Synthetic Glucocorticoids In The Aquatic Environment: Their Potential Impacts On Fish

A thesis submitted to the degree of Doctor of Philosophy

By

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Declaration

The work submitted in this thesis was carried out between 2007and 2011 at Brunel University, Uxbridge, UK. This work was carried out independently and has not been submitted for any other degree.

Abstract

Human pharmaceuticals have been shown to be entering the aquatic environment in quantities sufficient to produce adverse effects to aquatic organisms, particularly fish. The impacts of synthetic oestrogens have been well documented, but other groups of steroidal pharmaceuticals have not yet been studied. Hence, the present research was designed to study synthetic glucocorticoids (GCs), which are used in large amounts as immunosuppressive and anti-inflammatory drugs. This study involved different approaches, including *in silico, in vitro, in vivo* and *genomics*, to assess the effects of GCs on fish.

Using reliable data on consumption of GCs in the UK and the LF2000-WQX hydrological model, mean concentrations of GCs in the river Thames were predicted to be in the range from 30 ng/L to 850 ng/L. Mammalian cell lines were transiently transfected with trout corticosteroid receptors (GR1, GR2 and MR) and the transactivation abilities of ten of the most prescribed GCs in the UK were measured *in vitro*. All tested GCs showed significantly higher activity with GR2 than with GR1. In order to assess the impact of low concentrations of GCs *in vivo*, two chronic exposure experiments were conducted with adult fathead minnows (*Pimephales promelas*). Both experiments showed potency-related and concentration-related impacts on various endpoints. There was a concentration-related increase in plasma glucose concentrations and a decrease in blood lymphocyte count. Induction of secondary sexual characters in females suggests a concentration-related masculinisation of fathead minnows. There was a decreasing trend in plasma vitellogenin concentrations in female fish with increasing exposure concentration of GCs. Expression profiles of selected genes (PEPCK, GR and Vtg) in liver also demonstrated concentration-related effects at all three tested concentrations. Hence, it was not possible to define a no effect concentration for the tested GCs.

This study probably provides reliable estimates of the likely range of concentrations of GCs in a typical river, impacted by effluent from many sewage treatment plants. The i*n vitro* results indicate that all tested GCs bind to fish GR in a similar manner to that reported for mammalian receptors. The *in vivo* results suggest that GCs could cause effects at very low (as low as 100 ng/L) concentrations that could be environmentally-relevant. The immunosuppresive effects could make fish susceptible to disease and the reproductive effects may have population-level impacts. It is very likely that the effects of different GCs will be additive, as has been shown for oestrogenic chemicals. Therefore, this study warrants further environmental risk assessment of GCs, especially in mixture scenarios.

Acknowledgements

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List of Abbreviations

11-KT:	11-ketotestosterone.
11βHSD:	11β hydroxysteroid dehydrogenase.
ACTH:	Adrenocorticotrophic hormone.
ANOVA:	Analysis of Variance.
API:	Active pharmaceutical ingredient.
AR:	Androgen receptor.
BLAST:	Basic Local Alignment Search Tool.
BNF:	British National Formulary.
CAS:	Chemical Abstracts Service.
CBG:	Corticosteroid Binding Globulin.
COS-7:	CV-1(simian) in Origin and carrying the SV-40 genome – 7 cell line.
COX:	Cyclooxygenase.
CRH:	Corticotrophin Releasing Hormone.
DDT:	Dichloro diphenyl trichloroethane.
DHT:	Didydrotestosterone.
DMEM:	Dulbecco's Modified Eagle Medium.
DOC:	11-deoxycorticosterone.
EDC:	Endocrine Disrupting Chemical.
EE2:	17-ethynylestradiol.
ELISA:	Enzyme linked immunosorbent assay.
EMEA:	European Medicines Agency.
ER:	Oestrogen receptor.
GC/MS/MS:	Gas chromatography-tandem mass spectrometry.
GC:	Glucocorticoids.
GH:	Growth hormone.
GR:	Glucocorticoid receptor.
GR-CALUX:	Glucocorticoid receptor-chemical activated luciferase gene expression.
GRE:	Glucocorticosteroid Response Elements.
GREAT-ER:	Geography-Referenced Regional Exposure Assessment for European Rivers.
HPA:	Hypothalamus – Pituitary – Adrenalin axis.
HPI:	Hypothalamus – Pituitary – Interrenal axis
IGF:	Insulin-like Growth Factor.
LC/MS/MS:	Liquid chromatography-tandem mass spectrometry.
LC50:	50% lethal concentration.
LOEC:	Lowest observed effect concentration.
MAO:	Mechanisms of action.
MIH:	Maturation Inducing Hormone.
MR:	Mineralocorticoid receptor.
NHS:	National Health Services of the UK.
NOEC:	No Observed Effect Concentration.

NSAID:	Non-steroidal anti-inflammatory drug.
OECD:	Organisation for Economic Co-operation and Development.
ONGP:	Otho-Nitrophenyl β-Galactopyranoside.
PCA:	Prescriptions Cost Analysis.
PCB:	Polychlorinated biphenyls.
PCR:	Polymerase Chain Reaction.
PEC:	Predicted environmental concentration.
PEPCK:	Phosphoenolpyruvate carboxykinase.
PNEC:	Predicted no effect concentration.
PPAR:	Peroxisomal Proliferator Activated Receptor.
PR:	Progestin receptor.
qRT-PCR:	quantitative Real-Time PCR.
SSC:	Secondary Sexual Characteristics.
SSRI:	Selective Serotonin Reuptake Inhibitor.
STP:	Sewage treatment plant.
TBE:	Tris Borate EDTA buffer.
Vtg:	Vitellogenin.

Chapter 1.0

General Introduction

1.1 Anthropogenic impacts on the environment

The ever increasing human population inevitably increases the production and usage of chemicals that have negative impacts in the environment. Ecotoxicology, the study of the effects of toxic chemicals on biological organisms, especially at the population, community or ecosystem level, has grown rapidly over the past few decades. However, the evidence for impacts of man-made chemicals on wildlife came from the industrial and agricultural revolution era, and the best known examples were bioaccumulation of organochlorine pesticides in birds (thereby causing eggshell thinning that reduced the population of higher trophic level birds) and the development of insecticide resistance in insect pest populations. Unlike man-made catastrophic incidents, these ecotoxicological impacts are silent but long lasting and probably lead to more damage to the environment (Sumpter, 2005).

The chemicals that affect organisms via mimicking endogenous hormones are called endocrine disrupting chemicals (EDCs), and the concept of 'endocrine disruption' was introduced to explain the mode of action of such chemicals (Colborn *et al*., 1993). The International Programme for Chemical Safety (IPCS - which involves WHO, UNEP and ILO) has, together with Japanese, USA, Canadian, OECD and European Union experts, developed a definition for endocrine disrupters that was also adopted as a working definition in the European Community Strategy for Endocrine Disrupters: "*An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations*" (International Programme on Chemical Safety, 2002). These EDCs have many member chemicals, including industrial chemicals such as polychlorinated biphenyls (PCB), phthalates, styrene and bisphenol-A, agrochemicals such as dichloro diphenyl trichloroethane (DDT) and atrazine, surfactants such as alkylphenols, personal care products such as parabens and polycyclic musk, metals and pharmaceuticals. These chemicals in their

original forms or in their transformation forms can have activity in the environment (Ternes, 2001; Sumpter, 2005).

The impacts of EDCs on wildlife had already been reported before the term EDC was introduced. One of the earliest examples was the appearance of a rudimentary penis in female molluscs exposed to tributyltin, the active ingredient of antifouling paints used on ships (Smith 1981). Another example of endocrine disruption was reported in alligators from polluted lakes, which had significantly elevated plasma estradiol concentrations in females and significantly reduced plasma testosterone concentrations in males (Guillette *et al*., 1994).

In recent years, a large number of man-made chemicals has been shown to be able to mimic endogenous hormones, and the abnormal reproductive development in some populations of both humans and wildlife has been explained by endocrine disruption. Exposure to PCBs has been related to the increased incidence of endometriosis in human females and impaired neurobehavioral development in children. Similarly, exposure to PCBs and DDT is thought to be the reason for the increased cases of breast cancer and reduced immunity in humans (Carpenter, 2006). Global reductions in human semen quality over time are related to increasing exposure to oestrogenic and anti-androgenic chemicals (Mocarelli *et al*., 2007). Exposure to organochlorine contaminants has also been related to reproductive malfunction in Baltic seals, eggshell thinning in colonial water birds, reproductive failure in Ontario Lake trout and limb malformations in North American frogs (Bernanke and Kohler, 2009). However, there are critiques against these examples of endocrine disruptions and several other reasons have been proposed for these observations. For example, increased facilities for the detection and diagnosis of human cancer is said to be the reason for the apparent increased incidence of cancer.

The best known, evidence based, example of endocrine disruption came from the UK aquatic environment, where male rainbow trout (*Oncorhynchus mykiss*) caged downstream from sewage treatment plant (STP) outlets were found to be producing the egg yolk precursor protein, vitellogenin (Vtg) (Purdom *et al*., 1994). Further studies have confirmed a widespread feminisation of fish due to exposure to sewage effluent (Jobling *et al.,* 1998). Natural and synthetic oestrogens were identified as the cause of this feminisation (Desbrow *et al*., 1998). Later, some xenoestrogens such as alkylphenols were also reported as oestrogenic (Sumpter and Johnson, 2008). Recently, anti-androgens in surface waters have been identified and they are thought to be enhancing the incidence of intersex in fish (Jobling *et al*., 2009). Masculinization of wild fish has also been observed where pulp mill effluents are discharged into the habitats of such fish (Larsson and Forlin, 2002).

Another prominent example of endocrine disruption came from the Indian subcontinent with diclofenac, a non-steroidal anti-inflammatory drug (NSAID), and the decline of the vulture population. These predatory birds accumulated diclofenac via their food from diclofenac-treated cattle. Diclofenac is a cyclooxygenase (COX) inhibiter which in turn inhibits the synthesis of prostaglandins. Prostaglandins are the mediators for pain and inflammation and the inhibition of the synthesis of prostaglandin causes anti-inflammatory and analgesic effects (Vane, 1971). In the kidney, prostaglandins are involved in maintenance of the equilibrium between vasoconstriction and vasodilatation of the blood vessels that are involved in glomerular filtration. Lack of prostaglandins causes renal damages and renal failure and death of the birds. Thus the residues of diclofenac are the cause of the almost complete loss of the populations of three species of vultures in the Indian sub-continent (Oaks *et al*., 2004).

These two examples are probably the most cited and clearly demonstrated problem of endocrine disruption in the wild. Both of them had a human pharmaceutical as the causative

agent. In parallel, there have been lots of analytical chemistry papers reporting detectable amounts of human pharmaceuticals in many environmental samples (Appendix-1). These findings, and the fact that the targets of almost every human pharmaceuticals (it may be a receptor or enzyme) in the human body can be found in many wild organisms, led scientists to research the impacts of many other human pharmaceuticals on other organisms.

1.2   Pharmaceuticals in the environment

Pharmaceuticals are biologically active compounds, intended to affect the structure or function of the body for the purpose of cure, mitigation, treatment or prevention of a disease via a specific mode of action in the body (Directive 2004/27/EC of the European Parliament and of the Council of 31 March 2004). They are used in human and veterinary medicine, agriculture and aquaculture. Kummerer (2009) estimated that about 900 different active substances account for 90% of the total consumption of pharmaceuticals in Germany, a country that is probably representative of many others (including the UK). It has been estimated that over 3000 pharmaceutical substances are licensed for use in the UK, and major classes of drugs include NSAIDs, antibiotics, lipid regulators, β-blockers, cytotoxic drugs, psychiatric drugs and steroids. The top selling drugs in the UK are probably paracetamol, metformin and ibuprofen (Bound and Voulvoulis, 2004). Pharmaceuticals are usually lipophilic and often have a low biodegradability and have the potential for bioaccumulation and persistence in the environment (Fent *et al*., 2006).

Pharmaceuticals are taken orally, by injection, via an inhaler or applied on skin as cream and patches. They are absorbed into the blood and may be excreted unchanged, as a glucuronide or sulphate conjugate, as a major metabolite or as a complex mixture of many metabolites. Most pharmaceuticals undergo hepatic metabolism, which is a two phase reaction. In phase-I, oxidation, reduction and/or hydrolysis occur and in phase-II, conjugation such as addition of

glucuronic acid, sulphate, acetic acid or amino acid occurs. Through this hepatic metabolism, more soluble, less harmful by-products are released into the environment via excretion (Cunningham, 2009). Some drugs are resistant to hepatic metabolism and can be excreted in their pure, biologically active form (Ternes, 1998). In the case of a topical preparation, a large proportion is washed out directly into the drainage. These, together with the drugs disposed of directly (unused and expired) into the drainage system, reach the STPs.

Many of the drugs are removed in STPs by microbes or by adsorption to suspended solid sludge particles. STP removal rates vary depending on the nature of the pharmaceutical, technology and performance of STPs, temperature and the loading variations due to seasonal changes (Kummerer, 2009). STP removal rates have been reported in a range from about 10% (e.g. carbamazapine) to about 99% (e.g. most NSAIDs; Heberer, 2002). For some drugs, phase-II resulting conjugates are hydrolysed back to the parent drug by the microbes in STPs and the amount of active form is increased (Gros *et al*., 2006). For example, metabolites of the lipid regulator, clofibric acid, have been found to be hydrolysed back to their parent compound, so that their effluent concentrations were found to be higher than the influent concentrations (Daughton and Ternes, 1999). Nevertheless, STPs cannot remove all the pharmaceuticals, so their effluents remain the major source of human pharmaceuticals in the aquatic environment. Other point sources may be drug manufacturing site effluents (Larsson *et al*., 2007) and hospital waste waters (Kummerer, 2001) that contain high levels of human pharmaceuticals that are sufficient to produce adverse effects on biota. Veterinary medicines and the drugs used in agriculture enter the aquatic environment through non-point sources (Boxall *et al*., 2003). Livestock manure, which may contain active pharmaceutical ingredients, is used as agricultural fertiliser and STP sludge is spread as soil conditioner. Therefore there is the potential for pharmaceuticals to enter the aquatic environment via leaching and run-off (Ternes, 1998).

A variety of pharmaceuticals have been detected in many environmental samples worldwide (Jones *et al*., 2002; Kolpin *et al*., 2002; Heberer, 2002; Zuccato *et al*., 2006). They have been reported in STP effluents, surface water, seawater, groundwater, soil, sediment and fish. Geographically, most studies have been carried out in the USA, Germany, Switzerland, Denmark, the Netherlands and France, and for the most part in densely populated areas. However, high concentrations of some pharmaceutical have also been reported from waters around drug manufacturing sites in India (Larsson *et al*., 2007), which suggests that some drugs already have a global environmental dimension. The effluent concentrations can be diluted in surface waters and the concentrations found in the surface waters vary greatly depending on the flow rate, distance from STP and season. They also depend on the amount of hydrolysis and photolysis of the drugs in surface water (Johnson, 2010).

As large proportion of human pharmaceuticals can end up in aquatic environments via STPs, studies on the impacts of pharmaceuticals have focused mainly on aquatic organisms (Halling-Sørenson *et al*., 1998; Matthiessen, 2003; Khetan and Collins, 2007; Palermo *et al*., 2008 and the reviews of Fent *et al*., 2006; Corcoran *et al*., 2010; Burkhardt-Holm, 2010). Even if biologically active chemicals such as pharmaceuticals are present in the aquatic environment, this does not mean that they pose a threat to aquatic organisms. To do so, they must first get into those organisms, and then reach (internal) concentrations high enough to elicit effects. Recently, a study in the United States assessed the accumulation of pharmaceuticals in fish sampled from five effluent-dominated rivers receiving discharge from STPs. Sample analyses showed the presence of norfluoxetine, sertraline, diphenhydramine, diltiazem, carbamazepine, fluoxetine and gemfibrozil detected at high concentrations (Ramirez *et al*., 2009).

Little is known about how readily pharmaceuticals can get into aquatic organisms; only a little information on a few of the pharmaceuticals is available. However, some general 'rules' (McKim *et al.*, 1985) are available that can be used to predict whether or not a chemical will readily cross the gills of a fish. Key characteristics include the hydrophobicity of a chemical, its charge, and its size. In general, the more hydrophobic a chemical (which is usually expressed as log Kow or log P), the more readily it bioconcentrates in aquatic organisms. As far as size is concerned, if the molecular weight is less than 600, the chemical will cross the cell membrane unhindered; above this value, and its passage could be hindered to varying degrees (the larger the molecule is, the less easily it passes through cell membranes) (Zitko and Hutzinger 1976).

Some differences in the metabolism of drugs between humans and fish have been reported. In humans, the main enzymes involved in Phase-I of the hepatic metabolism are cytochrome P450 monoxygenase enzymes (P450 cytochromes). There are several forms, including CYP2C9, CYP3A4, CYP1A2, CYP2C19 and CYP2D6 (Gagne *et al.*, 2006). Fish have cytochrome P4503A but not any of the CYP2C family. Lack of these forms of cytochrome P450s is thought to be a reason for accumulation of drugs in fish (Richardson and Bowron, 1985). Moreover, it has been shown that steroids in water can be readily taken up by fish (Maunder *et al.*, 2007). Therefore plasma concentrations of human pharmaceutical could reach the therapeutic concentrations that occur in humans. A recent study (Fick *et al.*, 2010) has reported that plasma concentrations of levenogestrel in caged fish maintained in a STP effluent can reach the therapeutic levels observed in humans taking that drug.

Pharmaceuticals are designed to act via specific mechanisms of action (MOA) with a specific target in the human body. Examples of such targets are receptors (as in steroidal pharmaceuticals; see chapter 3 for their MOA), enzymes (e.g. NSAID), ion channels (e.g.

cypermethrin) and transporters (e.g. omeprazole). Recent molecular-biological and bioinformatics analyses have revealed that fish are closer to humans among several other aquatic organisms studied, with regard to the similarities of these drug targets (Gunnarsson *et al*., 2008). In other words, fish possess most of these drug targets. Huggett *et al*. (2004) indicated that overall receptor and enzyme identities in fish were 31 – 88% similar to mammalian targets. Therefore it is theoretically possible that fish (and possibly other aquatic organisms) could be affected by the pharmaceuticals present in rivers via the same MOA as occurs in humans.

1.2.1 Non-steroidal pharmaceuticals in the environment

Appendix-1 presents the amounts of different pharmaceuticals reported very recently in the surface waters of different countries. NSAIDs and antibiotics have been frequently reported. It should be noted that the same drug has been detected in different concentrations in different places. This is because drug use policies differ from country to country, the different sensitivity of analytical methods and the differences in the STP technologies (explained in detail in chapter 2). Some earlier reports with measured concentrations reported even higher concentrations of pharmaceuticals present in the surface water, however the advancement of analytical chemistry in recent years has enabled reliable concentrations to be determined, which are presented in appendix-1.

1.2.1.1 Laboratory evidence for impacts of human pharmaceuticals.

There have been several laboratory studies reporting the effects of different human pharmaceuticals known to be present in the environment, most frequently NSAIDs, fibrates, β-blockers, selective serotonin reuptake inhibitors (SSRIs), azoles, and antibiotics at low concentrations (reviewed by Fent *et al*., 2006; Corcoran *et al*., 2010; Burkhardt-Holm, 2010). Several fish species have been reported to be affected and most reported biological effects in

fish in the laboratory correlate with known effects of pharmaceuticals in mammals (Corcoran *et al*., 2010). NSAIDs, which may have similar effects to those described for Indian vultures, have been studied frequently. Brown trout (*Salmo trutta*) were exposed to 0.5 (probably an environmentally-relevant concentration), 5 and 50 µg/L diclofenac for 7, 14 and 21 days to show that exposure of brown trout to diclofenac at environmentally relevant concentrations can result in adverse effects to various organs and may compromise the health of affected fish populations (Hoeger *et al*., 2005). A recent study demonstrates the uptake and sub-lethal tissue damage in trout exposed to environmental concentrations of diclofenac and highlights further concern about this pharmaceutical in the aquatic environment (Mehinto *et al*., 2010). Other NSAIDs such as indomethacine (Lister and Van der Kraak, 2008), and ibuprofen (Flippin *et al*., 2007) have also been reported to have adverse effects on fish at low concentrations

Another large group of pharmaceuticals that has been tested on fish is SSRIs, which are used in large amounts as antidepressants. Serotonin is a neurotransmitter that appears to have a major role in depression. SSRIs inhibit the reuptake of serotonin by the nerve cells, so that serotonin stays in the synaptic gap longer than it normally would, and hence may repeatedly stimulate the receptors of the recipient cell. Serotonin receptors have been reported in several fish (Yamaguchi and Brenner, 1997) and some behavioural and reproductive impacts involving SSRI have been reported (Winberg *et al*., 1997). Western mosquito fish exposed to fluoxetine, at environmentally-relevant concentrations, did show some behavioural changes (Henry and Black, 2008). Exposing medaka to fluoxitine caused an increase of plasma oestrogen (E2) concentrations and growth impairement (Brooks *et al*., 2003).

Statin drugs, which are used to control blood cholesterol levels and as preventitive drugs of cardiac diseases, also have been tested with fish. These drugs act via the peroxisomal

proliferator activated receptor (PPARα) and induce the expression of genes which code for the enzymes of lipid metabolism. These enzymes, together with peroxisomes, remove the fatty acids and cholesterol from blood (Kota *et al*., 2005). There is evidence for the presence of PPAR in fish (Ruyter *et al*., 1997). Exposure of goldfish to environmental levels of a lipid regulatory drug, gemfibrozil, leads to bioconcentration of the drug in plasma and the potential for endocrine disruption (reduction of plasma testosterone over 50%) in fish (Mimeault *et al*., 2005). Runnalls *et al*. (2007), however, did not find any effect on lipid metabolism after exposing fathead minnows to 0.01 to 1 mg clofibric acid/L, instead they found reproductive impacts in male fish.

β-blockers are used to treat hypertension, angina, glaucoma and similar conditions and are known to act via β-adrenergic receptors (β-AR) to diminish the effect of epinephrine. These receptors have been found in fish heart, gills, liver and brain (Nickerson *et al*., 2001). Fathead minnows exposed to the β-blocker propranolol at 1 mg/L concentrations showed decreased egg counts (Giltrow *et al*., 2009). Growth impairment in larval fathead minnows exposed to another β-blocker, atenolol, has also been reported, although only at very high concentration of 10 mg/L (Winter *et al*., 2008). Similar effects of propanalol have been found in medaka and rainbow trout (Hugget *et al*., 2002; Owen *et al*., 2007).

Although fish are the most studied organisms, invertebrates and microbial populations have also been reported to be affected by the pharmaceuticals known to be present in the aquatic environment. Effects of three pharmaceuticals, fluoxetine, ibuprofen and carbamazepine, were examined on the activity of the benthic invertebrate *Gammarus pulex,* and the LOEC was reported to be as low as 10 ng/L for fluoxetine and ibuprofen. Carbamazepine showed a similar response, however, the differences were not significant (De Lange *et al*., 2006). Carbamazepine inhibited growth of Daphnia at 12.7 mg/L and midges at 9.2 mg/L in an acute

toxicity test and had sublethal effects at 92 µg/L in a chronic exposure experiment (Thacker, 2005). Invertebrate LC50 values for metoprolol and propranolol range from 64 to >100 mg/L and 0.8 to 29.8 mg/L, respectively, showing that propranolol is most harmful among the β-blockers studied so far (Hugget *et al*., 2002; Cleuvers, 2005). The lipid regulator gemfibrozil (Mimeault *et al*., 2005), abamectin (Tisler and Kozuh Erzen, 2006) and fluoxetine (Nentwig, 2007) are some other human drugs reported to have acute and chronic toxicity to aquatic invertebrates. For the phytoplankton, the SSRI fluoxetine is the most acutely toxic human pharmaceutical reported by far, with an EC50 of 24 µg/L (48 h, alga; Brooks *et al*., 2003). Exposure of marine microalgae to the fungicide clotrimazole resulted in a decrease in primary productivity which may in turn have adverse effects on community structure (Porsbring *et al*., 2009). In a recent study with a mixture of 13 pharmaceuticals commonly found in the aquatic environment, it has been shown that algae are sensitive to these drugs, which caused damage to their chloroplast (Vannini *et al*., 2011).

Antibiotics are used in high quantities by human and veterinary medicine and considerable amounts are released into the aquatic environments. The major concern with this group of pharmaceuticals is the development of resistance among microbial populations. Resistance has already been identified in aquatic biota (Larsson *et al*., 2007). However, there is still a lack of understanding and knowledge about sources, presence and significance of resistance of bacteria against antibiotics in the aquatic environment (Kummerer, 2009) and whether or not their resistance has any relevance to human health.

1.2.1.2   Evidence from field studies

In a recent study of two STP effluent-impacted streams in the USA, samples of water, bed sediment, and brain tissue of white suckers *Catostomus commersoni* were collected and tested for the presence of antidepressants. Downstream water was found to contain fluoxetine,

norfluoxetine, sertraline, norsertraline, paroxetine, citalopram, fluvoxamine, duloxetine, venlafaxine, and bupropion. Venlafaxine, bupropion, and citalopram were present at the highest measured concentrations found. Venlafaxine and fluoxetine in bed sediment were the predominant pharmaceuticals observed. Fluoxetine, sertraline and their degradates were the principal antidepressants observed in fish brain tissue (Schultz et *al*., 2010). The sex ratio of fish upstream from a wastewater treatment plant was 47% female to 53% male, while the ratio downstream from the plant was 83% female to 17% male. Researchers conclude that this disturbance could be associated with endocrine-disrupting compounds, including a synthetic oestrogen, found in the treatment plant effluent (Woodling et *al*., 2006).

1.2.1.3 Potential Human Health Impacts.

Risks from pharmaceuticals present in the environment to humans has been a vigorous debate (Dorne et *al*., 2007; Sanderson et *al*., 2007; Jones et al., 2005) and some authors (Sherer, 2006; Cunningham, 2009) take the view that the amounts detected in the environment are far below those likely to produce any adverse impact on humans. Rahman et *al*. (2009) reviewed the data on the pharmaceuticals in drinking water and concluded that the human health risk is minimal. However, there is limited information available about the potential long-term health effects of consuming low concentrations of pharmaceuticals, such as could occur through drinking water containing pharmaceuticals.

One study found some cause for concern about the exposure of vulnerable sub-populations, such as pregnant women and their foetuses, to drinking water containing very small amounts of chemotherapy drugs (Johnson et *al*., 2008). Another study looked at the effect of environmentally relevant levels of a mixture of 13 drugs on human cell function. Human embryonic cells were exposed to a mixture of atenolol, bezafibrate, carbamazepine, cyclophosphamide, ciprofloxacin, furosemide, hydrochlorothiazide, ibuprofen, lincomycin,

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ofloxacin, ranitidine, salbutamol, and sulfamethoxazole. The drug mixture inhibited the growth of human embryonic cells, with the highest effect observed as a 30% decrease in cell proliferation compared to controls. Results suggest that a mixture of drugs at ng/L levels can inhibit cell proliferation by affecting their physiology and morphology (Pomati *et al.*, 2006).

1.2.2   Steroid pharmaceuticals in the environment.

Steroid pharmaceuticals include oestrogens (such as the natural oestrogens, estrone and 17ß-estradiol, and the synthetic oestrogen, EE2), androgens (such as testosterone), progestins (such as levenogestrel and norethindrone) and corticosteroids (glucocorticoids and mineralocorticoinds). Steroids can also be hormone antagonists. For example, the drugs flutamide and dutasteride are both androgen antagonists. Steroid pharmaceuticals tend to be highly effective *in vivo* as they are designed specifically to elicit an acute response. One of the key lessons (Sumpter and Johnson 2005) to come out of all the environmental research on EE2 is that very biologically active oestrogens can be present in the environment, and they have the capability to cause effects at extremely low concentrations. This seems to be due to a combination of factors, which can be applied not only to oestrogens but to any steroidal pharmaceuticals, in particular 1) that EE2 is readily taken up by fish from the water (Scott *et al.,* 2005; Maunder *et al.,* 2007), 2) that it bioconcentrates to a reasonable degree in fish (Lange *et al.,* 2001), 3) that oestrogen receptors to which it binds very avidly exist in fish, just as they do in human patients taking the drug as a contraceptive, and 4) that these receptors play key roles in regulating reproduction. In a study of rainbow trout exposed to sewage effluent from a small Swedish treatment plant, it was found that the concentration of oestrogens in their bile was between one-hundred thousand and one million times higher than the surrounding water and increased with time. This showed a very efficient bioconcentration of oestrogen (Larsson *et al.*, 1999).

1.2.2.1 Oestrogens/anti-estrogens

Thousands of research papers have been dedicated to the varied aspects – both chemical and biological – of the oestrogenic chemicals, especially EE2 (Caldwell *et al*., 2008), now known to be present in the aquatic environment. There are many different oestrogenic chemicals present in the aquatic environment, ranging from 'real' oestrogens such as estradiol to oestrogen mimics (so-called xenoestrogens), such as the industrial chemicals nonylphenol and bisphenol-A. The 'real' oestrogens comprise both natural steroid oestrogens, such as estrone and estradiol, and synthetic oestrogens. The latter group is dominated by EE2, one of the active ingredients of the contraceptive 'Pill'. EE2 is an extremely potent oestrogen; concentrations less than 1 ng/litre have adverse effects on reproduction of fish, and only slightly higher concentrations prevent fish reproducing (e.g. Lange *et a.l,* 2001; Nash *et a.l,* 2004; Parrott and Blunt 2005), leading to population crashes (Kidd *et al.,* 2007).

Both natural and synthetic oestrogens are widely used. Oestrogens are prescribed alone or in combination with progestogens in hormone replacement therapy and as a treatment for oestrogen deficiency symptoms in postmenopausal women. Aromatase inhibitors (which inhibit the synthesis of oestrogens) are used principally in the treatment of breast and ovarian cancer (BNF 2006). Oestrogen is excreted from the human body in the form of conjugates with water-soluble groups such as glucuronides. Steroids in effluent, however, are mainly unconjugated (Routledge *et al*., 1998; Larsson *et al*., 1999), i.e. in the biologically active and fat soluble form, which facilitates their uptake by organisms. Deconjugation probably takes place with the help of bacteria (e.g. E.coli), which are abundant in sewage treatment plants. EE2 is more resistant to degradation during sewage treatment and in the environment than are the natural oestrogens. In Germany, EE2 has been found in drinking water in concentrations high enough to affect fish. Natural oestrogen often occurs at levels from a few ng/L to several

tens of ng/L in sewage effluent, while levels of EE2 are often between 0.5 and 3 ng/L, although levels several times higher have been measured (Heberer, 2002).

