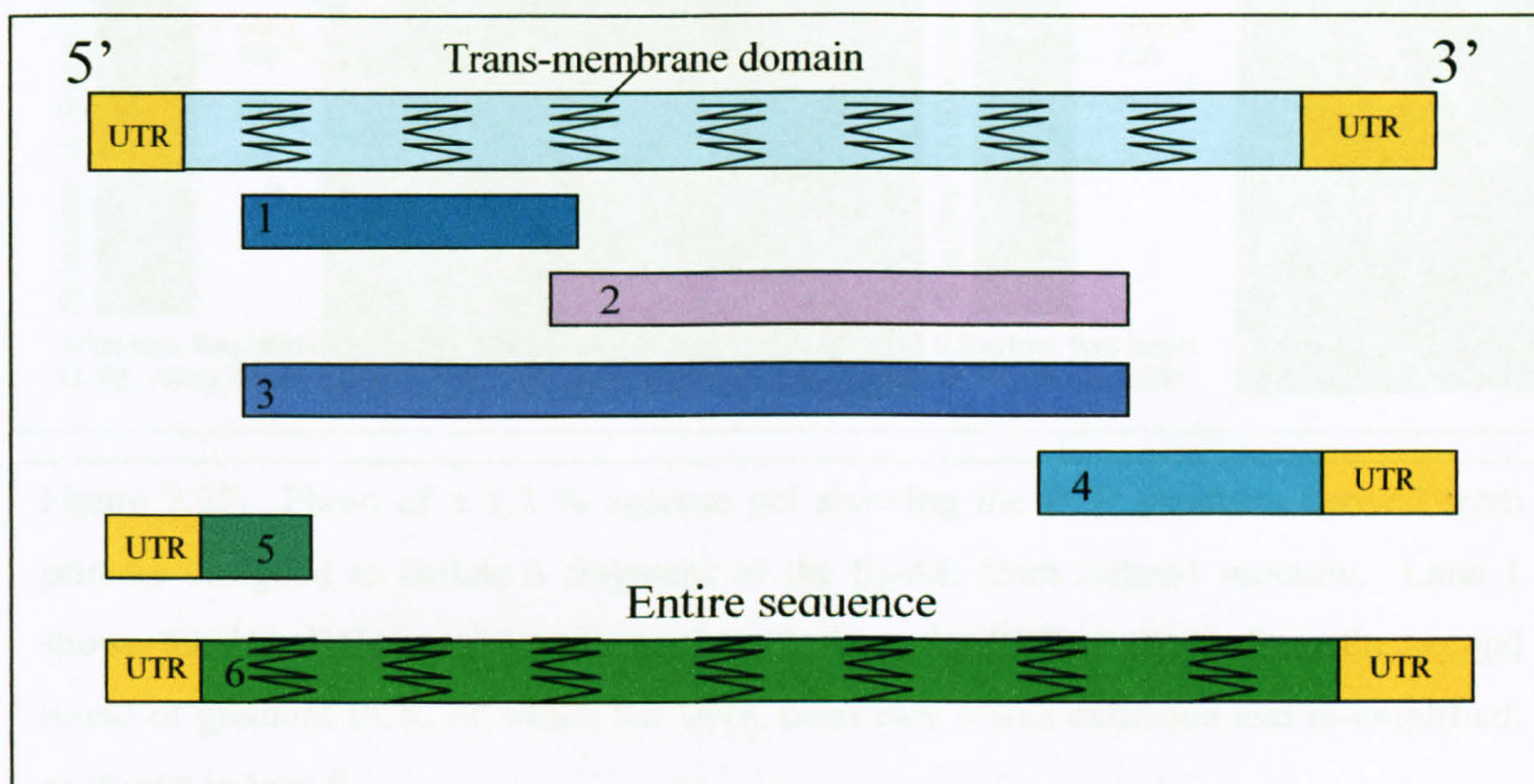


Figure 2.26. A diagrammatic representation of the 5 different fragments that were obtained to complete the entire molecular sequence of the β2-AR. Fragment 6 was the amplification of the whole receptor sequence. UTR: Un-translated region.

Fragment 1 was obtained by gradient PCR and despite using mRNA in the PCR reactions, a strong band was only obtained by extracting the DNA from the second round of PCR and re-amplifying it, as shown in Figure 2.27. This was done by running 0.5 μl of the extracted product in another round of PCR with nested primers.

Fragment 2 was also obtained by PCR, as was fragment 3, which was used to confirm that fragments 1 and 2 were from the same receptor. Fragment 4 was obtained by 3' RACE PCR and fragment 5 from 5'RACE PCR. Fragment 6 was obtained from a PCR run using proof reading taq and primers designed to amplify the whole receptor. Three individual clones were then taken from cloning the PCR product and 'primer walked' to verify the receptor sequence. On primer walking the three clones it was found that 2 variations of the same receptor existed. Figure 2.28 and Figure 2.29 compares the nucleotide and amino acid receptor sequences respectively, for both β2-AR sequences. From location of the initiator sequence and the ribosomal binding site, it is possible that the sequences for definitely one, and possibly two, functional β2-ARs were found.

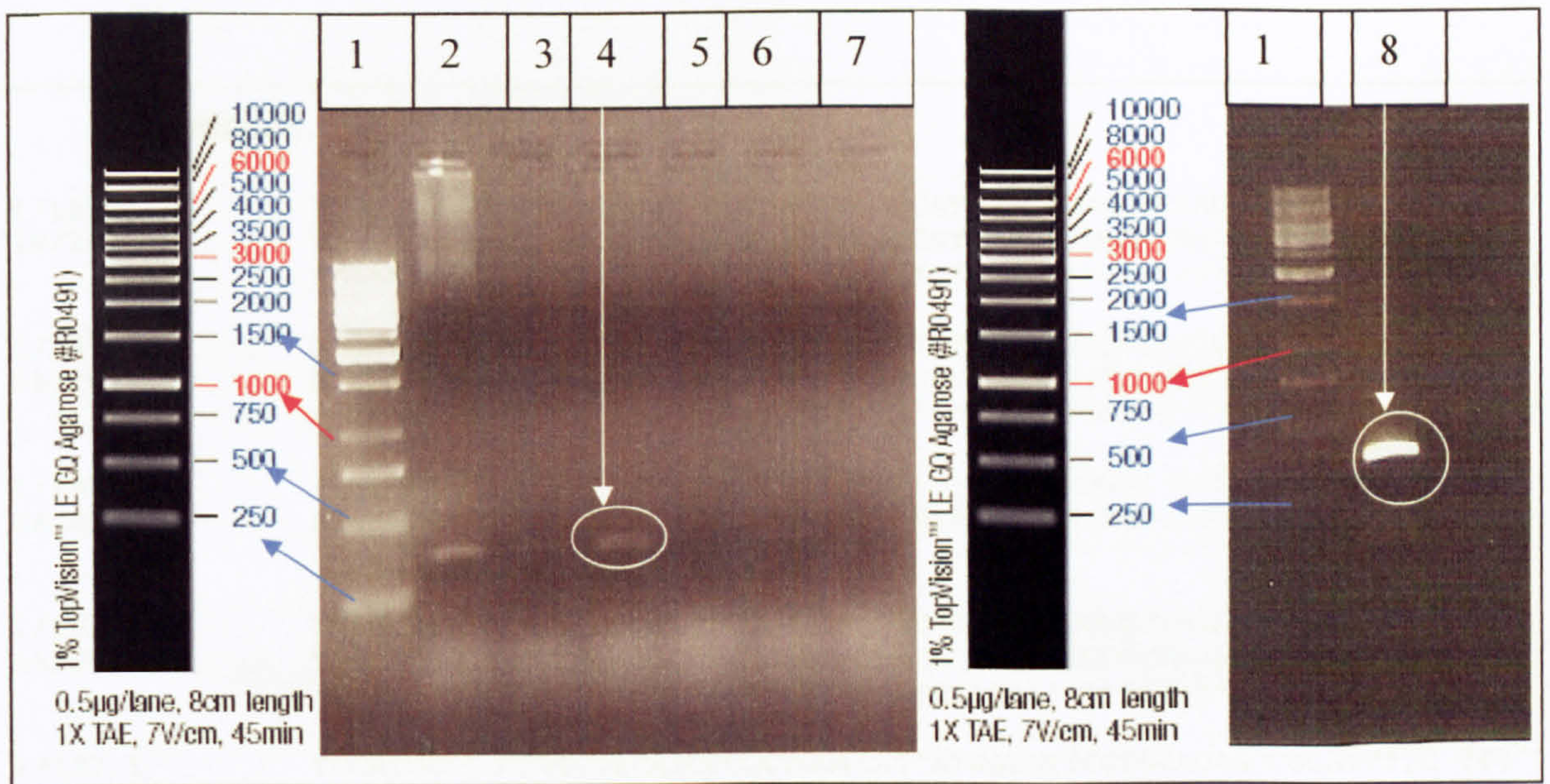


Figure 2.27. Photo of a 1.2 % agarose gel showing the PCR products derived from primers designed to isolate a fragment of the β_2-AR from fathead minnow. Lane 1 shows the 1kb DNA marker. Lanes 2 to 7 show the PCR products from the second round of gradient PCR, of which the DNA from lane 4 was extracted and re-amplified, as shown in lane 8.

```
1/197.1         ---------------------------------------------------CATGACCAG 9
18/207          CGACATTTAGTCTACAGCCGAGAGTGCTGTGCACATGAGAGTAAAAAAAAACATGACCAG 60
                                                                   *********

1/197.1         GTGATCAAGAGTCGAGTGAAAGAACACTATGTGAGACCAGAACACTTCCTAAGAGAAAAG 69
18/207          GTGATCAAGAGTCGAGTGAAAGAACACTATGTGAGACCAGAACACTTCCTAAGAGAAAAG 120
                ************************************************************

1/197.1         GATTATTTTTGGATAGTGGACTCCTAATTTTAGTCAAAGCTCATGGAGCGAGGGGATAGG 129
18/207          GATTATTTTTGGATAGTGGACTCCTAATTTTAGTCAAAGCTCATGGAGGGAGGGGATAGG 180
                ************************************************ ***********

1/197.1         TTGAGCGCGGAGAACACCTCCCTGCACATGAATGTTTCATCTGGGCTAAACGACTCTTCT 189
18/207          TTGAGCGTGGAGAACACCTCCCTGCACATGAATGTTTCATCTGGGCTAAACGACTCTTCT 240
                ******* ****************************************************

1/197.1         CCGGTGTCCGAATATAGCGACGCAGAGGTGGTCTTAATTAGCATCTTAATGGGGCTTCTG 249
18/207          CCGGTGTCCGAATATAGCGACGCAGAGGTGGTCTTAATCAGCATCTTAATGGGGCTTCTG 300
                ************************************** *********************

1/197.1         GTTCTAGGCATCGTCTTTGGCAACGTGCTGGTCATCAGCGCCATTGTACGATTTCAACGC 309
18/207          GTTCTAGGCATCGTCTTTGGCAACGTGCTGGTCATCAGCGCCATTGTACGATTTCAACGC 360
                ************************************************************

1/197.1         TTGCAGACGGGCACCAACTACTTCATCAGCTCCCTTGCCTGCGCCGACTTGGTCATGGGT 369
18/207          TTGCAGACGGGCACCAACTACTTCATCAGCTCCCTTGCCTGCGCCGACTTGGTCATGGGC 420
                ***********************************************************

1/197.1         CTCATGGTGGTGCCGTTCGGCGCGTGCTACATCCTCCTCAACACGTGGCACTTCGGAAAC 429
18/207          CTCATGGTGGTGCCGTTCGGCGCGTGCTACATCCTCCTCAACACGTGGCACTTCGGAAAC 480
                ************************************************************

1/197.1         TTCTTCTGCGAGTTTTGGACGGCTACGGACGTGTTGTGCGTGACCGCTAGCATCGAGACG 489
18/207          TTCTTCTGCGAGTTTTGGACGGCTACGGACGTGTTGTGCGTGACCGCTAGCATCGAGACG 540
                ************************************************************
```

See next page for continuation

Continued…

```
1/197.1         TTGTGCGTGATCGCCCTGGACCGGTACGTCGCCATCATGTGGCCTCTACGCTATCAGTCG 549
18/207          TTGTGCGTGATCGCCCTGGACCGGTACGTCGCCATCATGTGGCCTCTACGCTATCAGTCG 600
                ************************************************************

1/197.1         ATGCTTACGAAGCGGAAGGCGTGCGGGATAGTCCTCGCGGTGTGGGCGGTG--------- 600
18/207          ATGCTTACGAAGCGGAAGGCGTGCGGGATAGTCCTCGCGGTGTGGGCGGTGGCCGCCCTG 660
                ***************************************************

1/197.1         ------------------------------------TCCGACGACCCGGCTGCGCGGAGT 624
18/207          ATCTCTTTCCTGCCCATCCACATGGAGTGGTGGGTGTCCGACGACCCGGATGCGTTGAGT 720
                                                    ************* ****  ****

1/197.1         TGCTTGAAGAACCCGACCTGCTGCGACTTCAACACCAACGCCGCCTACGCCGTGACCTCC 684
18/207          TGCTTGAAGAACCCGACCTGCTGCGACTTCAACACCAACGCCGCCTACGCCGTGACCTCC 780
                ************************************************************

1/197.1         TCCATCGTCTCCTTCTACATCCCGCTGGTCATCATGGTGTTCGTGTACAGCCGGGTTTTC 744
18/207          TCCATCGTCTCCTTCTACATCCCGCTGGTCATCATGGTGTTCGTGTACAGCCGGGTTTTC 840
                ************************************************************

1/197.1         CAGGAGGCCCGTCGGCAGCTTCAGAAAATCGATCGCATTGAGGGACGCATACGCACGCAG 804
18/207          CAGGAGGCCCGTCGGCAGCTTCAGAAAATCGATCGCATTGAGGGACGCATACGCACGCAG 900
                ************************************************************

1/197.1         AGCTTCAGCACCCAGGAGGGGAAC------------CGGAGGACCAAGTTCGGCATGAAG 852
18/207          AGCTTCAGCACCCAGGACGGGAACGAGACGAAGAACCGGAGGACCAAGTTCGGCATGAAG 960
                ***************** ******            ************************

1/197.1         GACCACAAAGCTCTGAAGACGTTGGGGATCATCATGGGAACATTCACCCTCTGCTGGCTG 912
18/207          GACCACAAAGCTCTGAAGACCTTGGGGATCATCATGGGAACATTCACCCTCTGCTGGCTG 1020
                 ******************* ****************************************

1/197.1         CCGTTTTTCGTGCTTAACGTGGCGGCGGCCATCTGGAAGATGGAGAACATCATGTTGCCG 972
18/207          CCGTTTTTCGTGCTTAACGTGGCGGCGGCCATCTGGAAGATGGAGAACATCATGTTGCCG 1080
                ************************************************************

1/197.1         TTCAGGATCTTGAACTGGATCGGATACGCCAACTCCGCTTTCAATCCCCTCATCTACTGC 1032
18/207          TTCAGGATCTTGAACTGGATCGGATACGCCAACTCCGCTTTCAATCCCCTCATCTACTGC 1140
                ************************************************************

1/197.1         AGGAGTCCTGAGTTCAGGTGCGCCTTTCAGGAGATCCTGTGCCGGAGAACTTCTCACCTG 1092
18/207          AGGAGTCCTGAGTTCAGGTGCGCCTTTCAGGAGATCCTGTGCCGGAGAACTTCTCACCTG 1200
                ************************************************************

1/197.1         CCCTCCACGAGGAATAACAAGGGATTTATATACAGCGGGCACAGCTGGAAAGTGCACACG 1152
18/207          CCCTCCACGAGGAATAACAAGGGATTTATATACAGCGGGCACAGCTGGAAAGTGCACACG 1260
                ************************************************************

1/197.1         AAGACCGCCAGACAACGAGAGCCCTCGCCAGCTTGCGAAACCGAAATGGGTGCGGGCAAC 1212
18/207          AAGACCGCCAGACAACGAGAGCCCTCGCCAGCTTGCGAAACCGAAATGGGTGCGGGCAAC 1320
                ************************************************************

1/197.1         GGGAATTGTAATAAAGCTGTGACGTCCGATTTTTAGCCAATTTTGATCGTTTTGAATATT 1272
18/207          GGGAATTGTAATAAAGCTGTGACGTCCGATTTTTAGCCAATTTTGATCGTTTTGAATATT 1380
                ************************************************************

1/197.1         CCCTGGAGCACTTGAGAGATTTCTCG---------------------------------- 1298
18/207          CCCTGGAGCACTTGAGAGATTTCTGCAACCGCTTTTGTCTGTGACAAAACATGGTTTTTA 1440
                ************************

1/197.1         ------

18/207          GATGTCTTTGAAACCATGAAGGTTTTGCCCAAAAAAAAA 1445
```

Figure 2.28. Comparison of the probable β2-AR nucleotide sequences of the fathead minnow. One sequence has been called 1/197.1 and the other 18/207. Indicates weak groups of alignment. **Blue** nucleotides indicate start and stop codons. The ribosomal binding site is highlighted in yellow. The Initiator sequence (or TATA box) is highlighted in **red** or **pink.** Large capitals at the 3' UTL represent nucleotides not tested by proof reading taq. The 3' UTR motif is highlighted in green.

The initiator sequence (or TATA box) has the sequence - 5' **Y-Y-A^{+1}-N-T/A-Y-Y-Y** 3' (Y = C or T). It is usually found 25-30bp from the start codon. However, in both instances where the potential initiator sequence is expressed in the β2-ARs, this is not the case. **CTAATTTT** is found 11 bp from the start codon, and **TTATTTTT** is 32bp from the start codon.

```
1/197.1       MERGDRLSAENTSLHMNVSSGLNDSSPVSEYSDAEVVLISILMGLLVLGIVFGNVLVISA 60
207           MEGGDRLSVENTSLHMNVSSGLNDSSPVSEYSDAEVVLISILMGLLVLGIVFGNVLVISA 60
              ** *****.***************************************************

1/197.1       IVRFQRLQTGTNYFISSLACADLVMGLMVVPFGACYILLNTWHFGNFFCEFWTATDVLCV 120
207           IVRFQRLQTGTNYFISSLACADLVMGLMVVPFGACYILLNTWHFGNFFCEFWTATDVLCV 120
              ************************************************************

1/197.1       TASIETLCVIALDRYVAIMWPLRYQSMLTKRKACGIVLAVWAVS---------------D 165
207           TASIETLCVIALDRYVAIMWPLRYQSMLTKRKACGIVLAVWAVAALISFLPIHMEWWVSD 180
              *******************************************:               *

1/197.1       DPAARSCLKNPTCCDFNTNAAYAVTSSIVSFYIPLVIMVFVYSRVFQEARRQLQKIDRIE 225
207           DPDALSCLKNPTCCDFNTNAAYAVTSSIVSFYIPLVIMVFVYSRVFQEARRQLQKIDRIE 240
              ** * *******************************************************

1/197.1       GRIRTQSFSTQEGN----RRTKFGMKDHKALKTLGIIMGTFTLCWLPFFVLNVAAAIWKM 281
207           GRIRTQSFSTQDGNETKNRRTKFGMKDHKALKTLGIIMGTFTLCWLPFFVLNVAAAIWKM 300
              ***********:**    ******************************************

1/197.1       ENIMLPFRILNWIGYANSAFNPLIYCRSPEFRCAFQEILCRRTSHLPSTRNNKGFIYSGH 341
207           ENIMLPFRILNWIGYANSAFNPLIYCRSPEFRCAFQEILCRRTSHLPSTRNNKGFIYSGH 360
              ************************************************************

1/197.1       SWKVHTKTARQREPSPACETEMGAGNGNCNKAVTSDF 378
207           SWKVHTKTARQREPSPACETEMGAGNGNCNKAVTSDF 397
              *************************************
```

Figure 2.29. Comparison of the amino acid sequences for the two β2-AR sequences found in the fathead minnow. (*) Indicates identical amino acid sequence in that column. (:)Means conserved substitutions have been made. (.) Indicates that semi-conserved substitutions are observed

2.3.5 Hydropathy analysis

To find out where the TMDs were located in each β-AR sequence for the fathead minnow, hydropathy analysis was undertaken. Using a search engine on the internet, many different hydropathy programmes were found. Unfortunately, none of them gave the same results. In order to find the 'best fit', certain criteria were drawn-up based on information from other GPCRs and their motifs; these are summarised in Table 2.7. In addition to this, hydrophobic amino acids were identified and used to weight the results obtained from the different software programmes. Because TMDs are often areas of high conservation, other fish that had the same receptor sequences were aligned against the fathead sequence for each particular β–AR and used to compare amino acid sequences in each area. Figures 2.30 and 2.31, and Tables 2.8 and 2.9, show the results of this analysis.

Motif	Amino acid	Reference
Asparagine-linked glycosylation site in the NH_2 terminal region (before TMD1).	**N**	Emorine et al., 1989
Cysteine residues for disulfide bond formation	**C, C**	Cao et al., 1998
Aspartate residue in 2nd TMD, found in most serpentine receptors regardless of their natural ligand.	**D**	Devic et al., 1997
Motif at border between TMD III and IL2. This is characteristic of the α rhodopsin group. However, it is also quoted as occurring at the end of the 3rd TMD of other GPCRs.	**DRY**	Fredriksson et al., 2003, Devic et al., 1997
Ligand binding residues, found in TMD III (D), TMD V (S and S), and TMD VI (F).	**D, S, S, F**	Cao et al., 1998, Freddolino et al.,2004
Tryptophan residue found in the middle of 4th and 6th TMDs. This is found in most serpentine receptors, regardless of ligand.	**W**	Devic et al., 1997
Serine and Threonine residues in 3rd cytoplasmic loop (between TMD V and TMD VI) and COOH terminal region (after TMD VII).	**S** or **T**	Emorine et al., 1989
Found in TMDVII and is characteristic of the α rhodopsin group.	**NSxxNPxxY**	Fredriksson et al., 2003
Found in β1-ARs at the start of the COOH loop after TMD VII.	**RSPDFRKA** **FKR**	Mason et al., 1999

Table 2.7. Summary of the different motifs found commonly in GPCRs.

```
Fathead          ------------------------------------------MEALHTGPEV-LNERASF 17
Fathead          ------------------------------------------MEALHTGPEV-LNERASF 17
Fathead          ------------------------------------------MEALHTGPEV-LNERASF 17
Fathead          ------------------------------------------MEALHTGPEV-LNERASF 17
Fathead          ------------------------------------------MEALHTGPEV-LNERASF 17
Fathead          ------------------------------------------MEALHTGPEV-LNERASF 17

Fathead          LHTMGDGLPS--VNYSNDSKRTPDNL--SEQWLVGMGIIMGLVVIVIVVGNILVIVAIAR 73
Fathead          LHTMGDGLPS--VNYSNDSKRTPDNL--SEQWLVGMGIIMGLVVIVIVVGNILVIVAIAR 73
Fathead          LHTMGDGLPS--VNYSNDSKRTPDNL--SEQWLVGMGIIMGLVVIVIVVGNILVIVAIAR 73
Fathead          LHTMGDGLPS--VNYSNDSKRTPDNL--SEQWLVGMGIIMGLVVIVIVVGNILVIVAIAR 73
Fathead          LHTMGDGLPS--VNYSNDSKRTPDNL--SEQWLVGMGIIMGLVVIVIVVGNILVIVAIAR 73
Fathead          LHTMGDGLPS--VNYSNDSKRTPDNL--SEQWLVGMGIIMGLVVIVIVVGNILVIVAIAR 73

Fathead          NQRLQTLTNVFIVSLACADLIMGLLVVPFGADLEVRGSWMYGSFFCEFWISLDVLCVTAS 133
Fathead          NQRLQTLTNVFIVSLACADLIMGLLVVPFGADLEVRGSWMYGSFFCEFWISLDVLCVTAS 133
Fathead          NQRLQTLTNVFIVSLACADLIMGLLVVPFGADLEVRGSWMYGSFFCEFWISLDVLCVTAS 133
Fathead          NQRLQTLTNVFIVSLACADLIMGLLVVPFGADLEVRGSWMYGSFFCEFWISLDVLCVTAS 133
Fathead          NQRLQTLTNVFIVSLACADLIMGLLVVPFGADLEVRGSWMYGSFFCEFWISLDVLCVTAS 133
Fathead          NQRLQTLTNVFIVSLACADLIMGLLVVPFGADLEVRGSWMYGSFFCEFWISLDVLCVTAS 133

Fathead          IETLCVIAIDRYIAITSPFRYQSLLTKARAKVVVCAVWAISALVSFPPILMHWSRDTVDT 193
Fathead          IETLCVIAIDRYIAITSPFRYQSLLTKARAKVVVCAVWAISALVSFPPILMHWSRDTVDT 193
Fathead          IETLCVIAIDRYIAITSPFRYQSLLTKARAKVVVCAVWAISALVSFPPILMHWSRDTVDT 193
Fathead          IETLCVIAIDRYIAITSPFRYQSLLTKARAKVVVCAVWAISALVSFPPILMHWSRDTVDT 193
Fathead          IETLCVIAIDRYIAITSPFRYQSLLTKARAKVVVCAVWAISALVSFPPILMHWSRDTVDT 193
Fathead          IETLCVIAIDRYIAITSPFRYQSLLTKARAKVVVCAVWAISALVSFPPILMHWSRDTVDT 193

Fathead          SCYNEPECCDFITNREYAISSSVISFYIPLIVMIFVYARVYREAKQQLKKINKCEGRFYN 253
Fathead          SCYNEPECCDFITNREYAISSSVISFYIPLIVMIFVYARVYREAKQQLKKINKCEGRFYN 253
Fathead          SCYNEPECCDFITNREYAISSSVISFYIPLIVMIFVYARVYREAKQQLKKINKCEGRFYN 253
Fathead          SCYNEPECCDFITNREYAISSSVISFYIPLIVMIFVYARVYREAKQQLKKINKCEGRFYN 253
Fathead          SCYNEPECCDFITNREYAISSSVISFYIPLIVMIFVYARVYREAKQQLKKINKCEGRFYN 253
Fathead          SCYNEPECCDFITNREYAISSSVISFYIPLIVMIFVYARVYREAKQQLKKINKCEGRFYN 253

Fathead          N-GTNCKPNRKRTTKILALKEQKALKTLGIIMGTFTLCWLPFFIVNVVRVFGKEVVKKEL 312
Fathead          N-GTNCKPNRKRTTKILALKEQKALKTLGIIMGTFTLCWLPFFIVNVVRVFGKEVVKKEL 312
Fathead          N-GTNCKPNRKRTTKILALKEQKALKTLGIIMGTFTLCWLPFFIVNVVRVFGKEVVKKEL 312
Fathead          N-GTNCKPNRKRTTKILALKEQKALKTLGIIMGTFTLCWLPFFIVNVVRVFGKEVVKKEL 312
Fathead          N-GTNCKPNRKRTTKILALKEQKALKTLGIIMGTFTLCWLPFFIVNVVRVFGKEVVKKEL 312
Fathead          N-GTNCKPNRKRTTKILALKEQKALKTLGIIMGTFTLCWLPFFIVNVVRVFGKEVVKKEL 312

Fathead          FVFLNWLGYVNSAFNPIIYCRSPDFRKAFKRLLCCPRQADRRLHVSSCDLSRCTGGFVNS 372
Fathead          FVFLNWLGYVNSAFNPIIYCRSPDFRKAFKRLLCCPRQADRRLHVSSCDLSRCTGGFVNS 372
Fathead          FVFLNWLGYVNSAFNPIIYCRSPDFRKAFKRLLCCPRQADRRLHVSSCDLSRCTGGFVNS 372
Fathead          FVFLNWLGYVNSAFNPIIYCRSPDFRKAFKRLLCCPRQADRRLHVSSCDLSRCTGGFVNS 372
Fathead          FVFLNWLGYVNSAFNPIIYCRSPDFRKAFKRLLCCPRQADRRLHVSSCDLSRCTGGFVNS 372
Fathead          FVFLNWLGYVNSAFNPIIYCRSPDFRKAFKRLLCCPRQADRRLHVSSCDLSRCTGGFVNS 372

Fathead          MEQSMLGTWSDCNGTDSRDCSLERNGRVSHSESQL 407
Fathead          MEQSMLGTWSDCNGTDSRDCSLERNGRVSHSESQL 407
Fathead          MEQSMLGTWSDCNGTDSRDCSLERNGRVSHSESQL 407
Fathead          MEQSMLGTWSDCNGTDSRDCSLERNGRVSHSESQL 407
Fathead          MEQSMLGTWSDCNGTDSRDCSLERNGRVSHSESQL 407
Fathead          MEQSMLGTWSDCNGTDSRDCSLERNGRVSHSESQL 407
```

Figure 2.30. Results of the hydropathy analysis of the fathead minnow β1-AR sequence. Different results were obtained from hydropathy analysis using different software packages, with the predicted topology highlighted in the following colours; TMPRED, ConPred_all, nuap.nagoya-u.ac, TMHMM-2.0/, miklos/DAS In the final sequence of each block, the red highlighted letters show hydrophobic amino acids. Blue highlighted areas show the presence of GPCR motifs, as set out in Table 2.37

Table 2.8 identifies the number of TMDs predicted by each programme and summarizes whether the different motifs associated with GPCRs are predicted to be present.

Motif	TMPRED	ConPred_all	bp.nuap.nagoya-u.ac.	TMHMM-2.0	miklos/DAS / tmdas.cgi
Number of TMDs	7	7	6	7	7
N	Yes	Yes	Yes	Yes	Yes
C, C	Yes	Yes	Yes	Yes	Yes
D	Yes	Yes	Yes	Yes	Yes
DRY	Yes	Yes	Yes	Yes	No
W in middle of 4th and 6th TMDs	TMD IV: YES TMD VI: YES	TMD IV: YES TMD VI: YES	TMD IV: YES TMD VI: YES	TMD IV: Yes TMD VI: YES	TMD IV: Yes TMD VI: YES
S or T	Yes	Yes	No	Yes	Yes
Ligand binding residues in TMD III, V, VI	Yes	Yes	No	Yes	No
NsxxNPxxY	Yes	Yes	No	Yes	No
RSPDFRKAFKR	Yes	Yes	Yes	No	Yes

Table 2.8. A summary showing how the different hydropathy programmes predict TMDs and the motifs associated with GPCRs for the fathead minnow β1-AR.

As Table 2.8 shows, TMPRED and **ConPred_all** show the highest amount of motif conformity out of all the programmes used.

Table 2.9 shows the percentage of hydrophobic residues in each TMD. This was calculated by the number of nucleotides that were hydrophobic within each predicted TMD, divided by the total number of nucleotides within that particular TMD. As shown, TMPRED scores the highest percentage of hydrophobic residues within the predicted TMDs. Therefore, TMPRED was used to designate the TMDs for the β1-AR, as it is one of the programmes to satisfy the motif criteria found in GPCRs and it is the only programme that predicts the highest percentage of hydrophobic residues, showing 4 out of 7 TMDs to contain the highest percentage of hydrophobic residues when compared to predictions made by the other programmes.

	TMD	TMPRED	ConPred_all	bp.nuap.nagoya-u.ac.	TMHMM-2.0	miklos/DAS/
Percentage of **hydrophobic residues** found in each TMD:	I	90	76	74	74	79
	II	76	71	74	74	73
	III	62	74	73	73	60
	IV	68	67	65	65	80
	V	95	62	91	91	71
	VI	68	67	48	70	93
	VII	77	77	0	77	74

Table 2.9. A comparison of the percentage of hydrophobic residues in each TMD as predicted by each programme. Green highlights show the highest score for each TMD.

The same type of analysis was carried out for both β2-AR sequences. Again, TMPRED was the most successful at predicting the TMDs with respect to the criteria set (data not shown). The hydropathy results (based on using TMPRED) predicting the topology of the TMDs for all the fathead minnow β-ARs sequenced are shown in Figure 2.31.

Figure 2.31. The amino acid sequences of the β1-AR and both versions of the β2-ARs of the fathead minnow presented as a clustal alignment. The **red letters** denote the designation of the TMDs as predicted using the hydropathy programme TMPRED. (*) Indicates identical amino acid sequence in that column. (:)Means conserved substitutions have been made. (.) Indicates that semi-conserved substitutions are observed.

	Fathead minnow B1-AR (%)								Fathead minnow B2-AR Version 207 (%)							
	Whole sequence	TMDs							Whole sequence	TMDs						
		I	II	III	IV	V	VI	VII		I	II	III	IV	V	VI	VII
Fathead minnow B2-AR version 197	46	62	52	84	33	65	86	72	98	100	100	100	92	100	100	100
Fathead minnow B2-AR version 207	45	65	82	84	60	61	88	76								

Table 2.10 Percentage identity for the pairwise alignment for the whole sequence, and each TMD, for the fathead minnow β1- and both versions of the β2-ARs.

As shown by Figure 2.31, there is quite a lot of conservation between the different β-ARs, especially within the TMDs. Table 2.10 reports the results of the analyses the percentage identity (pairwise alignment) between different fathead minnow β-ARs, with respect to the whole sequences and their TMDs. TMD pairwise alignment was worked out by counting the number of identical aligned amino acids in the TMD in the overlapping parts of the TMDs, divided by the number of nucleotides in the TMDs that overlap, rounded to a whole percentage.

These results show that the homology between each receptor for each TMDs on the whole is very high (i.e. >60 %), with the only exception being in TMD IV between the two versions of the β2-AR.

2.3.6 Comparison of fathead minnow β1- and β2-ARs and their similarity to β-ARs of other fish species and humans

β1- and β2-ARs have been characterised in other fish species, and Table 2.11 and Table 2.12 show the percentage identities between different β1-ARs and β2-ARs, respectively, at amino acid level.

Other species	Fathead minnow β1-AR
Zebrafish β1-AR	91 %
Tetraodon β1-AR	76 %
Takifugu β1-AR	73 %
Stickleback β1-AR	75 %
Human β1-AR	57 %

Table 2.11. Percent homology between β1-AR in the fathead minnow and the β1-AR of other species of fish and the human β1-AR.

Other species	Fathead minnow β2-AR (version 197)	Fathead minnow β2-AR (version 207)
Zebrafish β2-AR	87 %	88 %
Trout β2-AR	62 %	63 %
Tetraodon β2-AR	57 %	62 %
Takifugu β2-AR	57 %	58 %
Human β2-AR	53 %	52 %

Table 2.12. Percent homology between the β2-AR in the fathead minnow and the β2-AR of other species of fish and the human β2-AR.

For the β1-AR, other fish species show the highest amount of homology to the β1-AR in the fathead minnow. Human β1-AR shows the least homology of the species tested, although 57% is still a high degree of conservation.

There is a greater degree of homology between other fish β1-ARs and fathead minnows β1-ARs, than there is between the fathead minnow β2-AR and other fish β2-ARs (as shown in Tables 2.11 and 2.12). In the fathead minnow, the β2-AR 207 version shows a slightly greater degree of homology to the β2-AR of the other species than the β2-AR version 197. Both fathead minnow β1- and β2-ARs show the highest degree of homology to their counterparts in the zebrafish. They have significantly less homology to the β-ARs of other fish species (e.g. trout and pufferfish).

As can be seen from Figures 2.33 and 2.34, when sequences for fish and human β1- and β2-ARs are aligned respectively, the greatest amount of conservation is found within the TMDs.

```
Fathead      -------------------------------------------MEALHTGPEVLNERASF 17
Zebrafish    ------------------------------------------------------------
Gammarus     ------------------------------------------------------------
Tetraodon    ------------------------------------------------------------
Takifugu     MSPPALVSWPTAQLGQAEGESASPAGAGARRTSSWSLRDSLPKAALHCLDELDLATHTAQ 60
Human        -----------------------------------------MGAGVLVLGASEPGNLSSA 19

Fathead      LHTMGDGLPS--VNYSNDS--KRTPDNL-------SEQWLVGMGIIMGLVVIVIVVGNIL 66
Zebrafish    ---MGDGLPS--VNYSNDS--KRATVDLN-----VSEQWLVGMGILMGLIVFVIVVGNIL 48
Gammarus     ---MGDGGAHSALSLPNAS--GTEAPDAPDSATAASERWMAGMSVLMGLIVVSIVLGNIL 55
Tetraodon    ---MGDGDLSSAPNSHNGS--ASDASNA-----GASEHWVACLSLVMGSIVLFIVFGNIL 50
Takifugu     QPVMGDGDPSSPVNLQNSS--ASDAPDS-----AASEHWLACMSLVMGLIVLFIVFGNIL 113
Human        AP-LPDGAATAARLLVPASPPASLLPPASESPEPLSQQWTAGMGLLMALIVLLIVAGNVL 78
                : **           *                *::* . :.::*. :*. ** **:*

Fathead      VIVAIARNQRLQTLTNVFIVSLACADLIMGLLVVPFGADLEVRGSWMYGSFFCEFWISLD 126
Zebrafish    VIVAIARNQRLQTLTNVFIVSLACADLIMGLLVVPFGAALEVRGAWMYGSFFCEFWISLD 108
Gammarus     VIVAIAKTQRLQTLTNVFIVSLAGADLIMGLLVVPFGAALEVRGSWLYGSFFCEFWISVD 115
Tetraodon    VIVAIARTQRLQTLTNVFIVSLASADLIMGLLVVPFGAALEVRGSWQYGSFFCEFWISVD 110
Takifugu     VIVAIAKTQRLQTLTNVFIVSLASADLIMGLLVVPFGAALEVRGSWQYGSFFCEFWISVD 173
Human        VIVAIAKTPRLQTLTNLFIMSLASADLVMGLLVVPFGATIVVWGRWEYGSFFCELWTSVD 138
             ******:. *******:**:*** ***:********** : * * * *******:* *:*

Fathead      VLCVTASIETLCVIAIDRYIAITSPFRYQSLLTKARAKVVVCAVWAISALVSFPPILMHW 186
Zebrafish    VLCVTASIETLCVIAIDRYIAIISPFRYQSLLTKARAKVVVCAVWAISALVSFPPILMHW 168
Gammarus     VLCVTASIETLCVIAIDRYVAITSPFRYQSLLTKARARTVVCVVWAISALVSFLPILMHW 175
Tetraodon    VLCVTASIETLCVIAIDRYVAITSPFRYQSLLTKARARAMVCAVWAISALVSFLPILMHW 170
Takifugu     VLCVTASIETLCVIAIDRYVAITSPFRYQSLLTKARAKAMVCAVWAISALVSFLPILMHW 233
Human        VLCVTASIETLCVIALDRYLAITSPFRYQSLLTRARARGLVCTVWAISALVSFLPILMHW 198
             ***************:***:** **********:***: :**.********** ******

Fathead      SRDTVDTS--CYNEPECCDFITNREYAISSSVISFYIPLIVMIFVYARVYREAKQQLKKI 244
Zebrafish    SQDSEETS--CYENPECCDFVTNRAYAISSSIISFYIPLIVMIFVYARVYREAKQQLNKI 226
Gammarus     SRDSVDTA--CYDDPECCDFVTNRAYAISSSIISFYIPLLVMIFVYARVYREAKQQLRKI 233
Tetraodon    SRDSVHTS--CYQNPECCDFITNKAYAISSSIISFYIPLLVMIFVYARVYREAKMQLRKI 228
Takifugu     SRDSVHTA--CYEDPKCCDFITNKAYAISSSIISFYIPLVVMIFVYARVYREAKLQLRKI 291
Human        WRAESDEARRCYNDPKCCDFVTNRAYAIASSVVSFYVPLCIMAFVYLRVFREAQKQVKKI 258
              :   . :  **::*:****:**: ***:**::***:** :* *** **:***: *:.**

Fathead      NKCEGRFYNN-GTNCKPN-------------------------------RKRTTKILALK 272
Zebrafish    NKCEGRFYNNHGTNCKPT-------------------------------RKRTTKILALK 255
Gammarus     DKCEGRFHNTLTGLTSKF-------------------------------KKRPSKILALR 262
Tetraodon    DKCEGRFHNTLTGLTSKC-------------------------------KKRPSKMLALR 257
Takifugu     DKCEGRFHNTLTGLTSKC-------------------------------KKRPSKILALR 320
Human        DSCERRFLGGPARPPSPSPSPVPAPAPPPGPPRPAAAAATAPLANGRAGKRRPSRLVALR 318
             :.** **  .      .                                 ::*.::::**:

Fathead      EQKALKTLGIIMGTFTLCWLPFFIVNVVRVFGKEVVKKELFVFLNWLGYVNSAFNPIIYC 332
Zebrafish    EQKALKTLGIIMGTFTLCWLPFFIVNVVRVFCAQVVDKELFVFLNWLGYVNSAFNPIIYC 315
Gammarus     EQKALKTLGIIMGTFTLCWLPFFIVNVVRVFCAEVVDKDLFVFLNWLGYANSAFNPVIYC 322
Tetraodon    EQKALKTLGIIMGTFTLCWLPFFIVNVVRVFSAAMVDQDLFVFLNWLGYVNSAFNPIIYC 317
Takifugu     EQKALKTLGIIMGTFTLCWLPFFIVNVVRVFSAATVDKDLFVFLNWLGYVNSAFNPIIYC 380
Human        EQKALKTLGIIMGVFTLCWLPFFLANVVKAFHRELVPDRLFVFFNWLGYANSAFNPIIYC 378
             *************.*********:.***:.*    * . ****:*****.******:***

Fathead      RSPDFRKAFKRLLCCPRQADRRLHVSSCDLSRCTGGFVNSMEQS---------------- 376
Zebrafish    RSPDFRKAFKRLLCCPRQADRRLHVSSCDLSRCTGGFGNSMEQS---------------- 359
Gammarus     RSPDFRKAFKRLLCCPRQADRRLHASSCDLSRYSGGFVSALEPG---------------- 366
Tetraodon    RSPDFRKAFKRLLCCPRQADRRLHVSSCDLSRCSGGFASPL------------------- 358
Takifugu     RSPDFRKAFKRLLCCPRQADRRLHISSCDLSRCSGGFVSPLENG---------------- 424
Human        RSPDFRKAFQGLLCCARRAARRRHATHGDRPRASGCLARPGPPPSPGAASDDDDDDVVGA 438
             *********: ****.*:* ** * :  * .* :* :  .

Fathead      -----MLGTWSDCNG--TDSRDCSLERNGRV-SHSESQL-- 407
Zebrafish    -----MLGTWSDCNG--ADSSDCSLERNGRM-SHSESQL-- 390
Gammarus     -----ALGLWSDCAA--LD---------------------- 378
Tetraodon    -----------------------------------------
Takifugu     -----TLGMWSDCAA--LDNSDSSLVGTAKR-SHSESTM-  455
Human        TPPARLLEPWAGCNGGAAADSDSSLDEPCRPGFASESKV-  477
```

Figure 2.33. Clustal multiple sequence alignment of the amino acid sequences of fish and human β1-ARs. Red letters indicate TMDs in fathead minnow sequence as determined by the software programme TMPRED. (*) Indicates identical amino acid sequence in that column. (:)Means conserved substitutions have been made. (.) Indicates means that semi-conserved substitutions are observed

```
Fathead197     MERGDRLSAENTSLHMNVSSGLNDSSP----VSEYSDAEVVLISILMGLLVLGIVFGNVL 56
Fathead207     MEGGDRLSVENTSLHMNVSSGLNDSSP----VSEYSDAEVVLISILMGLLVLGIVFGNVL 56
Zebrafish      MEGDNTLITENTSLYMNISAGLNASSPVSLSVSEYSDAEVVLISILIGILVLVIVFGNAL 60
Trout          MENVSTPAVFNLS---DLSVEMNSSSR-QWSYSEYSEAVAVLLGILMALLVMCIVFGNVL 56
Tetraodon      ------------------------------------EVTMILLGIVMSFLVLCIVFGNIL 24
Takifugu       MGSTAPTGYLNAS-----TEKIPTSDP-TKQEEGYSEVTMILLGMVMSFLVLCIVFGNIL 54
Human          MGQPGNGSAFLLAP--NRSHAPDHDVT-----QERDEVWVVGMGIVMSLIVLAIVFGNVL 53
                                                   :.  : :.:::.::*: ***** *

Fathead197     VISAIVRFQRLQTGTNYFISSLACADLVMGLMVVPFGACYILLNTWHFGNFFCEFWTATD 116
Fathead207     VISAIVRFQRLQTGTNYFISSLACADLVMGLMVVPFGACYILLNTWHFGNFFCEFWTATD 116
Zebrafish      VISAIVRFQRLQTVTNYFISSLACADLVMGLMVVPFGACYILLNTWHFGNFFCEFWTATD 120
Trout          VITAIVRFQRLQTVTNMFITSLACADLVMGLLVVPFGACYILLNTWHFGSFLCEFWTAAD 116
Tetraodon      VITAIARFQRLQNITNCFITSLACADLVMGLIVIPFGACYIIFKTWHFGSFWCEFWTATD 84
Takifugu       VITAIARFQRLQNVTNCFITSLACADLVMGLIVIPFGACYIIFKTWHFGSFWCEFWTATD 114
Human          VITAIAKFERLQTVTNYFITSLACADLVMGLAVVPFGAAHILMKMWTFGNFWCEFWTSID 113
               **:**.:*:***. ** **:*********** *:****.:*::: * **.* *****: *

Fathead197     VLCVTASIETLCVIALDRYVAIMWPLRYQSMLTKRKACGIVLAVWAVS------------ 164
Fathead207     VLCVTASIETLCVIALDRYVAIMWPLRYQSMLTKRKACGIVLAVWAVAALISFLPIHMEW 176
Zebrafish      VLCVTASIETLCVIALDRYIAIMWPLRYQSMLTKRKACGMVIGVWAVAALISFLPIHMEW 180
Trout          VLCVTASIETLCVIALDRYLAITSPLRYPSLLTKRKACVVVVTVWGVAALISFLPIHMKW 176
Tetraodon      VLCVTASIETLCVIAIDRYLAITSPFRYQSMLTKCRARIVVVLVWVIAALISFLPIHMKW 144
Takifugu       VLCVTASIETLCVIAIDRYLAITSPFRYQSMLTKCRARIVVVLVWVIAALISFLPIHMKW 174
Human          VLCVTASIETLCVIAVDRYFAITSPFKYQSLLTKNKARVIILMVWIVSGLTSFLPIQMHW 173
               ***************:***.**   *::* *:***  :*   ::: ** ::

Fathead197     ---DDPAARSCLKNPTCCDFNTNAAYAVTSSIVSFYIPLVIMVFVYSRVFQEARRQLQKI 221
Fathead207     WVSDDPDALSCLKNPTCCDFNTNAAYAVTSSIVSFYIPLVIMVFVYSRVFQEARRQLQKI 236
Zebrafish      WVSEDPEALSCLEEPTCCDFNTNAAYAVPSSIISFYIPLVIMAFVYSRVFQEARRQLQKI 240
Trout          WVSDEPEALSCLEDAHCCDFNTNAAYAVASSVVSFYIPLAVMAFVYGRVFQEARKQLEKI 236
Tetraodon      WISDDEDAKRCIEDSSCCEFYTNMAYAITSSIISFYIPLVIMVFVYSRVFQEAKRHLKKI 204
Takifugu       WISDDEEAKRCIEDSSCCEFYTNMAYAITSSIISFYIPLVIMVFVYSRVFQEAKQQLKKI 234
Human          YRATHQEAINCYANETCCDFFTNQAYAIASSIVSFYVPLVIMVFVYSRVFQEAKRQLQKI 233
                 .  *  *  :  **:* ** ***:.**::***:**.:*.***.******:::*:**

Fathead197     DRIEGRIR---TQSFSTQEGN-------------RRTKFGMKDHKALKTLGIIMGTFTLC 265
Fathead207     DRIEGRIR---TQSFSTQDGNET---------KNRRTKFGMKDHKALKTLGIIMGTFTLC 284
Zebrafish      DRIEGRIR---TQSLSTQEGNEI---------KNRRTKFCMKDHKALKTLGIIMGTFTMC 288
Trout          RGSEGRFH---AQMIDNNQGQDGGDGSGGGGGNGKRPKFCLKEHKALKTLGIIMGTFTLC 293
Tetraodon      DQSVGRFQISDDSNLNSLEANSTR---------SQKKAKFCLREHKALKTLGIIMGIFTLC 256
Takifugu       DQSVGRFHNNDDNNLRSLDANSTR---------SQKKAKFCLREHKALKTLGIIMGIFTLC 286
Human          DKSEGRFH---VQNLSQVEQDGRTG-----HGLRRSSKFCLKEHKALKTLGIIMGTFTLC 285
                 **::     . :   : :                 : .** :::************ **:*

Fathead197     WLPFFVLNVAAAIWKMENIMLPFRILNWIGYANSAFNPLIYCRSPEFRCAFQEILCRRTS 325
Fathead207     WLPFFVLNVAAAIWKMENIMLPFRILNWIGYANSAFNPLIYCRSPEFRCAFQEILCRRTS 344
Zebrafish      WLPFFVLNVVAAIWKMDNIMLPFRILNWIGYANSAFNPLIYCRSPEFRCAFQEILCWRPS 348
Trout          WLPFFVLNVVVTIWKVDNIKMPFRILNWIGYANSAFNPLIYCRSPEFRYAFQEILCLRGA 353
Tetraodon      WLPFFLLNVVVAIWKEQHNDLAFRILNWIGYANSAFNPLIYCRSPEFRYAFKEILCLKRN 316
Takifugu       WLPFFLLNVVVAIWKEQHNDLAFRILNWIGYANSAFNPLIYCRSPEFRYAFKEILCMKRN 346
Human          WLPFFIVNIVHVIQDNLIRKEVYILLNWIGYVNSGFNPLIYCRSPDFRIAFQELLCLRRS 345
               *****::*:. .* .        : :******.**.**********:** **:*:** :

Fathead197     HLPSTRN---NKGFIYSGHSWKVHT--KTARQREPSPACETEMGAG-------------N 367
Fathead207     HLPSTRN---NKGFIYSGHSWKVHT--KTARQREPSPACETEMGAG-------------N 386
Zebrafish      HLPSTRS---KKGYLYSGHSWKVHT--KTTRPREPSPACETEIGAECLTAGTKN----KN 399
Trout          AFPTN-------GYIYRGHSLRLSP--KDKPGSLSNNVGTVELGSLSSVTN-------IN 397
Tetraodon      RLTNPAP---VNGYIYSKQGWQSEQQGRSKVSLEDSEANESYLG---------------- 357
Takifugu       RLTNPGP---VNGYIYSRHSWQSEQQGRSKVSLEDSEANESCLGKGMEVCTVIDKTVDPN 403
Human          SLKAYGNGYSSNGNTGEQSGYHVEQEKENKLLCEDLPGTEDFVGHQGTVPSDNIDSQGRN 405
                :          *        . :       .                     :*

Fathead197     GNCNKAVTSDF--- 378
Fathead207     GNCNKAVTSDF--- 397
Zebrafish      GNYNKTVTSI---- 409
Trout          GYCNNPPLASIV-- 409
Tetraodon      --------------
Takifugu       GNCNKGLTTVTTVL 417
Human          CSTNDSLL------ 413
```

Figure 2.34. Clustal 2.0.3 multiple sequence alignment of the amino acid sequences of fish and human β2-ARs. **Red letters** indicate TMDs in fathead minnow sequence as determined by the software programme TMPRED. (*) Indicates identical amino acid sequence in that column. (:)Means conserved substitutions have been made. (.) Indicates means that semi-conserved substitutions are observed.

2.3.7 Phylogenetic analysis

Figure 2.35 and Figure 2.36 show the results of the phylogenetic analyses for fish and human species in which β-ARs have been characterised.

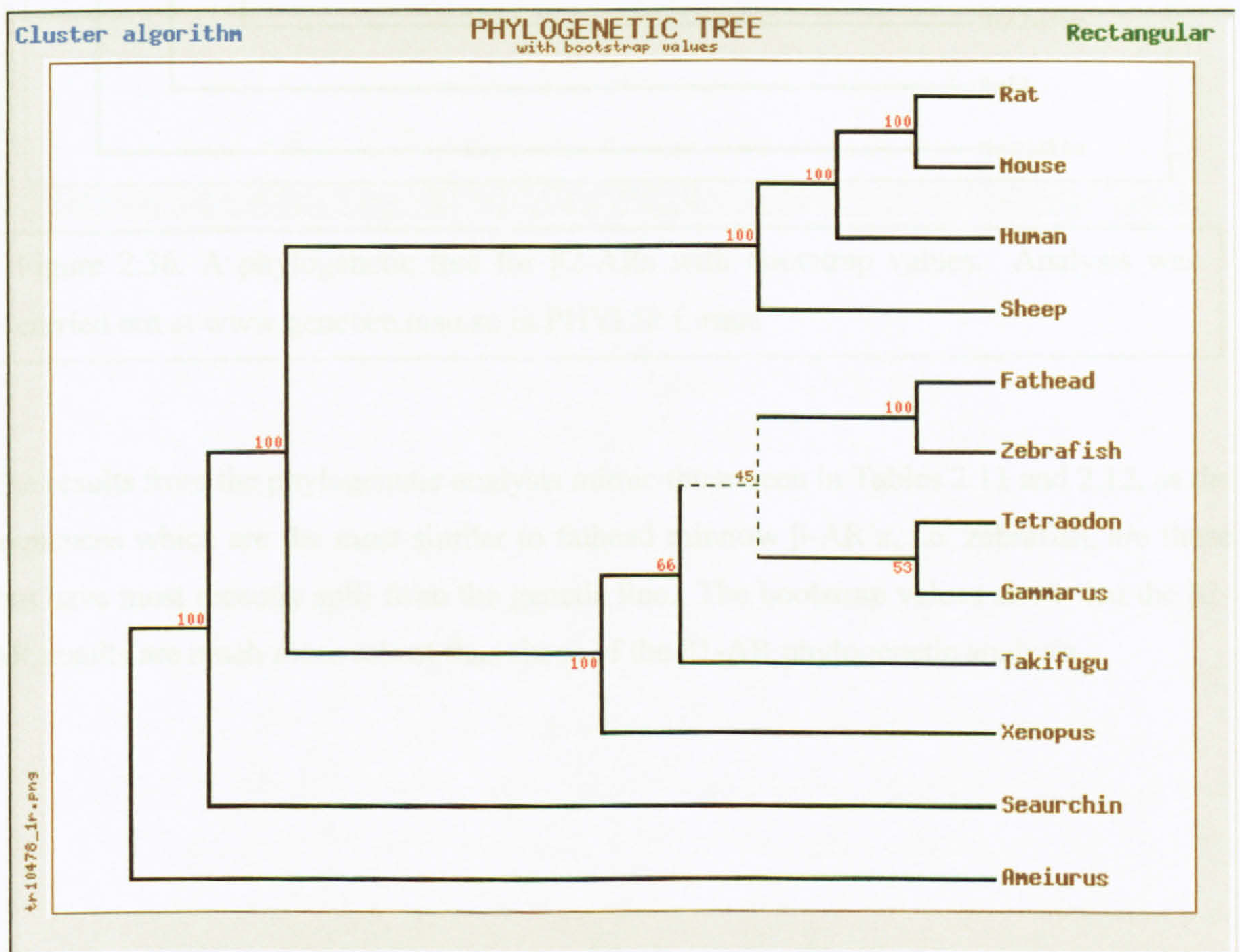


Figure 2.35. A phylogenetic tree for β1-AR with bootstrap values. Analysis was carried out at www.genebee.msu.su in PHYLIP format

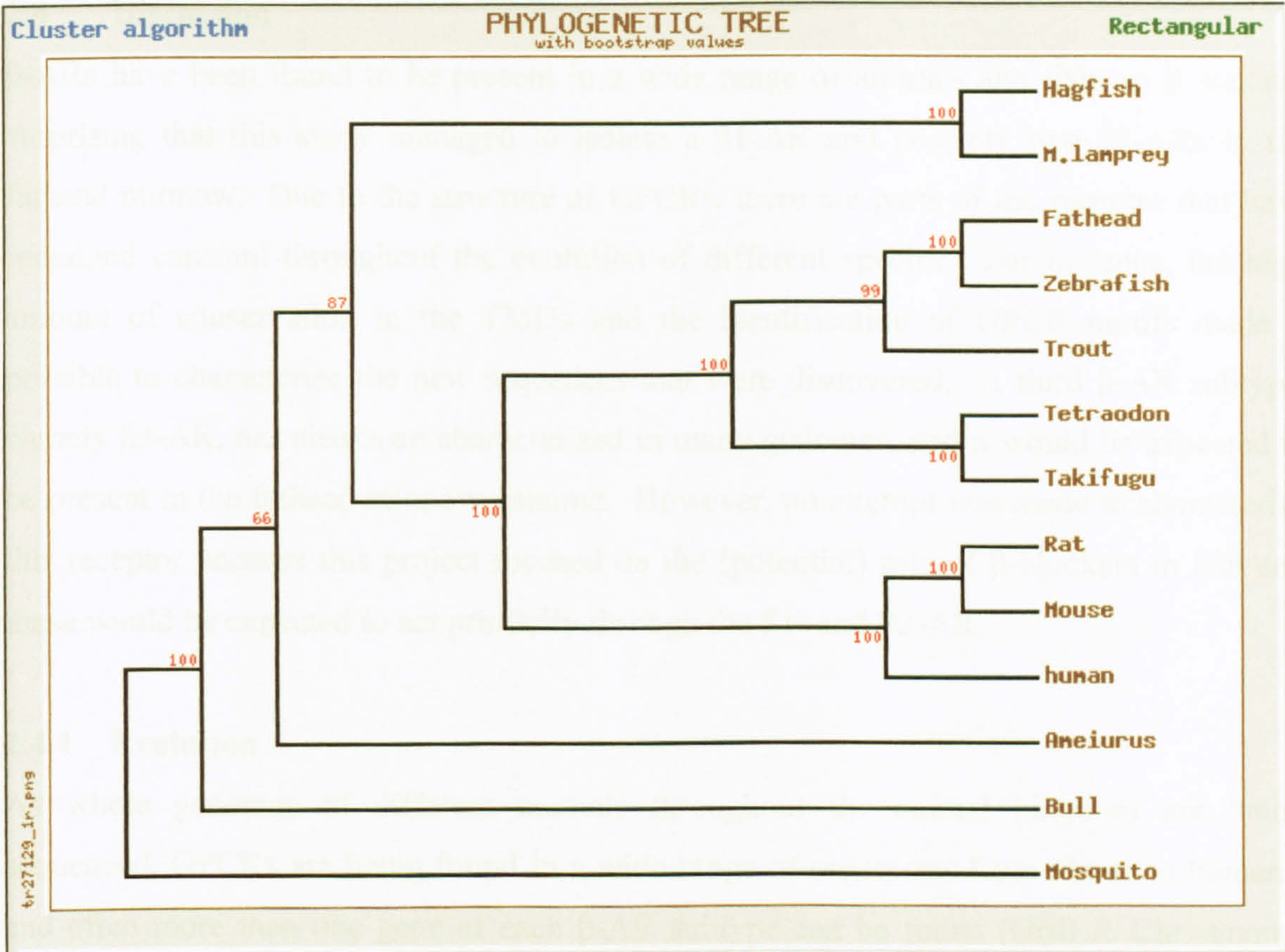


Figure 2.36. A phylogenetic tree for β2-ARs with bootstrap values. Analysis was carried out at www.genebee.msu.su in PHYLIP format

The results from the phylogenetic analysis mimic those seen in Tables 2.11 and 2.12, as the sequences which are the most similar to fathead minnow β-AR's, i.e. zebrafish, are those that have most recently split from the genetic line. The bootstrap values show that the β2-AR results are much more robust than those of the β1-AR phylogenetic analysis.

2.4 Discussion

β-ARs have been found to be present in a wide range of animals and fish, so it was not surprising that this study managed to isolate a β1-AR and possibly two β2-ARs in the fathead minnow. Due to the structure of GPCRs, there are parts of the receptor that have remained constant throughout the evolution of different species. For instance, the high amount of conservation in the TMDs and the identification of GPCR motifs made it possible to characterise the new sequences that were discovered. A third β-AR subtype, namely β3-AR, has also been characterised in many genomes, and it would be expected to be present in the fathead minnow genome. However, no attempt was made to characterise this receptor because this project focused on the (potential) role of β-blockers in fish and these would be expected to act primarily through the β1- and β2-AR.

2.4.1 Evolution

As whole genomes of different animals throughout the animal kingdom are being sequenced, GPCRs are being found in a wide range of organisms from plants to humans, and often more than one gene of each β-AR subtype can be found (Grill & Christmann, 2007). The presence of more than one gene for a particular subtype is due to different duplication events, such as the duplication of individual genes, chromosomal segments and even entire genomes that occurred through evolution (Gloriam et al., 2005). From the study of genomics it is now recognised that three whole genome duplications (WGDs) have taken place since the origin of vertebrates some 500 – 800 million years ago, as shown in Figure 2.37 (Froschauer et al., 2006). The first of these events (1R) happened after divergence of ciona and amphioxus from the vertebrate lineage but before the split between jawless (hagfish and lampreys) and jawed vertebrates. The second WGD (2R) happened after the split of the jawless vertebrates but before the split between cartilaginous (sharks, rays) and bony vertebrates (Froschauer et al., 2006). This means that teleosts and humans, although separated by approximately 450 million years of evolution, arose from the same vertebrate lineage and share a common ancestor from the Palaeozoic era (Froschauer et al., 2006). The discovery of this common ancestor facilitated the completion of the human genome (Jaillon et al., 2004). It is now possible by carrying out four-way comparisons with human, pufferfish, medaka and zebrafish genomes to establish that since the divergence of humans and teleosts, eight major interchromosomal rearrangements took place in a very short period of time (~50 million yr), to result in this evolutionary divergence (Kasahara et al., 2007).

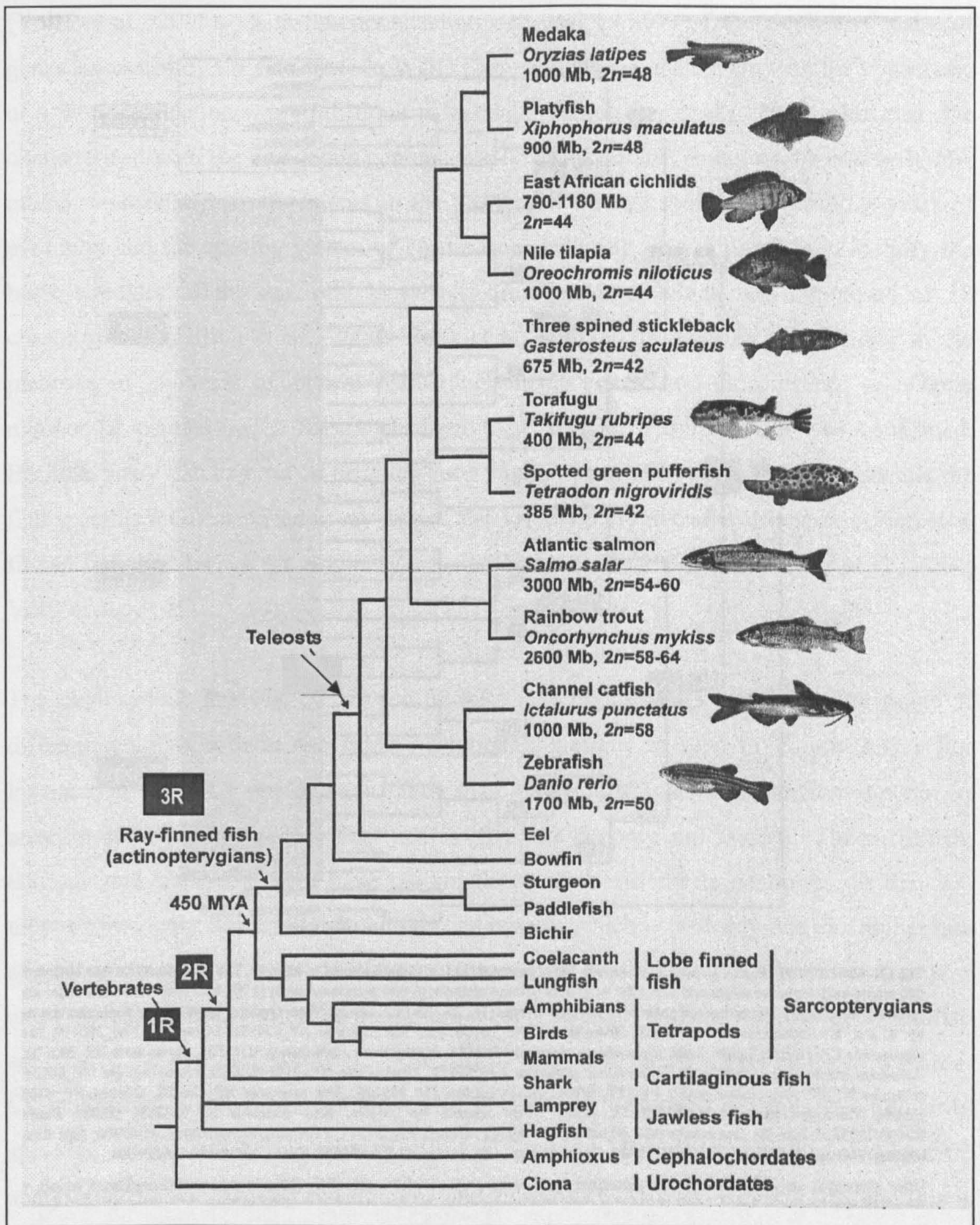


Figure 2.37. A diagrammatic explanation of the evolution of teleosts, depicting where each WGD occurred (Adapted from Froschauer, 2006)

The occurrence of a third WGD event (3R) was highly debated for a long time. During evolution, duplicated genes can undergo a range of processes and this makes the identification of the 3rd WGD a complicated process. Some duplicated genes are often deleted, some copies evolve at different evolutionary rates, whilst other duplicates become pseudogenes, whereby degenerative mutations cause the gene to become non-functional

(Volff et al., 2005). Non-functionalisation occurred to 90% of the duplicated pairs of genes generated by the fish specific WGD (3R), which was one reason why the hypothesis of a third duplication was so difficult to validate (Froschauer et al., 2006). However, the characterisation of the *Tetraodon* genome resolved this debate, as due to the relatively few interchromosomal rearrangements in the *Tetraodon* lineage during 100 millions years of evolution and the striking pattern of chromosomal pairing, it was possible to identify the basic structure of the ancestral bony vertebrate genome, which was composed of 12 chromosomes (Jaillon et al., 2004; Volff et al., 2005). In addition to this, due to the presence of hundreds of ancient pairs of duplicate genes, and the presence of a large number of paralogons, a feature distinctive of WGDs, a third WGD was confirmed. Because many fish express co-orthologs of a single mammalian gene, this suggests that the fish-specific WGD occurred in ray-finned fish (Actinopterygii) after divergence from lobe finned fish, and before the origin of teleosts (Hahn et al., 2006; Christoffels et al., 2004; Volff et al., 2005).