A 7-year, whole lake experiment at the Experimental Lakes Area in northwestern Ontario, Canada showed that chronic exposure of fathead minnow to low concentrations (5–6 ng/L) of the potent synthetic oestrogen, 17-ethynylestradiol (EE2) led to feminization of males, including impacts on gonadal development as evidenced by intersex in males and altered oogenesis in females, and, ultimately, a near extinction of this species from the lake. These observations demonstrate that the oestrogens and their mimics observed in freshwaters can impact the sustainability of wild fish populations (Kidd *et al*., 2007), depending of course on their environmental concentrations.

The consequences of intersexuality on reproductive performance in fish have been studied recently (Harris *et al*., 2011). In this study, mildly feminised fish were able to reproduce as well as unaffected fish. However, the intersex condition in severely feminised males reduced their reproductive performance by up to 76%. It should be emphasised that it is important in the future to investigate any effects over several generations and in the field at the level of the population, including the significance of genetic variations.

1.2.2.2 Androgens/anti-androgens

As with oestrogens/anti-oestrogens, this group includes agonists, receptor antagonists, and compounds that inhibit the synthesis of androgens. Androgen agonists are prescribed mainly as replacement therapy in males with a deficiency or absence of endogenous testosterone associated with hypogonadism. Much of the testosterone prescribed is in the form of esters. The aim of esterification is to improve the lipophilicity of the molecule, which slows the release of testosterone from the site of entry into the body. Two different sub-classes of anti-androgenic pharmaceuticals are available: androgen receptor antagonists (e.g. Bicalutamide)

and inhibitors of the enzyme 5-alpha reductase, which converts testosterone into dihydrotestosterone (e.g. Finasteride). The use of large amounts of anti-androgenic pharmaceuticals reflects the high incidence of prostatic diseases amongst the male population; in fact, prostate cancer is the most common cancer of men in the UK (Westlake and Cooper, 2008), and it is dependent on endogenous androgen for development, growth and survival (Culig and Bartsch 2006). All anti-androgenic pharmaceuticals on the market are synthetic.

Androgens have been identified in water from municipal treatment plants. Studies show that the amounts involved can vary considerably, and concentrations up to several tens of ng/L of testosterone and androstenedione, for example, have been reported (Kolodziej *et al*., 2003). The presence of androgens in municipal effluent has also been shown using *in vitro* tests with yeast cells containing the human androgen receptor (Svenson and Allard, 2004). In this way, androgens have been detected in concentrations of up to 100 ng/L of dihydrotestosterone equivalents. The majority of this is thought to have a natural origin, which is excretion by people, since men's urine contains significant amounts of androgens.

Farm animal also excrete substantial amounts of hormones. In the vicinity of stockbreeders and stud farms, therefore, one can detect hormonal disruptions in fish which has been attributed to natural oestrogens and androgens, although it is also suspected that synthetic hormone preparations may be the cause. In an American report, clear masculinisation was detected in fathead minnow in the vicinity of large breeders of beef cattle (Orlando *et al*., 2004). In some countries the use of hormone supplements is a common practise to stimulate growth and increase the transformation of feed into muscle mass. However, researchers have not yet been able to decide whether natural hormones or synthetic hormone preparations are the cause of masculinisation in fish.

Experimental studies of zebrafish show that exposure to methyltestosterone (Orn *et al*., 2003) and to the synthetic anabolic steroid trenbolone results in lower concentrations of Vtg and a higher proportion of males. Flutamide inhibits androgen-receptor binding (Ankley *et al*., 2004), while dutasteride is a α-reductase inhibitor, interfering with the production of the potent androgen dihydrotestosterone from testosterone. There are several ways of measuring the effects of androgens. Androgens affect primary (the sex organs) as well as secondary sex characteristics in many vertebrates, including fish. In guppies (*Lebistes reticulata*) and related fish species, a number of androgens may cause the anal fin to develop into something which resembles the male's breeding organ or gonopodium. For over 20 years female mosquito fish (*Gambusia sp*.) close to pulp and paper manufacturers in the south of the USA have been known to develop a gonopodium-like structure (Howell *et al*., 1980). We are still not certain what the cause is, but there is evidence which suggests that the wastewater contains wood-derived steroids which masculinise fish. It can be difficult to establish whether sexual development is affected in organisms in the wild. In the vicinity of a large Swedish pulp mill, however, a skewed sex ratio in favour of males has been observable for several years in eelpout embryos – an effect which can be linked to the degree of exposure to effluent (Larsson and Förlin, 2002). There are many indications that this skewed effect is caused by androgens, since effluent induces a number of male sex characteristics in female fish in laboratory experiments. Examples of this are colouring in guppies and the production of spiggin, an androgen-regulated protein, in the three-spined stickleback (*Gasterosteus aculeatus*). A recent study also identified and partly characterized a number of candidate androgens in untreated effluent from the Swedish pulp mill, including progesterone (Larsson *et al*., 2006).

1.2.2.3 Progestogens

Progestogens are most commonly and widely used for contraception, either alone or in combination with oestrogens. They are also used for treatment of a number of other conditions, including hormone replacement therapy, menstrual problems, endometriosis, anorexia, cancer, and assisted reproduction. They are administered via a variety of routes, including pills, patches, injection, implant, gels, creams, and suppositories. PR antagonists, such as mifepristone, are used as abortifacients during the first 2 months of pregnancy. It is also used in 'morning-after treatment', to prevent possible pregnancy.

In a study with caged fish (rainbow trout) exposed for 14 days to undiluted, treated sewage effluents from three sites in Sweden, 25 pharmaceuticals were evaluated. The progestin pharmaceutical levonorgestrel was detected in fish blood plasma at concentrations exceeding the human therapeutic plasma level, with the measured effluent level higher than water levels shown in laboratory experiments to reduce the fertility in fish (Zeilinger et al., 2009). In total, 16 pharmaceuticals were detected in fish plasma at concentrations higher than 1/1000 of the human therapeutic plasma concentration. This study shows that rainbow trout exposed to sewage effluents have blood plasma levels of pharmaceuticals similar to human therapeutic concentrations, suggesting a risk for pharmacological effects in the fish (Fick *et al*., 2010). The recent demonstration that synthetic progestogens can adversely affect fish (mainly egg production) at very low concentrations (Zeilinger *et al*., 2009; Paulos *et al*., 2010) has confirmed the need to closely evaluate the environmental effects of steroidal pharmaceuticals.

1.2.2.4 Corticosteroids

Corticosteroids are either glucocorticosteroids (GCs) or mineralocorticosteroids, with molecular weights ranging from 360 to 500. The generalised molecular structure of GCs and the properties related to the different carbon substitutions are given in Figure 1.1. In most of

the synthetic corticosteroids, H in the 9th carbon is substituted by F. In some cases it is substituted by Cl. This is to increase the stability of these compounds in the human body, so that frequent administration is avoided. Therefore it is anticipated that the corticosteroids with F or Cl substitution could resist degradation in STP and hence be found in measurable concentrations in environmental water. A detail review of the possible environmental concentrations of GCs is presented in Chapter 2.

In fish, the interrenal cells of the anterior kidney secrete corticosteroid hormones, including cortisol and corticosterone, which modulate functions such as glucose metabolism, mineral balance, and behaviour (explained in detail in section 1.4). Fish are lacking aldosterone and cortisol has been shown to have both gluco and mineralocorticoid activity (Sturm *et al*., 2005). Details of their mechanism of action and the differences in the potency of different GCs are presented in Chapter 3. Section 1.3 explains the physiology of GCs in human and their known side effects. As fish are known to have similar drug targets (Chapter 3), similar effects could be predicted in fish (explained in Chapter 4).
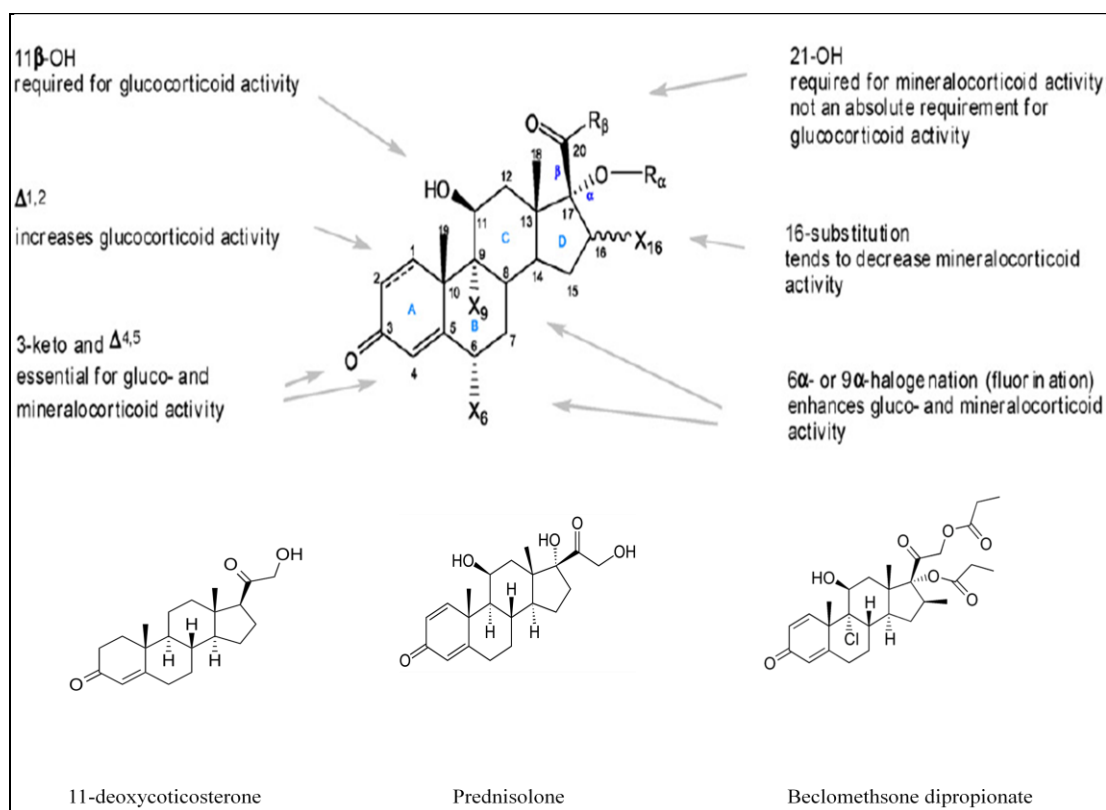
Figure 1-1. Molecular structure of corticosteroids. A generalised structure and the properties related to the different carbon substitutions (Buchwald, 2008) are shown.

1.2.2.5   Environmental Concentrations of steroid pharmaceuticals

A representative summary of current knowledge about the environmental concentrations of natural and synthetic steroid hormones and their antagonists is provided in Table 1.1. Effluent concentrations are reported much more frequently than surface water (river) concentrations. In both cases, variability in concentrations would be expected. In the case of effluents, the efficiency of the STP will be a major factor in determining effluent concentrations of "down-the-drain" micropollutants (Johnson *et al*., 2007). In the case of river water concentrations, the degree of dilution of the STP effluent in the river, which can be highly variable, will play a major role in determining concentrations.

Table 1-1. Representative concentrations of natural and synthetic steroid hormones and their antagonists in the aquatic environment

Steroids	Mode of Action	Name	Effluent (ng/L)	Surface water (ng/L)	Reference
OESTROGENS	Agonist	Ethinyl Estradiol	0.8-2.8		Johnson *et al*. (2005)
		Ethinyl Estradiol		4.6 max	Williams *et al*. (2003)
		Estradiol	<0.4-4.3	ND	Williams *et al*. (2003)
		Estrone	<0.4-12.2	0.32-2.12	Williams *et al*. (2003)
		Conjugated Oestrogens	0.07-2.6	Not measured	Tyler *et al*. (2009)
	Antagonists	Tamoxifen	146	27-212	Roberts and Thomas (2006)
	Aromatase Inhibitor		No data	No data	
ANDROGENS	Agonist	Testosterone	0.3 - 8	Not measured	Kolodziej *et al*. (2003)
		Testosterone	Not measured	2.8 - 6.0	Vulliet *et al*. (2009)
	Antagonists		No data	No data	
	5αReductase Inhibitor		No data	No data	
PROGESTOGENS	Agonists	Norethindrone	5.2-41	Not measured	Vulliet *et al*. (2007)
		Levonorgestrel	0.9-17.9	Not measured	Vulliet *et al*. (2007)
	Antagonists	Mifepristone	No data	No data	
GCs	Agonists	Triamcinolone	14±1	Not measured	Schriks *et al*. (2010)
			Not measured	<0.63	Tölgyesi *et al*. (2010)
		Cortisone	0.13-0.58	0.06-4.2	Chang *et al*. (2007)
		Prednisolone	0.47-0.72	0.03-0.64	Chang *et al*. (2007)
MINERALO-CORTICOIDS	Agonists		No data	No data	
	Antagonists		No data	No data	

Environmental concentrations of steroid hormone antagonists are currently very poorly documented. In most cases (e.g. antagonists of androgens, progestogens and mineralocorticoids) no data are available, either for effluents or rivers. A very limited amount of data is available for the oestrogen receptor antagonist Tamoxifen (Roberts and Thomas 2006), which suggests that the environmental concentration is in the low hundreds of ng/L range. In summary, aquatic wildlife are probably exposed, albeit continuously, to very low ng/L, or even sub-ng/L, concentrations of both natural and synthetic steroid hormones and their antagonists: these are the "environmentally-relevant" concentrations that need to be assessed for potential effects.

1.2.2.6 Effect Concentrations for steroid pharmaceuticals

Summarized in Table 1.2 are current knowledge on the effects of natural and synthetic steroid hormones and their antagonists on fish. When data on other groups of organisms are available, they seem to be considerably less sensitive to these pharmaceuticals than are fish (Caldwell *et al*., 2008). Effect concentrations of the different steroids and their antagonists appear to be reasonably variable, although all are relatively low (in the μg or ng/L ranges). Fish are extremely sensitive to EE2, with effects occurring in the very low ng/L range. For example, a number of comprehensive, thorough studies have shown that concentrations as low as a few ng/L prevent fish reproducing (Lange *et al*., 2001; Kidd *et al.,* 2007) , and even lower concentrations can produce biochemical changes. Similarly, recent data on synthetic progestogens (Zeilinger *et al*., 2009) show that at least some representatives of this class of pharmaceutical will inhibit egg laying of fish at extremely low concentrations, possibly less than 1 ng/L. Androgens may be somewhat less potent (effect concentrations in the low μg/L range), but not enough data are available to judge their potency with any degree of certainty. There are no published effects data for either synthetic GCs or mineralocorticoids at environmentally-relevant concentrations.

A reasonable amount of information is available on the effects of both oestrogen and androgen antagonists on fish. Both types of oestrogen antagonists (receptor blockers and aromatase inhibitors) have been studied in well executed studies (e.g. Williams *et al*., 2007; Sun *et al.,* 2007), and in both cases it has been found that µg/L concentrations are required to produce measurable responses in fish, with the LOEC being in the low µg/L, but not ng/L, range. The AR receptor blocker flutamide has also been reasonably well studied, and appears to be relatively inactive in fish, with the LOEC being in the high µg/L range (Table 1.2). In contrast, the AR antagonist Cyproterone Acetate appears quite potent, and has anti-androgenic effects in the low ng/L range.

In summary, plenty of high quality data show that µg/L concentrations will always cause major effects, ng/L concentrations often will (especially those of the synthetic steroids), and even sub-ng/L concentrations of some steroids can cause major adverse effects (for example, lack of reproduction). Recent evidence strongly supports the fact that amongst the pharmaceuticals in use today, steroid hormones merit particular attention from ecotoxicologists. Both Johnson *et al*. (2008a) and Besse and Garric (2009) have identified progestogens as requiring risk assessment for the aquatic environment. Their concerns are vindicated, because two very recent, independent studies have now demonstrated that some synthetic progestogens can inhibit fish reproduction at very low (ng/L, or even sub-ng/L) environmental concentrations (Paulos *et al*., 2010; Zeilinger *et al*., 2009). Even the very well researched "oestrogens in the aquatic environment" issue continues to produce surprises; for example, Tyler *et al*. (2009) showing that equine oestrogens used in hormone replacement therapy are present in the aquatic environment, and very potent in fish. Therefore the present study on GCs in the environment tries to fill some gaps in the knowledge of steroidal pharmaceuticals in the environment.

Table 1-2. Representative effect concentrations of natural and synthetic steroid hormones and their antagonists. All reliable data concerns effects on freshwater fish. LOEC = Lowest Observed Effect Concentration

Steroids	MOA	Name	Effects	LOEC (µg/L)	Species	Reference
OESTROGENS	Agonist	Ethinyl Estradiol	Secondary sexual characteristics / Altered sex ratio / feminisation / VTG	0.004	*Pimephales promelas*	Länge *et al*. (2001)
		Estradiol	Reduction in reproductive output	0.781	*Pimephales promelas*	Thorpe *et al*. (2003)
		Estrone	VTG / Sex ratio / Reproduction / secondary sexual characteristics	0.1 0.005-0.025 (VTG)	*Danio rerio*	Brion *et al*. (2004)
	Antagonist	Tamoxifen	Inhibition of Reproductive output/ VTG / Gonadal histology	5.6	*Pimephales promelas*	Williams *et al*. (2007)
	Aromatase inhibitor	Fadrozole	GSI / VTG	24.8	*Pimephales promelas*	Panter *et al* (2004)
		Letrozole	Fecundity / fertility / VTG	5	*Oryzias latipes*	Sun *et al*. 2007
ANDROGENS	Agonist	Testosterone	No data			
		5α-Dihydrotestosterone	Masculinisation of females/ Vtg induction in females	6.0	*Pimephales promelas*	Panter *et al*. (2004)
	AR Antagonist	Cyproterone	Plasma T and 11-KT reduction in males	0.1	*Fundulus heteroclitus*	Sharpe *et al*. (2004)
			Plasma T reduction in females	0.01		
		Flutamide	Spiggin inhibition	500	*Gasterosteus aculeatus*	Sebire *et al*. (2008)
			Nest building behaviour / Male courtship behaviour	100		
			Testis histopathology/ Ovary histopathology	62.7	*Pimephales promelas*	Jensen *et al*. (2004)
			Plasma E2 increase in males / Vtg induction in males and females / T increase in females / fecundity / hatching	651		
	5αReductase Inhibitor		No data			
	5αReductase Inhibitor		No Data			
PROGESTOGENS	Agonist	Norethindrone	Inhibition of reproduction/ masculinisation of females / steroid levels	25 1-10	*Oryzias latipes* *Pimephales promelas*	Paulos *et al*. (2010)
		Levonorgestrel	Inhibition of reproduction/ masculinisation of females	0.0008	*Pimephales promelas*	Zeilinger *et al*. (2009)
GLUCO-CORTICOID	Agonists		No Data			
MINERALO-CORTICOID	Agonists		No Data			
	Antagonist		No Data			

1.3   Corticosteroids in humans.

Corticosteroids are 21-carbon steroidal hormones synthesised from cholesterol (Figure 1.2). They have a wide spectrum of effects in almost every organ and in all stages of life. The HPA axis controls the synthesis and secretion of GCs from the adrenal cortex and GCs in turn regulate their own release through a negative feedback system. Environmental and internal stimuli induce the secretion of CRH from the hypothalamus. The portal system carries the CRH to the anterior lobe of the pituitary, where it stimulates the secretion of adrenocorticotrophic hormone (ACTH). ACTH in turn stimulates the synthesis of GCs in the adrenal glands. Endogenous GCs such as cortisol (primates, guinea pigs, sheep) and corticosterone (rodents) play important roles in animal physiology. GCs have effects on carbohydrate, lipid and protein metabolism; on body and fat mass and energy expenditure; on gastrointestinal, renal, pulmonary and cerebral function.

Synthetic GCs are used mainly for their anti-inflammatroy and immuno-supressive actions, in treating adrenocortical insufficiency, hypersensitivity, asthma, rheumatic disease, inflammatory bowl disease, inflammatory skin disorders like eczema, contact dermatitis, inflammatory eye and ear conditions, immunosuppression in lymphomas and leukaemia, allergic rhinitis, psoriasis, Cushing's disease and adrenal replacement therapy. These pharmaceuticlas are available in the form of tablets, capsules, inhalers, topical creams, ointments, eye/ear drops and injections. GCs can be administered as oral, nasal, topical, intramuscular injections, suppositories and ear/eye drops. The average daily dose of corticosteroids varies from 100 μg to500 mg, depending on the preparation and the route of administrations (BNF, 2006). Synthetic GCs share the same biochemical backbone as endogenous cortisol, but have modifications at some carbons in addition to other structural changes (added methyl/ hydroxyl groups). Fluorinated synthetics such as betamethasone and dexamethasone are structurally similar to natural GCs but fluorination at C9 is believed to

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prevent oxidative metabolism. Synthetic GCs have been designed to have higher glucocorticoid potency, reduced mineralocorticoid effects and a longer duration of action (Figure 1.1). The clinical potencies of the different synthetic GCs depend on the rate of absorption, the concentration in the target tissues, the affinity for the glucocorticoid receptor (GR), and the rate of metabolism and subsequent clearance. The plasma half-life ranges between 80 (cortisol) and 270 (dexamethasone) minutes (Sapolsky *et al.*, 2000).
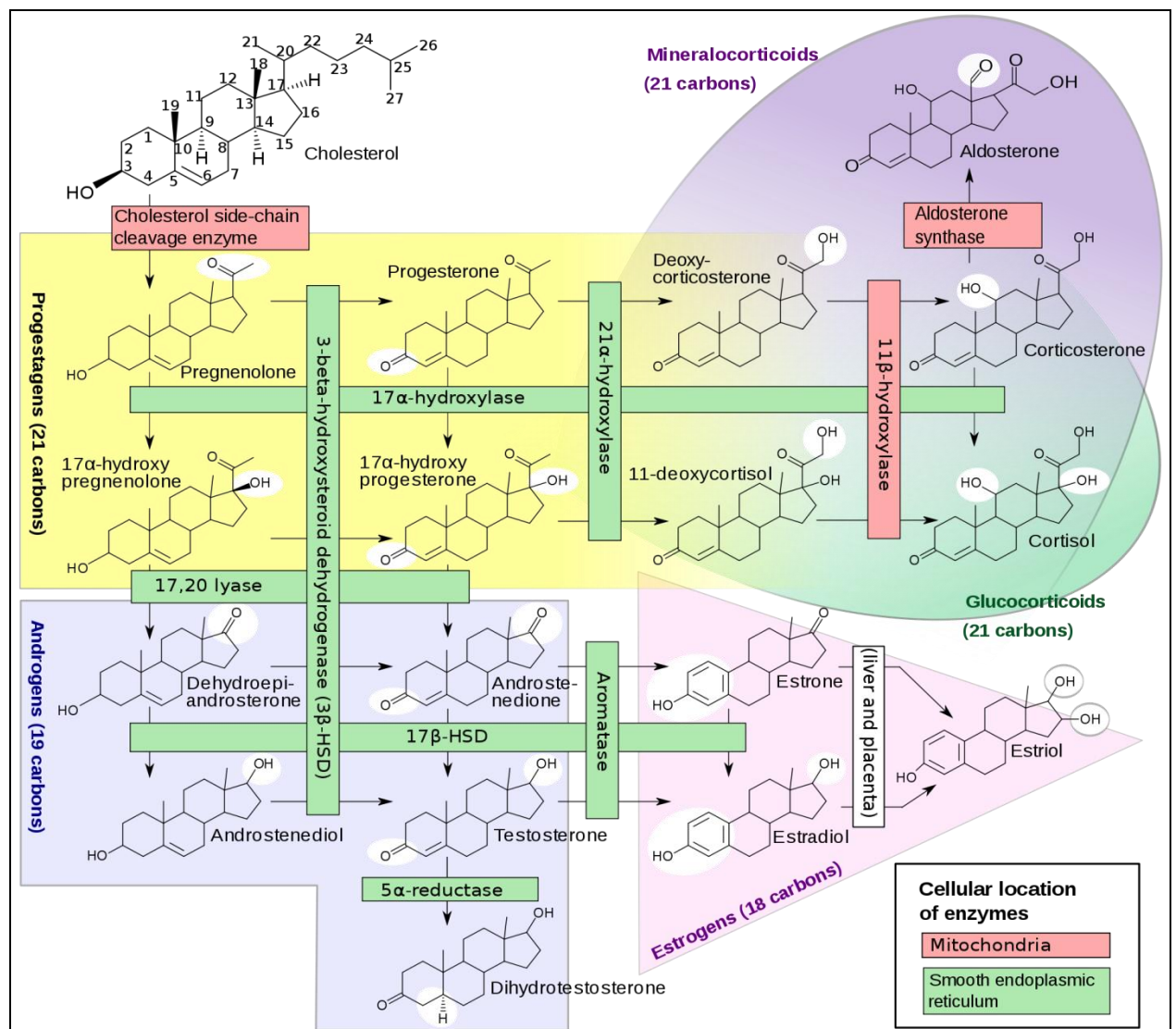
Figure 1-2. Pathways and enzymes involved in steroidogenesis in human. Key parts of the different groups of steroids are highlighted.

(Source:http://en.wikipedia.org/wiki/File:Steroidogenesis.svg)**.**

Pharmacological effects of GCs are mediated via the GRs. The MOA is described in detail in Chapter 3. The resulting biological effects can be anti-inflammatory/immunosuppressive, metabolic, and toxic. The anti-inflammatory and immunosuppressive effects of GCs include changes in the circulation/ migration of leukocytes and alterations in specific cellular functions (Sapolsky *et al.*, 2000). The MR was found to be expressed in Leydig and Sertoli cells, as well as in spermatozoa, and hence aldosterone is thought to be regulating spermatic fluid osmolarity and to play a role in spermatozoa motility. In addition, it appears that it can also stimulate testosterone production within the Leydig cells (Ge *et al.*, 2005).

The metabolism of GCs mainly occurs in the liver and a large proportion of GCs is eliminated via urine. Most cortisol is reduced to dihydrocortisol and then to tetrahydrocortisol by the enzyme 11β hydroxysteroid dehydrogenase (11βHSD), which is then conjugated to glucuronic acid. These derivatives are freely soluble and are rapidly excreted with urine. In mammals, at least 2 isoforms of this enzyme exist. The type 1 and type 2 11βHSD isoforms share only 14% homology and have distinctly different physiological roles and tissue distributions. 11βHSD -1 catalyzes the conversion of inactive 11-keto metabolites (cortisone/11-dehydrocorticosterone) into biologically active 11-hydroxylated corticosteroids (cortisol/ corticosterone). It is localized primarily to the liver but it is also present in the brain, pituitary, adrenal, lung, ovary and adipose tissue. 11βHSD-1 has also been identified in human decidua and fetal membranes, where it may act locally to increase bioactive GC concentrations to facilitate the process of parturition. 11βHSD-2 acts as a dehydrogenase, and it converts biologically active GCs into inactive metabolites (reviewed by Odermatt and Nashev, 2010). 11βHSD2 is present at high levels in the placenta, protecting the foetus from maternally derived GCs, but is also found in many other organs, such as the brain, pancreas and kidney. Many synthetic GCs are poor substrates for 11βHSD and are not oxidized by

these enzymes. Therefore these synthetic GCs can gain direct access to the GR without any significant reduction in their circulating or tissue levels (Siebe *et al.*, 1993).

In humans, a typical undesired side-effect of GCs is drug-induced Cushing's syndrome. Other important undesired effects are hyperglycemia and osteoporosis (Table 1.3). Typical mineralocorticoid side-effects are hypertension, hypokalemia, without causing peripheral edema, metabolic alkalosis and connective tissue weakness. Suppression of hypothalamic pituitary secretion by hyper secretion of cortisol is commonly reported and this phenomenon has negative impacts on growth (due to the lack of GH) and reproduction (due to the lack of gonodotropic hormones).

Table 1-3. Typical side effects of GCs in human (Schacke *et al.*, 2002).

Organ	Undesired side effect
Skin	Atrophy, striae rubrae distensae, Delayed wound healing Steroid acne, perioral dermatitis, Erythema, teleangiectasia, petechia, hypertrichosis
Skeleton and muscle	Muscle atrophy/myopathy, Osteoporosis, Bone necrosis
Eye	Glaucoma, Cataract
CNS	Disturbances in mood, behavior, memory, and cognition 'Steroid psychoses' steroid dependence, Cerebral atrophy
Electrolytes, metabolism, endocrine system	Cushing's syndrome, Diabetes mellitus, Adrenal atrophy Growth retardation, Hypogonadism, delayed puberty Increased Na + retention and K+ excretion
Cardiovascular system	Hypertension, Dyslipidemia, Thrombosis, Vasculitis
Immune system	Increased risk of infection (e.g., Candida) Re-activation of latent viruses (e.g., CMV)
Gastrointestinal	Peptic ulcer, Gastrointestinal bleeding, Pancreatitis

GCs inhibit testosterone production within the Leydig cells via a pathway mediated by the GR. In addition, GCs may induce spermatogonia and spermatocyte apoptosis and decrease sperm yield (Ge *et al.*, 2005). Stress-induced polycystic ovarian disease is thought to be due to the hyper secretion of cortisol and HPG suppression. Several animal studies have shown that GC excess during pregnancy, either from maternal stress or through exogenous

administration to the mother or fetus, reduces birth weight and causes lifelong hypertension, hyperglycaemia and behavioural abnormalities in the offspring. These effects are transmitted across generations without further exposure to GCs, which suggests an epigenetic mechanism (Drake *et al*., 2007). A series of studies in sheep have focused on the perinatal and life-long consequences of GC exposure in pregnancy. These studies in the sheep model have shown that maternal injections with GCs, in a manner similar to clinical treatment for women at risk of preterm birth, enhance foetal lung maturation, but were also associated with developmental and other functional alterations that are of concern. With weekly doses to the mother, there is restricted foetal growth, delayed myelination of the central nervous system, altered blood pressure soon after birth and increased insulin response to glucose challenge in early adulthood. The findings in experimental animals are supported by studies of children in the Western Australian preterm infant follow-up study, which indicated that increasing the number of GC exposures, for the purpose of enhancing lung maturation prior to preterm birth, is associated with reduced birth weight and behavioural disorders at 3 years of age (Newnham, 2001).