The phylogenetic analysis of β1- and β2-ARs (see Figures 2.35 and 2.36) in a range of different animals reflects the same evolutionary pattern as seen in Figure 2.37. For instance, in the β-AR phylogenetic trees, mammals and teleosts separate from a common ancestor after the divergence of the jawless fish, the lamprey and hagfish. The pufferfish, takifugu and tetraodon, both have the smallest known vertebrate genomes, yet they are more evolutionary divergent than human and mouse, which is probably due to a higher rate of gene mutation (Jaillon et al., 2004). However, as seen in the β-AR phylogenetic trees and Figure 2.37, both pufferfish β-ARs are closely related. Fathead minnow and zebrafish are part of the cyprinid family (Cyprinidae) of the super family Cyprinoidea in the order Cypriniformes, which mean that the zebrafish is one of the closest relations, evolutionarily speaking, to the fathead minnow (Volff et al., 2005). The results from the phylogenetic analysis also show that fathead minnow and zebrafish β-ARs are very similar, and this is probably why using primers based on the zebrafish sequence to amplify unknown fathead minnow β-AR sequences was successful. In the β2-AR analysis, the trout is shown as being the next closest family to the fathead minnow, which was why degenerative primers designed from zebrafish and trout β2-AR sequences were successful in amplifying unknown fathead minnow β2-AR fragments. The β1-AR phylogenetic analysis shows the sea urchin, an echinoderm, to have a closer evolutionary relationship with teleosts compared to *Ameiurus melas*, an Actinopterygian fish. This may be due to the conservation of some genes through evolution. The sea anemone, a cnidarian, has within its very simple and ancestral genome some blocks of DNA that have been found to have

the same compliment of genes as the human genome (Pennisi, 2007). The brown bullhead, *Ameiurus melas*, is divergent from the teleosts in both β-AR analyses. *Ameiurus* is an Actinopterygii (ray-finned fish), and as shown in Figure 2.37, this group of fish diverged before the major evolution of teleosts and this is reflected in the results of the β-AR analyses.

Teleosts are a large and very successful group of vertebrates and they constitute 99.8 % of ray-finned fish (Actinopterygians), which in turn make up 95% of all fish species (Volff et al., 2005). Because of the three rounds of WGD that have occurred in teleosts, multiple numbers of receptors can be found, and so if no gene deletions had occur, we could expect to find eight copies of each AR subtype. For example, in the pufferfish (*Fugu rubribes*) genome, eight α2-ARs can be identified (Ruuskanen et al., 2005). So, finding two different versions of the β2-AR in the fathead minnow is an indication that at least two β2-AR genes are present, and a further possible six may yet be found.

When genes become duplicated, one copy often evolves as a pseudogene. Pseudogenes are genes that accumulate disabling mutations in the absence of a selective pressure to maintain the additional duplicated gene on an evolutionary time scale. The mutations can vary from multiple disruptions of a coding sequence to just the loss of one nucleotide which results in a frame shift leading to a truncated protein, although care must be taken in this instance as a nucleotide change or loss could be a polymorphism or a sequencing error (Foord et al., 2005). Gene deletions may have occurred, for example, in the zebrafish suite of α2-ARs. Zebrafish have five distinct α2-ARs, however after three rounds of WGD it could be predicted that eight α2-ARs should be present (Ruuskanen et al., 2005). This suggests that three α2-ARs are either yet to be annotated or they have been deleted.

Genes that lack a ribosomal binding sequence and a TATA box or initiator sequence in the 5' UTR of a nucleotide sequence are often pseudogenes, as without these specific sequences, mRNA is unable to translate them into proteins. Of the two β2-ARs found in the fathead minnow, both genes have a ribosomal binding sequence and TATA box in the 5'UTR and this suggests that both these genes are functional. However, although the 1/197 β2-AR gene found in the fathead minnow was shorter in places than the 207 β2-AR gene, hydropathy analysis showed it to have seven TMDs with a non-disrupted reading frame, which is further evidence to suggest that this is not a pseudogene. The presence of two genes coding for the same protein may be beneficial to some organisms, as the genes can undergo functional divergence, whereby the function carried out by a single ancestral

gene is now carried out by two genes, a process known as subfunctionalisation, which is often found in teleosts. For example, in atlantic killifish, two distinct aryl hydrocarbon receptor genes were found, and although they were both functional, they showed different expression patterns throughout the fish, which may suggest they carry out the same function but in different tissues (Hahn et al., 2006).

The β1-AR in the fathead minnow lacked both a TATA box or an initiator sequence, however it does possess a ribosomal binding sequence. This could meant that this gene is a pseudogene or that the 5' UTR sequence, which was not confirmed by proof reading taq, is incorrect or incomplete. The reading frame of this gene is undisturbed and hydropathy analysis shows it to have seven TMDs, which suggest that this gene is not a pseudogene.

Another genetic evolutionary pathway is via transposable elements (TEs). These mobile sequences are most active in germ cells and are able to integrate into new sites within genomes. This can lead to major disruption from deletions, duplications, inversions, modification of flanking genes and translocations, but they are also big drivers of evolution (Froschauer et al., 2006; Volff et al., 2005). Teleosts, including pufferfish which have very compact genomes, have a much higher diversity of TEs than mammalian genomes (Volff et al., 2005).

In summary, teleosts have undergone an extra round of WGD compared to other animals, have a high turnover of mobile TEs, and often subfunctionalize duplicated genes. These processes are known to lead to major evolutionary transitions and it may be these factors that contributed to the vast diversity of fish, which is especially noticeable in the many possible forms of sex determination, and could be the reason why teleosts are a most diverse and successful taxa, with over 24,000 species (Froschauer et al., 2006; Volff et al., 2005).

2.4.2 Chromosomal organisation of receptors

The mapping of different receptors to specific chromosomes provides further information on the evolutionary pathways, for if genes are found in close proximity on a chromosome, then this strongly suggests that they are evolutionary related (Yang-Feng et al., 1990). For example, the genes coding for the human β1-AR and α2-AR are found on the same chromosome very close together (chromosome 10, q24 – q26), and β2-AR and α1-AR can both be mapped to chromosome 5 (q32 – q34) (Yang-Feng et al., 1990). Hence, it can be deduced from chromosomal locations that different receptor subtypes have been generated

by whole genome duplication. A third round of duplication in the teleost lineage brought the total number of α-ARs up to 8, as in the pufferfish (*Fugu rubribes*) (Ruuskanen et al., 2005).

It is most likely all ARs arose from a common ancestral receptor gene and subsequently diverged through gene duplication and chromosomal duplication to perform distinctive roles (Yang-Feng et al., 1990). For example, the β1-AR gene most likely arose from ancestral gene duplication, whilst the β2-AR may have arisen from chromosomal duplication (Yang-Feng et al., 1990).

2.4.3 GPCR structure

GPCRs are the largest and most diverse group of transmembrane receptor molecules, and occur in nearly every eukaryotic cell (Ranganathan, 2007). There are more than 1000 GPCRs in the human genome and these have been categorised into five families. β-ARs and rhodopsin are in the largest subgroup and are structurally and evolutionary related to each other (Libert et al., 1989). Rhodopsin is a light sensitive photoreceptor found in the mammalian eye and the crystalline structure for this GPCR has been determined. However, obtaining the three dimensional structure of other GPCRs has proven difficult, primarily because of their natural low abundance and secondly because, unlike the fixed inactive state of rhodopsin, other GPCRs have an inherent structural flexibility and instability (Rosenbaum et al., 2007). This has meant that although 50 % of modern drugs are targeted to a wide variety of GPCRs, they have all been designed on a rhodopsin-based model (Cherezov et al., 2007; Rosenbaum et al., 2007).

The ligand 11-cis-retinal binds to rhodopsin and it is the covalent bonds between this ligand and the receptor that keeps rhodopsin in a tightly locked inactive state (Ranganathan, 2007). However, it is the plasticity of many other GPCRs that enables them to elicit a range of responses from various different ligands. Hence, by determining the structure of human β2-AR, similarities and several differences between it and the structure of rhodopsin have come to light, as shown in Figure 2.38. The main structure of both receptors is very similar in that they have a roughly ellipsoid arrangement of seven membrane spanning alpha helical segments (TMDs) that surround a ligand binding site (Ranganathan, 2007).

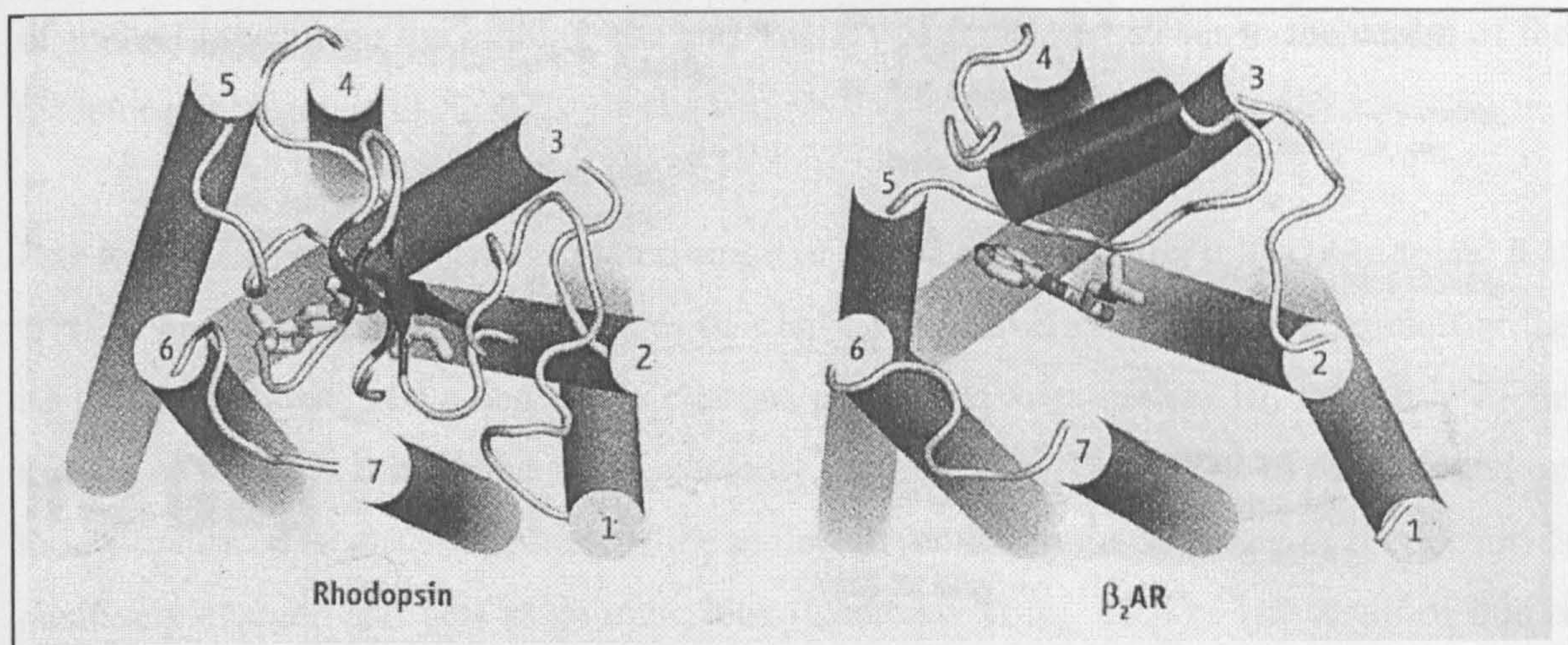


Figure 2.38. The crystalline structure of rhodopsin (left) and β2-AR (right) with a ligand bound in the ligand binding pocket. The numbers 1 to 7 refer to each helical segment (TMD) (Ranganathan, 2007).

There are a lot of similarities between the ligand binding pockets in that both receptors have a tryptophan side chain and all amino acids deep within the binding pocket are conserved between the two receptors (Rosenbaum et al., 2007). The main differences are found in the cytoplasmic and extracellular loops. For example, when carazolol is bound to β2-AR to stabilize it, some amino acids in the helical structure of the second extracellular loop make direct contact with the ligand, which does not occur in rhodopsin (Ranganathan, 2007).

It may be that this sequence variability in the second extracellular loop in the β2-AR accounts for the receptor's affinity for different ligands (Cherezov et al., 2007; Ruuskanen et al., 2005). Some differences are also found in the amino acids present at the entrance to the binding pocket. This variation in β2-ARs compared to rhodopsin may be especially important to larger ligands, such as peptide molecules, that bind closer to the membrane surface near the extracellular loops, and may account for the plasticity of β-ARs to bind a large variety of ligands (Cherezov et al., 2007).

Subtle differences in the orientation of the helices are also found between the two GPCRs (Rosenbaum et al., 2007). The main difference is that TMD I in the β2-AR, compared to rhodopsin, lacks a proline-induced kink and angles away from the centre of the receptor, due to a need for an accessible binding site in β2-AR, which is provided in part by lack of interactions between the N terminus and extracellular loops (Cherezov et al., 2007). Additionally, TMDs II and V are moderately divergent to those in rhodopsin in that TMD

II pivots further from the binding site and TMD V is translated closer to the centre of the receptor (Cherezov et al., 2007).

Due to the successful, recent characterisation of the β2-AR structure, it has been found that GPCRs are highly polarised molecules that have a negatively charged binding cleft at the tip of the molecule, and a negatively charged groove between helices III, IV and V in the middle of the lipid membrane. Unexpectedly, the Asp-Arg-Tyr (E/DRY) motif found on the cytoplasmic face at the bottom of the molecule (unlike in the structure of rhodopsin) is positively charged and acts as an ionic lock (Cherezov et al., 2007). The result of this is that the overall negative charge of the receptor facilitates the binding of catecholamine ligands to GPCRs.

All three β-ARs genes isolated in the fathead minnow did not contain introns (non-coding regions of DNA) in their sequence. This is like many GPCRs, eg rat β1-AR, whose coding sequence is not interrupted by introns (Machida et al., 1990). The length of each fathead β-AR also compared favourably to β-ARs in other species, with the total β1-AR being 1412bp, 1/197 β2-AR totalling 1298 bp and 207 β2-AR equalling 1445 bp. Each sequence contained 7 TMDs and the motifs that are associated with GPCRs and β-ARs.

With such complexity it is remarkable that despite 350 million years of evolution, the ligand binding characteristics, the order of potency and efficacy of tested agonists of GPCRs, for example between the zebrafish and human α-ARs, is so highly conserved (Ruuskanen et al., 2005)

2.4.4 Concluding remarks

From the work carried out in this chapter, two (possibly three) genes were isolated and characterised in the fathead minnow, namely the β1-AR gene, and two possible β2-AR genes. All of these genes (the β1-AR and the two β2-AR genes) had an uninterrupted reading frame with no introns, a ribosomal binding site, a start and stop codon and a 3' UTR motif of AAUAAA. Both the β2-AR genes had a TATA box but the β1-AR lacked this in the 5' end of the UTR sequence, this may be due to it not being present or due to the 5' end of this gene not being sequenced in totality. The seven TMDs (an integral element of GPCRs) were revealed in hydropathy analysis in all the characterised genes, and showed a high homology within the TMDs to other fish and human TMD sequences. In addition the above genes showed a high homology to other fish and vertebrate homologues, with the fathead β1-AR and β2-AR genes having 91 % and 88 % homology to zebrafish β1-AR

and β2-AR genes respectively. Phylogenetic analysis revealed the closest ancestor to the fathead minnow with respect to β1-AR and the β2-AR genes to be the zebrafish (*Danio rerio*).

Chapter 3 Exposure of fathead minnows to propranolol

3.1 Introduction

Propranolol-HCl is a non-specific β-blocker that is prescribed to humans to treat high blood pressure, heart failure and other heart-related diseases by targeting β1- and β2-ARs. Due to its incomplete removal at STWs, propranolol has been detected in the aquatic environment in the high ng/L levels, and thus may be reaching and having effects on non-target organisms.

3.1.1 Pharmokinetic action of propranolol

Propranolol is a prescribed pharmaceutical that is used to treat angina and hypertension in humans by binding to β-ARs and blocking catecholamine binding. Propranolol therefore acts as an antagonist that binds to the receptor, and in essence acts as an inhibitor by competing with the natural hormone for binding sites. This blocks the physiological activity of the natural ligand, adrenaline or noradrenaline, and does not activate hormone-induced effects. This is opposite to the reaction that an agonist type drug would have, which would mimic the function of the hormone by binding to the receptor and causing the normal hormonal response (Lodish et al., 2000).

3.1.2 Propranolol in the aquatic environment

Propranolol and other human and animal pharmaceuticals reach the aquatic environment through a variety of different routes, including soil and ground water runoff from the application of digested sludge onto farmland, industrial waste, leaching of domestic waste at landfill sites and by disposal of raw sewage or, more usually, STP effluents (Ternes, 1998). However the majority of pharmaceuticals are present in the aquatic environment due to incomplete removal at sewage treatment works (STWs). In Germany it was found that 96 % of propranolol was removed from the influent compared to the effluent, yet the 4 % remaining is largely why propranolol has been found ubiquitously in all rivers and streams in Germany at concentrations in the ng/l range, with maximum and median concentrations reaching 590 ng/L and 12 ng/L, respectively (Ternes 1998). In studies conducted by Ternes (1998), concentrations of propranolol were much higher in rivers around STWs and in small rivers and streams receiving effluent where this effect was magnified due to the low dilution factor of the water body. However this is not a problem unique to Germany; Table 3.1 details concentrations of propranolol found in estuaries, rivers, effluents and streams around America, Europe and the U.K. About 12 tonnes of propranolol is prescribed each year in the UK, and as shown in Table 3.1, small quantities

of propranolol are routinely discharged into our rivers and streams through STW effluent alone (Ashton et al., 2004).

Water body	Maximum concentration (ng/L)	Median concentration (ng/L)	Reference
U.S. effluent samples	2200	≤ 1900	Huggett et al., 2003a
German STW effluents	290	170	Ternes, 1998
German rivers	590	12	
STW effluents around Europe	90	10	Andreozzi et al., 2003
U.K. estuaries	56	13	Thomas & Hilton, 2004
U.K. STW effluent	-	76	Ashton et al., 2004
U.K. STW effluent	280	90	
U.K. rivers upstream of STW	-	< 10	
U.K. Rivers	-	29	

Table 3.1. Maximum and median concentrations of propranolol found in rivers, streams, estuaries and STW effluents throughout America, Europe and the U.K.

Once propranolol has been ingested, it undergoes extensive first pass metabolism by cytochrome P450 enzymes, and it is excreted via urine largely as metabolites, with only 10 % being excreted as the original parent compound (Huggett et al., 2003; Laville et al 2004; Thomas & Hilton, 2004). The major metabolite is 4-hydroxypropranolol, and little information exists on the fate and concentrations of 4-hydroxypropranolol in the environment (Ternes, 1998).

The chemical structure of propranolol-HCL is shown in Figure 3.1. Propranolol has a log P of 3.67, and has a half-life of 16.8 day at 50 °N in winter, suggesting that is has the potential to bioaccumulate in biota or partition into the sediment, which would consequently prolong its residence time in rivers and streams (Andreozzi et al., 2003; Huggett et al., 2003). Propranolol is also unlikely to degrade through hydrolysis, since pharmaceuticals made for oral consumption are usually water resistant (Andreozzi et al., 2003). Possible natural degradation processes include photolysis, where the direct action of sunlight and the indirect action of natural photo-sensitizers such as nitrates and humic acids cause the degradation of propranolol.

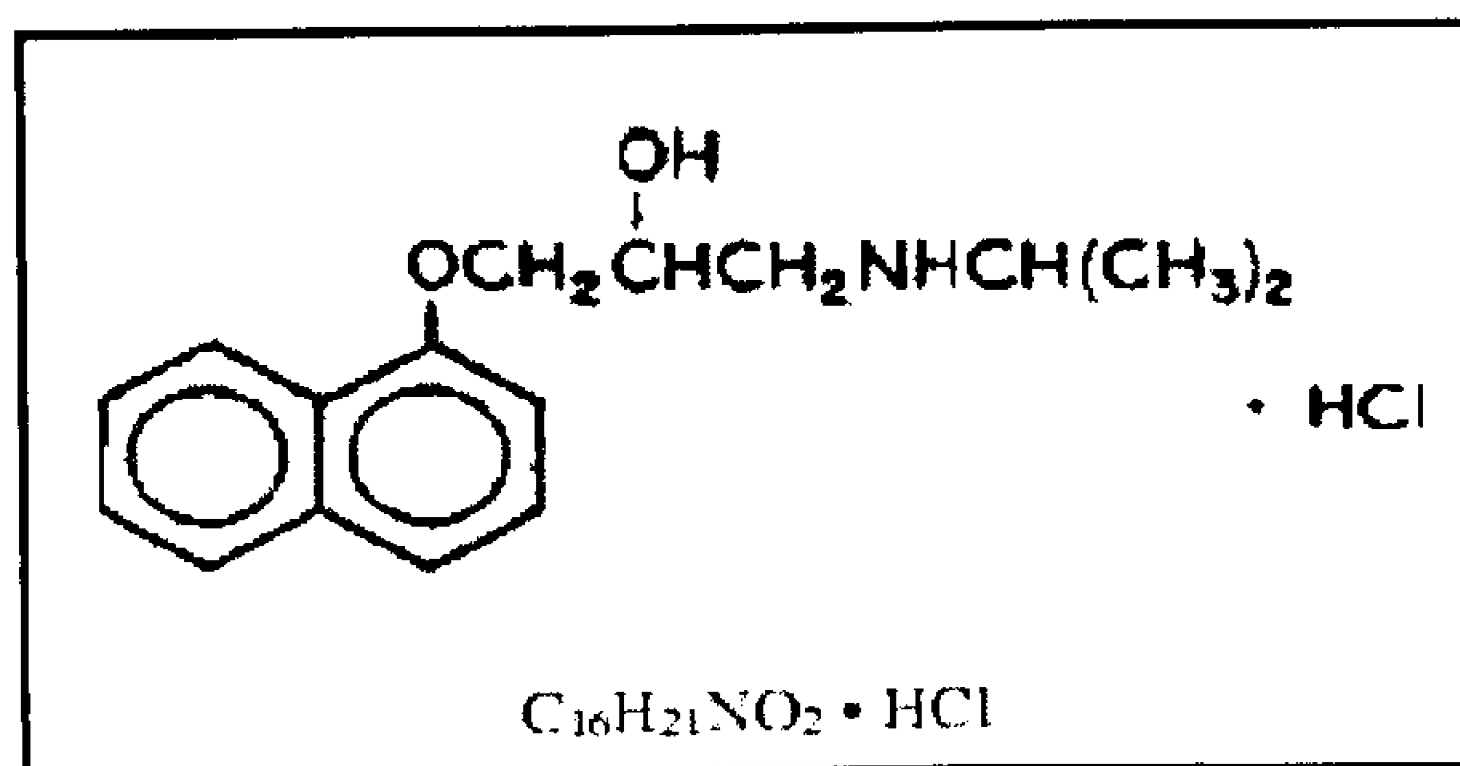


Figure 3.1. The chemical structure of propranolol-HCl. Obtained from (www.rxlist.com/cgi/generic/propran.htm)

3.1.3 Biological effects of propranolol

Propranolol acts upon human β-ARs in order to treat angina and hypertension. However what is not known is how propranolol may act upon β-ARs of fish once it reaches the aquatic environment. β-ARs in fish have been shown to mediate responses to stress through the same GPCR mechanism that is found in humans. The effects may be chronic rather than acute toxic effects, since environmental concentrations of propranolol are nowhere near the 24-hour EC_{50} limit of 2 mg/L recorded by Lilius et al. (1995), although there are insufficient data to predict whether environmental concentrations would cause any long-term effects. Since little is known about the comparability of the functions of β-ARs between teleost and mammalian systems, suggestions as to the effects caused may not be straightforward. Further, the non-specific antagonist nature of propranolol may induce effects in fish that are totally different from its supposed primary pharmocodynamic activity.

Tables 3.2 to 3.6 summarise the *in vitro* and *in vivo* ecotoxicology work in the literature that has been carried out using β-blockers. The initial observation is that in experiments carried out with all β-blockers, the work has on the whole been carried out in the low to mid mg/L range. This suggests that in the majority of experiments, effects were seen only in the mg/L range, which is much higher than the environmental concentrations of β-blockers. Hence, any acute effects would not be seen in the environment. However, this is not true for the work carried out with propranolol, for in particular the work carried out with invertebrates and fish shows environmental concentrations to have fundamentally life-threatening toxic effects.

Compound	Species	EC$_{50}$: (mg/L)	Exposure duration	Observations	Reference
Propranolol	*Desmodesmus subspicatus*	5.8	3 d	Reduced growth	Cleuvers, 2003
	Pseudokirchneriella subcapitata	7.4	96 hr		Ferrari et al., 2004
	Synechococcus leopolensis	0.67	96 hr		
	Lemna minor	114	7 d		Cleuvers, 2003
Metoprolol	*Desmodesmus subspicatus*	7.3	3 d	Reduced growth	
	Lemna minor	>320	7 d		

Table 3.2. Summary of the available *in vivo* data on β-blocker activity in plants. Green alga: *Desmodesmus subspicatus* and *Pseudokirchneriella subcapitata.* Blue-green alga: *Synechococcus leopolensis.* Duckweed:*Lemna minor*

Compound	Species	EC$_{50}$:mg/L	Exposure duration	Observations	Reference
Atenolol	*Vibrio fisheri*	1304	30 min	Bioluminescence	Escher et al., 2006
	Desmodesmus subspicatus	1335	24 hour	Photosynthesis inhibition	
Metoprolol	*Vibrio fisheri*	144	30 min	Bioluminescence	
	Desmodesmus subspicatus	40	24 hour	Photosynthesis inhibition	
Propranolol	*Vibrio fisheri*	61	30 min	Luminescence	Ferrari et al., 2004
		81	30 min	Bioluminescence	Escher et al., 2006
	Deomodesmus subspicatus	4.1	24 hour	Photosynthesis inhibition	
Sotalol	*Vibrio fisheri*	>1000	30 min	Bioluminescence	
	Deomodesmus subspicatus	>3000	24 hour	Photosynthesis inhibition	

Table 3.3. Summary of the available *in vitro* data on β-blocker activity in bacteria and plants. Photosynthesis inhibition was measured by chlorophyll fluorescence. Bacterium: *Vibrio fisheri* Green algae: *Desmodesmus subspicatus*

The *in vivo* experiments carried out with plants (Table 3.2), show propranolol to inhibit growth at lower mg/L concentrations than metoprolol. In addition to this, algae appear to be more sensitive to β-blockers than duckweed. Liu et al. (2007) report that they found 25 candidate GPCRs in the genome of the plant *Arabidopsis thaliana* for which they have found a ligand, a phytohormone abscisic acid, which is an important developmental hormone for a candidate GPCR in this plant. If the plants tested by Cleuvers (2003) and Ferrari et al. (2004) possess GPCRs, this could explain why plant growth is affected by β-blockers. When bacteria and plants were exposed *in vitro* to β-blockers (Table 3.3), there was approximately a 1000-fold difference in the sensitivity of the bacteria to different β-blockers, in that propranolol caused effects at much lower concentrations than sotalol. The order of sensitivity of the tested β-blockers was propranolol < metoprolol < atenolol < sotalol.

Compound	Species	Effect level		Exposure duration	Observations	Reference
		Value	(mg/L)			
Metoprolol	*Hyalella azteca*	LC_{50}	>100	48 hr	Mortality	Huggett et al., 2002
	Daphnia magna	EC_{50}	> 100	48 hr	Immobilization	Cleuvers, 2003
		LC_{50}	63.9	48 hr	Mortality	Huggett et al., 2002
		LC_{50}	76.21	48 hr	Mortality	Villegas-Navarro et al., 2003
		LOEC	12.5	9 d	Growth	Dzialowski et al., 2006

Table 3.4. Summary of the available *in vivo* data on metoprolol activity in invertebrates. Amphipod: *Hyalella azteca*, Crustacean: *Daphnia magna*,

Tables 3.4 and 3.5 summarise *in vivo* data from the testing of β-blockers in invertebrates. At a quick glance, propranolol has been used in more experiments than any other β-blocker. The LC_{50} for metoprolol and propranolol in invertebrates ranges from 64 to >100 mg/L and 0.8 to 29.8 mg/L, respectively, showing that propranolol is harmful to invertebrates at much lower concentrations than metoprolol.

Compound	Species	Effect level (mg/L)		Exposure duration	Observations	Reference
		Value	(mg/L)			
Propranolol	*Hyalella azteca*	LC_{50}	29.8	48 hr	Mortality	Huggett et al., 2002
			0.1	27 d	Reduced fecundity	
	Cyclotella meneghiniana	EC_{50}	0.244	96 hr	Growth	Ferrari et al., 2004
		NOEC	0.094			
	Daphnia magna	EC_{50}	7.5	48 hr	Immobilization	Cleuvers, 2003
		EC_{50}	2.75	48 hr	Mortality	Ferrari et al., 2004
		LC_{50}	1.6	48 hr	Mortality	Huggett et al., 2002
		LOEC	0.44	9 d	Growth	Dzialowski et al., 2006
		LOEC	0.11		Reduced fecundity	
			0.88		Reproductive failure	
		LOEC	0.055		Heart rate	
	Ceriodaphnia dubia	EC_{50}	1.51	48 hr	Mortality	Ferrari et al., 2004
		NOEC	0.009	7 d	Reproduction	
		LC_{50}	0.8	48 hr	Mortality	Huggett et al., 2002
			0.25	7 d	Reduced fecundity	
	Brachionus calyciflorus	NOEC	0.18	48 hr	Reproduction	Ferrari et al., 2004

Table 3.5. Summary of the available *in vivo* data on propranolol activity in invertebrates. Some effects are seen at environmentally relevant concentrations of propranolol. Amphipod: *Hyalella azteca*, Diatom: *Cyclotella meneghiniana*, Crustacean: *Daphnia magna, Daphnia pulex, Ceriodaphnia dubia*, Rotifer: *Brachionus calyciflorus*

Compound	Species	Effect level		Exposure duration	Observations	Reference
		Value	(mg/L)			
Propranolol	Medaka	LC_{50}	24.3	48 hr	Mortality	Huggett et al., 2002
		LOEC	0.5	14 d	Reduced growth	
		LOEC	0.001 *	14 d	Reduced testosterone & increased estradiol concentrations	
		LOEC	0.0005*	4 week	Reduction in egg production	
		LOEC	0.0005*	4 week	Reduction in hatchability	
	Zebrafish *ELS*	NOEC	2	10 d	Mortality	Ferrari et al., 2004
		NOEC	< 4	24 hr	Decrease in number of spontaneous movements	Fraysse et al., 2006
		NOEC	4	48 hr	Heart rate reduction	
			32	80 hr	No hatching observed	
Metoprolol	Medaka	LC_{50}	>100	48 hr	Mortality	Huggett et al., 2002
Nadolol	Medaka	LC_{50}	>100	48 hr	Mortality	
Atenolol	Fathead minnow *ELS*	NOEC	10	5 days	Hatching	Winter et al., 2008
		LOEC	>10			

Table 3.6. Summary of the available *in vivo* data on β-blocker activity in fish. *ELS*: early life stage. Medaka: *Oryias latipes*, Zebrafish: *Danio rerio*, Fathead minnow: *Pimephales promelas*. Some queries exist over the reproducibility of some of the Huggett et al., (2002) data as shown by *.

Some queries exist over the reproducibility of the medaka data (*) reported by Huggett et al. (2002), for in some experiments only three pairs of medaka were used at each concentration, and no repeat test was carried out for any of the experiments. In addition to this, the exposures were not carried out in a flow through system, but instead the set up consisted of beakers with the test solutions being replenished daily. Further, the propranolol was dissolved in ethanol, which seems unnecessary at such low concentrations, as propranolol is readily soluble in water. Lastly, the dose-response curves reported are unusual in that there were no effects at high concentrations but there were effects observed at low concentrations, i.e. the response seen did not increase with concentration.

However, even if the Huggett et al. (2002) data are discounted, in other experiments where fish have been exposed to β-blockers (see Table 3.6), propranolol, in all studies, had an effect at lower concentrations than other β-blockers. This may be because propranolol has a log Kow of 3.48, reflecting its lipophilic nature, and so has the potential to bioaccumulate in the fatty reserves of fish (Frielle et al., 1987). Therefore, in summary, from these data collected, propranolol appears to be the most bio-toxic β-blocker and is observed to cause chronic and acute effects to some invertebrates and fish at concentrations as low as 0.0005 mg/L (if the Huggett et al., 2002 data are included), which is within the concentration range reported for effluents and even some river waters.

3.1.4 Fish as ecotoxicological models

Fish have been used in toxicity tests for the last 140 years, and since the Second World War their use has increased in acute and chronic testing (Ankely & Villeneuve 2006). Today, it is generally the smaller fish such as zebrafish (*Danio rerio*), Japanese medaka (*Oryias latipes*) and the fathead minnow (*Pimephales promelas*) that are most commonly used in laboratory ecotoxicology tests. Such tests include acute 48 hour or 96 hour lethality tests using juvenile fish to screen toxicity of new and existing drugs, or as a range finder for longer chronic tests. Partial life-cycle tests are also conducted to measure endpoints at different stages of a life-cycle. These tests are advantageous in that they are shorter and less expensive than conducting whole life-cycle tests. Examples of established partial life cycles tests include the 30 day early life-stage test, where embryos are grown to assess survival, hatching success and growth. Alternatively, a seven day larval survival and growth test can be conducted using less than 24 hour old embryos. Another partial life-cycle test is the 42 day reproduction (or pair breeding) test, which is used to assess any effects on fecundity, fertility, hatchability, plasma sex steroids, vitellogenin production,

gonadal histopathology, alterations in secondary sexual characteristics, growth and overall condition of the fish (Ankely & Villeneuve 2006).

Fathead minnows belong to the Cyprinidae family of fish, which contains 21,000 species distributed in North America, Eurasia and Africa, and can be found in many habitats ranging from big lakes to small streams. They are opportunistic omnivores and can tolerate a wide range of water qualities including variation in pH, alkalinity, hardness, turbidity and temperature (Ankely & Villeneuve, 2006). Fathead minnows are widely used in laboratory chronic reproductive toxicology tests, as they can easily be induced to reproduce in captivity (Thorpe et al., 2007). They have also been shown to be good predictive species in that results obtained can often be extrapolated to other fish species, (Ankely & Villeneuve, 2006). The consistency in reproductive capacity between spawning pairs can be less than 30 % when eggs are collected correctly, using a mesh and collection tray underneath the spawning tile, irrespective of their original source, age or body size, and this consistency has been demonstrated to be maintained over the course of a 42 day pair breeding study period (Thorpe et al., 2007).

3.1.4 Aim of exposure experiment

The aim of the experiment was to see if environmentally relevant concentrations of DL-propranolol-HCl had any effect on the fathead minnow (*Pimephales promelas*), either in respect to the reproductive performance, or with respect to other parameters, such as the overall condition of the fish, by conducting two reproductive partial life cycle tests. The production of viable offspring is crucial to the survival of any population and so hatchability trials were also conducted using the eggs from the adults exposed to the test concentrations of propranolol (Thorpe et al., 2007).

3.2 Materials and Methods

3.2.1 Experimental set up

Prior to setting up the exposure room, the walls and surfaces of the room were washed down with Pinefresh and hosed down with water. The header tanks were bleached, and the bleach left overnight to kill any algae that may have accumulated in the system from previous experiments. Each glass fish tank was scrubbed down with Scale-Free and washed and rinsed in the tank washing machine. Each flow meter was dismantled and put through the washing machine, after which fresh PTFE tape was put around the screws. Before introducing any fish, water was run through the tanks for 48 hours.

The exposures were carried out using a flow through system in a room that contained two series of identical tanks. Thermostatically heated, dechlorinated tap water from a header tank flowed through 6 flow meters into 6 mixing chambers, via medical grade silicon tubing (VWR, Leicestershire). From these the water went to 6 fish tanks through silicone rubber tubing (VWR, Leicestershire). In total there were 36 fish tanks which each had a working volume of 10.5 litres. Each tank was aerated (see Figure 3.2). The flow meters were set to deliver 400ml of header tank water per minute to each mixing chamber, and by regulating the flow of water into each fish tank via a clamp on the connecting tubing from the mixing chamber, each tank received 66 ml of water per minute. This equated to each tank receiving roughly 9 tank renewals per day.

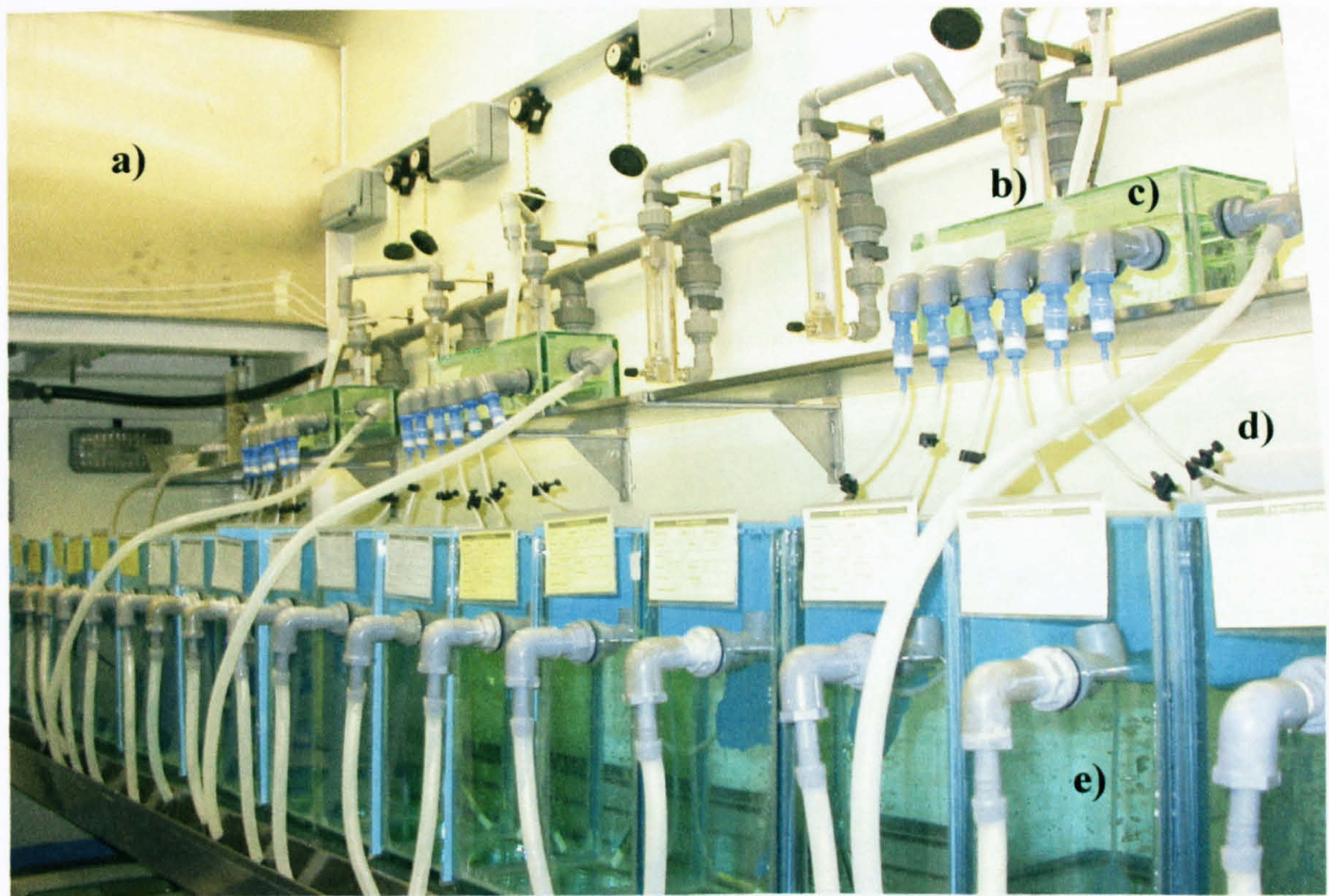


Figure 3.2. A photograph of one side of the experimental room. **a)** shows the header tank. **b)** indicates one of the flow meters. **c)** shows a distribution chamber. **d)** shows one of the clamps regulating the flow from the distribution chamber to the fish tanks. **e)** indicates a fish tank.

To regulate the dosing of the test substance, propranolol-HCL, during the exposure period, a peristaltic pump was set up and 4L amber stock bottles (Sword Scientific, Warwickshire), containing different stock concentrations of propranolol HCl, were prepared. These stock concentrations were pumped from the stock bottles through 0.16mm tubing (Watson Marlow, Cornwall) to a peristaltic pump set at 56 rpm, and out through peroxide cured silicon tubing (Watson Marlow, Cornwall) to the distribution chambers (see Figures 3.3 and Figure 3.4). Magnetic stirrers (Fisher Scientific, Leicestershire) were placed under each stock bottle, to ensure continual mixing of the contents of the stock bottles, and under each mixing chamber, to keep a constant concentration of propranolol HCl entering the fish tanks (i.e. to mix the header tank water (40 ml/min) and the propranolol HCl (1 ml/min) in the mixing chamber). In the second experiment, the peristaltic pump was switched on one week prior to dosing and header tank water was pumped through the system. This ensured a more stable flow and hence less fluctuation in the flow was encountered during the second experiment.

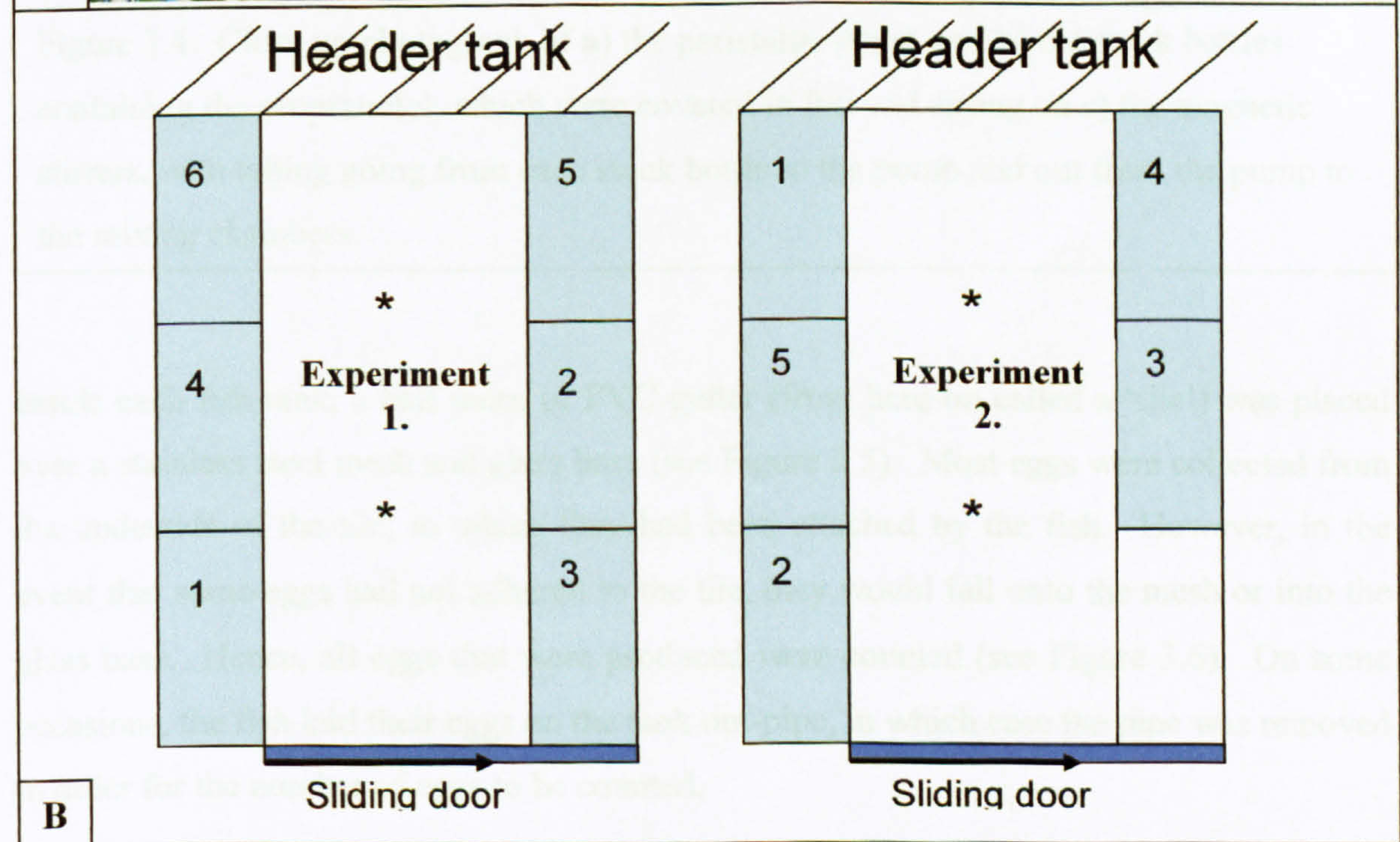


Figure 3.3. The design and lay out of the exposure room. **A.** Photograph of the exposure room with the peristaltic pump and stock dosing bottles shown by the arrow →
B. Diagram of the location of each test concentration in the exposure room for each experiment. Each block ( ) represents 6 tanks. 1 = DWC, 2 = 0.001mg/L, 3 = 0.01mg/L, 4 = 0.1 mg/L, 5 = 1.00mg/L, 6 = 10 mg/L (10mg/L was only tested in the first exposure). * shows the location of the air vents in to the room.

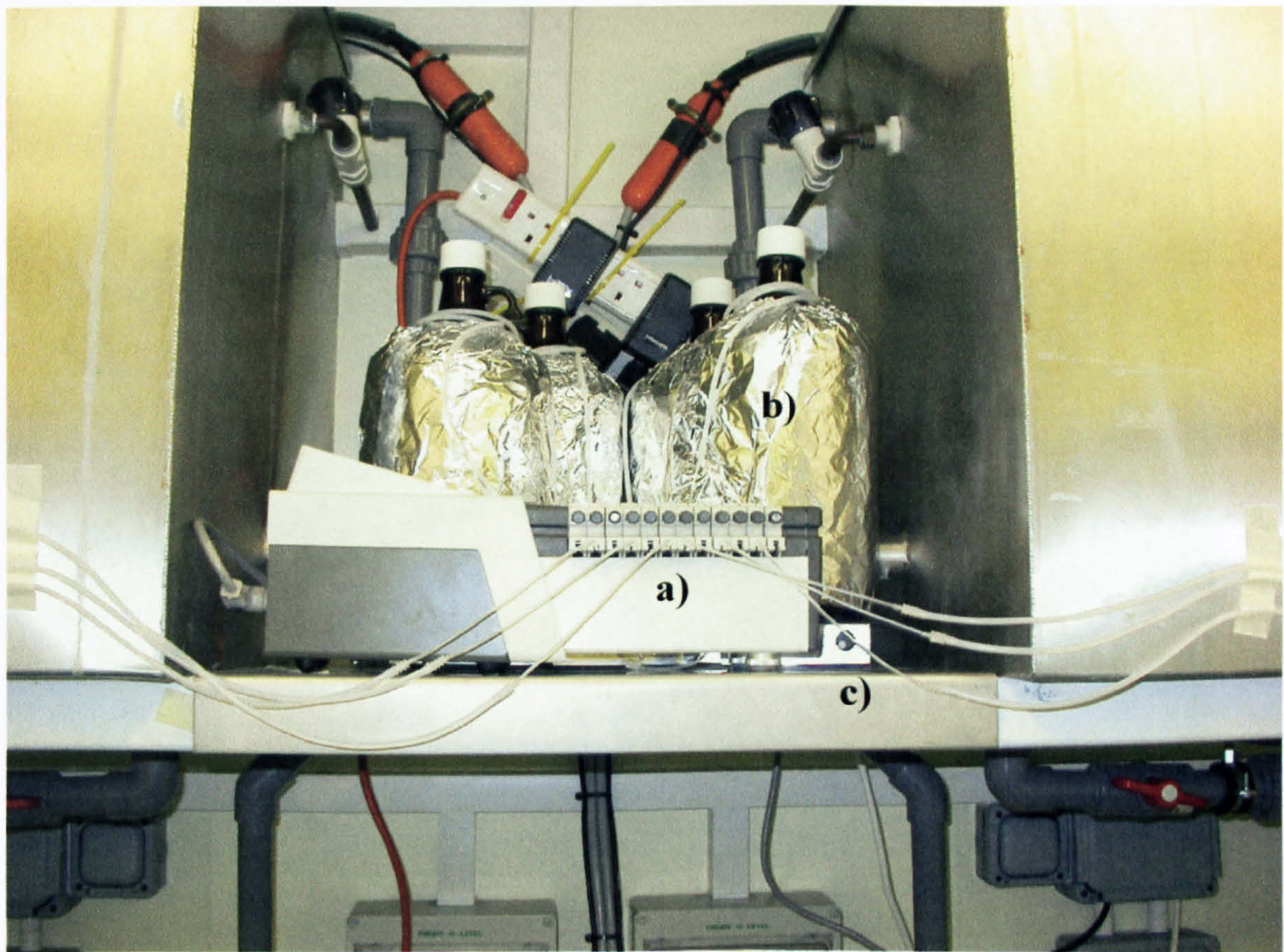


Figure 3.4. Close up photograph of **a)** the peristaltic pump and **b)** the stock bottles containing the propranolol, which were covered in foil and sitting on **c)** the magnetic stirrers, with tubing going from each stock bottle to the pump and out from the pump to the mixing chambers.

Inside each fish tank, a half piece of PVC gutter (from here-on called a 'tile') was placed over a stainless steel mesh and glass base (see Figure 3.5). Most eggs were collected from the underside of the tile, to which they had been attached by the fish. However, in the event that some eggs had not adhered to the tile, they would fall onto the mesh or into the glass base. Hence, all eggs that were produced were counted (see Figure 3.6). On some occasions, the fish laid their eggs on the tank out-pipe, in which case the pipe was removed in order for the number of eggs to be counted.

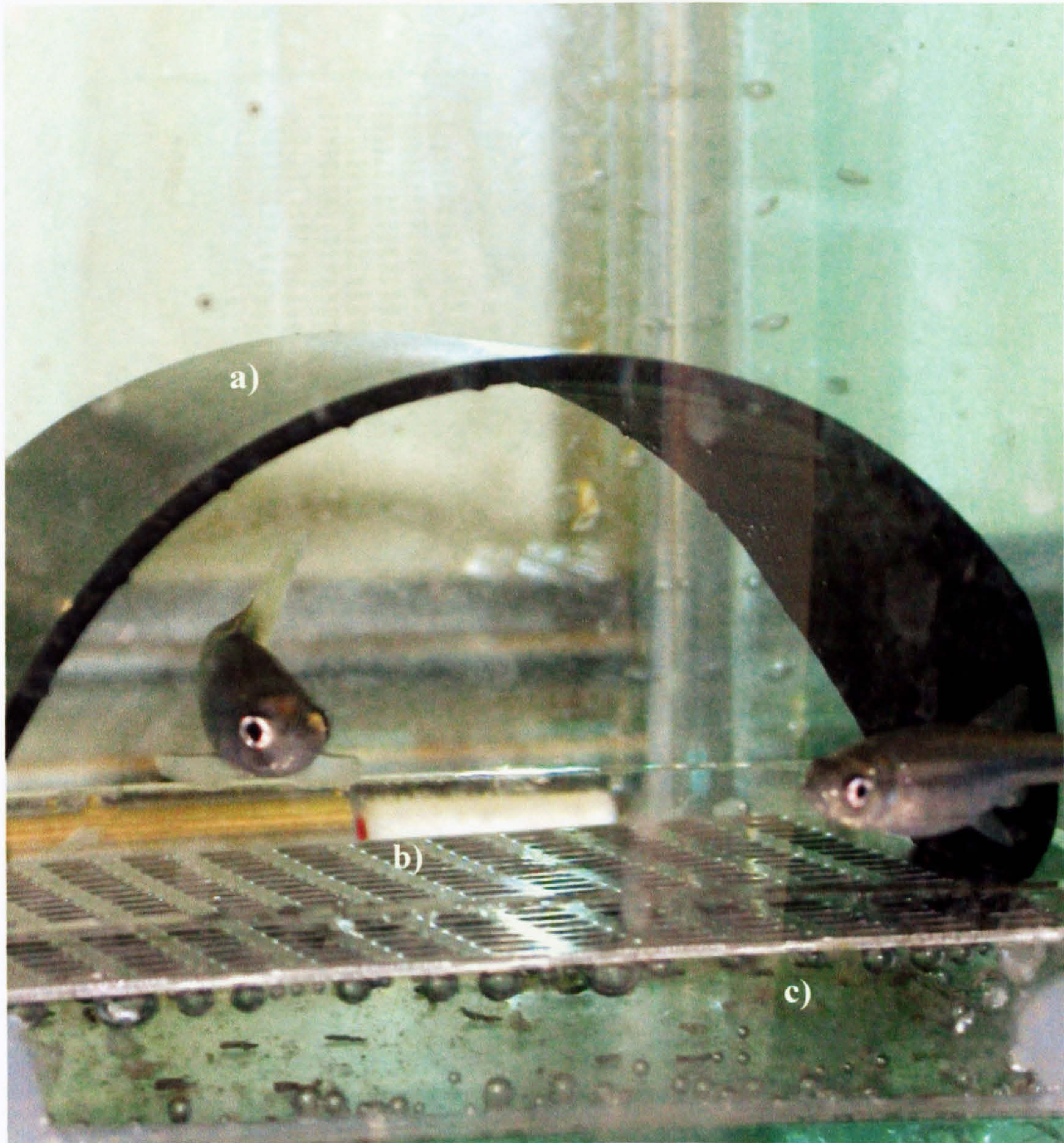
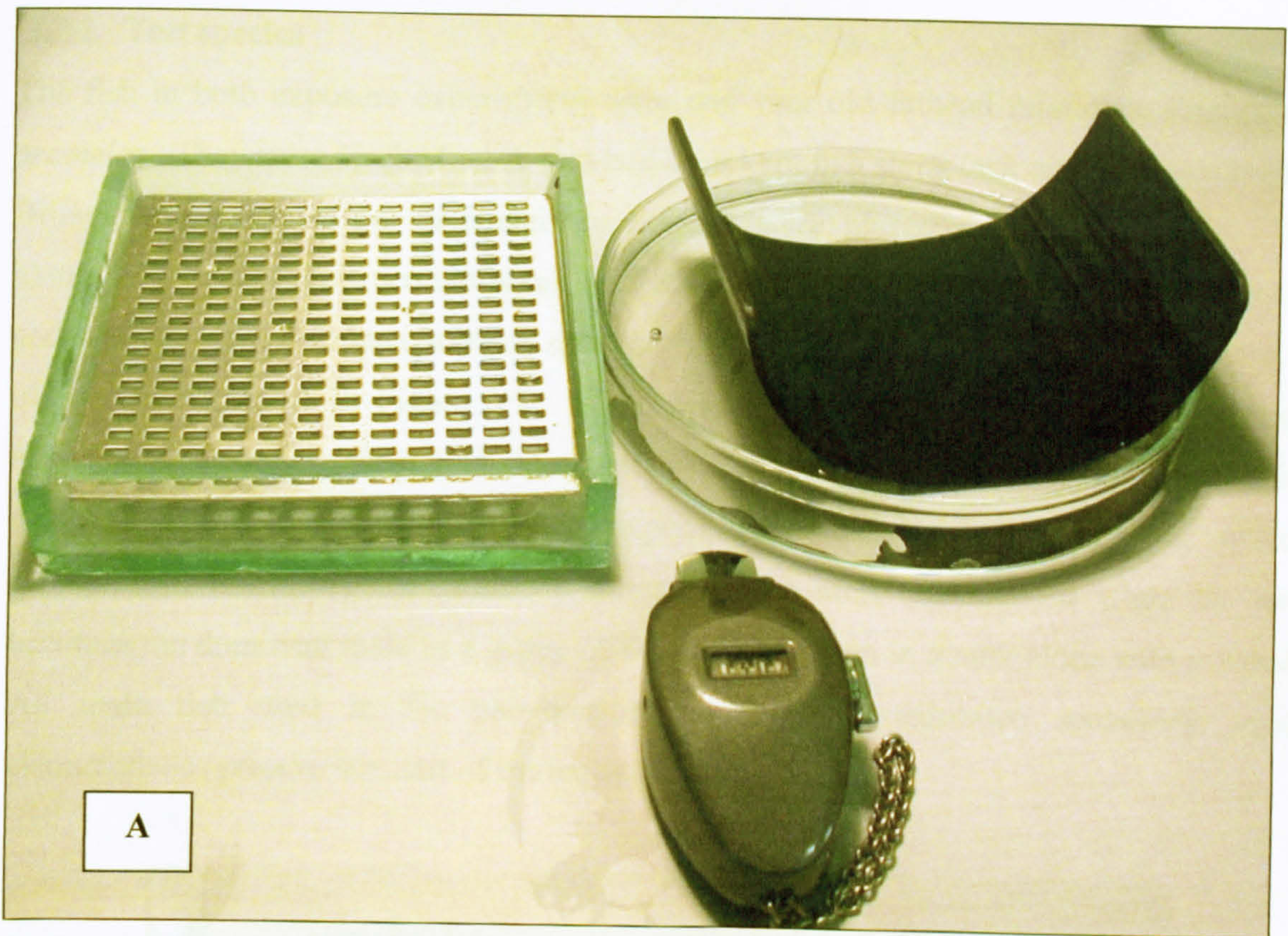


Figure 3.5. Male and female fathead minnow. In each fish tank there was **a)** a half piece of gutter (tile), **b)** steel mesh and **c)** a glass base. Collectively this apparatus allowed all the eggs produced to be recovered and counted.



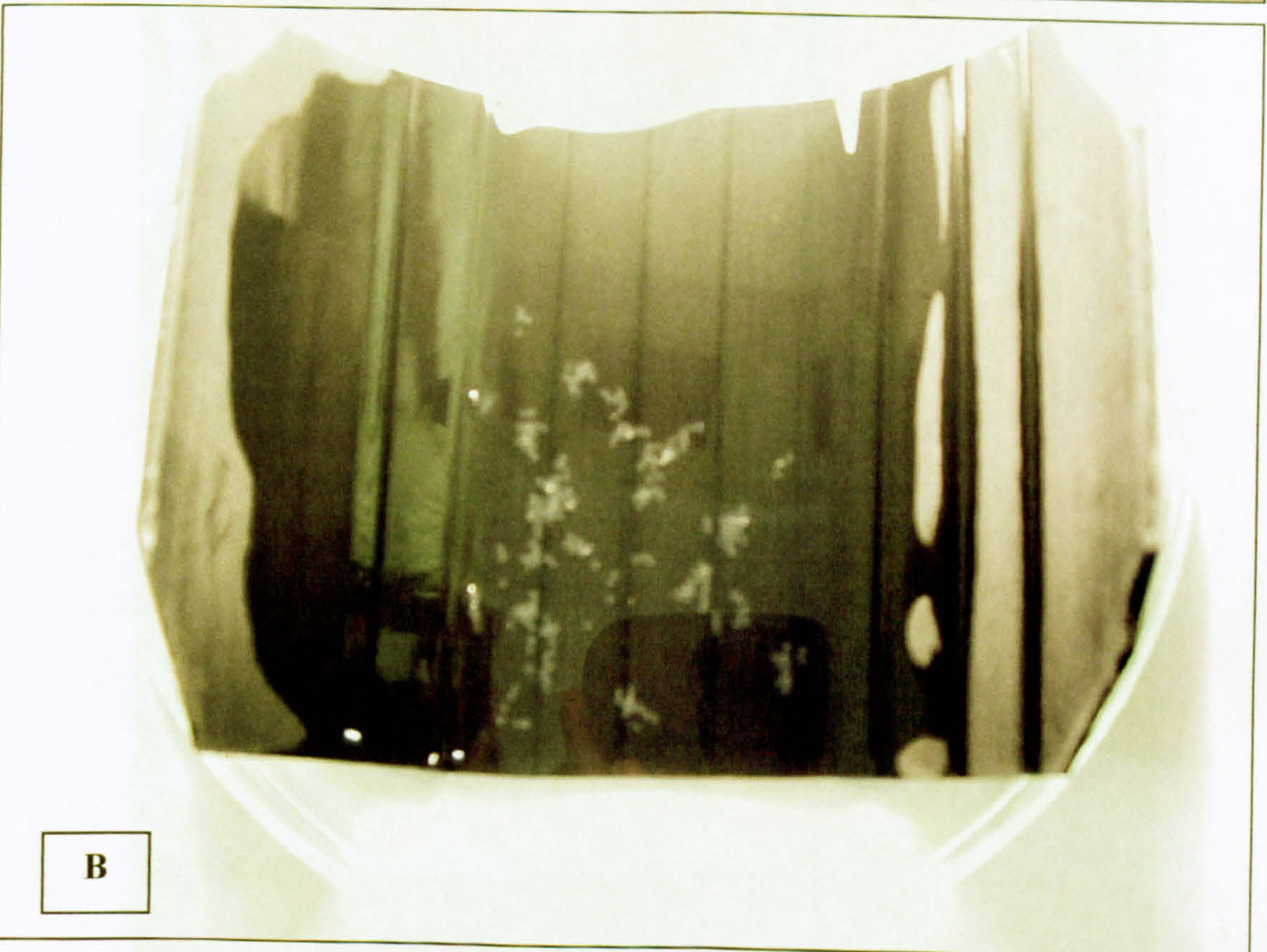


Figure 3.6. **A.** The eggs from the tile were counted under water using a ticker counter. The eggs from the mesh and the base were also included in the count.
B. A close up of eggs on the tile.

3.2.2 Test species

The fish in both exposure experiments were one year old fathead minnows, *Pimephales promelas*. They were virginal stock that had been kept in a stock tank and had been bred at Brunel University. One male and one female were placed in each fish tank. The identifying features between the sexes are that the males tend to be larger than the females and when not stressed have a black spot on their pectoral fin, whilst the females have an ovipositor, as shown in Figure 3.7. When sexually mature, the male shows secondary sexual characteristics; these include a vertical dark stripes, and a soft, mucus–secreting dorsal pad, known as a fatpad, with large nuptial tubercles on the front of his head (Ankley et al., 2001, Smith, 1978). These features become more pronounced when the male becomes the dominant male in a group of fatheads, or when in a tank alone with a female. All male fish used in the pair-breeding experiments exhibited secondary sexual characteristics prior to the start of the experiments.

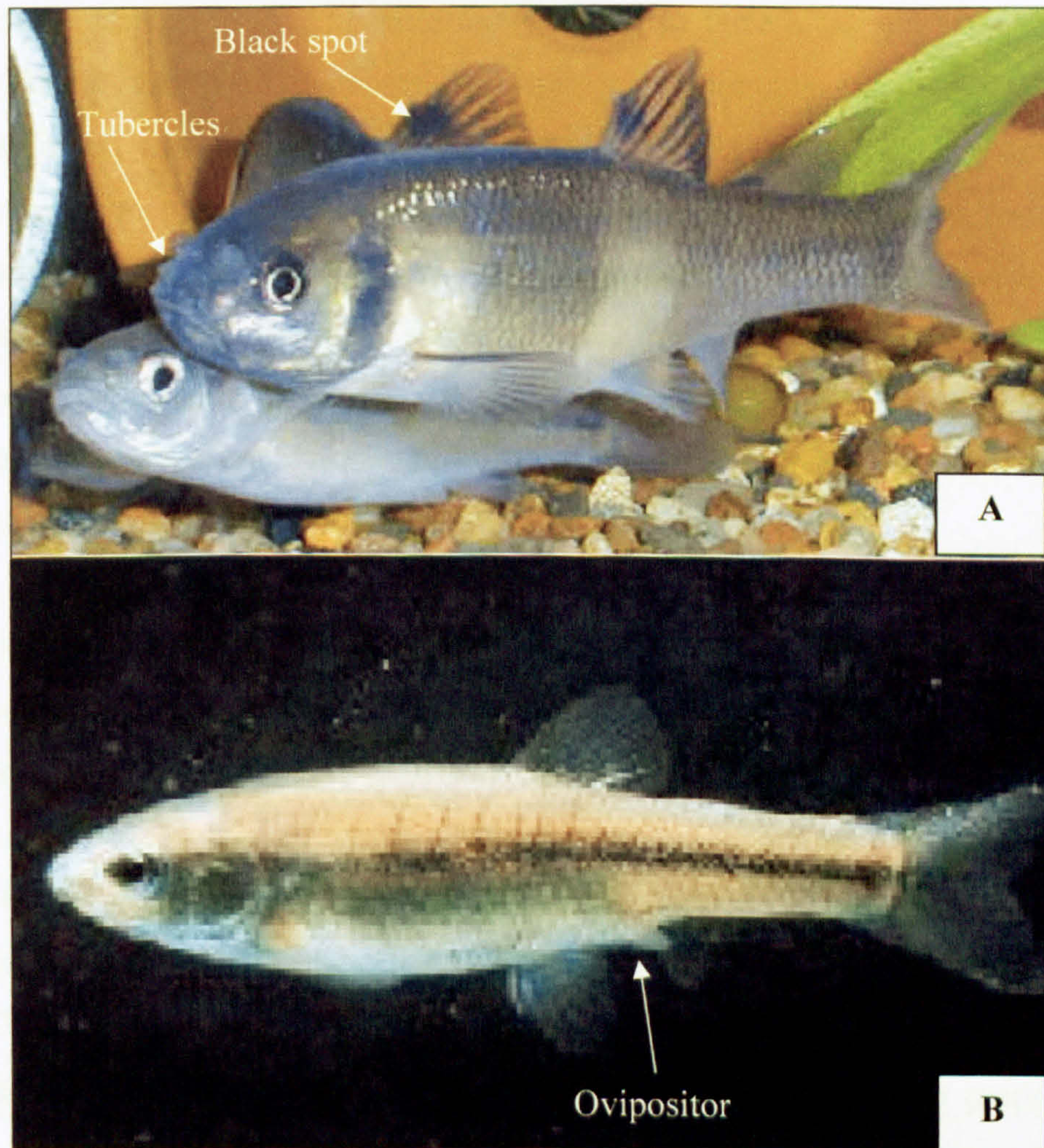


Figure 3.7. **A.** Two male fathead minnows. The male in the forefront is exhibiting secondary sexual characteristics and has the characteristic black spot on his pectoral fin. Image from www.aqualandpetsplus.com/Live%20Food,%20Fathead%20Minnows.htm

B. A female fathead minnow with ovipositor.

Image from www.dnr.state.wi.us/org/water/wm/ww/biomon/

During the breeding season, a sexually mature male will use his tubercles to deter intruders in an effort to defend his territory, and his fatpad to clean the spawning surface, in this case the tile (Smith, 1978). When the female is ready to spawn, the male and female move round the tank together, accompanied by close lateral contact and body vibration between the male and female (Ankley et al., 2001). After sufficient stimulation, the male lifts and presses the female's ventral surface against the underside of the spawning object (Thorpe et al., 2007). The female then releases her eggs on the underside of the tile and the male fertilises them simultaneously. The male then expels the female from the area and stays underneath the tile, guarding and preening the eggs, removing any dead ones and any air bubbles with his fatpad (Harries et al., 2000). If the eggs are removed, the pair will breed again, usually within 3 to 7 days.

3.2.3 Test substance and concentrations

The experimental design incorporated two separate replicate experiments. This was partly due to a lack of space in the exposure rooms, but also for ethical and statistical reasons. From an ethical standpoint to repeat two smaller experiments, rather than conduct one large experiment, was preferable because it meant that if no effect whatsoever was found in the first experiment, then the second experiment need not be undertaken, hence reducing the number of fish used. Secondly, on ethical grounds, if a particular concentration of the test substance evoked a toxic effect, then that particular concentration need not be repeated in the second experiment, hence less fish are put under duress. For statistical reasons, two separate experiments allow the results of the two experiments to be compared, so if any effects occur, and are repeatable, the results will be more robust.

Propranolol (DL-Propranolol hydrochloride, *1–isopropylamino-3-(1-naphthyloxy) proprano-2-ol hydrochloride*) (CAS 318-98-9) is a racemic mixture that is 99 % pure. Propranolol was obtained from AstraZeneca, Brixham, and was stored in a plastic container in a fridge. Since it is light sensitive, a sample was taken and stored in a foil wrapped beaker from which the stock solutions were made. A new sub-sample was set up for each experiment. The amount of propranolol added to a 4 L stock bottle was as set out in Table 3.7. Once measured out, header tank water was added to the bottle to make it up to 4 L, and the bottle swirled to mix the propranolol and water. Because propranolol-HCl is light sensitive, each bottle was wrapped in foil before being placed on a stirrer. The tubing was placed in the bottom of each bottle so that the stock solution could be pumped by the peristaltic pump into the mixing vessel.

Nominal concentration of Propranolol-HCl (mg/L) 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

Table 3.7. Amount of propranolol-HCl added to each 4L stock bottle to make up the different stock concentrations of propranolol-HCl. The stock was then diluted prior to delivery to the fish tanks

In the first experiment, five concentrations of propranolol-HCl, namely 0.001, 0.01, 0.1, 1.0 and 10 mg/L, were used, together with a water control. In the second experiment this was reduced to 4 concentrations of propranolol-HCl, which were 0.001, 0.01, 0.1, and 1.0 mg/L, together with a water control. There were six fish tanks per concentration; four of these had a male and female pair in them and the remaining two tanks were used for egg hatchability trials.

3.2.4 Pair-breeding test

One male and one female fish were placed in each tank and left largely undisturbed for 3 days, during which the only disturbance was for feeding; this was known as the acclimation phase. Following on from this, breeding compatibility was determined, during which fish were monitored and daily records made of when each pair spawned. Not all pairs spawned, and if this was the case, then they were swapped for another male and female pair. More fish were paired up than were actually required for the experiment, and so criteria were needed to select the pairs that were to be used for the experiment. These criteria were that the pair needed to have spawned at least twice, with a gap between successive spawning being not greater than seven days. The pairs of fish that were the first to meet these criteria in each treatment group were selected for the experiment and the 21-day baseline period began. During the baseline period, only header tank water entered the tanks and the number of eggs produced during each spawning was recorded. Data and reproductive output of each pair were collected at the same time daily, at 12 am, noon (±1 hour). After 21 days, a transitional phase occurred when propranolol-HCl was introduced to the tanks. The tanks containing breeding pairs were divided into groups of six tanks, which was done by drawing paper out of a hat to ensure random placement of individual

pairs within the treatment groups. The transitional phase lasted for three days and allowed the concentration of propranolol to reach the desired value, after which the fish were maintained for a further 21 days at these concentrations. During the transitional phase no eggs were collected or counted and the only disturbances to the fish were feeding and close external monitoring in case signs of distress developed from the introduction of the drug. Following this period, the 21 day exposure period started, during which the same parameters were measured as in the baseline period. Figure 3.8 summarises the pair-breeding test protocol. There were four pairs of fish in each treatment group.

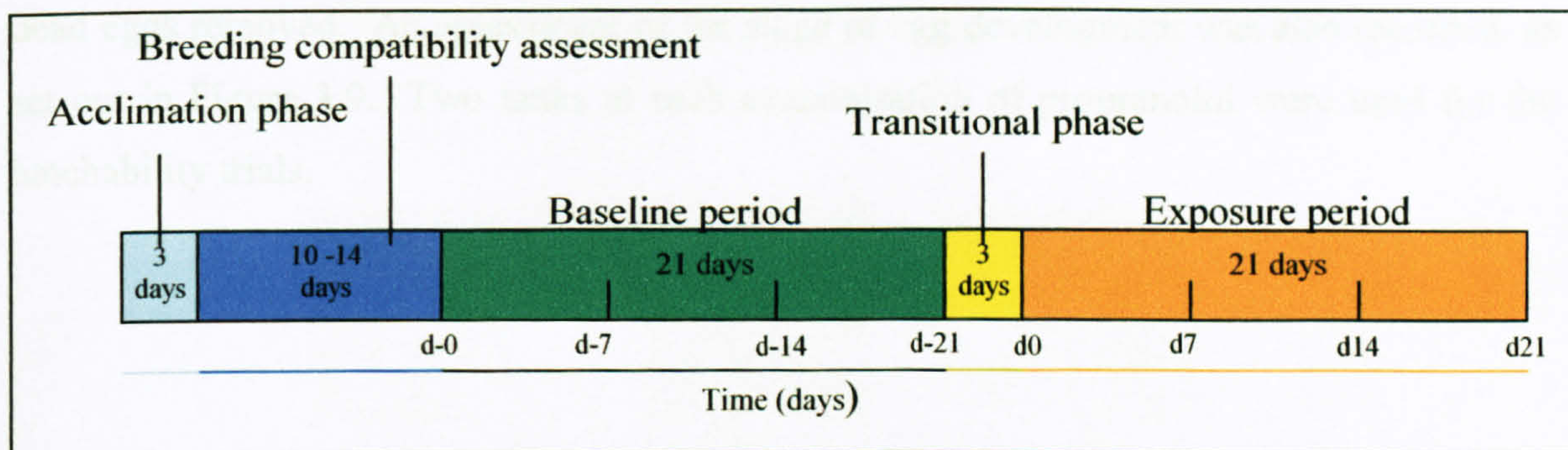


Figure 3.8. Protocol of the pair-breeding test, with the different phases shown.

After 21 days of exposure to propranolol-HCl, the fish were terminated using an overdose of ethyl 3-aminobenzoate methanesulfonate (MS-222) (Sigma, Dorset), under a schedule I procedure. Tissue samples (liver, gonad, brain, heart) were taken for RT-PCR analysis and blood samples were pooled at each concentration for analysis of the concentration of propranolol-HCl in the plasma. Wet weight, fork length, liver weight, gonad weight and fatpad weight were recorded. The number of tubercles and the prominence of tubercles were determined using the qualitative scheme set out by Smith (1978) (see Table 3.8 for details).

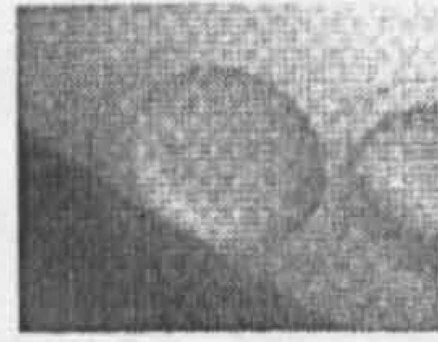
Denotation	Description
0	No visible sign of tubercles
+	Tubercles visible as white disks, not protruding above body surface
++	Tubercles project above body surface
+++	Tubercles are prominent, but not sharp
++++	Tubercles are prominent and sharp

Table 3.8. The qualitative scheme (as devised by Smith, 1978) used to assess the degree of tubercle development at the end of the 21d exposure period.

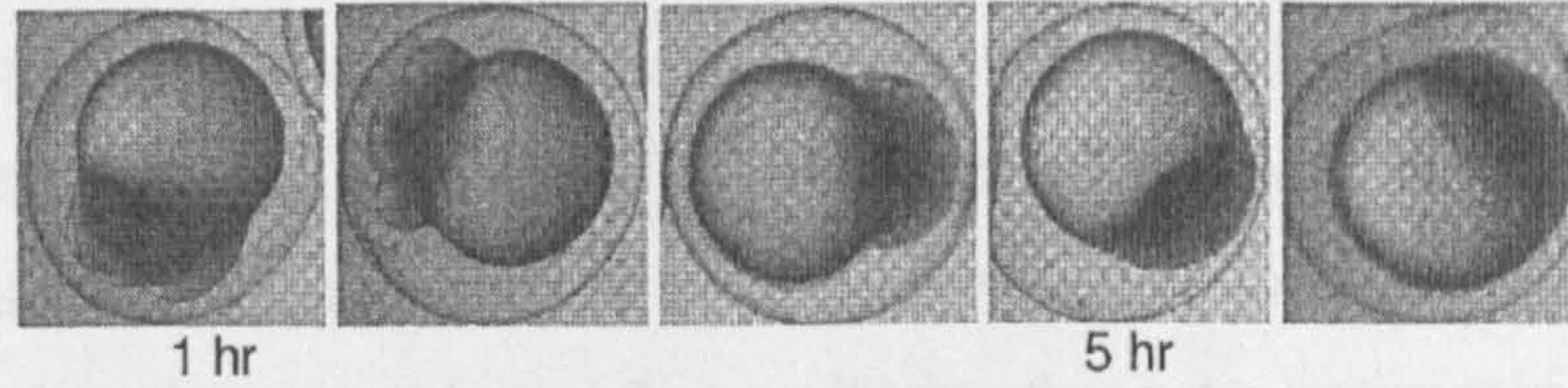
3.2.5 Hatchability trails

To assess whether the eggs produced by each pair were viable (i.e. successfully produced live young), hatchability trials were undertaken. After the first successful spawn in the baseline period, fifty eggs were left on the tile from the next spawn and this tile submersed in another tank in a wire meshed cage that was suspended in the tank, so that the eggs were covered by water. The new tank was from the same treatment group, i.e. the developing eggs were exposed to the same concentration of propranolol as were the adult fish that laid the eggs. The eggs were checked and counted daily and any abnormalities recorded and dead eggs removed. An assessment of the stage of egg development was also recorded, as set out in Figure 3.9. Two tanks at each concentration of propranolol were used for the hatchability trials.

0) Unfertilised egg

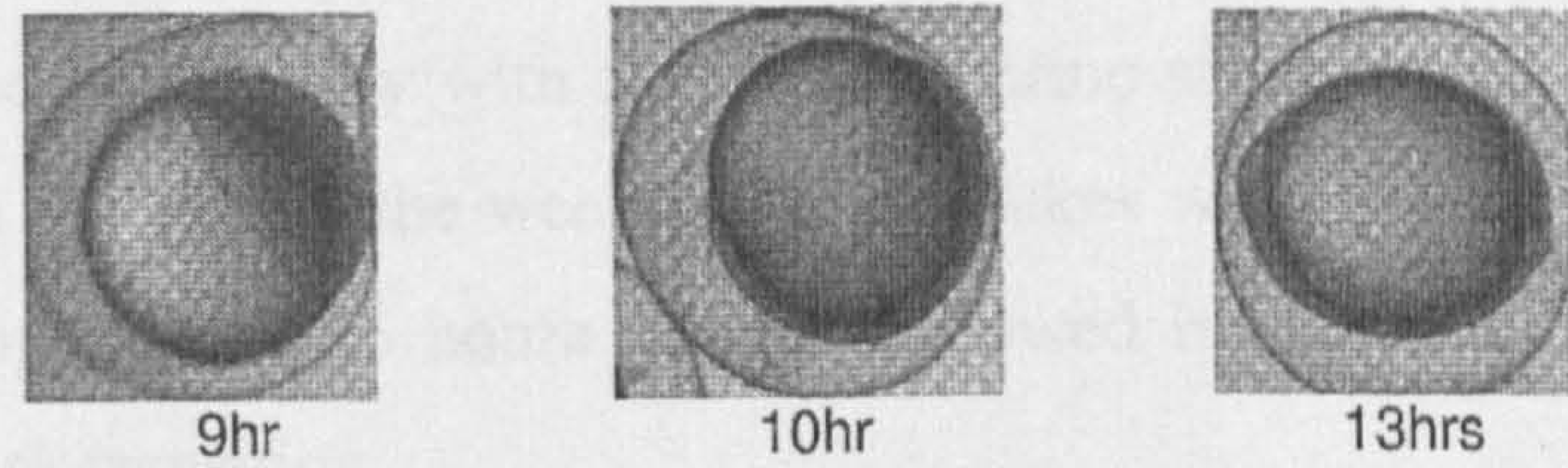


1) Blastulation



1 hr 5 hr

2) Completion of gastrulation

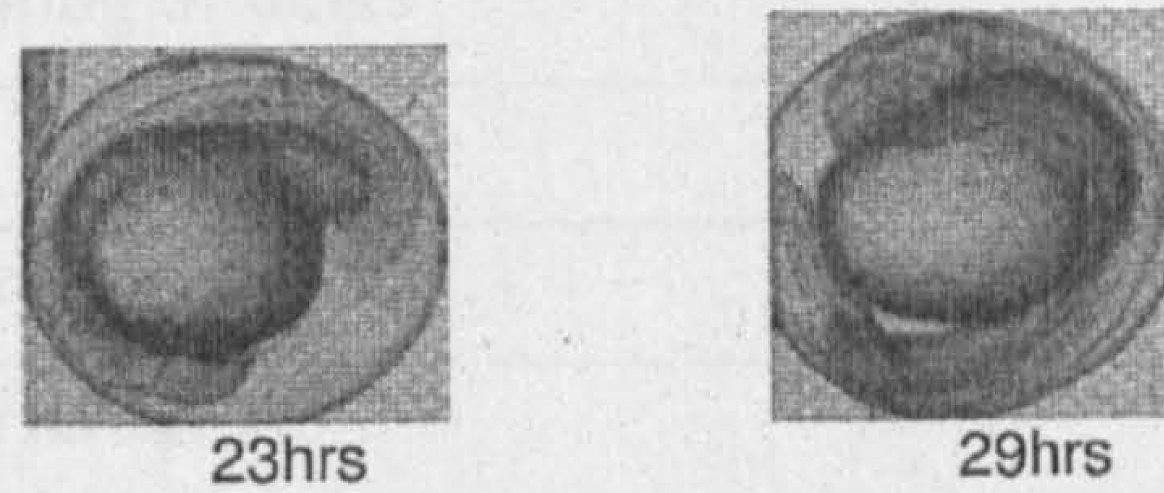


9hr 10hr 13hrs

3) Completion of somites and optic cup formation

15hrs 17hrs

4) Spontaneous movement and tail detachment



23hrs 29hrs

5) Retinal pigmentation/heart beating/body pigmentation

46hrs 69hrs 78hrs

6) Commencement of hatching

Figure 3.9. Photographs of the different stages of egg development. (Taken from the PhD thesis of Tamsin Runnalls, 2005).

3.2.6 Water, light and feeding parameters

The water temperature was set at 25°C ± 1, and this was monitored twice weekly. Flow into the mixing chamber was set at 400 ml/min and was checked twice weekly. The flow into the fish tanks was 66 (± 10 %) ml/min, which was checked twice weekly, and was altered by a clamp on the connecting tubing. The dissolved oxygen concentration was monitored weekly and kept above 75 % saturation. Water hardness and pH were also checked weekly and recorded. Tanks were cleaned twice weekly, during which the base, mesh and tile were removed and cleaned in hot water, and any debris siphoned out. This meant that at least half a tank of water was changed every time the tank was cleaned. Table 3.9 summarises the protocol used for monitoring the water parameters.

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 fish received a photoperiod of 16 hours of light followed by 8 hours of darkness, with a 20 minute dawn: dusk transition.

Parameter	Frequency parameter was monitored
Header tank flow	Daily
Peristaltic pump flow (during dosing)	
Temperature	Twice weekly
Flow into tanks	
Cleaning of tanks	
pH	Weekly
Dissolved oxygen	
Hardness	

Table 3.9. Summary of the monitoring and maintenance of water parameters throughout the pair-breeding experiments.

3.2.7 Collection of water and plasma for analysis of propranolol concentrations

20ml water samples were collected from 3 tanks per treatment group on days 0, 4, 7, 14 and 21 during the exposure period in 40 ml amber glass vials (Sigma, Dorset) that had been washed twice with methanol and once with ethanol. These were stored in the freezer until analysis, which was carried out by AstraZeneca, Brixham, UK.

The plasma was collected on termination of the fish at the end of the 21-day exposure period. Blood was pooled at each concentration, per sex, to ensure there was enough plasma for analysis. Each sample was then spun in a centrifuge for 10 minutes at 2,500rpm, after which the plasma was carefully pipetted off and stored at –80 ° C until analysis, which was carried out by AstraZeneca, Brixham, UK.

3.2.8 Statistical analyses

Statistical analysis of the water chemistry results, using a t-test or Mann-Whitney rank sum test, showed that there were no statistically significant differences at each concentration between experiments. Hence, the results from the first and second exposure experiments were pooled for each concentration and the data checked for normal distribution and equal variance using the Kolmogorov-Smirnov test and Levene Median test, respectively.

To test the null hypothesis that there were no statistically significant differences in cumulative egg production between the baseline and exposure periods within each treatment group, a parametric t-test was used. In cases where data were not normally distributed or of equal variance, a non-parametric Mann-Whitney rank sum test was performed.

To test the second hypothesis that during the exposure period there were no differences in cumulative egg production between any of the different treatment groups, a One Way Repeated Measures ANOVA was used. This test is a parametric test that assumes that all treatment effects are normally distributed with the same standard deviations (variances). Lots of individual t-tests could have been used to test this hypothesis, but by using an ANOVA, it avoids introducing a Type II error, otherwise known as an α error. A 5 % error is introduced during each test, whereby there is a 5 % chance of concluding that there is no difference between treatments when one exists. So instead of carrying out lots of t-tests, a one-way ANOVA was carried out on the exposure data. If a statistical difference was found, the ANOVA was followed by a post-hoc test. The test chosen in this case was a Holm-Sidak post-hoc test, to show which treatment groups were different from each other. The Holm-Sidak test can be used for both pairwise comparisons and comparisons between an exposed group versus a control group. It is more powerful than the Tukey and Bonferroni tests and is recommended as the first-line procedure for pairwise comparison testing; consequently, it is able to detect differences that these other tests do not.

Data obtained from the sampling of fish at the end of the 21-day exposure period were also analysed for significant statistical differences between treatments. Table 3.10 summarises the tests used to prove or disprove null hypotheses set up for each data group. The gonad somatic index (GSI) and the condition index (CI) were calculated as set out below.

$$\frac{\text{Gonad weight (mg)}}{\text{Wet body weight (mg)}} \times 100 = \text{GSI}$$

$$\frac{\text{Wet body weight (mg)} \times 100}{\text{Fork length (mm)}^3} = \text{CI}$$

Hatchability data were analysed for normality and equal variance. The hatchability data did not show normal distribution and so a Mann Whitney rank sum test was used to compare baseline and exposure data for each treatment group. The data for 1.0 mg/L could not be analysed as there were fewer than 2 data points

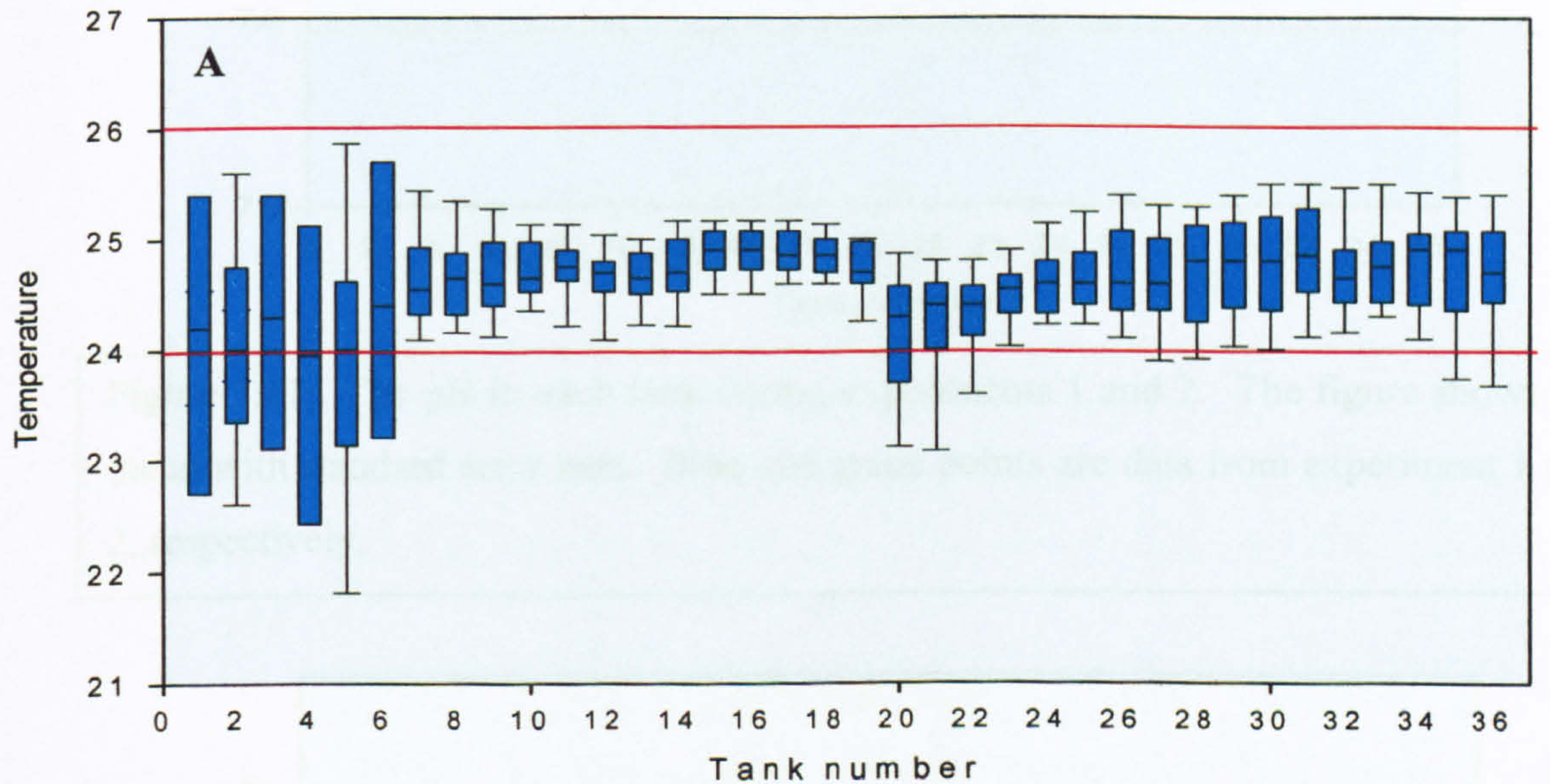
Data set	Null hypothesis	Test used	Post-hoc test used
Wet Weight	There are no differences in wet weight between treatment groups.	Parametric ANOVA	Holm-Sidak
Fork length	There are no differences in fork length between treatment groups	Parametric ANOVA	N/A
Fat pad weight	There are no differences in fat 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

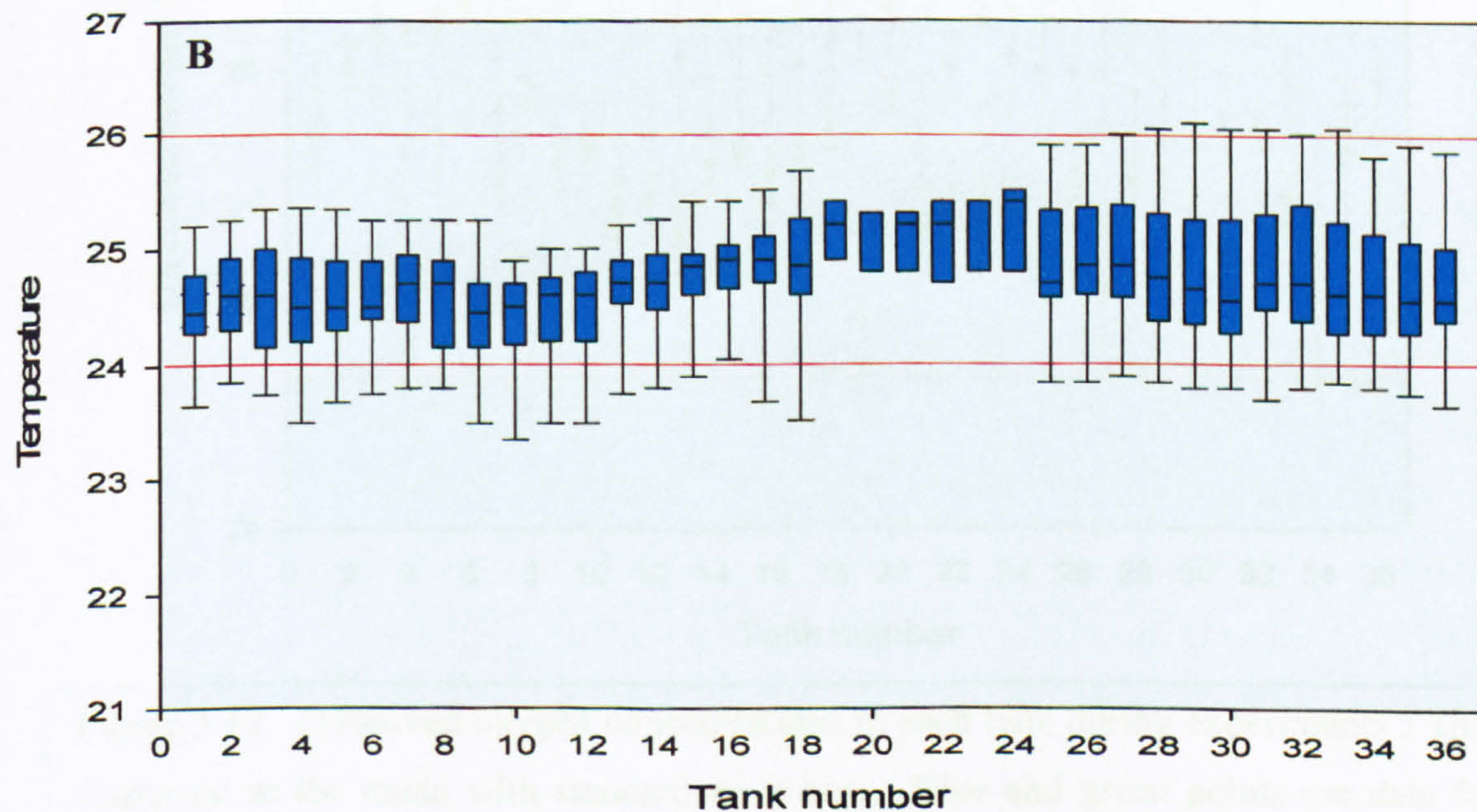
Table 3.10. A summary of the null hypothesis for each parameter assessed, together with the statistical test that was applied, and where applicable, the post-hoc test used to accept or reject each null hypothesis.

3.3 Results

3.3.1 Water parameters

Figures 3.10 to 3.13 show the data on the different water parameters that were measured routinely during both experiments. In some tanks the temperature dropped below the set limits, which was either due to hot weather, which caused the in-house chiller units to start working, which in turn lowered the room and hence the tank water temperatures, or because an air bubble got stuck in the tube running from the distribution chamber to the tank, which stopped the flow of heated water to that particular tank.





Figures 3.10. Boxplots of the tank water temperatures in experiments 1 (**A**) and 2 (**B**). The box plots show the median, 10th, 25th, 75th and 90th percentiles. Red lines represent the set temperature i.e. 25 ± 1°C.

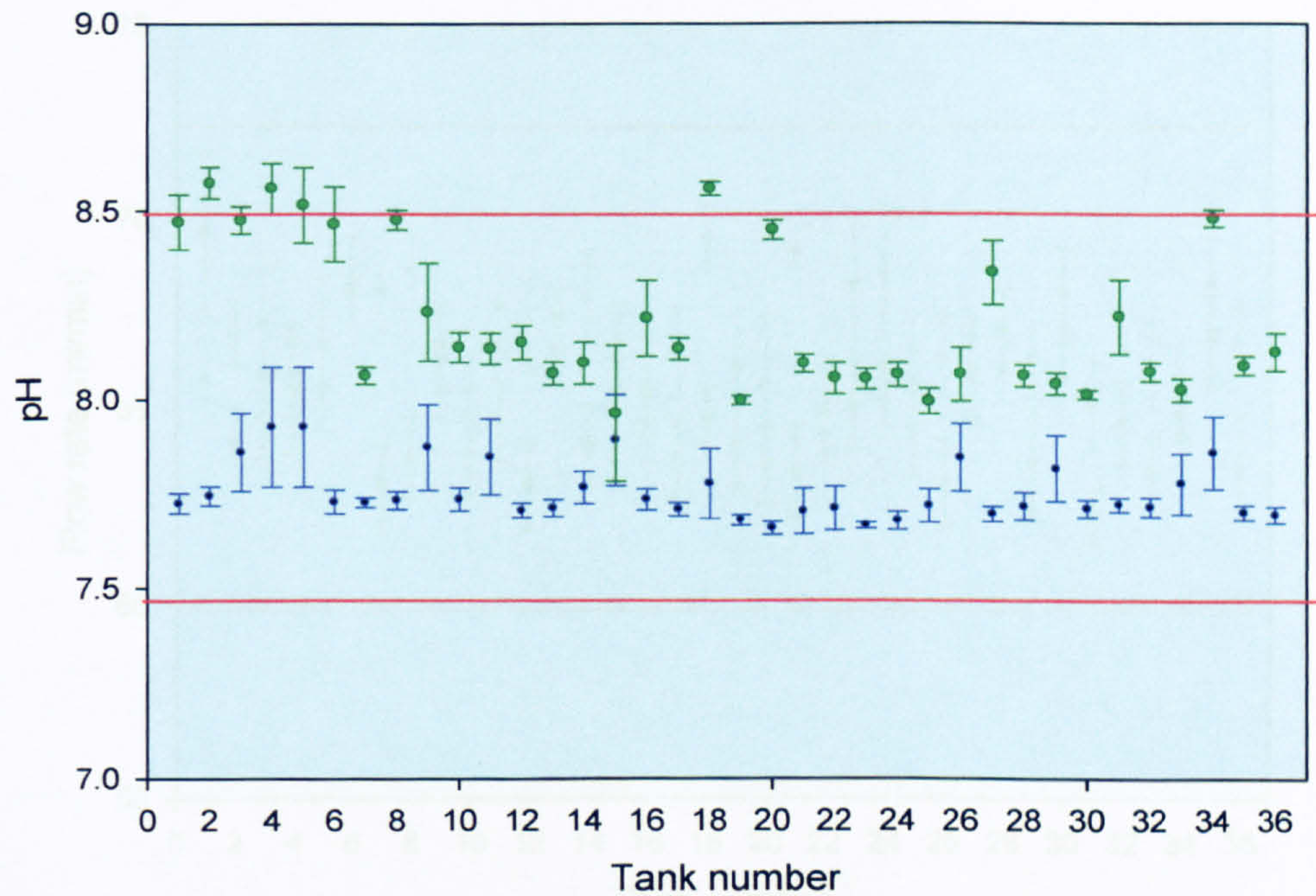


Figure 3.11. The pH in each tank during experiments 1 and 2. The figure shows the mean with standard error bars. Blue and green points are data from experiment 1 and 2, respectively.

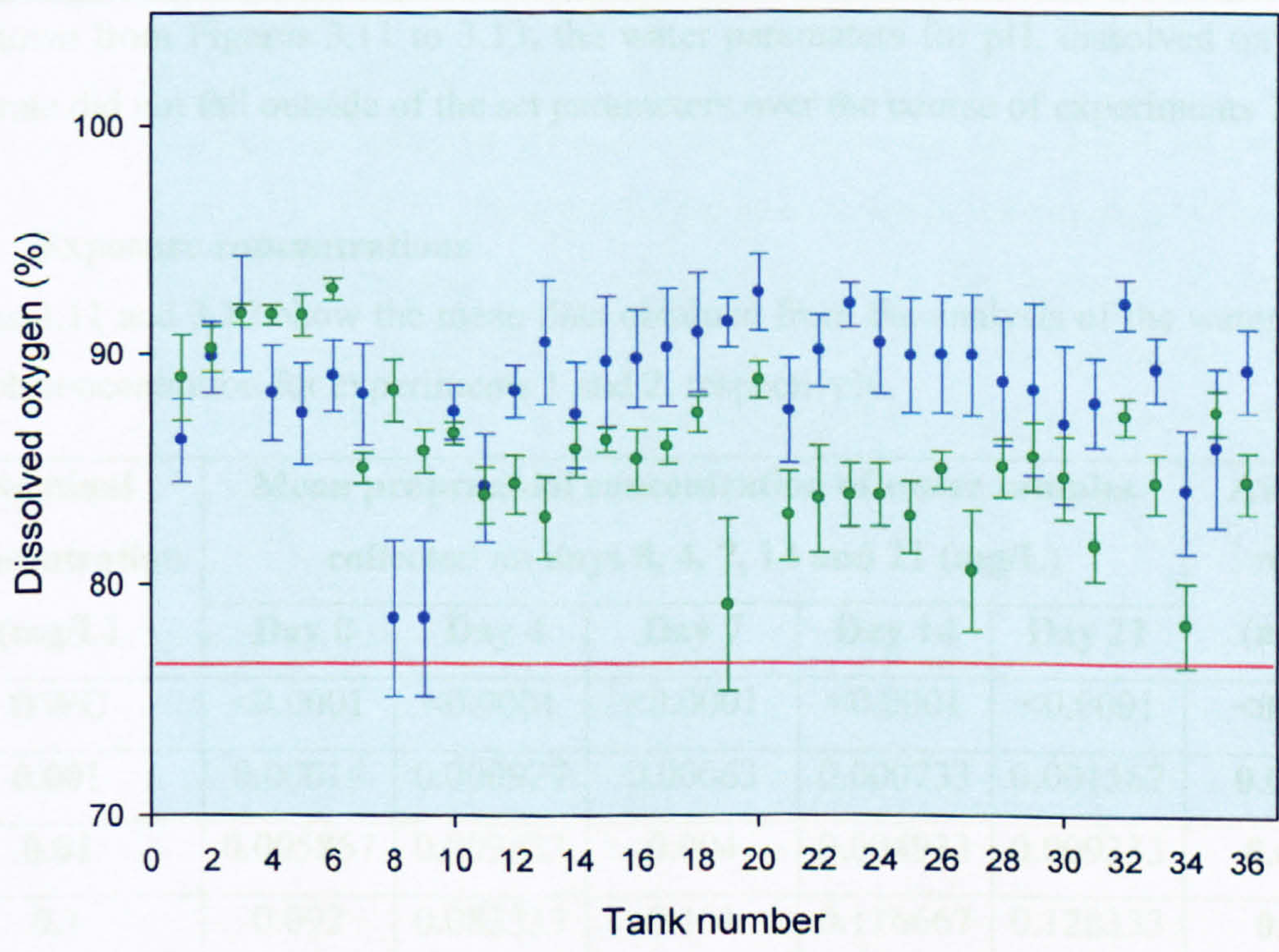


Figure 3.12. Dissolved oxygen concentrations in each tank during experiments 1 and 2, displayed as the mean with standard error bars. Blue and green points are data from experiment 1 and 2, respectively. Red line shows the limit below which the dissolved oxygen should not fall.

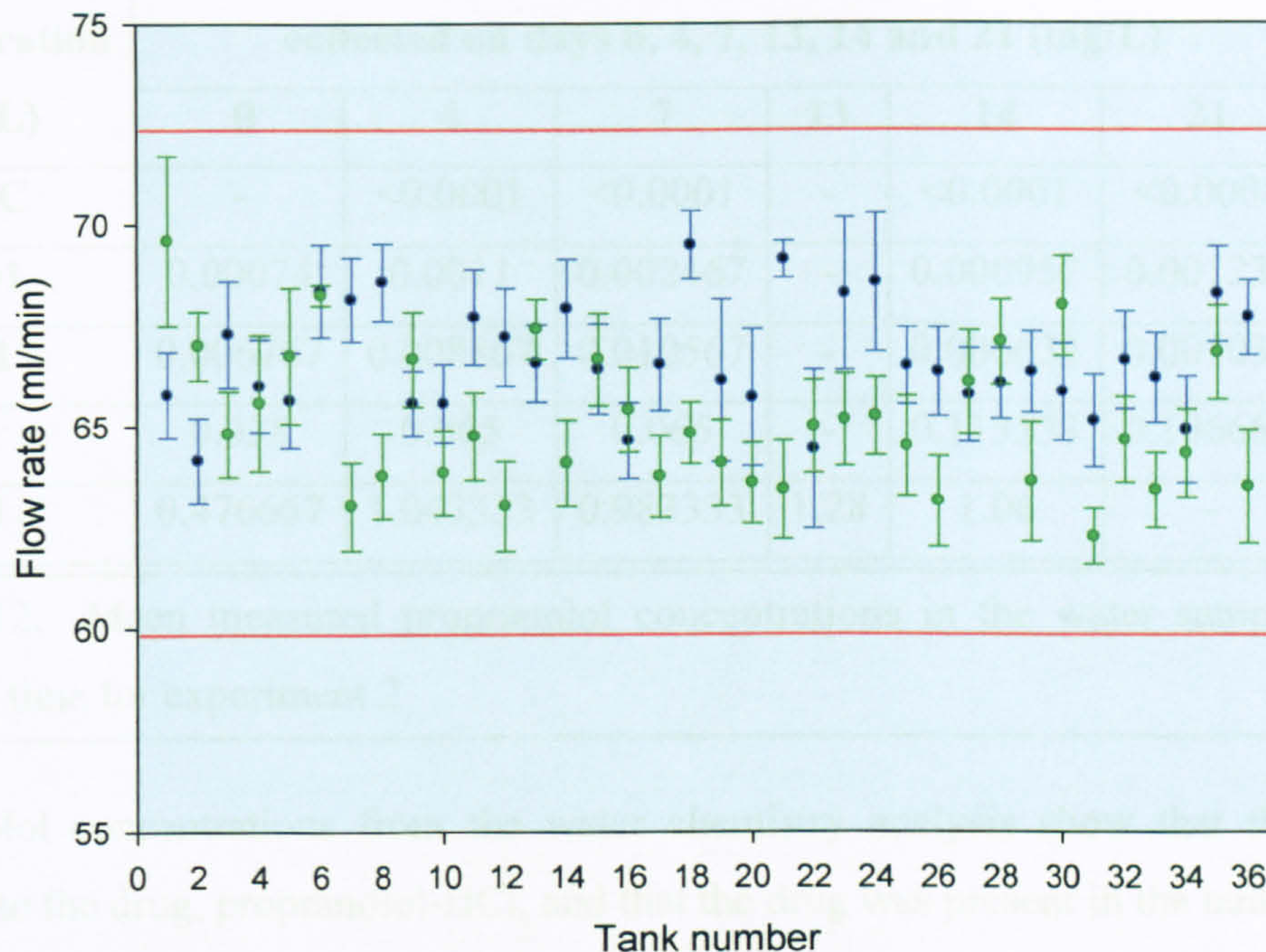


Figure 3.13. Flow rates in each tank during experiments 1 and 2, displayed as the mean with standard error bars. Blue and green points are data from experiment 1 and 2, respectively. Red lines shows the defined parameters to which the flow was set (66 ml/min ± 10 %)

As shown from Figures 3.11 to 3.13, the water parameters for pH, dissolved oxygen and flow rate did not fall outside of the set parameters over the course of experiments 1 and 2.

3.3.2 Exposure concentrations

Tables 3.11 and 3.12 show the mean data obtained from the analysis of the water samples at each concentration for experiments 1 and 2, respectively.

Nominal Concentration (mg/L)	Mean propranolol concentration of water samples collected on days 0, 4, 7, 14 and 21 (mg/L)					Average result (mg/L)
	Day 0	Day 4	Day 7	Day 14	Day 21	
DWC	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	**<0.0001**
0.001	0.00014	0.000927	0.00063	0.000733	0.001567	**0.00084**
0.01	0.005867	0.003933	0.004	0.004933	0.009333	**0.0053**
0.1	0.092	0.083333	0.101	0.116667	0.128333	**0.098**
1.0	1.1	1.133333	1.0366667	1.166667	2.133333	**1.16**
10	3.4	-	-	-	-	

Table 3.11. Mean measured propranolol concentrations in the water samples at each sampling time for experiment 1. Dilution Water Control (DWC)

Nominal Concentration (mg/L)	Mean propranolol concentration of water samples collected on days 0, 4, 7, 13, 14 and 21 (mg/L)						Average result (mg/L)
	0	**4**	**7**	**13**	**14**	**21**	
DWC	-	<0.0001	<0.0001	-	<0.0001	<0.0001	**<0.0001**
0.001	0.00074	0.0011	0.002467	-	0.000957	0.001233	**0.0013**
0.01	0.006767	0.008867	0.010567	-	0.006633	0.007033	**0.0080**
0.1	0.038	0.065	0.065	-	0.113333	0.106667	**0.078**
1.0	0.476667	1.043333	0.983333	1.28	1.06	-	**0.97**

Table 3.12. Mean measured propranolol concentrations in the water samples at each sampling time for experiment 2

Propranolol concentrations from the water chemistry analysis show that the fish were exposed to the drug, propranolol-HCl, and that the drug was present in the tanks containing fish that were being dosed, and absent from dilution water control (DWC) tanks. This showed that there was no contamination of propranolol into the control tanks. The propranolol concentration for each treatment was in the expected concentration range, apart from the 10 mg/L tanks in experiment 1. At a nominal concentration of 10 mg/L, only 34 % of the nominal dose was measured in the tanks. This may have been to solubility issues, and/or uptake of the drug by these fish in the tanks. In all other treatments, the average propranolol concentrations in experiments 1 and 2 ranged from 53 to 112 % and from 78 to 130 % of the nominal concentrations, respectively, demonstrating that the measured concentrations were relatively close to the nominal concentrations. Analysis of the water chemistry results showed that there were no statistically significant differences in the mean results at each concentration between the two experiments, and so biological data from each experiment were combined to make one data set.

3.3.3 Blood plasma analysis

Propranolol concentrations were measured in the plasma collected from the sampled fish and Table 3.11 shows these data. As the Table shows, as the concentration of propranolol increased in the water, so did the plasma concentration of propranolol. It is also shown that fish in the control tanks (DWC) had no measurable propranolol in their plasma. At exposure concentrations of 0.1 and 1.0 mg/L, the fish started to rapidly bioaccumulate propranolol in their blood, and this was especially obvious in male fish.

Nominal Concentration (mg/L)	Actual mean Concentration (mg/L)	Mean propranolol concentration in plasma (mg/L)		Plasma concentration as percentage of actual mean water concentration (%)	
		Male	Female	Male	Female
DWC	<0.0001	<0.00025	<0.00025	<0.00	<0.00
0.001	0.0013	0.000555	0.000690	42.69	53.07
0.01	0.0080	0.00605	0.00855	75.62	106.88
0.1	0.078	0.34	0.21	435.89	269.23
1.0	0.97	15.00	5.75	1546.39	592.78

Table 3.11. Mean measured propranolol concentrations in the pooled plasma samples collected from fish at each concentration. Letters in red show where the internal plasma concentration of propranolol exceeded the external actual water concentration.

3.3.4 Fish mortality

At the start of the addition of propranolol to the tanks there were four pairs of fathead minnow in each concentration. Unfortunately during the three day transition period, in the 10 mg/L tanks (actual concentration was measured to be 3.4 mg/L) during experiment 1, the fish died or had to be euthanized before the exposure period began. In all cases the males became ill before the females and before termination showed a lack of orientation and were noted to be swimming on one side or nosing the bottom of the tank. Therefore no egg production data or sampling data were collected for the 10 mg/L concentration. Because of these unexpected results and because it was not intended to collect acute toxicity data for propranolol, the 10 mg/L dosing was not repeated in experiment 2.

At the 1.0 mg/L concentration on day 5 in experiment 1, two males started to show slightly similar behaviour to those that died in the 10 mg/L and they were therefore euthanized. During experiment 2, the fish at the 1.0 mg/L concentration were closely observed during the exposure period. It was noted on day 2 that the fish in all the 1.0 mg/L dosing tanks had a reduced appetite, as a noticeable quantity of food could be seen on top and around the spawning tiles. By day 4 two males were euthanized as they had started to swim on one side with an apparent loss of orientation and the pairing females were terminated on day 5 as they also showed the same symptoms. The remaining fish at this concentration, despite having a reduced appetite, showed no signs of distress. On day 11 during experiment 2 of the exposure period, one of the males at the 1.0 mg/L concentration was

found dead outside of the tank. This was most unexpected as no signs of distress had been observed in the days prior to this incident. The remaining fish being maintained at 1.0 mg/L were then culled after this event and tissue and sampling data were collected. Hence cumulative egg data were collected only for 11 days in experiment 2 at this concentration of propranolol.

Four other deaths occurred during both experiments and in all cases it was because the female had become egg-bound. Tables 3.12 and 3.13 show the number of fish that were used to collect data at each concentration in each experiment. In experiment 2 it was possible to collect data from five fathead minnow pairs in the control tanks, because extra tanks had become available as the 10 mg/L concentration was not being undertaken.

Concentration (mg/L)	Number of fish pairs used in experiment 1	Number of fish pairs used in experiment 2	Total number of fish pairs
DWC	3	5	8
0.001	4	3	7
0.01	4	4	8
0.1	4	3	7
1.0	2	3 for 11 days	5 (for 11 days)
10	0	n/a	0

Table 3.12. Number of fish pairs used to collect cumulative egg data in experiments 1 and 2, and the total number of fish that were used for data analysis of the combined dataset.

Concentration (mg/L)	Number of fish used in experiment 1		Number of fish used in experiment 2		Total number of fish	
	Male	Female	Male	Female	Male	Female
DWC	4	3	5	5	9	8
0.001	4	4	4	4	8	8
0.01	4	4	4	4	8	8
0.1	4	4	4	3	8	7
1.0	3	3	1	3	4	6

Table 3.13. Number of male and female fish used to collect data parameters in experiments 1 and 2, and the total number of fish that were used for data analysis.

3.3.5 Cumulative egg production

Table 3.14 shows cumulative egg production and a summary of egg production data during the baseline period from each experiment. Across both experiments, the total number of eggs per female in the baseline period ranged from 444 to 1068. The mean number of eggs per female per reproductive day ranged from 21 to 51. These data compare favourably to pair breeding assay data reported by Winter et al., (2008), where the number of eggs per female per reproductive day ranged from 27 to 50, but are slightly lower than those produced by Thorpe et al. (2007) (80 to 93 eggs/female/day). The mean number of eggs per spawn was between 125 and 251 in the baseline period. This range is slightly higher than data reported by Winter et al. (2008) (103 to 161 eggs/spawn) and lower than that reported by Thorpe et al. (2007) (358 ± 14 eggs/spawn).

Treatment (mg/L)	Number of fish pairs in each experiment		Cumulative egg production		Mean number of eggs/female		Mean number of eggs/female/ reproductive day		Mean number of eggs/ spawn	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
DWC	4	5	4270	4702	1068	940	51	45	251	194
0.001	4	4	3246	2893	812	723	39	34	180	223
0.01	4	4	2359	2447	590	612	28	29	147	144
0.1	4	4	4297	3450	1074	863	51	41	195	230
1.0	4	3	1774	1755	444	585	21	28	136	125

Table 3.14. Summary of baseline period egg production data for experiments 1 and 2. Data in pink includes data from fish that died during the exposure period but completed the baseline period (i.e. fish that are not included in Figure 3.13)

Table 3.15 shows cumulative egg production and a summary of egg production data in the exposure period from both experiments. The mean eggs per female in the exposure period ranged from 49 to 887, the mean number of eggs per female per reproductive day were from between 0 to 42 and the mean eggs per spawn were from between 25 to 238. In all egg production data for the exposure period the minimum value is a lot lower compared to the baseline period in this study and in comparison to data sets from other studies. The minimum value in all these exposure egg production data is from the 1.0 mg/L treatment groups in both experiments. All other treatment group data in the exposure period were comparable to the baseline egg production data in both experiments.

Treatment (mg/L)	Number of fish pairs in each experiment		Cumulative egg production		Mean number of eggs/female		Mean number of eggs/female/ reproductive day		Mean number of eggs/ spawn	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
DWC	3	5	2662	4046	887	809	42	39	205	238
0.001	4	3	3185	2006	796	669	38	32	187	167
0.01	4	4	2210	2866	553	717	26	34	138	159
0.1	4	3	3294	2540	824	847	39	40	165	212
1.0	2	3	98	0	49	0	2.3	0	25	0

Table 3.15. Summary of exposure period egg production data for experiments 1 and 2. Data in blue shows where data were only taken for 11 days as fish had to be euthanized.

Figure 3.13 shows the pooled data from experiments 1 and 2 of cumulative egg production during baseline and exposure periods. Comparison of the baseline and exposure periods can only be made at <u>each concentration</u> since the number of pairs differs between concentrations, which therefore automatically effects the cumulative number of eggs produced, and/or the slope of the line. For example, 1.0 mg/L had the least number of fish pairs (n = 5), so the cumulative egg production is, as expected, much lower than for the DWC and 0.01 mg/L treatments, which each have the most amount of fish pairs (n = 8).

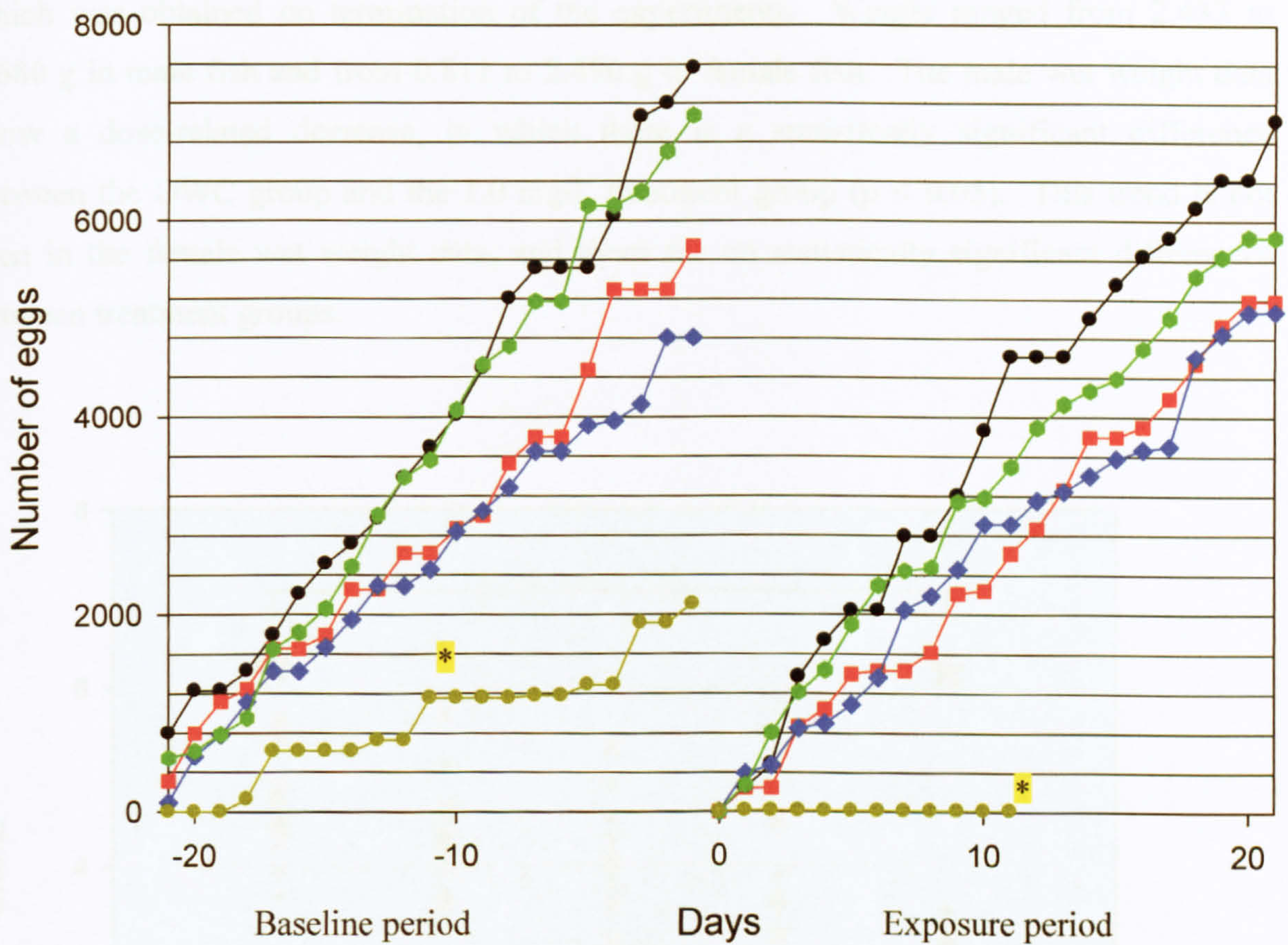


Figure 3.13. Cumulative egg production at each concentration during the 21 day baseline and exposure periods. The data from experiments 1 and 2 have been pooled.
● DWC. ■ 0.001 mg/L. ◆ 0.01 mg/L. ● 0.1 mg/L. ● 1.0 mg/L
* shows a statistically significant difference ($p = < 0.05$) after 11 days of each time period.

On comparison of the cumulative egg production during the baseline and exposure periods within each treatment group, there is a statistically significant difference (a decrease) in the cumulative egg production between the baseline and exposure periods for the 1.0 mg/L treatment group ($p = < 0.05$). There is no statistically significant difference between baseline and exposure periods for any of the other treatments.

3.3.6 Body and organ size

Figures 3.14 and 3.15 show the analysis of male and female wet weight data, respectively, which was obtained on termination of the experiments. Weight ranged from 2.433 to 5.680 g in male fish and from 0.811 to 2.490 g in female fish. The male wet weight data show a dose-related decrease, in which there is a statistically significant difference between the DWC group and the 1.0 mg/L treatment group ($p < 0.05$). This trend is not seen in the female wet weight data, and there are no statistically significant differences between treatment groups.

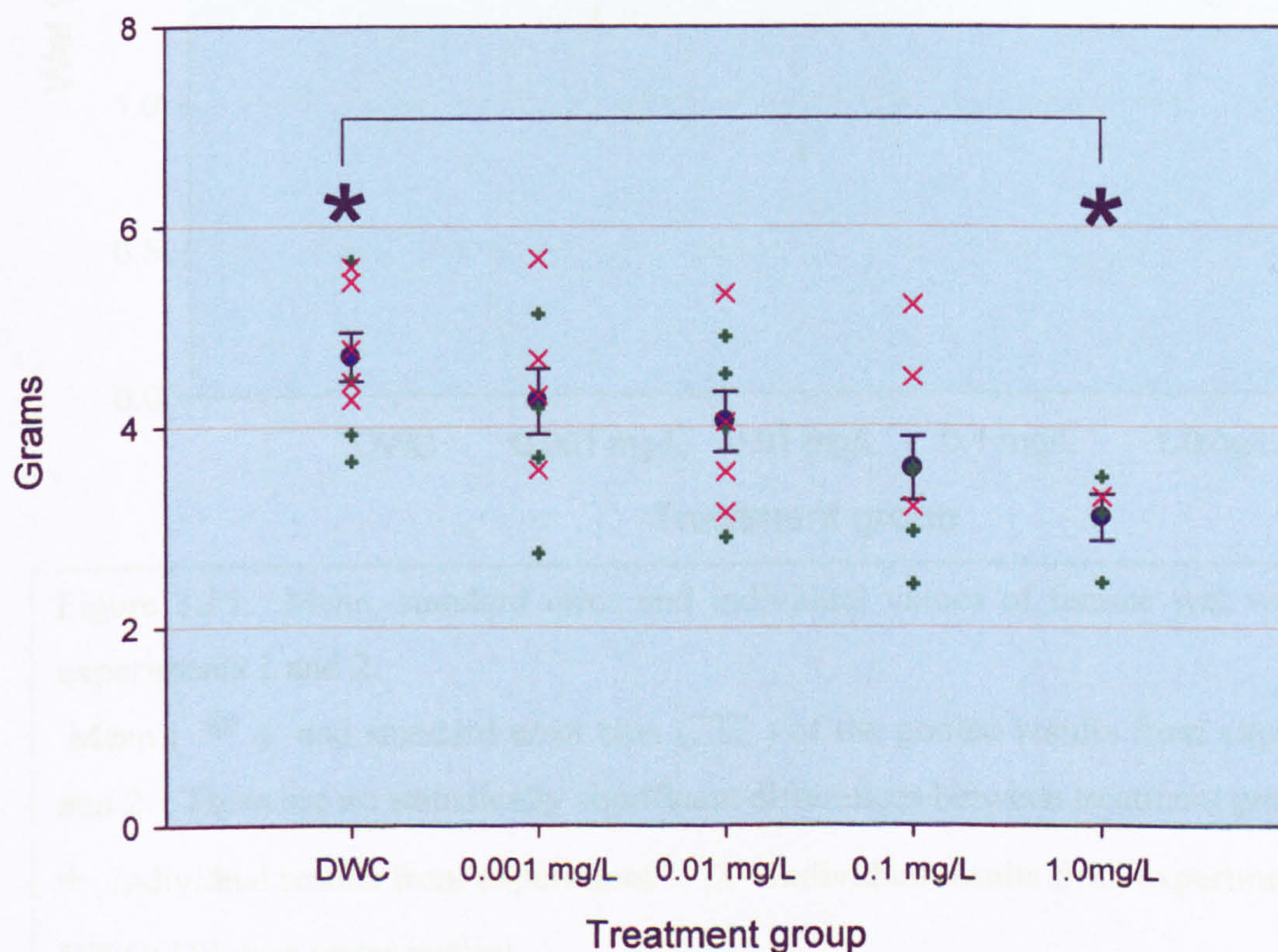


Figure 3.14. Mean, standard error and individual values of male wet weight from experiments 1 and 2.

Mean (●) and standard error bars (⌶) of the pooled results from experiments 1 and 2. * denotes a statistically significant difference between treatment groups ($p < 0.05$).