1.4   Corticosteroids in fish.

Cortisol is the principal glucocorticoiod in fish, and one of the most commonly measured indicators of environmental stress in fish is the plasma concentration of cortisol (reviewed in Mommsen *et al*., 1999). Aldosterone has not been reported in fishes and cortisol is thought to have both GC and mineralocorticoid actions. However, characterisation of the corticoid receptor in fish revealed that one MR and two GR forms exist in many species (details in Chapter 3) and 11-deoxycortisol acts as the major mineralocorticoid (Sturm *et al*., 2005). In the stressed situation, elevated cortisol is important for activation of the central nervous system, increasing blood glucose concentration and elevating blood pressure, by which fish can cope with stress. Cortisol is also thought to reduce the stress-induced

inflammatory/immune reaction in order to avoid tissue damage due to the inflammation. Apart from the stress response, cortisol plays important roles in the metabolism of carbohydrates, protein and lipid. It also plays a significant role in osmoregulation, growth and reproduction.

An adrenal gland is not found in fish and instead cortisol synthesis occurs in interrenal cells, which are found in the head-kidney region. Cortisol production by fish has been reported from very early life stages, for example, 36 h after fertilization in the common carp (Stouthart *et al*., 1998). The Hypothalamus-Pituitary-Interrenal axis (HPI) coordinates the synthesis and release of cortisol, in a similar way to how the HPA axis does in mammals (reviewed by Mommsen *et al*., 1999). Adrenocorticotrophic hormone (ACTH) released from the anterior pituitary gland induces the secretion of cortisol. ACTH secretion is also controlled by the negative feedback mechanism of cortisol itself. In addition, several internal and external factors control ACTH secretion. For example, 11-ketotestosterone (11-KT) suppresses ACTH-induced cortisol production in rainbow trout *in vitro* (Young *et al*., 1996) and *in vivo* (Pottinger *et al*., 1996).

Generally, cortisol is hyperglycaemic via hepatic gluconeogenesis (Wendelaar Bonga, 1997; Mommsen *et al*., 1999). Genomic studies revealed up-regulation of genes involved in many aspects of hepatic energy metabolism, including glucose and protein metabolism, in response to stressor-induced elevation of plasma cortisol concentrations (reviewed by Prunet *et al*., 2008). For example, an increase in liver PEPCK mRNA levels was observed both *in vivo* as well as *in vitro* in trout hepatocytes stimulated with cortisol. This cortisol mediated PEPCK gene expression was inhibited in the presence of RU486, a GR antagonist (Sathiyaa and Vijayan, 2003; Vijayan *et al*., 2003; Aluru and Vijayan, 2007; a detailed review is presented in Chapter 5). In trout, cortisol elevated some of the genes involved in protein catabolism

(Barton and Iwama, 1991). These studies support a direct role for cortisol in the regulation of liver metabolism in fish.

There have been several studies to elucidate the impacts of stress in fish, with the use of cortisol or a few of the many synthetic GCs (e.g. dexamethasone, triamcinolone), but the concentrations used were very much higher than the expected concentrations of GCs present in the aquatic environment (reviewed by Mommsen *et al*., 1999). GCs have been applied to fish via different routes such as single or repeated injection at differing sites (intramuscular, subcutaneous), oral application, various oil deposits, implantation of silicon tubes containing GCs or osmotic mini-pumps. Other methods involved in increasing endogenous cortisol, such as overcrowding, handling stress, temperature or exposure to air, have also been tested. Although the experimental design is likely to strongly influence the outcome of a particular experiment and there have been some species specific results, some of the common impacts on metabolism have been frequently reported. A clear differentiation has been reported between short-term, acute effects and long-term treatment with cortisol. Under acute stress situations, there is a plasma cortisol surge within a minute to an hour, followed by a gradual decrease to basal levels within a day or two (Pankhurst, 2011).

Long-term exposure to exogenous cortisol with slow releasing implants usually results in chronically elevated plasma cortisol concentrations. Impacts on carbohydrates, protein turnover, amino acid dynamics and lipids have been consistently identified. Cortisol treatment significantly increases the activities of all key gluconeogenic enzymes, namely glucose 6-phosphatase, fructose 1,6-bisphosphatase and PEPCK. Increased activities of these gluconeogenic enzymes support an increased liver capacity for gluconeogensis in cortisol-treated fish (Vijayan *et al*., 2003). Additional support comes from experiments on isolated liver systems. Significantly enhanced rates of gluconeogenesis from lactate were noted in

isolated carp liver cells maintained in culture (Janssens and Waterman, 1988) and incubated with cortisol *in vitro*, and in hepatocytes isolated from Gulf toadfish (*Opsanus beta*) previously injected with dexamethasone. A strong peripheral and hepatic lipolytic action of cortisol has been reported, resulting in increases in plasma fatty acid concentrations. European eels showed increased peripheral lipolysis in the presence of elevated cortisol (Sheridan, 1986). Cortisol induces proteolytic action, especially on fish white muscle and possibly also in the liver. Andersen *et al*. (1991) found increased concentrations of free amino acids in plasma of cortisol-implanted fish, suggesting peripheral proteolysis in response to cortisol. In many cases both *in vivo* and *in vitro* observations have been confirmed with the use of cortisol bloking agents (e.g. metyrapone, RU486) where the observed impacts of cortisol were reversed (Mommsen *et al*., 1992).

Cortisol-treated fish showed more tolerance to sea water and this has been explained by the fact that cortisol adapts fish to salinity by cellular differentiation of chloride cells and by stimulating branchial Na+/K+-ATPase activity (reviewed by McCormick, 1996). Up-regulation of GR in seawater-acclimated fish gills has been reported (Weisbart *et al*., 1987), which means seawater tolerance is a receptor-mediated action. Synergy between cortisol and other osmoregulatory hormones such as growth hormone (GH) and insulin-like growth factor-1 (IGF-1) has also been reported in brown trout (Madsen, 1990).

GCs are known to suppress reproductive functions as an adaptive response to divert metabolic building blocks away from biosynthetic pathways (reviewed by Milla *et al*., 2009). GCs cause follicular atresia, advance or delay oocyte maturation and ovulation or affect egg size, fertilization success, spawning behaviour and progeny quality (Clearwater and Pankhurst, 1997). Cortisol has been found to suppress plasma Vtg levels (Carragher *et al*., 1989; more

details are given in Chapter 5), and it brings about a 30% decrease in liver estradiol-binding capacity (Pottinger and Pickering, 1990).

The reported effects of corticosteroids on the production of GnRH and gonadotropins are also consistent with interference of the HPG axis by GCs. In brown trout, the levels of both pituitary and plasma gonadotropins were reduced by cortisol treatment (Carragher *et al*., 1989). In female fish, androgens are involved in the regulation of the final stages of GnRH/gonadotropin secretion (Nagahama *et al*., 1994). The negative effects of cortisol or stress on plasma androgen suppression (Carragher *et al*., 1989; Campbell *et al*., 1994) also support the idea that corticosteroids indirectly disrupt the HPG axis.

Conversely, sex-steroids regulate corticosteroid production, further supporting their reciprocal interaction during the reproductive cycle. Estradiol suppressed cortisol production in interrenal cells *in vitro* (McQuillan *et al*., 2003), which confirms the reciprocal antagonism between both steroids, even if that result was not observed in immature rainbow trout (Barry *et al*., 1997; McQuillan *et al*., 2003). By contrast, *in vivo*, oestrogens were reported to promote cortisol production in immature trout (Pottinger *et al*., 1996). Low plasma cortisol concentrations were observed during spermatogenesis in male fish (Pickering and Christie, 1981). This could be a physiological adaptation to protect the testes against the adverse effects of cortisol (Pottinger *et al*., 1995). Indeed, this steroid has numerous deleterious effects on male reproduction. When facing stressful situations or cortisol treatment, a delay in testicular development was observed, marked by smaller gonads, retardation in spermatogenesis and lower sperm quality (Campbell *et al*., 1992).

After stressor application or cortisol administration during spermatogenesis, the plasma androgen levels were lower in treated fish than in controls (Pickering *et al*., 1987; Carragher *et al*., 1989; Pottinger, 1999). These observations suggest that the effect of cortisol on

spermatogenesis retardation is partly caused by inhibition of androgen production (Consten *et al*., 2002; Pickering *et al*., 1987). During the spermiation period, cortisol has also negative effects on some reproductive parameters, such as testis growth (Carragher *et al*., 1989). In rainbow trout, a direct negative effect of cortisol on the maturation-inducing hormone was reported (Milla *et al*., 2008). Overall, it has been clearly demonstrated that GCs can have negative impacts on fish reproduction. However, none of the studies used environmentally-relevant concentrations of any GC (which might be very low compared to the reported stress-related plasma cortisol levels) and exposure via water. We do not know if the GCs present in the aquatic environment can reach concentrations high enough to cause any adverse effects.

As explained in stress-related studies with fish, suppression of the HPI axis is possible with exposure to synthetic GCs, as GCs could mimic the higher concentrations of endogenous cortisol. However, presently there are no data about the possible bioaccumulation of GCs into fish, potency differences among the GCs on fish, and the possible effects of environmentally relevant concentrations of GCs on fish. Therefore, the present study was designed in order to obtain some of these data before conducting any actual exposure studies aimed at assessing the impacts of the synthetic GCs present in the environment.

1.5   Objectives

My research was carried out to test the following null hypothesis:

"The low concentrations of synthetic glucocorticoid drugs present in the aquatic environment do not have any adverse impacts on fish"

Experiments were carried out with the following objectives:

- To predict environmental concentrations of GCs (Chapter 2).

- To estimate the potency of different GCs *in vitro* with the fish corticoid receptors (Chapter 3).

- To assess the impacts of low concentrations of synthetic GCs *in vivo* (Chapter 4).

- Study the expression profile of selected genes in the liver of fathead minnows exposed to synthetic GCs (Chapter 5).

- To assess the risk of GCs in the aquatic environment (Chapter 6).
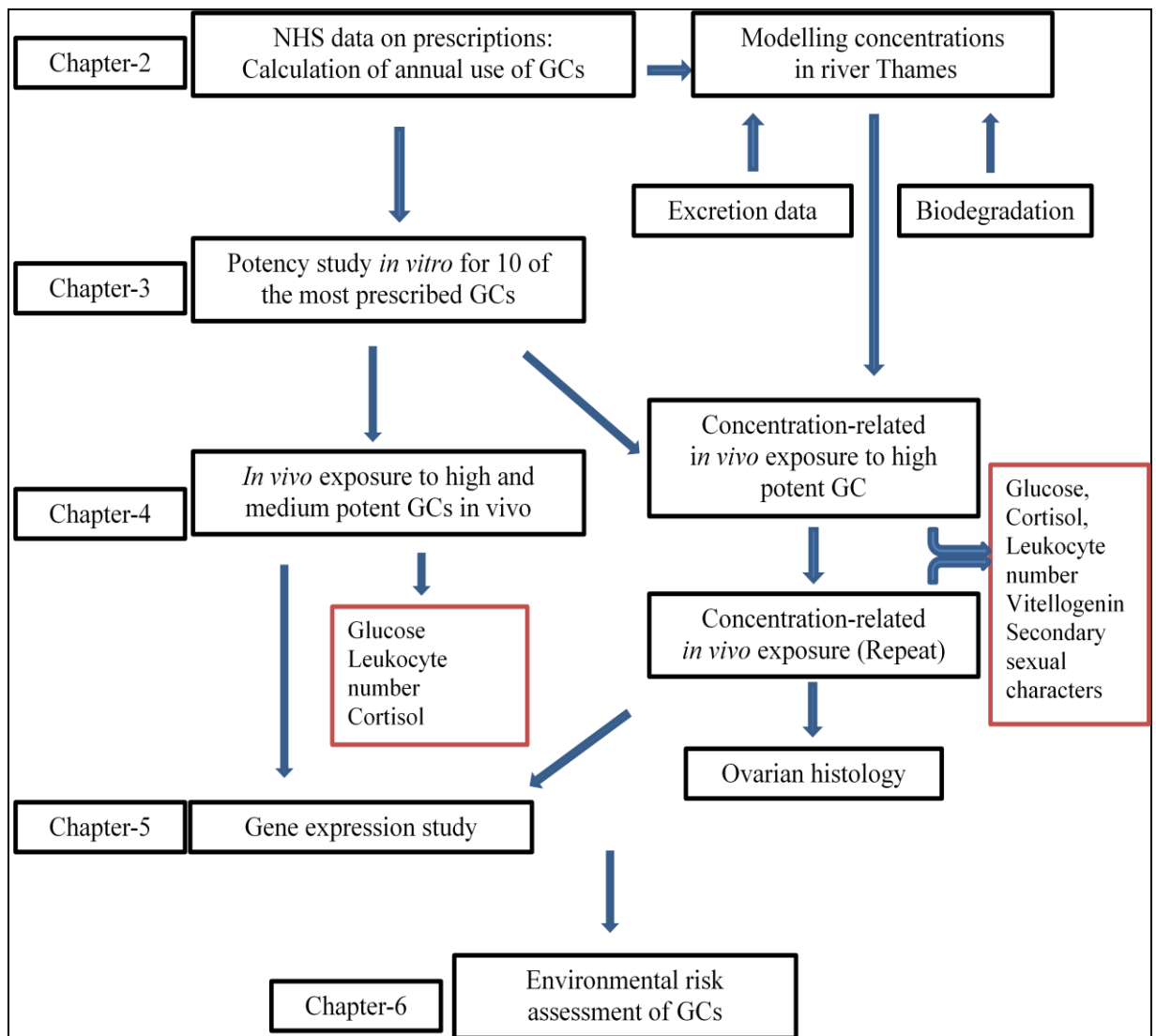
Figure 1.3. Schematic outline of my research.

Chapter 2.0

Prediction Of The Environmental Concentrations Of Synthetic Glucocorticoids

2.1 Introduction

Pharmaceuticals and personal care products are extensively and increasingly used and hence are released continuously into the environment. The fate of pharmaceutical products that are used for human and farm animals varies, depending on the route of administration, their dosage form and the physicochemical properties of the active pharmaceutical ingredient (API). Drugs that are not absorbed (for example, various dosage forms designed to be applied to the skin to give a local effect) remain in the actual pharmaceutical product. In these forms (e.g. ointments, powders, and dermal patches), the majority of the drug is often not absorbed. It is either washed off the skin or remains inside the patch. In the case of oral administration, a drug passes through the gastro-intestinal tract and leaves the body via urine and faeces, in unchanged form or after it has been metabolised to more water-soluble forms (conjugates or breakdown products). Hence, human drugs, once used, or expired and disposed into sewage, nearly always end up in the sewage system. It is generally accepted that synthetic steroids are relatively poorly removed in sewage treatment plants (STP) (Johnson and Sumpter, 2001), and some experimental evidence also supports this fact (Kanda and Churchley, 2008; Ternes *et al*., 1999). Another potential source of drugs in the receiving waters is leakage from landfill sites and agricultural land on which sludge from STP has been spread.

Therefore it is not surprising that a variety of pharmaceuticals have been detected in many environmental matrices worldwide. They have been reported to be present in sewage treatment plant effluents, surface water, seawater, groundwater, soil, sediment and fish (Ternes, 1998; Halling-Sørensen *et al*., 1998; Heberer, 2002; Jones *et al*., 2001; Zuccato *et al*., 2006; Gros *et al*., 2010). It has also been reported that drug manufacturing site effluents (Larsson *et al*., 2007) and hospital waste waters (Kummerer, 2001) can contain high levels of human pharmaceuticals. STPs with advanced technologies, such as granular activated carbon, membrane technology, ozonation, and ultraviolet radiation have been used to remove

pharmaceuticals present in the sewage. However, several pharmaceutical products, for example anti-epileptics (carbamazepine), serotonin reuptake inhibitors and lipid regulators (clofibric acid, gemfibrozil) are known to be reasonably resistant to such treatments, because of their high solubility and/or poor degradability in water. Hence, concentrations of pharmaceutical products in receiving water have been shown to reach nanogram/L levels, and even microgram/L levels (Gros *et al*., 2010).

2.1.1   Need for quantification

Pharmaceuticals present in the aquatic environment may have adverse effects on living organisms (Jobling *et al*., 1998; Matthiessen, 2003; Corcoran *et al*., 2010; Burkhardt-Holm, 2010).   In order to assess the risk posed by pharmaceuticals to aquatic organisms, we need to know if they are present in surface waters (and hence aquatic organisms will be exposed), and if they are, is the concentration of a particular pharmaceutical in the surface water sufficient to produce a potential impact on the aquatic organism, and what organisms are the most sensitive? Currently, the environmental risk assessment of pharmaceuticals for human use is based on the guidelines of the European Medicines Agency (EMEA, 2006), which is a tiered process. The first tier consists of deriving a predicted environmental concentration (PEC) in surface waters using the input data on maximum daily dose of active ingredient consumed per inhabitant, market penetration of drug, volume of wastewater per person per day (set as 200 litres) and the dilution factor from STP effluent to surface water (set as 10). In this phase, the calculation is based on assumptions such as no metabolism, biodegradation or retention of the drug, which leads to worst case estimates of risk. If the PEC is above 10 ng/ L, aquatic fate and effect studies using exposure experiments have to be conducted in higher tier risk assessment phases. Higher tier phases will determine the lowest observed effect concentration (LOEC), no observed effect concentration (NOEC), and the predicted no effect concentration (PNEC). These values are important in predicting the risk of pharmaceuticals to the

environment. Hence, PEC values provide important information for the prioritization of pharmaceuticals for environmental monitoring strategies. There are two approaches, namely analytical chemistry and mathematical modelling, available to derive the PEC of a chemical in the aquatic environment. This chapter explains how and why the modelling approach was used in this project to estimate the concentrations of GCs in the UK.

2.1.2 Measurements with analytical chemistry

Chemical methods such as gas chromatography (GC) or liquid chromatography (LC) coupled with mass spectrometry (MS) or tandem mass spectrometry (MS/MS), as well as biological methods such as glucocorticoid receptor-chemical activated luciferase gene expression (GR-CALUX) and enzyme linked immunosorbent assay (ELISA) methods, can be used to measure precise concentrations (see table 2.1). These are very useful methods when the chemical is present in microgram/L concentrations and when there is an established protocol for appropriate extraction from water and subsequent clean-up procedure. These methods are very specific to a particular chemical (in the case of GR-CALUX, for a group of chemical) and in theory more accurate. But these methods have their own drawbacks (reviewed in Johnson *et al*., 2008). Since the pharmaceuticals are often found in the environmental samples in low nanogram per litre concentrations, and they might be present in a mixture of similar compounds, extraction and quantification will be expensive and time consuming.

The occurrence of pharmaceutical residues in rivers and streams can vary at different timescales (high flow vs low flow) and at different locations (due to differences of population density, available dilution, and distance from STP effluent). The day (for example, weekend or working day will influence the use of stress-related drugs), or month (school holidays), or the year (any outbreak of disease) the sample is taken could also have a large influence on the concentrations present in the environment. They can also vary from country to country,

depending on their drug use policy and the available natural flow per person. It would probably be impractical, using analytical chemistry, to design a sampling strategy that would account for all these factors, and hence provide a range of concentrations representative of those that occur in a river of interest. Moreover, many individual GCs are used in low amounts and hence the surface water concentrations could be in the pg/L range (example, halcinonide; Figure 2.6 and 2.7), which is under the limit of detection for most analytical techniques (though these are decreasing). For a group of pharmaceuticals with many individual products (for example, about 30 different GCs are in regular use in the UK), analytical measurement has another drawback as it will measure only one at a time; validated methods need to be established for each and every single GC.

Despite these difficulties, there are a number of reports available from different parts of the world with measured concentrations of GCs (Table 2.1). These reports provide only a partial picture of the concentrations of GCs in the aquatic environment, but they do suggest that the measured concentration varies from 0.3 ng/L to up to 1900 ng/L in different environmental samples. Therefore, the modelling approach is justified as it is possible to predict the environmental concentration throughout a river catchment, and for different seasons. It is also possible to predict not only the concentrations of one particular GC of interest, but also the overall concentration of all GCs.

Table 2-1. Available data on measured concentrations of GCs in environmental samples and the detection methods used to obtain the data.

GC	Influent (ng/L)	Effluent (ng/L)	Surface water (ng/L)	Method	Reference
Triamcinolone	14 -41	-	-	LC-high resolution MS	Schriks *et al*., 2010
	-	-	0.2-0.5	LC/MS	Tölgyesi *et al*., 2010
	31	30	-	LC/MS/MS	Piram *et al*., 2008
Cortisone	381- 472	-	-	LC-high resolution MS	Schriks *et al*., 2010
	174	229	-	LC/MS/MS	Piram *et al*., 2008
	9 - 51	0.16-0.36	4.2	LC- ESI- MS/MS	Chang *et al*., 2007
	-	31.9	-	LC/MS	Vulliet *et al*., 2007
Prednisolone	315 -1918	-	-	LC-high resolution MS	Schriks *et al*., 2010
	1.4 - 4.6	0.5 - 0.62	0.64	LC- ESI- MS/MS	Chang *et al*., 2007
	-	-	0.04-0.58	LC/MS	Tölgyesi *et al*., 2010
Cortisol	275 -301	-	-	LC-high resolution MS	Schriks *et al*., 2010
	53	53-63	-	LC/MS/MS	Piram *et al*., 2008
	13 -65	0.17–0.83	3.4	LC- ESI- MS/MS	Chang *et al*., 2007
	0.26-120	-	0.17-2.67	LC/MS	Tölgyesi *et al*., 2010
Prednisone	117 – 545	-	-	LC-high resolution MS	Schriks *et al*., 2010
	0.6 -4.5	-	0.86	LC- ESI- MS/MS	Chang *et al*., 2007
Dexamethasone	15	7	-	LC/MS/MS	Piram *et al*., 2008
	0.5 – 1.9	-	-	LC- ESI – MS/MS	Chang *et al*., 2007
	-	-	0.01-0.07	LC/MS	Tölgyesi *et al*., 2010
Flumethasone	-	-	0.02-1.43	LC/MS	Tölgyesi *et al*., 2010
Glucocorticosteroid (undefined)	-	390	52	LC- ESI – MS/MS	Chang *et al*., 2009
	-	11 -243	0.39 – 1.3	GR - CALUX	Van der Linden *et al*., 2008

2.1.3   Modelling the concentrations of GCs

In the past few decades, several models have been developed and subsequently modified in order to predict the fate of point source chemicals in the environment, based on their biological and physico-chemical characteristics. The PhATE (Pharmaceutical Assessment and Transport Evaluation) in US waters (Anderson *et al*., 2004), GREAT-ER (Geography-Referenced Regional Exposure Assessment for European Rivers) in Europe (Feijtel *et al*., 1997), and LF2000-WQX in the UK (Keller and Young, 2004; Williams *et al*., 2009) are some examples of models widely used to predict the concentration of chemicals that reach the water body via STP. In the present study, predictions were made using the LF2000-WQX model, which was developed from the Low Flows 2000 geographic information systems (GIS) hydrological model to predict concentrations of chemicals in real catchments. Low Flows 2000 (Young *et al*., 2003) was developed by the Centre for Ecology and Hydrology at Wallingford and it has been widely used by the Environment Agency of England and Wales and by the Scottish Environment Protection Agency. The LF2000-WQX can predict statistical distributions of concentrations of down-the-drain chemicals in river stretches downstream of all major STPs in England and Wales. The LF2000-WQX model is also based on GREAT-ER, which has been applied to a number of rivers across Europe and has been shown to give reasonable estimates of measured concentrations of down-the drain chemicals (Feijtel *et al*., 1997). GREAT-ER and LF2000 – WQX are similar in many aspects and they use Monte-Carlo simulation to generate different scenarios from distributions describing the river and effluent flow rates, and the final model results (PECs) are expressed as distributions. Thus, many decades of flow, or rainfall, data are collected and different values assigned a different probability (explained in 2.2.4).

All the above models require an individual loading input per capita for the pharmaceutical, which depends on the total consumption of a particular pharmaceutical of interest in the

region of interest (country), the degree of metabolism and excretion from the patients, and the population density. The amounts present in the surface water mainly depend on the rate of removal at STPs, seasonal flow rate of a river, in stream sorption and biodegradation in river. Values for all these parameters are entered into the models. These models use a digitized river network incorporating the STP discharge points.

2.1.4   Objectives

The main objective of this part of my project was to predict the range of possible concentrations of GCs in the aquatic environments of the UK.

Specific objectives

1. Calculate the annual consumption of GCs in the UK, in order to obtain the per capita load into the sewage system.
2. Search literature for information on the metabolism and excretion of GC in patients.
3. Estimate the removal rate in STPs based on the available physicochemical properties of GCs.
4. Predict the concentrations of GCs in the river Thames for different scenarios.

This chapter presents predictions of environmental concentrations of a total of 28 GCs using the LowFlow2000-WQX model (Williams *et al*., 2009) in the River Thames, estimated from data on the prescription of these drugs in the UK. It also presents the concentrations of highest and lowest consumed GCs for high flow and low flow conditions of the river.

Cortisol   Prednisolone   Betamethasone   Dexamethasone   Fluticasone

Beclomethasone (dipropionate)   Mometasone (furoate)   Clobetasone   Budesonide   Methyl prednisolone
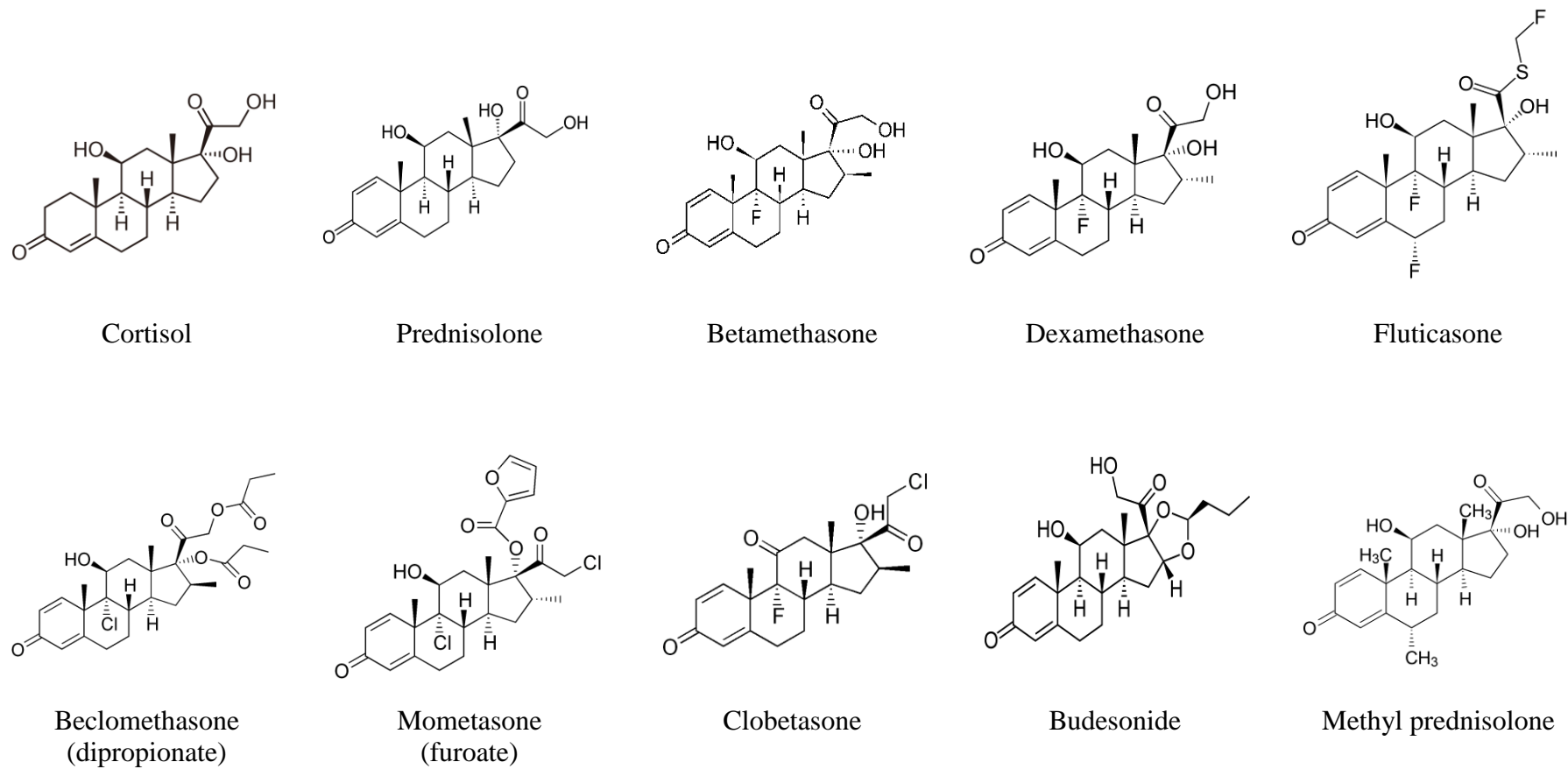
Figure 2-1. Chemical structures of the 10 most prescribed GCs in the UK. Note the fluorine substitution in betamethasone, dexamethasone, fluticasone and clobetasone and chlorine substitution in beclomethasone, mometasone and clobetasone.

Table 2-2. Some physico-chemical and biological information on five of the most prescribed GCs in UK.