+ Individual results from experiment 1. X Individual results from experiment 2

DWC: Dilution water control

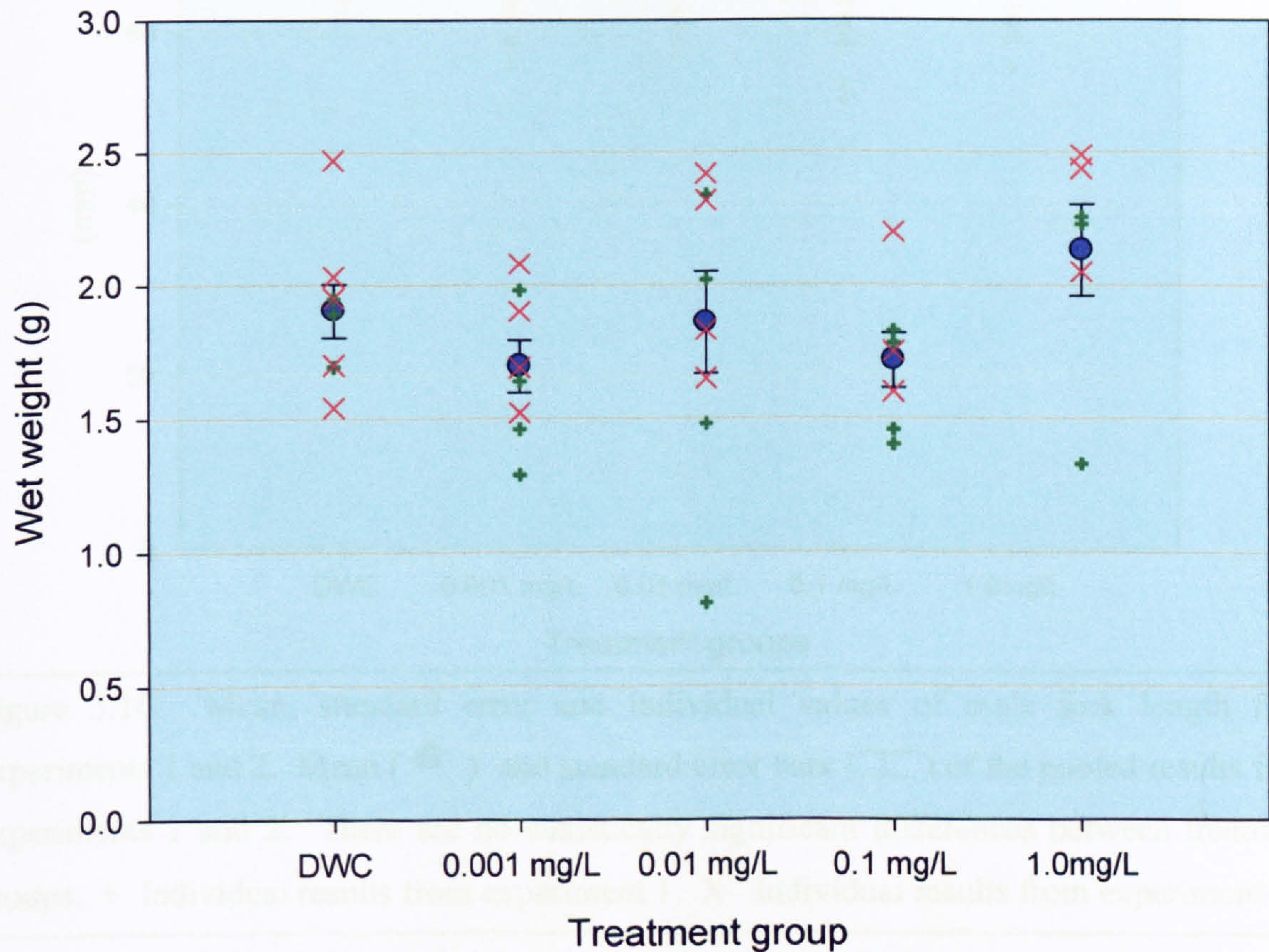


Figure 3.15. Mean, standard error and individual values of female wet weight from experiments 1 and 2.
Mean (●) and standard error bars ( ) of the pooled results from experiments 1 and 2. There are no statistically significant differences between treatment groups
+ Individual results from experiment 1. X Individual results from experiment 2.
DWC: Dilution water control

Male fork length ranged from 52 to 71 mm and female fork length ranged from 42 to 65 mm. Figures 3.16 and 3.17 show results of male and female fork length, respectively. No statistically significant differences are found between treatment groups. However, a slight downward trend is seen in male fork length as the concentration of propranolol increases.

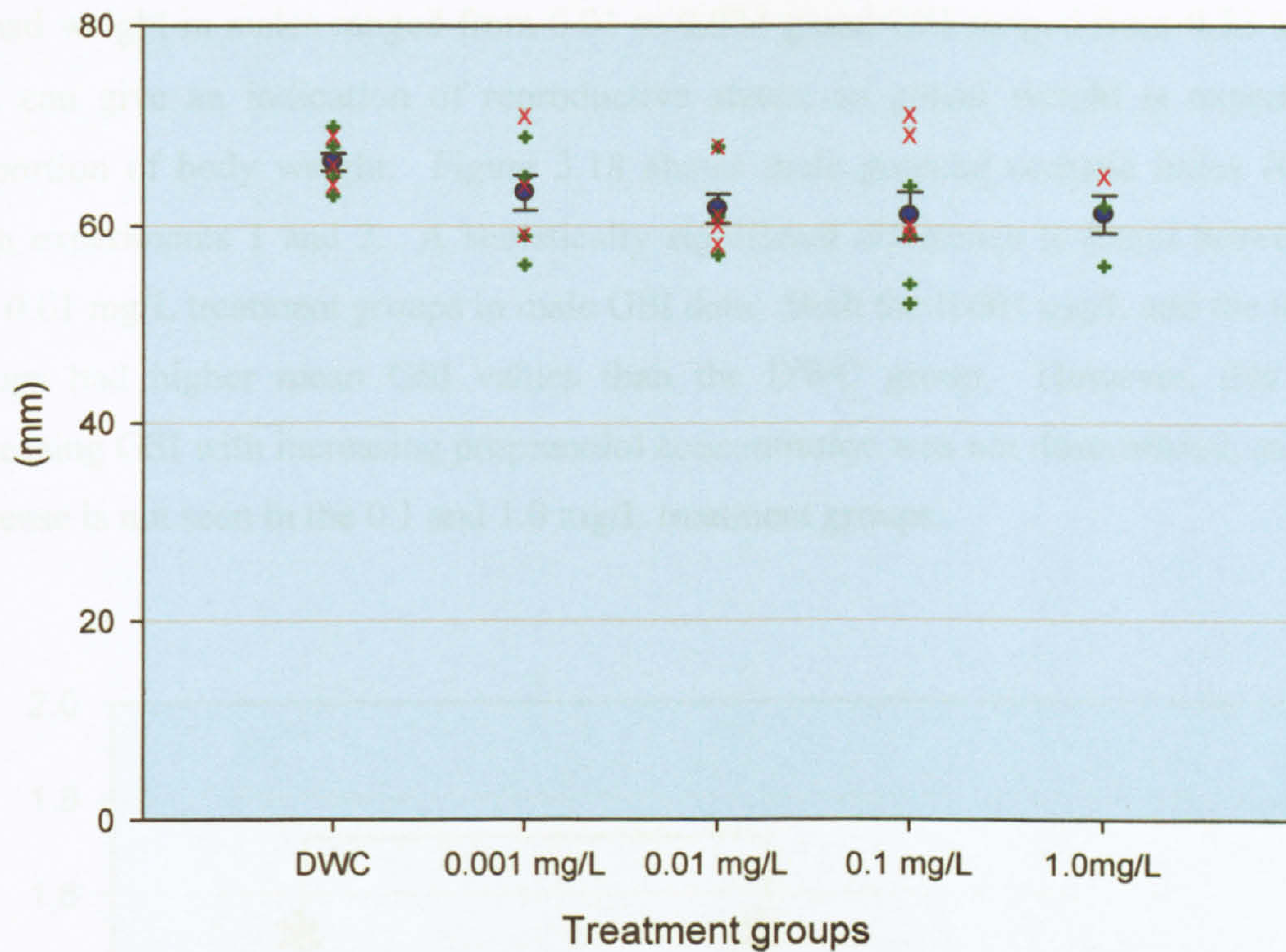


Figure 3.16. Mean, standard error and individual values of male fork length from experiments 1 and 2. Mean (●) and standard error bars (⌶) of the pooled results from experiments 1 and 2. There are no statistically significant differences between treatment groups. + Individual results from experiment 1. X Individual results from experiment 2.

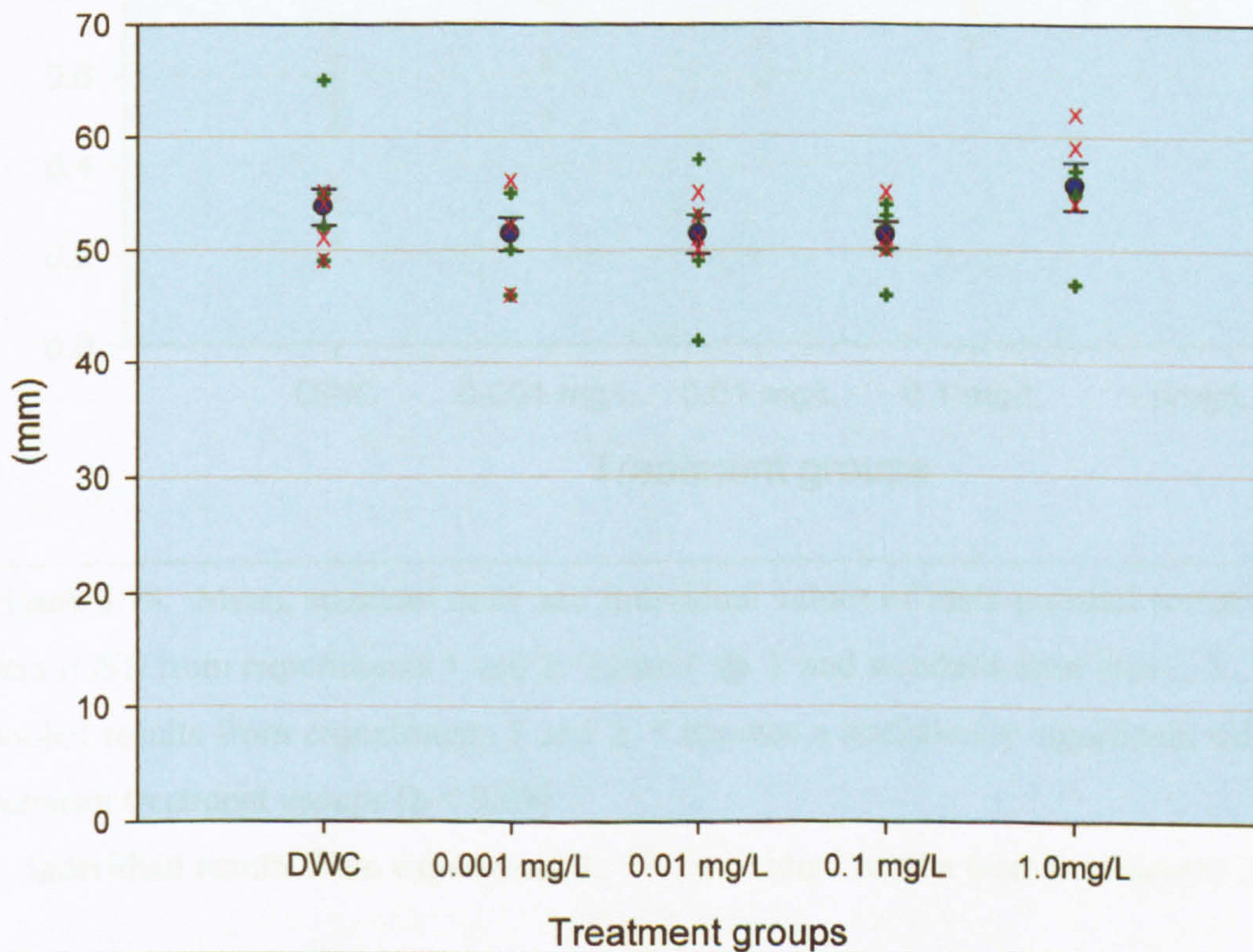


Figure 3.17. Mean, standard error and individual values of female fork length from experiments 1 and 2. Mean (●) and standard error bars (⌶) of the pooled results from experiments 1 and 2. There are no statistically significant differences between treatment groups. + Individual results from experiment 1. X Individual results from experiment 2.

Gonad weight in males ranged from 0.01 to 0.074 g and GSI ranged from 0.22 to 1.39 %. GSI can give an indication of reproductive status, as gonad weight is expressed as a proportion of body weight. Figure 3.18 shows male gonadal somatic index (GSI) data from experiments 1 and 2. A statistically significant difference is found between DWC and 0.01 mg/L treatment groups in male GSI data. Both the 0.001 mg/L and the 0.01 mg/L groups had higher mean GSI values than the DWC group. However, this trend of increasing GSI with increasing propranolol concentration was not dose-related, as the same increase is not seen in the 0.1 and 1.0 mg/L treatment groups.

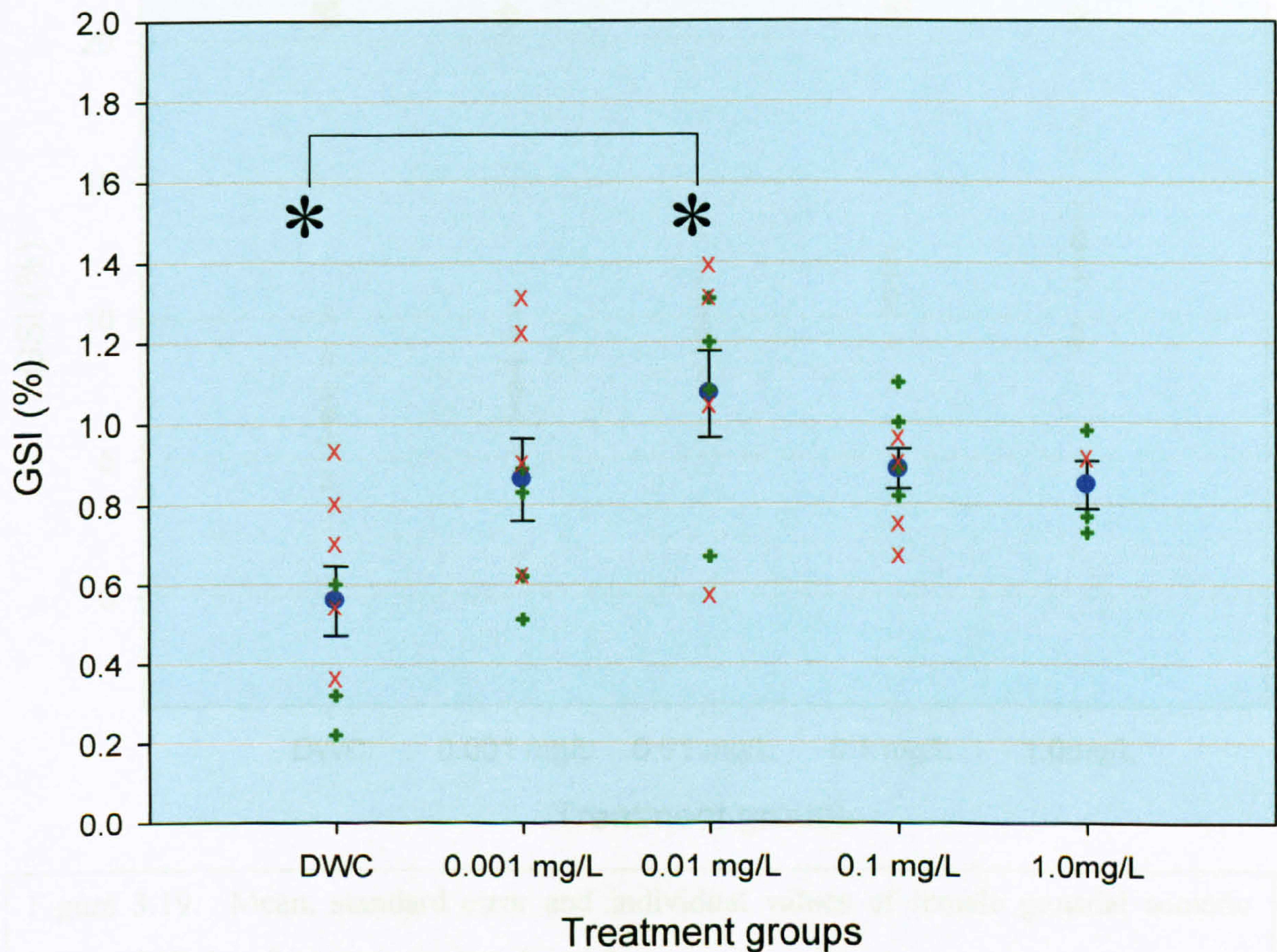


Figure 3.18. Mean, standard error and individual values of male gonadal somatic index data (GSI) from experiments 1 and 2. Mean (●) and standard error bars (⌶) of the pooled results from experiments 1 and 2. * denotes a statistically significant difference between treatment groups ($p < 0.05$)

+ Individual results from experiment 1. X Individual results from experiment 2.

Female gonad weight ranged from 0.01 to 0.357 g and GSI ranged from 1.32 to 17.14 %. The female GSI data exhibits a dose-related effect, for as the concentration of propranolol increases, so does the GSI, as shown in Figure 3.19. This produces statistically significant differences between treatment groups DWC and 0.1 mg/L, DWC and 1.0 mg/L, and between 0.001 and 1.0 mg/L.

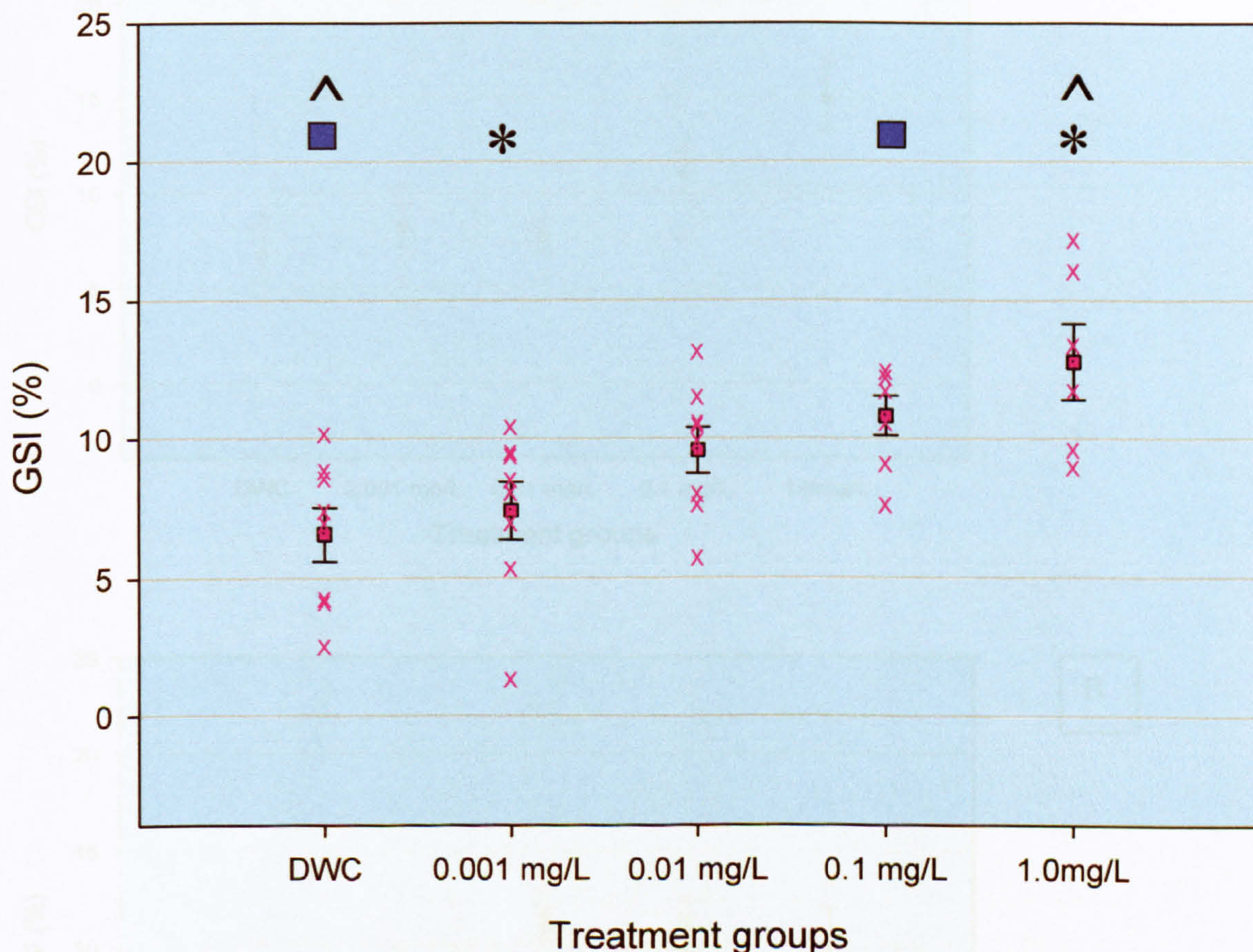


Figure 3.19. Mean, standard error and individual values of female gonadal somatic index (GSI) data from experiments 1 and 2.

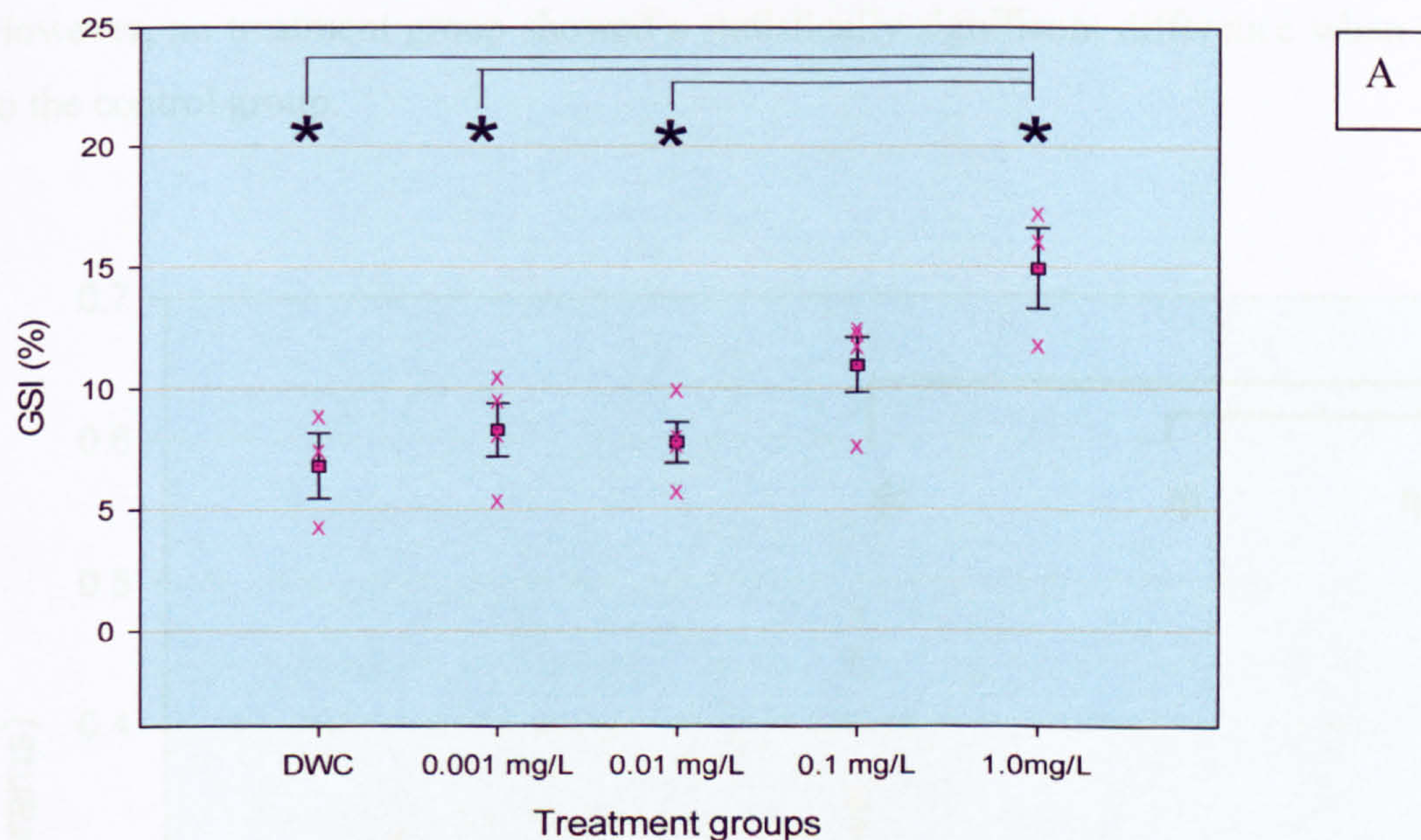
■ Mean. ⌶ Standard error bars. × Individual result.

* denotes a statistically significant difference between treatment groups 0.001 and 1.0 mg/L ($p < 0.05$).

^ denotes a statistically significant difference between DWC and treatment group 1.0 mg/L ($p < 0.05$).

■ denotes a statistically significant difference between DWC and treatment group 0.1 mg/L ($p < 0.05$).

The same relationship is seen in the female GSI when data from experiments 1 and 2 are viewed separately, as shown in Figures 3.20. The statistically significant differences are still seen in experiment 1, however in experiment 2 a similar trend is apparent but the differences are not statistically significant when $p < 0.05$.



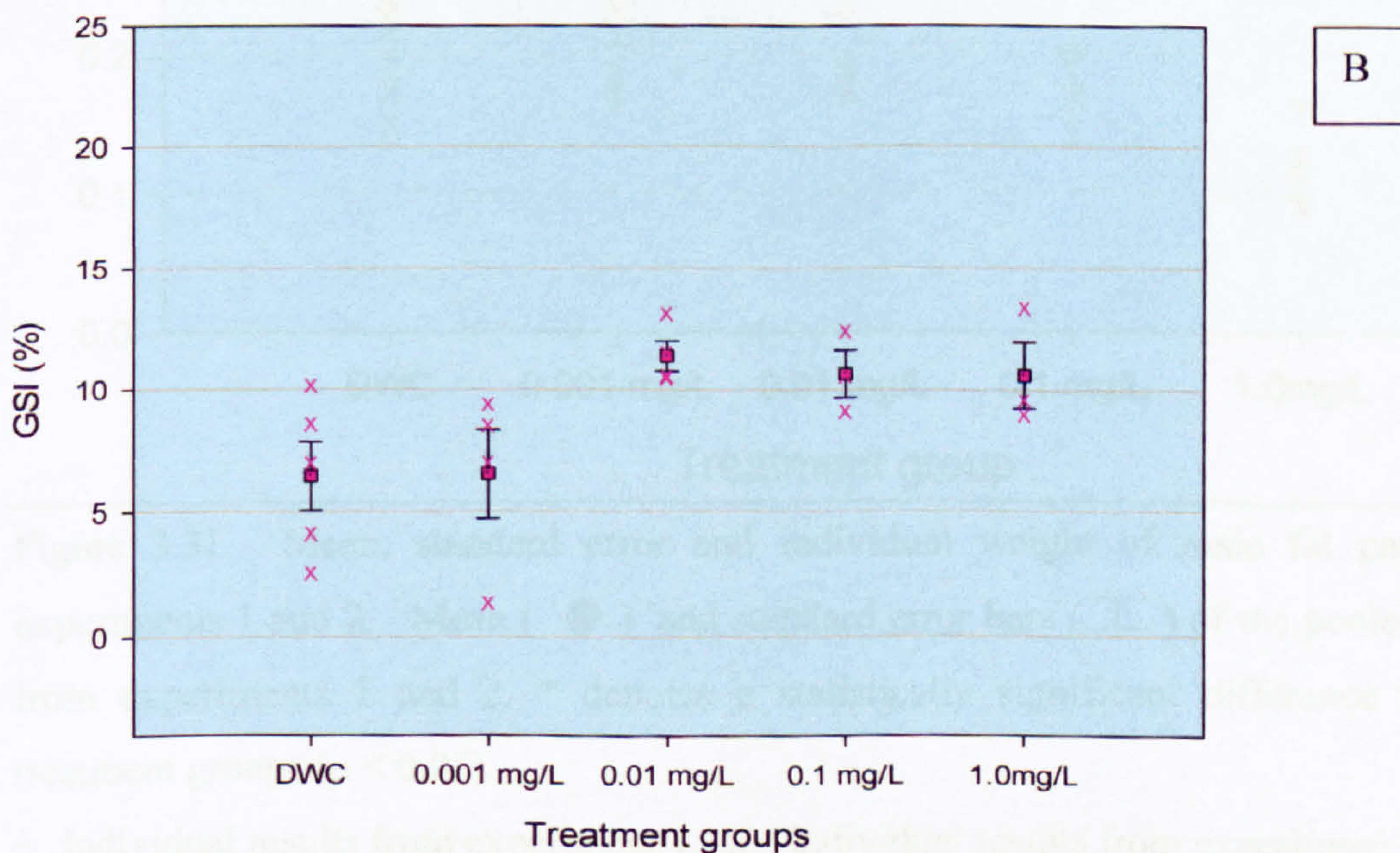


Figure 3.20. Mean, standard error and individual values of female gonadal somatic index (GSI) data from experiment 1 (**A**) and experiment 2 (**B**). * denotes a statistically significant difference between treatment groups ($p < 0.05$).

Mean. Standard error bars. X Individual result.

Figure 3.31 shows the weight of the fatpads from male fish at each concentration of propranolol. The male fatpad weight ranged from 0.090 to 0.477 g. As the concentration of propranolol increases from 0.01 mg/L, there is a dose-related decrease in the weight of the fatpad, and a statistically significant difference (*) was found between treatment groups 0.01mg/L and 1.0 mg/L and also between groups 0.1 mg/L and 1.0 mg/L ($p < 0.05$). However, no treatment group showed a statistically significant difference when compared to the control group.

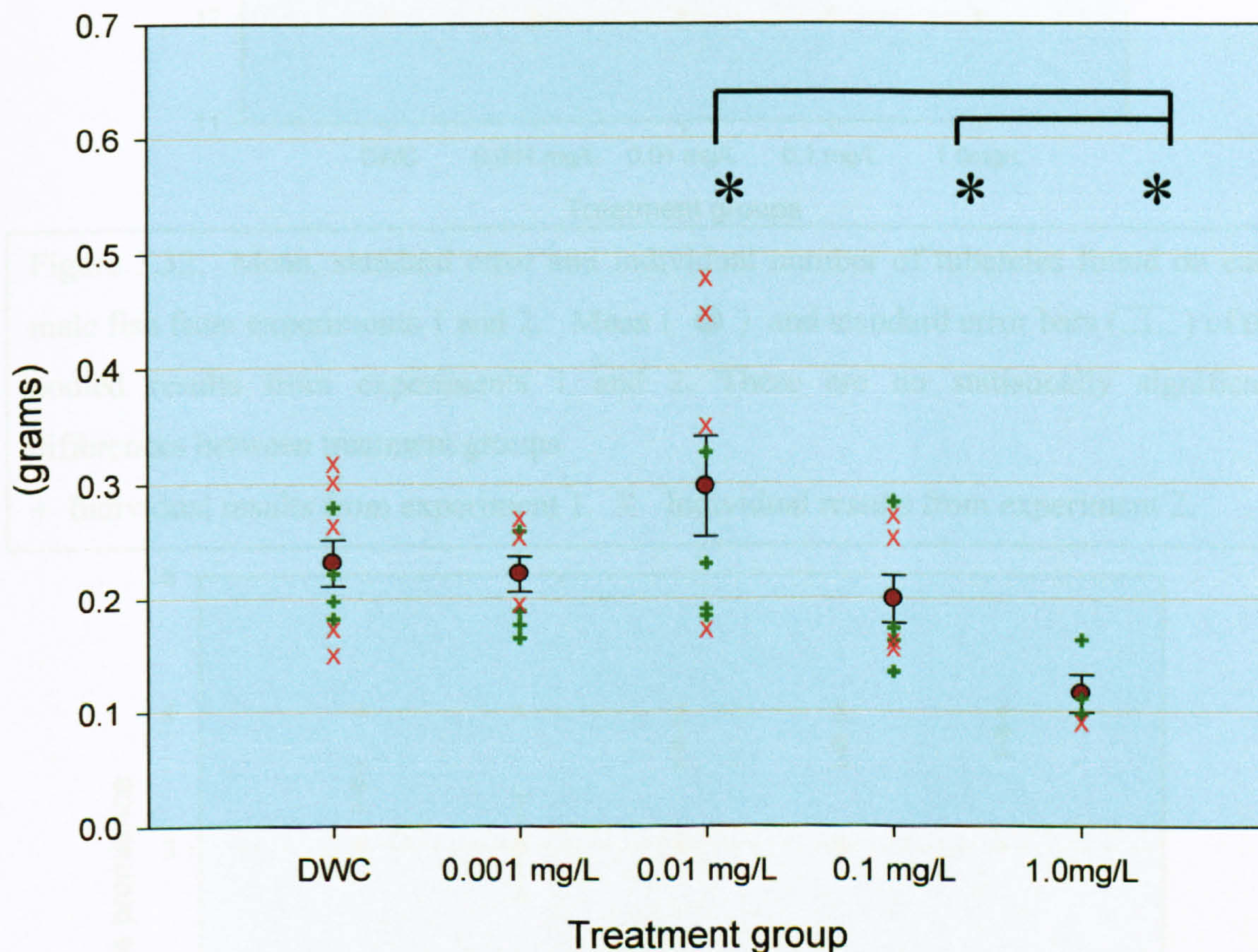


Figure 3.31. Mean, standard error and individual weight of male fat pads from experiments 1 and 2. Mean (●) and standard error bars (⌶) of the pooled results from experiments 1 and 2. * denotes a statistically significant difference between treatment groups ($p < 0.05$)

+ Individual results from experiment 1. X Individual results from experiment 2.

Figures 3.32 and 3.33 show data on the number of tubercles and the prominence of these tubercles, as assessed using the qualitative scheme devised by Smith (1978), respectively. The number of tubercles ranged from 12 to 16, and the tubercle prominence score from 1 to 4. There are no statistically significant differences between treatment groups in either of these sets of data. No secondary sexual characteristics were observed in any female fish sampled.

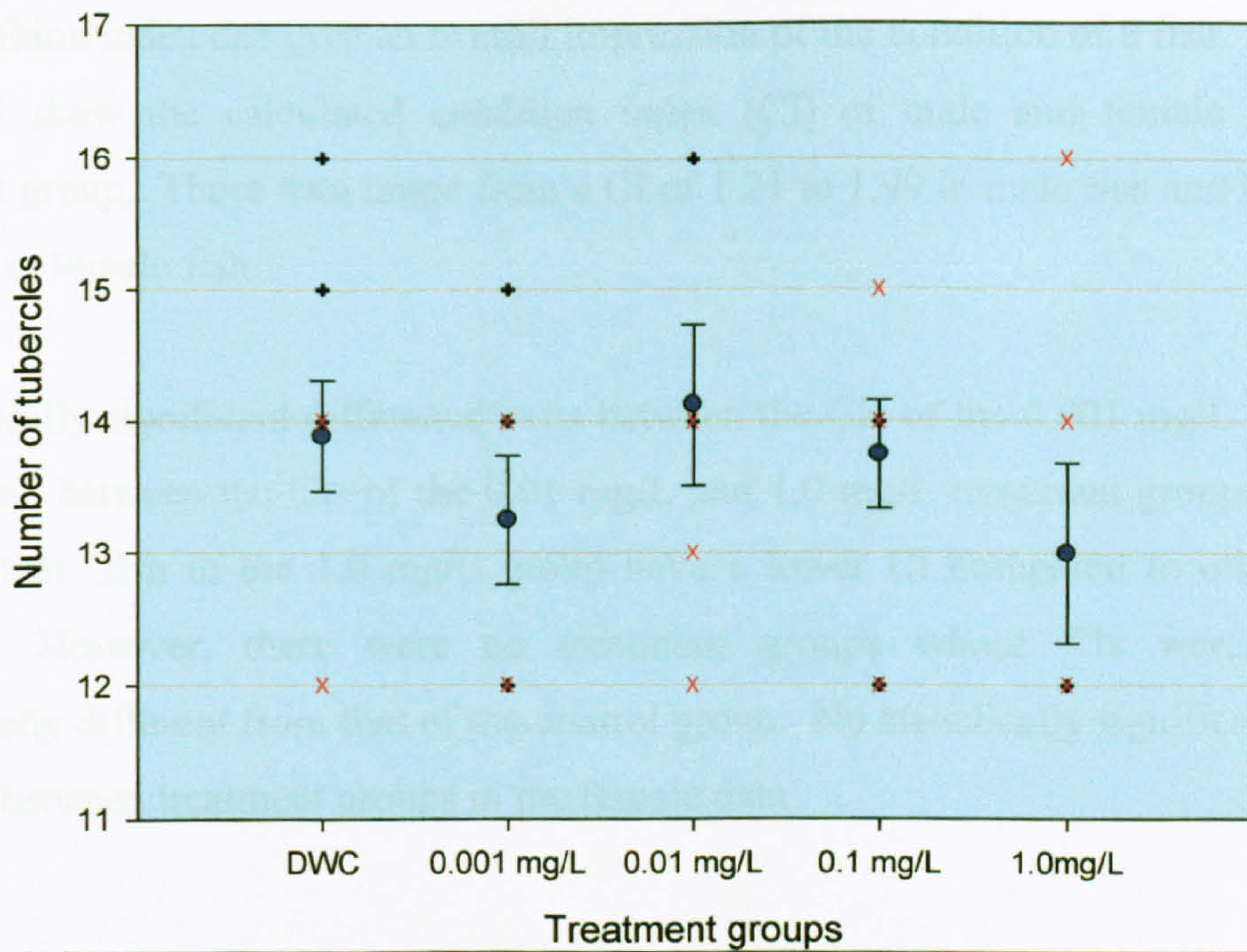


Figure 3.32. Mean, standard error and individual number of tubercles found on each male fish from experiments 1 and 2. Mean (●) and standard error bars (⌶) of the pooled results from experiments 1 and 2. There are no statistically significant differences between treatment groups

+ Individual results from experiment 1. X Individual results from experiment 2.

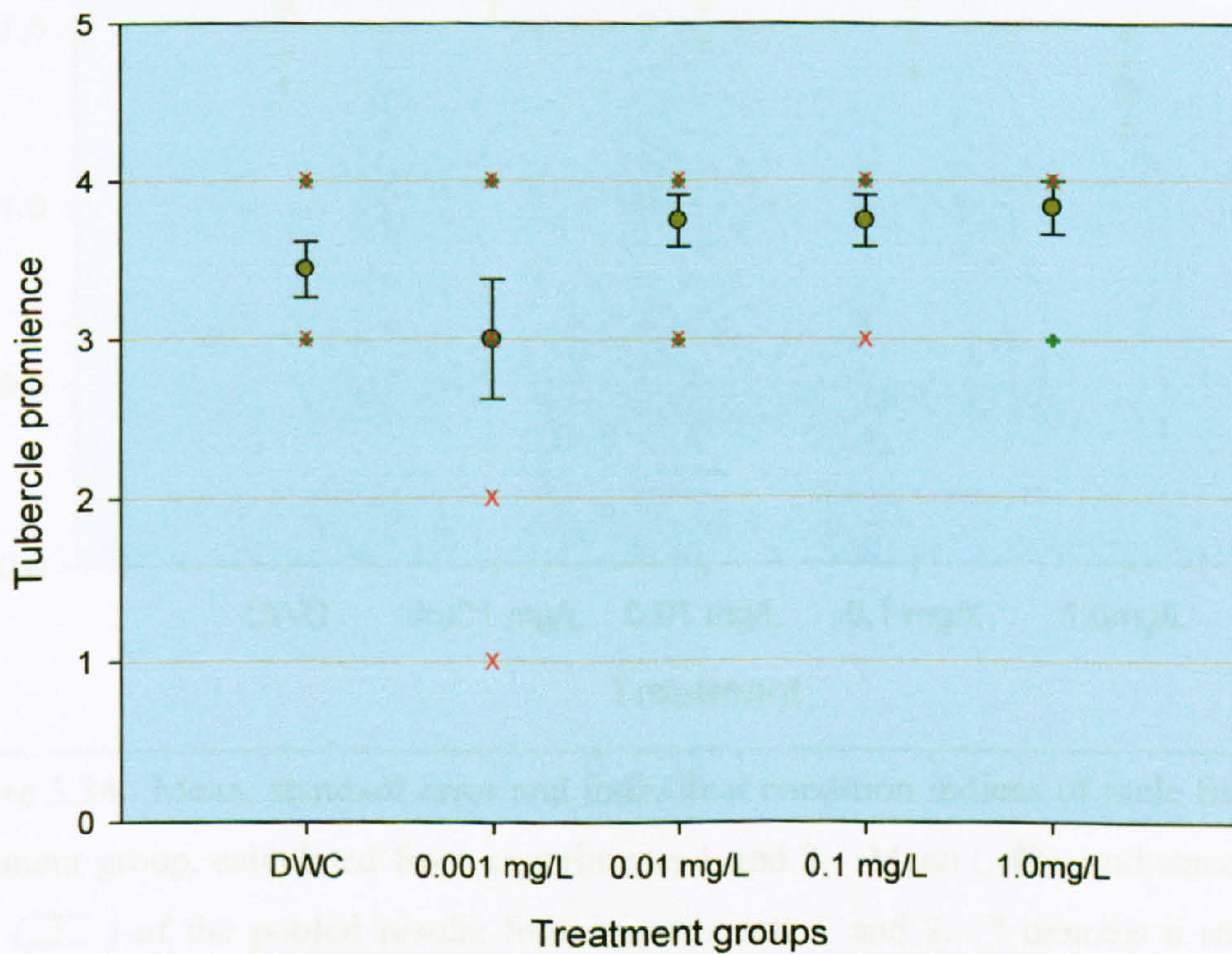


Figure 3.33. Mean, standard error and individual results of tubercle prominence on male fish from experiments 1 and 2. Mean (●) and standard error bars (⌶) of the pooled results from experiments 1 and 2. There are no statistically significant differences between treatment groups + Individual results from experiment 1.

X Individual results from experiment 2.

The condition index can give an overall impression of the condition of a fish. Figures 3.34 and 3.35 show the calculated condition index (CI) of male and female fish in each treatment group. These data range from a CI of 1.21 to 1.99 in male fish and between 1.00 and 1.58 in female fish.

A statistically significant difference exits between the CIs of the 0.001 mg/L and 1.0mg/L groups and between the CIs of the 0.01 mg/L and 1.0 mg/L treatment groups in the male data, in that fish in the 1.0 mg/L group have a lower CI compared to other treatment groups. However, there were no treatment groups whose CIs were statistically significantly different from that of the control group. No statistically significant difference is found between treatment groups in the female data.

Figure 3.34. Mean, standard error and individual condition indices of male fish in each treatment group, calculated from experiments 1 and 2. Mean (●) and standard error bars (⌶) of the pooled results from experiments 1 and 2. * denotes a statistically significant difference between treatment groups ($p < 0.05$)

+ Individual results from experiment 1. X Individual results from experiment 2.

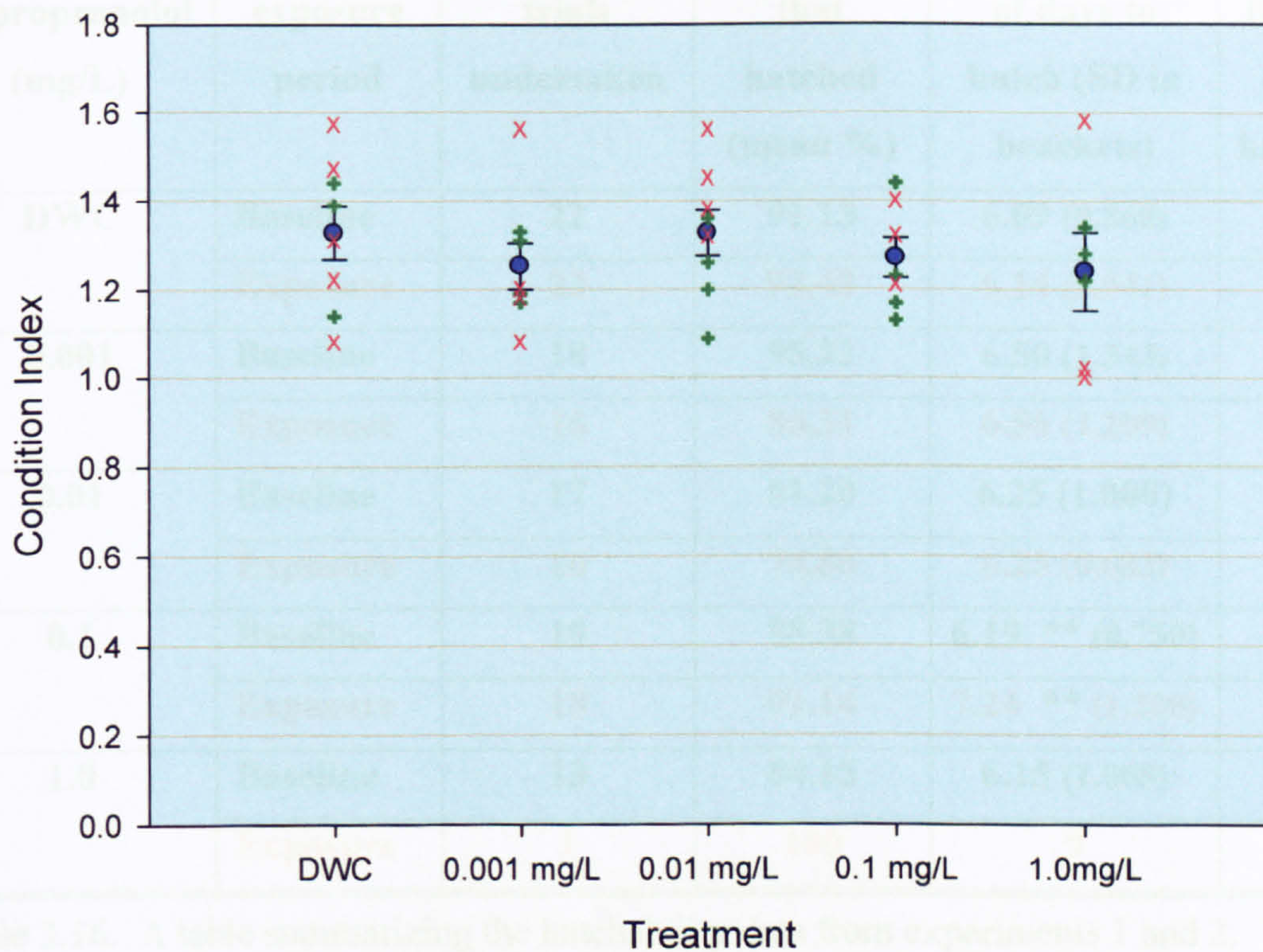


Figure 3.35. Mean, standard error and individual condition indices of female fish in each treatment group, calculated from experiments 1 and 2. Mean (●) and standard error bars (⌶) of the pooled results from experiments 1 and 2. There are no statistically significant difference between treatment groups.
+ Individual results from experiment 1. X Individual results from experiment 2.

3.3.7 Hatchability trials

The data from the hatchability trials are summarized in Table 3.16, and Figures 3.36 and 3.37.

Concentration of propranolol (mg/L)	Baseline or exposure period	Number of trials undertaken	Percentage that hatched (mean %)	Mean number of days to hatch (SD in brackets)	Number that died during hatching*
DWC	Baseline	22	91.13	6.09 (0.868)	0
	Exposure	22	92.42	6.14 (0.941)	0.36
0.001	Baseline	18	95.22	6.50 (1.543)	0
	Exposure	16	86.31	6.56 (1.209)	0.31
0.01	Baseline	17	81.20	6.25 (1.000)	0
	Exposure	16	74.86	6.25 (0.683)	0.19
0.1	Baseline	19	88.38	6.19 ** (0.750)	0
	Exposure	18	91.14	7.24 ** (1.200)	0.33
1.0	Baseline	13	84.15	6.15 (1.068)	0
	Exposure	1	100	9	0

Table 3.16. A table summarizing the hatchability data from experiments 1 and 2.

* calculated as the total number of progeny that died during hatching in each treatment group period, divided by the number of trials undertaken in that treatment group.

** statistically significantly difference between the baseline and exposure period in the 0.1 mg/L treatment group ($p<0.05$)

The mean percentage of eggs that hatched in the 0.001 mg/L and the 0.01 mg/L was lower in the exposure period compared to the baseline period, as shown in Figure 3.36. This observation was not seen in the control group, the 0.1mg/L group and the one hatching test that was carried out in the 1.0mg/L group, where the mean percentage of eggs that hatched was higher in the exposure period compared to the baseline period.

The mean number of days that it took for the eggs to hatch in each treatment group increased during the exposure period compared to the baseline period in all treatment groups, except in the 0.01mg/L group where it remained constant. In the 0.1mg/L treatment group this increase between the baseline and exposure periods was statistically significant, as shown in Figure 3.37. The 1.0 mg/L data could not be statistically analysed because there were fewer than 2 data points. It was sometimes observed that the progeny died during hatching. This happened only during the exposure period and was observed in all treatment groups (except 1.0 mg/L group where only 1 batch of eggs was produced), including the control group, in which it happened with the most frequency.

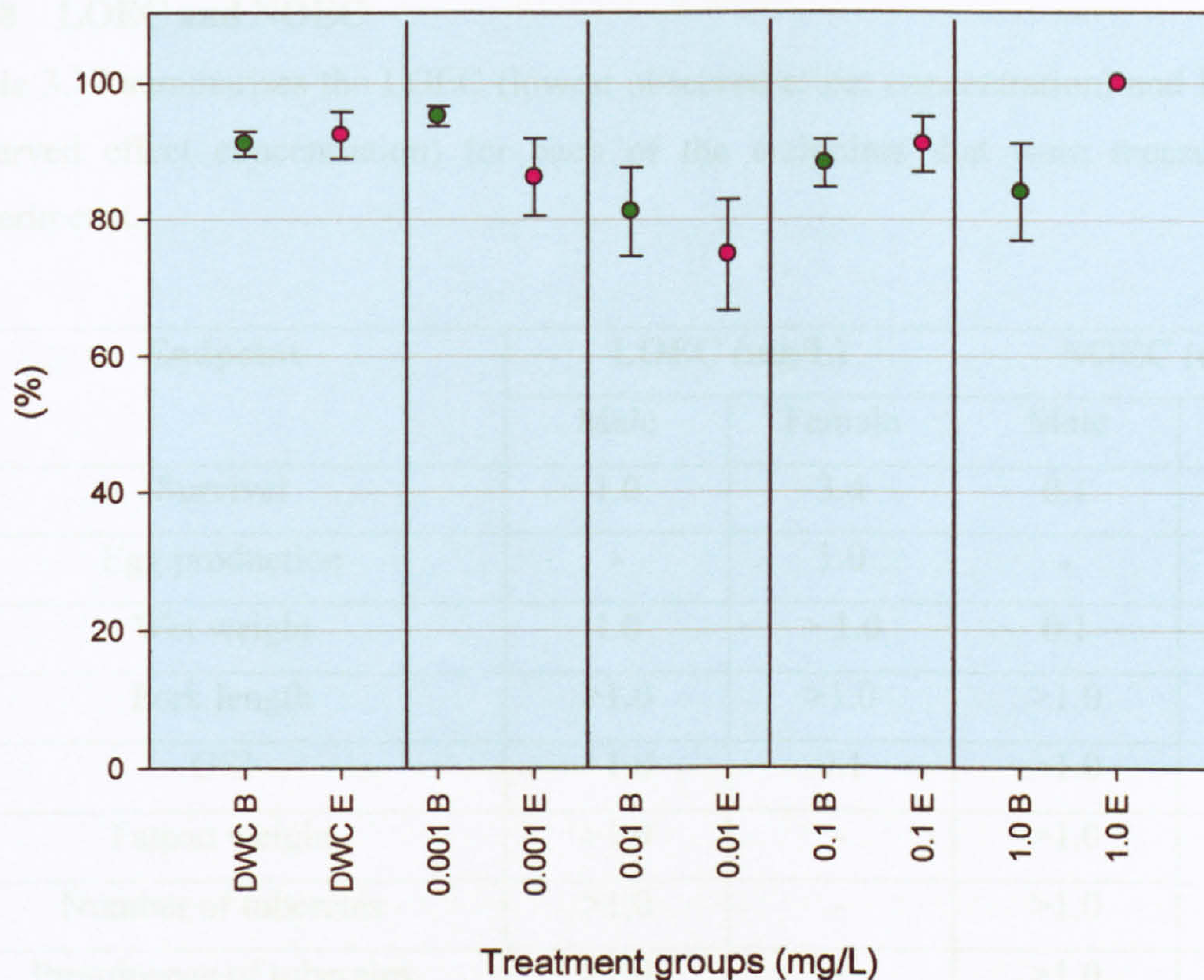


Figure 3.36. Results of hatchability trials with regard to the percentage of eggs that hatched. B: Baseline period. E: Exposure period. ● Mean baseline period ● Mean exposure period ⌶ Standard error bars.

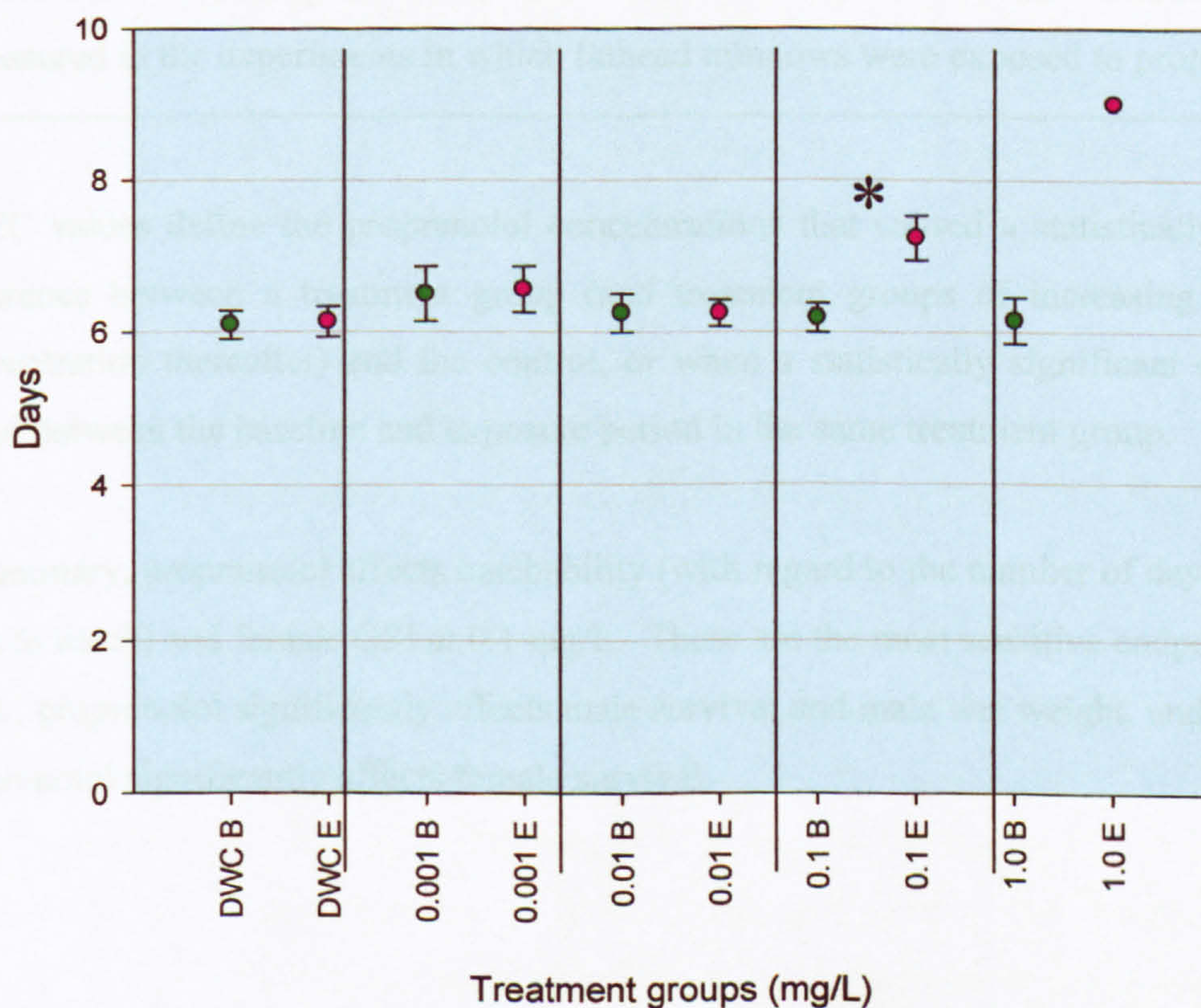


Figure 3.37. Mean and standard error data from hatchability trials with regard to the number of days it took for 50 eggs, in each hatchability trial, to hatch. B: Baseline period. E: Exposure period. * denotes statistically significant difference between baseline and expose period in 0.1mg/L treatment group ($p<0.05$)

3.3.8 LOEC and NOEC

Table 3.17 summarises the LOEC (lowest observed effect concentration) and NOECs (no observed effect concentration) for each of the endpoints that were measured in the experiments.

Endpoint	LOEC (mg/L)		NOEC (mg/L)	
	Male	Female	Male	Female
Survival	1.0	3.4	0.1	1.0
Egg production	-	1.0	-	0.1
Wet weight	1.0	> 1.0	0.1	>1.0
Fork length	>1.0	>1.0	>1.0	>1.0
GSI	>1.0	0.1	>1.0	0.01
Fatpad weight	>1.0	-	>1.0	-
Number of tubercles	>1.0	-	>1.0	-
Prominence of tubercles	>1.0	-	>1.0	-
Condition index	>1.0	>1.0	>1.0	>1.0
Hatchability (Days to hatch)	0.1	0.1	0.01	0.01

Table 3.17. Summary of the LOEC and NOEC values for the various endpoints measured in the experiments in which fathead minnows were exposed to propranolol

LOEC values define the propranolol concentrations that caused a statistically significant difference between a treatment group (and treatment groups of increasing propranolol concentration thereafter) and the control, or when a statistically significant difference is found between the baseline and exposure period in the same treatment group.

In summary, propranolol affects hatchability (with regard to the number of days it takes for eggs to hatch) and female GSI at 0.1 mg/L. These are the most sensitive endpoints. At 1.0 mg/L, propranolol significantly affects male survival and male wet weight, and at 3.4 mg/L propranolol significantly affects female survival.

3.4 Discussion

Propranolol has been found to be toxic to other organisms and fish (see references in Introduction), however it was most surprising that when fathead minnows were exposed to the test concentrations used in these experiments, severe disruption to their health was caused at concentrations of 1.0 mg/L propranolol. This acute toxicity of propranolol to fish, and its other effects, might be explained by the reasoning below.

3.4.1 Side effects of propranolol

In humans, propranolol acts by blocking adrenaline and noradrenaline from binding with β1 and β2-ARs. The (S)-enantomer of propranolol is the active form of the drug and is a hundred times more potent in blocking the β-ARs than the (R)-enantomer, hence it is responsible for almost all of the drug's pharmacological effects (Egginger, 1994; Masubuchi et al., 1993). However, these effects also include ones which the drug was not necessarily designed for. For example, one side effect of propranolol in mammals is the drug's ability to inhibit sperm motility and fertilizing ability, and this spermicidal action has even been investigated as a potential way to control sea lamprey populations in the Great Lakes, Canada (Ciereszko et al., 2004).

Propranolol is lipophilic (log Kow 3.48), and due to this physical property it can easily move across the blood-brain barrier in organisms (Vu & Beckwith 2007). Studies have shown that propranolol can be found in human brain tissue at concentrations ten to twenty six times higher than in plasma (Cruickshank & Neil-Dwyer, 1985; Westerlund, 1985). This is a high degree of bioaccumulation, especially when compared to brain concentrations of the hydrophilic β-blocker atenolol, which has a brain: plasma ratio of 0.2 (Cruickshank & Neil-Dwyer, 1985). Similar accumulation can also be found in the brains of rabbits and monkeys that were administered propranolol (Srivastava & Kapoor, 1983).

Propranolol is also known to act as a serotonin receptor antagonist, by interacting with the 5-hydroxytryptophan receptor (5-HT) in the brain (Dzialowski et al., 2006; Westerlund, 1985). Serotonin is synthesised in serotonergic neurons in the central nervous system (CNS) from the amino acid tryptophan, and its functions are numerous. For example, serotonin controls appetite, sleep, memory, learning behaviour, temperature regulation, mood, sexual behaviour, cardiovascular function, muscle contraction, endocrine regulation, and depression (http://serendip.brynmawr.edu/). Some patients prescribed propranolol have been known to suffer adverse CNS effects such as bad nightmares, hallucinations, fatigue, impaired short term memory and ability to concentrate, reduced anxiety, and

alterations in mood (Vu & Beckwith 2007; Currie et al., 1988). Less CNS-related side effects are reported from patients prescribed the hydrophilic β-blocker atenolol (Westerlund, 1985).

The effects of propranolol on catecholamine levels in the brain seem to be localised. In rats, propranolol does not alter catecholamine levels in the brain stem, yet in the hypothalamus, levels of adrenaline and noradrenaline decrease. However, levels of the enzyme dopamine β-hydroxylase in rats were decreased by 23 and 29 % in both the brain stem and hypothalamus, respectively (Srivastava & Kapoor, 1983). Dopamine β-hydroxylase is the enzyme that converts dopamine to noradrenaline, and so the decreased activity of this enzyme could account for the lower levels of noradrenaline in the hypothalamus (Srivastava & Kapoor, 1983). Hence, the disorientation and reduced appetite observed in fathead minnows on exposure to toxic concentrations of propranolol may have been due to CNS effects of propranolol. In studies where rats were exposed to propranolol, an obviously decreased locomotor activity was also observed due to the local anaesthetic action of propranolol as a consequence of its CNS toxicity (Lemmer & Saller, 1974).

In these propranolol experiments the GSI of the female fish showed a dose-related increase. This was observed in conjunction with a statistically significant decrease in cumulative egg production data in the 1.0 mg/L group between baseline and exposure periods. However, although spawning was much reduced at the highest concentrations of propranolol, on termination it was observed that the female fish had continued to produce eggs and the gonads of the female fish at the highest concentration were full of eggs. It seems that due to the lack of mating behaviour by both fish, no spawning behaviour occurred to induce spawning. One of the CNS side effects of propranolol in humans is decreased sexual behaviour, and this may be due to the antagonistic affects of propranolol on serotonin receptors. Serotonin has been shown to be an important neuromodulator of sexual function in vertebrates and invertebrates and the reproductive status in fish has been correlated to serotonin levels (Foran et al., 2004). For example, serotonin in fish modulates the secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus, and also induces oocyte maturation in teleost fish (Foran et al., 2004). Hence the lack of reproduction in the 1.0 mg/L tanks and the dose-related increase in GSI in female fish is probably not due to a reproductive toxic effect (i.e. a specific and direct effect on the reproductive system), but it is more likely that propranolol had CNS effects on serotonin levels, which consequently affected fathead reproduction through lack of

mating behaviour and lack of oocyte maturation in the female fish. If oocytes grew to a specific stage, but then did not mature and so were not ovulated, this could account for the increase in GSI in females.

3.4.2 Lipid metabolism and β-blockers

When fathead minnows were exposed to propranolol, a dose-related, statistically significant reduction in the weight of the male fat pad was observed in the 0.01, 0.1 and 1.0 mg/L treatments. However, although fatpad weight decreased between 0.01 and 1.0 mg/L, nevertheless no treatment group had a fatpad weight that was significantly less than that of the control group. Fatpads and tubercles in male fathead minnows are an expression of their secondary sexual characteristics, and when a reduction in the weight of male fatpads is observed, it is usually associated with anti-androgenic effects of a chemical (Villeneuve et al., 2006). In these experiments, a significant reduction in male wet weight at the highest propranolol treatment was evident (together with a dose-related decreasing trend as the propranolol concentration increased), yet the fork length did not change significantly between any treatment group. In addition, the condition index in males decreased as the propranolol concentration increased. These changes, taken together, suggest that the effects seen in these experiments were not due to anti-androgenic changes in secondary sexual characteristics, but could instead be due to modifications of the lipid metabolism in the fish that utilised the fat in the fatpad, decreased the weight, and consequently the condition index, of the fish at the highest propranolol concentrations.

When β-blockers are administered to treat hypertension (i.e. high blood pressure) in humans, β-blockers are normally used as a long-term and often life-long therapy; hence studies have been carried out to assess the affects of β-blockers on lipid metabolism in humans (Pasotti et al., 1982). Adrenaline and noradrenaline are known to strongly stimulate lipolysis in mammals by targeting β-ARs, resulting in increased plasma fatty acid levels (van Heeswijk et al., 2005; Vianen et al., 2002). Studies in humans found that the effects of non-selective β-AR antagonists, such as propranolol and pindolol, significantly increased plasma levels of high density lipid cholesterol, compared to selective β1-AR antagonists such as metoprolol and atenolol, which did not affect plasma lipids or lipoproteins to such an extent (Pasotti et al., 1982; Bielmann et al., 1979). Since β-blockers work to stop the affects of endogenous catecholamines, this suggests that β1-ARs are targeted to a greater extent than β2-ARs in humans by adrenaline and/or noradrenaline to stimulate lipolysis.

As in humans, teleost fish also release catecholamines to regulate the mobilisation of energy. However unlike humans, lipid metabolism in teleosts dominates over carbohydrate metabolism, since very small quantities of carbohydrates are ingested and stored (Vianen et al., 2002; Fabbri et al., 1998). Adrenaline in fish acts as it does in mammals, in that it stimulates lipolysis (Van Den Thillart et al., 2001). However in some species of fish, such as tilapia, goldfish, bream and carp, noradrenaline has been found to inhibit lipolysis via β-ARs (Vianen et al., 2002; Van Den Thillart et al., 2001; Van Raajj et al., 1995). Furthermore, in tilapia it was found that the effects of the non-selective β-AR antagonist timolol could block the inhibitory effects of noradrenaline (Vianen et al., 2002). In studies by Van Den Thillart et al. (2001), when selective β1-AR or β2-AR antagonists were infused together with isoproternol (β-agonist), they caused either an increase or decrease, respectively, of plasma free fatty acids in carp. This suggests that catecholamines cause either an inhibition or stimulation of lipolysis when they bind to β1- or β2-ARs, respectively. Another explanation is that noradrenaline may preferentially bind to β1-ARs, thus causing an inhibition of lipolysis, whilst adrenaline preferentially binds to β2-ARs, which in turn results in the stimulation of lipolysis, as depicted in Figure 3.38. Results of binding studies used in the characterisation of β-ARs support this explanation (Bristow, 2000; Kawai & Arinze 1983). In fact, adrenaline has up to 100-fold selectivity for β2-AR compared to noradrenaline (Filadelfi et al., 2002).

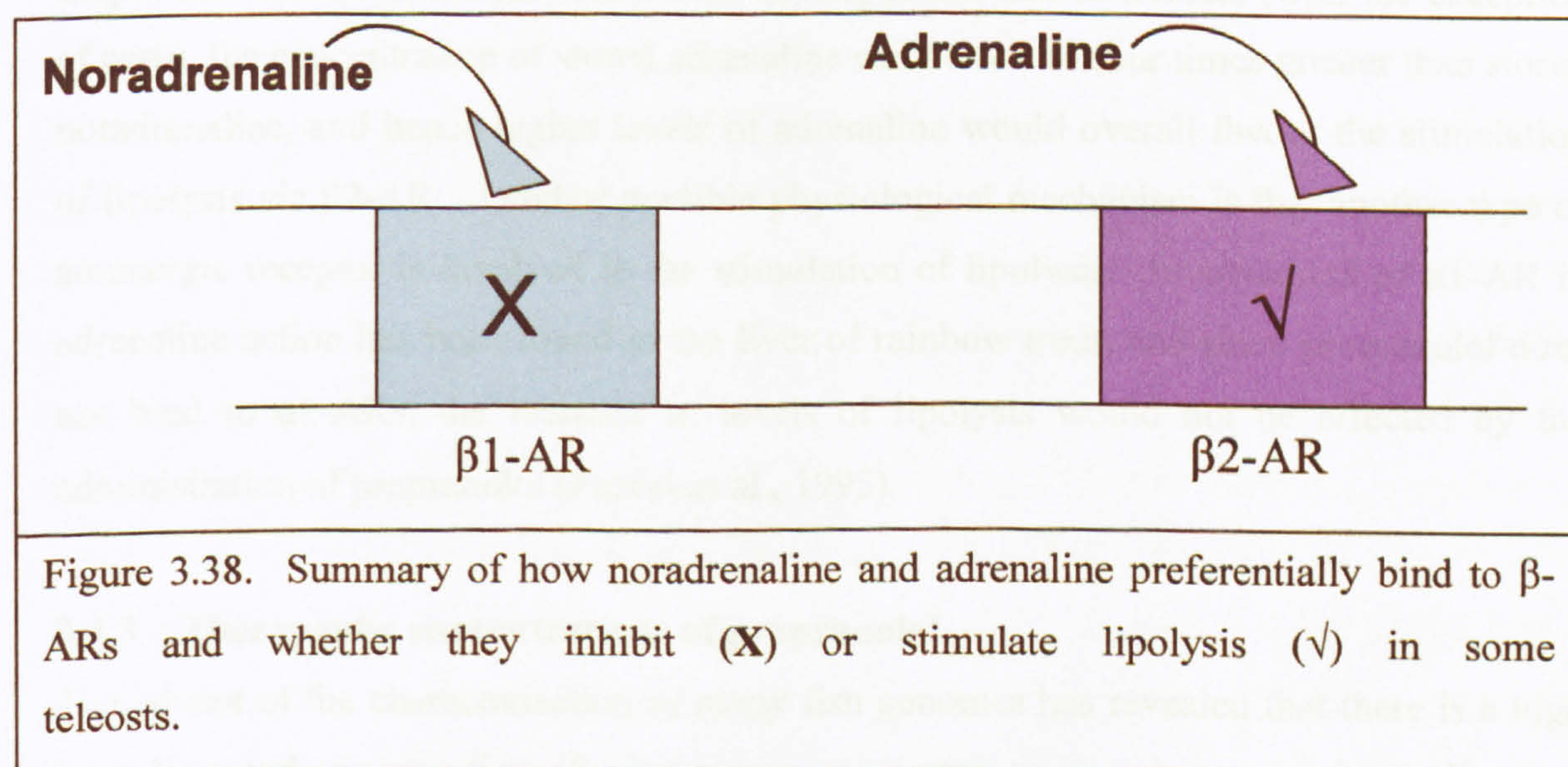


Figure 3.38. Summary of how noradrenaline and adrenaline preferentially bind to β-ARs and whether they inhibit (**X**) or stimulate lipolysis (√) in some teleosts.

Previous studies hence demonstrate that lipid metabolism in fish may be under a different mechanism of control compared to the one found in mammals. A possible reason for the suppression of lipolysis in fish, compared to mammals, may be to prevent the build up of fatty acids during hypoxic conditions, which could potentially disrupt bio-membranes (Van Den Thillart et al., 2001).

From the effects seen in the propranolol experiments with respect to the probable mobilisation of lipid reserves, and presuming that fathead minnows respond to catecholamines in a similar way to carp, propranolol would have to enable the stimulation of lipolysis and prevent or reduce the inhibitory effects of noradrenaline on β1-ARs. Possible mechanisms of action to facilitate this would be for propranolol to block more β1-ARs compared to β2-ARs. It is known that propranolol has a 2.1 greater affinity for β1-ARs compared to β2-ARs. However, this is a minor difference compared to metoprolol and bisoprolol, which have 75 and 120 times greater selectivity for β1-ARs compared to β2-ARs, respectively (Bristow, 2000). Alternatively, propranolol may compete more successfully with noradrenaline than adrenaline for β-AR binding sites, or desensitization of β1-ARs may have occurred, or on the other hand, stimulation of lipolysis could have been caused by an up-regulation in the expression of β2-ARs. It would require a considerable amount of research to discover the exact mechanisms of action of propranolol on lipid metabolism.

However, if fathead minnows do not respond in the same manner as do carp, other mechanisms of action may be occurring. It would seem that propranolol was acting in a similar way to timolol in studies by Vianen et al. (2002), for both propranolol and timolol (non-selective β-antagonistic drugs) stopped the normally low levels of lipolysis in both tilapia and fathead minnows. Reid et al. (1998) found that in teleosts (with the exception of carp), the concentration of stored adrenaline can be two to four times greater than stored noradrenaline, and hence higher levels of adrenaline would overall favour the stimulation of lipolysis via β2-AR. Another possible physiological mechanism is that another type of adrenergic receptor is involved in the stimulation of lipolysis. Involvement of α1-AR in adrenaline action has been found in the liver of rainbow trout, and since propranolol does not bind to α1-ARs, the increase in levels of lipolysis would not be affected by the administration of propranolol (Fabbri et al., 1995).

3.4.3 Therapeutic concentrations of propranolol

The advent of the characterisation of many fish genomes has revealed that there is a high homology and conservation of many enzyme/receptor systems between mammalian and teleost systems (Huggett et al., 2003). When pharmaceuticals are taken by patients, the most relevant concentration that describes the quantity of drug needed to interact with a specific drug target, and initiate a physiological response, is the human plasma concentration, as it is plasma that brings the target and drug into contact (Williams & Cook, unpublished). Since a certain plasma concentration of pharmaceutical is required to

affect target receptors and enzymes in humans, it has been hypothesised that approximately the same concentration would be required to affect another species sharing the same target (Brown et al., 2007) The 'fish plasma model' hypothesised by Huggett et al. (2003) compares estimated or actual drug concentrations in fish plasma with human therapeutic plasma concentrations, in order to assess whether it is likely that environmental or experimental concentrations of a drug would produce therapeutic levels in fish (Brown et al., 2007). The effect ratio (ER) is calculated as shown in Figure 3.39, and the lower the ER, the greater the potential there is for a pharmacological response to occur in fish (Huggett et al., 2003).

$$ER = \frac{\text{Human therapeutic plasma concentration } (H_TPC)}{\text{Fish steady state plasma concentration } (F_{SS}PC)}$$

Figure 3.39. Calculation used to obtain the effect ratio (ER) of a pharmaceutical.
If the ER is ≤ 1, the predicted drug concentration in the fish plasma is equal to or greater than the drug concentration in human plasma that elicits a therapeutic effect.
If the ER is > 1, the fish drug plasma concentration is lower than the plasma drug concentration in humans that elicits a therapeutic effect.

Due to the different physical properties of pharmaceuticals, some drugs are more likely to reach human plasma therapeutic levels from waterborne exposure than are others. Propranolol has a log Kow of 3.48, and since it is relatively lipophilic it is likely to bioaccumulate in fish from exposure via water. The propranolol concentrations in the plasma in fathead minnows used in the experiments conducted here show a dose-related relationship in that as the concentration of propranolol in the water increased, propranolol plasma concentrations also increased. This agrees with data by Wong et al. (1979), who established that propranolol plasma concentration and daily dose were significantly correlated. Table 3.18 shows the ER at each concentration of propranolol when compared to human therapeutic plasma levels measured in patients prescribed 80 mg/day of propranolol, which would be a sufficient therapeutic dose in the majority of patients with hypertension (www.pbs.gov.au). All of these patients had been on this medication for at least one week (Wong et al., 1979). It should also be noted that the internal plasma concentration of propranolol often exceeds the water concentration, showing bioaccumulation of the drug in the fish.

Propranolol concentration (mg/L)	Log Kow	H_TPC (µg/ml)	$F_{SS}PC_{(actual)}$ (µg/ml)		ER	
			Male	Female	Male	Female
DWC	3.48	0.146	<0.00025	<0.00025	>584	>584
0.001			0.000555	0.000690	263.06	211.59
0.01			0.00605	0.00855	24.13	17.08
0.1			0.34	0.21	**0.43**	**0.70**
1.0			15.00	5.75	**0.0097**	**0.025**

Table 3.18. Effect ratios (**ER**) of male and female fish exposed to different concentrations of propranolol. **H_TPC** calculated from Wong et al. (1979) from patients that had been prescribed a daily dose of 80 mg of propranolol for at least one week. **$F_{SS}PC$** is the actual plasma concentrations of male and female fish from the different propranolol treatments

From the ERs shown in Table 3.18, male and female fish exposed to 0.1 and 1.0 mg/L of propranolol have ER values less than 1. Hence, the plasma concentration of the drug in these fish is greater than the drug concentration in human plasma which is needed to elicit a therapeutic effect. This could explain why fish exposed to concentrations of 0.1mg/L of propranolol, and higher, showed statistically significant effects from the control in some endpoints.

When compared to other pharmaceuticals, as shown in Table 3.19, it can be seen that like propranolol, diclofenac and gemfibrozil also have ER values less than 1, unlike atenolol, which does not reach human plasma therapeutic levels in fish plasma. Although there is a high homology in receptor systems between humans and fish, it must however be remembered that the response to a pharmaceutical could be greater in non-target organisms than in humans, as they could have higher receptor density, or less receptors are needed to trigger a response.

Drug	Log K_{OW}	H_TPC(µg/ml)	$F_{SS}PC$ (µg/ml)	ER
Ibuprofen [1]	3.80	10	4.68	2.14
Naproxen [1]	3.10	20	3.64	5.49
Diclofenac [1]	4.02	0.42	3.44	0.12
Ketoprofen [1]	3.00	5	0.06	83.3
Gemfibrozil [1]	4.80	15	32.07	0.47
Atenolol [2]	0.23	1,000 (maximum)	51.8	19.3

Table 3.19. **H_TPC, $F_{SS}PC$** and **ER** values of different pharmaceuticals. Values in red show where the ER is less than 1.

[1]: Data taken from Brown et al., 2007.

[2]: Data taken from Winter et al., 2008.

However, despite a few drawbacks, the 'fish plasma model' uses intelligent science, based on the wealth of mammalian pharmacology available, to predict potential drugs that may cause harm to fish, and hence this approach can highlight where additional testing may be required.

3.4.4 Comparisons to other studies

The most sensitive endpoints in this study were shown to be hatchability (with regard to the number of days it took for eggs to hatch) and female GSI. Both of these endpoints had LOEC values of 0.1 mg/L. Huggett et al. (2002) also found hatchability to be a sensitive endpoint in medaka ($LOEC_{\text{hatchability \& egg production}}$) = 0.0005 mg/L). However, the data from this study, with fathead minnows, does not support the Huggett et al. (2002) data in that the difference in LOEC values between these two studies is 5,000-fold. The effects found by Huggett et al., (2002), although statistically significant when compared with the controls, did not exhibit a dose-response relationship, unlike the data from this study. In addition, the data from Huggett et al. (2002) were not repeated and the sample size at each treatment was relatively small. With respect to survival rates, fathead minnows ($LOEC_{\text{male survival}}$ = 1.0 mg/L, $LOEC_{\text{female survival}}$ = 3.2 mg/L) were shown to be more sensitive to propranolol than medaka (LC_{50} = 24.3 mg/L).

A parallel pair breeding assay using fathead minnows was conducted with another β-blocker, atenolol (Winter et al., 2008), and the results differed ten-fold from those obtained in this study. The most sensitive endpoint on exposing fathead minnows to atenolol was the male condition index (LOEC = 3.2 mg/L). Fathead minnows were also able to tolerate

high concentrations of atenolol (LOEC $_{survival}$ >10 mg/L), unlike in this study, where acute toxicity occurred at surprisingly low concentrations. The main reason for this could be due to the difference in the lipophilicity of these two β-blockers. Atenolol has a much lower log Kow, compared to propranolol, and therefore reaches much lower plasma concentration in fish. This was reflected in the measured plasma concentrations of these two pharmaceuticals. Plasma concentrations of atenolol in relation to the corresponding measured water levels only ranged from 1.8 to 12.2 %, unlike propranolol that bio-accumulated up to 1550 % of the surrounding water concentration of propranolol in male fish at 1.0 mg/L treatment (Winter et al., 2008).

Chapter 4 Expression of β1- and β2-ARs

4.1 Introduction

Adrenergic receptors are not just found in the heart, they are also be found in many different tissues. Using real time polymerase chain reactions (RT-PCR) and sequencing information from the β1- and β2-ARs, the expression levels of these adrenergic receptor subtypes in different tissues can be evaluated.

4.1.1 Expression of β-ARs in mammals and fish

Table 4.1 and table 4.2 show the expression of β1-ARs and β2-ARs in different tissues and in different species, respectively. This is not an exhaustive list, but instead represents a glance at the literature to show where expression levels have been researched. However, a variety of methods have been used to conclude that β-ARs are expressed in these particular tissues. As science and research techniques move on, it becomes apparent that some of the older techniques used, such as identification of a biomarker (e.g. changes in glucose levels), are not specific methods in determining expression of a particular gene, as too many other factors can alter such biomarkers (Van den Thilliart et al., 2001). Other older techniques, such as radio binding assays, could also provide misleading results. For example, if a β-blocker prevents the binding of radio-labelled adrenaline, you could conclude, maybe rightly, that the tissue contains β-ARs (GarciaSainz et al., 1996). However, some β-blockers, such as propranolol and endogenous catecholamines, can also bind to other receptors, such as seretonin receptors, and it could be these receptors, not β-ARs, that were involved in the binding.

In addition, a response of a tissue to a β-blocker does not necessarily mean that the tissue expresses β-ARs, as the response could be controlled by another organ. For example, it may be thought that changes in reproduction were brought about via β-ARs in the gonad. However, effects on reproduction could be affected by a β-blocker acting on the brain, which then controls reproduction (Huggett et al., 2002). With regard to the specificity of some results, jumps in knowledge can mean that some data were incorrectly interpreted. For example, the fairly recent characterisation of the β3-AR meant that the specificity of some techniques had to be revised. For example, Nickerson et al. (2003) found the predominant β-subtype to be β3-AR in fish red blood cells, and not β1-AR, as had been previously surmised by Perry et al. (1991). These problems demonstrate that some methods do not allow the unequivocal demonstration of the expression of β-ARs in particular tissues, and also that due to new information, the β-AR sub-type may have been incorrectly identified. Hence, in Tables 4.1 to 4.4, an asterix has been added where more

robust techniques (RT-PCR, northern blots, RNAse protection assays) have been used to identify β-AR expression in different tissues.

Animal	Tissue showing β1-AR Expression	Reference
Human	Heart, placenta, cerebral cortex and lung	Ellis and Frielle, 1999 *
	Pancreas, liver, heart, kidney, thalamus, adrenal glands	Evanko et al., 1998
	Heart	Hoffman,2001
		Strosberg, 1997
		Lemoine et al., 1988
	Heart and fatty cells	Nagatomo & Koike, 2000
Mice	Pulmonary lymphocytes	Jain et al., 2003 *
Rat	Pineal gland of the brain, heart	Machida et al., 1990 *
Xenopus	Mature oocyte	Devic et al., 1997 *
Trout	Liver	Van den Thilliart et al., 2001

Table 4.1. Overview of the expression of β1-ARs in different animals. * highlights where more robust techniques have been used to determine expression of β-ARs.

Animal	Tissue showing β2-AR Expression	Reference
Human	Heart	Lemoine et al., 1988
	Trachea (bronchi) and capillary vessels	Nagatomo & Koike, 2000
	Bronchial muscle	Hoffman, 2001
	Heart	Ellis and Frielle, 1999
		Hoffman, 2001
	Uterine muscle, detrusor muscle of the bladder	Hoffman, 2001
	Uterus, skeletal muscle, lungs	Strosberg, 1997
Dog	Liver	GarciaSainz et al., 1996
Channel catfish	Head kidney, spleen, leukocytes	Finkenbine et al., 2002
Trout	Liver, red muscle , white muscle, gills, heart, kidney, spleen	Nickerson et al., 2001 *
	Liver	Reid et al., 1992
		Van den Thilliart et al., 2001

Table 4.2. Overview of the expression of β2-ARs in different animals. * highlights where more robust techniques have been used to determine expression of β-ARs.

Tables 4.3 and 4.4 show that other β-AR subtypes (in some cases the subtype is not stated), have also been found in a variety of tissues in mammals and fish.

Animal	β-AR type	Tissue	Reference
Human	β3-AR	Adipose tissue	Lemaire & Rockman, 2004
		Brown and white adipose tissue	Strosberg, 1997
		Gastro-intestinal tract	
		Digestive tract, urinary bladder	Nagamoto & Koike, 2000
		Detrusor smooth muscle of urinary bladder	Takeda et al., 1999 *
	β4-AR	Heart	Nagamoto & Koike, 2000
	β-ARs	Smooth muscle	Hoffman, 2001
	ARs	Virtually every cell type	Small et al., 2003
Rat	β3-AR	Digestive tract, urinary bladder	Nagamoto & Koike, 2000
		Adipose tissue, stomach fundus, colon, ileum, heart	Evans et al., 1996 *
		Adipose tissue, illeum	Zaagsma & Nahorski, 1990

Table 4.3. Overview of the expression of other β-ARs in mammals.

Animal	β-AR type	Tissue	Reference
Rainbow trout	β3a-AR	Gill, heart, red muscle	Nickerson et al.,2003 *
	β3b-AR	Red blood cells	Nickerson et al.,2003 *
	β-ARs	Cardiac tissue	Keen et al., 1993
		Red blood cells	Wang et al., 1999
			Reid & Perry, 1991
			Thomas & Perry, 1994
		Female gonads.	Dzialowski et al., 2006
Zebrafish	β-ARs	Heart	Fraysse et al., 2006
Medaka	β-ARs	Gonad	Huggett et al., 2002

Table 4.4. Overview of the expression of other β-ARs in fish. * highlights where more robust techniques have been used to determine expression of β-ARs.

Data in Tables 4.1 to 4.4 helped decide which tissues would be selected to evaluate the expression of β1 and β2-ARs in the fathead minnow. Huggett et al., (2002), in a four week exposure of medaka to propranolol, found that the total number of eggs and the number of viable eggs that hatched were significantly reduced at concentrations as low as 0.5μg/L. Because of this result, gonadal tissue was selected for expression analysis to test the hypothesis that propranolol caused an alteration in the expression of β-ARs in the gonads, which could explain the results found by Huggett et al., (2002). Heart tissue was chosen for analysis, for as shown in Tables 4.1 to 4.4, both β1 and β2-ARs are found in the hearts of mammals and fish, and it is this major organ that β-blockers are designed to target. Liver was also selected for this study as the liver is a major site for catecholamine-stimulated glycogenolysis (glycogen metabolism) and gluconeogenesis (glucose metabolism) and triglyceride synthesis (lipogenesis) in humans (Dentin et al., 2006; Fabbri et al., 1998). Hence, if metabolic changes were occurring because of propranolol, changes in the β-AR expression may be seen in this organ. The brain was chosen for β-AR expression analysis because in human's, β1- and β2-ARs are found in smooth muscle around hollow organs that have to contract (i.e. not skeletal muscle) and so by choosing the brain, which is not skeletal muscle, it tests the hypothesis that there would be no expression of β1- or β2-ARs because the brain is not made of muscle. Hence, heart, brain, gonad and liver were the tissues used for expression analysis. These tissues were obtained from female fathead minnows that had been used in the propranolol experiments (see chapter 3 for full details of these experiments).

4.1.2 Absolute and Relative RT-PCR

Two types of RT-PCR methodology exists. One is known as absolute RT-PCR and the other is relative RT-PCR. Both methods allow gene expression quantification and unlike northern blots are exquisitely sensitive and permit the analysis of gene expression from very small amounts of RNA (Freeman et al., 1999). The difference between relative and absolute RT-PCR lies in how results are quantified. With relative RT-PCR, a housekeeping gene, such as β-actin, is used to help quantify results. The theory behind this method is that expression of a housekeeping gene is constant throughout all tissues and will not vary in response to external pressures, such as chemical exposure, hypoxia, starvation, etc. Hence the change in expression of a gene of interest can be measured relative to the constant expression of a housekeeping gene. This was a popular method in the past, as the methodology was slightly easier than absolute RT-PCR. However, it has recently been discovered that the expression of housekeeping genes can vary, sometimes

quite drastically, which will of course invalidate the use of that gene in quantification methods.

Absolute RT-PCR uses a standard which is an amplicon of the target gene that is being measured. Once the number of molecules in a defined aliquot of external standard is measured, a standard curve, covering a known concentration, is run on each assay plate. Providing the samples fall within the standard curve, the number of molecules expressed per μl of sample can be calculated. This method is a much more robust way of quantifying the expression of a particular sequence, since the strand of RNA, or in this case mRNA, of the standard is the same as the mRNA strand that is being analysed. Hence both strands simultaneously undergo reverse transcription and amplification under the same conditions, using the same primers, thus introducing less error in quantification.

4.1.3 Use of RNA in RT-PCR

Three classic types of RNA exist, messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA), and recently a fourth category of micro-RNAs has also been discovered (Groβhans & Filipowicz, 2008). Most RT-PCR methods use total RNA, as the conversion of RNA to mRNA is an extra step and could result in possible loss of material. Further, the fact that problems include not all mRNA molecules have poly(A) tails, normalisation against total RNA or rRNA is not possible when using mRNA, and the concentration of mRNA may be insufficient to allow quality assessment using some machines (Bustin, 2000). However, even if total RNA is used in RT-PCR, it is only actually mRNA that is being measured, as it is only mRNA that contains the coded information stored in DNA and which is attained during transcription (Lodish et al., 2004). Hence, the use of total RNA when amplifying small copy numbers will result in a lot of background noise, and if maximum sensitivity is required, mRNA is the preferred choice of starting material.

4.1.4 Aim of RT-PCR

The up or down regulation of receptors can be affected by chemicals in the environment, which can result in a change in the physiological response. For example, exposure to environmentally relevant concentrations of ethinylestradiol alters the pattern of expression of estrogen receptor subtypes (ERα and ERβ) in the brain, testis and liver of roach (Katsu et al., 2007). The up-regulation of ERα during exposure to ethinylestradiol during early life stages induced gonadal feminization and vitellogenin induction in male roach, hence providing a molecular explanation for these changes (Katsu et al., 2007). Pharmaceuticals

are often administered to up or down regulate receptors. For example, a nasal contraceptive spray is being developed that releases gonadotropin-releasing hormone (GnRH) agonists. If GnRH receptors are down regulated by this spray, as anticipated, this will in turn prevent the production of gonadotrophins which induce ovulation (Kamran & Moghissi, 2000). Another example where gene expression is altered by a pharmaceutical is provided in a study by Villeneuve et al., (2007). Using quantitative real-time PCR, an increase in aromatase-A mRNA expression in fathead minnow ovary after exposure to fadrozole was observed. The up-regulation of this isoform was consistent with the direct mechanism of action of fadrozole. Hence, the hypothesis behind the RT-PCR work was to discover whether propranolol caused an up or down regulation of β1- and/or β2-AR expression levels that might explain any physiological responses observed.

4.2 Materials and Methods

4.2.1 Tissue acquisition

For the set up and validation of the RT-PCR technique, liver tissues from fathead minnows were used. Tissues used in the analysis of β1- and β2-ARs were obtained from female fathead minnows from the two propranolol experiments. All tissues were acquired by dissection as detailed in section 2.2.1, and mRNA was obtained as set out in sections 2.2.2 and 2.2.4. Because fathead minnow hearts are so small, to enable the analysis of β1- and β2-AR expression levels in heart tissue, all the hearts obtained from the propranolol experiments had to be combined into one sample for each concentration, to obtain a high enough concentration of mRNA for testing. All other tissues were tested per individual fish, making the number of samples tested a lot higher than for the heart.

4.2.2 RT-PCR Overview

Absolute RT-PCR was used to quantitatively assess the expression levels of β1- and β2-ARs in the heart, liver, gonad and brain of female fathead minnows. The starting material for the samples, internal standard and the external standards was mRNA. During the assay, mRNA was reversed transcribed into cDNA and subsequently the target DNA was amplified using gene specific primers. QuantiTect SYBR Green (Qiagen, Crawley) was added to each well in the master mix and this bound to any double stranded DNA produced during the assay. The amount of DNA at the end of the assay was measured in each sample by the amount of fluorescence emitted from the SYBR Green. Care had to be taken to ensure that no non-specific primer annealing occurred during the assay, which would produce extra DNA products as these non-target products would be included in the measurement of SYBR Green fluorescence, leading to an incorrect result. Additionally, all traces of contaminant DNA were properly removed from samples and standards before the assay began.

4.2.3 RT-PCR preparation of primers, target DNA and standards

For a high efficiency in RT-PCR it is recommended that each amplicon (piece of target DNA that will be amplified) is between 75 to 150 bp in length, as the shorter the amplicon, the greater the efficiency of the amplification. However, the amplicon must be longer than 75 bp to distinguish it from primer dimers. Amplicons must not have any secondary structures and it is recommended that the guanine: cytosine (G:C) content should be between 50 to 60 % and that repeats of a single base pair should be avoided. Figure 4.1 shows the location of the amplicon within the β1-AR. The amplicon is 185 bp long and has a G:C content of 55%.

5'ACGCGGGGGGGCTGGAGACgagagcgcgg**atg**gaagcgctccacacaggacctgaagttctgaatgagc
gcgcttcatttctccacaccatgggagacggtttaccgtctgtaaactacagcaatgactctaaacgcaccccggataacttatcaga
acagtggctcgtgggcatgggaatcatcatgggtctggtagtaattgtcattgtagtggggaatatactggttatagtcgccatagcg
cggaatcagaggctccagacgctcaccaatgttttcatagtgtctctggcgtgcgcagaccttatcatggggttactggtggtgccat
ttggcgcagacttggaggtcagaggatcttggatgtatggatcgttcttctgtgaattctggatatctcttgatgtactttgcgtcacggc
gagcatcgagaccctgtgcgtaattgcaattgacaggtacatcgccatcacctctccatttcgctatcaaagccttttaacgaaagca
cgagccaaggtggtggtgtgtgcagtttgggctatatcagctcttgtgtcatttccacccatcctaatgcactggtcccgggacaccg
tggatacatcatgctataacgaacccgagtgctgtgacttcatcaccaaccgtgaatatgccatctcatcctccgttatatcgttttacat
ccctttaatagtcatgatattcgtctatgccagggtatacagagaagccaaacaacaactgaaaaaaattaacaaatgtgagggaag
attctacaacaatggtactaattgcaaacctaaccgaaaacgaaccaccaagatcctggctttaaaagagcagaaggcgttgaaaa
cgttgggaataatcatgggaacattcactctctgctggctgccgttcttcatcgttaacgtggtgcgggtgtttggcaaagaggtggt
gaaaaaggaact*cttcgtattttgaactggctggggtacgtcaactccgcctttaaccccatcatatactgtcggagtcccgacttt*
aggaaagcctttaagaggctgttgtgttgtccgaggcaggcggaccgcaggttgcacgtgagctcgtgcgatctgtcgcgctg
*caccgggggctttgtgaactcaatgg*agcagagcatgctcgggacctggtcggactgtaacggcacggacagccgcgactgca
gtctagagaggaacggaagggtgtcccattcagagtctcagctg**taa**aagtacatctgcgaatatcaagtcaatattgcacgagct
gtttgttcgattggaaatcaggttatagcctactggtgcgctagtgatatgtgagtgtgattgtttcagtgaacgtttcagacaaattcga
aaatatttccACAAAAAAAAGAAAAAAAA 3'

Figure 4.1. Nucleotide sequence of the β1-AR in the fathead minnow. The amplicon (185 bp) is shown in *italics* between the two primers highlighted in blue. Amplicon length: 185 bp. G:C content: 55 % The amplicon has no secondary structure. Nucleotides in orange represent the UTRs. **atg** and **taa** are the start and stop codons, respectively.

Figure 4.2 shows that the location of the amplicon in the β2-AR. A part of the β2-AR amplicon was within the 5' UTR of the receptor. Because the primers match both types of β2-AR characterised (i.e.197 and 207), the RT-PCR will amplify and quantify both types. The amplicon selected is 226 bp long, has a G:C content of 46%, and no secondary structure.

There are also criteria that are used when designing primers that will select the amplicon from all the cDNA present during the assay. The most important feature is that the primers should anneal specifically to the target and no other piece of DNA. Both primers should have similar melting temperatures of 50 to 60°C, and should be 18 to 30 nucleotides in length. The G:C content should be between 40 to 60 % and no more than two guanine or cytosine nucleotides should be in the last five bases, yet one of these nucleotides should be at each end of the primer. Additionally it is recommended that there are no repeats of any single nucleotide in a row and checks should be made to ensure that no primer dimers or secondary structures are formed, either within the primer or with the primer pair. To help ensure that the primers designed fell inside these criteria, each primer pair was run through a programme (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) to assess its fit to the criteria.

```
197        5'  ---------------------------------------------------CATGACCAG 9
207            CGACATTTAGTCTACAGCCGAGAGTGCTGTGCACATGAGAGTAAAAAAAAACATGACCAG 60
                                                                  *********

197            GTGATCAAGAGTCGAGTGAAAGAACACTATGTGAGACCAGAACACTTCCTAAGAGAAAAG 69
207            GTGATCAAGAGTCGAGTGAAAGAACACTATGTGAGACCAGAACACTTCCTAAGAGAAAAG 120
               ************************************************************

197            GATTATTTTTGGATAGTGGACTCCTAATTTTAGTCAAAGCTCATGGAGCGAGGGGATAGG 129
207            GATTATTTTTGGATAGTGGACTCCTAATTTTAGTCAAAGCTCATGGAGGGAGGGGATAGG 180
               ************************************************ ***********

197            TTGAGCGCGGAGAACACCTCCCTGCACATGAATGTTTCATCTGGGCTAAACGACTCTTCT 189
207            TTGAGCGTGGAGAACACCTCCCTGCACATGAATGTTTCATCTGGGCTAAACGACTCTTCT 240
               ******* ****************************************************

197            CCGGTGTCCGAATATAGCGACGCAGAGGTGGTCTTAATTAGCATCTTAATGGGGCTTCTG 249
207            CCGGTGTCCGAATATAGCGACGCAGAGGTGGTCTTAATCAGCATCTTAATGGGGCTTCTG 300
               ************************************** *********************

197            GTTCTAGGCATCGTCTTTGGCAACGTGCTGGTCATCAGCGCCATTGTACGATTTCAACGC 309
207            GTTCTAGGCATCGTCTTTGGCAACGTGCTGGTCATCAGCGCCATTGTACGATTTCAACGC 360
               ************************************************************

197            TTGCAGACGGGCACCAACTACTTCATCAGCTCCCTTGCCTGCGCCGACTTGGTCATGGGT 369
207            TTGCAGACGGGCACCAACTACTTCATCAGCTCCCTTGCCTGCGCCGACTTGGTCATGGGC 420
               *********************************************************** 
```

Figure 4.2. A comparison of the initial part of the sequence for 197 and 207 versions of the β2-ARs isolated in the fathead minnow. The amplicon (224bp) is shown in *italics* between the two primers highlighted in blue. Nucleotides in orange represent the 5' UTR. **ATG** is the start codon.

Once the amplicon and primers had been designed, they were run on a PCR machine with fathead minnow liver cDNA to ensure that the primers were specific to the target amplicon and only amplified one product. The PCR products were run on a 2 % agarose gel to verify this, as shown in Figure 4.3.

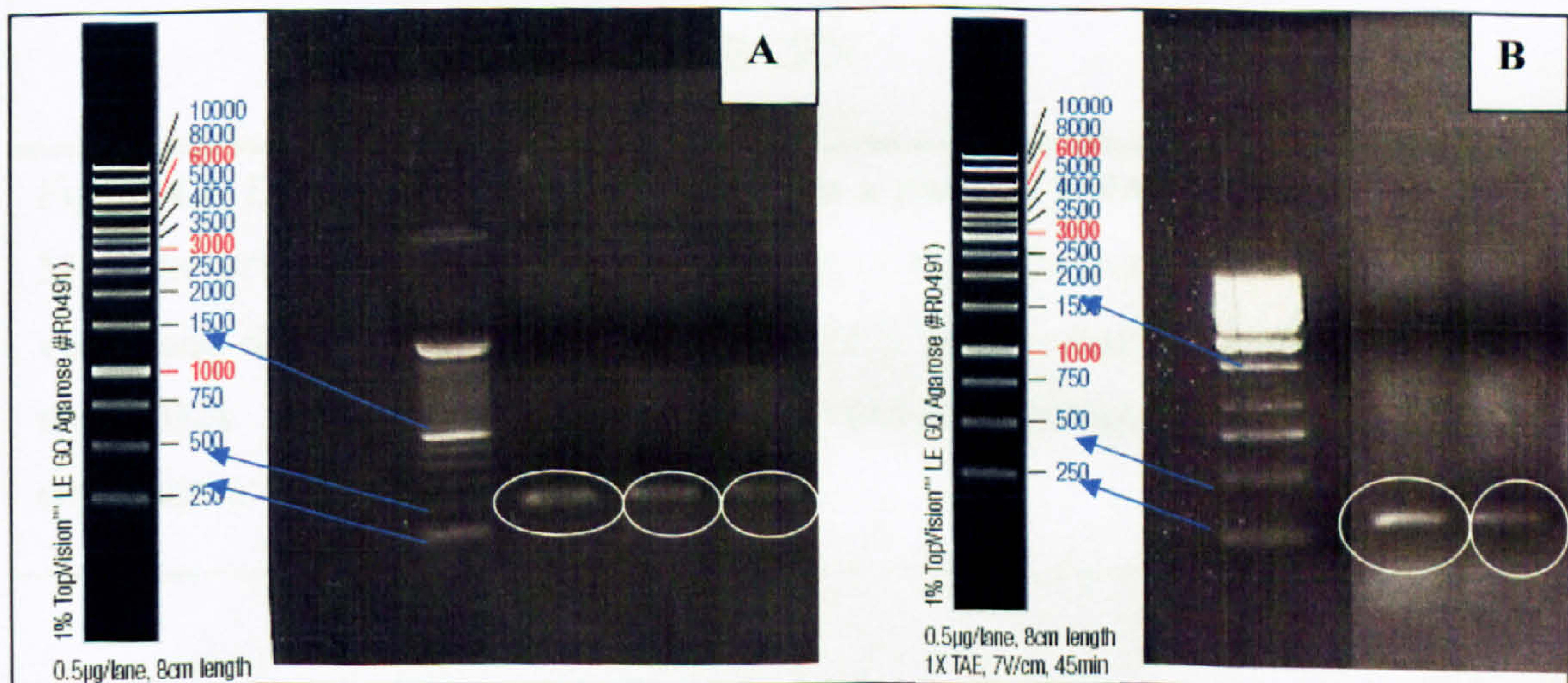


Figure 4.3. Photographs taken under UV light of PCR products run on a 2 % agarose gel. A 1Kb DNA ladder is shown on the left of each photograph and the white circles highlight the fact that only one PCR product was amplified. **A**. PCR products obtained by using β1-AR specific primers. **B.** PCR products obtained by using β2-AR specific primers.

External standards were made from cloning vectors that had the relevant transcript inserted, as detailed in section 2.2.9. Instead of using the whole vector to make the external standards, a PCR was run to amplify only the relevant part of the vector. This was done using M13R and M13F primers, which matched sequences in the vector. Hence the piece of DNA amplified in the PCR contained the DNA insert (the part of the β-AR), called the amplicon, SP6 and T7 promoters and a small part of the vector, as detailed in Figure 4.4. This PCR reaction was carried with proof reading taq to ensure correct amplification of the product.

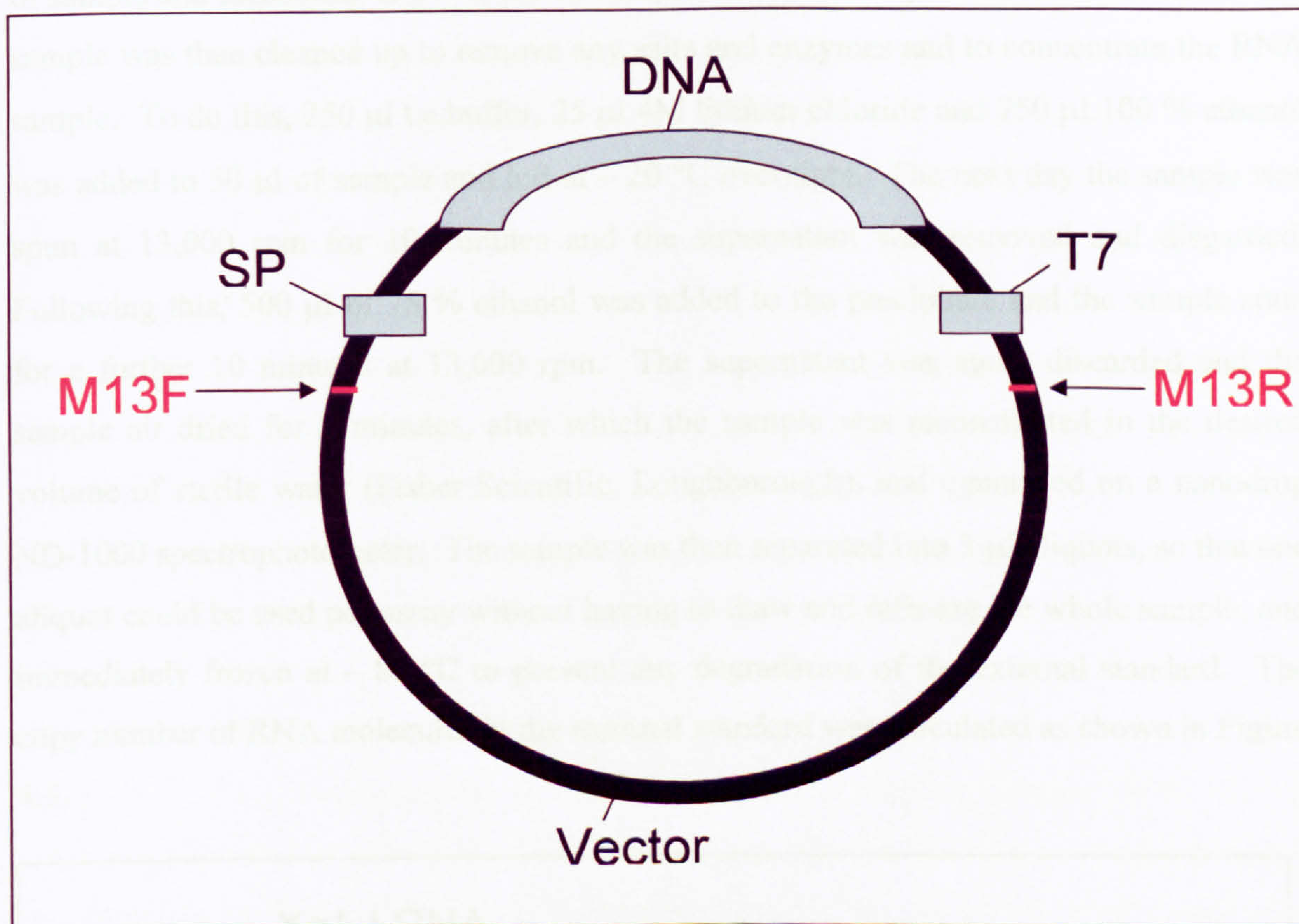


Figure 4.4. Diagram of a vector that contains a piece of DNA inserted into it. Using M13F (5' GTTTTCCCAGTCACGAC 3') and M13R (5' CAGGAAACAGCTATGAC 3') primers, the vector was cut almost in half, so that during PCR the part that was amplified contained the DNA insert and SP6 (5' CATTTAGGTGACACTATAG 3') and T7 (5' GTAATACGACTCACTATAG 3') promoters

The PCR products were run on a gel and the DNA extracted, as set out in section 2.2.8 and 2.2.9. The DNA sample was cleaned up using MinElute PCR purification kit (Qiagen, Crawley) to remove any salts and enzymes and to recover and concentrate the DNA. The end product was then quantified using a nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Loughborough) against a blank reading of 1.5μl elution buffer.

The extracted and cleaned DNA underwent in-vitro transcription (whereby RNA was generated from the DNA sample) using the Riboprobe in-vitro transcription system (Promega, Southampton). The orientation of the DNA insert in the vector dictates which enzyme is used in the reaction. For instance, if the transcript is in the anti-sense direction, SP6ase is used, whereas if it is orientated in the sense direction, T7ase is used. The DNA sample was incubated for 2 hours at 37 °C with the correct enzyme and a mix of other components including dNTPs, according to the protocol. The newly made RNA sample was DNAse treated (Invitrogen, Paisley) by adding 2 µl of DNAse I enzyme to every 50 µl of sample and incubating at 37 °C for 15 minutes to remove any DNA contamination. The sample was then cleaned up to remove any salts and enzymes and to concentrate the RNA sample. To do this, 250 µl t.e.buffer, 25 µl 4M lithium chloride and 750 µl 100 % ethanol was added to 50 µl of sample and left at – 20 °C overnight. The next day the sample was spun at 13,000 rpm for 10 minutes and the supernatant was removed and disgarded. Following this, 500 µl of 75 % ethanol was added to the precipitate and the sample spun for a further 10 minutes at 13,000 rpm. The supernatant was again discarded and the sample air dried for 5 minutes, after which the sample was reconstituted in the desired volume of sterile water (Fisher Scientific, Loughborough), and quantified on a nanodrop ND-1000 spectrophotometer. The sample was then separated into 3 µl aliquots, so that one aliquot could be used per assay without having to thaw and refreeze the whole sample, and immediately frozen at – 80 °C to prevent any degradation of the external standard. The copy number of RNA molecules in the external standard was calculated as shown in Figure 4.5.

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

Figure 4.5. Equation used to determine the number of RNA molecules per µl of sample. Xg/µl refers to the concentration of the RNA sample. The transcript length is the length of the whole transcript from (and including) M13F to M13R.

To normalise each plate, an internal standard made from fathead minnow liver mRNA and diluted to 5 ng/µl was made and stored in 20 µl aliquots. The internal standard was run in triplicate on all assay plates.

4.2.4 RT-PCR assay

All surfaces, pipettes and the centrifuge were wiped down with RNase away to remove any sources of contamination. All the components were defrosted on ice, and whilst this occurred the RT-PCR machine was set up with the plate details and assay parameters. The external standards were made up by serial dilution so that the working range of the standards was from 10^7 to 10^1 molecules/ µl. For each dilution the starting sample was flicked, spun for 10 seconds, added to sterile dilution water and mixed by repeat pipetting. The diluted sample was then left on ice for 3 minutes before the process was repeated for the next serial dilution. The samples were thawed on ice and diluted to 5 ng/µl using sterile water. The master mix for each assay plate was made up by adding SYBR Green, target specific primers, reverse transcriptase and RNase free. These components were briefly spun to ensure proper mixing and then 20 µl was pipetted into each well with a 200 µl pipette using reverse pipetting to reduce error from air bubbles and film retention. This was not carried out on ice, as some methods suggest, as ice can create primer dimers in the mix. Triplicate 5 µl samples of RNA sample, standard or sterile water (non-template control, NTC) were added to the designated wells and a clear cover was stuck to the plate, pushed down with a scraper and a compression pad was placed over the top of the plate. The plate was placed in a centrifuge and spun for 15 seconds to mix the contents of each well, after which the plate was placed in the RT-PCR machine and the assay started.

4.2.5 Interpretation of the results

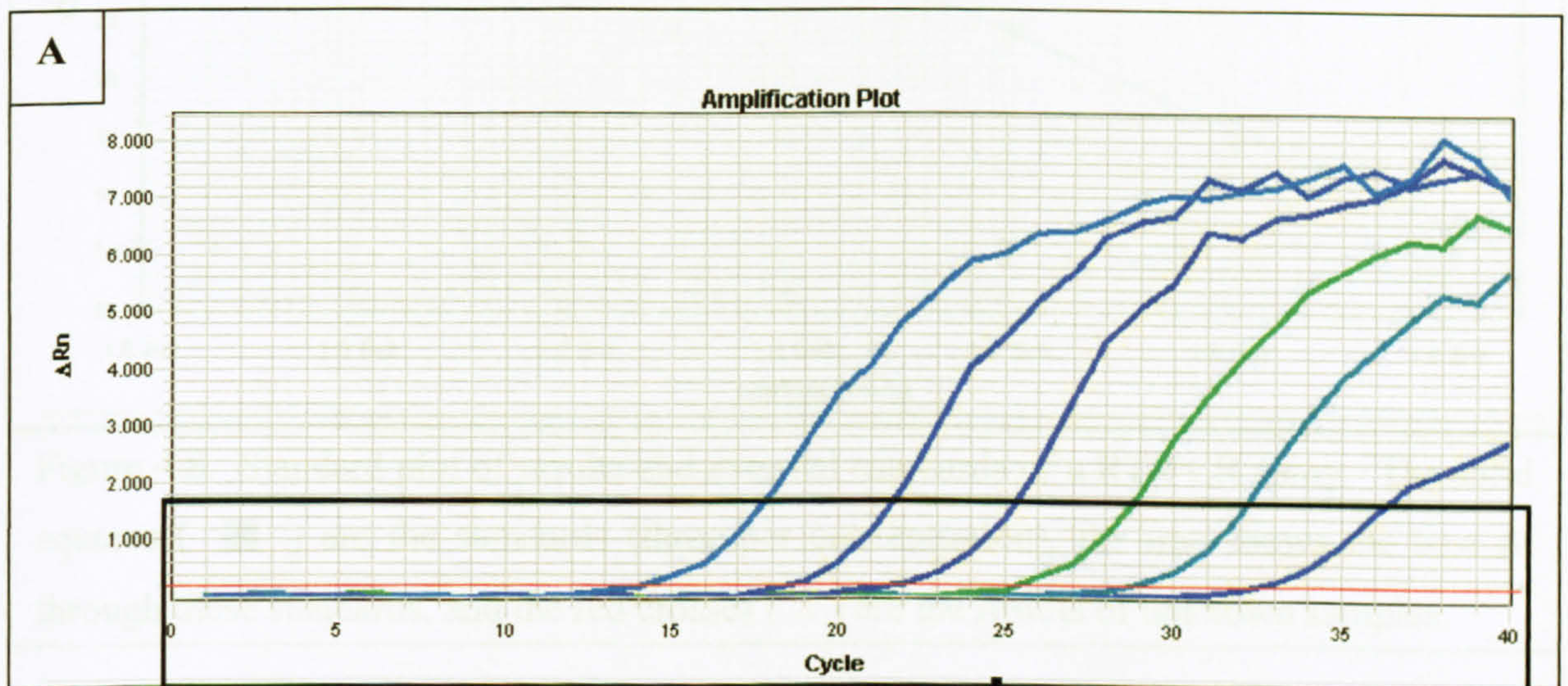
To ensure there was no contamination in the assay and to determine whether primer dimers had formed within the master mix, the non-template control (NTC) was analysed. Any fluorescence seen in the NTC wells is deducted from the results for all other wells. If a DNA product is detected in these wells, it would show that contamination had occurred. The efficiency of each reaction is calculated as shown in Figure 4.6, and should be greater than 90 %.

$$\frac{1}{-\text{slope}} = a$$

$$10\ x^y\ a - 1 \times 100 \qquad = \text{Efficiency (\%)}$$

Figure 4.6. Calculation used to work out the efficiency of a RT-PCR reaction.

The actual results produced are sigmoidal-shaped amplification plots, in which the number of cycles is plotted against fluorescence. In each sample, the cycle number in which the amount of fluorescence emitted by the SYBR Green reaches a threshold limit represents the desired result for that sample. The threshold limit is a preset limit in which a significant increase in fluorescence occurs; this limit is known as the C_T line (threshold cycle) and from this the amount of starting template can be deducted. Figure 4.7 explains how this theory works in practise.



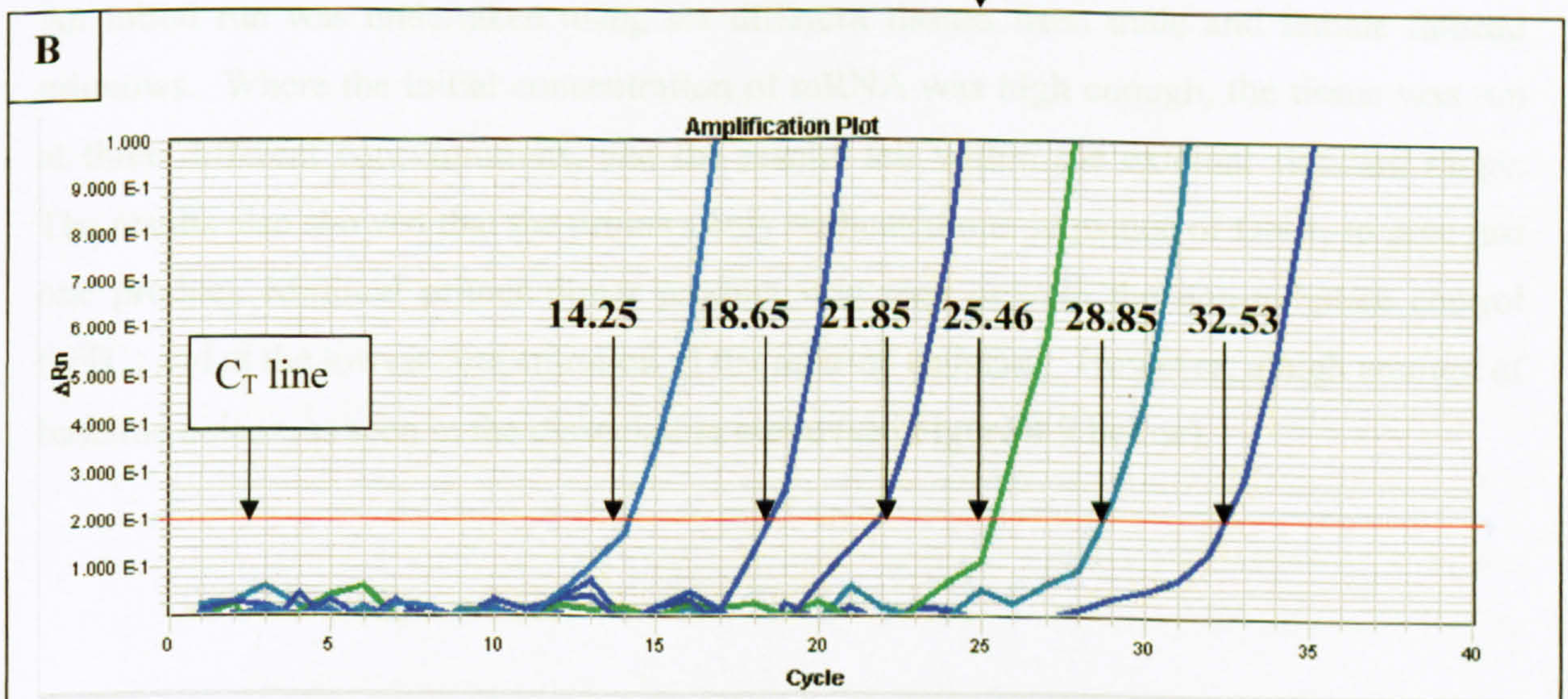


Figure 4.7. Amplification plots from a RT-PCR assay. Red horizontal line is the automatically pre-set C_T line. **A**. Amplification plot. **B.** is a close up of **A** to show that the result for each sample is the cycle number when the sample line (the amplification plot) crosses the C_T line.

Because each assay is run with external standards of known concentrations, the C_T results can be converted into the number of molecules of RNA /μl (see Figure 4.8).

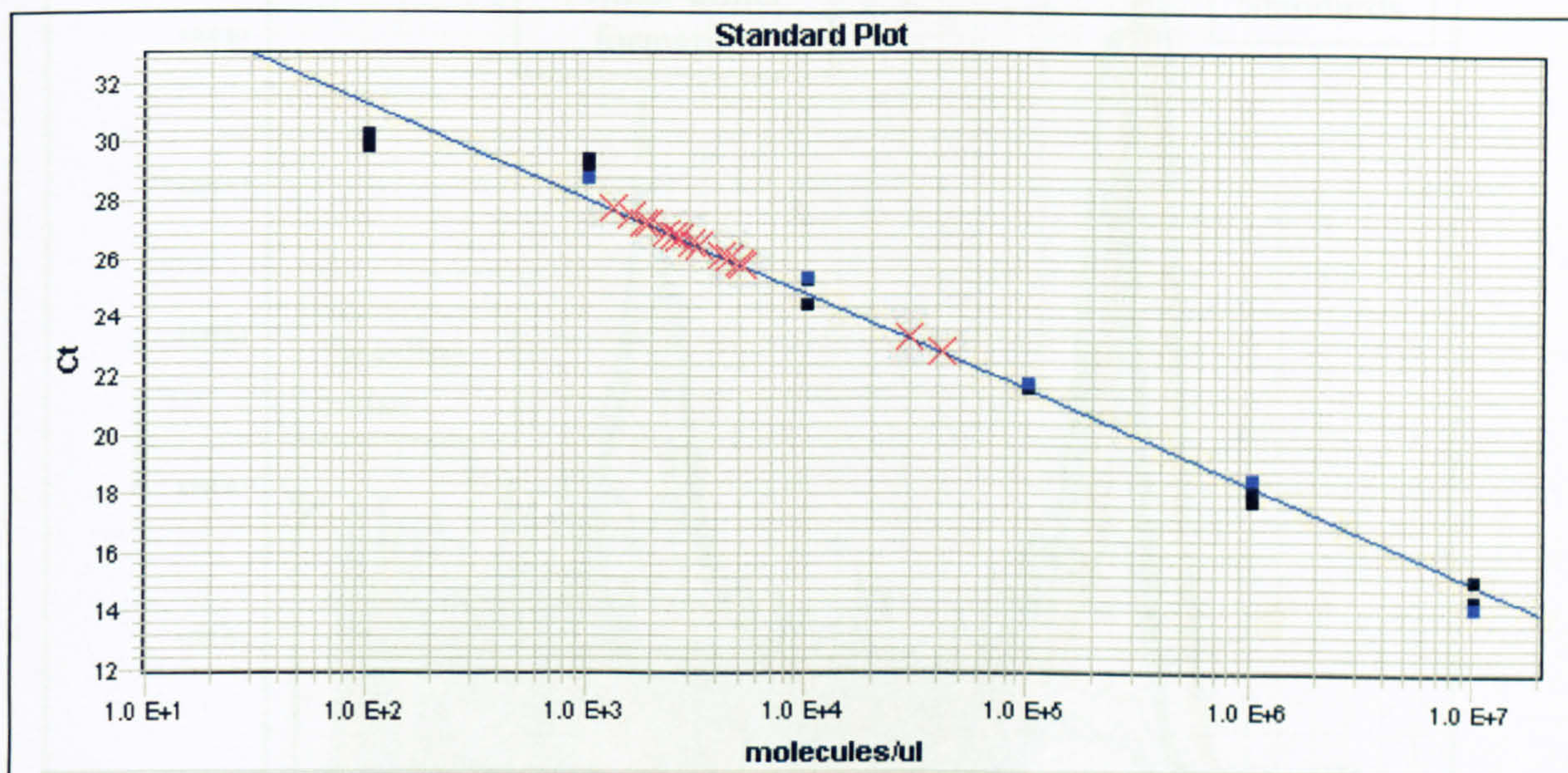


Figure 4.8. Standard plot of results and external standards of a RT-PCR assay. The solid squares (■) are the standards (three per concentration), the line shows the best fit through these standards, and the red crosses (X) are the results of unknown samples.

4.2.6 Optimisation of β1-AR RT-PCR

An initial run was undertaken using six different tissues from male and female fathead minnows. Where the initial concentration of mRNA was high enough, the tissue was run at three different concentrations, and the results fell within the external standard range. The results also showed that the primers only replicated one sequence of DNA, to give just one product. Minimal primer dimer product was seen only in the non-template control (NTC) and at the lowest concentration of the internal standard. However, a high amount of baseline noise was seen in the dissociation curve (see Figure 4.9 below).

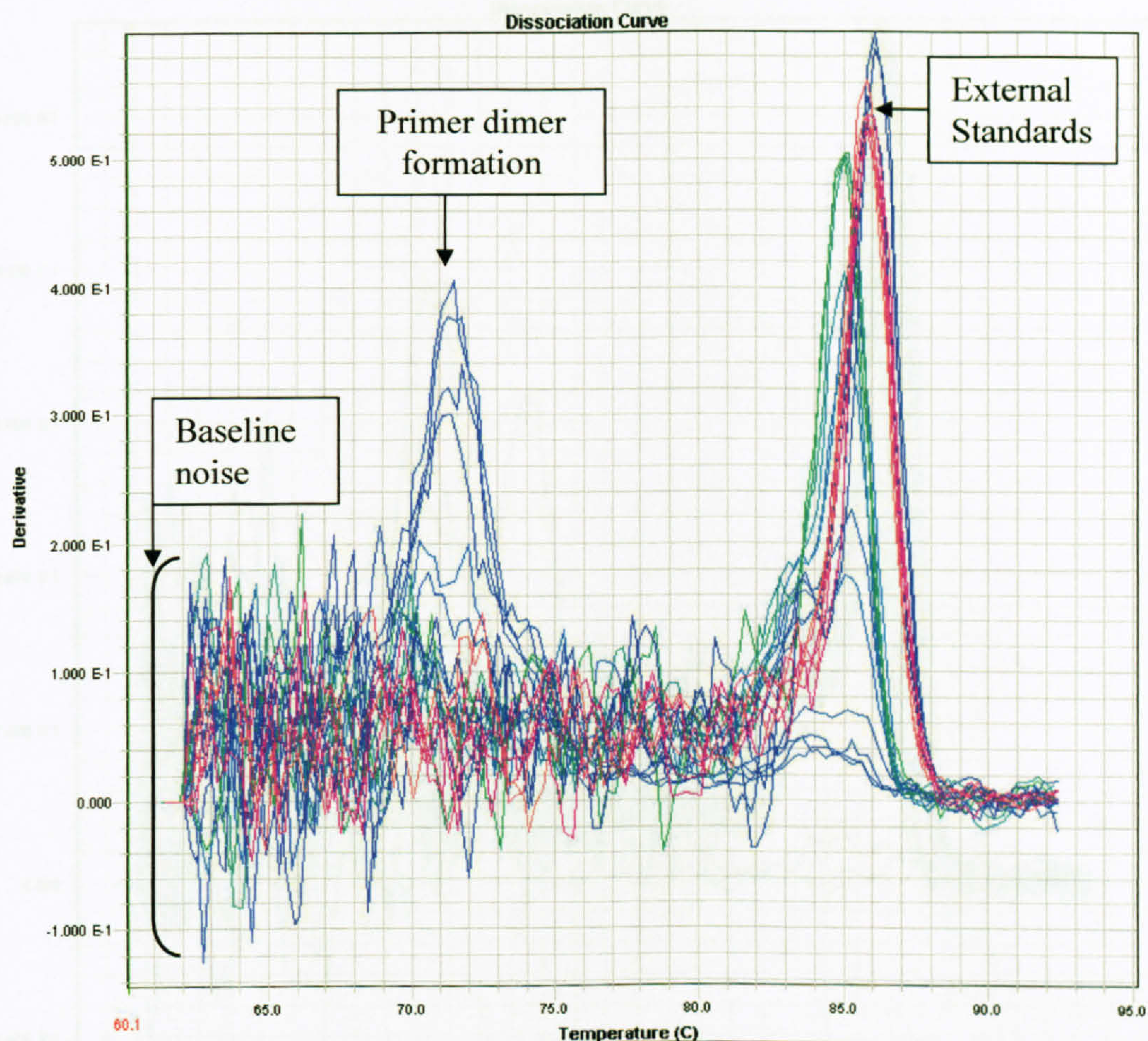


Figure 4.9. Dissociation curve for the β1-AR RT-PCR assay, NTC and external standards. This assay was run with an annealing temperature of 53°C and an extension time of 15 seconds over 40 cycles. There is a large amount of baseline noise seen in this assay.

A series of individual changes were made to the assay in an effort to reduce the amount of baseline noise, as part of an optimisation of the assay. Initially the extension time of the assay was reduced from 15 to 10 seconds, in an aim to reduce the amount of baseline noise by reducing the amount of non-specific amplification that could be caused by a longer extension time in the PCR cycle. The dissociation curve of this assay, as shown in Figure 4.10, shows the background noise to be worse after reducing the extension time, compared to the previous assay.

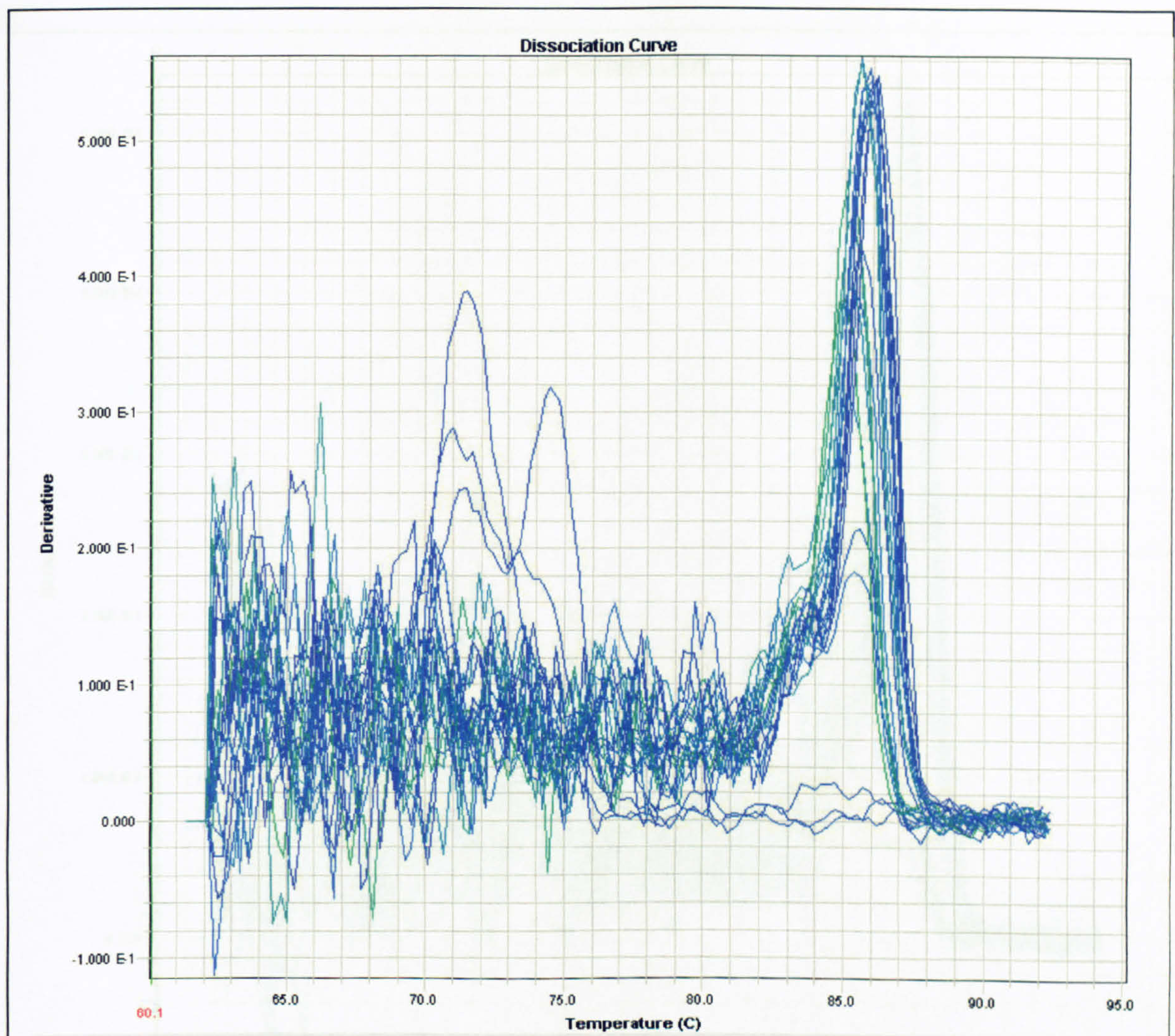


Figure 4.10. The dissociation curve for the NTC and internal standards for the β1-AR RT-PCR assay. This assay was run with an annealing temperature of 53°C and a reduced extension time of 10 seconds over 40 cycles. The extension time had been reduced in this assay but the background noise is still present.