Name of GC	Hydrocortisone	Prednisolone	Betamethasone	Beclomethasone	Fluticasone
Reasons for use	Adrenocortical insufficiency Hypersensitivity Asthma Rheumatic disease Inflammatory bowel disease Eczema	Rheumatic disease Immunosuppression in lymphoma/leukaemia Asthma Inflammatory bowel disease Inflammation of eyes /ears	Eczemas Psoriasis Eczematous Inflammation of external ear Inflammation of eyes	Prophylaxis of asthma Acute ulcerative colitis	Prophylaxis of asthma Eczema Prophylaxis & treatment of allergic and perennial rhinitis
Route and Average daily dose	Oral 30 mg Intravenous/ Intramuscular -500 mg , 6 hourly Topical 15-30 mg for 2weeks	Oral(95%) 20-40 mg (maximum 60 mg/day) Rectal enemas/suppositories 20 mg Ophthalmic/Oral drops 2-3 drops-3hourly	Topical (30%) 1-2 times/day Oral 2-3drops/day	Nasal inhalation (70%) 400-1000µg /da y Topical (15%) 5 mg	Nasal inhalation (70%) 100-500 µg/day Topical cream or ointment 1-2times/day 100 -200 µg/day
Molecular formula (and molecular weight)	$C_{21}H_{30}O_5$ (362.4)	$C_{21}H_{26}O_5$ (358.4)	$C_{22}H_{29}FO_5$ (392.5)	$C_{22}H_{29}ClO_5$ (408.9)	$C_{22}H_{27}F_3O_4S$ (444.5)
Log P	1.61	1.66	1.93	2.12	3.70
Water solubility (mg/L) at 25°C	219.6	221.4	75.14	49.39	27
Vapour pressure (mmHg at 25°C)	3.44E-15	2.13E-15	2.81E-15	6.19E-17	2.79E-15
Henry's constant (atm m^3 mol^{-1} at 25°C)	5.77E-8	2.71E-8	7.15E-8	1.27E-8	3.15E-8

2.2   Materials and Methods

2.2.1   Calculation of annual consumption of GCs in the UK

The data in this chapter were obtained from a publicly accessible and free database (http://www.ic.nhs.uk/) maintained by the National Health Services (NHS) of the UK. Prescriptions Cost Analysis (PCA) data for England, Wales, Scotland, and Northern Ireland were obtained from the regional NHS websites. All regional data referred to the year 2006, except the data from Scotland, which referred to the year ending on 31 March, 2007. According to the NHS, the PCA data 'were based on information obtained from prescriptions sent to the Prescription Pricing Division of the Business Services Authority (PPDBSA) for payment'. PCA data covered all prescriptions dispensed to the community; that is, by community pharmacists, appliance contractors, dispensing doctors, and items personally administered by doctors.

As the NHS provides healthcare for the vast majority of citizens of the UK, it is generally considered that most medicines prescribed in the UK will be prescribed within the NHS. Medicines prescribed in hospitals, by private doctors, or purchased via the internet, will not feature on the database used here. Nor will drugs taken illegally (e.g. anabolic steroids taken by athletes). Although it is not possible to know with a high degree of accuracy what proportion of pharmaceuticals used in the UK are prescribed or otherwise obtained from outside the NHS (and hence are not covered by this database), it is thought that the proportion will be relatively low. Thus, although the information on amounts prescribed in this chapter will be under-estimates of actual use, they will probably not compromise the predicted concentrations to any significant extent.

The PCA data are based on the therapeutic grouping used in the British National Formulary (September 2005; edition 50). For each macro-region, the following data were considered (the NHS definition is provided for each element);-

Drug name: The drug was shown by individual preparation name, which may be proprietary or generic, followed by form and strength.

Items dispensed (PXS): A prescription item referred to a single item prescribed by a doctor (or dentist/nurse) on a prescription form.

Quantity (QTY): The quantity of a drug dispensed was measured in units specified on the information supplied with the product.

Standard quantity unit (SQU): The code indicated the form of the drug and the units in which quantity is measured:

Code 1 – a unit (e.g. one tablet, capsule, pack, aerosol, etc.)

Code 3 – millilitres

Code 6 – grammes

Code 0 – individually formulated (unit varies)

The total amount of API prescribed was calculated for each individual preparation, multiplying the value in the quantity column by the amount of API in each unit. For example, 58140200 tablets of 'prednisolone Tab 1 mg' were prescribed by the NHS in England during 2006. According to present methodology, 58.14 kg of this formulation was dispensed. Similarly, other formulations, such as 2.5 mg, 5 mg, and 25 mg tablets, together with other generic formulations such as prednesol and deltacortil, used in England, Wales, Scotland and Northern Ireland, resulted in 1488.3 kg of API prednisolone in total being prescribed in 2006.

If the API was conjugated with further chemical groups, the parental API only was considered. When the drug was dispensed as a gel or topical cream, the percentage of API was calculated. For example, the amount of anugesic HC-cream dispensed in England was 1025.9 kg, but this preparation is of 0.5% hydrocortisone. Therefore the amount of API, hydrocortisone, from this preparation was 5.13 kg. In the case of multiphase drugs, containing units with different strengths and compositions in each pack, the different compositions were taken into account. When the value in the quantity column referred to a blister or a pack, that value was multiplied by the number of tablets or patches contained in each blister or pack, according to the British National Formulary, 2006. Similarly, the value was multiplied by the number of doses in a single prescription for the inhaler drugs (examples include Beclomethasone dipropionate and Fluticasone propionate). By combining the data obtained for each API, the total amount of API prescribed during 2006 was obtained, and expressed in kilograms.

2.2.2   Metabolism and excretion

Data on the metabolism and excretion of GCs in humans were obtained from the world wide web (www.drugbank.ca; www.drugs.com), and from medical and veterinary literature (Bahr *et al*., 2000; Boobis, 1998; Edsbacker *et al*., 1987; Feher *et al*., 1975; Goyal and Bishnoi 2009; Harper *et al*., 2000; Jjemba 2006; Munck *et al*., 1984; Mostl *et al*., 1999; Pozo *et al*., 2009; Martinelli *et al*., 1979; Sparagana *et al*., 1970; Liu *et al*., 2009). The percentage of excretion (of original drug or metabolite) of each individual GC was taken into account and summarised as a range of percentages for total GCs, in order to predict a maximum and a minimum load that might enter the sewerage system from each person (per capita load).

2.2.3   Removal in STP

Direct measurement of removal rates of GCs in STP is available from only two publications (Chang *et al*., 2007; Piram *et al*., 2008). These reports are contradictory, as Chang *et al*. (2007) report more than 95% removal, while Piram *et al*. (2008) report below 5% removal (Table 2). Therefore, SimpleTreat was used for estimating the removal rate. This is a spreadsheet to predict the distribution and elimination of chemicals by sewage treatment. SimpleTreat 3.0 is an improved version compared to the original version, accounting for the different modes of operation of sewage treatment plants and for the descriptions of the interactions between the chemical and its engineered environment, in particular biodegradation (Struijs, 1996). Most of the input data for SimpleTreat were obtained from World Wide Web search (www.chemspider.com; http://pubchem.ncbi.nlm.nih.gov/; table 1). Biodegradation constant (per hour) in an activated sludge was either obtained from a web search or estimated from BIOWIN (biowin v 4.10). If particular input data were not available, the default value was used in the spreadsheet. Removal data for each individual GC were taken into account and summarised as a range of percentages for total GCs in order to predict a maximum and a minimum per capita load.

2.2.4   Prediction of environmental concentrations

The prediction of concentrations of GCs in the River Thames was made using the LowFlow2000-WQX model which has previously been used to assess concentrations of steroid oestrogens (Williams *et al*., 2009), triclosan (Price *et al*., 2010) and cytotoxic drugs (Rowney *et al*., 2009) in the UK. Details of the model are given in these publications and in Keller and Young, 2004. Briefly, the LF2000-WQX software (Keller and Young, 2004; Williams *et al*., 2009) is a geographical information-based system that combines hydrological models with a range of water-quality models, including a catchment-scale water-quality

model. This model generates spatially explicit statistical distributions of down-the-drain chemicals for both conservative and degradable compounds. It uses a Monte Carlo mixing-model approach to combine statistical estimates of chemical loads at specific emission points (e.g. Sewage Treatment Works) with estimated river flow duration curves for the whole river network of interconnected model reaches (a reach is the river stretch between model features e.g. major tributaries, sewage treatment works). Thus working from the low order streams at the head of the river network to the outlet from the river basin, the model accounts for the accumulation of point loads and the accumulation of water in which these loads are diluted. Degradable chemicals are removed from the river water by a non-specific dissipation process assuming first-order kinetics.

For this case study, per-capita input loads of GCs for the UK were estimated from the prescription data and were adjusted to take account of the removal rate in sewage treatment works and the excretion from patients. In order to quantify the uncertainty in these calculations, the lowest removal rate together with the highest excretion rate was taken as worst case scenario and vice-versa as the best case scenario. In calculating the per-capita loads, it was assumed that the GCs are consumed by all age groups of people and that they are released to the environment only via urinary and faecal route excretion, that all the sewerage is treated before entering the river and that there were no seasonal overflows from the STP. It was also assumed that patients are equally distributed all over the UK. The estimated UK population was 60587300 in 2006 (http://www.statistics.gov.uk) and this number was used to calculate the per capita consumption of GCs.

The LF2000-WQX model does not run at a particular flow rate, rather it samples the distribution of flows that are likely to occur in each river reach and then calculates a distribution of concentrations through a series of mass balance calculations. Therefore the 90th

percentile concentration can be interpreted as being typical of low flow conditions and the 10th percentile flow as typical of high flow conditions. In order to show the differences due to the flow rate of the river, the 10th and 90th percentile concentrations of hydrocortisone (CAS number: 50-23-7) and halcinonide (CAS number: 3093-35-4), which are the highest and lowest consumed GCs respectively in the UK, were predicted.

The river Thames was chosen as a case study because it has previously been identified as a river with the highest proportion of its catchment at risk from oestrogenic pharmaceuticals (Williams *et al*., 2009).

2.3   Results

A total of 28 different GCs were prescribed in UK in 2006 and the estimated total amount was 4370 kg, of which hydrocortisone and halcinonide accounted for 1811 kg and 900g, respectively (Table 2.3). The percentage composition of individual GCs prescribed in the UK and the percentage of total GCs prescribed in different parts of the UK in 2006 are presented in Figures 2.2 and 2.3.

Biodegradation constants in an activated sludge STP for different GCs were in the range of $0.3 – 1$ hr^{-1} means 2.3 hours to 0.69 hour half life (EU Technical Guidance Document). Removal rates of different GCs in STPs vary between 11% - 76%. Excretion (either as original drug or metabolite) of GCs was in the range of 28% - 54%. Calculated input load for hydrocortisone and halcinonide in the LF2000-WQX model were 5.503 microgram/head/day and 10.1 ng/head/day, respectively. For the best case scenario the calculated input value for total GCs was 13.3 microgram/head/day and for the worst case it was 96.2 microgram/head/day.
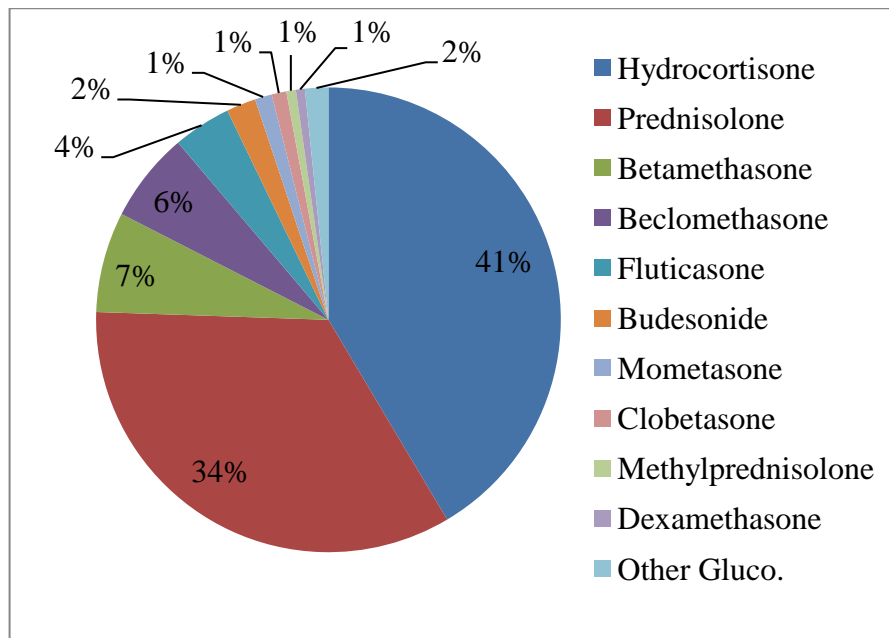
Figure 2-2. Percentage composition of individual GCs prescribed in the UK 2006. Other Glucocorticoids: Deflazacort, Flumetasone, Fluocinolone, Diflucortolone, Fluocinonide, Fluocortolone, Rimexolone, Alclometasone, Fluorometholone, Flunisolide, Fludroxycortide, Ciclesonide, Fluprednidene, Desoximetasone and Halcinonide.
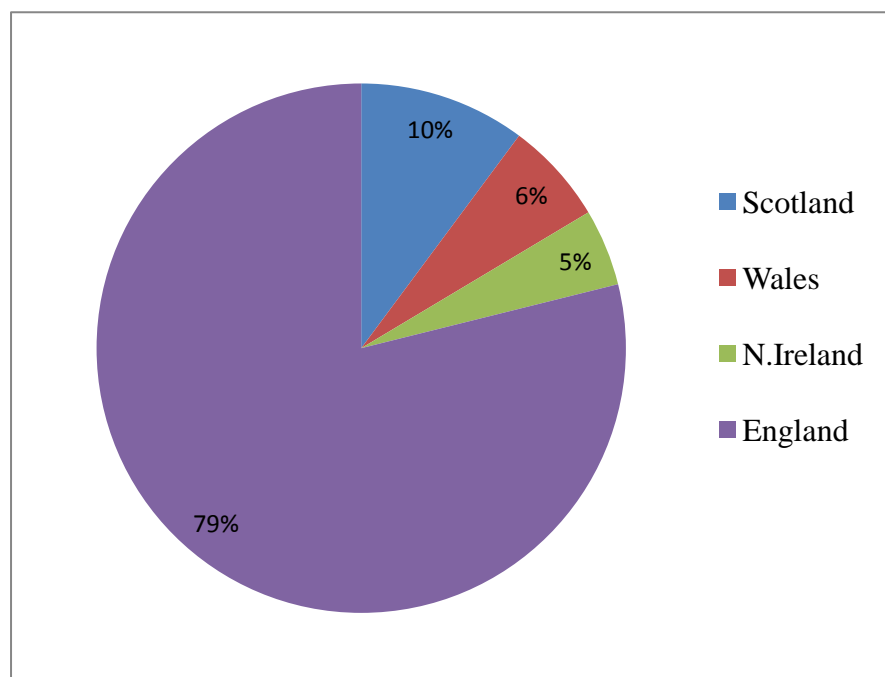
Figure 2-3. Percentage of total GCs prescribed in different parts of the UK in 2006.

Table 2-3. Glucocorticoids prescribed in 2006 in the UK for clinical use.

Principal Name	CAS Number	Log P	Amount Prescribed (kg)
Hydrocortisone	50-23-7	1.61	1810.91
Prednisolone	50-24-8	1.62	1488.30
Betamethasone	378-44-9	1.94	305.33
Beclometasone	4419-39-0	2.12	273.96
Fluticasone	90566-53-3	2.69	176.29
Budesonide	51333-22-3	2.42	89.46
Mometasone	105102-22-5	2.81	51.81
Clobetasone	54063-32-0	2.61	44.72
Methylprednisolone	83-43-2	2.06	28.98
Dexamethasone	50-02-2	1.83	27.26
Clobetasol	25122-41-2	2.48	21.60
Triamcinolone	124-94-7	1.16	16.61
Cortisone	50-06-5	1.47	15.72
Others*	/	/	16.77
Total			**4367.72**

* Others include: Deflazacort, 14484-47-0; Flumetasone, 2135-17-3; Fluocinolone, 807-38-5; Diflucortolone, 2607-26-9; Fluocinonide, 356-12-7; Fluocortolone, 152-97-6; Rimexolone, 49697-38-3; Alclometasone, 66734-13-2; Fluorometholone, 426-13-1; Flunisolide, 3385-03-3; Fludroxycortide, 1524-88-5; Ciclesonide, 141845-82-1; Fluprednidene, 1255-35-2; Desoximetasone, 382-67-2; Halcinonide, 3093-35-4.

The output of the LF2000-WQX model is in the form of colour-coded river maps, with each colour indicating a different predefined concentration range. These maps can be used to identify the 'hot spots', those locations where maximum concentrations are predicted. 10th and 90th percentile concentrations of hydrocortisone are presented in Figures 2.4 and 2.5, respectively, which reveal that due to changes in river flow rate, the concentration of hydrocortisone is predicted to reach maximum values up to 8 ng/L (high flow) and 24 ng/L (low flow). Similarly, 10th and 90th percentile concentrations of halcinonide (Figures 2.6 and

2.7) were very much lower, being up to 20 pg/L in high flow conditions and up to 80 pg/L in low flow conditions.

When the model was run assuming that of all the consumed drugs are excreted and there is no removal in STP, mean concentrations of total GCs were predicted to be up to 1732 ng/L. However, only a percentage of consumed drugs are excreted and there is likely to be some removed in STPs. Therefore more realistic concentrations estimates are presented here. Predicted mean concentrations of all GCs combined, assuming the lowest excretion rate and the highest removal rate in the STP (best case scenario) are presented in Figure 2.8, where the concentrations of total GCs in the river Thames were up to 30 ng/L. This value was increased to 854 ng/L at the 'hot spots' of the river Thames, when the highest excretion rate and the lowest removal in STP were used in the modelling (worst case: Figure 2.9).

There are many stretches on the River Thames that do not receive effluent from sewage treatment works which are not shown on the maps, and of course all of these are predicted to have zero concentration. In fact, there are about 7600 km of rivers in the Thames catchment, of which only about 1350 km are likely to be contaminated with GCs (stretches below major STPs). In the best case scenario, about 99% of the contaminated river length would be predicted to have mean concentrations of between 0.1 and 50 ng/L. In the worst case scenario, only about 54% of the contaminated river length would be expected to show this lower concentration (0.1 to 50 ng/L) range and the rest would be expected to have higher concentrations. In this worst case scenario, about 28% of the contaminated river length falls in the 50 to100 ng/L range, about 12% within the 100-200 ng/L range, and about 5% was predicted with greater than 200ng/L total GCs concentrations. This 5% of the river length could be considered as the 'hotspots'.

Figure 2-4. Distribution of predicted concentrations (pg/L) of Hydrocortisone (highest prescribed GC) during high flow conditions of the river Thames.
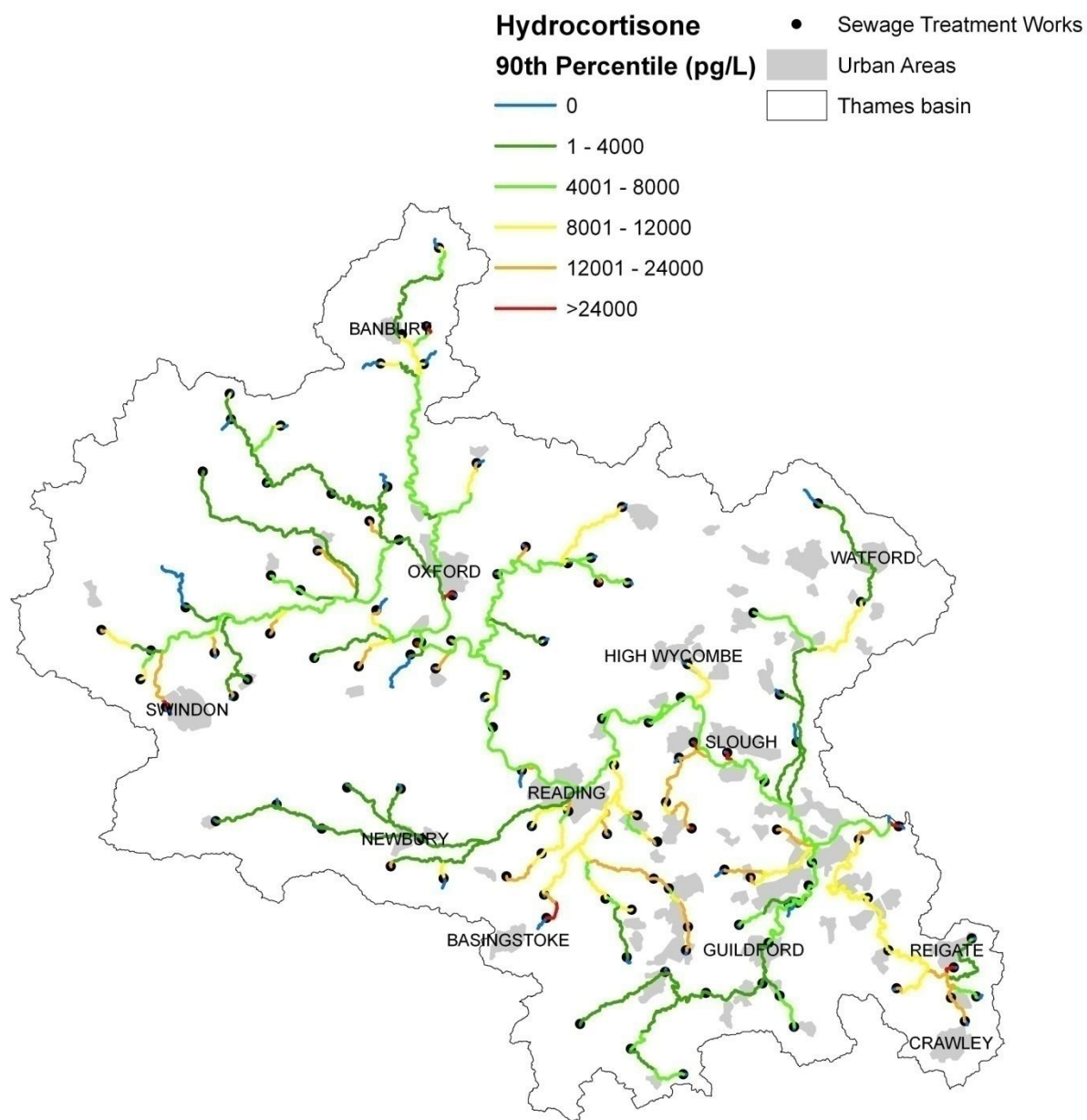
Figure 2-5. Distribution of predicted concentrations (pg/L) of Hydrocortisone (highest prescribed GC) during low flow conditions of the river Thames.
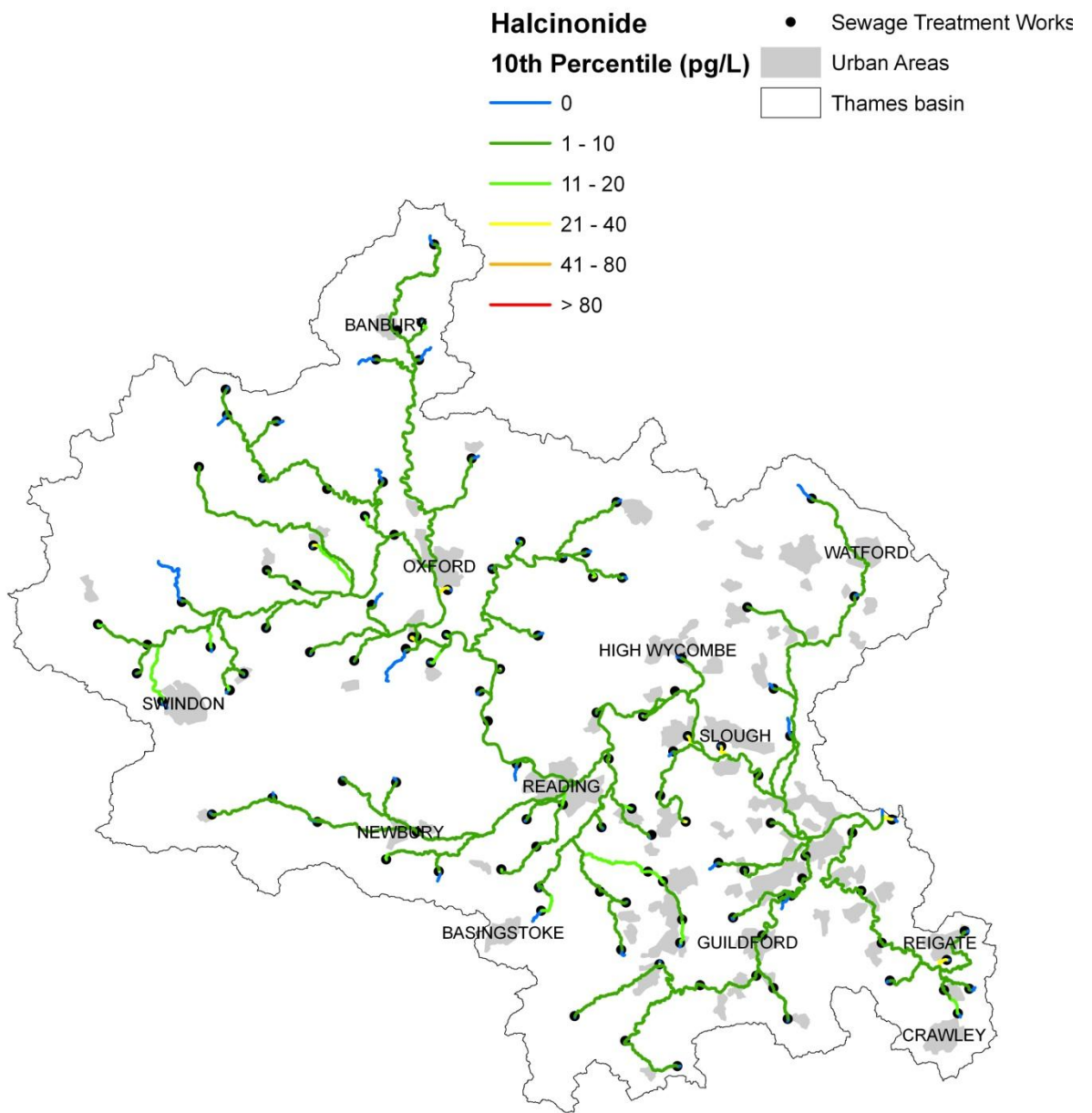
Figure 2-6. Distribution of predicted concentrations (pg/L) of Halcinonide (lowest prescribed GC) during high flow conditions of the river Thames.
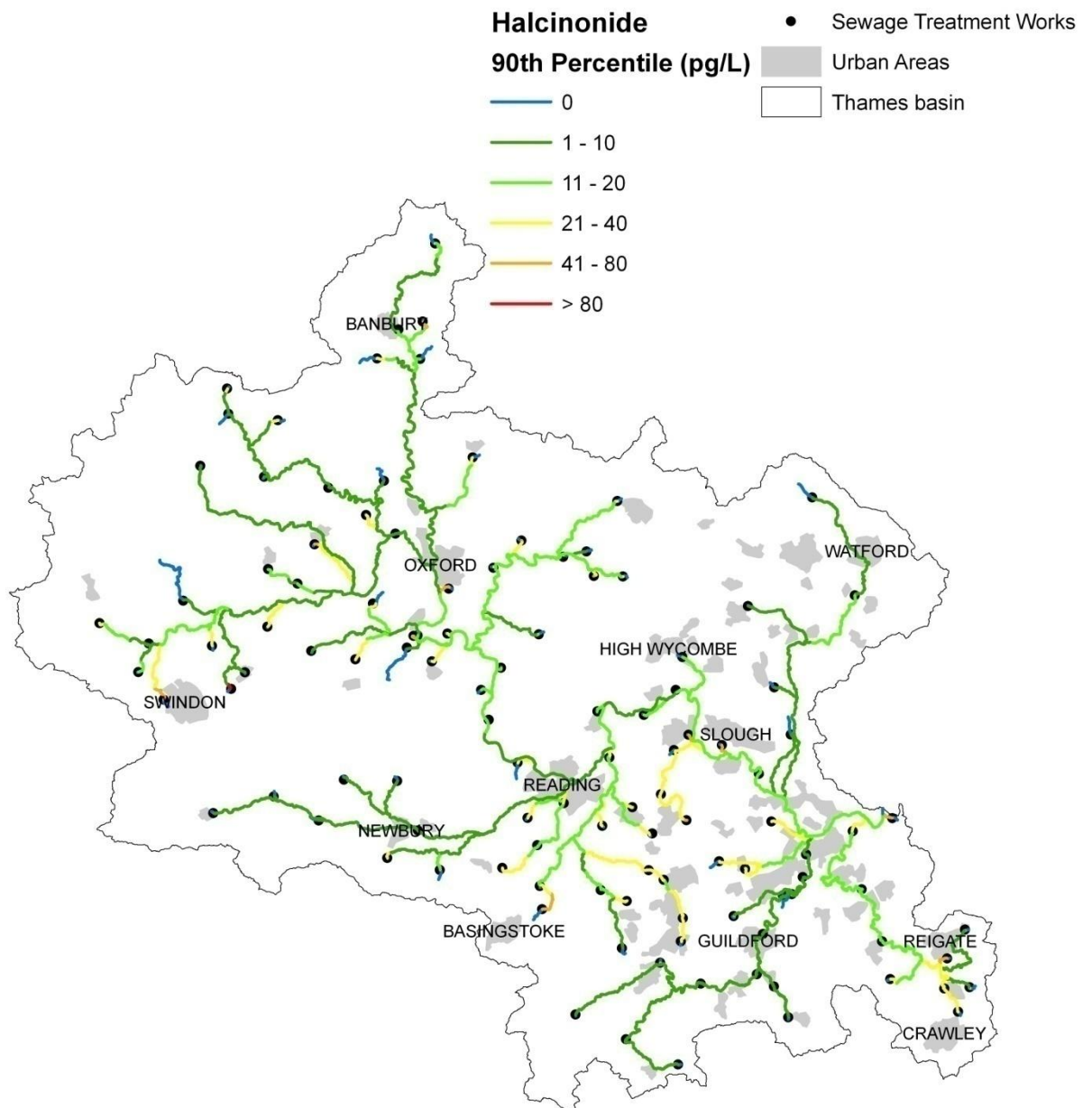
Figure 2-7. Distribution of predicted concentrations (pg/L) of Halcinonide (lowest prescribed GC) during low flow conditions of the river Thames.
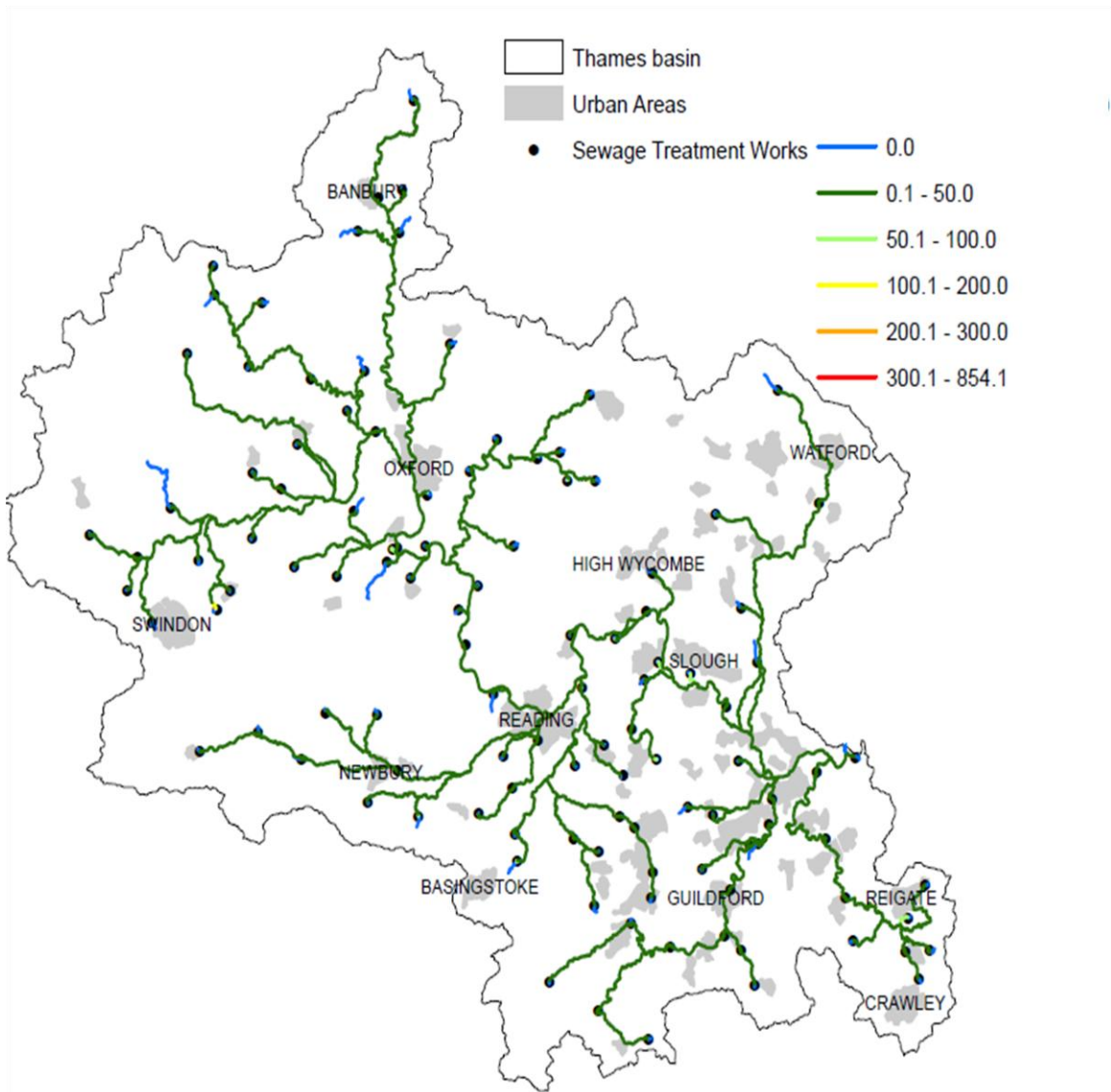
Figure 2-8. Distribution of predicted (50th percentile) concentrations (ng/L) of total GCs along the river Thames basin (best case scenario). The predicted concentrations were obtained with mean flow conditions of the river, maximum predicted removal rate at STP, and minimum excretion from patients.
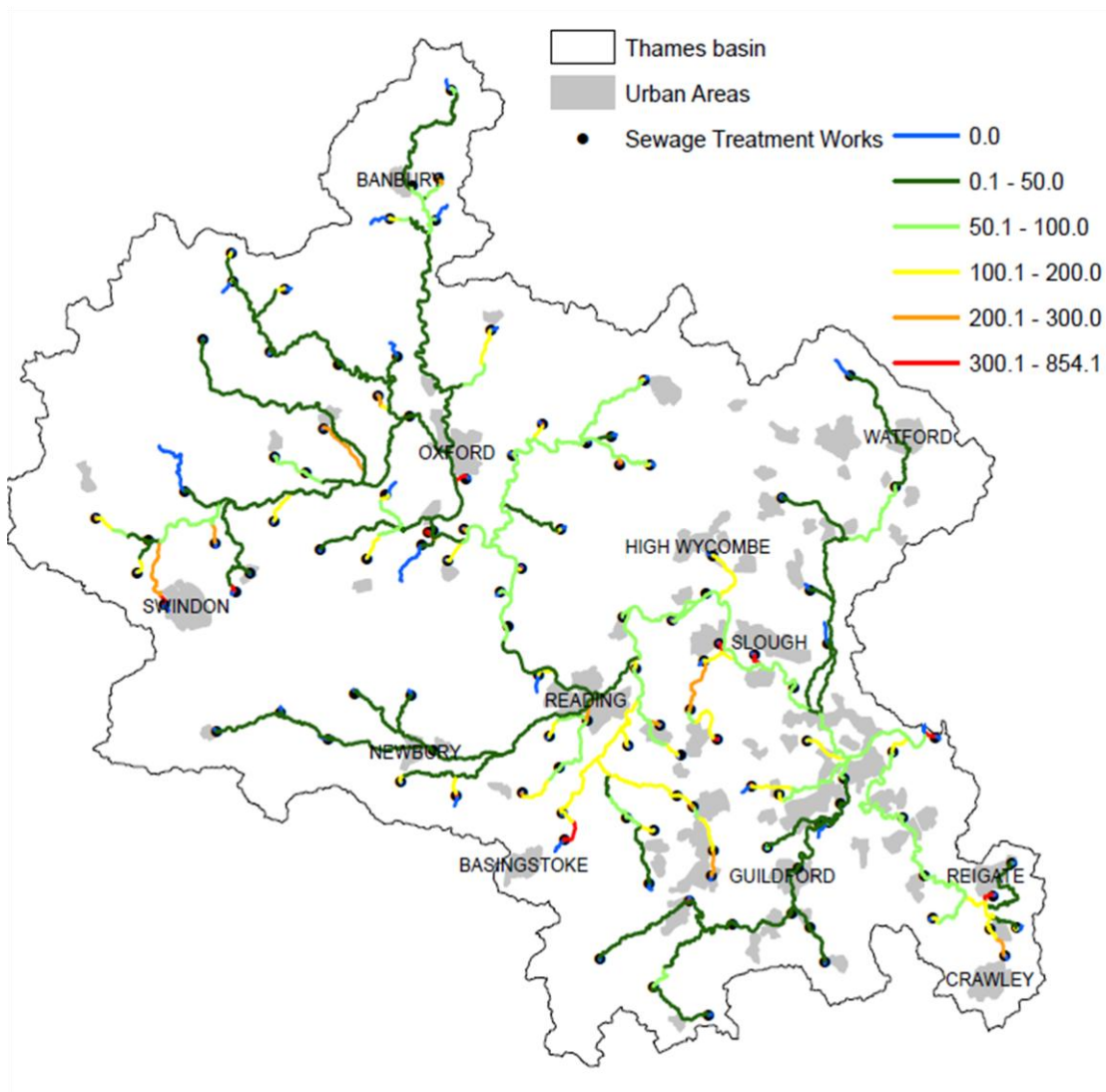
Figure 2-9. Distribution of predicted (50th percentile) concentrations of total GCs along the river Thames basin (worst case scenario). Predicted concentrations were obtained with mean flow conditions of the river, minimum predicted removal rate at STP, and maximum excretion from patients.