It was concluded that reducing the extension time from 15 seconds to 10 seconds actually increases the amount of baseline noise, and hence was undesirable.

The next alteration to the assay in an effort to reduce the amount of baseline noise was to increase the annealing temperature from 53 to 55 °C. The theory was that a higher annealing temperature would produce more specific PCR products and less non-specific products that might be the cause the background noise.

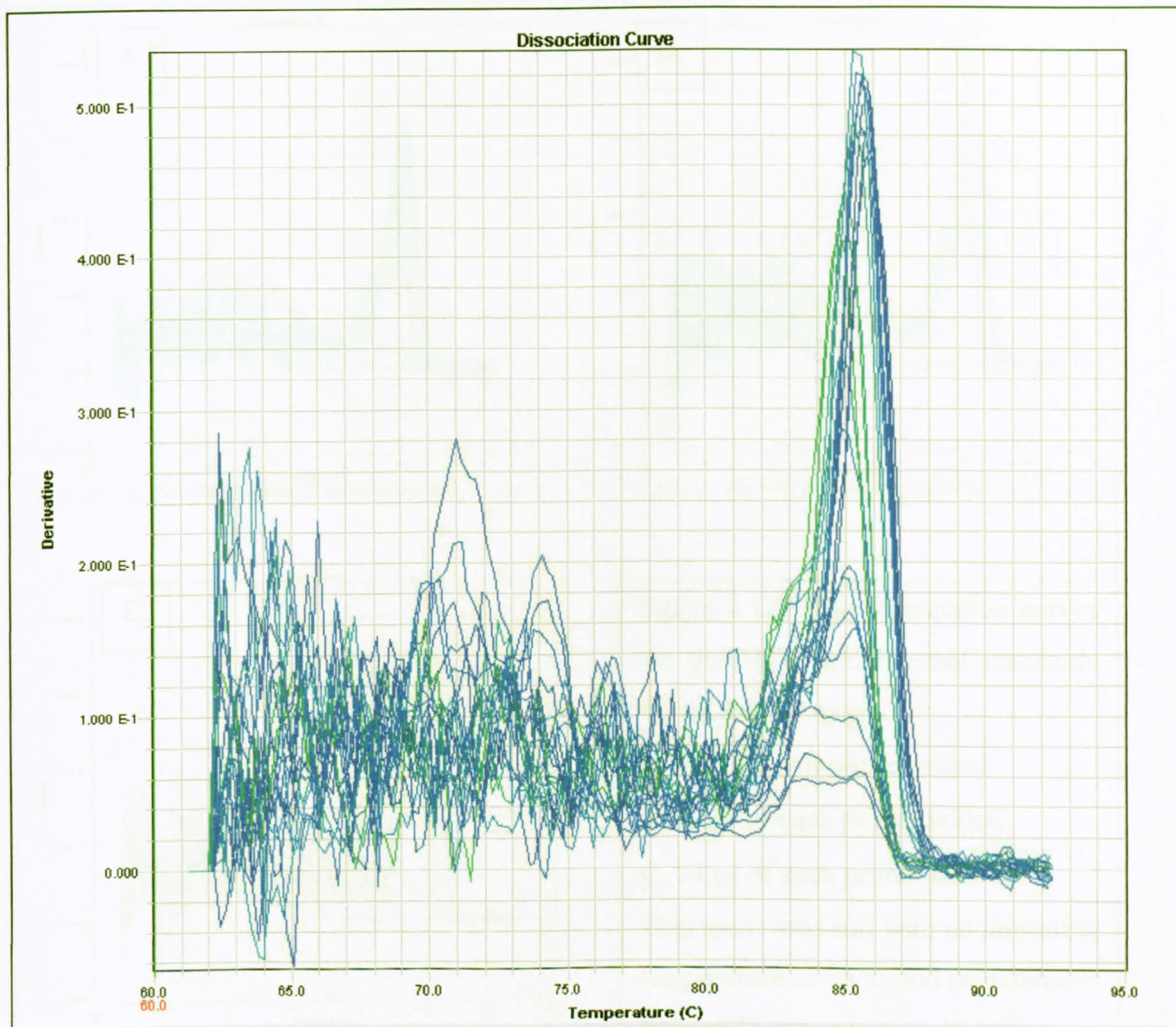


Figure 4.11. The dissociation curve for the NTC and internal standards for the β1-AR assay. This assay was run with an increased annealing temperature of 55°C and an extension time of 15 seconds over 40 cycles. The annealing temperature had been increased in this assay and the background noise is slightly reduced.

As Figure 4.11 shows, the amount of background noise is slightly reduced after this change in the assay. The amount of primer dimer is also reduced. Hence, increasing the annealing temperature of the assay appeared to reduce the amount of background noise and definitely reduced primer dimer formation.

To further reduce the amount of baseline noise, the amount of primer available was reduced so that there was less primer available to form primer dimers and non-specific products. As Figure 4.12 shows, the amount of background noise was slightly reduced when 6 and 10 μl of each primer is added to the master mix, with the best reduction in baseline noise being achieved with 6 μl of each primer added to the master mix.

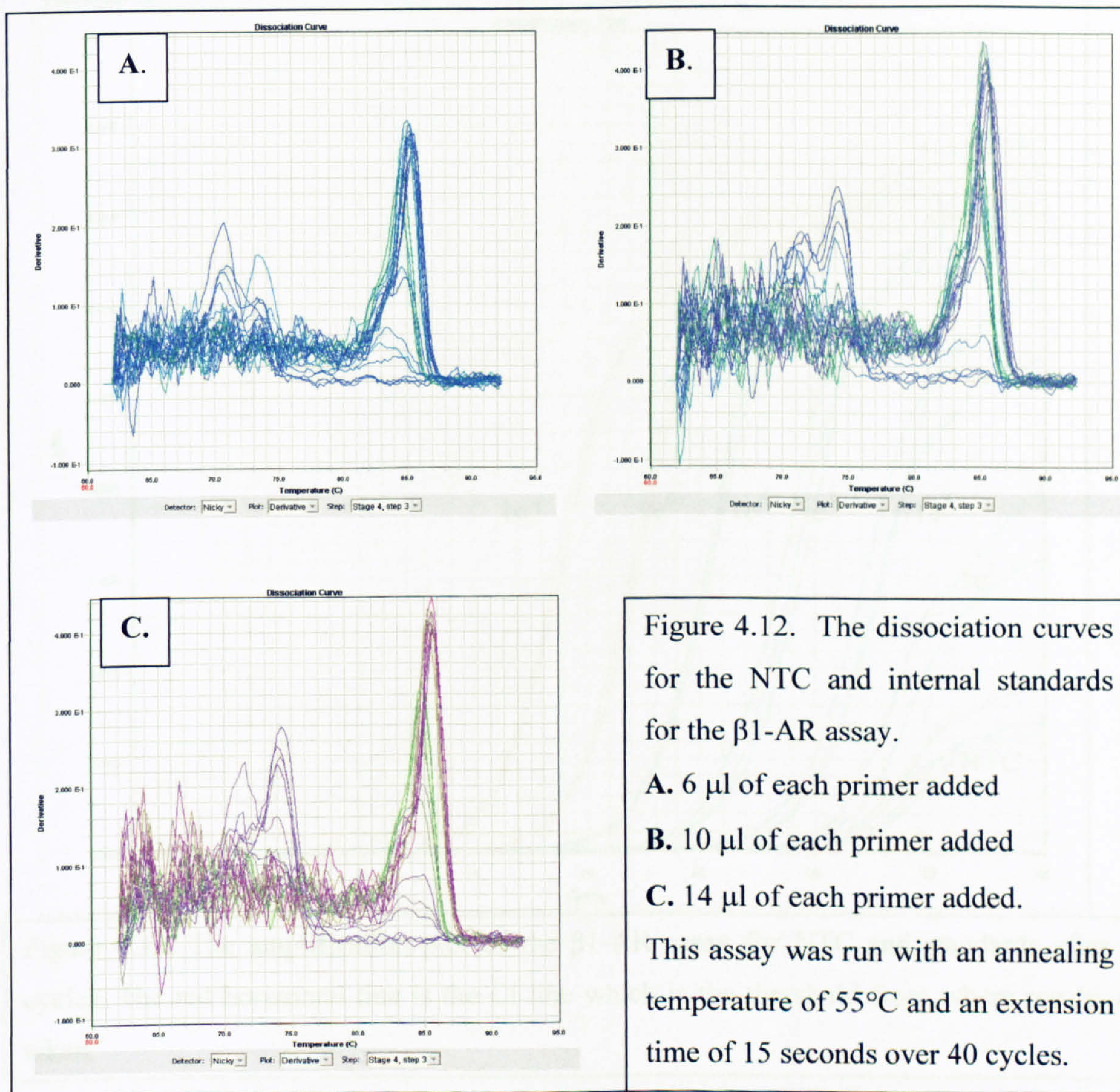


Figure 4.12. The dissociation curves for the NTC and internal standards for the β1-AR assay.

A. 6 μl of each primer added

B. 10 μl of each primer added

C. 14 μl of each primer added.

This assay was run with an annealing temperature of 55°C and an extension time of 15 seconds over 40 cycles.

However, as well as reducing the baseline noise, when 6 μl of each primer is added to the master mix, the amplification of target DNA is also reduced, and it may be that with such a small amount of primer available this may be becoming a limiting factor, which would make the assay less sensitive. Hence, it was decided that 10 μl was the optimal amount of each primer that needs to be added to the master mix for this assay, as DNA amplification is not affected but the baseline noise is somewhat reduced.

The next optimisation step in an attempt to reduce the amount of baseline noise was to decrease the amount of cycles from 40 to 30. As Figure 4.13 shows, the primer dimer is formed after 30 cycles, so by reducing the number of cycles, the formation of primer dimer and perhaps background noise may be reduced.

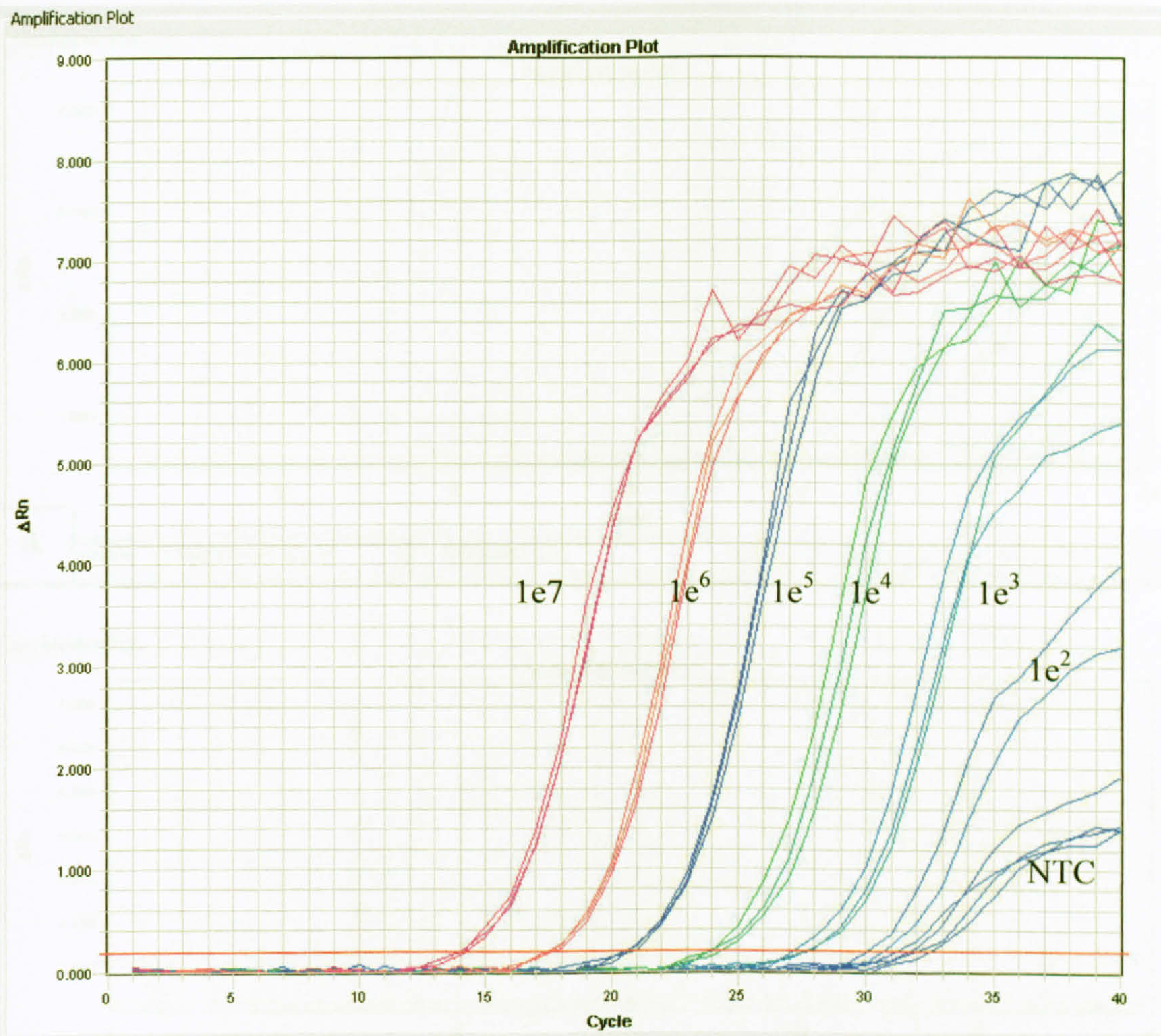
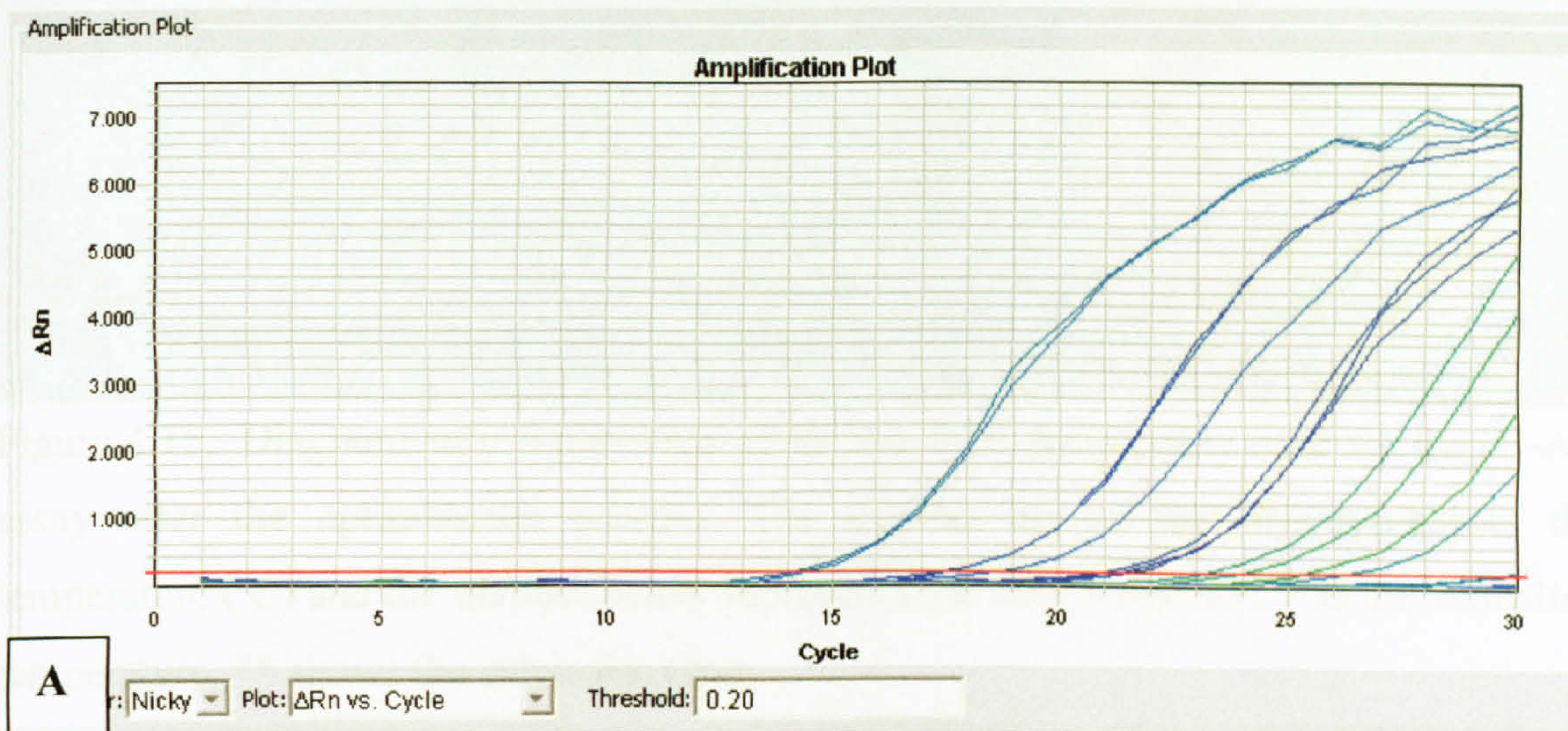


Figure 4.13. The amplification plot of the β1-AR assay for NTC and standards after 40 cycles. The red horizontal line is the Ct line which is the threshold from where results are taken.

However, although the baseline noise was much reduced, when a liver sample was run for only 30 cycles of amplification, no result was obtained. This was probably because there were not enough rounds of amplification to create a measurable amount of target DNA (as shown in Figure 4.14). The number of cycles of amplification was therefore set back to 40 cycles.



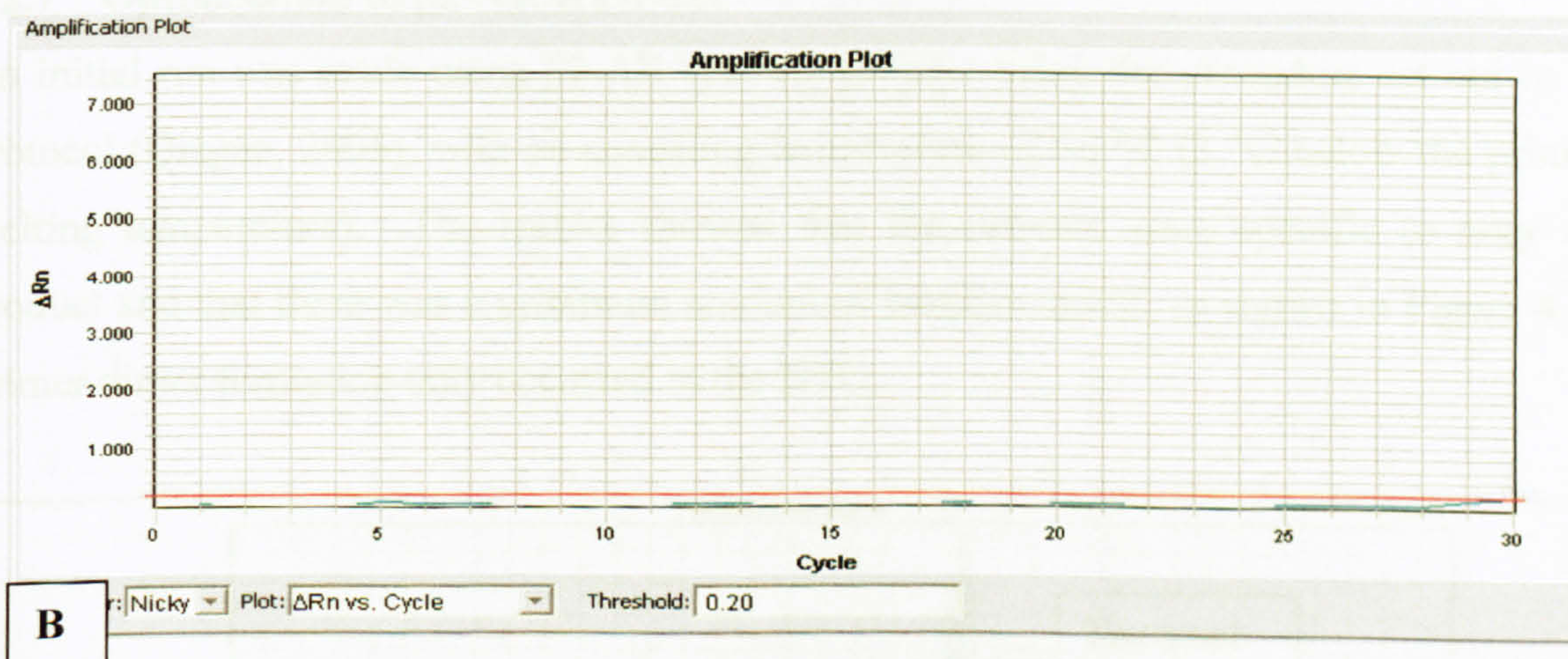


Figure 4.14. Amplification plots of RT-PCR β1-AR assay after 30 cycles of amplification. This assay was carried out with an annealing temperature of 55°C, an extension time of 15 seconds and with 10μl of primer added to the master mix . **A.** Results of standards and NTC. **B.** Results of liver control sample. As shown, the control liver sample has not been amplified enough times to obtain a measurable amount of target DNA.

So, in summary, the β1-AR RT-PCR assay was run over 40 cycles at an annealing temperature of 55 °C, with an extension time of 15 seconds (Figure 4.15) and with 10 μl of each primer (100 μM) added to the master mix (1400μl SYBR Green, 28 μl reverse transcriptase and 784 μl RNase free water).

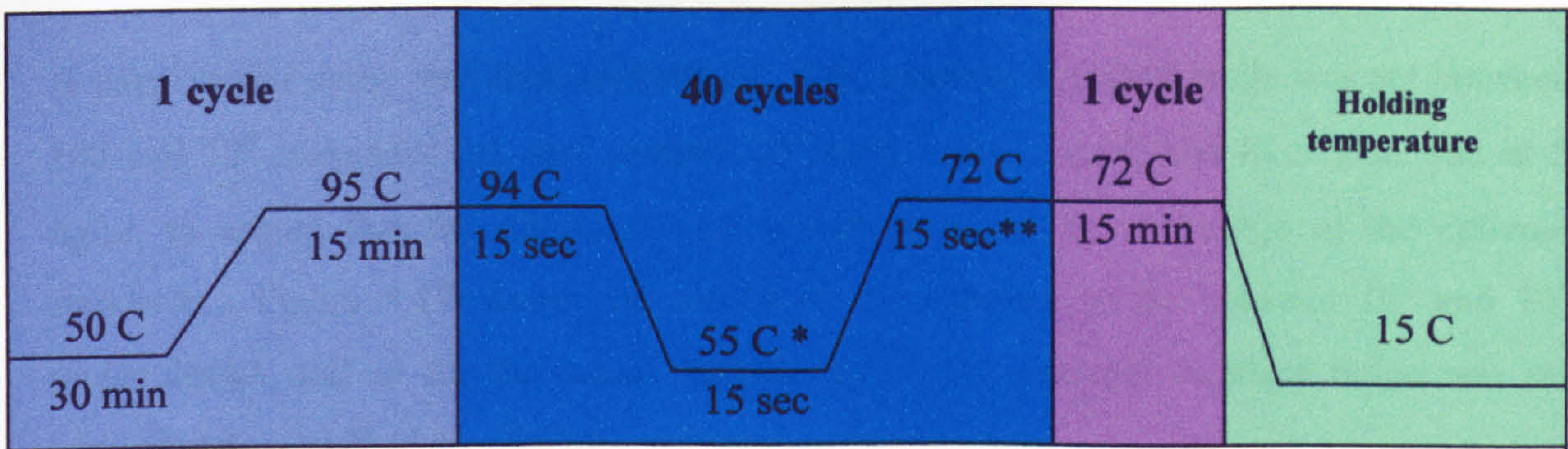


Figure 4.15. Diagrammatic explanation of the RT-PCR programme used for the β1-AR assay after the optimisation process. The number above the line represents the temperature (°C) and the number below represents the time (minutes). * is the annealing temperature. ** shows the extension time.

4.2.7 Optimisation of β2-AR RT-PCR

An initial run was made using β2-AR specific primers using the procedure set out in the protocol (Qiagen, 2005), with an annealing temperature of 56 °C (3 °C below the primers melting temperature). The results showed that the primers were specific to only one product and that there was a minimum amount of baseline noise, as shown in Figure 4.16. Primer dimer formation only occurred in the NTC.

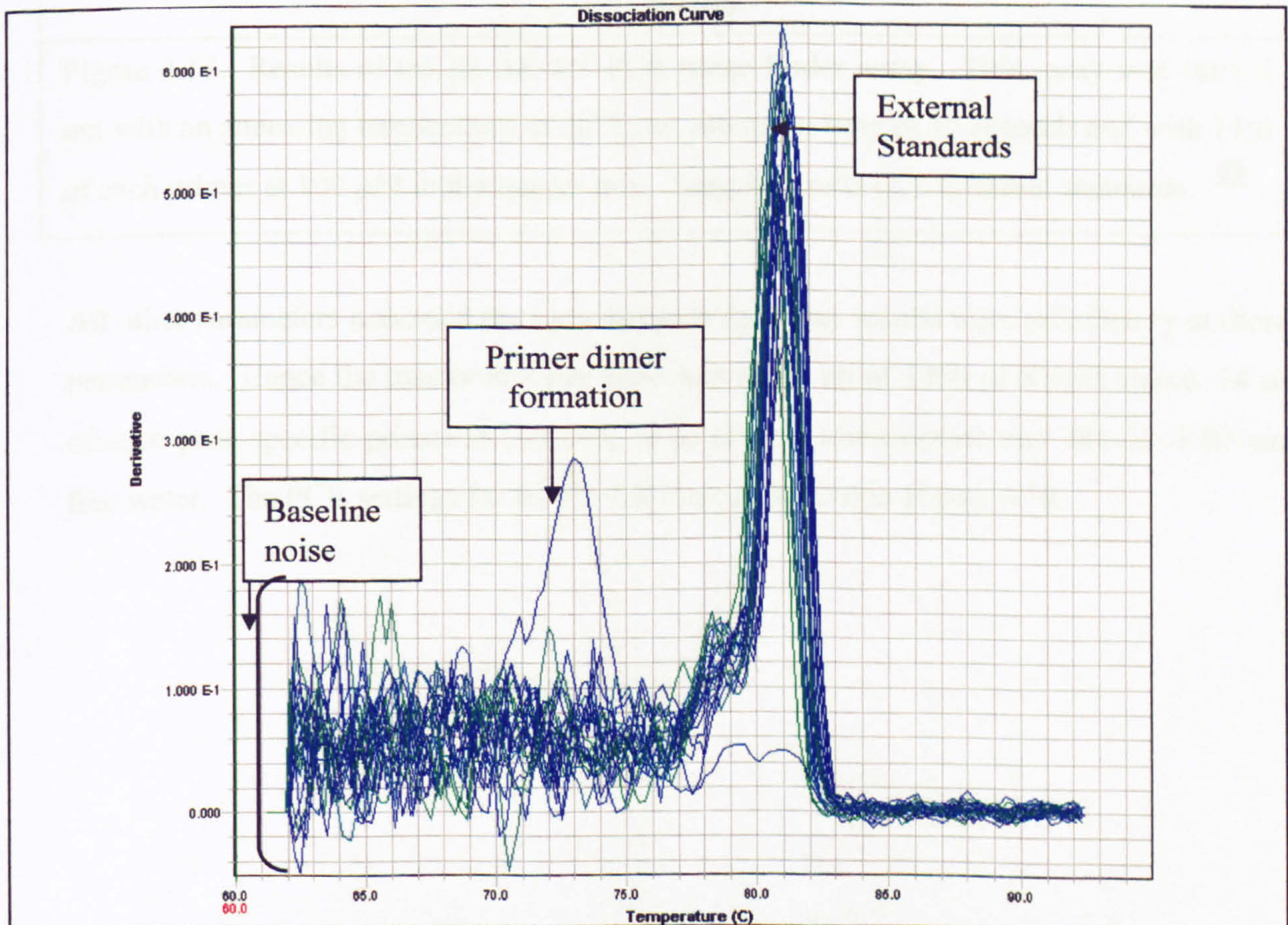


Figure 4.16. Dissociation curve for the β2-AR assay. This assay was carried out with an annealing temperature of 56°C, an extension time of 15 seconds ad with 14μl of each primer at 100 μM in the master mix. Only one product peak is evident with minimal baseline noise.

A range finder assay was then undertaken. The external standard range was set between 100 and 10^8 molecules /µl, and samples of brain, heart, gonad, and liver were run at 5 ng/µl, to ensure that the sample β2-AR results were in the mid-range of the external standards. Figure 4.17 shows the results of the samples to lie between 10^2 and 10^4 molecules/µl, and so for the actual sample assays, the external standard range was set between 10 and 10^6 molecules/µl.

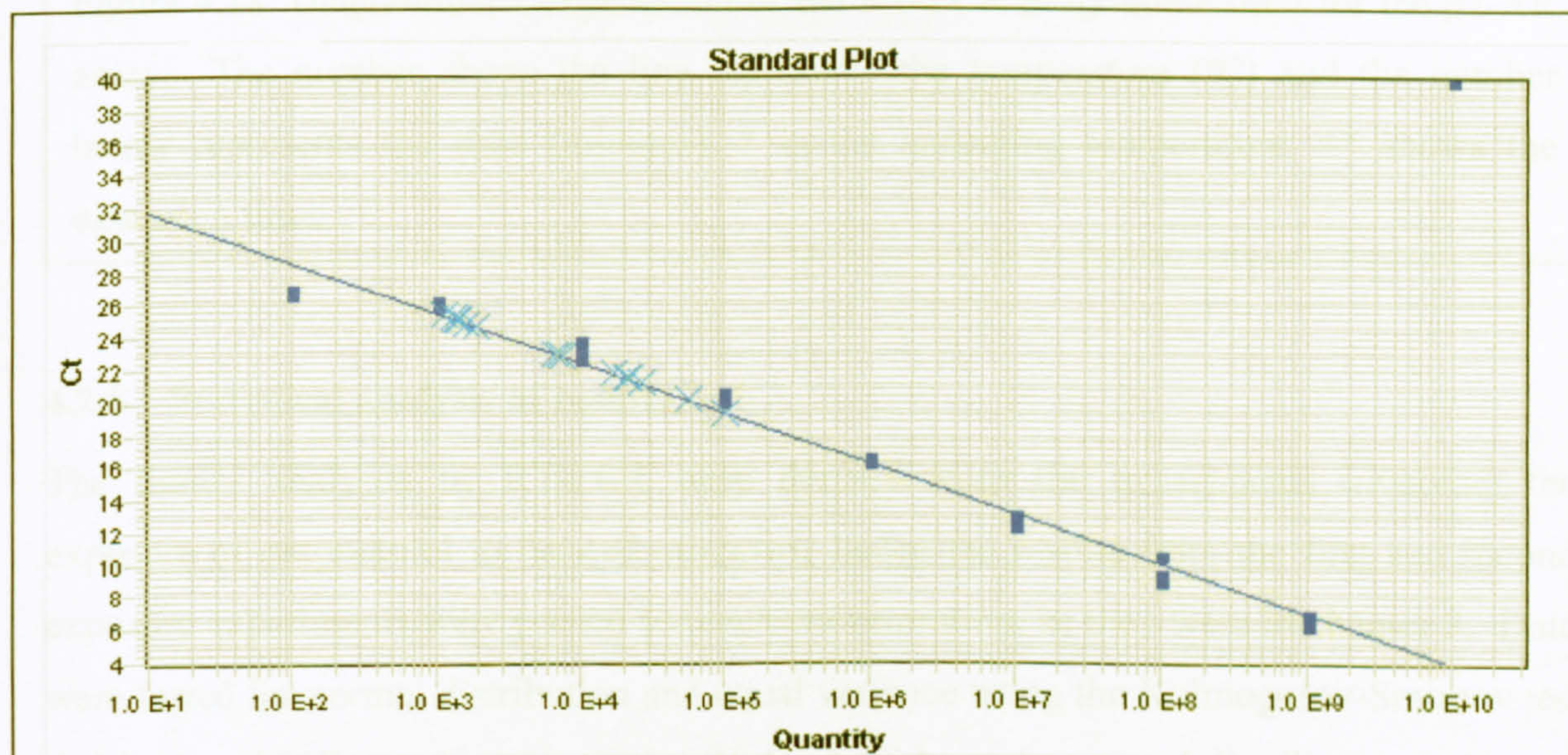


Figure 4.17. Results of the β2-AR RT-PCR range finder assay. This assay was carried out with an annealing temperature of 56°C, an extension time of 15 seconds and with 14µl of each primer at 100 µM in the master mix. Sample results (X). External standards ■

All other parameters remained the same because the assay results were satisfactory at these parameters. Hence the master mix per plate was made up of 1400 µl SYBR Green, 14 µl of each gene specific primer at 100 µM, 28 µl reverse transcriptase and 784 µl of RNase free water. The PCR settings for the β2-AR were as set out in Figure 4.18.

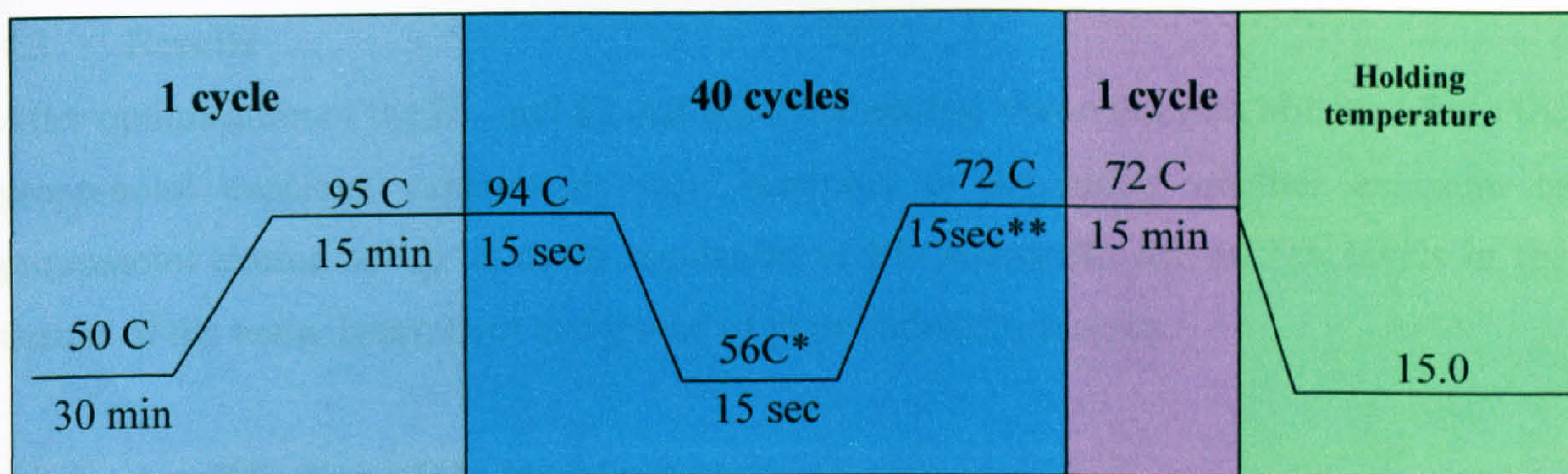


Figure 4.18. Diagrammatic explanation of the RT-PCR programme used for the β2-AR assay. The number above the line represents the temperature (°C) and the number below represents the time (minutes). * is the annealing temperature. ** shows the extension time.

4.2.8 Statistical analysis of results

The tissues analysed by RT-PCR were derived from the experiments involving the exposure of propranolol to fathead minnows, hence the results from the first and second exposure experiments were pooled for each concentration, as they were in chapter 3. Data were tested for normal distribution and equal variance using the Kolmogorov-Smirnov test and Levene Median test, respectively. If the data showed a normal distribution and equal variance, a One Way Repeated Measures Analysis of Variance (One Way ANOVA) was carried out to determine whether there were any statistically significant differences between the data sets. If a difference was found, this was followed by a post-hoc All Pairwise Multiple Comparison Procedures (Holm-Sidak method).

If the data failed normality and/or equal variance tests, then a Kruskal-Wallis One Way ANOVA on ranks was carried out to determine whether there were any statistically significant differences between the data sets. If a difference was found this was followed by a post hoc All Pairwise Dunn's test.

4.3 Results

After optimisation of the β1- and β2-AR RT-PCR assays, tissue samples obtained from the propranolol exposure experiments were analysed to determine whether exposure to propranolol caused an up or down regulation of β1- and/or β2-AR mRNA levels in the tissues of the brain, heart, liver and gonad of these fathead minnows.

4.3.1 Amplification of the target sequence

For each primer pair, an aliquot of amplified product (post RT-PCR) from wells from the internal standard at 1e^{7} molecules/µl, and the liver control, were sent for sequencing (Dundee University, Dundee) to determine if the correct sequence was being amplified. The sequence of amino acids that the primers should have amplified was 185bp and 226bp for the β1-AR and the β2-AR, respectively, as set out in Figure 4.19 below.

A.

CTTCGTATTTTTGAACTGGCTGGGGTACGTCAACTCCGCCTTTAACCCCATCATATACTGTCGGAGT
CCCGACTTTAGGAAAGCCTTTAAGAGGCTGTTGTGTTGTCCGAGGCAGGCGGACCGCAGGTTGCACG
TGAGCTCGTGCGATCTGTCGCGCTGCACCGGGGGTTATGTGAACTCAATGG

B.

AGGTGATCAAGAGTCGAGTGAAAGAACACTATGTGAGACCAGAACACT
TCCTAAGAGAAAAGGATTATTTTGGATAGTGGACTCCTAATTTTAGTC
AAAGCTCATGGAGGGAGGGGATAGGTTGAGCGTGGAGAACACCTCCCT
GCACATGAATGTTTCATCTGGGCTAAACGACTCTTCTCCGGTGTCCGAA
TATAGCGACGCAGAGGTGGTCTTAATCAGCAT

Figure 4.19. Sequences of the β1- and β2-AR amplicons. **A.** Nucleotides in the β1-AR amplicon. Nucleotides highlighted in blue are the primers (β1.15F and β1.16R) used for the β1-AR RT-PCR assay. Red amino acids are those in the seventh TMD. **B.** Nucleotides in the β2-AR amplicon. Nucleotides highlighted in green are the primers (β2.22F and β2.22R) used for the β2-AR RT-PCR assay. Nucleotides highlighted in orange show the 5' UTR and those highlighted in red are those in the first TMD.

The sequencing results were compared to the supposed sequence (see chapter 2) in a clustal alignment (www.ebi.ac.uk/Tools/clustalw2/index.html). The results are shown in Figures 4.20 and 4.21. For each primer pair, Liver F and Liver R are the same sample, and Std F and StdR are also the same sample, but each was run with either the forward (β1.15F or β2.22F)) or reverse (β1.16R pr β2.23R) primer. The first part or last parts of the sequence are missing from each sample because at the start of every run the sequence quality is not good enough for the nucleotides to be read. These data show that the DNA

obtained from the transcription and amplification of mRNA in the RT-PCR assay are indeed fragments of either the β1-AR or the β2-AR.

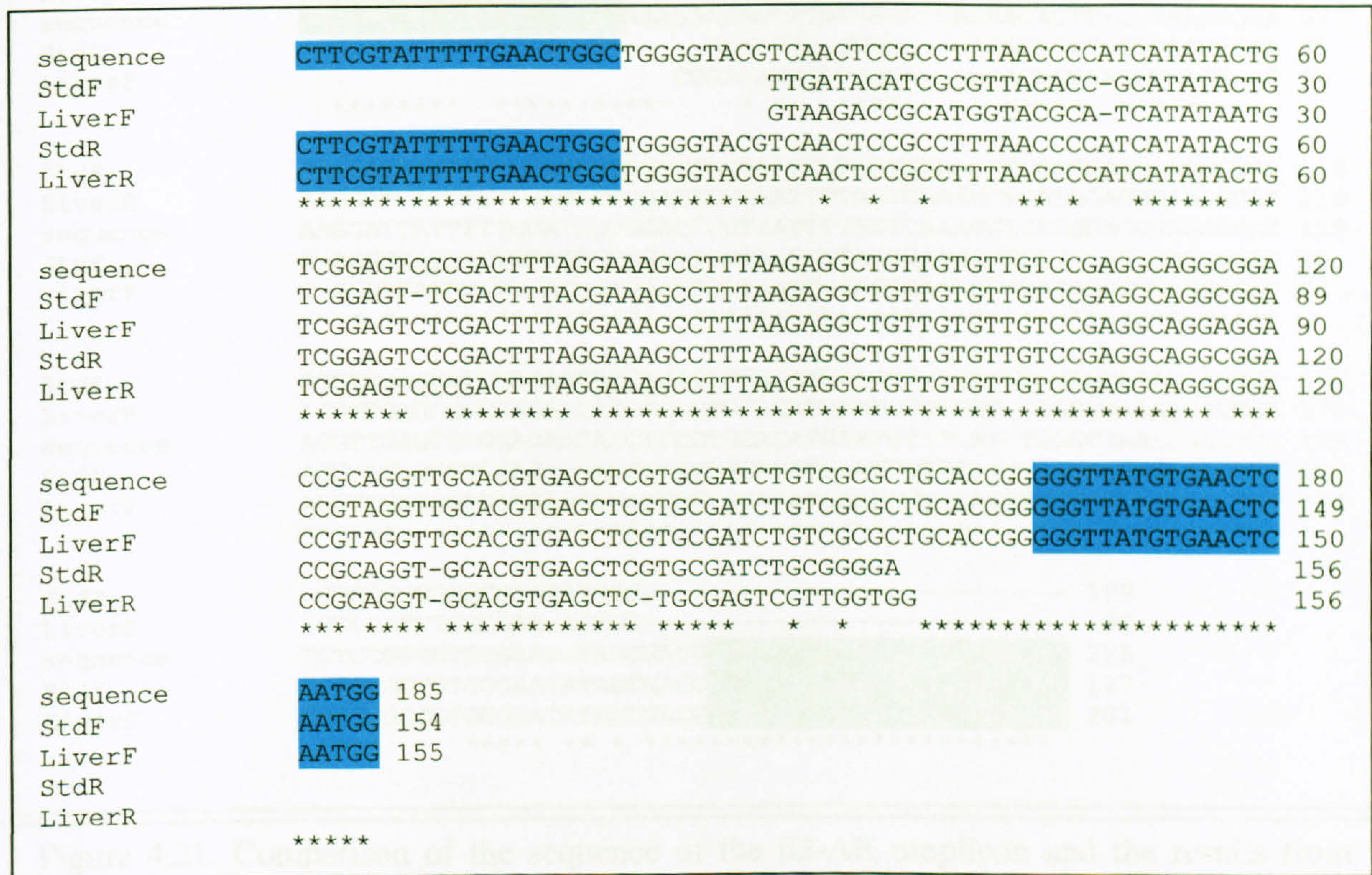
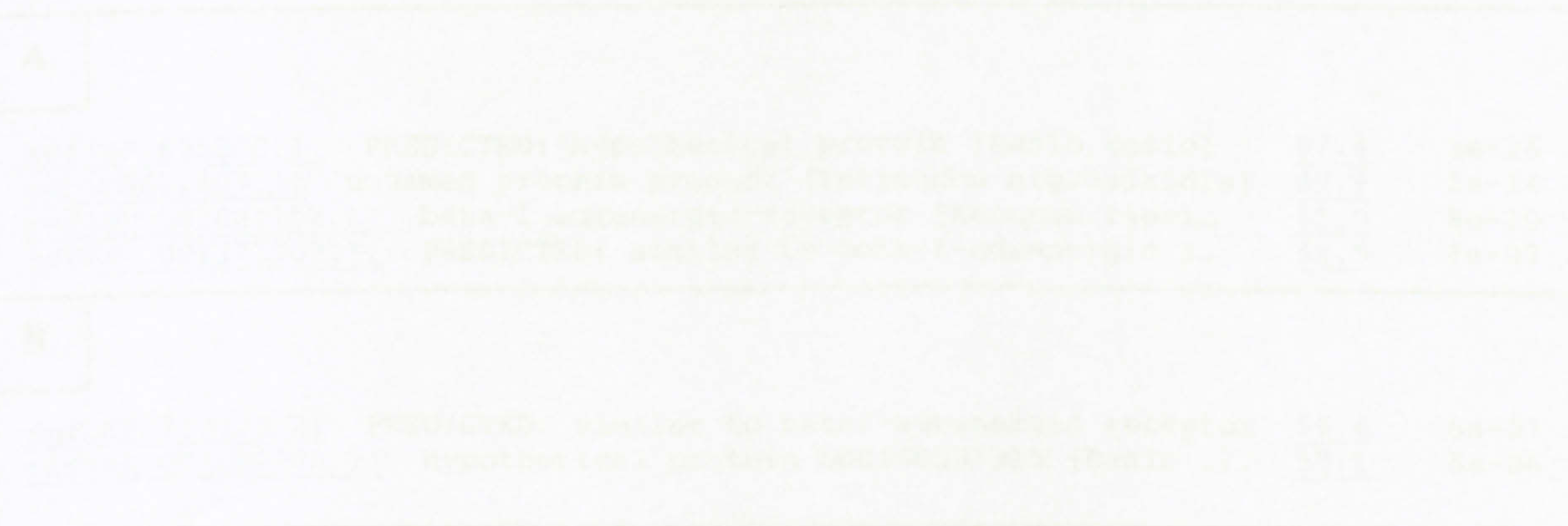


Figure 4.20. Comparison of the sequence of the β1-AR amplicon to the results from liver and standard samples in both the forward and reverse directions. Nucleotides highlighted in blue are the β1.15F and β1.16R primers. Alignment was carried out using CLUSTAL W (1.83) multiple sequence alignment.



```
StdR            TTGTGATCAAAGGTCGAGTGAAAGAACACTATGTGAGACCAGAACACTT-CCTAAGAGAA 59
LiverR          AGGTGATCAA-GGTCGAGTGAAAGAACACTATGTGAGACCAGAACACTT-CCTAAGAGAA 58
sequence        AGGTGATCAAGAGTCGAGTGAAAGAACACTATGTGAGACCAGAACACTT-CCTAAGAGAA 59
StdF                                     GCAATTG-TGAGA--CAGACACTT-CCTAAGAGAA 31
LiverF                                 CGGGAATGGGTGAGA--AGGACACTAACCTAAGAGAG 35
                  ********  ***********    * *   *****     *****  *********

StdR            AAGGATTATTTTTGGATAGTGGACTCCTAATTTTAGTCAAAGCTCATGGAGGGAGGGGAT 119
LiverR          AAGGATTATTTTTGGATAGTGGACTCCTAATTTTAGTCAAAGCTCATGGAGGGAAGGGAT 118
sequence        AAGGATTATTTTTGGATAGTGGACTCCTAATTTTAGTCAAAGCTCATGGAGGGAGGGGAT 119
StdF            A-AGGTTATTTTTGGATAGTGGACTCCTAATTTTAGTCAAAGCTCATGGAGGGAGGGGAT 90
LiverF          C-GGATTATTTTTGGATAGTGGACTCCTAATTTTAGTCAAAGCTCATGGAGGGAGGGGAT 94
                   * ************************************************* *****

StdR            AGGTTGAGCGTGGAGAACACCTCCCTGCACATGAATGTTTCATCTGGGCTAAACGACTCT 179
LiverR          AGGTTGAGCGTGGAGAACACCTCCCTGCACATGAATGTTTCATCTGGGCTAAACGACTCA 178
sequence        AGGTTGAGCGTGGAGAACACCTCCCTGCACATGAATGTTTCATCTGGGCTAAACGACTCT 179
StdF            AGGTTGAGCGTGGAGAACACCTCCCTGCACATGAATGTTTCATCTGGGCTAAACGACTCT 150
LiverF          AGGTTGAGCGTGGAGAACACCTCCCTGCACATGAATGTTTCATCTGGGCTAAACGACTCT 154
                ***********************************************************

StdR            -CTCCCGGGCCCGAATATCGG-------------------------- 199
LiverR          -CTCCATTTCCCGAA-ATTGG-------------------------- 197
sequence        TCTCCGGTGTCCGAATATAGCGACGCAGAGGTGGTCTTAATCAGCAT 226
StdF            TCTCCGGTGTCCGAATATAGCGACGCAGAGGTGGTTTTAATTAGCAA 197
LiverF          TCTCCGGTGTCCGAATATAGCGACGCAGAGGTGGTTTTAATTAGCAA 201
                 ****     ***** ** * *************************
```

Figure 4.21. Comparison of the sequence of the β2-AR amplicon and the results from liver and standard samples in both the forward and reverse directions. Nucleotides highlighted in green are the β2.22F and β2.23R primers. Alignment was carried out using CLUSTAL W (1.83) multiple sequence alignment.

These sequences were run through a blastx database (www.ncbi.nlm.nih.gov/blast) to retrieve annotation to the sequences. The results (shown in Figure 4.22) show that the fragments from the β1 and the β2-AR assays are recognised as being fragments of the β1-AR or β2-AR, respectively, and that the correct fragments are being amplified and measured in the RT-PCR reaction for both assays

A.

ref|XP_685300.1|	PREDICTED: hypothetical protein [Danio rerio]	87.4	3e-16
emb|CAG12607.1|	unnamed protein product [Tetraodon nigroviridis]	80.9	2e-14
ref|NP_001084152.1|	beta-1 adrenergic receptor [Xenopus laevi...	65.9	8e-10
ref|XP_001377707.1|	PREDICTED: similar to beta-1-adrenergic r...	58.5	1e-07

B.

ref|XP_700720.2|	PREDICTED: similar to beta2-adrenergic receptor	56.6	5e-07
ref|NP_001082940.1|	hypothetical protein LOC100037315 [Danio ...	53.1	5e-06

Figure 4.22. Results from running each sequence from **(A)** β1-AR and **(B)** β2-AR RT-PCR assay in a blastx analysis. These demonstrate that the DNA fragments are from β1 and β2-ARs, respectively.

4.3.2 The efficiency of each assay

As shown in Tables 4.4 and 4.5, the efficiency ranges for all assays were greater than 90 %, with efficiency values ranging from 93 and 117 % for the β1-AR assay and between 91 and 128 % for the β2-AR assay.

RT-PCR assay: β1-AR				
Plate number	**Samples**	**R_2**	**Slope**	**Efficiency (%)**
1	Liver Exposure 1	0.996	-3.410	96.45
2	Brain Exposure 1	0.945	-3.202	105.26
3	Gonad Exposure 1	0.967	-3.175	106.52
4	Heart Exposures 1 & 2	0.966	-3.493	93.32
5	Liver Exposure 2	0.972	-3.342	99.17
6	Brain Exposure 2	0.966	-2.956	117.92
7	Gonad Exposure 2	0.967	-3.175	106.52

Table 4.4. Efficiency, slope and R_2 values for all the β1-AR RT-PCR assays. Efficiency values are all > 90%.

RT-PCR assay: β2-AR				
Plate number	**Samples**	**R_2**	**Slope**	**Efficiency (%)**
1	Liver Exposure 1	0.995	-3.553	91.18
2	Brain Exposure 1	0.971	-3.101	110.12
3	Gonad Exposure 1	0.970	-3.113	109.52
4	Heart Exposures 1 & 2	0.966	-3.107	109.82
5	Liver Exposure 2	0.978	-3.269	102.26
6	Brain Exposure 2	0.939	-2.801	127.52
7	Gonad Exposure 2	0.9320	-3.157	107.38

Table 4.5. Efficiency, slope and R_2 values for all the β2-AR RT-PCR assays. Efficiency values are all > 90%.

4.3.3 Internal standard results

A liver sample was run in triplicate on each plate at a concentration of 5ng/μl to act as an internal control between the different plates. These data were plotted on a graph, as shown below in Figures 4.23 and 4.24. These data show that although the same sample was run on each plate, some results are statistically significantly different from the other internal standard results on different plates. For example, in the β1-AR assay, the internal standard results for plate 5 are 3-fold different to those for plate 6, and in the β2-AR assay, internal standard data for plate 7 are statistically different to those of all other plates except plate 3. This is not ideal. However, unlike the results of the expression of β-ARs between tissue types, the results for the internal standards for each assay are all within the same order of magnitude and overall, although there is a small amount of internal variability between plates, the variability in expression levels of the β-ARs in different tissues is very much greater. With regard to comparing data from the same tissue type, each tissue type from each exposure was analysed on the same plate in each assay, and because the efficiency of each assay was >90 %, the comparability of data within each tissue type is validated.

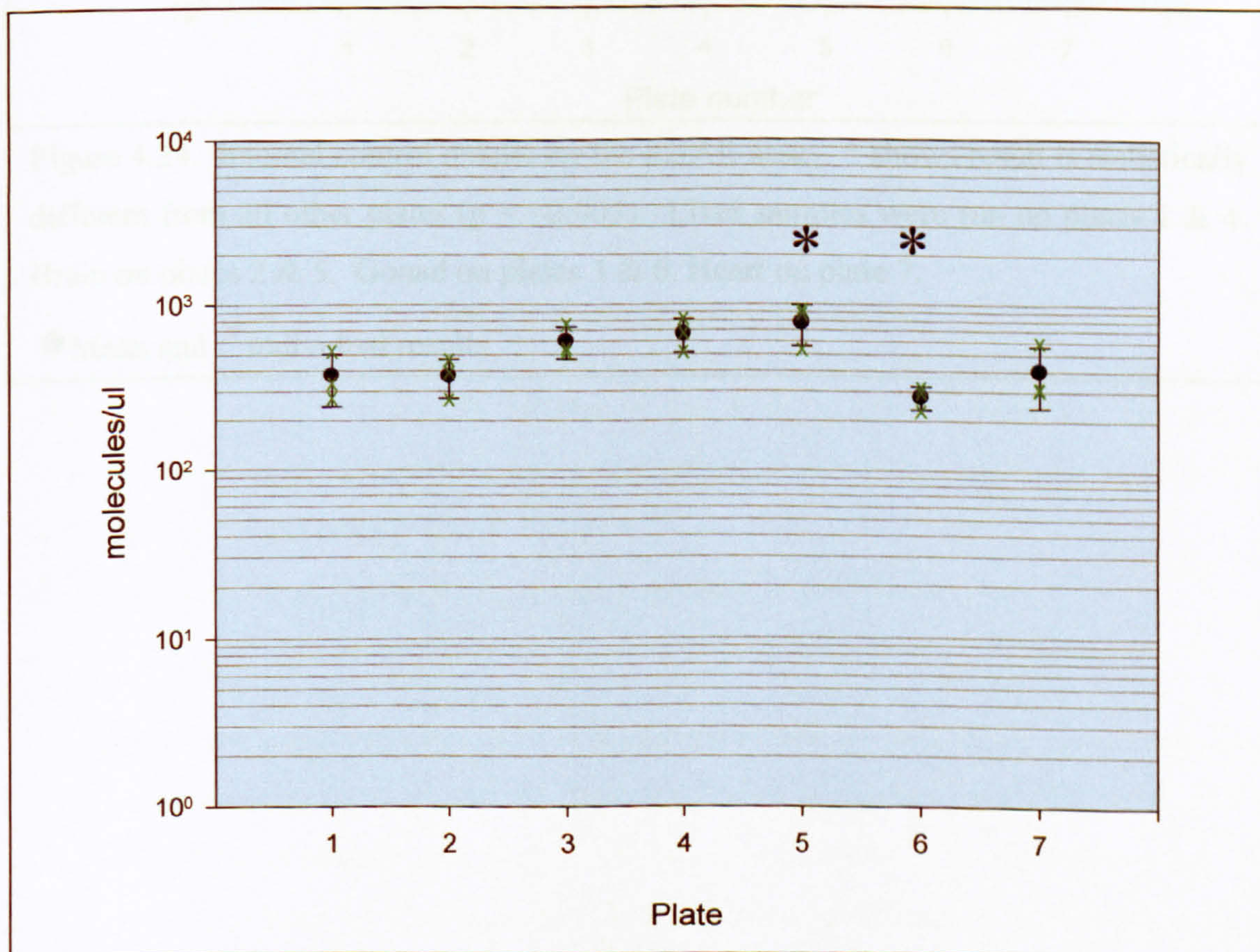


Figure 4.23. Internal control results for each plate in the β1-AR assay. * shows a statistically significant difference between the two data points (p = <0.005). Liver samples were run on plates 1 & 4. Brain on plates 2 & 5. Gonad on plates 3 & 6. Heart on plate 7. ● Mean and X individual results

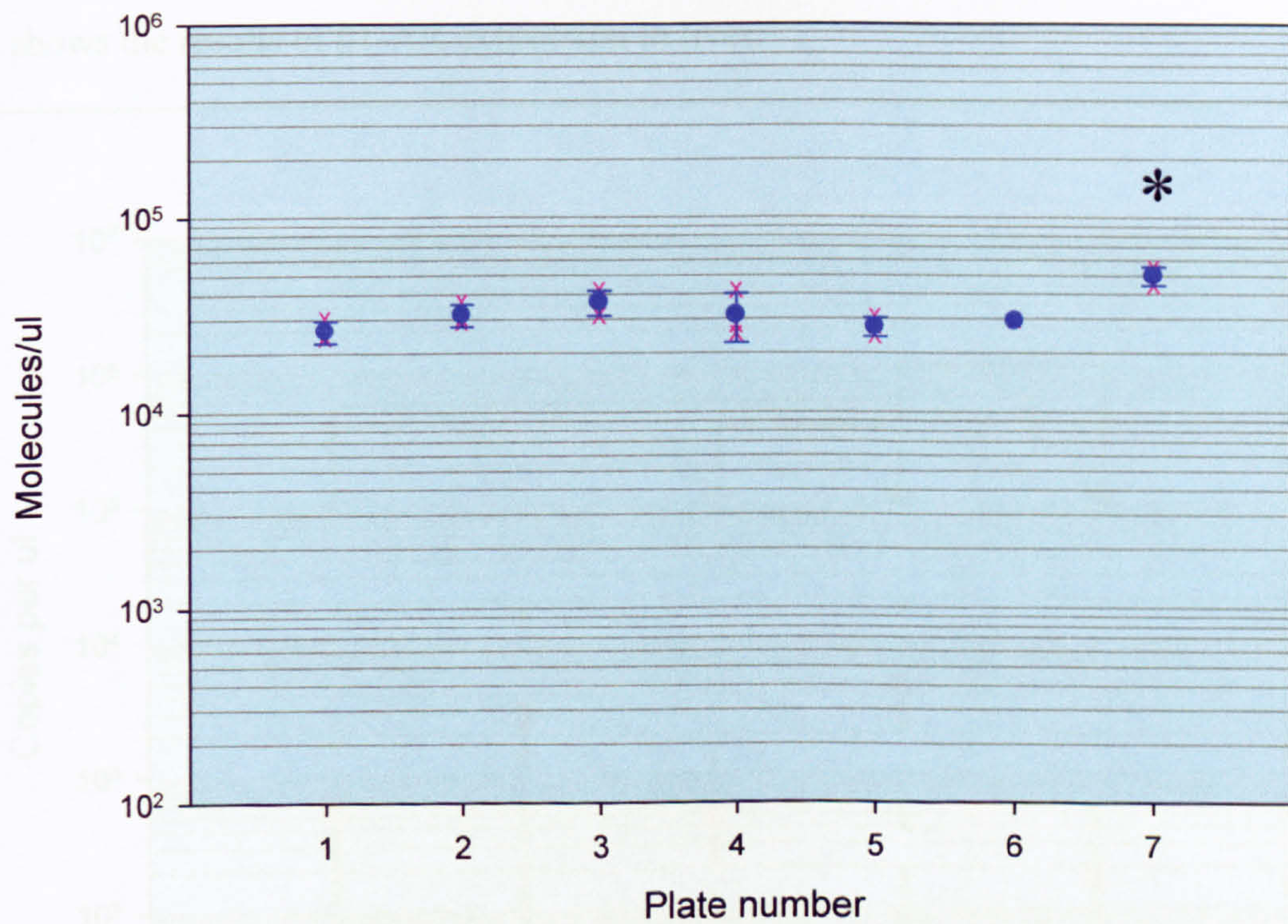


Figure 4.24. Internal control results for the β2-AR assay. * shows result is statistically different from all other plates (p = <0.005). Liver samples were run on plates 1 & 4. Brain on plates 2 & 5. Gonad on plates 3 & 6. Heart on plate 7.

● Mean and X individual results

4.3.4 Expression levels of β1-AR in different tissues

Expression levels of β1-AR mRNA in different tissue types was tested under the null hypothesis that stated there were no differences in the expression level of β1-AR (measured as β1-AR mRNA) between treatment groups within each tissue type. Figure 4.25 shows the results of β1-AR expression in liver.

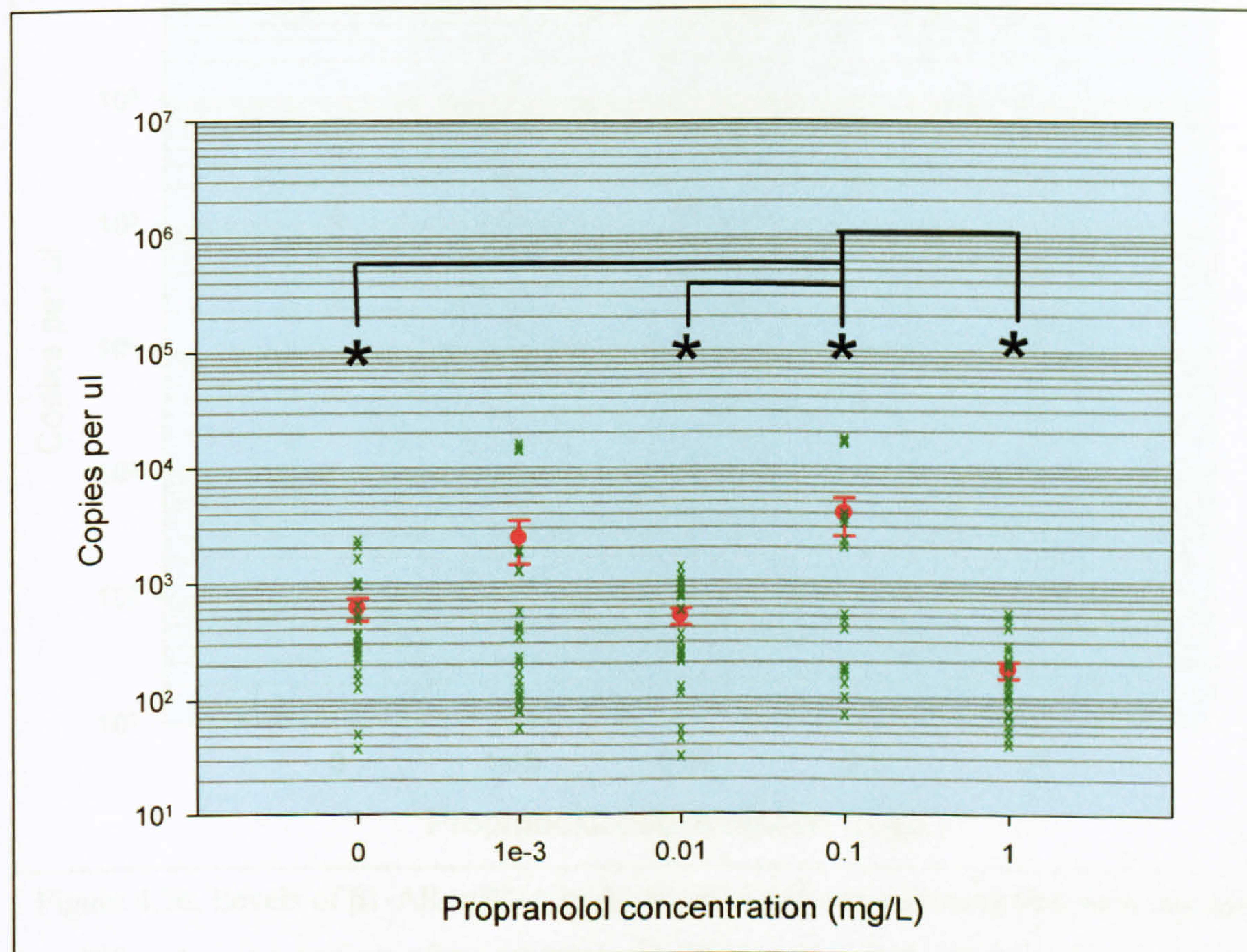


Figure 4.25. Levels of β1-AR mRNA in the liver of fathead minnows that were exposed to different concentrations of propranolol. * shows a statistically significant difference between data groups (p = <0.05).