2.4   Discussion

As discussed in chapter 1, many different groups of pharmaceuticals are present in the aquatic environments and the concentrations of some non-steroidal anti-inflammatory drugs can reach high levels. Studies show that many of the highly consumed pharmaceutical substances, such as paracetamol and aspirin, are readily biodegradable. In contrast, the impacts of EE2 in the environments have been well documented (reviewed in Caldwell *et al*., 2008) and the annual

usage of EE2 in the UK (about 25 kg) is well below that of most of the GCs used in this study. Therefore priority in the research of pharmaceuticals present in the environment should be given to steroids and anti-steroids (Runnalls *et al.*, 2010), which are both highly potent groups that can be persistent in nature. GCs were not included in a previous study on pharmaceuticals used in the UK, involving a similar calculation of annual usage and an assessment of the environmental risk posed by human pharmaceuticals (Sebastine and Wakeman 2003). They are not in the list of the 25 most-used pharmaceuticals in the UK (Jones *et al.*, 2002).

There are approximately 30 different GCs that are currently licensed for use in the UK (BNF 2006). Statistics on how much is spent on different pharmaceuticals by the National Health Service, via the 'Prescription Cost Analysis 2006', have been produced by the Department of Health. Though this database does not cover private hospital prescriptions, it is estimated that it accounts for about 80% of use for the whole of the UK. Thus, this database probably provides a reliable assessment of the amounts of the different GCs used annually in the UK. In order to prioritize those GCs most likely to be present at measurable concentrations in the UK environment, and hence those that may present a risk to fish, it is important to establish which GCs are used to the greatest extent in the UK.

 The top most used GC in the UK, hydrocortisone, is reported to be readily degradable (Chang *et al.*, 2007). The second most used GC, prednisolone, has been reported by Chang *et al.* (2007) to be the most frequently detected GC in effluent in China. This in turn was found to be a result of its relatively low efficiency of biodegradation. Although other GCs are used in comparatively low amounts compared to prednisolone, their structural modifications, designed to make them more stable in patients, could mean that they are present in the environment at concentrations higher than might otherwise be expected.

With no data available on the concentrations of GCs in UK waters, the modelling approach is probably the best way to start in order to predict a range of possible concentrations in a river catchment and to identify the 'hot spots'. In the past, various models have been successful in predicting concentrations of oestrogens (Williams *et al.*, 2003; Balaam *et al.*, 2010) and beta-blockers (Alder *et al.*, 2010) in the environment. Price *et al.* (2010, 2010a) have reported using the LF2000-WOX for predicting triclosan and decamethylcyclopentasiloxane concentrations in UK waters. Model predictions of oestrogens tallied well with measured concentrations (reviewed in Hannah *et al.*, 2009). The main difference from oestrogens is that GCs are available in different routes of administration (not only orally) and the excretion and removal rates for individual GCs vary considerably. This is the reason for the wide range of concentrations predicted for the GCs in river Thames.

Unlike oestrogens, considerable amounts of GCs are used as topical creams that may be washed off directly into wastewater. Therefore present predictions may be underestimates, because a high proportion, or even all, of the topical GCs may reach the aquatic environment. Another reason for any underestimation is that GCs used as veterinary medicines were not included. Farm animals may also contribute by excreting natural GCs and their metabolites, in exactly the same way that they excrete other steroidal hormones (reviewed by Johnson *et al.*, 2006). It is currently impossible to accurately determine the amount released into the environment for the reasons cited above, and also because unused or expired medications may be disposed of in a manner that could contribute more (Bound and Voulvoulis, 2005; Tong *et al.*, 2010). This underestimation is compensated for in part by the amount of biodegradation in the river and in stream sorption to the sediment, two factors not taken into account in the present calculations (because no data are available). Nevertheless, results presented will be useful to identify the hotspots for further quantification and risk assessment. The range of concentrations predicted could be used in laboratory in-vivo experiments. It is also possible to

model the effects of mixtures of GCs, in a manner similar to the approach of Sumpter *et al.* (2006) for mixtures of oestrogenic chemicals.

The concentrations of GCs in the environment seem to exceed those of oestrogens, as indicated by pharmaceutical use profiles and the concentrations detected in surface waters. For example, the usage profile in Denmark shows that total consumption of GCs and ethinyl estradiol were 361 and 0.7 kg/year, respectively (Ingerslev *et al.*, 2003). The present estimate of over 4000 kg/year of GC consumption in UK is justified as the population of UK is slightly more than 10 times of the population of Denmark. Using liquid-chromatography tandem mass spectrometry, Chang *et al.* (2009) reported concentrations of GCs and oestrogens as 52 and 9.8 ng/L, respectively, from surface waters in China. But we don't know the usage profile of GCs in China, so direct comparison with the situation in the UK is not possible. For a comparison of a similarly behaving steroid, we can take ethinyl estradiol, 25 kg of which is used annually in the UK, and surface water concentrations are about 0.5 ng/L. This ratio is in agreement with the present estimate for hydrocortisone, where the usage is 1810 kg/year and the 90th percentile concentration is up to 24 ng/L. It should be noted that excretion and STP removal rates are not exactly the same for these two compounds, and that probably accounts for their differences. Johnson (2010) has recently described how flow rate can influence the concentration of point source chemicals in a catchment, with oestrogen as his example. The natural variation in flow from winter to summer typically produced a 20 to 30-fold difference in predicted oestrogen concentration over the course of a year. The predictions for halcinonide reveal a 10 to 12-fold change on the average concentrations between high flow (10th percentile: Figure 2.6) and low flow (90th percentile: Figure 2.7) conditions. Similarly predictions for hydrocortisone reveal a 6 to 8-fold change on the average concentrations between high flow (10th percentile: Figure 2.4) and low flow (90th percentile: Figure 2.5) conditions.

The concentrations of GCs in STP influents and effluents have been measured only in two reports (Chang *et al*., 2007; Piram *et al*., 2008). The former reports more than 95% removal in STP (except for prednisolone: 60%) and the latter reports below 5% removal in STP (see table 2.1 for details). Sewage treatment plants differ widely in the treatment process technology they employ (Johnson and Sumpter 2001). The efficiency of removal depends on the age of the activated sludge, the hydraulic retention time, the organic loading, the cultivated microorganisms, the number of inhabitants and the season (Johnson *et al*., 2007). Both Chang *et al*. (2007) and Piram *et al*. (2008) reports could be right, due to different sampling strategies and the technology behind the STPs. However, this uncertainty is the reason I choose to use the SimpleTreat 3.0 model (Struijs, 1996), a model widely accepted as a tool to predict the fate of a chemical in an STP. Moreover, this model allowed me to predict the removal rate for each individual GC and summarise a range of removal rates for the prediction of total GC concentrations.

Several analytical methods have been developed to determine GCs in the environment (Table 2.1). Liquid/Gas chromatography coupled with mass spectrometry is most commonly used. It has been emphasized that the environmental concentrations are often close to the limit of detection of these methods (Sumpter & Johnson, 2005), so that it is often difficult to get a reliable value of the environmental concentration of a pharmaceutical. It is noted that the values presented in Table 2.1 (maximum of 53 ng/L for surface water) are for individual GCs. Because a total of 28 individual GCs are in regular use, the maximum value (854 ng/L) is much higher. All the individual GCs have been combined as they have a similar mode of action and the risk posed to the environment could be additive (Brian *et al*., 2005). This approach has been reported previously for similarly acting chemotherapy drugs (Johnson *et al*., 2008). Therefore from the present study, it is proposed the dose-response exposure studies should be carried out in the range of concentrations between 10 ng to 1000 ng/L for GCs.

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Present predicted concentrations are specific for the UK situation, as the LF2000-WQX models all STPs along the river catchment of interest and examines dilution factors in real locations. It does not use the standard dilution factor of 10. Because of the high population density of the UK (especially close to the metropolitan cities), many of the rivers that receive STP effluent dilute it by a factor less than 10. England has very low natural flow available per person compared to, for example, the USA. Britain is a relatively densely populated country: it is more than twice as densely populated as France (106 people per sq.km), nine times as densely populated as the USA (27 people per sq.km) and 100 times as densely populated as Australia (2 people per sq.km).This means that for a particular amount of consumption of GCs, other countries may have lower surface water concentrations. This low dilution factor is one of the reasons for comparatively higher concentrations of oestrogens predicted for the UK compared to the USA (Hannah *et al*., 2009).

The elderly population of the UK is increasing (Figure 2.10a). Over the last 25 years the percentage of the population aged 65 and over increased from 15 per cent in 1984 to 18 per cent in 2009, an increase of 1.7 million people. This trend is projected to continue. By 2034, 23 per cent of the population is projected to be aged 65 and over. By 2034, the number of people aged 85 and over is projected to be 2.5 times larger than in 2009, reaching 3.5 million. The number of prescription items dispensed annually per person in England, from 1978 to 2007 (Figure 2.10b), indicates that older patients (age 65 and over) are being prescribed three times more pharmaceuticals than are the average population. Therefore, the above projections reveal that the use of pharmaceuticals will most likely increase substantially in the next 50 years. It has been predicted that in the future, flows in the River Thames will reduce, especially in late summer and autumn (Diaz-Nieto and Wilby, 2005). This might be the case in many parts of the world (Dai, 2010), and together with an ever-increasing use of glucocorticoids, the environmental concentrations could increase.
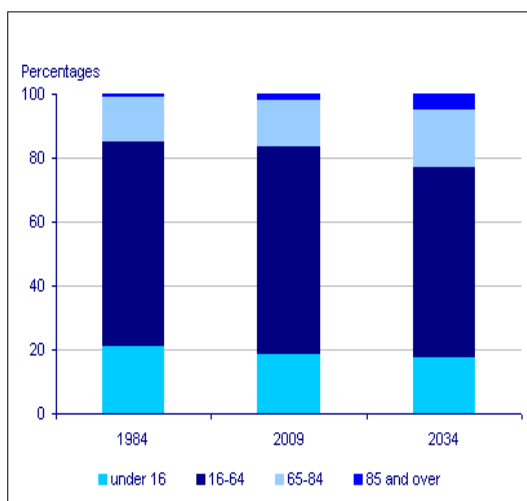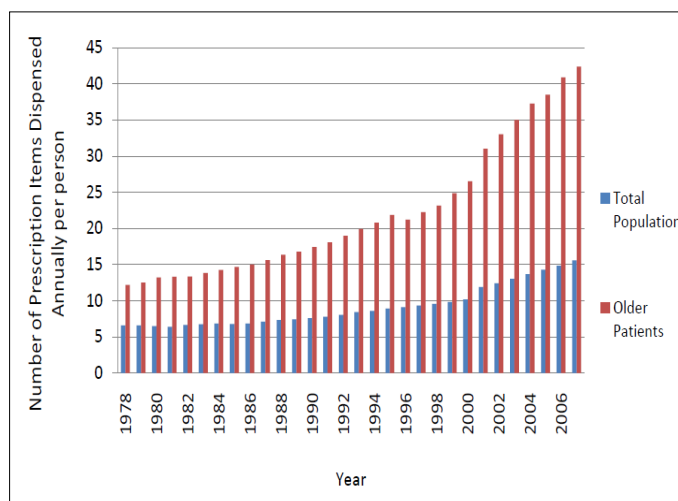
(a)   (b)

Figure 2-10. Prediction of increasing use of pharmaceuticals due to increasing elderly population in the UK. (a) Percentage of population by age, UK, 1984, 2009 and estimation for 2034 shows that percentage of elderly population (age 65 and over) is increasing. (b) Number of prescription items dispensed annually per person in England, from 1978 to 2007, indicates that pharmaceuticals have been prescribed to older patients (age 65 and over) three times more frequently than that of an average population (Source: Office for National Statistics; http://www.statistics.gov.uk/CCI/nscl.asp?ID=5014).

The European Medicines Agency, which was set up in 1995, is responsible for the risk assessment of any drug introduced in Europe, in order to protect and promote public and animal health. But it is not clear whether any kind of risk assessment has been conducted for the glucocorticoids, which have been in use for the last six decades (David *et al*., 1970). The EMEA (2006) has proposed that a PEC of 10 ng/L for an individual drug should be a trigger value for further environmental risk assessment. Currently there are no biological studies reported for GCs using environmentally-relevant concentrations. Therefore, having predicted the environmental concentrations, the next step in the risk assessment of GCs will be to undertake further research, to establish a range of concentration-relationships, thus enabling the determination of the LOEC, NOEC, and the PNEC for a range of aquatic species, often

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algae, Daphnia and a fish species. These values are important in predicting the risk of such chemicals to the environment.

Synthetic GCs have been reported to have many side effects in humans, mainly hyperglycemia, osteoporosis and muscle wasting (David *et al*., 1970; Melby 1977; Mahajan and Tandon 2005). Therefore environmentally available GCs could cause such effects to vertebrates, such as fish. Anti-inflammatory/ immunosuppressive properties of these drugs mean that they might make fish more susceptible to disease. Given the fact that the GCs do not have the hydroxy group of the oestrogens, and that side-chain cleavage resulted in C_{19} compounds (similar to 11KT of fish), it has been argued that the metabolic transformations of GCs may more likely result in the formation of substances with androgenic activities. It has also been reported that GCs oppose the action of oestrogens in mammals (Rhen *et al*., 2003). Therefore reproductive endpoints also have to be assessed. Modelled predictions of environmental concentrations have been previously reported to correlate with the impacts of pharmaceuticals in the environment (Tyler *et al*., 1998; Jobling *et al*., 2006), thus field studies should be focused on the reported 'hot spots'.

2.5   Conclusions

The range of concentrations of total GCs along the river Thames, predicted by the LF2000-WQX model, is between 0 and 850 ng/L. Therefore, it is proposed that laboratory dose-response exposure studies on fish be conducted in the range of concentrations between perhaps 10 ng to 1000 ng/L, which is considered as environmentally relevant. Studies to understand the removal rates of GCs in STP will make the present results more precise. If field studies to investigate possible effects of GCs on wild fish are conducted, they should be focused on the reported 'hot spots'.

Chapter 3.0

Estimating The Potency Of Different Corticosteroids *In vitro*

3.1 Introduction

The usage profile and the physicochemical properties of synthetic GCs, together with the river flow data, allowed the prediction of concentrations of GCs in the aquatic environment, which together with the chemical quantification data available in the literature (reviewed in Chapter 2), suggest that it is possible that GCs can be present in the aquatic environment in the ng/L range. Because of their biological effects in almost every organ, GCs are one of the most widely used drug classes and their release into the environment is inevitable. Aquatic vertebrates, mainly fish, are known to be the most sensitive species to the pharmaceuticals present in the environment (Gunnarsson *et al*., 2008). However, it is not known whether synthetic GCs have any impacts on fish, and if they do, which of them are the most potent GCs among the many different GCs presently in use. Before using fish to examine the impacts of GCs present in the environment, it is ethical and useful to apply *in vitro* (use of cells or tissues, cultured under controlled conditions in the laboratory, and where possible derived from animals of interest) methods to see if the fish receptors are responding to synthetic GCs, and to determine the differences in their potencies. A major advantage of the *in vitro* approach is that it can be used to test many different GCs (10 in this case) at a time, so that the number of animals in research is reduced.

3.1.1 Alternative approaches to animal testing

There is an increasing interest over the past few decades in the use of alternative testing methods that reduce the use of animals in regulatory testing. Several organisations around the world currently dedicate large amount of funds to develop alternative methods. A European Directive on the protection of laboratory animals for experimental purposes, introduced in 1986, promotes the development and validation of alternative techniques to animal testing. These principles have been introduced in subsequent legislation, such as that advocating a ban

on the marketing of cosmetics containing ingredients tested on animals. The European Partnership for Alternative Approaches to Animal Testing (EPAA) is collaboration between the European Commission, European trade associations, and industry sectors, aiming to accelerate the development, validation and acceptance of alternative approaches to further the reduction, refinement and replacement of animal use in regulatory testing. The Organization for Economic Co-operation and Development (OECD)'s Test Guideline Programme has recently adopted an *in vitro* method to detect skin and eye irritants in consumer products. Beside these, the European Centre for the Validation of Alternative Methods (ECVAM) and the European Union regulation concerning the registration, evaluation, authorization and restriction of chemicals (REACH) are also working to promote alternatives to the use of animals. The UK Government, together with industry and other bodies, is funding a centre to promote alternative test (NC3R's – National Centre for the Replacement, Refinement and Reduction of Animals in Research).

One major group of animal alternative techniques is cell-based assays, such as the Yeast Oestrogen Screen (YES), Yeast Androgen Screen (YAS) and Glucocorticoid Receptor – Chemical Activated Luciferase gene expression (GR-CALUX). Yeast screens are widely used to detect the oestrogenic or androgenic chemicals present in water samples (Routledge and Sumpter, 1996) and GR-CALUX is used to detect glucocorticoid activity in water samples (Van-der Linden *et al*., 2008). A similar approach is also available for oestrogenic (Legler *et al*., 2002) and androgenic (Sonneveld *et al*., 2005) detection. The experimental approach described in the present chapter is the basic technique used in the development of the GR-CALUX. The present experiment utilizes an alternative technique to animal experimentation to determine the potency of different GCs prescribed in the UK, in order to see whether the GCs can bind to fish receptors to produce receptor-mediated impacts of GCs on fish, and if so, which are the more potent GCs that could pose a threat to wild fish.

3.1.2 Corticosteroid receptors of fish

The effects of corticosteroid hormones are mediated through intracellular receptors that act as ligand-dependant transcription factors. Studies on the steroid receptors (Thornton *et al*., 2003), which includes corticosteroid receptors (CR: GR and MR), oestrogen receptors (ERα and ERβ), progesterone receptor and androgen receptor have been of considerable interest in the past decade. The presence of steroid receptors in vertebrates has been known for a long time, and more recently steroid receptors resembling vertebrate ERs have been identified in cephalopods (Keay *et al*., 2006) and gastropods (Thornton *et al*., 2003). The phylogenetic analysis of vertebrate steroid receptors indicates that they could arise through serial gene duplications in the chordates from this ancestral steroid receptor (Thornton, 2001).

Earlier evidence for CR in fish was reported from cortisol binding studies and some studies using synthetic GCs, such as triamcinolone acetonide and dexamethasone. These studies indicated the presence of a single class of high affinity, low capacity, binding sites in the cytosol from various tissues, including gill, intestine, liver, brain, hypothalamus, leucocytes, and erythrocytes (reviewed in Mommsen *et al*., 1999). These binding studies also showed that CR concentrations and affinity can be altered by experimental manipulations, including hormonal treatments and stress induction. Molecular characterization of the fish GR is available for many species of teleosts, such as rainbow trout (Ducouret *et al*., 1995), tilapia (Tagawa *et al*., 1997) and flounder (Tokuda *et al*., 2005). Later, two isoforms of GR, namely GR1 and GR2, have been described in trout (Bury *et al*., 2003) and cichlids (Greenwood *et al*., 2003). Although fish lack aldosterone, the presence of a MR has been reported, with characterization of cDNA encoding a MR in trout (Colombe *et al*., 2000; Sturm *et al*., 2005) and cichlid (Greenwood *et al*., 2003). This MR shows (Figure 3.1) high homology with other known MRs and it has been shown to have a higher affinity for cortisol than aldosterone. In contrast, mammalian MR has equal affinity for both cortisol and aldosterone. Therefore it was

concluded that in fish, cortisol has both gluco and mineralocorticoid activities (Sturm *et al*., 2005).

GR and MR share the same basic structure, with the following functional domains: the amino terminal A/B domain that modulates the transcriptional activity, the C domain which is responsible for DNA binding and receptor dimerization, the D domain which is the hinge region and the E domain which is the ligand binding domain. Figures 3.1 and 3.2 show the amino acid identities of different domains in selected GRs and MRs, and reveal that the A/B domain is variable across species whereas the C domain is highly conserved among species.

Figure 3-1. Amino acid identity between selected domains (A/B, C, D and E) of the trout MR (rtMR) compared to trout GRs (rtGR1, rtGR2) and human MR (hMR) (Figure modified from Prunet *et al*., 2006).
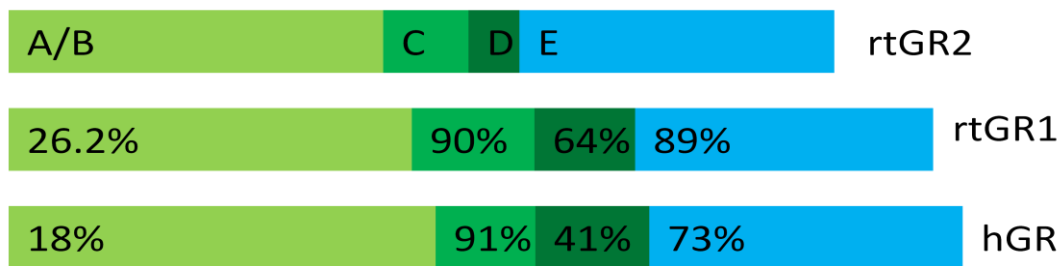
Figure 3-2. Amino acid identity between selected domains (A/B, C, D and E) of the trout GR2 (rtGR2) compared to trout GR1 (rtGR1) and human GR (hGR) (Figure modified from Bury *et al*., 2003).

In the absence of hormone, the glucocorticoid receptor (GR) is present in the cytoplasm, complexed with a variety of proteins. The GCs diffuse through the cell membrane into the cytoplasm and bind to the GR, resulting in release of the proteins. The resulting activated form of the GR has two principal mechanisms of action, transactivation and transrepression, as described below (Figure 3.3).

Transactivation: A direct mechanism of action involves homodimerization of the receptor, translocation via active transport into the nucleus, and binding to specific DNA responsive elements, thereby activating gene transcription. The biological response depends on the cell

type. Transrepression: In the absence of activated GR, other transcription factors, such as NF-kB or AP1, are able to transactivate target genes. However, activated GR can complex with these other transcription factors and prevent them from binding to their target genes, and hence repress the expression of genes that are normally up-regulated by NF-kB or AP-1. This indirect mechanism of action is referred to as transrepression.
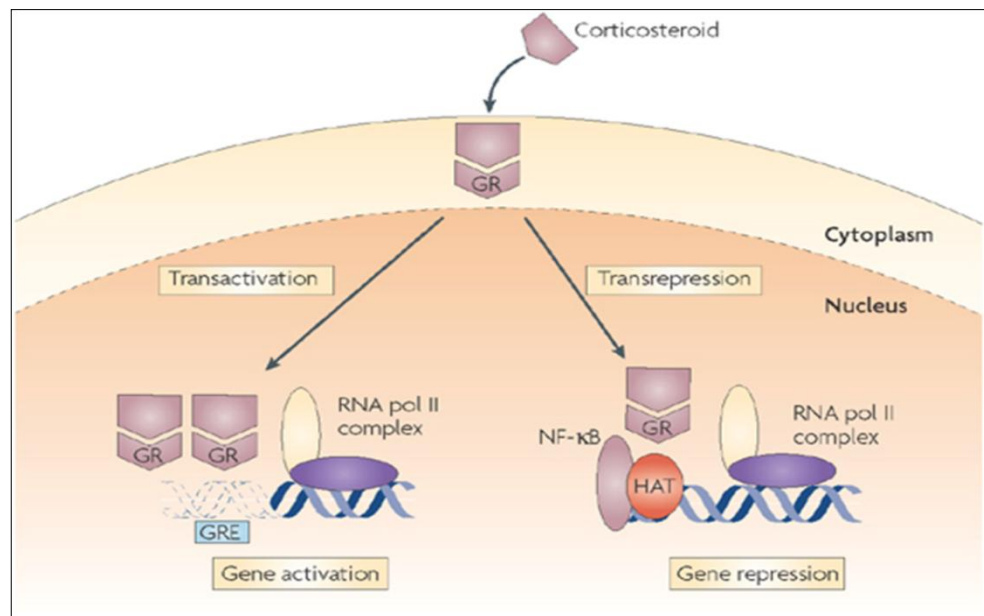
Figure 3-3. Mechanism of corticosteroid action as an example of anti-inflammatory gene transactivation and inflammatory gene transrepression.Corticosteroids enter the cell, bind to the glucocorticoid receptor (GR) in the cytoplasm and translocate to the nucleus, where the transcription of target genes is initiated. Many genes contain glucocorticosteroid response elements (GREs) in their promoters. Through transactivation, binding of the activated glucocorticoid receptor homodimer to a GRE in the promoter region of steroid-sensitive genes leads to local unwinding of the DNA Structure, allowing recruitment of large protein complexes, including RNA Polymerase II (RNA Pol II), resulting in the transcription of genes encoding anti-inflammatory mediators such as annexin-1, secretory leukoprotease inhibitor (SLPI), interleukin-10 (IL-10) and the inhibitor of nuclear factor-κB (IκBα). Through transrepression, the glucocorticoid receptor–corticosteroid complex interacts with large co-activator molecules with intrinsic histone acetyltransferase (HAT) activity (such as cyclic AMP response element binding protein, CBP), which are activated by pro-inflammatory transcription factors (such as NF-κB and AP1), thus switching off expression of the inflammatory genes that are activated by these transcription factors. (Figure taken from Holgate and Polosa, 2008).

3.1.3   Transactivation assays

Naturally occurring transactivation of a gene can be induced *in vitro* by inserting a transactivator gene and special promoter regions of DNA into the genome at the appropriate position. The transactivator gene expresses a transcription factor that binds to a specific promoter region of DNA. By binding to the promoter region of a gene, the transcription factor causes that gene to be expressed. If this specific promoter region is also attached to a reporter gene, it is possible to determine when the transactivator is being expressed and the expression can be measured to assess the potency/efficacy of the ligands of interest. In this study, COS-7 (**C**V-1(simian) in **O**rigin and carrying the **S**V-40 genome) cells, which are derived from the African green monkey and lack any endogenous CRs, were transiently transfected with trout CRs and GC-responsive luciferase and galactosidase reporter genes, and then exposed to synthetic GCs of interest. This exposure results in transactivation of the CR and consequent expression of the luciferase gene, which can be measured by lysing the cells and adding luciferin substrate and measuring the light output with a luminometer. Similarly, galactosidase expression can be measured with the substrate otho-nitrophenyl β-galactopyranoside (ONGP) and formation of the yellow colour measured spectrophotometrically.

 A highly potent drug produces a larger response at low concentrations. Below a certain concentration of a ligand, the transactivation is too low to measure, but with increasing concentration it rises until at sufficiently high concentrations it can no longer be increased and the maximum effect will be reached, and thus a sigmoid curve (dose-response curve) could be plotted for the activity and concentration. The concentration of ligand at which activity is 50% of maximum activity is termed the half maximal effective concentration (EC50). The lower the EC50, the less the concentration of a ligand required to produce 50% of maximum activity and hence the higher the potency of that ligand. Potency is a measure of drug activity expressed in terms of the amount required to produce an effect of given intensity (Figure 3.4).

It is proportional to affinity and efficacy. Affinity is the ability of the ligand to bind to a

receptor. Efficacy is a measure of the activity for a particular concentration of a ligand.
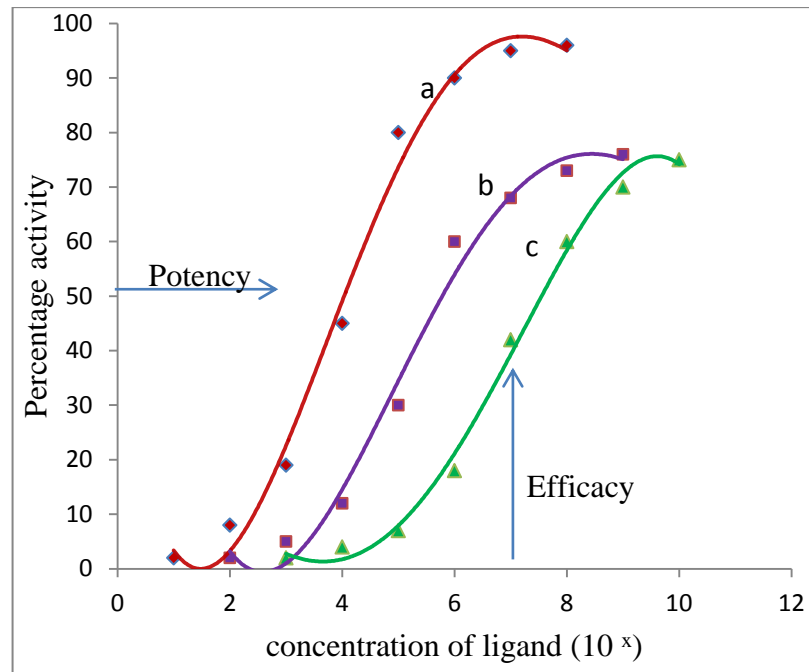
Figure 3-4. Hypothetical dose-response curves for three different ligands, namely a, b and c. Ligand-a is highly potent and it has high efficacy at all concentrations. Ligand-b is of medium potent but its efficacy is similar to that of ligand-c in higher concentrations. Thus ligand-a, which is more active at low concentrations, could be more unfavorable from the environmental perspective.

3.1.4 Objectives

The objective of the part of my project presented in this chapter was to perform *in vitro*

transactivation assay with ten of the most prescribed GC in the UK, in order to see if all these

GCs bind to Fish CRs, and also to estimate their potencies in order to prioritize them in the

exposure experiments I conducted with adult Fathead minnows. Objectives also included an

analysis of the GR sequences of selected groups of animals and selected fish species available

in the open literature, in order to investigate their similarities.

3.2   Materials and Methods

The total amount of synthetic corticosteroids used in the UK was calculated as described in Chapter 2. The ten GCs prescribed in the highest amounts were chosen and their potencies/ efficacies with fish GR and MR were studied *in vitro*.

Hydrocortisone (CAS: 50-23-7), prednisolone (CAS: 50-24-8), dexamethsone (CAS: 50-02-2), fluticasone propionate (CAS: 90566-53-3) and mometasone furoate (CAS: 105102-22-5) were purchased from Sigma (UK). Betametnasone (CAS: 378-44-9), beclomethasone (CAS: 4419-39-0), clobetasone (CAS: 54063-32-0), budesonide (CAS: 51333-22-3) and methyl prednisolone (CAS: 83-43-2) were purchased from Steraloids Inc, USA. Unless otherwise stated, all the bench chemicals were purchased from Sigma. All the weight measurements of chemicals were done with an electronic balance (CAHN 21 automatic electrobalance), using a range from 1 mg to 200 mg. After dissolving the GC in ethanol, all solutions were stored in the fridge (4°C).

3.2.1   Propagation of plasmid DNA

All the plasmids containing the appropriate cDNA (GR1, GR2, MR, luciferase and β-galactosidase) were provided by Dr. Nicolas Bury from King's College, London. Details about the construction of these plasmids are available in their literature (Bury *et al*., 2003; Sturm *et al*., 2005). Briefly, for GR2, the full clone encoding part of the 5'-untraslated region, the entire coding region and part of the 3'-untranslated region was excised from pGEM4Zf plasmid by EcoR1 (endonuclease enzyme) and ligated into EcoR1 cut dephosphorilated expression vector pCMV5, thus named as pCMrtGR2. Orientation of the insert was confirmed by restriction enzyme profile.

In the present study, receptor expression vector (pCMrtGR2: 50ng) was transformed with 100μl of chemo-competent E-coli strain (J109 - Promega) by the heat shock method and

propagated in super-optimal broth with catobolite repression (SOC medium). Different dilutions of the above culture were spread on lysogeny broth (LB) agar plates together with ampicillin (as the plasmid contains an ampicillin resistance gene, any bacteria that are not transformed with plasmid will be killed). After overnight incubation at 37°C, isolated colonies were transferred to 5ml LB (with ampicillin) for shaking incubation overnight. 300µl of this culture was added to 150ml LB (with ampicillin) the next day and incubated overnight. Receptor plasmid DNA was extracted using Hi speed plasmid purification kits (Qiagen) according to the manufacturer's instructions. Final elution using the buffer provided was done twice and separately, to avoid any loss of plasmid on the column.

Integrity of the plasmid DNA was confirmed with agarose gel electrophoresis (Figure 3.5) and the concentration was measured spetrophotometrically (nanodrop) in ng/µl. A ratio of the absorbances at 260nm and 280nm was found to be between 1.9 and 2.0 for all extracted plasmids, which excludes the possibility of contamination of solvents or by any proteins. The same procedure was repeated for pFC31Luc, which contains the mouse mammary tumor virus promoter upstream of the luciferase gene, and pSVβ, which contains the gene coding for the β-galactosidase enzyme. All plasmids were kept in a -20°C freezer until used. During all the above procedures, standard microbiological techniques, such as sterilizing containers, cleaning all the work surfaces with 70% IMS (industrial methylated spirit), transfer of materials under flame, opening of lids as little as possible, heating the inoculation loop to red hot before and after inoculation and incubation of spread Petri dishes in inverted position, were followed.
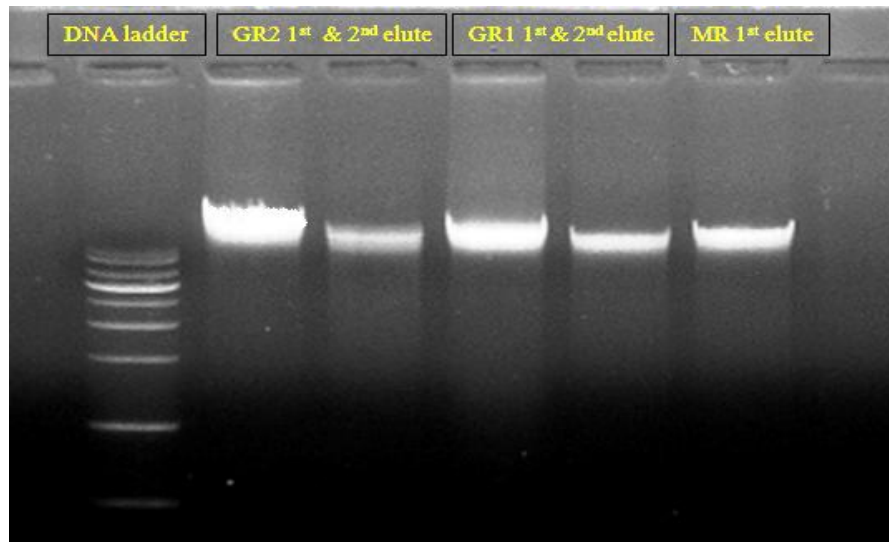
Figure 3-5. Gel electrophoresis for testing the integrity of propagated plasmid DNA. A 1% agarose gel with ethidium bromide was prepared and 10μl of elute from plasmid purification with loading dye were run for 45 minutes. A 1kb ladder was run for comparison (left hand lane) and all three receptor plasmids were observed to be purified and they were found to be of the appropriate length.

3.2.2 Transfection assay

Throughout the experiment standard aseptic techniques were followed. The mammalian cell line COS-7, which doesn't have any CR in the cells, was grown in Dulbecco's Modified Eagle Medium (DMEM-Invitrogen) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin (Sigma), 2mM glutamine (Sigma), and 10% denatured fetal calf serum (Invitrogen) in a humidified atmosphere with 5% CO_2. For recovery from liquid nitrogen storage, cells were propagated two times a week for two weeks (4 passages) in 75 cm^3 flasks with 20 ml medium inoculated with 0.5 million cells.

For the transfection assay, 20000 cells were inoculated in 1ml medium in each well of 12-well plates. Four hours before transfection and throughout the rest of the experiment, cells were maintained in DMEM nutrient mixture F-12 Ham (no phenol red, Sigma) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 3.7 g/liter NaHCO3, and

2.5% desteroided denatured fetal calf serum (Sigma). Cells were transiently transfected by the Gene juice transfection reagent (Novagen). The transfection mixture, prepared for a 12-well plate, contained 2 μg of the receptor expression vector (pCMrtGR2), 4 μg pFC31Luc and 1 μg pSVβ. Twelve hours after transfection, the medium was renewed and the GCs (Sigma) were added from 1000-fold concentrated stock solutions in ethanol. After 36h incubation, cells were harvested with lysis buffer (Promega) and extracts were analyzed for luciferase (Promega) and β-galactosidase activities. Solvent-controls (receiving ethanol instead of hormone), with triplicate cell cultures per treatment, were included in each assay.

For the luciferase assay, 10 μl of cell extract were placed in a well of a 96-well plate and read by the luminometer (Glo max – Promega) with setting of 10 second reading and 2 seconds delay time using single injector for addition of 50 μl reagent buffer to each well, so that the luminometer can perform readings immediately after the addition of reagent. This ensures loss of activity is prevented.

For the β-Galactosidase assay, KP buffer at pH 7.3 was prepared with $K_2HPO_4.3H_2O$ and KH_2PO_4 (Fisher, UK). ONGP and 2-mercaptoethanol were purchased from Sigma. For a 96-well plate, 13.7 ml KP buffer, 240 μl $MgCl_2$/ 2-mercaptoethanol and 5.3 ml ONGP stock were mixed. 200 μl of this mixture was added to 50 μl of cell extract in clear 96-well plates and incubated at 37°C. After yellow colour formation, reaction time was noted and the plate was read by the spectrophotometer (Spectramax 340PC) at 420 nm.

Luciferase activity was corrected for well-specific transfection efficiency (as determined by β-galactosidase activity) and transcriptional activity was expressed as the percentage of the luciferase activity, considering the activity observed in cells treated with 10^{-6} M hydrocortisone as 100% activity. Third order polynomial curves were obtained with Excel and were optimised with data-fit software (version 9: oakdale engineering-

www.oakdaleengr.com) in order to calculate the EC50 (concentration of hormone to produce half maximal activity) values. Each assay was repeated three times with freshly prepared ingredients.

3.2.3 Optimization of assay

Transfection efficiency depends on the amount of each different plasmid present in the transfection mixture. Therefore preliminary assays in triplicates were performed with different ratios of receptor plasmids, luciferase reporter plasmid and β-galactosidase reporter plasmid with hydrocortisone and solvent control. The ratio that produced maximum transcriptional activity was chosen for the assays described above.

3.3 Results

Figure 3.6 reveals the relative potencies of the top 10 most used corticosteroids with trout GR2. Dexamethasone was found to be most potent and had high efficacy for most of the concentrations tested, followed by beclomentasone and betamenthasone. EC50s for the above three GCs were not significantly different from each other, but were significantly different from the rest of the tested GCs. Similarly, clobetasone had significantly lower potency and efficacy than any of the other GCs. Fluticasone, momethasone, budesonide, prednisolone, methyl prednisolone and hydrocortisone were in that order of potency with the GR. However, their EC50 values did not differ significantly.
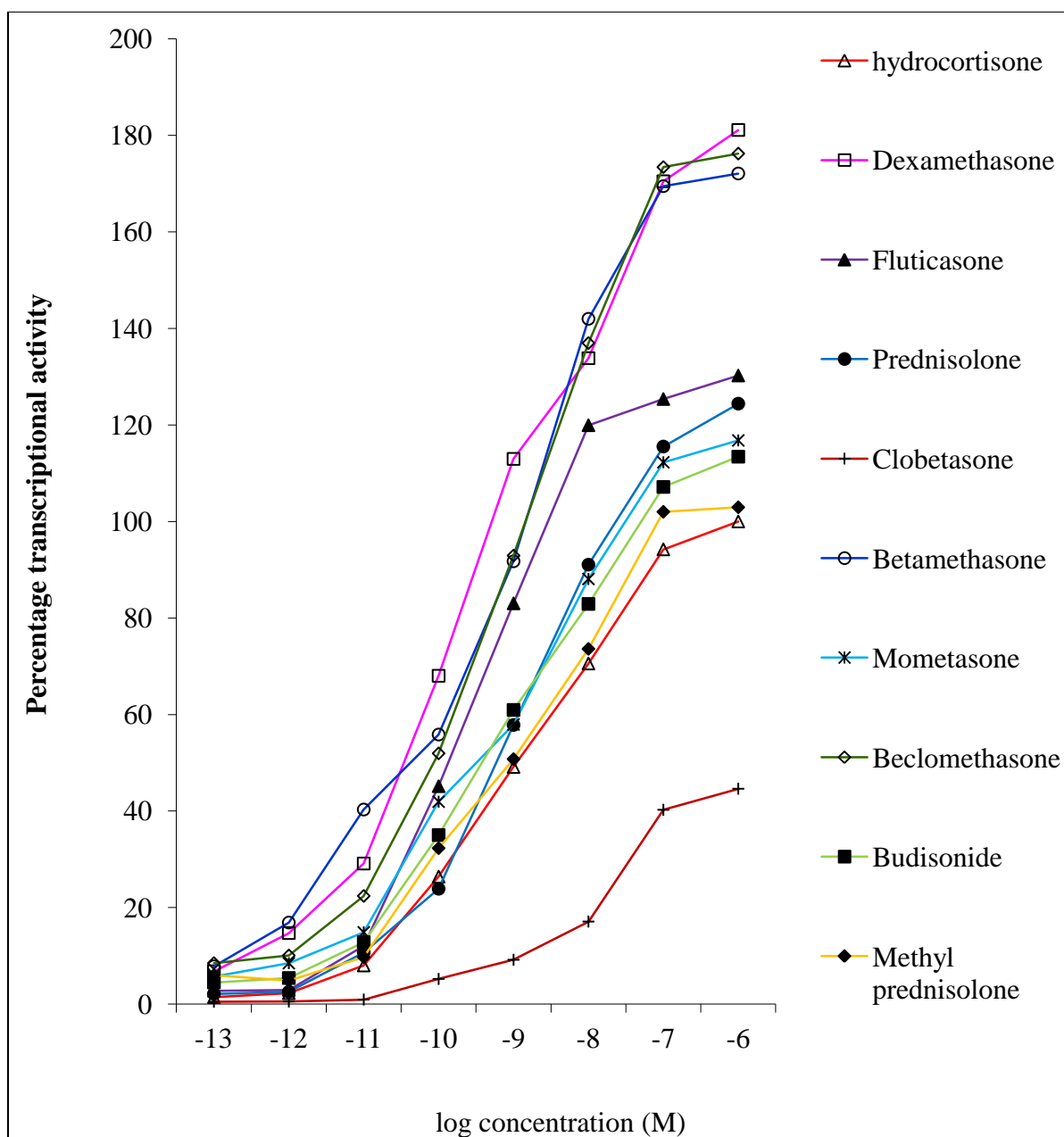
Figure 3-6. Percentage transactivation activity (as luciferase activity normalised against β-galactosidase activity for the transfection efficiency) of COS-7 cells co-transfected with trout GR2 expression vector plasmid as well as with luciferase reporter plasmid and β-galactosidase expression plasmid. After transfection, cells were treated with different GCs over a range of concentrations. Luciferase activity of 10^{-6} M Hydrocortisone was considered as 100% activity. Values are the average of three replicates.

The EC50 values of the binding of each of the ten GCs to both GR1 and GR2 were

determined, and are given in Table 3.1. The 10 GCs showed no activity to the MR, and hence

no EC50 values could be calculated. Therefore, as is the case in mammals (BNF 2006), these ten GCs could be classified as low, medium and high potency for GR2. Trout GR1 also responded to all the corticosteroids, but with lower transcriptional activity. Potencies with GR1 and GR2 are in a similar, although not identical, order.

Table 3-1. EC50 values of tested GCs with trout GR2 and GR1. Values are the mean and standard deviation of three independent experiments performed in duplicate

Corticosteroid	EC50 with trout GR2 (nM)	EC50 with trout GR1 (nM)
Dexamethasone	0.386 ± 0.02	40.98 ± 2.43
Beclomethasone	0.737 ± 0.07	44.78 ± 1.98
Betamethasone	0.771 ± 0.09	42.34 ± 2.78
Fluticosone	0.805 ± 0.02	45.09 ± 4.56
Mometasone	0.816 ± 0.01	47.24 ± 3.90
Budisonide	0.856 ± 0.11	52.64 ± 6.32
Prednisolone	1.12 ± 0.03	52.09 ± 7.98
Methyl prednisolone	1.15 ± 0.12	52.13 ± 4.41
Hydrocortisone	1.22 ± 0.12	50.21 ± 2.54
Clobetasone	1.86 ± 0.91	61.09 ± 9.02

The average of the maximal level of transcription of each of the GCs with all three CR was calculated from three replicates. None of the GCs produce significant activity with MR (Figure 3.7) although cortisol, prednisolone and methyl prednisolone had slightly higher activity with MR than that of the solvent control. Transcription activity of GR2 was higher than that of GR1 with all tested GC.
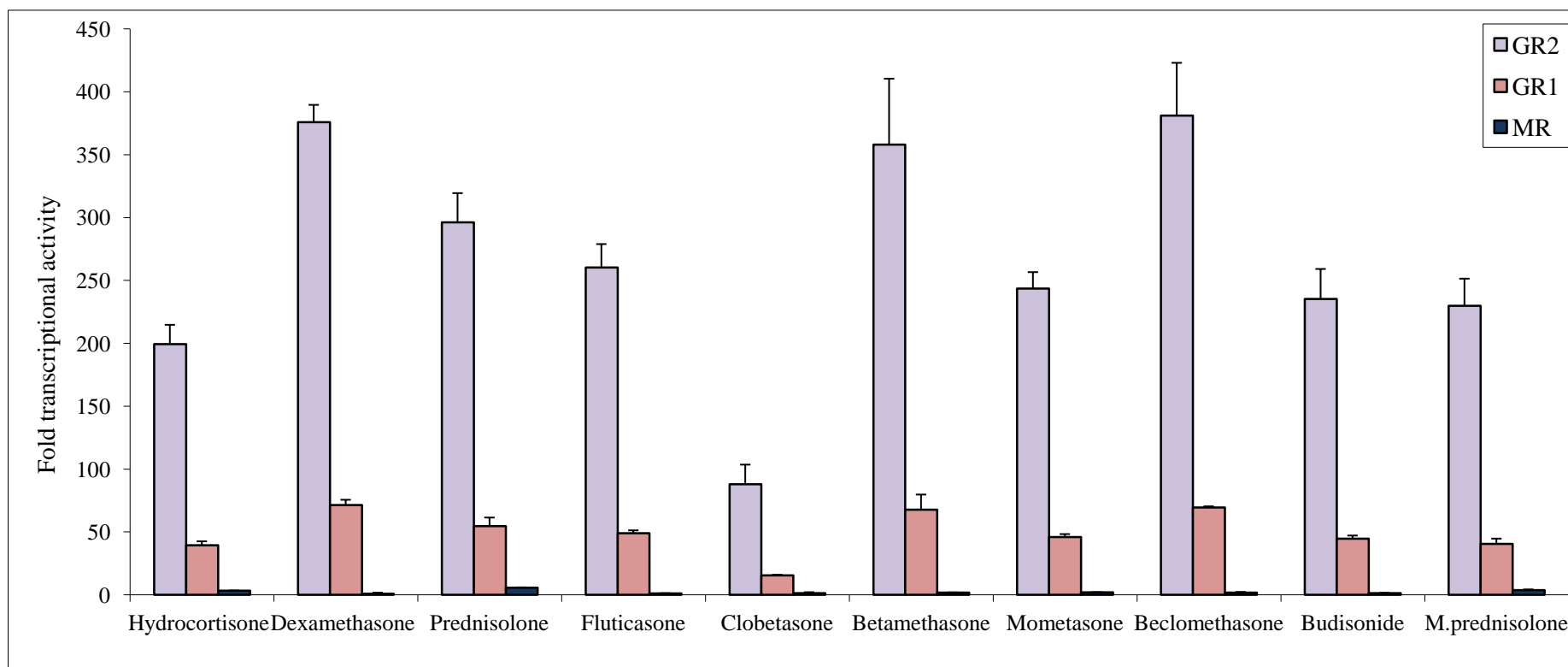
Figure 3-7. Transcriptional activities (as measured by luciferase activity normalised to β-galactosidase activity and presented as fold of activity of the solvent control) of the ten most highly consumed GCs in the UK, measured at 1μM concentrations, in COS-7 cells co-transfected with trout GR2, GR1 or MR expression vector plasmid as well as with luciferase reporter plasmid and β-galactosidase expression plasmid. Values are the mean of three replicates and the standard deviation.

3.4   Discussion

The present study is the first to report the comparison of the potencies of a significant number of the synthetic GCs used in the UK. Dexamethasone, as in human (*in vivo*; BNF 2006), was found to be a high potency GC. Although beclomethasone dipropionate is second in the list, the amount (274 kg/year compared to 28 kg/year for dexamethasone) used in the UK makes it environmentally significant. Another GC that falls into the high potency group was betamethasone, which is also environmentally significant as the usage calculated as 305 kg/year. In the medium potency group, prednisolone is an important GC, with 1880 kg calculated as the annual usage. Human GR transfected in COS-7 cells showed higher transcriptional activity for betamethasone esters compared to dexamethasone and prednisolone (Spika *et al.,* 2003). However, physico-chemical properties of individual chemical and their behavior in STPs vary and it is difficult to conclude which chemical is of the greatest threat to aquatic organisms. Nevertheless, the relative potencies of different GCs obtained from this experiment can be used to predict the effect of a mixture of GCs, as might occur in the environment, in a similar way that the effects of mixtures of oestrogens have been predicted (Thorpe *et al.*, 2003a; Brian *et al.*, 2005). This is because all GCs have a similar GR-mediated mode of action.

The results described in previous transfection studies with trout GR and cortisol are consistent with the results of the present study. Bury et al. (2003) have also reported higher luciferase activity for trout GR2 than for GR1, as occurred in this study. The differences in the transcriptional sensitivities were explained by the differences in the amino acid sequences in the regions of DNA binding and transactivation between GR1 and GR2 (Bury *et al.*, 2003). EC50 values of hydrocortisone for GR2 and GR1 fell within the range previously reported (Prunet *et al.*, 2006; Sturm *et al.*, 2005) employing similar assays using COS-7 cells.

Another recent detailed study on trout GRs provide more evidence for the higher sensitivity of GR2 (Sturm *et al*., 2011). In this study, GR mutants were constructed with different combinations of domains from GR1 and GR2 and it was found that the presence of the E domain of GR2 reduced the EC50 values of tested corticosteroids compared to constructs containing the E domain of GR1(Figure 3.8). Further cloning of subsequences of E domain was also carried out and all results with these confirmed that GR2 is more sensitive to GCs.
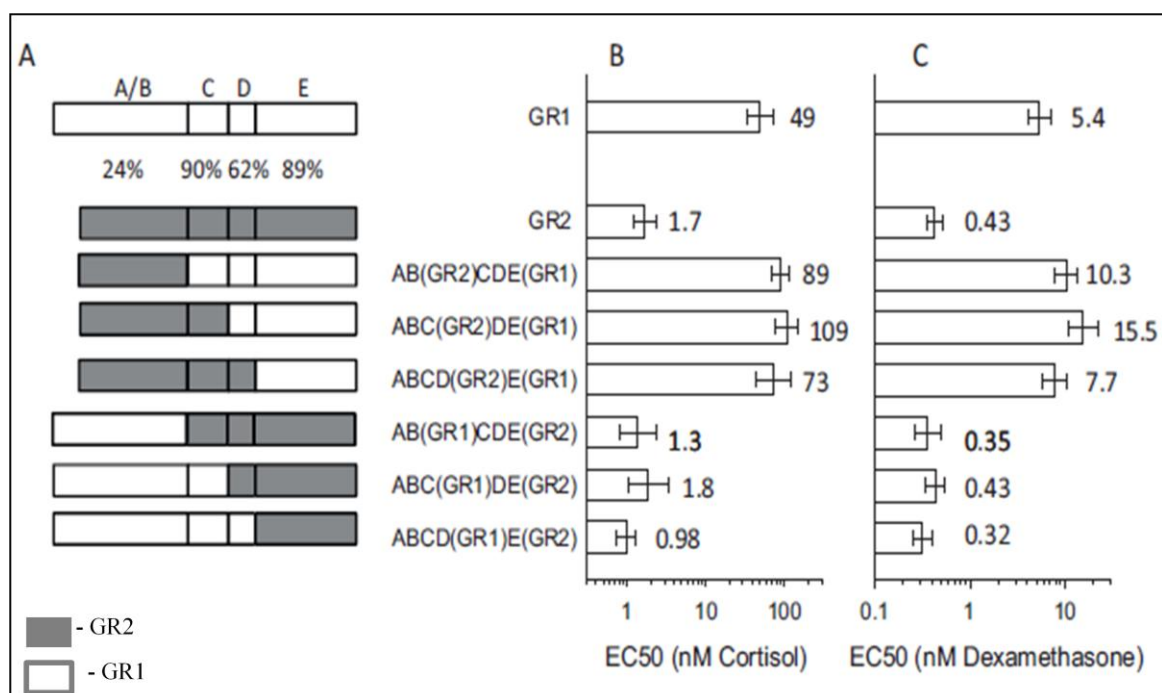
Figure 3-8. Transactivation properties of domain-swap chimeras between rtGR1 and rtGR2. Transactivation assays were carried out in COS-7 cells. (A) Graphical representation of the domain composition of recombinant domain-swap mutants. Percentages indicate amino acid identities between rtGR1 and rtGR2 for the main receptor domains. (B) and (C) Median effective concentrations (EC50s) and 95% confidence limits of the stimulation of transactivation activity by cortisol (B) and dexamethasone (C). Numbers at bars denote the EC50 in nM. EC50s are considered significantly different if confidence limits do not overlap (Sturm *et al*., 2011).