● Mean and X individual results. Error bars show standard error.

The data show that the null hypothesis is rejected for the liver data, as there is a statistically significant difference between the 0.1 mg/L treatment group and the DWC, 0.01 and the 1.0 mg/L groups. However, this apparent response to propranolol is not dose-related. The mean expression level of β1-AR in the 1.0 mg/L treatment group is the lowest of all the mean data points.

Figure 4.26 shows the expression level of β1-AR mRNA in the brain at different concentrations of propranolol.

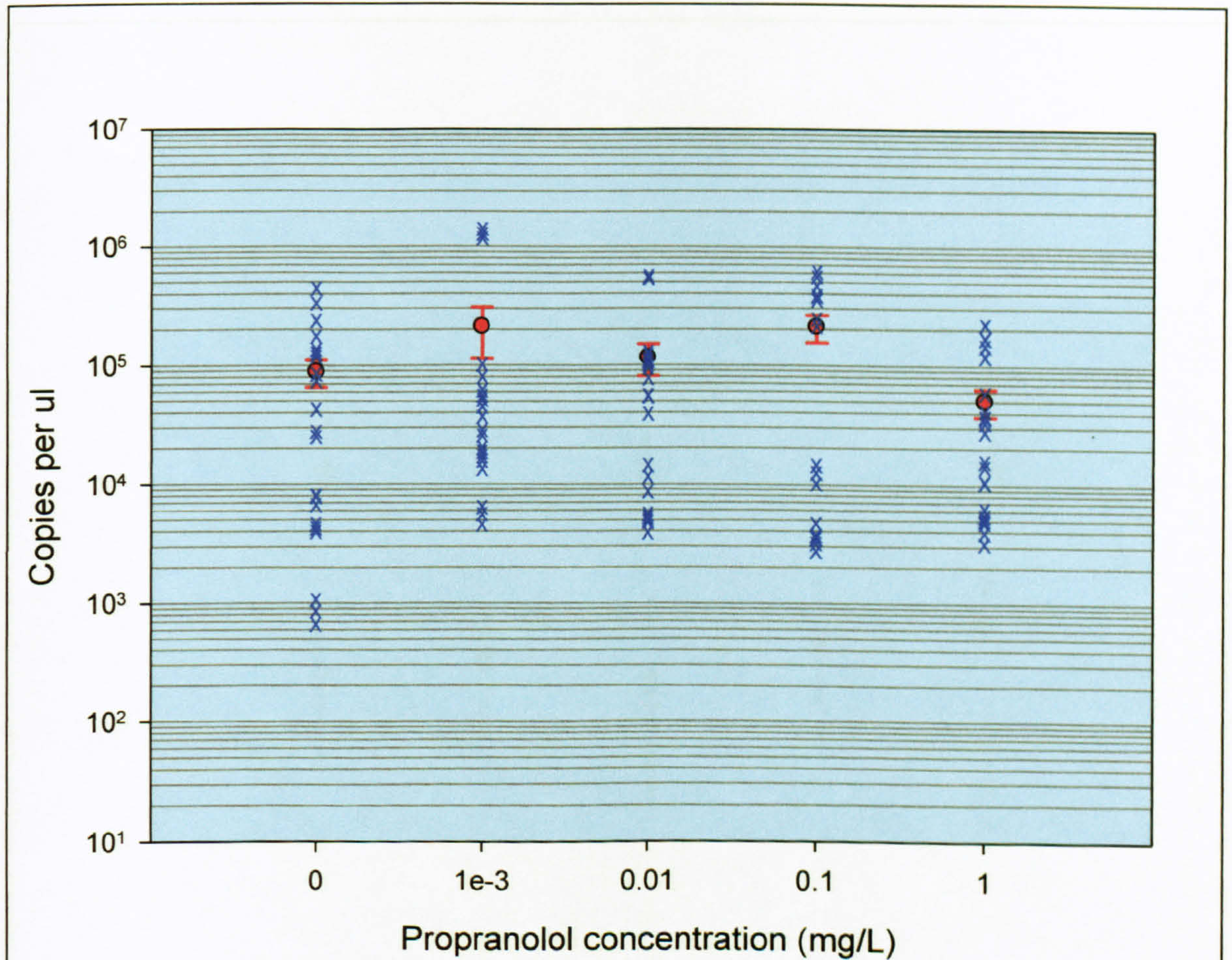


Figure 4.26. Levels of β1-AR mRNA in the brain of fathead minnows that were exposed to different concentrations of propranolol. ● Mean and X individual results. Error bars show standard error.

For brain tissue, the null hypothesis is not rejected, as there are no statistically significant differences in the expression levels of β1-AR between any of the treatment groups. However, although not statistically significant, the level of β1-AR mRNA in the brain at 1.0 mg/L is again the lowest of all the treatment groups.

Figure 4.27 shows the results of the level of β1-AR expression in the gonad at different exposure concentrations of propranolol.

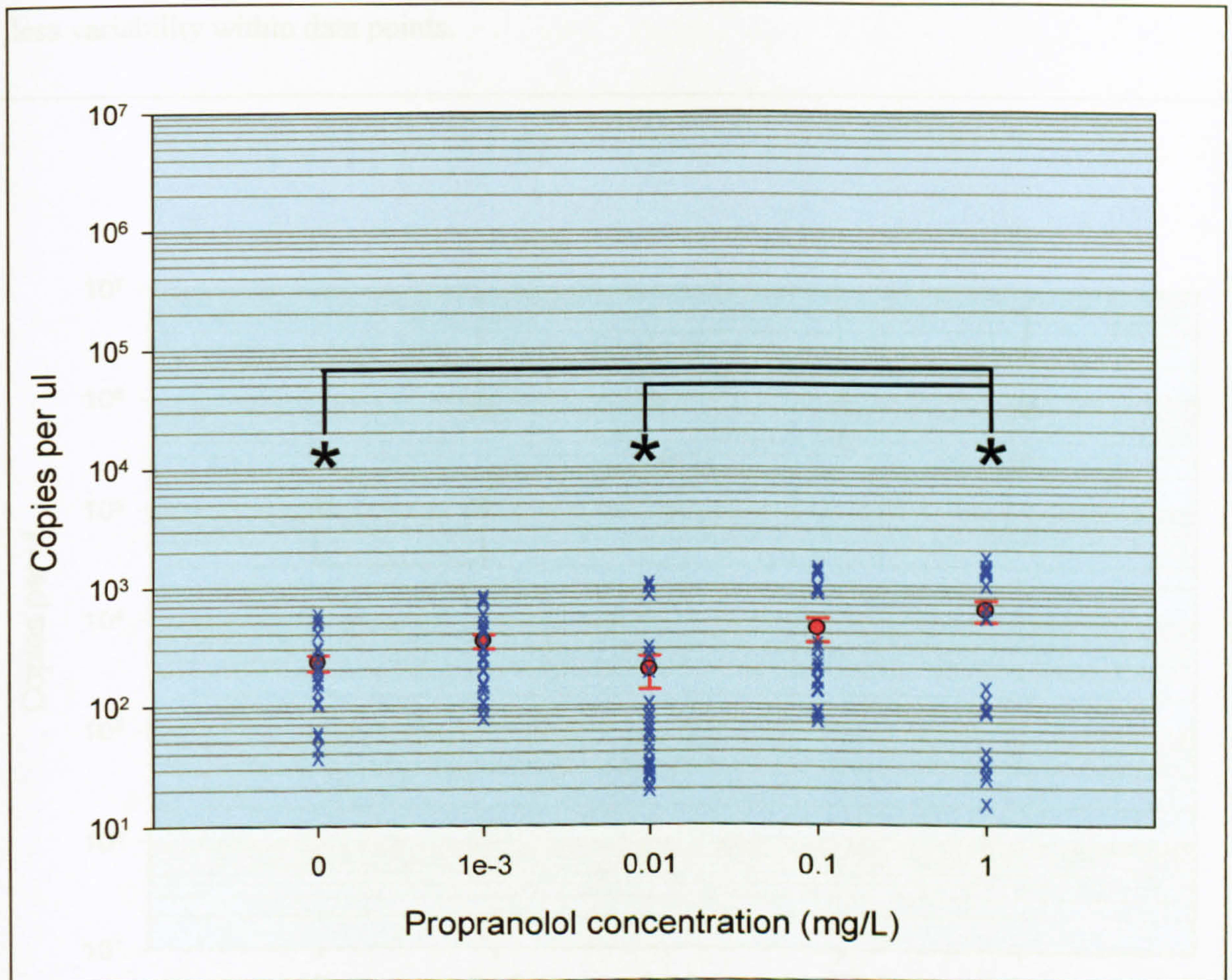


Figure 4.27. Levels of β1-AR mRNA in the gonad of fathead minnows that were exposed to different concentrations of propranolol. * shows a statistically significant difference between data groups (p = <0.05).
● Mean and X individual results. Error bars show standard error.

The null hypothesis is rejected as there is a statistically significant difference between the 1.0 mg/L treatment group and the DWC and 0.01 mg/L treatment groups. However, this is clearly not dose-related, although there is a statistical difference between the control group and the 1.0 mg/L treatment group, as there is an increase in the expression level of β1-AR in the gonad at 1.0 mg/L compared to the control group. This trend is also apparent from the 0.01 and 0.1 mg/L data. In other words, there appears to be some limited evidence of an increase in the amount of β1-AR mRNA with increasing propranolol concentration.

Figure 4.28 shows the expression levels of β1-AR in the heart of fathead minnows exposed to different concentrations of propranolol. As stated in the Material and Methods section, there were less samples to analyse for the heart (due to pooling of samples) and so there is less variability within data points.

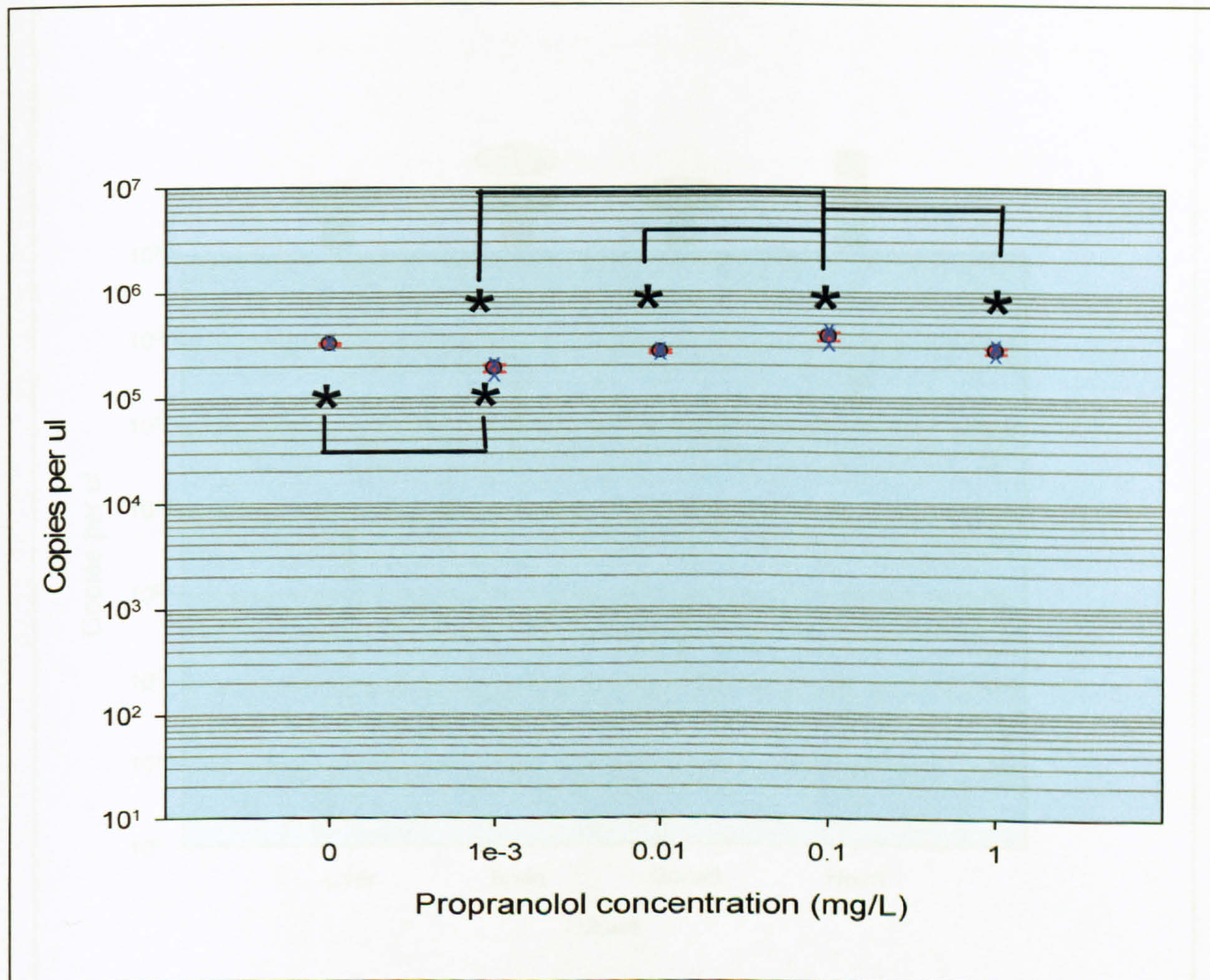


Figure 4.28. Levels of β1-AR mRNA in the heart of fathead minnows that were exposed to different concentrations of propranolol. * shows a statistically significant difference between data groups (p = <0.05).

Mean and **X** individual results. Error bars show standard error.

The null hypothesis is rejected as there is a statistically significant difference between the 0.1 mg/L treatment groups and the 0.001, 0.01, 1.0 mg/L treatment groups. There is also a statistically significant difference between the DWC and 0.001 mg/L treatment groups. However, overall there does not appear to be a dose-related effect.

Analysis was also conducted to determine whether there was a difference in β1-AR expression levels between tissue types. The null hypothesis tested was that there was no difference in the expression levels of β1-ARs between different tissues.

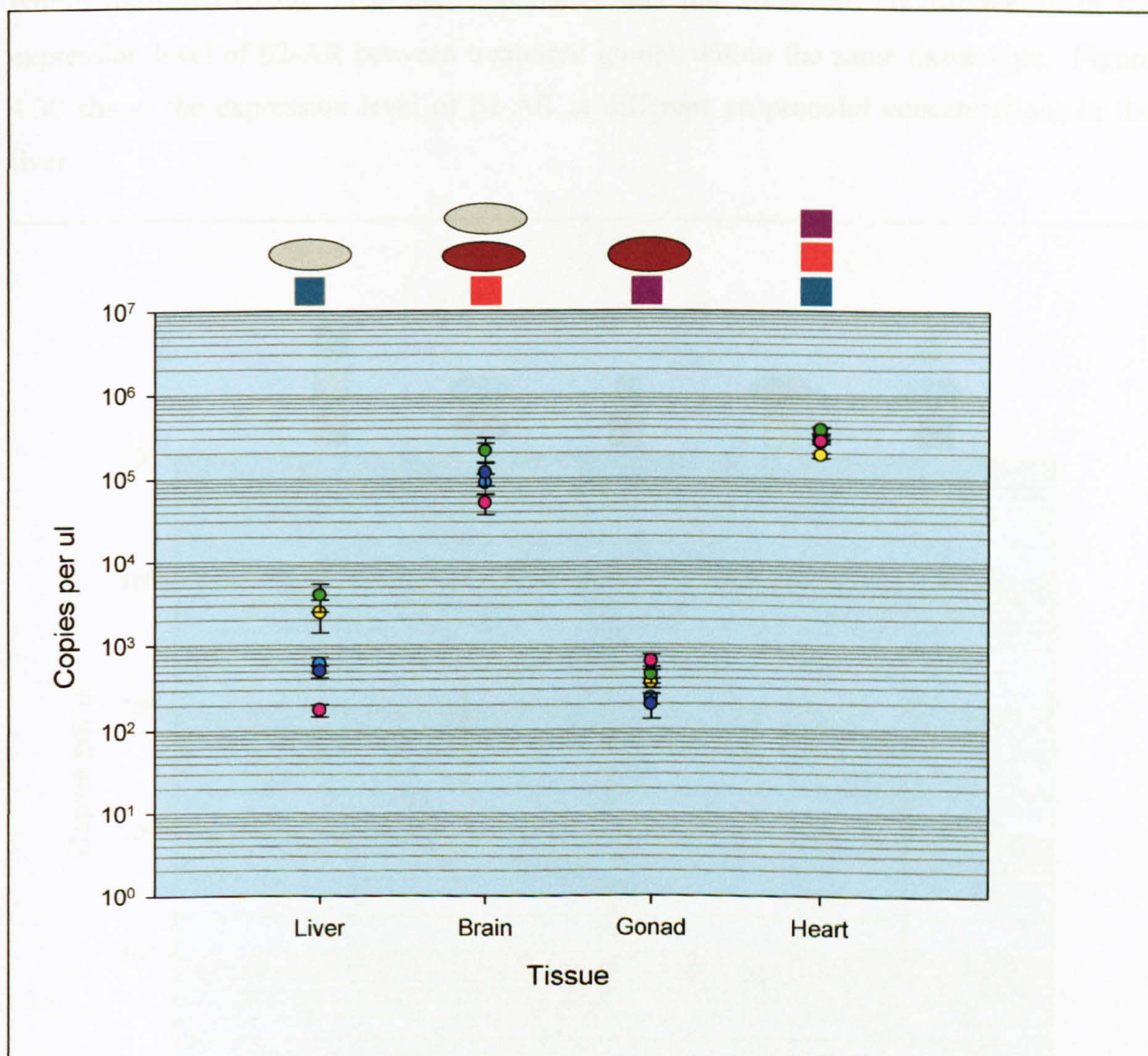


Figure 4.29. Levels of β1-AR mRNA in all tested tissue types of fathead minnows that were exposed to different concentrations of propranolol. Matching coloured squares and ovals at the top of the graph show where statistically significant difference between data groups exist ($p = <0.05$). Mean results shown as coloured circles. Error bars show standard error.

= DWC, = 0.001 mg/L, = 0.01 mg/L, = 0.1 mg/L, = 1.0mg/L

As Figure 4.29 shows, the null hypothesis is rejected. There is a statistically significant difference in the expression level of the β1-AR between the heart and all other tissues. There is also a statistically significant difference between the expression level of the β1-AR in the brain compared to liver and gonad tissues. The heart shows the greatest amount of β1-AR expression, followed by brain tissues. There is no statistically significant difference in β1-AR expression level between liver and gonad tissues.

4.3.5 Expression level of β2-AR in different tissues

Analysis of results obtained in the β2-AR RT-PCR was carried out to see whether exposure to different concentrations of propranolol caused a change in the level of β2-AR mRNA within the same tissue. The null hypothesis was that there are no differences in the expression level of β2-AR between treatment groups within the same tissue type. Figure 4.30 shows the expression level of β2-AR at different propranolol concentrations in the liver.

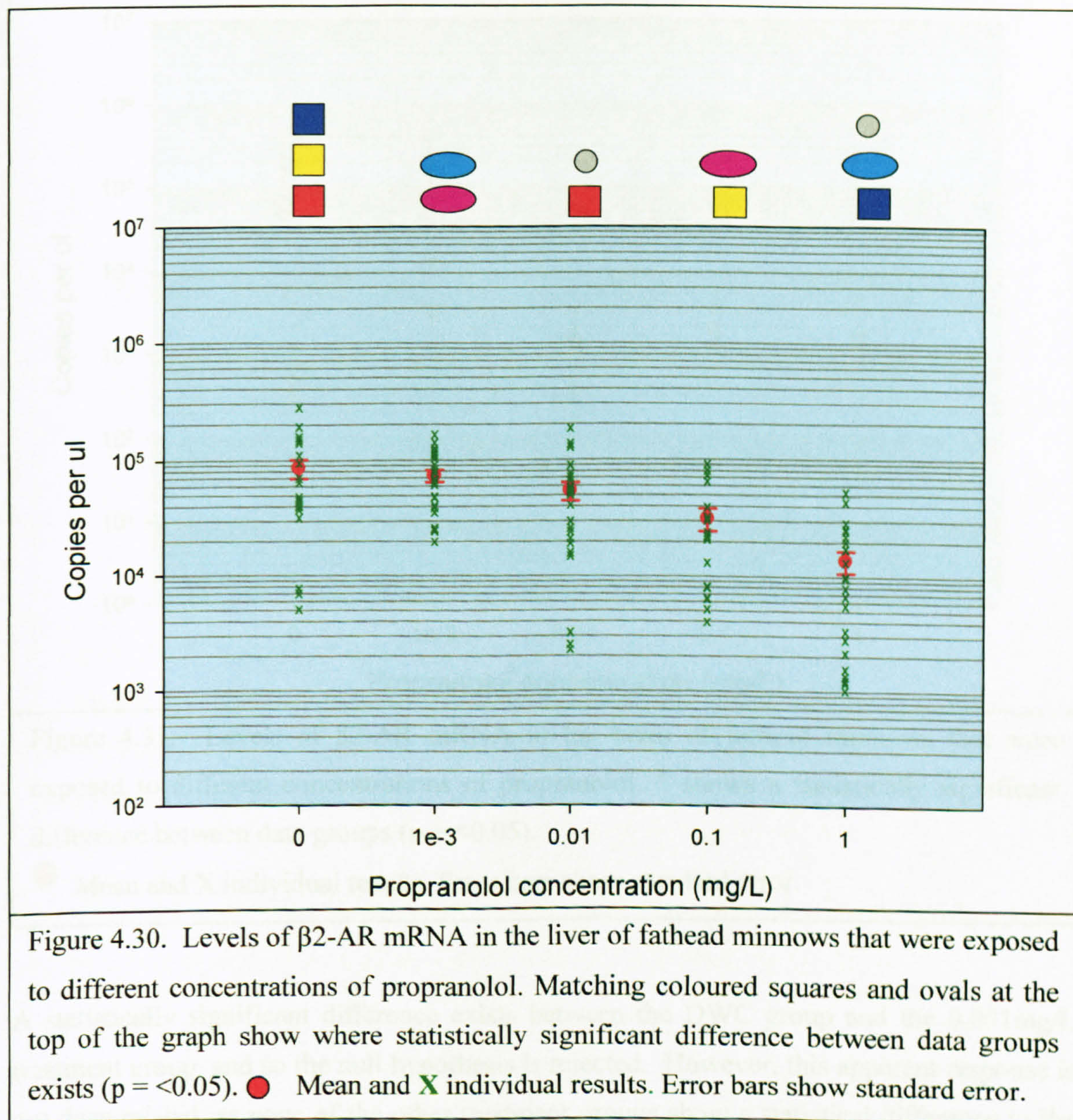


Figure 4.30. Levels of β2-AR mRNA in the liver of fathead minnows that were exposed to different concentrations of propranolol. Matching coloured squares and ovals at the top of the graph show where statistically significant difference between data groups exists (p = <0.05). ● Mean and X individual results. Error bars show standard error.

The null hypothesis is rejected. There are statistically significant differences in the expression level of β2-ARs in the DWC treatment group compared to the 0.01, 0.1 and 1.0 mg/L treatment groups. There are also significant differences between the 0.001 mg/L treatment group and the 0.1 and 1.0 mg/L treatment groups. Another statistically significant difference also exists between 0.01 and 1.0 mg/L treatment groups. This

response to propranolol seems to be dose-related, because as the concentration of propranolol increases, the expression level of β2-AR in the liver decreases.

Figure 4.31 shows the expression level of β2-AR in the brain of fish exposed to different concentrations of propranolol.

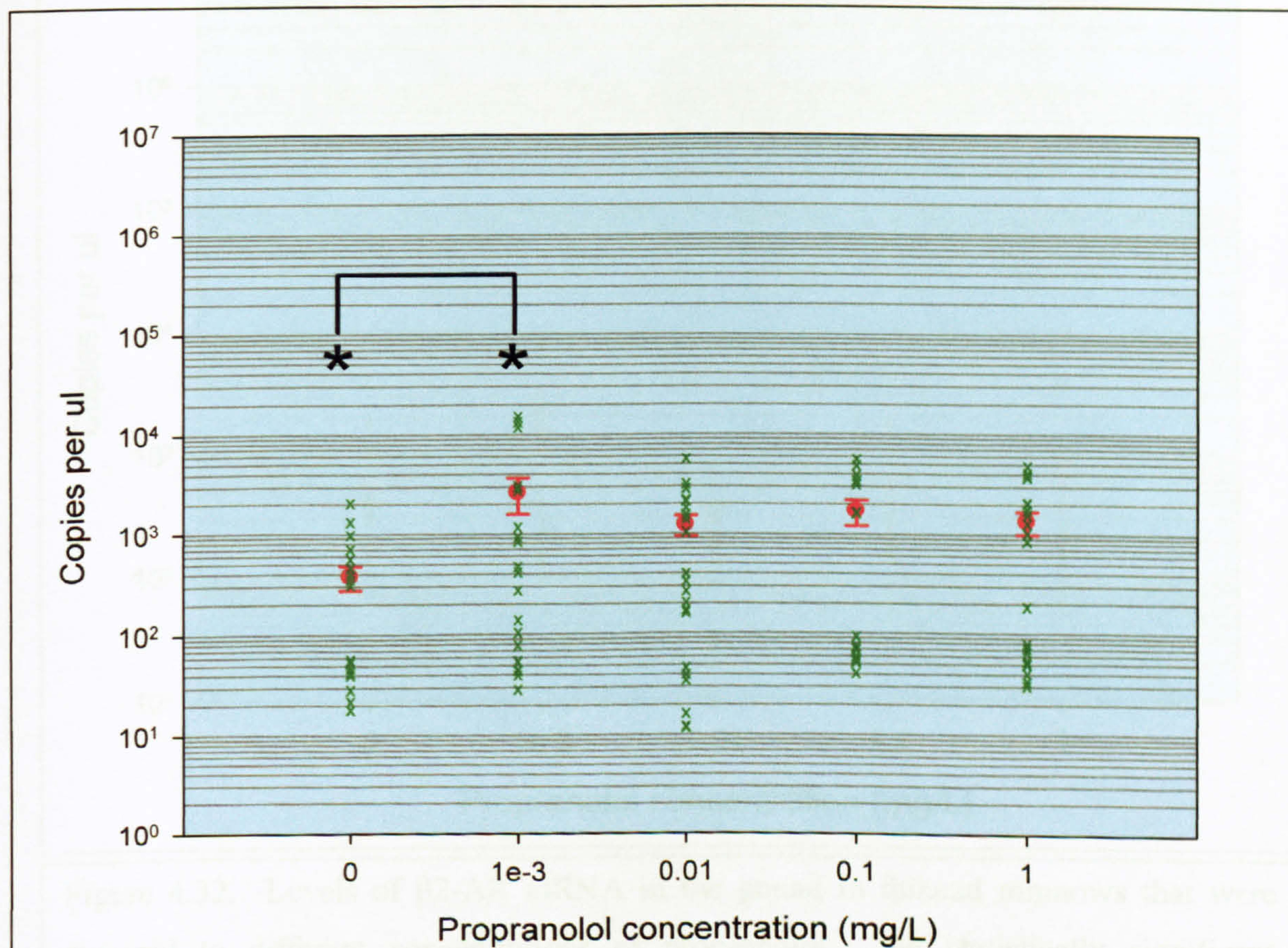


Figure 4.31. Levels of β2-AR mRNA in the brain of fathead minnows that were exposed to different concentrations of propranolol. * shows a statistically significant difference between data groups (p = <0.05).

Mean and X individual results. Error bars show standard error.

A statistically significant difference exists between the DWC group and the 0.001mg/L treatment group, and so the null hypothesis is rejected. However, this apparent response is not dose-related, as none of the other treatment groups show a statistical difference to the control group.

Figure 4.32 shows the expression level of β2-AR in female gonadal tissue of fish exposed to different concentrations of propranolol.

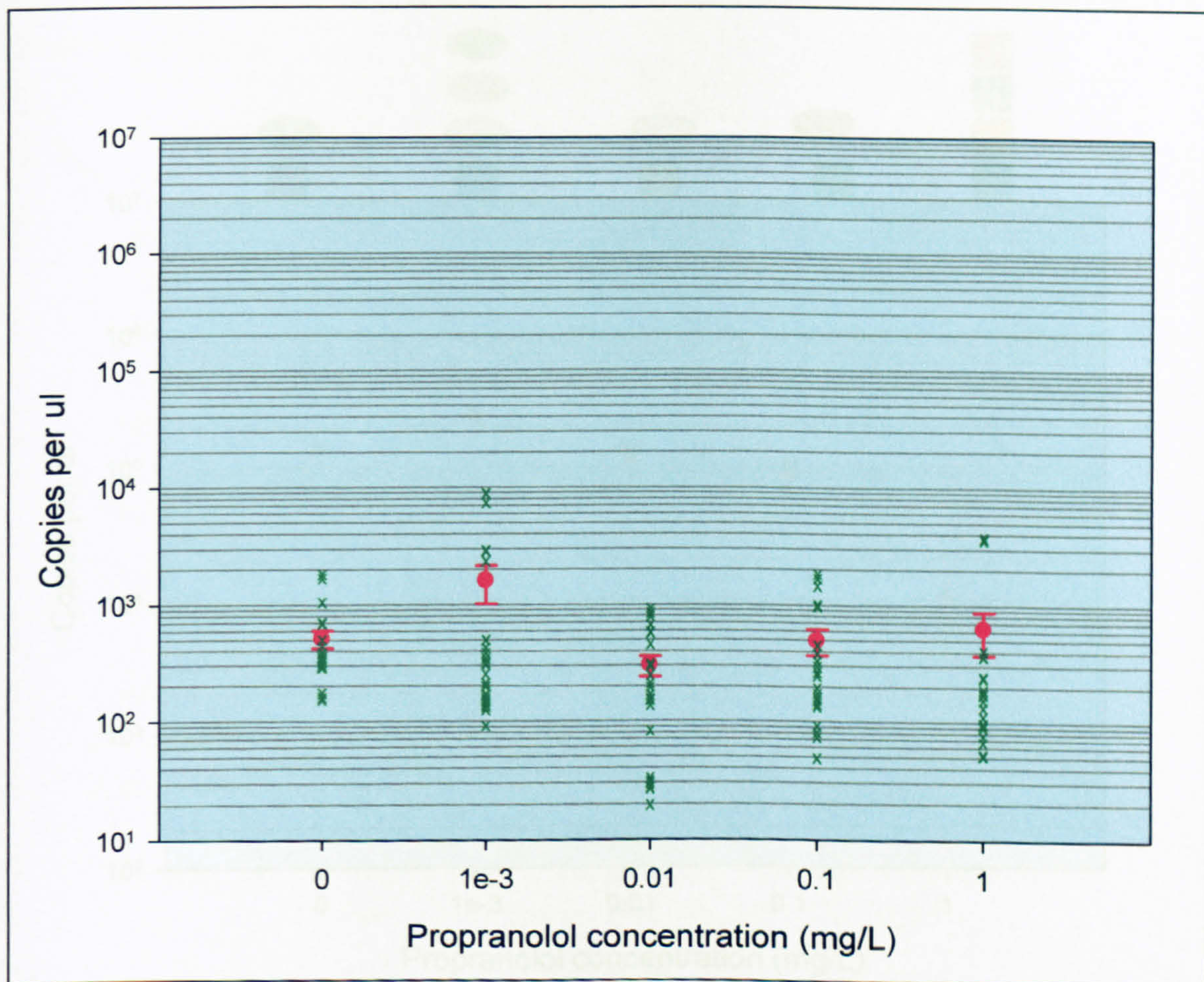


Figure 4.32. Levels of β2-AR mRNA in the gonad in fathead minnows that were exposed to different concentrations of propranolol. No statistically significant differences are found between treatment groups. ● Mean and **X** individual results. Error bars show standard error.

No statistically significant differences are found between treatment groups in the expression level of β2-ARs in brain tissue. The null hypothesis is accepted.

Figure 4.33 shows the expression level of β2-AR mRNA in the heart of fish exposed to different concentrations of propranolol.

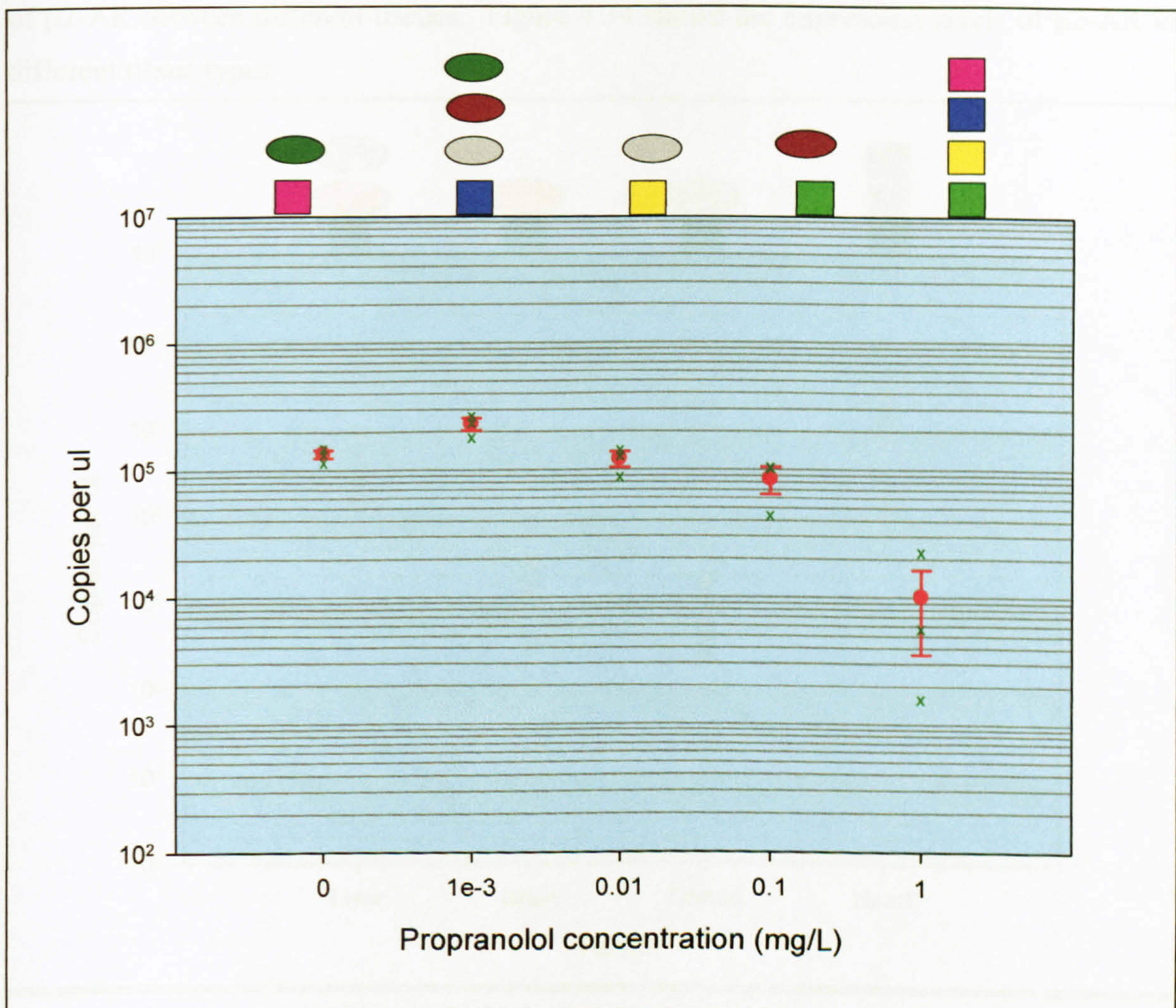


Figure 4.33. Levels of β2-AR mRNA in the heart of fathead minnows that were exposed to different concentrations of propranolol. Matching coloured squares and ovals at the top of the graph show where statistically significant difference between data groups exists (p = <0.05). ● Mean and **X** individual results. Error bars show standard error.

The null hypothesis is rejected. There are statistically significant differences between the expression levels of β2-ARs at 1.0mg/L and all other treatment groups, and also differences between the 0.001mg/L treatment group data and the control, 0.01, and 0.1 mg/L treatment groups. The β2-AR expression level decreases in a dose-dependent manner from the lowest dose.

To test whether there were statistical differences in β2-AR expression levels between tissue types, a null hypothesis was set that stated that there were no differences in the expression of β2-AR between different tissues. Figure 4.34 shows the expression levels of β2-AR in different tissue types.

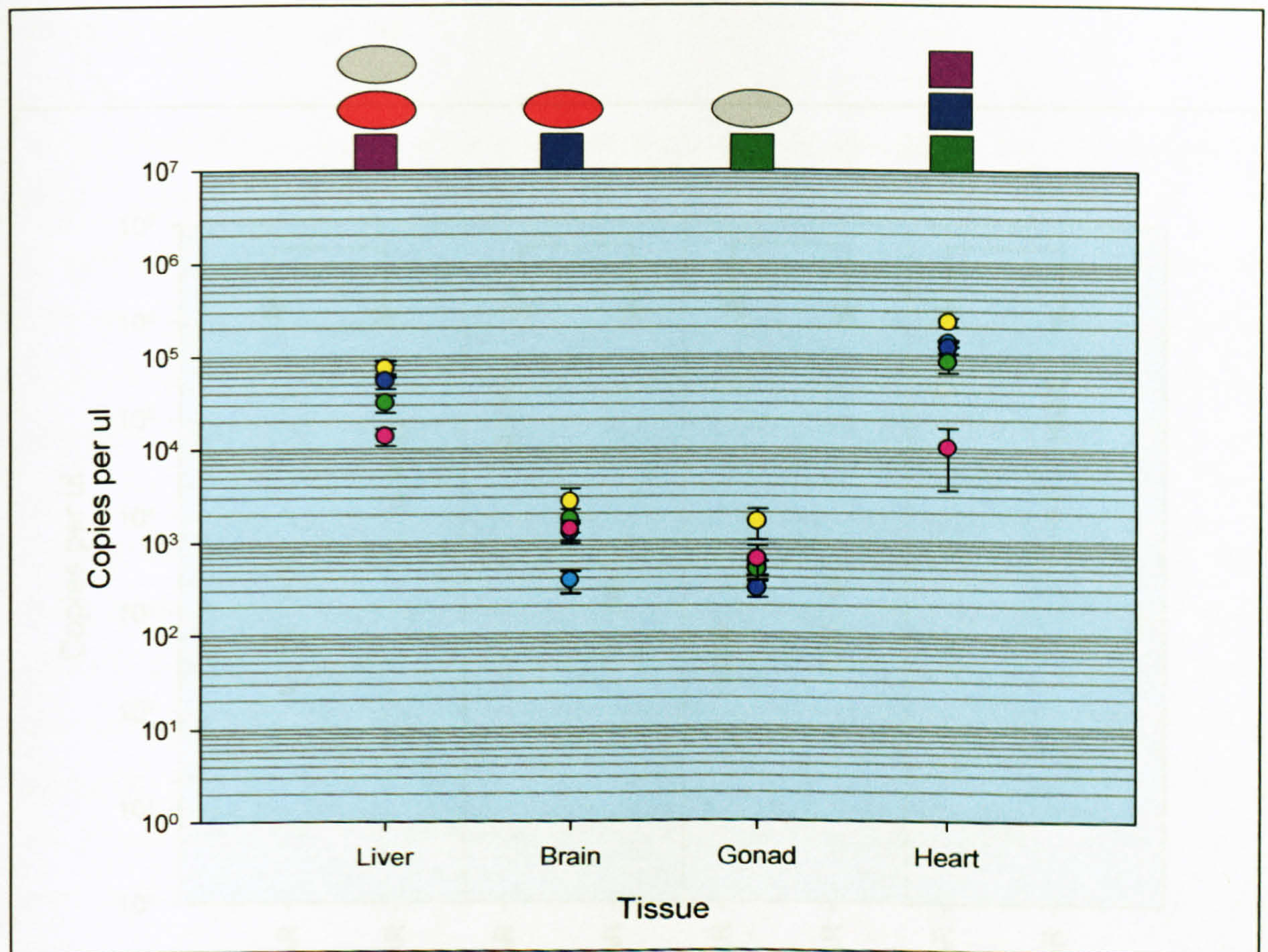


Figure 4.34. Levels of β2-AR mRNA in all tested tissue types of fathead minnows that were exposed to different concentrations of propranolol. * shows a statistically significant difference between data groups (p = <0.05).

Mean results are shown as coloured circles. Error bars show standard error.

● = DWC, ● = 0.001 mg/L, ● = 0.01 mg/L, ● = 0.1 mg/L, ● = 1.0mg/L

The null hypothesis is rejected. There is a statistically significant difference in the expression level of the β2-AR between the heart and all other tissues. There is also a statistically significant difference between the expression level of the β2-AR in the liver and brain and gonad tissues. The heart shows the greatest amount of β2-AR mRNA, followed by liver tissue. There is no statistically significant difference in β2-AR expression level between brain and gonad tissues. The dose-related decreases in the expression level of β2-AR in both the liver and the heart is again quite apparent in this Figure.

4.3.5 Comparison of β1- and β2-AR expression levels

Figure 4.35 shows the expression levels of β1- and β2-ARs in different tissues. The null hypothesis that was tested was that there was no difference in the expression levels of β1- and β2-ARs in each tissue.

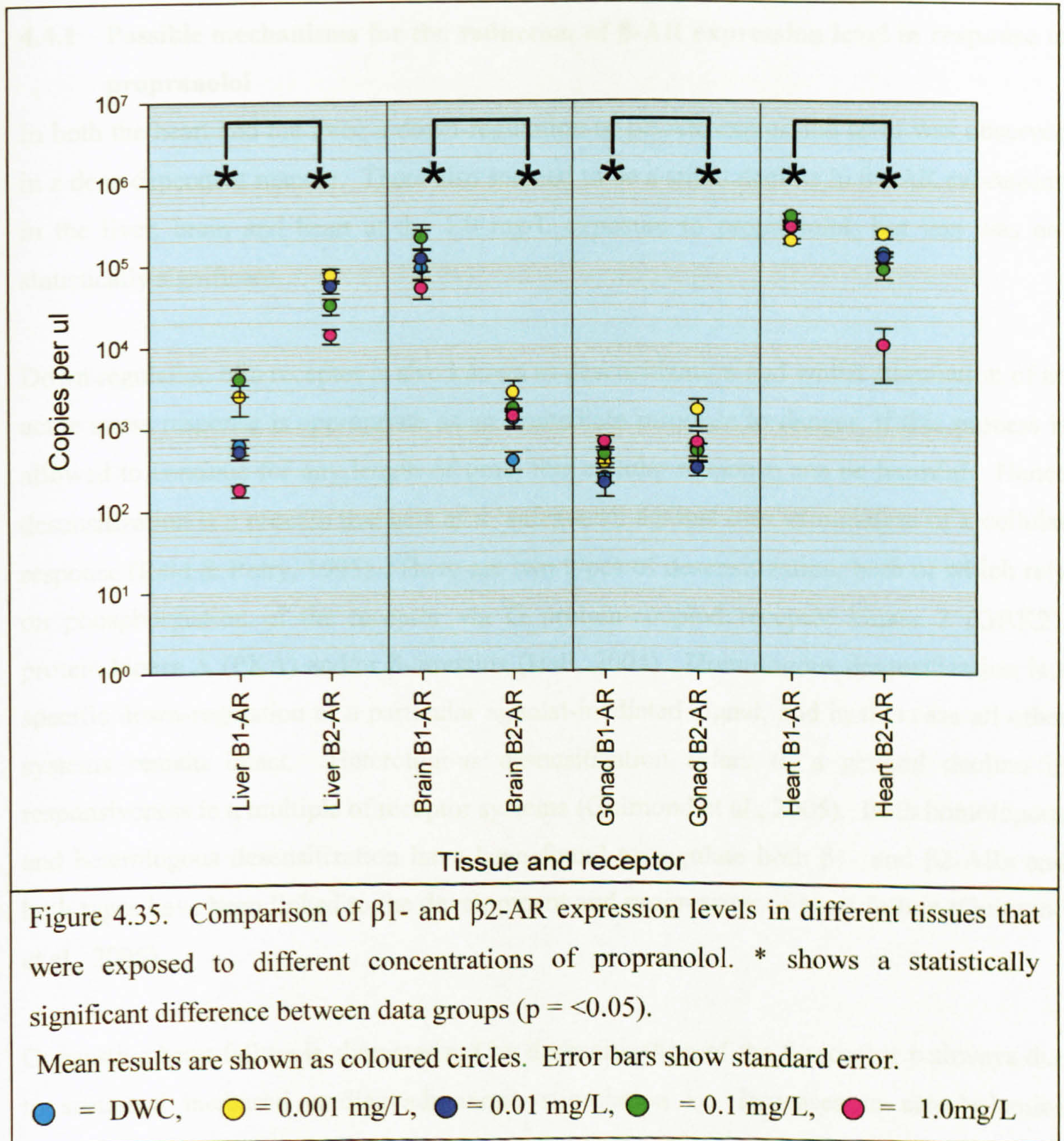


Figure 4.35. Comparison of β1- and β2-AR expression levels in different tissues that were exposed to different concentrations of propranolol. * shows a statistically significant difference between data groups (p = <0.05).

Mean results are shown as coloured circles. Error bars show standard error.

● = DWC, ● = 0.001 mg/L, ● = 0.01 mg/L, ● = 0.1 mg/L, ● = 1.0mg/L

The null hypothesis is rejected, for in every tissue tested there is a statistically significant difference between β1- and β2-AR expression levels. In fathead minnows, the highest levels of β1-AR expression are found in the brain and heart, whilst the liver and heart show high expression levels of β2-ARs.

4.4 Discussion

Analysis of the heart, liver, brain and gonads of female fathead minnows showed the expression levels of β1- and β2-ARs to be different in these tissues. Effects of different concentrations of propranolol also showed a dose-related decrease of β2-AR mRNA level in the liver and heart tissues.

4.4.1 Possible mechanisms for the reduction of β-AR expression level in response to propranolol

In both the heart and the liver, a down-regulation of β2-AR expression level was observed in a dose-dependent manner. There also seemed to be a small decline in β1-AR expression in the liver, brain and heart at the 1.0 mg/L exposure to propranolol, but this was not statistically significant.

Down regulation of a receptor is also known as desensitization and whilst stimulation of an acute stress response is appropriate as an immediate response to danger, if this process is allowed to continue for any length of time, this cellular response can be harmful. Hence desensitization is a process that acts as a 'safeguard' against over-stimulation of a cellular response (Reid & Perry, 1995). There are two types of desensitization, both of which rely on phosphorylation of the receptor via G protein-coupled receptor kinase 2 (GRK2), protein kinase A (PKA) and/or β-arrestins (Hall, 2004). Homologous desensitization is a specific down-regulation of a particular agonist-mediated signal, and in this case all other systems remain intact. Heterologous desensitization refers to a general decline in responsiveness in a multiple of receptor systems (Guimond et al., 2005). Both homologous and heterologous desensitization have been found to regulate both β1- and β2-ARs and both types have been linked to the development and progression of heart failure (Guimond et al., 2005).

Congestive heart failure is characterized by desensitization of the β-receptor pathways due to sustained increased cardiac adrenergic stimulation i.e. increases in catecholamine concentrations (Lowes et al., 2000). In the failing heart, concentrations of noradrenaline are markedly increased as the sympathetic nervous system is activated to compensate for decreased cardiac function (Ellis & Frielle, 1999). However, years of work clearly demonstrate that high levels of catecholamines are extremely toxic to myocardium, and to overcome this, the heart down-regulates catecholamine responses (i.e. expression of β-ARs), in an effort to protect myocardial tissue damage (Port & Bristow, 2001). So in essence when catecholamine stimulation carries on in the long term, this is a poorly

designed homeostatic regulation of the cardiac system, as chronic increases in noradrenaline, instead of helping the failing heart, actually compound the situation by causing a decrease in receptor availability (Ellis & Frielle, 1999). Since noradrenaline acts mainly through β1-ARs, the desensitization response is seen mainly in β1-ARs (Lemoine et al., 1988). In a healthy adult (less than 50 years old) human heart, the percentage of β1-ARs in the ventricular myocardium is between 70 to 80 % of total ARs, however in a failing heart this is reduced such that the proportion of β1- and β2-ARs more closely approximates 50:50 ratio (Port & Bristow 2001). β2-ARs occupy only 20 to 25 % of the AR population in a healthy heart and this percentage shows little or no change in a failing heart (Lowes et al., 2000).

However, β2-ARs are six times more efficient in transducing a given receptor occupancy compared to β1-ARs, and hence β1-ARs have a lower efficacy (intrinsic activity) than β2-ARs (Birnbaumer et al., 1994). So, a small change in β2-AR expression will have a much larger effect than a small change in β1-AR expression. In addition, the heart contains few spare β-ARs, as binding and activation of nearly all the receptors is required for the maximal physiology and precise response mechanisms. Hence in order to maintain normal cardiac function, it is critical that no small changes in expression of the β-ARs occurs (Ellis & Frielle, 1999).

The down regulation of the β2-ARs seen in the fathead minnow at the higher concentrations of propranolol could suggest that propranolol was behaving in a similar fashion to chronic release of catecholamines in that it was acting as a toxin. If there are less receptors available for propranolol to bind to, the effects of the drug are reduced, hence the number of β-ARs became down-regulated as the fish tried to desensitise themselves to propranolol. Because both β2-ARs in the heart and the liver showed a down-regulation of β2-ARs, the mechanism in action could be homologous desensitization, as the response is specific (as far as we know) to just one receptor type.

A study using atenolol also showed β-AR density to be significantly reduced in the hearts of rats exposed to that β-blocker (Vonhoff et al., 2006). In that study, atenolol was used as a reference substance against extracts from the plant *Lycopus europaeus*, which also reduced β-AR density in the heart.

4.4.2 Expression level changes of β-ARs

β-ARs have been found to mediate the adrenergic regulation of trout erythrocyte (red blood cell) volume and intracellular pH, which consequently affects the oxygen-carrying capabilities of erythrocytes (Reid et al., 1991). During short term catecholamine release and subsequent activation of β-ARs in erythrocytes, this would help the fish flee from any imminent danger by quickly providing a greater uptake of oxygen in the blood, which would then be transported to tissues and organs. However, during chronic catecholamine exposure, this response could become damaging. Reid and Perry (1995) report the down-regulation of β-ARs on the surface of red blood cells in trout after large chronic increases in plasma catecholamine levels. Initially it was supposed that β1- ARs were the dominant β-AR subtype of erythrocytes, however after the advent of more specific quantification techniques and the discovery of the β3-AR, it was found that β3b-ARs are in fact the main β-AR subtype on erythrocytes in trout which controls Na^+/H^+ exchange (Nickerson et al., 2003; Perry et al., 1991). Unlike the situation with β1- and β2-ARs, the desensitization of β3-ARs does not occur via phosphorylation by G protein-coupled receptor kinase 2 (GRK2), protein kinase A (PKA) and β-arrestins, and as such does not contain target sequences for phosphorylation by these kinases (Strosberg, 1997). Instead, insulin and the thyroid hormone T3 have been found to down regulate β3-AR in some human tissues (Strosberg, 1997).

Unlike catecholamines, cortisol is reported to have an opposing effect on β-AR expression. Cortisol is a corticosteroid hormone produced in the cortex of the adrenal gland and is released in humans and fish as a response to stress. It causes an increase in blood pressure, blood sugar level and is a known immunosuppressant. Hence, cortisol has been found to up-regulate the number of functional surface β-ARs in the liver, so that accelerated energy production can occur as a response to stress (Reid et al., 1992).

4.4.3 Expression levels of β-ARs in other animals and fish

By comparing the expression levels of β1- and β2-ARs in the same tissue and in different tissues from the fathead minnow (Figure 4.41), it is apparent that there are large differences in the expression levels of these two receptors. For example, in the brain, β1-ARs are predominant over β2-AR expression by over 1000-fold. In the liver, β2-ARs are expressed about 1000-fold times more than β1-ARs. In the gonad and heart there is not as much differentiation in the expression of these two receptors, however the difference is still statistically significant. β2-ARs are expressed at slightly higher concentrations than β1-ARs in the gonad, and in the heart β1-ARs are expressed more than β2-ARs. Overall,

β1- and β2-ARs are expressed most highly in the heart, have the lowest levels of expression in the gonads, and the expression of β1-ARs in the brain and heart are very similar

In the livers of most species, including humans, the numbers of both α1 and β2-ARs are high and glycogenolysis occurs primarily via β2-ARs in normal conditions (Erraji-Bencheckroun et al., 2004). Dog livers also have been found to predominantly express the β2-AR subtype (Garcia-Sainz et al., 1996). Nickerson et al. (2001) and Moon (2004) both found that, by using an RNase protection assay, β2-AR to be highly expressed in the liver of rainbow trout. The data from the RT-PCR work carried out in fathead minnow also demonstrates high β2-AR expression in the liver and shows that, of the β1 and the β2-AR subtypes, it is the principal one, hence the fathead minnow data are comparable to those in other species. It is evident from the above data that β1-ARs are expressed at much lower levels than β2-ARs in the livers of most species. Mersmann (2002) states that β1-ARs occupy only 20 % of the β-AR population in human livers. In the fathead minnow this difference is even greater, as the β1-AR population was found to be less than 1 % of the combined β1- and β2-AR population.

β-blockers mainly target the heart, and so it would be expected that the heart has a high expression level of β-ARs. This is true of most animals; β1- and β2-ARs are found to be highly expressed in the hearts of many animals. The predominant β-AR subtype of hearts in mammals is β1-AR. For example, rat heart is predominantly β1-AR, pig heart is made up of 72 % β1-ARs,and in humans β1-ARs are mostly expressed in the heart, with β1-ARs making up 70 to 80 % of total ARs in the ventricular myocardium (Hall, 2004; Port & Bristow 2001; Mersmann 2002; Strosberg, 1997; Machida et al., 1990). The fathead minnow RT-PCR assay also showed β1 and β2-ARs to be highly expressed in the heart, with β1-ARs expressed 71% more than β2-ARs, which is very comparable to data from pig and human hearts.

β1-ARs are also found in high levels in the brain of animals (Hall, 2004). Frielle et al. (1987) state that the pineal gland and cerebral cortex have the highest RNA levels of β1-ARs in the brain of rats. Machida et al. (1990) also found, using northern blot analysis, β1-AR mRNA levels to be the highest in the pineal gland, thalamus, amygdale, septum, hippocampus and anterior basal ganglia, which are all parts of the brain. Again, the results from the fathead minnow RT-PCR are comparable to these reports. Fathead minnow β1-AR mRNA was found at high concentrations in the brain, which when calculated showed

brain β1-AR levels to be 21 % of heart β1-AR expression. This is a comparatively high level of expression, as β2-AR representation in the brain was only 0.12 % of β1-AR heart expression.

By using RT-PCR, β1-ARs have been found to be expressed in mature oocytes of Xenopus (Devic et al., 1997). These levels change significantly during early development, and the catecholamine concentration in mature oocytes has been found to follow a similar trend to β1-AR mRNA expression (Devic et al., 1997). Because β-ARs have been found in mature oocytes provides evidence to support their presence in non-fertilised oocytes, and this may explain why β-ARs were found in the female gonads of fathead minnows, albeit at fairly low expression levels.

β-ARs have also been found in other fish tissues that were not tested in this study. In trout, β2-ARs were also found to also be highly expressed in red and white muscle, with lower expression levels in the gills, kidney and spleen (Moon, 2004; Nickerson et al., 2001). However, in summary, the β1- and β2-AR expression levels, particularly those in the heart, liver and brain tissues of fathead minnows, resemble the pattern of expression found in mammals and other fish.

Chapter 5 General discussion

5.1 Theories to explain the effects of propranolol on fish

Exposure of propranolol to fathead minnows caused an acute toxicity at 10 mg/L. Before the fish at 10 mg/L were euthanized, they were observed to be very disorientated and swimming on one side or nosing the bottom of the tank. Propranolol also caused adverse effects to the health of fish at 1 mg/L, and a loss of appetite was noted together with slight disorientation. Other effects of propranolol included a statistically significant decrease in reproduction at 1.0 mg/L, dose-related decreases in male weight, CI and fatpad weight, and a dose-related increase in female GSI. Molecular analysis of β1- and β2-AR expression revealed a dose-related decrease in β2-AR expression in the liver and hearts of fathead minnows exposed to propranolol.

The loss of appetite together with the disorientation of the fish and lack of sexual behaviour indicates that propranolol was acting as a CNS toxin. It is possible that loss of appetite caused the fish to mobilise fat stores, which resulted in reduction of CI and weight of the fish and fatpad. However, the desensitization of β2-ARs in the heart and liver do not support this theory, for if this was the case, a down-regulation of β-ARs in the brain may have been observed.

Another likely theory is that the effects seen when fish were exposed to propranolol are due to energy re-allocation. If propranolol caused the heart rate to be reduced sufficiently, there would have been a decrease in oxygen delivery to tissues and hence a reduced energy supply to vital tissues and organs. This would account for the observed disorientation of the fish and could equate to light-headiness and fainting experienced in humans through lack of oxygen. In an effort to survive, the fathead minnows stopped reproducing and allocated the remaining energy to survival (Dzialowski et al., 2006). In addition, propranolol increased lipolysis, which caused the mobilisation of fat stores, a primary store of energy in fish, which resulted in decreased weight, CI and fatpad weight (Vianen et al., 2002). This theory is supported by the desensitisation of β2-ARs in the heart and liver. If both of these organs were targeted by propranolol, which caused a decreasing heart rate and increasing lipolysis, the fish, in an effort to survive, down regulated β-ARs in these organs to try to minimise the toxic effects of propranolol.

Of the two theories, the later is more probable as the molecular data indicate that the heart and liver were affected by propranolol. Without much more research, the mechanisms of action of propranolol underpinning the effects observed will remain theories only. As most

organs have ARs, often more than one type, propranolol (a non-selective β-blocker) could cause direct effects on almost any organ and hence any physiological process. The indirect effects (due, for example, to changes in energy metabolism) are almost infinite. However, the aim of the project was to conduct an environmental risk assessment of propranolol on fish, not to tease out the exact mechanisms of action behind any effects.

5.2 Aquatic risk assessment of propranolol

The detection of pharmaceuticals in a variety of water bodies at a range of concentrations has raised concern worldwide. The testing that these bioactive ingredients undergo before they are released into the environment has, up until recently, not sufficiently attempted to address the chronic effects of these drugs. By carrying out the pair-breeding assays in this study, a lot of data were collected that assessed the chronic effects of propranolol on fathead minnows. These included data not only on the reproductive capabilities of the fish, but also provided information on general health parameters and also appropriate molecular information (on quantification of β-AR expression) that helped in understanding the mechanisms underlying changes in the holistic parameters. Molecular work can also help explain data collected from physical parameters. Propranolol targets β1- and β2-ARs in humans, and since the homology between human and fathead β-ARs is very high, it can be assumed that propranolol will also target β-ARs in fathead minnows. Using RT-PCR, the tissues that express these receptors can be identified and hence enable us to predict which organs might be affected by the drug. This intelligent-based testing strategy uses the mode of action of the drug to aid in hypothesis testing and in predicting where effects may be occurring. This approach should help identify chronic effects which may have been overlooked if standard Organization for Economic Cooperation and Development (OECD) ecotoxicology tests had been used alone.

Propranolol has been detected in surface waters at concentrations ranging from 0.00001 to 0.00056 mg/L and in wastewater effluent at concentrations ranging from 0.0022 to 0.000002 mg/L (Gros et al., 2007; Ashton et al., 2004; Huggett et al. 2003; Ternes 1998). In this study, dose-related effects of propranolol on fathead minnows were observed at concentrations as low as 0.1 mg/L, with severe disruption to health occurring at 1.0 mg/L. In determining the level of risk of propranolol to fathead minnows, this study has shown that at environmental concentrations, propranolol poses no risk to fathead minnows. When using these data obtained in the pair-breeding assays to calculate a PEC:PNEC ratio, the risk characterisation ratio equals 0.056, as shown in Figure 5.1. The ratio is less than 1, which means propranolol is classified in this assessment as not being of potential concern.

$$\text{Risk characterisation ratio} = \frac{\text{PEC}}{\text{PNEC}} = \frac{0.00056}{0.01} = \mathbf{0.056}$$

Figure 5.1. Calculation of the PEC/PNEC ratio for propranolol using data obtained from the pair-breeding assays with fathead minnows.

With respect to assessing the risk of propranolol to all aquatic life, published literature so far shows no effects in plants, bacteria, invertebrates or fish at concentrations that are low enough to be found in the environment. The only data to suggest anything to the contrary are data published by Huggett et al. (2002), where it was reported that Japanese medaka showed reduced hatchability and egg production at 0.0005mg/L. If data from Huggett et al. (2002) are included in the ratio calculation, the risk characterisation ratio is calculated as being **1.12,** showing that propranolol is a chemical that is of potential concern. However, as discussed in chapter 3, there are queries over the reproducibility of these data. This example highlights how the ratio is only as accurate as the data used in the calculation and if, as proposed by Ioannidis (2005), that 'most published research findings are false', then this ratio can often be incorrect.

$$\text{Risk characterisation ratio} = \frac{\text{PEC}}{\text{PNEC}} = \frac{0.00056}{0.009} = \mathbf{0.062}$$

Figure 5.2. Calculation of the PEC/PNEC ratio for propranolol using data from the literature

The PEC/PNEC ratio calculated from published data listed in this thesis, without including the Huggett et al. (2002) data, is 0.062, as shown in Figure 5.2. This ratio is less than 1, and comparable to the risk characterisation ratio obtained from the fathead minnow pair-breeding assays. Hence, based on what is known currently, it can be concluded that propranolol is not a pharmaceutical of potential concern to the aquatic environment.

5.3 Propranolol and other β-blockers

β-blockers are often grouped together in one class of pharmaceuticals, and although they all act on β-ARs, they can differ greatly in their specificity and lipophilic properties. The differences in physical parameters between β-blockers means that the toxicity to aquatic

organisms also differs between each individual β-blocker. For example, propranolol, due to its relatively high log Kow of 3.48, can easily cross the blood: brain barrier and act as a serotonin receptor antagonist, whereas atenolol, which has a log Kow of 0.23, cannot cross the blood: brain barrier. This means that atenolol is less toxic to the brain and consequently has fewer CNS side effects in humans compared to propranolol.

The high log Kow of propranolol means that it has the potential to bioaccumulate in tissues and organs, at greater concentrations than other less lipophilic β-blockers, and hence the internal concentrations of propranolol can often exceed those in the water surrounding the organism. This study provides an example, as propranolol plasma concentrations of fathead minnows placed in 0.1 and 1.0 mg/L of propranolol far exceeded the water concentrations of the drug. In the 1.0 mg/L tanks, the concentration in the plasma of male fish was as much as 1546 % greater than the actual mean water concentration. A parallel pair-breeding assay has been conducted with atenolol and the plasma concentrations of atenolol ranged from 1.8 to 12.2 % of the water concentrations (Winter et al., 2008). This is a huge difference between the two β-blockers and another reason why they should not be classed as the same kind of pharmaceutical when considering the risks they may pose to the environment. The metabolism of β-blockers in humans is also very different, in that 90 % of propranolol is metabolised, which differs greatly to atenolol, of which only 10 % is metabolised (Maurer et al., 2007). Currently nothing is known about the metabolism of β-blockers in fish.