Trout GR and MR were used in the present study despite the fact that fathead minnows were used in the *in vivo* exposure experiments (Chapter 4). This is because the trout CR plasmids

were available at the time of this study, whereas those for the fathead minnow were not. There have been small differences in susceptibility to oestrogenic chemicals reported between small fish species, such as medeka, zebrafish and fathead minnow, *in vivo* (Seki *et al*., 2006). However, in the same study there were no differences in the response to androgenic compounds. It has been reported that there were no significant differences in the affinity of 4-nonylphenol and p-octylphenol for oestrogen receptors (ERβ) among human, quail and medaka *in vitro* (Nishizuka *et al*., 2004). Therefore, it is assumed that the differences in sensitivity between different species of fish are very small and hence, the potency of different drugs for a particular receptor can probably be assessed using any species of fish. However, recent results on the midshipman GR and sensitivity to GCs, as explained above, need some verification, and if repeatable, could change the current view that different species of fish show very similar responses to the same chemical.

Another interesting finding from the present study is the fact that none of the tested GCs produced significant activity with trout MR. Although there were slightly higher activities found for hydrocortisone, prednisolone and methyl prednisolone compared to the solvent control, none of them were significant. *In vitro* ligand binding and transactivation of the human GR and MR were found to be similar (Rupprecht *et al*., 1993), and mammalian MR has been found to bind cortisol (a glucocorticoid) and aldosterone (a mineralocorticoid) with equal affinity (Funder *et al*., 1988). But in fish the situation is different. Fish tissues are unable to produce aldosterone and instead cortisol regulates both glucocorticoid and mineralocorticoid activities (reviewed in Bury *et al*., 2003). But the *in vitro* sensitivity of MR for different corticoids (both mineralocorticoid and glucocorticoid) has been a debate for a long time, and it has not been sorted out yet. For example, Colombe *et al*. (2000) reported that fish MR binds cortisol or corticosterone rather than aldosterone, *in vitro*. Sturm *et al*. (2005) suggested 11-deoxycorticosterone but not cortisol or aldosterone is the major agonist for fish

MR. Recent results on midshipman MR (Arterbery *et al*., 2011) suggests that Aldosterone and cortisol bind well to fish MR. Interestingly, midshipman MR too did not bind to cortisone. Sturm *et al*. (2005) also found that the affinity of dexamethasone with trout MR was weaker than the affinities of cortisol and aldosterone. There was no evidence for other synthetic GCs with fish MR. This inability of synthetic GC to induce MR *in vitro* could be an interesting factor when the fish are exposed to GCs. Whether GCs have an impact on osmoregulation and other MR-mediated physiology has to be studied *in vivo*.

In vivo potencies for many different GCs in human volunteers have been assessed by several methods. They greatly vary with the method of administration and the type of assay performed. After topically administrated, budesonide was 2-3 times more potent than beclomethasone dipropionate in inducing vasoconstriction, whereas after oral administration budesonide was 2-4 times less potent than beclomenthasone in depressing plasma cortisol and reducing the total WBC (Johansson *et al*., 1982). A medium potent GC, hydrocortisone, was found to have similar neurotoxic side-effects as that of the highly potent dexamethasone in a recent investigation (Aden *et al*., 2008). Although there is no comparable *in vitro* study involving all these 10 GCs, a combination of several studies using various methods to estimate the potencies of GCs (such as vasoconstrictor assay/ skin blanching assay) with mammalian and human GR (BNF, 2006) indicates that the potencies of these GCs in mammals/humans are in a similar order as is reported in the present study for the fish GR.

Recombinant yeast screen assays have a number of advantages, such as the absence of other endogenous receptors, comparatively easy to establish, and no cell lysis is needed (Routledge and Sumpter, 1996). However, the transfection assay in the form of ER-CALUX has been shown to be 20 times more sensitive than the yeast oestrogen screen, possibly due to the fact that the yeast cell membrane permeability is low compared to mammalian cell membrane

permeability (Legler *et al*., 2002). Therefore, this transfection assay, based on mammalian cells rather than yeasts, should be a useful tool to compare the activity of different synthetic corticosteroids in fish. Mammalian cell lines (CHO, COS-7) transiently transfected with fish GR and MR have been shown to respond to natural and some synthetic corticosteroids (Prunet *et al*., 2006; Sturm *et al*., 2005). Specifically, cortisol binds to and induces the transcriptional activity of both GR and MR. The very low rate of GC metabolism in COS-7 cells (Spika *et al*., 2003) makes COS-7 cells an ideal model for transfection studies.

The results presented here are specific for this cell line and for the assay conditions specified. In a similar transfection study with human CRs, relative transactivation was found to vary among three different cell lines tested (Lim-Tio *et al*., 1997). However, the order of potency, whether high, medium or low, of different ligands was consistent between cell lines (i.e. whole sets of dose-response curves were shifted to the left or right, changing the EC50 values). Therefore, results on the order of potency in the present experiment are probably repeatable in any cell line.

There are two basic requirements for a chemical to have the potential to induce adverse effects in fish; specifically regarding GCs, they should be:

1) Released to the environment, and resistant enough to degradation to be present in rivers.

2) Highly potent, with the ability to bind strongly to fish GR, and produce GR-mediated effects, at the low concentrations present in the aquatic environment.

Bearing this in mind, ten of the most prescribed GCs were chosen for the *in vitro* study. It was assumed that even though GCs of lesser usage volumes might be of higher potency than any of these ten, they may not be available to fish because environmental concentrations would be extremely low. It should be noted, though, that from a concentration addition (Brian

et al., 2005) point of view, all the 28 GC presently in use in the UK could contribute to the total concentration of GCs present in the aquatic environment. However, it is difficult to correlate differing *in vitro* sensitivities with the situation *in vivo.* A first line hypothesis would be that, at low environmental concentrations of GCs, GR2 will bind and transcribe a set of genes to produce GR-mediated impacts, while as environmental concentrations increase, GR1 will also participate to enhance the impacts. Given the fact that the presence of GR1, GR2 and MR is organ-specific (for example, Stolte *et al*., 2008, report higher levels of GR1 in fish brain, while higher GR2 levels exist in the pituitary), it will be interesting to study the *in vivo* effects on fish exposed to different concentrations of GCs.

3.5 Conclusions

The present study reveals that fish GRs respond to synthetic GCs and transcribe GR-responsive genes that can produce significant effects in fish. Fish GR2 is more sensitive to all the tested GCs compared to GR1. Close similarities of the amino acid sequences of the GRs of many different species of fish suggest that the potencies of GCs will be similar in any fish of interest. Although these results, with the support of the read-across hypothesis, confirm that synthetic GCs can affect fish, some complicating issues arise due to the sensitivity differences among receptors and the organ-specific presence of receptor variants. Therefore *in vivo* experiments with different concentrations of GC should be conducted in order to provide the data needed for robust environmental risk assessment.

Chapter 4.0

The Potential Impacts Of Glucocorticoids On Adult Fathead Minnows (*Pimephalas Promelas*)

4.1 Introduction

Pharmaceuticals and personal care products are extensively and increasingly used and hence are released continuously into the environment. A variety of pharmaceuticals have been detected in many environmental samples worldwide (Chapter 1). They have been reported to be present in sewage treatment plant effluents, surface water, seawater, groundwater, drinking water, soil, sediment and fish (Ternes, 1998; Jones *et al*., 2001; Heberer, 2002; Zuccato *et al*., 2006; Lopez-Serna *et al*., 2010). It has also been reported that drug manufacturing site effluents (Larsson *et al*., 2007) and hospital waste waters (Kummerer, 2001) can contain high concentrations of human pharmaceuticals that are sufficient to produce adverse effects on biota. Pharmaceuticals, their metabolites and transformation products in the environment may have adverse effects on living organisms (Khetan and Collins, 2007; Corcoran *et al*., 2010; Burkhardt-Holm, 2010). For example, the classic non-steroidal anti-inflammatory drugs ibuprofen (De Lange *et al*., 2006) and diclofenac (Triebskorn *et al*., 2004) have been reported to have acute toxicity to algae and invertebrates and to fish at environmentally-relevant concentrations, and residues of diclofenac are the cause of the almost complete loss of the populations of three species of vultures in the Indian sub-continent (Oaks *et al*., 2004).

Pharmaceuticals present in the environment may or may not adversely affect the aquatic organisms. It depends on many different physico-chemical properties of drugs, such as water solubility, partition coefficient, bioavailability, bioconcentration factor, receptor binding affinity and interaction with other co-factors. It is important to emphasize that currently it is not possible to know whether or not the concentrations of the human pharmaceuticals present in the aquatic environment are adversely affecting aquatic organisms. This is because reliable measurements of concentrations of pharmaceuticals in rivers (where fish live) are very sparse and because few of the reported effects of pharmaceuticals on various species of aquatic organisms have been independently verified.

For more than a decade, the issue of oestrogenic chemicals in the aquatic environment, and their possible effects on fish and other wildlife, has been a topic of very considerable interest. EE2 is an extremely potent oestrogen; concentrations below 1ng/litre have adverse effects on reproduction of fish, and only slightly higher concentrations prevent fish reproducing (e.g. Länge *et al*., 2001; Nash *et al.,* 2004; Parrott and Blunt, 2005), leading to population crashes (Kidd *et al*., 2007). Thousands of research papers have been dedicated to the various aspects - both chemical and biological – of the oestrogenic chemicals in the aquatic environment issue (reviewed by Caldwell *et al*., 2008). However**,** none of the other classes of steroidal pharmaceuticals have received much attention. Given the ease with which steroids can enter fish from the environment (Maunder *et al*., 2007), this represents a significant knowledge gap. The recent demonstration that synthetic progestogens can adversely affect fish at very low concentrations (Zeilinger *et al*., 2009; Paulos *et al*., 2010) has confirmed the need to closely evaluate the environmental effects of steroidal pharmaceuticals. GCs are also a group of steroidal chemicals with a similar mode of action to EE2. Currently nothing is known about the impacts of synthetic GCs in the environment. Previous chapters have described that amount of GCs present in the aquatic environment could be many-fold higher than that of EE2. Further, GCs can bind to fish GRs as they do with human GR. These conclusions together form the basis for a scientific hypothesis that GCs present in the environment may have adverse impacts on fish. The present chapter describes the experiments conducted to test the null hypothesis that environmentally relevant concentrations of GCs do not have adverse impacts on fish.

4.1.1   Test Species

Chemical toxicity results obtained with one species are often used to extrapolate to other species. In ecological risk assessments this is a challenging task because toxicity tests with

one species need to be predictive of possible effects on thousands of untested species. Therefore it is wise to use a species that is very sensitive to toxicity and can act as representative of many other aquatic species. Fish have been reported to be the most sensitive organisms for many aquatic pollutants (Gunnarsson *et al*., 2008), and many of the toxicity pathways in fish which are comparable to those in humans have been well studied. Fish have been used in toxicological studies over the past century. In year 2009, a total of 438000 toxicology procedures, including those for safety and efficacy evaluation, were conducted in the UK, of which 20% used fish as experimental animal (statistics on animal research published by Home Office: http://www.homeoffice.gov.uk). In the early days of ecotoxicology, the most common species involved in such tests were trout, salmon, bluegill and goldfish. But using large fish, with long life spans, make the full life cycle tests tedious and expensive. Being larger, they also may be able to withstand the negative impacts of environmentally-relevant concentrations of chemicals that could harm other smaller species and their susceptible early life stages. Therefore it became reasonable to use the early developmental stages (embryo studies) and smaller species with relatively shorter life cycles in order to assess the impacts of chemicals that are present in the environment at trace amounts.

Small fish, commonly used to accurately assess potential chronic risks of chemicals via life cycle tests or embryo tests incorporating sub-lethal endpoints, are largely freshwater species, such as the fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) (Ankley and Johnson, 2004). Recently, the three-spined stickleback (*Gasterosteus aculeatus*) has been reported to be a successful fish model for testing androgenic endocrine disruptors, as it has a quantifiable *in vivo* androgen and anti-androgen endpoint, the production of the glue protein, spiggin (Katsiadaki *et al*., 2002).

The fathead minnow is classified phylogenetically under Actinopterygii, Cypriniformes, and is in the Family Cyprinidae. Wild fathead minnows inhabit muddy pools, small rivers and ponds. The species is an opportunistic omnivore that feeds on detritus and algae, and spawns in still water habitats along the banks. These fish can tolerate a wide range of poor water quality characteristics, including pH, turbidity, hypoxia and temperature (www.fishbase.org). The adult fish clearly show sexual dimorphism with pronounced secondary sexual characteristics (SSC; Figure 4.1). The male fathead minnow is larger than the female (3–5 g versus 2–3 g, respectively) and, when reproductively active, exhibits dark banding over the entire body, a dorsal fat pad, a black spot on the dorsal fin, and nuptial tubercles, all of which are not normally seen in females. Females have slightly protruding ovipositors. These SSC can be qualitatively and quantitatively characterised in order to see the feminisation or masculinisation effect of a chemical. In the fathead minnows SSC are under endocrine control. Body colour (light or dark), coloration patterns (presence of vertical bands), body shape (head and pectoral region), and SSC (size of dorsal pad, number of nuptial tubercles in male fathead minnow, ovipositor length in females) are all reported to be affected by EDCs. For example, androgen receptor agonists, such as methyltestosterone and dihydrotestosterone, can cause female fathead minnows to develop pronounced nuptial tubercles (Smith, 1974; Ankley *et al*., 2001; Panter *et al*., 2004). It has been reported that oestrogen receptor agonists and androgen receptor antagonists can decrease nuptial tubercle number and size of the dorsal fat pad in adult males (Miles-Richardson *et al*., 1999).
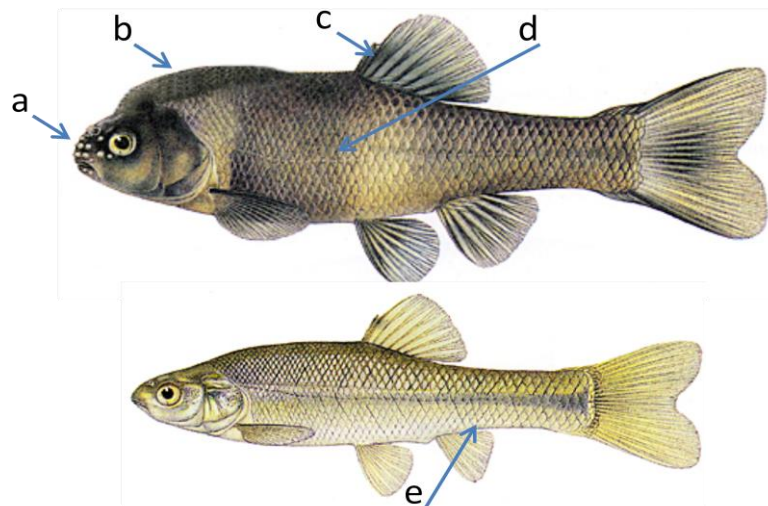
Figure 4-1. External morphology of male (upper) and female (lower) Fathead minnows showing secondary sexual characters. a-nuptial tubercle, b-dorsal fat pad, c-dark dorsal fin spot, d-dark banding, e-ovipositor. (http://aquaticpath.umd.edu/fhm/intro.html)

Courtship behaviour in the fathead minnow is elaborate and relatively well-defined. Following spawning, males are highly territorial, chasing other males and actively guarding nest sites where the adhesive eggs have been deposited by the females. In the laboratory by providing an artificial nest with an egg-depositing surface, daily egg counts can be made in a reproductive performance assay. The fathead minnow short term reproductive assay was one of the three assays recommended for initial screening of EDCs (the other two are rodent and amphibian assays) by the USEPA.

Fathead minnows were chosen for the present study because they have a short life cycle (maturity in 4-5 month post-hatch), their basic reproductive and endocrine physiology are well reported (Jensen *et al*., 2001; Leino *et al*., 2005), many toxicity endpoints have been well defined (example: vitellogenin (Vtg), a biomarker for oestrogenic chemicals; Sumpter and Jobling, 1995) and still being scrutinised (Watanabe *et al*., 2007; Dang *et al*., 2011), and they are relatively easy to culture and the reproductive cycle can be controlled by altering temperature and photoperiod (reviewed in Ankley and Villeneuve, 2006). Compared to other

small fish species, fathead minnows provide enough blood to enable one to measure the plasma concentrations of glucose, cortisol and Vtg. The only shortcoming of the fathead minnow compared to the zebrafish is that the molecular biomarkers are not well defined, whereas in the zebrafish the whole genome has been reported. However, fathead minnows have been used very successfully in the past for assessing the impacts of various endocrine disrupters (Panter *et al*., 2002; Lange *et al*., 2001; Brian *et al*., 2005; Williams *et al*., 2007; Giltrow *et al*., 2009; Paulos *et al*., 2010).

4.1.2   Fish Toxicity Tests

Many different types of toxicity tests with fish are performed for different purposes. For example, the European commission has set requirements for any chemical of which marketing quantity exceeds one ton per year per manufacturer. These requirements include acute toxicity for freshwater fish (96h, LC50), acute toxicity for daphnids (48h, EC50) and growth inhibition test on freshwater algae (growth rate: 72h, ErC50 and/or biomass: 72h, EbC50). Similar requirements have been in place in USA with fish, amphibian and rodent toxicity assays. The data obtained are used for deciding on the classification of the chemical and for hazard and risk assessment (calculation of PNEC) of the substance. These short term toxicity tests are also used as preliminary test for finding the range of concentrations for the chronic exposure experiments. Fish toxicity testing is also an important element of Whole Effluent Toxicity (in USA) testing and Whole Effluent Assessment or Direct Toxicity Assessment (in Europe; Chapman, 2000). These assessments recommend short-term toxicity testing with fish. However, some European countries are now moving towards alternative test methods, such as the fish embryo test (reviewed by Nagel, 2002).

The OECD has established test guidelines using fish as test organisms for the testing of acute toxicity (OECD 203), prolonged toxicity test (OECD 204), early life-stage toxicity (OECD

210), short term toxicity test on embryo and sac-fry stages (OECD 212), and juvenile growth test (OECD 215). There is increasing concern about water quality in developed countries, particularly in North America and in Europe, not only concerned with acute toxicity of single compounds but also with the sub-lethal effects of complex mixtures of compounds at much lower concentrations. With respect to potentially adverse effects following long-term exposure to sub-lethal concentration of chemicals (chemical mixtures), more emphasis has been given to the development of methodologies to identify more specific modes of toxic action, e.g. endocrine disruption. Thus, OECD expert groups are currently developing modified test guidelines, which incorporate more sophisticated endpoints.

One of the very successful tests for assessing the impacts of EDCs is the partial life cycle reproduction test, the so-called pair breeding test (Harries *et al*., 2000), which is usually a 21-day assessment of egg production and terminal comparison of metabolic, morphological and histological endpoints. In this test, each pair of fish is assigned a separate tank, so that the egg production endpoint per pair of fish is accurately measured. This is extremely labour-intensive and expensive, hence it is only suitable if the EDC being tested is expected to affect reproduction, preferably egg production.

The other test method involves a chronic exposure of adults in groups placed in separated tanks, each tank receiving a different concentration of chemical, together with a control tank (OECD 230). This test guideline describes an *in vivo* screening assay for certain endocrine active substances where sexually mature male and spawning female fish are held together and exposed to a chemical during a limited part of their life-cycle (in this experiment at least 3 weeks exposure is considered as chronic). This assay covers the screening of oestrogenic and androgenic activity, and also aromatase inhibition. The assay was validated by the OECD on the fathead minnow, the Japanese medaka and the zebrafish; however, androgenic activity is

not tested in zebrafish. At the end of the 21-day exposure period, depending on the species used, one or two biomarker endpoint(s) are measured as indicators of oestrogenic, aromatase inhibition or androgenic activity of the test chemical; these endpoints could be Vtg (fathead minnow, Japanese medaka and zebrafish) and SSCs (fathead minnow and Japanese medaka only). Another test method is the full life cycle test (Lange *et al.*, 2001), which is tedious and expensive, but the results can be more convincing. Therefore before any regulatory decisions are made, all or most of the tests discussed above could be conducted with a range of concentrations of a chemical of interest.

Although the group exposure (OECD 230) protocol is often criticised for its 'pseudo-replicate' nature (unless repeated results are obtained, their validity is in question), the advantage of this test is that large number of fish can be exposed and an endpoint that has a relatively narrow range of variability between different concentrations assessed. The experiments described in this chapter have adopted this group exposure scenario, mainly because the main endpoint expected, plasma glucose concentration, would probably not vary more than 2 to 3-fold between different tested exposure concentrations. Another endpoint is related to immune-suppression, and the reproductive endpoint had least priority.

4.1.3   Possible Endpoints

Predicting the potential impacts of pharmaceuticals present in the environment that have not been tested previously with fish is challenging. Because most pharmaceuticals are designed to affect biological receptors/enzymes that are mostly conserved across vertebrate families, the read-across hypothesis has been suggested as a promising approach (Huggett *et al.*, 2004; Owen *et al.*, 2009). This involves an efficient use of mammalian data to better understand and predict the potential for a given pharmaceutical to impact the environment. For most pharmaceuticals, mode of action, therapeutic target, plasma concentrations, metabolism,

excretion and therapeutic dosage data for humans are available from the literature and/or manufacturer. Across-species extrapolation of these data suggests there is potential for impacting amphibian and fish in the aquatic environment, depending, of course, on the level of contamination of the environment.

The impacts of GCs on fish could be predicted from their therapeutic target, their side effects in humans after long term treatment, the effects of natural GCs (cortisol and corticosterone) on human physiology on human physiology and from the responses of fish to stress. GCs are mainly used as anti-inflammatory and immunosuppressive drugs. Their target receptors are present in almost all parts of the body. A major biochemical effect of GCs is increasing hepatic glucose output by stimulating hepatic gluconeogenesis, while depressing protein synthesis or stimulating protein catabolism in muscle. A number of hepatic enzymes concerned with gluconeogenesis exhibit marked increases in activity after the administration of GCs. Among these enzymes are glucose-6-phosphatase, fructose-6-diphosphatase, and phosphoenolpyruvate carboxy kinase (Mommsen *et al*., 1999).

Suppression of the inflammatory response by corticosteroids has long been investigated. This usually results in reduction of the number of circulating lymphocytes. Suppression of the hypothalamic-pituitary-adrenal system is another frequently reported impact induced by corticosteroids in human. Regulation of ACTH-dependent steroidogenesis is accomplished by long or external loop negative feedback. Corticotropin-releasing factor from the hypothalamus activates ACTH release by the anterior pituitary, and ACTH stimulates the conversion of cholesterol to cortisol in the adrenal cortex. As cortisol concentrations rise in the blood, binding sites in the hypothalamus are occupied, and corticotropin-releasing factor is no longer synthesized and released, until concentrations of cortisol in the extracellular fluid decline. In fact, the standard procedure to evaluate the effects of GCs or to compare their *in vivo*

potencies is the measurement of the reduction in GC production via urinary cortisol concentrations or salaivary cortisol concentrations. Significant reduction in adrenal weight within 5 to 10 days after the beginning of corticosteroid therapy has been reported from earlier studies on the side effects of GCs in human and it is generally acknowledged that adrenal atrophy is apparent in nearly all species tested after 10 days of high dose corticosteroid therapy (Salassa *et al*., 1953).

Other side effects of GCs often reported are Cushing's syndrome, myopathy, osteoporosis-vertebral compression, fractures, aseptic necrosis of bone, peptic ulceration gastric haemorrhage, intestinal perforation, pancreatitis, psychiatric disorders (often called steroid psychosis), pseudo cerebral tumour, glaucoma, hypertension, thrombosis, vasculitis, sodium and water retention-oedema, hypokalemic alkalosis, ketoacidosis, diabetes mellitus, hyperlipidemia, obesity, growth failure, secondary amenorrhea, inhibition of fibroplasias, impaired wound healing, subcutaneous tissue atrophy, suppression of the immune response, superimposition of a variety of bacterial, fungal, viral, and parasitic infections, hypogonadism and hirsutism (reviewed in Melby, 1977; Schacke *et al*., 2002).

Physiological impacts of endogenous cortisol are similar to the reported stress responses, since it is well-known that stressed animals have elevated cortisol concentrations. Stress responses have been classified as primary response (increased cortisol, catecholamines), secondary response (metabolic changes: increased glucose, lactate, and decreased tissue glycogen; cellular changes: increased heat shock proteins; osmoregulatory disturbances: Na, K, water; haematology: leucocrit, haemoglobin, lysozyme activity) and tertiary response (on growth, swimming performance, feeding and reproduction: reviewed in Sapolsky *et al*., 2000). Elevated cortisol as a consequence of stress has been reported to affect the HPG axis

via a negative feedback loop and inhibit the synthesis of gonodal steroids (Pickering *et al*., 1987; Campbell *et al*., 1992).

In vivo studies on GCs and fish are very rare. Most studies reported so far have applied treatments via implanted capsules or food, and involved high concentrations of GC compared to the expected environmentally-relevant concentrations (Pickering *et al*., 1987; Carragher *et al*., 1989). No studies have been reported using the environmentally-relevant exposure route, via the water, so there is no established endpoint corresponding to Vtg for oestrogen or egg production for progestins. Therefore several endpoints should be studied before adopting a reliable endpoint. The present study is the first report on the effects of chronic exposure of fish to low concentration of synthetic GCs.

4.1.4 Objectives

The main objective of the experiments present in this chapter was to assess the impacts of environmentally-relevant concentrations of GCs on Fathead minnows.

Specific objectives were

- To assess the potential impacts of two different GCs (prednisolone and beclomethasone dipropionate) at the same concentration on fathead minnows, in order to see if the potency differences observed *in vitro* could be verified *in vivo*, and also to determine the range of variations in the endpoints analyzed.
- To assess the potential impacts of beclomethasone dipropionate in a range of different concentrations, in order to see if the responses of fish are concentration-related.
- To suggest a LOEC for beclomethasone dipropionate on fathead minnows.

4.2 Materials and Methods

4.2.1 Experiment-1: Mixed Sex Adult Fathead Minnows Exposed to Two Different GCs

In order to assess the impact of low concentrations of GCs *in vivo*, a 21-day exposure experiment was conducted with adult fathead minnows, in 30 L glass tanks, using a continuous flow-though system. Fish (12 months old) were selected from a breeding stock maintained at Brunel University and were fed three times per day, once with adult brine shrimp (Tropical Marine Centre, Gamma irradiated) and twice with flake food (King British Tropical flake food, Lillicos, Surrey). One group of 10 fish was exposed to 1µg prednisolone/L, another group was exposed to 1µg beclomethasone dipropionate/L and the third group served as control. Although egg production was not assessed, fish were provided with plastic tiles (3 in each tank) as a spawning surface, in order to reduce the competition and stress level. Aerators with air stones were used to maintain sufficient dissolved oxygen concentrations.

Prednisolone (CAS no: 50-24-8, 99% purity, Sigma-Aldrich, UK) and Beclomethasone dipropionate (CAS no: 5534-09-8, 99% purity, Sigma-Aldrich, UK) were dissolved in ethanol and stock solutions (1mg/L) were freshly prepared in 2.5 L amber bottles every 4th day, by dissolving the GC in double-distilled water and stirring vigorously overnight. Stock solutions were dosed at 18 ml/h, using a Watson Marlow (Cornwall, UK) multi-channel peristaltic pump, into glass mixing vessels (aspirator bottles), where they mixed with dechlorinated tap water (at 18 L/h) before delivery to each fish tank to produce the desired concentrations. Flow rates and dosing efficiency were monitored daily to ensure that GC entered the fish tanks at the expected rates. All tubing within the system was medical grade silicon. Dosing of the tanks with GCs was carried out for a week prior to fish being put into the tanks, to allow the

system to equilibrate. During this equilibration period, fish were acclimatised in similar experimental conditions. Temperature (25.2 ± 1.02°C) and dissolved oxygen (7.12 ± 0.92 mg/L) were monitored daily throughout the experiment. Water samples (500ml) were taken 3 times on the days when the stock solutions were changed and kept frozen for analysis.

4.2.1.1   Terminal Sampling of Fish

After 21 days of exposure, fish were terminally anaesthetised using 200 mg Tricaine Methane sulphonate/ L (MS-222; Sigma, Poole, UK) that was buffered with sodium bicarbonate (1:1). Netting to blood sampling was completed as quickly as possible, to avoid elevation of the plasma cortisol concentration. During the procedure, fish were treated humanely with minimal suffering. Weight in grams and fork length in mm were measured. The tail was then removed from each fish and blood was withdrawn using 75μl capillary tubes, then decanted into eppendorf tubes (on ice) which contained an enzyme inhibitor, aprotinin (Sigma). The cut edge of the tail was used to make a blood film on a microscopic slide for staining and differential cell counting. 25μl fresh blood was transferred into 1975μl Natt-Herrick's stain solution (NaCl, 1.94 g; Na_2SO_4, 1.25 g; Na_2HPO_4, 0.87 g; KH_2PO_4, 0.125 g; 37% formalin, 7.5 mL; and methyl violet 0.05 g made up to 500 mL in distilled water and filtered) in an eppendorf tube for total cell count using a haemocytometer. Blood samples were kept on ice until plasma was separated by centrifugation for 4 min at $14,000 \times g$, and this was then stored at -20°C for glucose, cortisol and Vtg measurements.

Fish were dissected to obtain the liver and gonads. Weights of these organs were measured and livers were snap-frozen in liquid nitrogen and then placed in a -80°C freezer. Gonads were fixed in 5 ml Bouin's solution (Sigma) for histological analysis. It was noted that 3 to 4 minutes were taken to complete each fish sampling (from netting to dissection).

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Condition factor, K, was calculated from the equation $K = 100 \times W/L^3$, where W is the weight in grams and L is fork length in cm. Liver-somatic index (LSI), which expresses the size of the liver in relation to the body weight, was calculated by using the equation LSI = (liver weight in gram/body weight in gram) × 100. Gonadosomatic index (GSI) expresses the size of the gonad in relation to the body weight, and was calculated by using the equation GSI = (gonad weight in gram/body weight in gram) × 100.

4.2.1.2 Plasma Glucose Measurement.

Blood glucose concentration was determined using a quantichrome kit (Universal Biological, UK). This method uses as little as 5 µL sample volume, which is an advantage for fathead minnow as this species provides only gives small volumes of blood sample. It has a linear detection range from 0.7 mg/dL (39 µM) to 300 mg/dL (16.6 mM) and is conducted in 96-well plates. All the procedures were carried out under a chemical fume hood. Standards were prepared in distilled water as shown in Table 4.1.