Further examples of the differences between β-blockers are seen in ecotoxicological data. Propranolol, when tested on plants, bacteria, algae, invertebrates and fish, has lower EC_{50} values compared to other β-blockers, and this difference can be as much as a 1000-fold. The general toxicity trend of some β-blockers is propranolol > metoprolol > atenolol > sotalol. This trend is also reflected in the PEC/PNEC values of atenolol and metoprolol, which have PEC/PNEC ratios much less than 1 (0.00077 and 0.28, respectively; Cleuvers, 2005).

Hence, one very compelling, and important message, is that one β-blocker is not the same as another β-blocker when considering their ecotoxicities. However, it would be a huge task to treat every pharmaceutical separately from all others and in addition, with regard to ecotoxicology, it would be ethically unsound to do so. It may be that physical properties of drugs within each type of pharmaceutical should have some bearing as to how they are

classified. For example, drugs with similar log Kow values, within the same class of drug, could be combined.

5.4 Reproduction as an ecotoxicological endpoint

The propranolol pair-breeding tests conducted in this study used reproduction as the main endpoint. Reproduction is a vital process and when an animal has a reduced energy supply or is under stress, then that animal may forgo reproduction in order to survive (Dzialowski et al., 2006). Hence, reproduction is a good indicator that may highlight when something is not right. However, had reproduction been the only endpoint in the pair-breeding assays, it may have been concluded that propranolol was having a reproductive toxic effect (i.e. a specific and direct effect on the reproductive system). Instead, by having additional endpoints, such as fatpad weight, weight of fish, weight of gonads and molecular endpoints, a better understanding of the internal mechanisms at work can be gained. Other studies also show that reproduction is not always the most sensitive biomarker; for example, in a pair-breeding assay with atenolol, fathead minnow reproduction showed a LOEC of >10mg/L, yet the most sensitive fathead minnow chronic test endpoint was male condition index, which had a LOEC of 3.2 mg/L (Winter et al., 2008).

Many pharmaceuticals are designed with a specific mode of action and by transferring existing knowledge about drugs and mechanisms of action across taxonomic classes, a wealth of information can be gained. As this study demonstrates, it is only by taking these mechanisms into account when carrying out ecotoxicology testing that it is observed that these drugs are capable of having an effect at much lower concentrations than would be observed if reproduction was the only endpoint. It could be argued that if the fish is in relatively good health, it still has the energy reserves available to reproduce. However, as the hatchability data from this study shows, the number of days for eggs to hatch was significantly increased in the 0.1 mg/L treatment tanks (fish exposed to 1.0 mg/L did not spawn enough to analyse their eggs), so although the fish were still reproducing, the progeny may not have survived, as they took longer to hatch and hence many have been under increased predation threat. Hence, intelligent testing could be a basis for environmental risk assessment.

5.5 The 'fish plasma model' approach

The results from this study provide support for the 'fish plasma model' proposed by Huggett et al. (2003). In this study, the effect ratios for the 0.1mg/L and 1.0 mg/L treatment tanks are less than 1, indicating that drug plasma concentrations in these fish are

greater than the drug concentration in human plasma which is needed to elicit a therapeutic effect. This corresponds well with the statistically significant effects that were observed in the fish exposed to these concentrations of propranolol. The model also predicts that at 0.01 mg/L of propranolol, no effects should be observed in fathead minnows, because the propranolol plasma levels are not greater than the drug concentration in human plasma which would be needed to obtain a response. The results are consistent with the model predictions, as no effects of propranolol were observed below 0.1 mg/L.

This model has proved more sensitive compared to a traditional reproductive endpoint, as it predicts a response at the 0.1 mg/L concentration, which the pair-breeding assay alone would have failed to detect. Hence, the transfer of knowledge from humans to fish is very effective in this case, and very accurately supports the fish plasma model hypothesis of Huggett et al. (2003).

5.6 Further work

With respect to areas of work to concentrate on if the research was continued, there are several points for consideration.

Having characterised the molecular sequence for the β1- and β2-ARs, the characterisation of the β3-AR should be carried out. Nickelson et al. (2003) isolated 2 subtypes of the β3-AR, of which the β3a-AR was highly expressed in the heart and gill, whilst β3b-AR was found to be highly expressed in red blood cells in rainbow trout (*Oncorhynchus mykiss*). In humans, β3-AR has a markedly different expression pattern to β1- and β2-ARs in that it is highly expressed in brown and white adipose tissue, and research now provides a consistent picture of the role of β3-ARs in humans with respect to lipid metabolism (Strosberg, 1997). β3-AR has also been detected in the left atrium of the human heart, and if β3a-AR is also found in the heart of trout, it raises questions as to the purpose it has there. Is it involved in heart function and if it is, does it interact in any way with β1- and β2-ARs (Strosberg, 1997).

A very interesting study would be to determine whether propranolol actually binds to the fathead minnow β1- and β2-ARs, and to what degree i.e. to find the binding affinity of propranolol to fathead minnow β-ARs. Similar work has been carried out by Ruuskanen et al. (2005), whereby α2-ARs were expressed in Chinese hamster ovary (CHO) cells and competitive ligand binding assays were carried out using adrenaline and phentolamine. This approach would be most interesting, as it would add weight to the evidence already

collected in this study that propranolol was binding to β-ARs in the fathead minnow, and causing its effects through this binding.

Besides binding to β-ARs, propranolol is also known to act as a serotonin receptor antagonist, by interacting with the 5-hydroxytryptophan receptor in the brain (Dzialowski et al., 2005; Westerlund, 1985). Since one hypothesis to explain many of the effects observed in this study was that propranolol was causing CNS side-effects, it would be interesting to investigate whether propranolol was acting as a serotonin antagonist. This could be done by first determining whether or not propranolol binds to the fish 5-HT receptor. If binding is possible, serotonin concentrations in the plasma and brain could be measured and it could be determined whether propranolol was causing an up or down regulation of 5-hydroxytryptophan receptors in the brain. The higher the affinity propranolol has for the 5-HT receptor in fish compared to the β-ARs, the more likely proranolol's effects are mediated not through the β-AR, but instead the 5-HT receptor. Another interesting endpoint would be to follow up the second hypothesis and determine whether propranolol did actually physically decrease heart rate in the fathead minnow at high concentrations of propranolol.

The issue of mixtures of pharmaceuticals in ecotoxicology is a very relevant one, for it is not the case that a fish or any aquatic organism is exposed to only one pharmaceutical at a time. The more realistic scenario is that aquatic organisms are exposed to a variety of different chemicals at the same time, of which some combinations of chemicals can exponentially increase the biological activity of some drugs. A mixture can include a range of pharmaceuticals and their metabolites that could target a multitude of different receptors, enzymes and proteins. As this study shows, a range of different β-blockers have been detected in the environment and hence a number of them would be present in the aquatic environment at the same time. Further work could be to combine concentrations of all β-blockers, or at least a mixture of those that are found at the highest concentrations in the environment, to determine the effects of this mixture on fish.

Pharmaceuticals in the environment is an emerging topic that has highlighted that many of the pharmaceuticals and personal care products that are used end up in aquatic and terrestrial environments. The design of these drugs means that they are often persistent in the environment in biologically active forms. However, as this study demonstrates, many of the effects of these drugs on aquatic organisms occur at concentrations that are above those found in the environment. Despite this, there is a wealth of evidence that illustrates

how some drugs, such as ethinylestradiol and diclofenac, can sometimes cause catastrophic effects on wildlife at concentrations present in the environment. So the issue is, if we are to unintentionally add these chemicals to the environment, we must first be certain and responsible enough to ensure that repeats of these tragic incidences (many millions of vultures have been killed by diclofenac in the last few years) do not occur. In particular, thought must be given to ensure that new drugs are tested thoroughly with intelligent testing in mind that includes the mode of action of the drug, hopefully to ensure that pharmaceuticals do not enter our environment at concentrations able to cause harm to the organisms living there.

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www.pbs.gov.au

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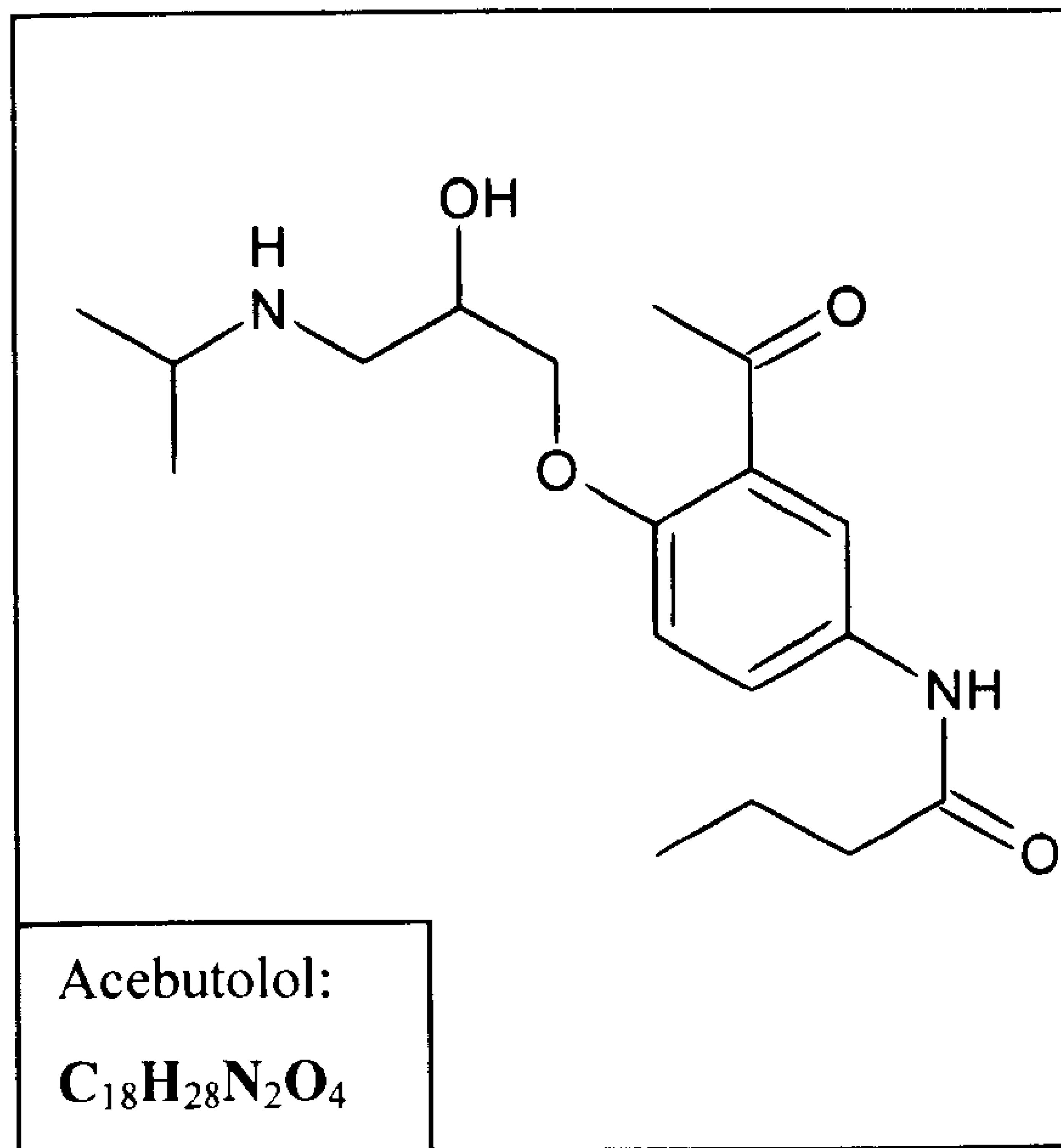
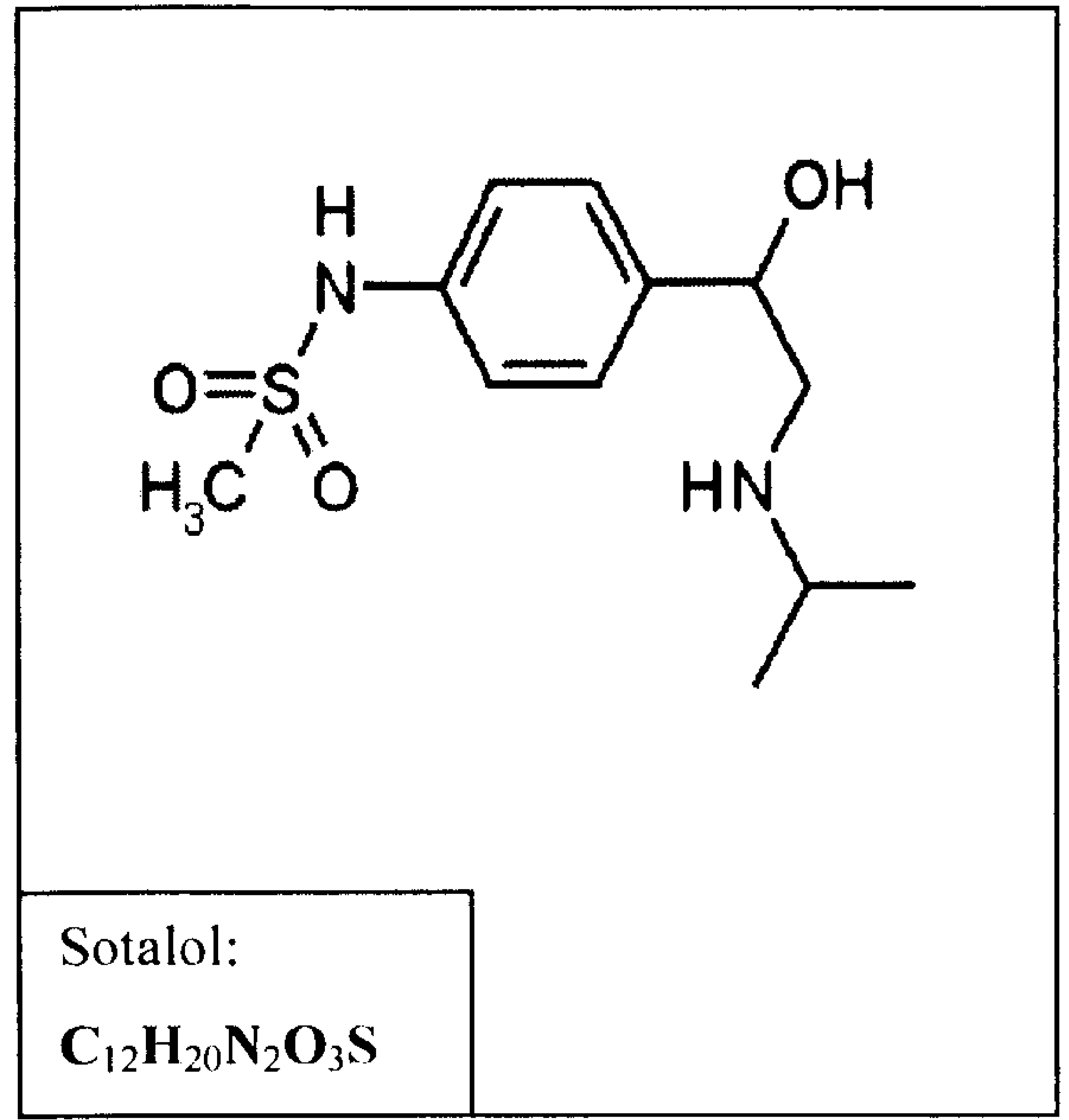
Appendix A.

Chemical structures of commonly used β-blockers.



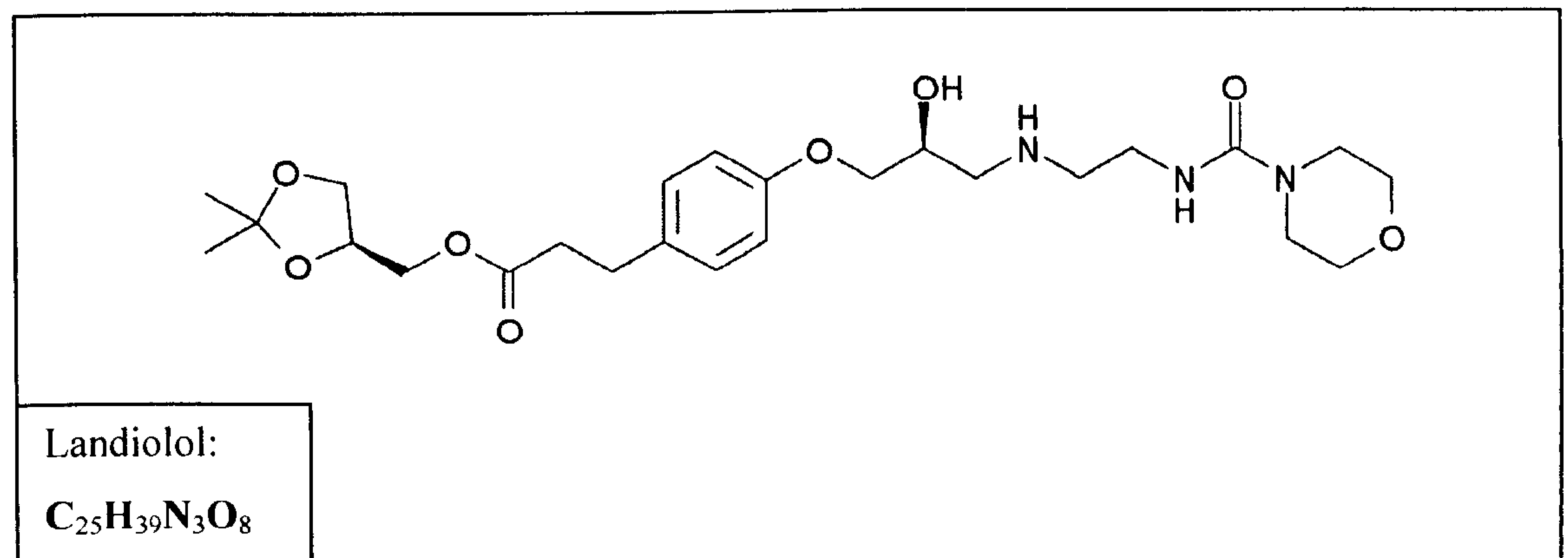
Acebutolol:

$C_{18}H_{28}N_2O_4$



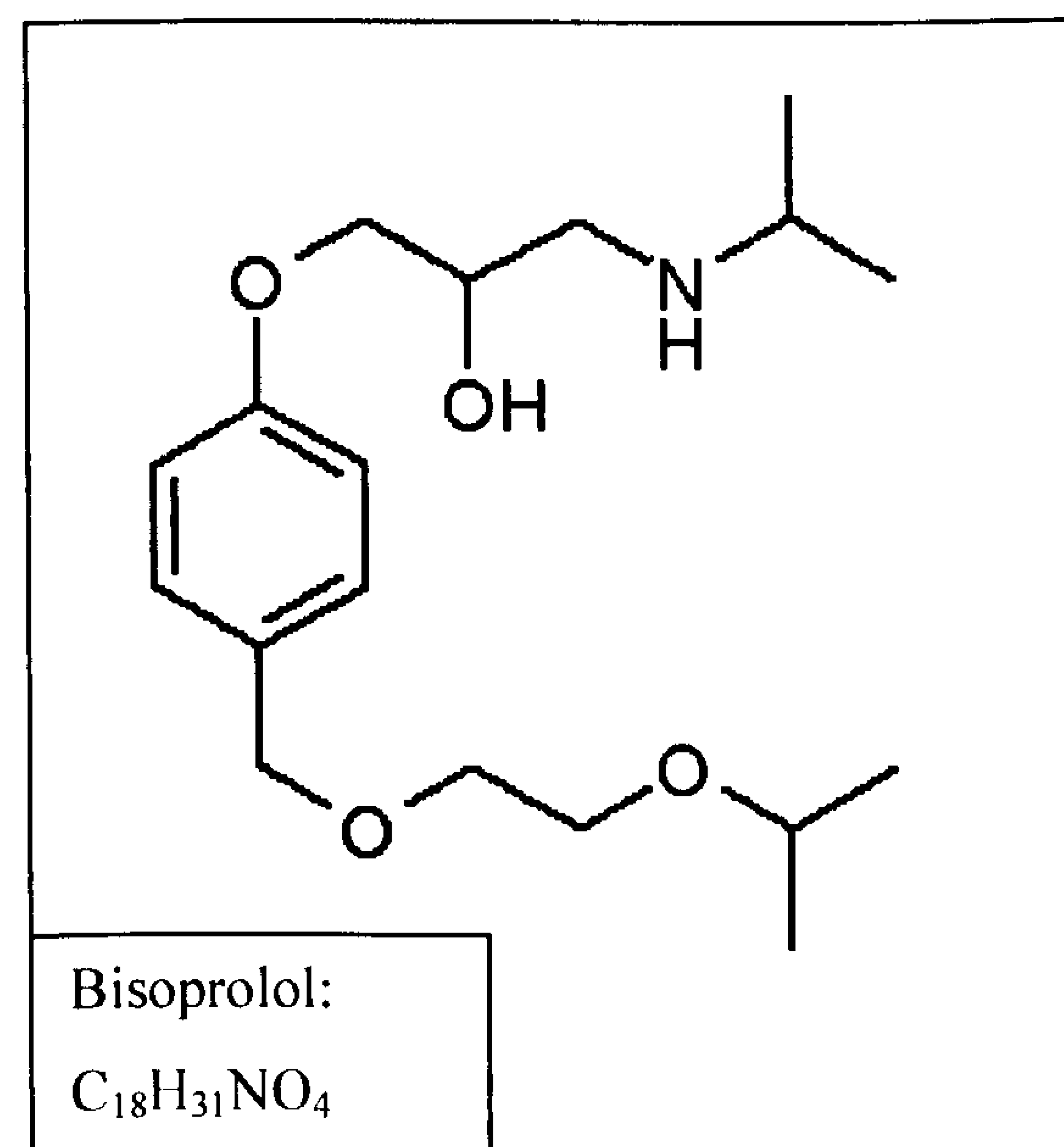
Sotalol:

$C_{12}H_{20}N_2O_3S$



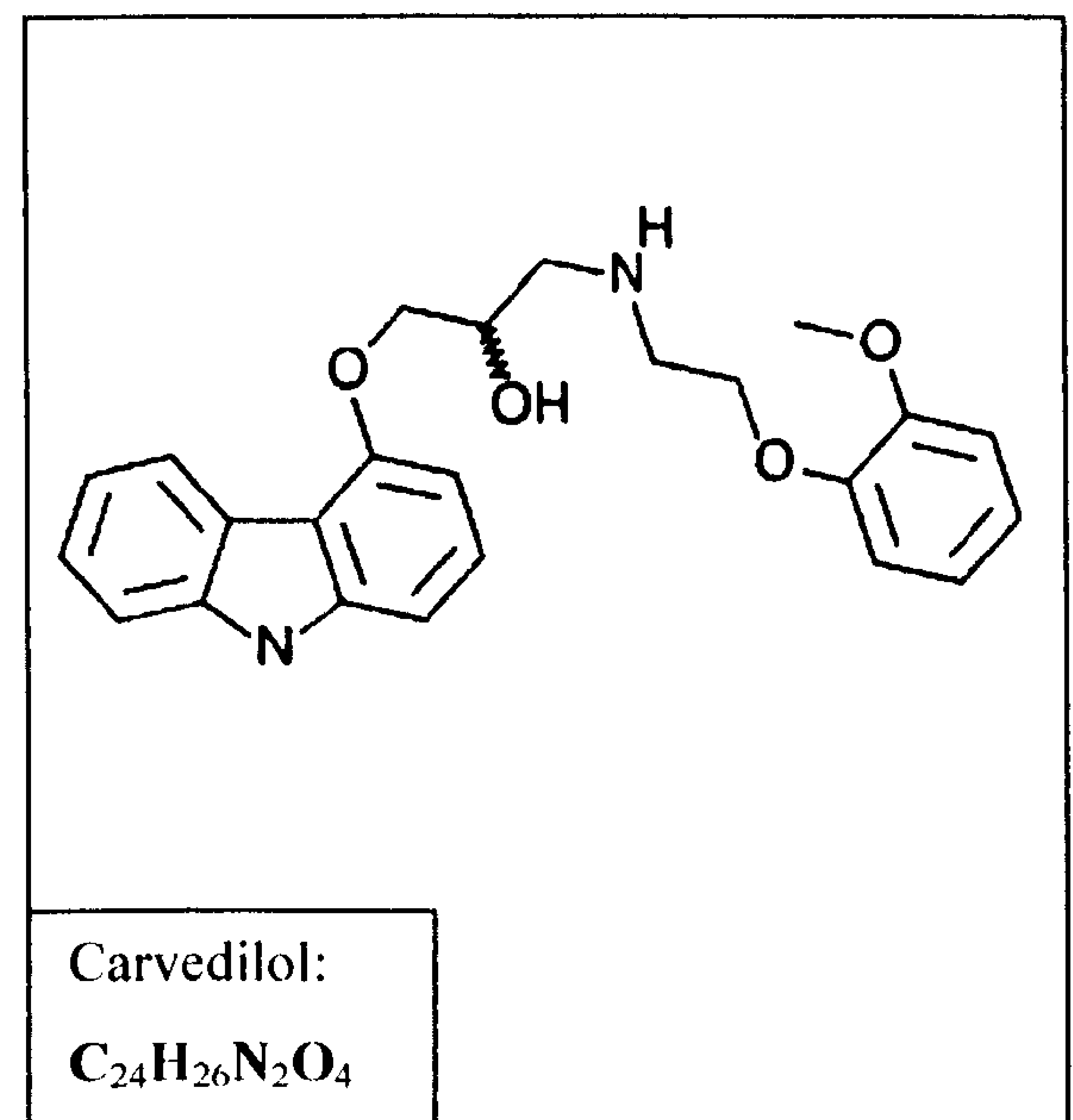
Landiolol:

$C_{25}H_{39}N_3O_8$



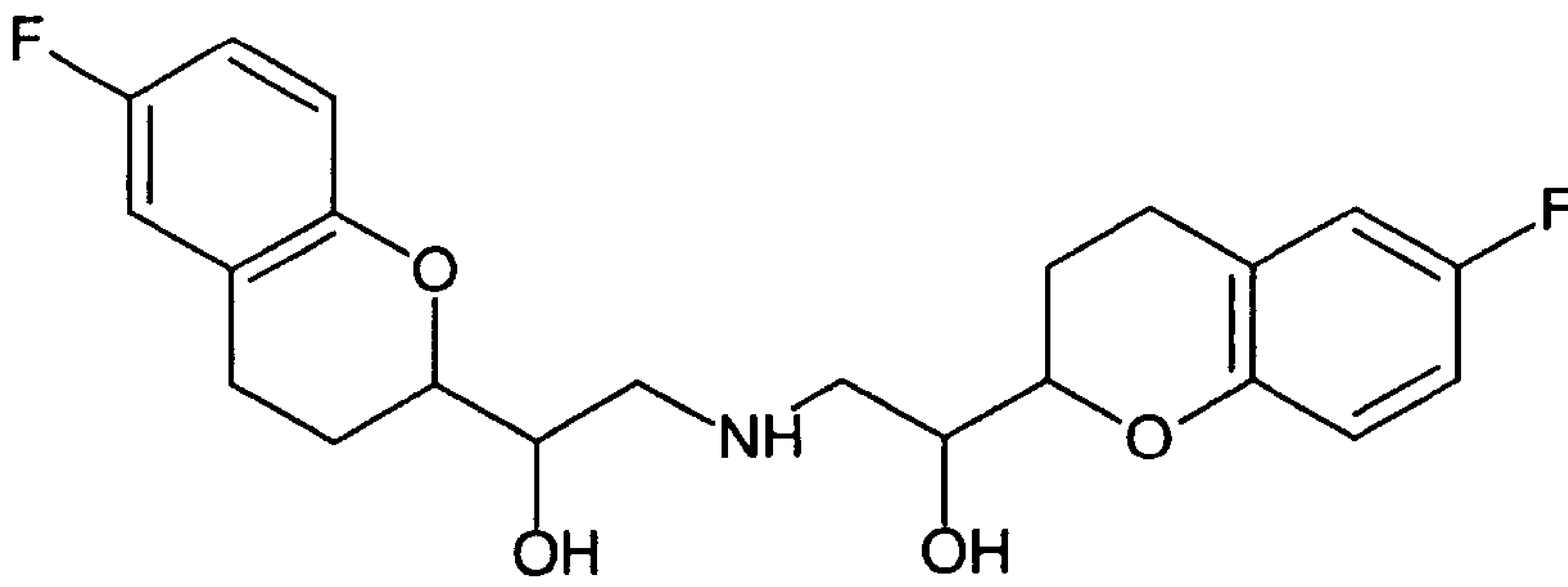
Bisoprolol:

$C_{18}H_{31}NO_4$



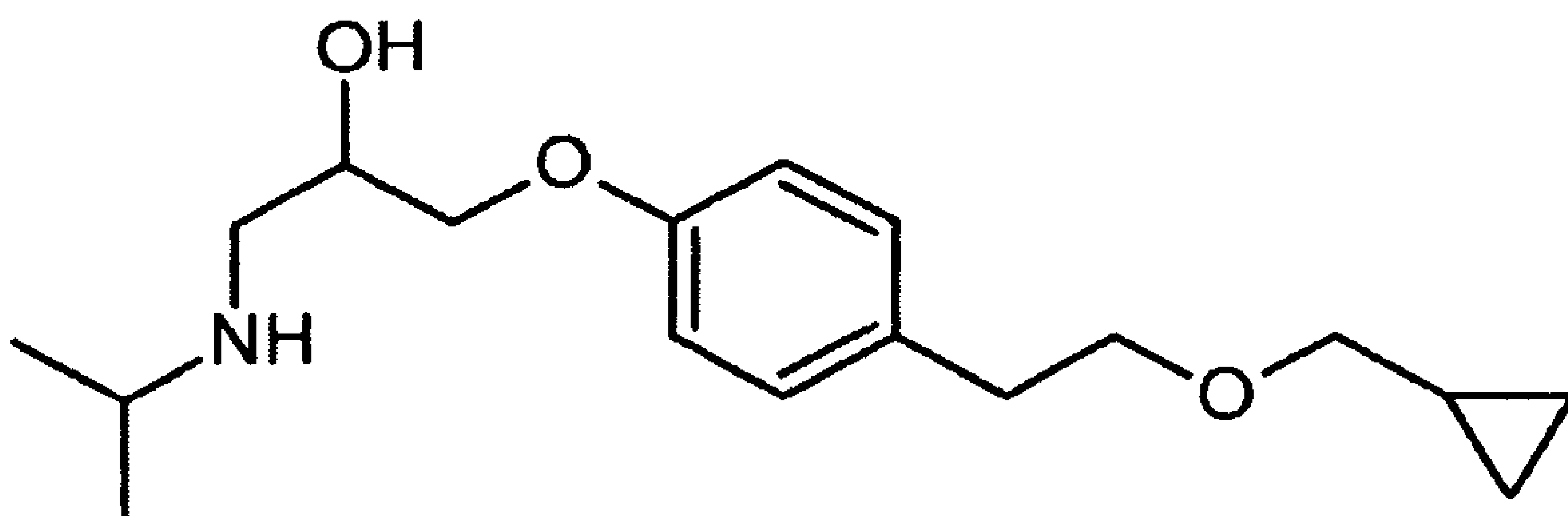
Carvedilol:

$C_{24}H_{26}N_2O_4$



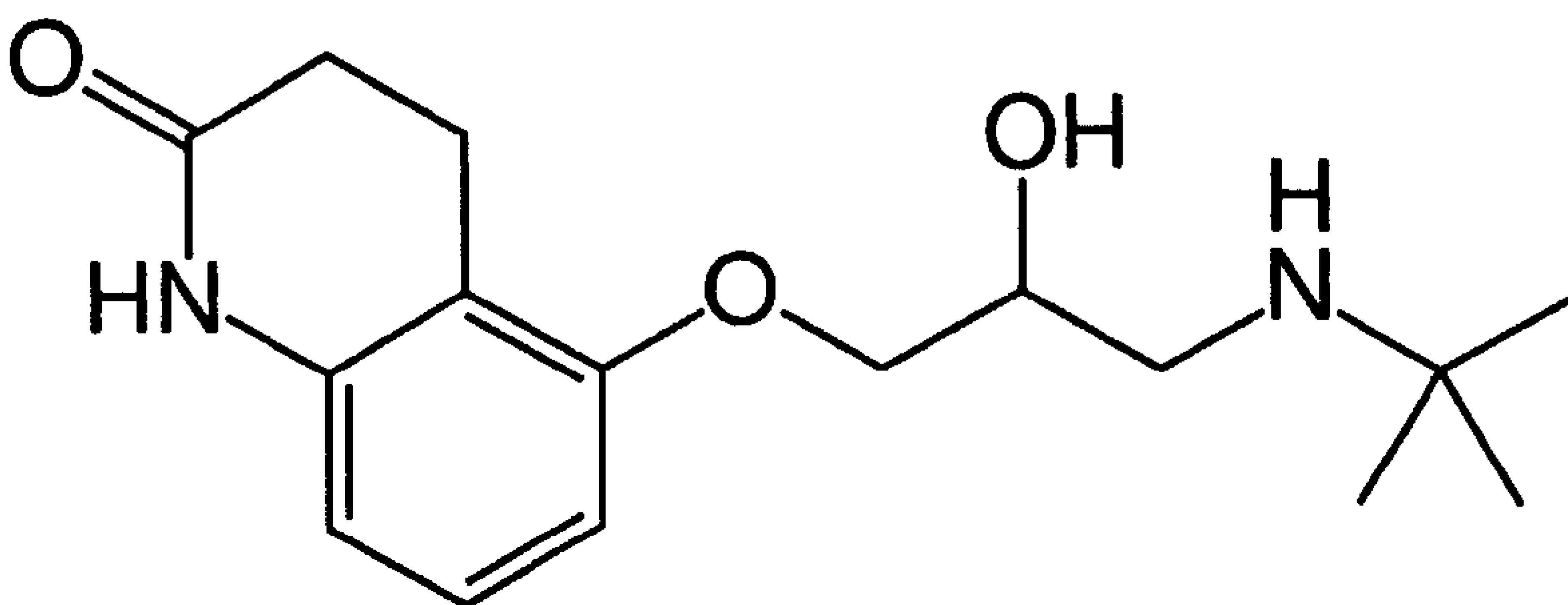
Nebivolol:

$C_{22}H_{25}F_2NO_4$



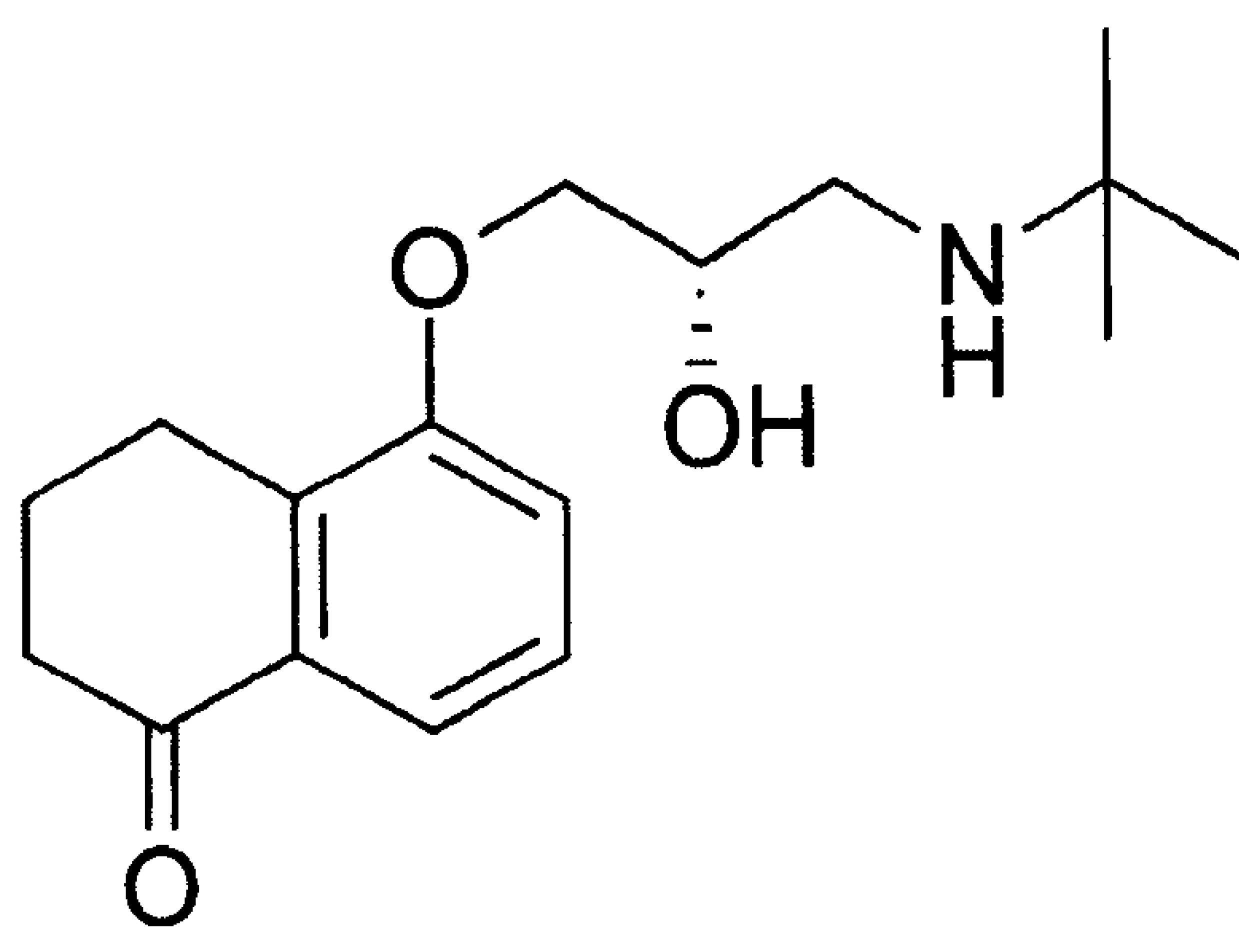
Betaxolol:

$C_{18}H_{29}NO_3$



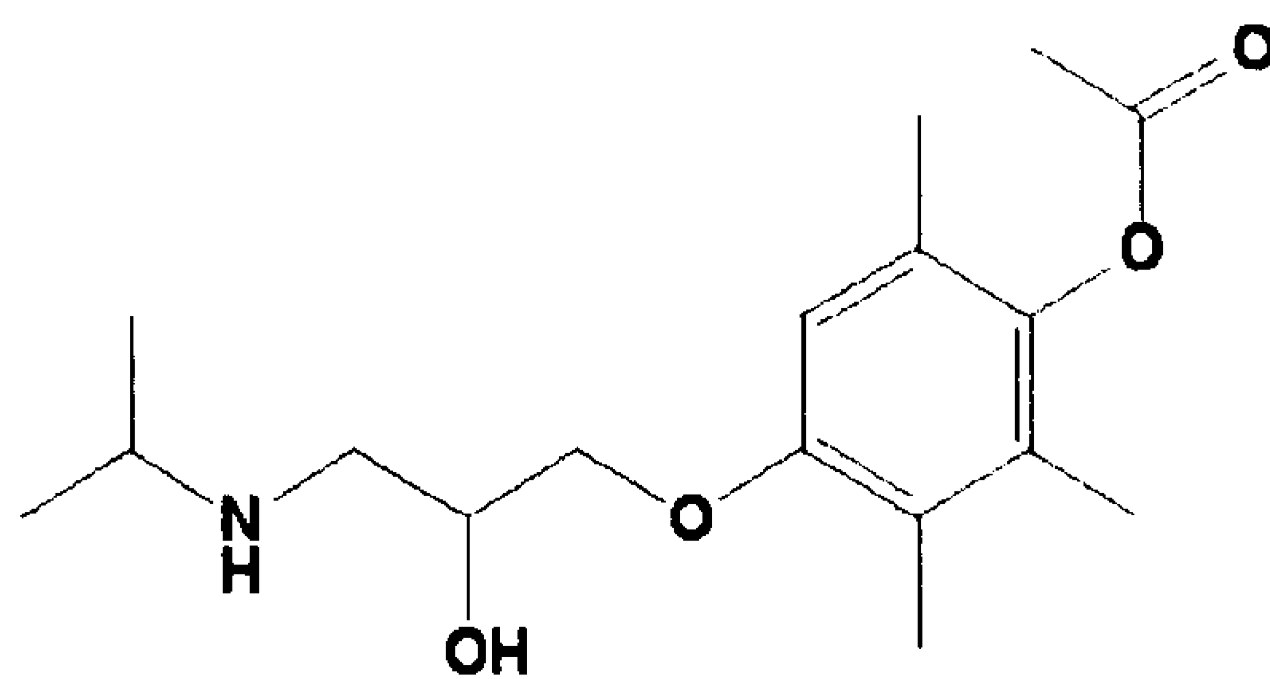
Carteolol:

$C_{16}H_{24}N_2O_3$



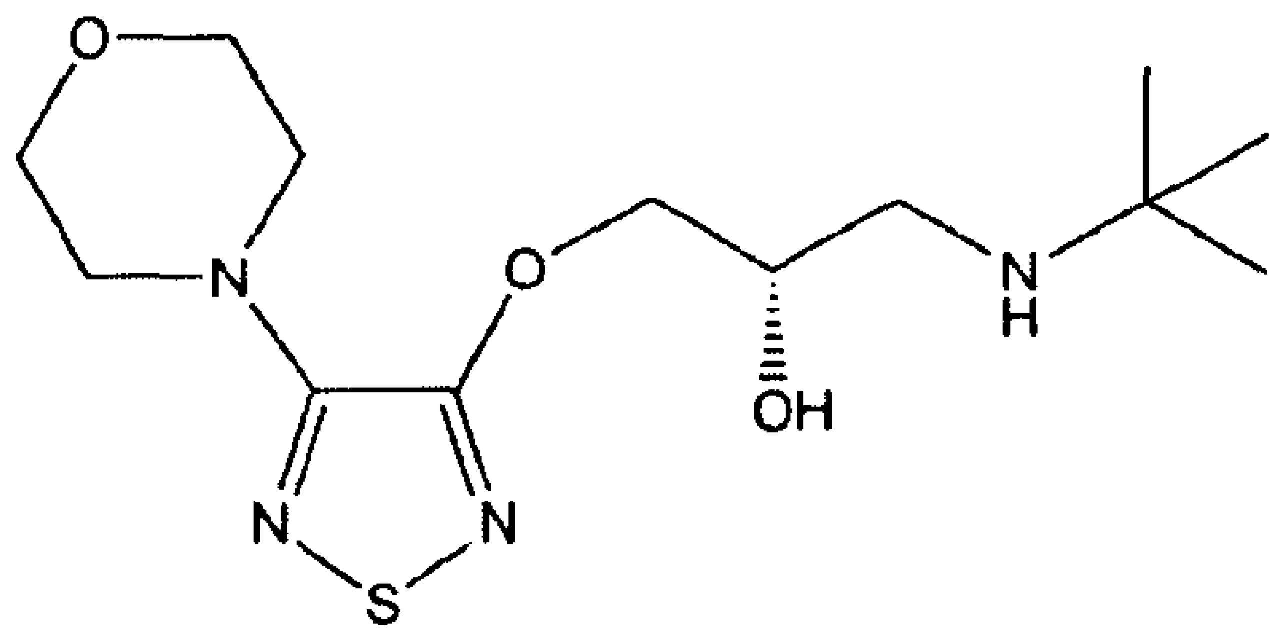
Levobunolol:

$C_{17}H_{25}NO_3$



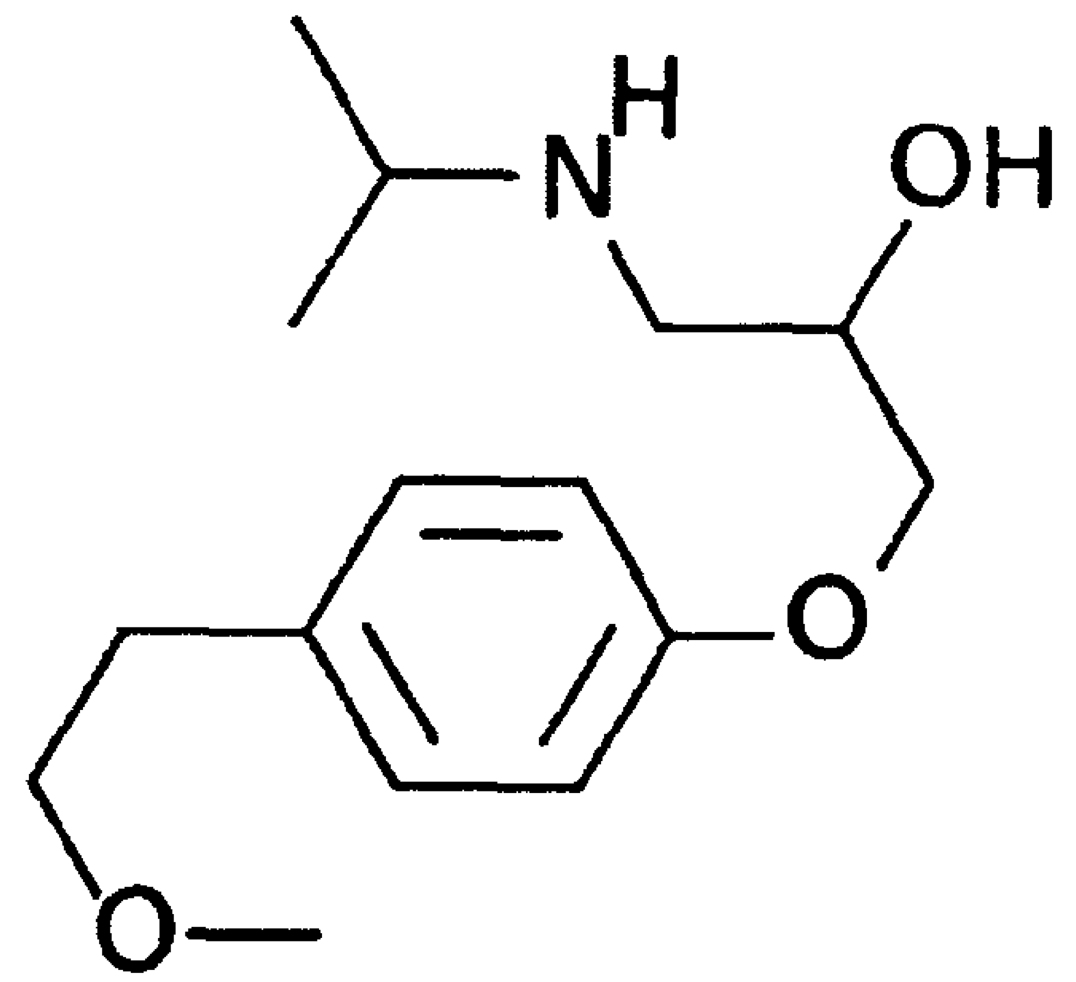
Metipranolol:

$C_{17}H_{27}NO_4$



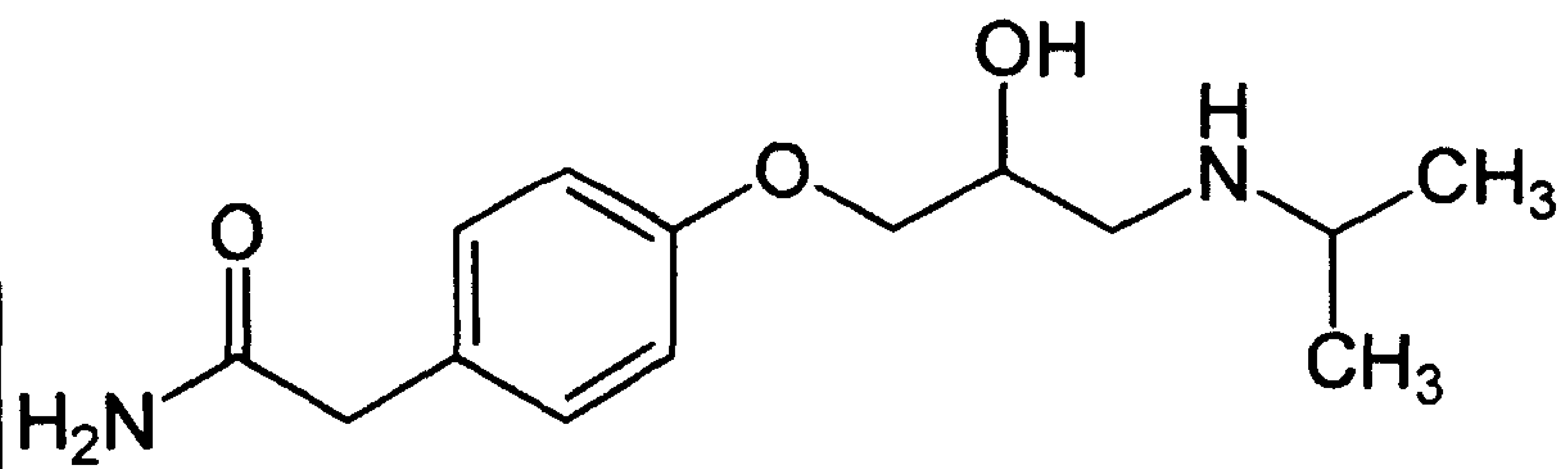
Timolol:

$C_{13}H_{24}N_4O_3S$



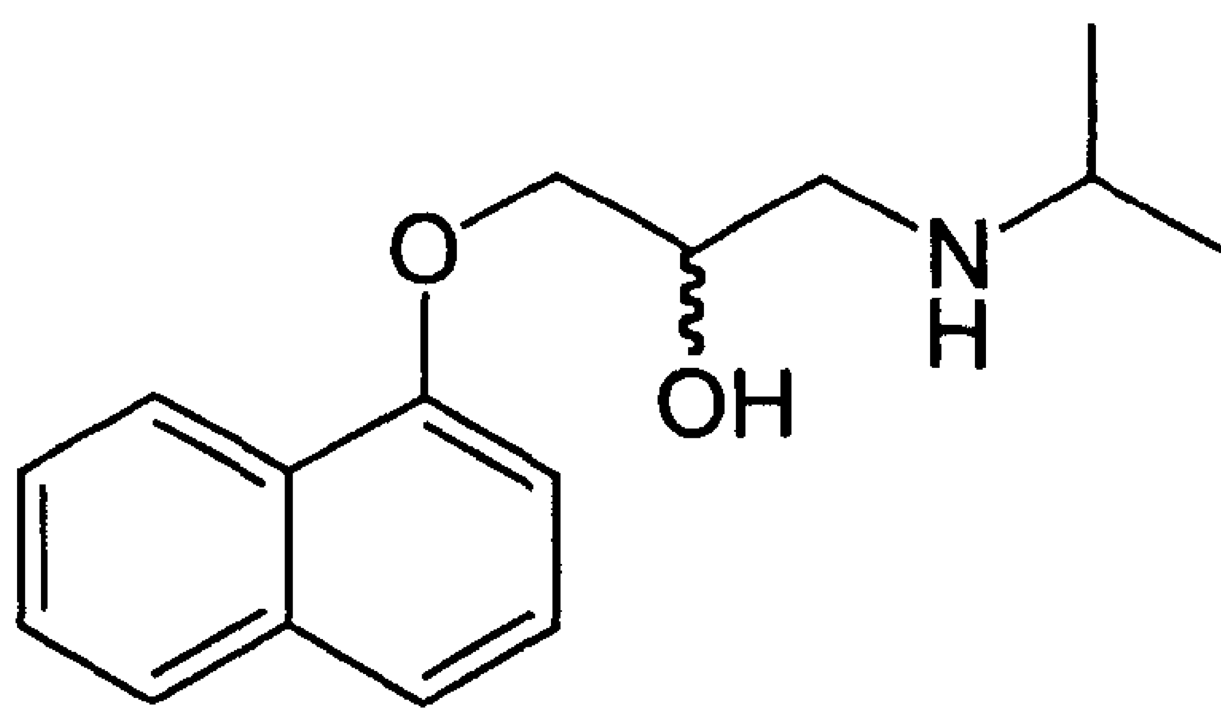
Metoprolol:

$C_{15}H_{25}NO_3$



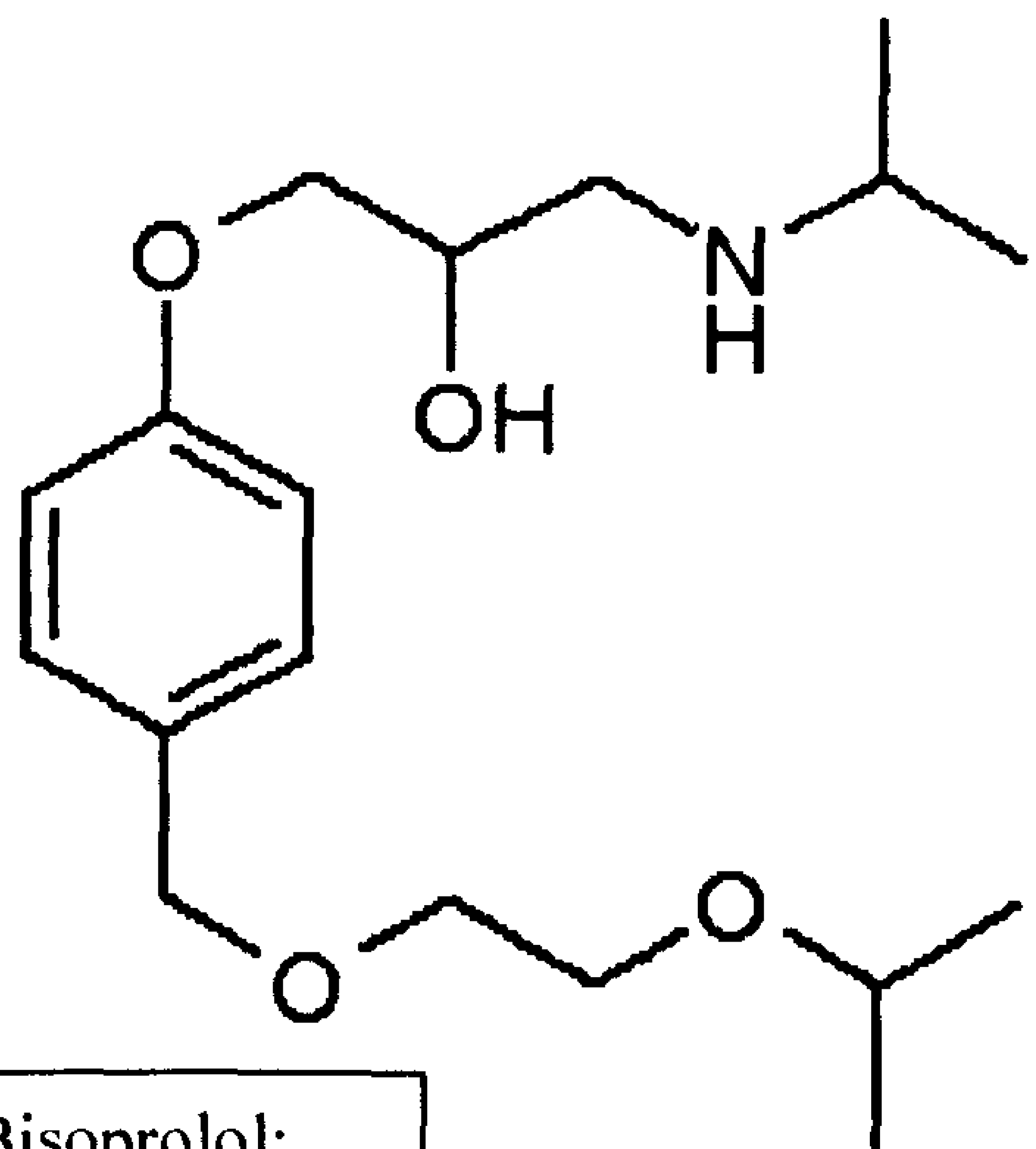
Atenolol:

$C_{14}H_{22}N_2O_3$



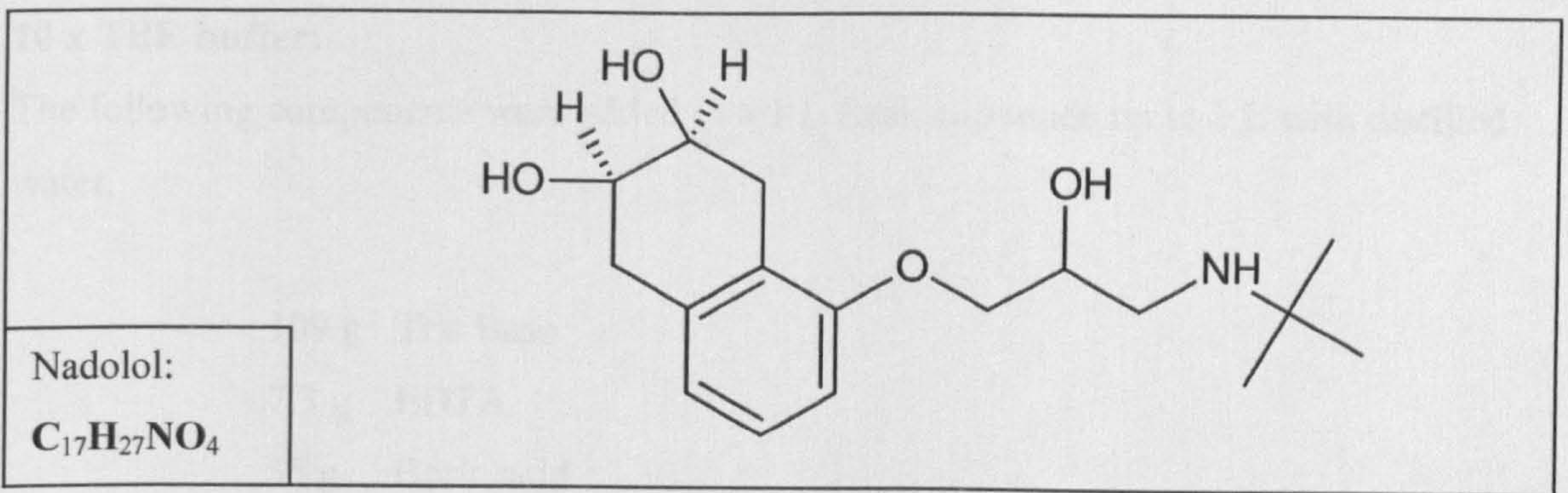
Propranolol:

$C_{16}H_{21}NO_2$



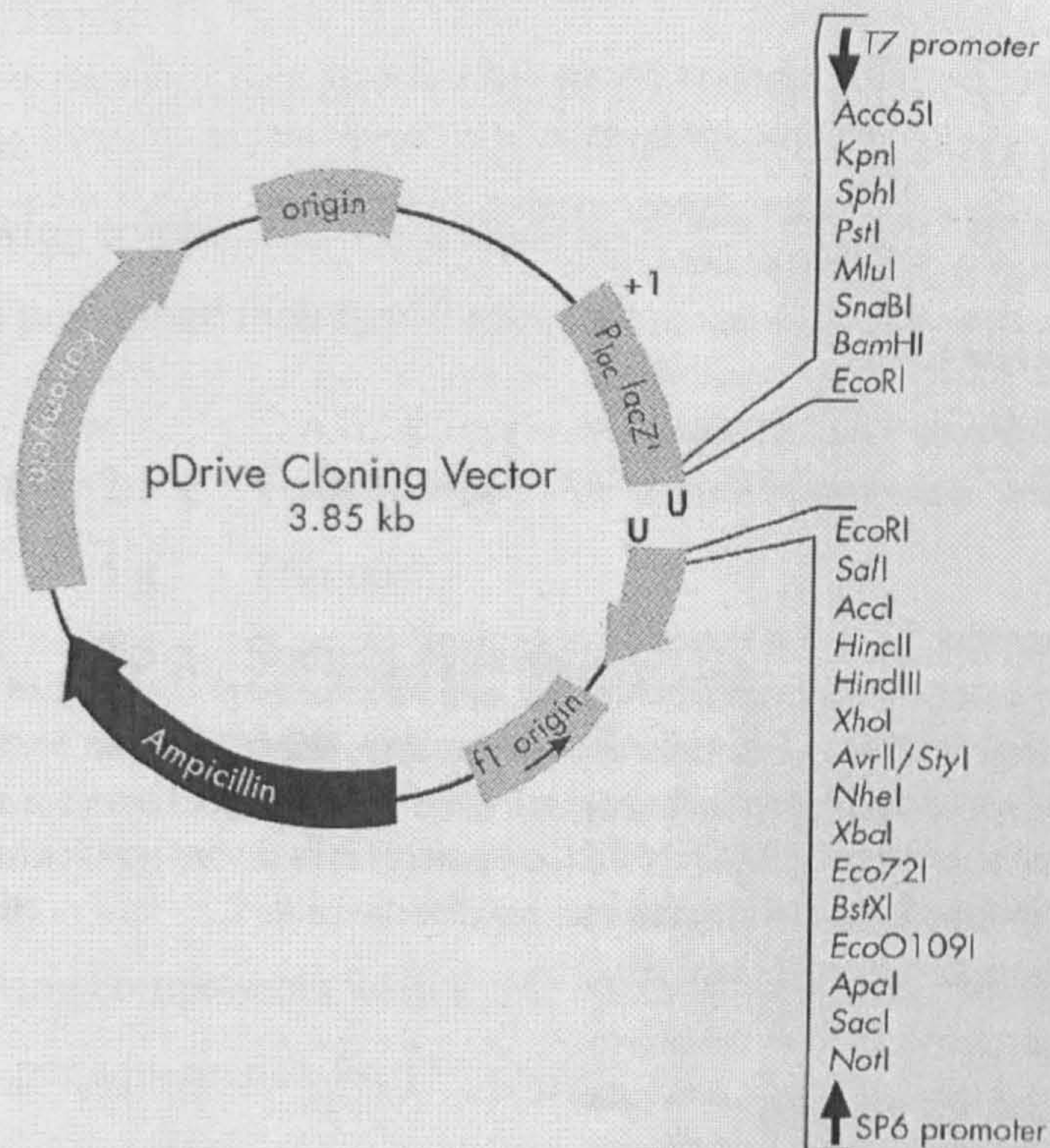
Bisoprolol:

$C_{18}H_{31}NO_4$



The pDrive cloning vector referred to in 2.2.9.

A



B

Element	Position (bp)
Multiple cloning site	266–393
LacZ α-peptide	216–593
T7 RNA polymerase promoter	239–258
T7 transcription start	256
SP6 RNA polymerase promoter	398–417
SP6 transcription start	400
Ampicillin resistance gene	1175–2032
Kanamycin resistance gene	2181–2993
pUC origin	3668
Phage f1 origin	588–1043
Primer binding sites:*	
M13 forward (–20)	431–447
M13 forward (–40)	451–467
M13 reverse	209–224
T7 promoter primer	239–258
SP6 promoter primer	400–418

10 x TBE buffer:

The following components were added to a 1 L flask and made up to 1 L with distilled water.

109 g	Tris Base
7.3 g	EDTA
55 g	Boric acid

1 x TBE buffer:

100ml of 10 x TBE buffer was made up to 1L with distilled water.

L.B. Agar:

The following components were made up to 500 ml with Millipore water and autoclaved at 15 pounds per square inch for 15 minutes.

2.5 g	Yeast Extract
5 g	Triptone
2.5 g	Sodium chloride
7.5 g	Agar

L.B. Broth:

The following components were made up to 500 ml with Millipore water and autoclaved at 15 pounds per square inch for 15 minutes.

2.5 g	Yeast Extract
5 g	Triptone
2.5 g	Sodium chloride

Loading buffer:

The following components were made up to volume with purite water, shaken and aliquoted into 5 ml tubes for storage at –20 °C until needed.

Component	For 100 ml	For 50 ml
20 % Ficoll 400	20 g	10 g
0.1 M sodium EDTA pH 8.0	20 ml	10 ml
1 % S.D.S	1 g	0.5 g
0.25 % Bromophenol Blue	0.25 g	0.125 g
0.25 % Xylene cyanol	0.25 g	0.125 g

Coding translation for degenerative primers:

R = A + G   Y = C = T   M = A + C

K = G + T   S = G + C   W = A + T