Table 4-1. Glucose standards for plasma glucose assay

No	Standard (provided from kit) + Water	Total volume (µL)	Glucose concentration (mg/dL)
1	150 µL + 0 µL	150	300
2	100 µL + 50 µL	150	200
3	50 µL + 100 µL	150	100
4	25 µL + 125 µL	150	50
5	0 µL + 150 µL	150	0

5 µL diluted standards and samples were transferred into appropriately labelled 1.5-mL eppendorf tubes. 500 µL reagent was added to each tube. Tubes were closed tightly and the contents were mixed well. Tubes were placed in a boiling water bath for 8 minutes and then cooled in a cold water bath for 4 minutes. 200 µL of the resultant solution was transferred in duplicate into a clear bottom 96-well plate. Optical density at 650 nm was recorded using a

spectrophotometer (Spectramax 340PC). Plasma glucose concentrations were obtained directly from the linear plot equation and are presented here as mean ± SD for control and treatment groups of fish.

4.2.1.3 Leukocyte Counts

Total blood cell count and leukocytes counts were performed based on the method described by Morgan *et al*. (1993). For total cell counts, a thin film of Natt-Herrick's stained blood was made on a haemocytometer and an average of 5 square counts was taken to calculate total cell numbers. A micropipette was used to load the counting chamber of the haemocytometer.

Air-dried blood smears were fixed in methanol for 5 minutes and stained with acid haematoxylin and eosin for differential cell counts. Due to the difficulties in differentiating sub-populations of leukocytes under a light microscope, it was decided to differentiate RBCs from WBCs and the proportion of WBCs was estimated from a count of a total of 200 cells. From this proportion, total WBCs were extrapolated from the haemocytometer counts and are presented as the number of WBCs (in thousands) / μL blood.

4.2.1.4 Plasma Cortisol Measurement

Plasma cortisol concentrations were measured with an Enzyme Immunoassay Kit (Cambridge Bioscience: Cat. No. 900-071), according to the manufacturer's instructions. Frozen plasma samples and the kit contents were allowed to reach room temperature while preparing the ELISA bench and automated plate washer. Cortisol standard solution (100,000 pg/mL) from the kit was serially diluted with assay buffer, and the standard solutions (10,000, 5,000, 2,500, 1,250, 625, 313 and 156 pg/mL, respectively) for the assay were prepared immediately before every assay. Wash Buffer was prepared by diluting 5 mL of the supplied concentrate with 95 mL of deionized water and it was then placed into the appropriate bottle in the automated plate washer. From the preliminary assay with different dilutions of samples, it was decided to

dilute the control group fish plasma 30-fold and the treated group fish plasma 20-fold, using 10 µL plasma and appropriate amount of assay buffer in order to keep the OD values within the working range of the ELISA.

All standards and samples were run in duplicate. For each plate, non-specific binding (NSB), total activity (TA) and blank were included. 100 µL of standards and diluted samples were pipetted into appropriate wells (except for TA, blank and NSB). 100 µL of assay buffer was pipetted into NSB wells. 50 µL of blue Conjugate was pipetted into each well, except the TA and Blank wells. 50 µL of antibody was pipetted into each well, except the Blank, TA and NSB wells. The plate was then sealed and incubated at room temperature on a plate shaker for 2 hours at ~500 rpm. After 2 hours, the contents of the wells were emptied and the plate was washed three times by adding 400 µL of wash solution to every well. After the final wash, the wells were emptied, and the plate was firmly tapped on a lint-free paper towel to remove any remaining wash buffer. 5 µL of the blue Conjugate was added to the TA wells. 200 µL of the substrate solution was added to every well using a multichannel pipette and the plate was incubated at room temperature for 1 hour without shaking. After the incubation, 50 µL of stop solution was added to every well using a multichannel pipette and the plate was read immediately for optical density at 405 nm using a spectrophotometer (Spectramax 340PC).

The mean OD value of both blank wells was subtracted from the mean OD values of each duplicate of samples and standards. The average net OD bound for each standard and sample was calculated by subtracting the average NSB OD from the average OD bound. For each pair of standard wells and sample wells, the percentage of the maximum binding was calculated. Using a Logit-Log paper plot, percent bound versus concentration of cortisol for the standards was plotted. A straight line through the points was approximated, and the unknowns were

determined by interpolation. The few samples which fell outside the working range of the assay were repeated in a similar assay with appropriate dilutions of sample.

The above kit had cross-reactivity for the following compounds: Cortisol (100%), Corticosterone (27.68%), 11-deoxycortisol (4.0%), Progesterone (3.64%), Prednisone (0.85%), Testosterone (0.12%), Androstenedione (<0.1%), Cortisone (<0.1%) and Estradiol (<0.1%).

4.2.1.5 Measuring Concentrations of GC in Experimental Tanks

Prednisolone concentrations in tank water samples were measured with a specific Enzyme Immunoassay Kit (Cambridge Bioscience: Cat. No. 900-071), according to the manufacturer's instructions. This kit was developed for cortisol, but prednisolone was reported to have 122% cross-reaction.

Prednisolone from water was extracted using diethyl ether. In a fume hood, 10 mL of Diethyl Ether was added to 10 mL of sample and then shaken well for 5 minutes. Layers were allowed to separate for 5 minutes. By placing the test tube into liquid nitrogen, the water phase was frozen solid, allowing the ether phase to be transferred into a clean test tube. The ether was evaporated to dryness under nitrogen stream. Extracted prednisolone was dissolved in 1 mL assay buffer and vortexed well. This was diluted 1:5 with assay buffer during the assay. The ELISA procedure was similar to that of cortisol, as described in section 4.2.1.4.

Beclomethasone dipropionate did not have any cross-reaction with the antibody in the kit described above, so no measured concentrations for beclomethasone dipropionate are presented for this experiment.

4.2.2　Experiment 2: Concentration-Related Exposure to Beclomethasone Dipropionate

In order to assess the concentration-related impacts of a highly potent GC, beclomethasone dipropionate, a 21-day exposure experiment was conducted with adult fathead minnows, in 30 L glass tanks, using a continuous flow-though system. A diagrammatic representation of the experimental set-up is presented in Figure 4.2. Briefly, three different concentrations (10 µg/L, 1 µg/L and 100 ng/L) and a control tank with no chemical added were run in duplicate. 10 males and 10 females were grouped into each tank. Fish (14 months old) were selected from a breeding stock maintained at Brunel University and were fed three times per day, once with adult brine shrimp (Tropical Marine Centre, Gamma irradiated) and twice with flake food (King British Tropical flake food, Lillicos, Surrey).
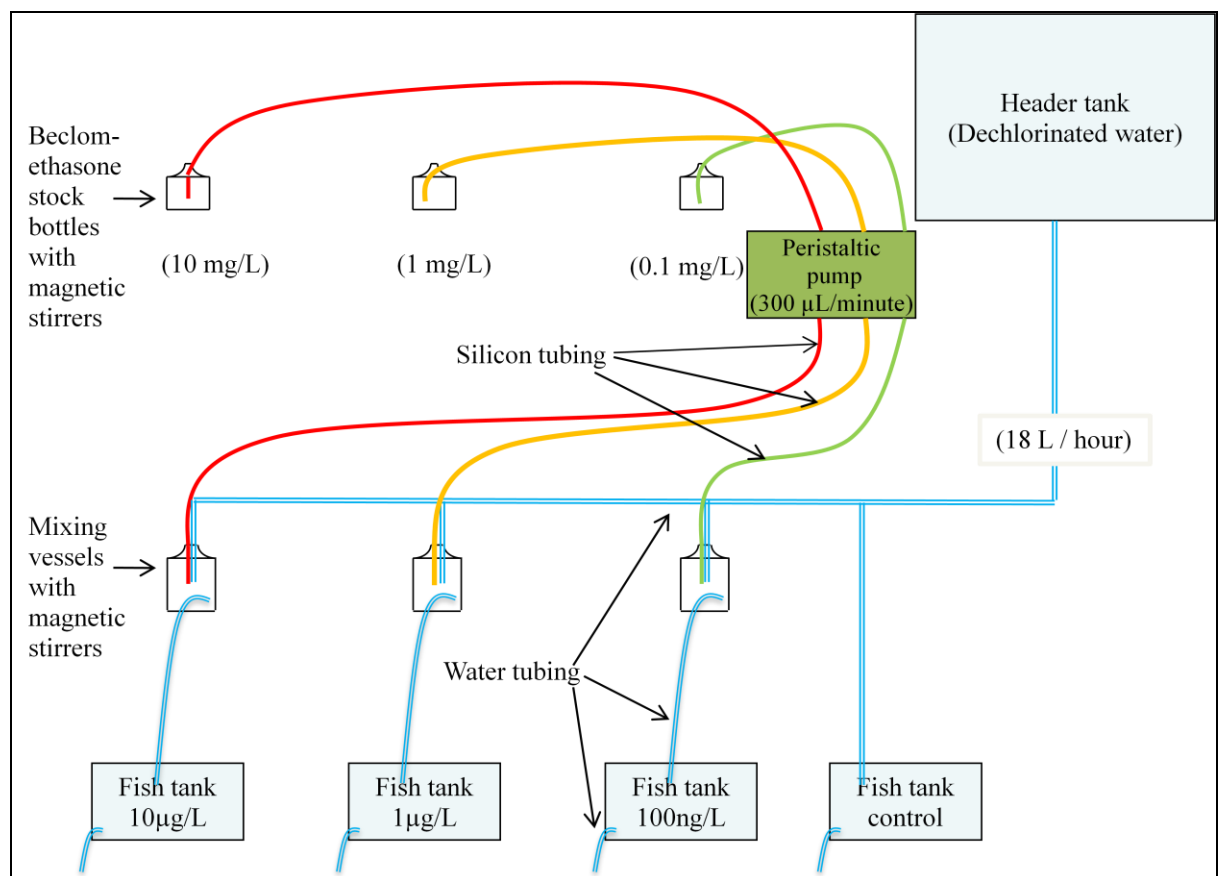
Figure 4-2. The experimental set-up used for the concentration-response exposure of fathead minnows to beclomethasone dipropionate for 21 days.

Beclomethasone dipropionate (CAS no: 5534-09-8, 99% purity, Sigma-Aldrich, UK) was dissolved in ethanol and stock solutions (10 mg/L, 1mg/L and 0.1 mg/L) were freshly prepared in 2.5 L amber bottles every 4th day, by dissolving the master stock in double-distilled water and stirring vigorously overnight. Stock solutions were dosed at 18 ml/h, using a Watson Marlow (Cornwall, UK) multi-channel peristaltic pump, into glass mixing vessels (aspirator bottles), where they mixed with dechlorinated tap water (at 18 L/h) before delivery to each fish tank to produce the desired concentrations. Flow rates and dosing efficiency were monitored daily to ensure that GC entered the fish tanks at the expected rates. All tubing within the system was medical grade silicon. Dosing of the tanks with GCs was carried out for a week prior to fish being put into the tanks, to allow the system to equilibrate. During this equilibration period, fish were acclimatised in similar experimental conditions. Temperature ($25.1 \pm 1.21°C$) and dissolved oxygen (7.02 ± 0.47 mg/L) were monitored daily throughout the experiment. Water samples (500 ml) were taken 3 times on the days when the stock solutions were changed and kept frozen for analysis.

4.2.2.1 Sampling

The sampling protocol was similar to that described in section 4.2.1.1. It was noted that 3 to 4 minutes were taken to complete each fish sampling (from netting to dissection).Sampling was started from control tanks and moved towards the higher concentration tanks on day one and vice-versa on day two inorder to avoid the diurnal variations in the endogenous steroids levels. Especially in this experiment, any RNA contamination across samples was excluded by wiping the dissection tools and table with RNAse away (Fisher) before and after every dissection. This is because the liver samples from this experiment were analysed for gene expression (Chapter 5). Immediately after the plasma separation, blood cells were sorted by percoll separation as described in Section 4.2.2.4.

4.2.2.2 Assessing Secondary Sexual Characters

After blood sampling, length and weight measurements, sex was recorded and SSC was qualitatively and quantitatively assessed by adopting the procedures of Smith (1974) and USEPA (2002). In males, nuptial tubercles were counted (the head was immersed into liquid nitrogen to make tubercles clearly visible) under a magnifying glass and they were graded from 1 to 3, where 1 = present, 2 = enlarged, 3 = pronounced. Fat pad height was measured in mm and then it was removed with a scalpel, taking care not to remove any underlying muscle tissue. The weight of each fat pad was measured and fat pad index (FPI = fat pad weight (mg)/ body weight (mg) $\times$ 100) was calculated using fat pad weight and body weight of each fish.

For females, ovipositor length was measured in mm with vernier callipers. The dorsal fin was examined for the presence of a black spot or band. Fat pad appearance was recorded and graded as follows:

1: No fat pad visible

2: Small fat pad evident

3: Fat pad is clearly visible and is just above body surface

4: Fat pad is prominent, and is clearly above the body surface, but not 'overhanging'

5: Fat pad is very prominent and is starting to 'overhang' the body surface

4.2.2.3 Plasma Glucose Measurement.

Blood glucose was determined using a quantichrome kit (Universal biological, UK) as described in Section 4.2.1.2.

4.2.2.4 Leukocyte Counts

Blood cell sorting and counting were carried out by percoll separation and flow cytometry according to the methods described by Inoue *et al*. (2002) and Morgan *et al*. (1993). These methods were slightly modified because of the low blood volume of the fathead minnow. Instead of using whole blood, blood cells after plasma separation (as described in section 4.2.1.1) were reconstituted with 200 µL Hank's balanced salt solution (HBSS; Sigma). This cell suspension was carefully overlaid onto 200 µL of 1.085 g/ml Percoll (Sigma) in 0.85% NaCl and centrifuged at 1400 g for 15 minutes. Under these conditions, erythrocytes and basophils form a pellet at the bottom of the tube, while the neutrophils, monocytes, lymphocytes and thrombocytes remained at the saline-Percoll interface. The interface layer was collected with a Pasteur pipette and reconstituted in HBSS. A stock solution of 3,3-dihexyloxacarbocyanine (DiOC6(3) dye; Sigma) was prepared in ethanol at 500 µg/ml. Ten times diluted stock solution in HBSS was prepared just before staining. 25µL of reconstituted leukocytes, 50µL of dye solution and 1925µL HBSS were mixed in a flow cytometer tube and left at room temperature for 10 minutes.

After staining with DiOC6(3), blood cells were analyzed using flow cytometry. From each sample, 40000 cells were sorted. Forward scatter (FS), side scatter (SS) and green fluorescence (FL) of each cell was measured. As an example (shown in Figure 4.3), FS vs SS plots were obtained for each sample and gates for different sub-populations of leukocytes were set as J, D and H. As lymphocytes and thrombocytes cannot be differentiated by flow cytometry, they were considered as single group (J). D and H correspond to neutrophils and monocytes. For each sample, the percentage of lymphocyte plus thrombocyte of the total number of blood cells was calculated.
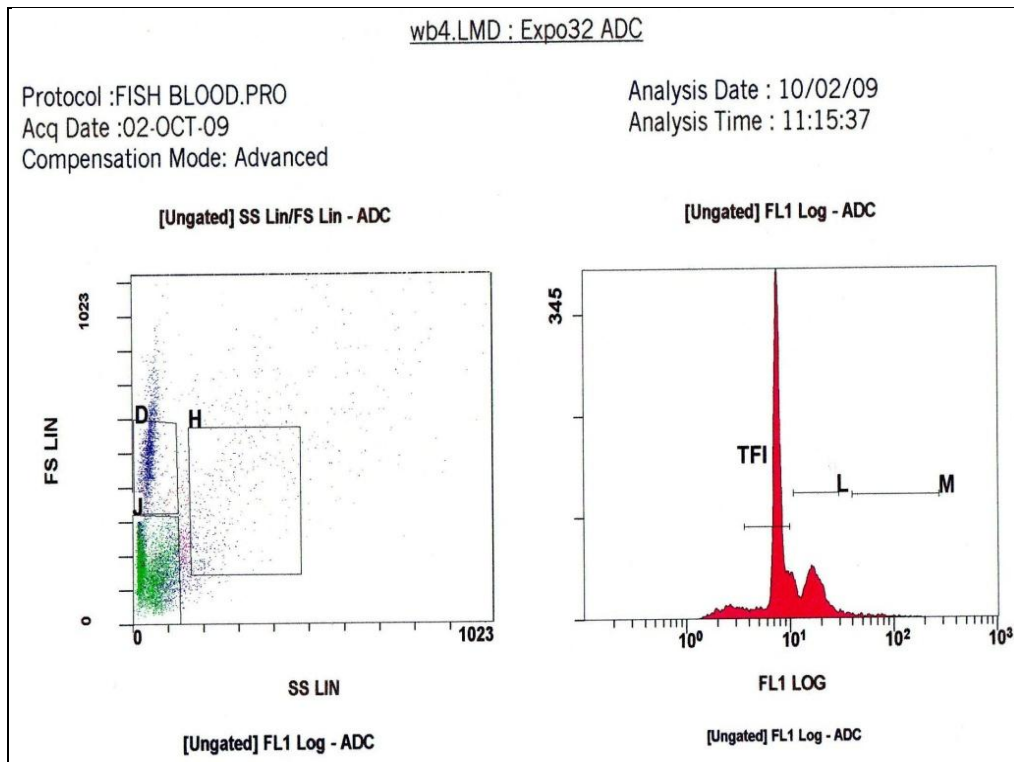
Figure 4-3. Flow cytometer generated dot plot of leukocyte cells stained with DiOC6(3). On the left: Forward light scatter vs side scatter plot where three sub-populations are gated as J (Lymphocytes and Thrombcytes), D (Neutrophils) and H (Monocytes). On the right: Florescence intensity (in logarithmic scale) vs frequency plot where florescence of three sub-populations are differentiated as TF1(Lymphocytes and Thrombcytes), L (Neutrophils) and M (Monocytes).

4.2.2.5 Plasma Cortisol Measurement

Plasma cortisol concentrations were measured with an Enzyme Immunoassay Kit (Cambridge Bioscience: Cat. No. 900-071), according to the manufacturer's instructions as described in section 4.2.1.4. Since most female fish provided very small volumes of blood, not all fish were included in this assay. The number of samples measured in each treatment is presented in the results.

4.2.2.6 Plasma Vtg Measurement

For all female fish, plasma Vtg concentration was measured by fathead minnow sandwich ELISA kit (Biosense, Norway). This ELISA utilises specific binding between antibodies and Vtg to quantify Vtg concentrations in samples from fathead minnow. The wells of the microplates have been pre-coated with a specific capture antibody that binds to Vtg present in standard and sample added to the wells. A different Vtg-specific detecting antibody, labelled with the enzyme horseradish peroxidase (HRP), is added to create a sandwich of Vtg and antibody. The enzyme activity is determined by adding a substrate that gives a coloured product, and the colour intensity is directly proportional to the amount of Vtg present. This ELISA has been validated and used by many laboratories for measurement of fathead minnow Vtg concentrations (Eidem *et al.*, 2006).

Throughout the assay, careful and precise pipetting at every step in the assay was maintained. Whenever a multichannel pipette was used, reverse pipetting was done to avoid the air bubble effect. In all dilutions a vortexer was used instead of shaking the sample to avoid foaming.

Since doing the dilutions of samples and preparation of standards needs time, part of the procedure was done the day before actual assay. Dilution buffer ($5\times$ concentrate) was made up into 1x concentrate with distilled water and stored in a 4°C fridge. A washing buffer (PBS, 0.05% Tween-20) tablet was dissolved in 1 L distilled water on a magnetic stirrer and placed into the appropriate bottle in the automated plate washer. The required amount of TMB substrate solution was measured out in a 15-mL falcon tube and kept in the dark at room temperature. The remaining TMB was placed in a fridge ar 4°C. 0.3M H_2SO_4 was prepared from the stock. All the eppendorf tubes were labelled for standards and sample dilution and arranged ready for use the next day.

Detecting antibody (vial E) was diluted 1:500 by adding 24 µl to 12 ml Dilution buffer for each plate immediately before running the assay. As Vtg is an unstable molecule, all standard and sample dilutions were done on ice. Frozen samples were thawed on ice. Using the fathead minnow Vtg standard (vial G) from the kit and cold dilution buffer, two-fold serial dilution of 11 Vtg standards were prepared from 50 ng/mL to 0.05 ng/mL.

As Vtg concentrations have been found to be highly variable in the past, each sample was diluted to 1:5000, 1:50000 and 1:500000 (Per Com: Nicola Beresford) in order that one dilution was on the linear part of the standard curve. Each standard and sample was run in duplicate, and NSB wells were included for each plate. These wells are used to determine Non-Specific Binding (unspecific background signal). 100 µl of each fathead minnow Vtg standard and sample dilution were added to appropriate wells and the plate was incubated at room temperature (20-25°C) for 1.5 hour.

The plate was then washed three times with 300 µl washing buffer per well. 100 µl of the diluted detecting antibody was then added to all wells. The plate was sealed and incubated at room temperature (20-25°C) for 30 minutes. The plate was then washed five times with 300 µl Washing buffer per well. 100 µl TMB substrate solution was then added to all wells. The plate was covered with aluminium foil and incubated at room temperature (20-25°C) for 20 minutes. The reaction was stopped by adding 100 µl 0.3M H_2SO_4 to all wells. At that satge absorbance at 450 nm was read with a spectrophotometer (Spectramax 340PC).

The mean of the absorbance values of the two NSB wells was calculated and this value was sustracted from the absorbance values of all other wells on the same plate. This gives the NSB-corrected absorbance values for standard and sample dilutions.

The mean of the NSB-corrected absorbance values for each set of standard duplicates was calculated and the standard curve was constructed using linear curve fit. A working range was

selected and the unknowns were interpolated from this and the concentration of Vtg in each sample was calculated according to the dilution of the sample.

4.2.2.7 Gonadal Histology

Histological procedures involved tissue fixation, dehydration, paraffin embedding, microtome sectioning, staining and finally differentiating cells under the microscope. Sections of ovary were stained first with haematoxylin, which stains nuclear material dark blue or purple, and then with eosin, which counter-stains cytoplasm and erythrocytes a pink colour.

On the sampling day, ovaries were dissected in a caudal-to-cranial direction while applying gentle traction to the oviduct in order to minimise trauma. The ovaries were fixed in 5 mL Bouin's solution (Sigma), which was replaced by 70% industrial methylated spirits (IMS) after 24 hours, and again after 48 hours. Each ovary was sliced into 3 transverse sections with a microtome blade and the sections were placed into a plastic tissue cassette submerged in 70% IMS for histological processing. Tissues were maintained in the following solutions for the period of time mentioned, using the automatic tissue processor (TP 1020, Leica Inc.).

70% IMS	3 hours
90% IMS	2.5 hours
95% IMS	1.5 hours
100 % IMS	6 hours
Histoclear	4.5 hours
Paraffin Wax	2.5 hours

Histomount and histoclear were purchased from National Diagnostics, USA. Paraffin wax and eosin were purchased from RA Lamb Inc. Haematoxylin was purchased from VWR Inc. and Li_2CO_3 was from Sigma-Aldrich, UK. Acid Alcohol was prepared from 70% HCl and IMS as 1:99.

After the tissue processing as described above, ovary slices were embedded into wax blocks.
Tissues were sectioned to 4 μm thickness using the RM 2235 microtome (Leica Inc.), and
placed onto microscope slides coated with Histobond (RA Lamb, UK). They were dried for
~48 h before staining with Eosin and Haematoxylin on an automated staining machine
according to the following protocol.

Histoclear	15 minutes (Dissolves wax)
100% IMS	2 minutes
90% IMS	2 minutes
70% IMS	2 minutes
Water	2 minutes (Rehydration)
Haematoxylin	15 minutes (Stains nuclear material)
Water	15 minutes (Wash)
Acid Alcohol	5-30 seconds (Resolves stain)
Water	20 seconds (Wash)
Saturated Li_2CO_3	20 seconds (Raises pH and removes any Bouin's residue)
Eosin	5-20 seconds (Counter-stains)
Water	5 minutes (Wash)
70% IMS	2 minutes
90% IMS	2 minutes
100% IMS	5 minutes (Tissue dehydration)
Histoclear	5 minutes (Helps mount cover slip)

Finally, the stained sections were mounted using histomount and covered with cover slips.
The slides were examined under an Olympus BX51 compound light microscope and
photographs were taken using a digital camera and the Q-Capture Pro v. 5.1.1.14 program
(Media Cybernetics Inc.).

Examination of all histological samples was conducted blindly, without any knowledge of the sample identity or its treatment. The ovaries were examined for the presence of oocyte atresia, a process in which oocytes (at any point in development) degrade and are resorbed. Atretic oocytes are characterized by clumping and perforation of the chorion, fragmentation of the nucleus, and disorganization of the ooplasm (EPA, 2009).

For each female fish, 6 cross sections of ovary were analysed. Cells were categorised as vitellogenic stage, cortical alveoli stage, bulbiani body stage and perinucleolar stage (Figure 4.4), and in each cross section, the number of cells falling into each category was counted on a Gateway 2000 computer using the free UTHSCSA ImageTool program (developed at the University of Texas Health Science Center at San Antonio, Texas and available from the Internet by anonymous FTP from ftp://maxrad6.uthscsa.edu). Reference pictures to support the classification of follicle types in fathead minnow were obtained from Jensen *et al*. (2001) and Wolf *et al*. (2004). The percentage of each cell type in every cross section examined was calculated and compared.
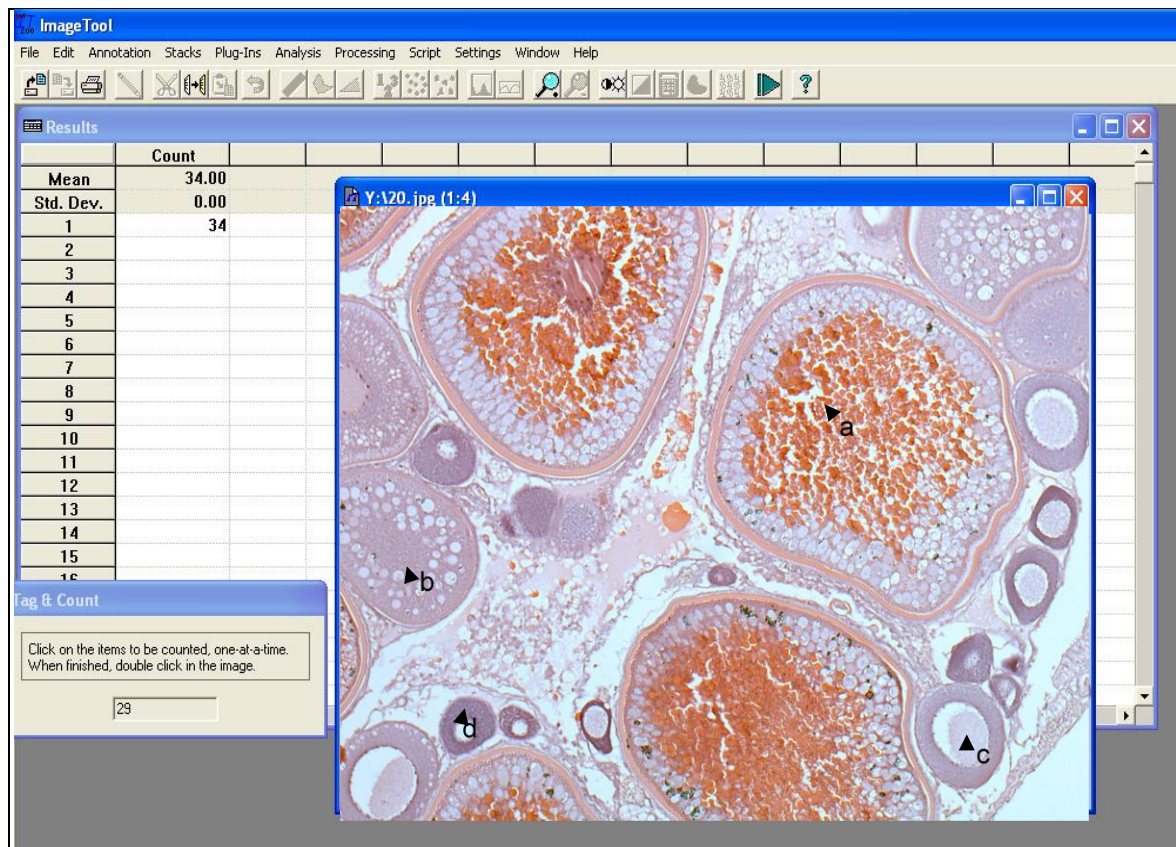
Figure 4-4. A light micrograph showing the four different stages oocytes. a: vitellogenic stage, b: cortical alveolar follicle, c: balbiani body stage and d: perinucleolar stage, counted with image tool software.

4.2.3 Statistical Analyses

Experimental results are presented as means ± standard deviations. All statistical analyses were carried out using SigmaStat 3.5. As appropriate, normality tests and equal variance tests were performed and statistical significances were tested with a t-test or ANOVA. If datasets passed the equal variance test, one way ANOVA followed by a pair-wise comparison (with Holm-Sidak test or Tukey test) was done. If the data failed an equal variance test, then non-parametric one way ANOVA on ranks (Kruskal-Wallis) followed by a Tukey test was done. In this case, median values are indicated in addition to means. $p < 0.05$ was considered to be significant.

For some dose-response effect in the second experiment, in addition to ANOVA, trend analysis (using SPSS: Jonckheere-Terpstra (JT) test) was done. JT test is a non-parametric test for ordered differences among classes. It tests the null hypothesis that the distribution of the response variable does not differ among classes. It is designed to detect alternatives of ordered class differences. For such ordered alternatives, the JT test can be preferable to tests of more general class difference alternatives, such as the Kruskal-Wallis test.

4.3   Results

Endpoints measured in both experiments (example, plasma glucose and cortisol) are presented together. In experiment-2, the group of fish exposed from day 0 to day 21 is named as set-1 and the duplicate set exposed from day 1 to day 22 is named as set-2. In experiment-2, data from set-1 and set-2 were analysed separately, and the results compared by student t-test. If there were no differences between set-1 and set-2, data were pooled (unless otherwise stated; as an example, Vtg concentrations were analysed only in females, so n = 20) to give n= 40 for each treatment. This approach provided more power in the statistical tests.

4.3.1   General Condition of Fish

There was no mortality observed due to exposure to GCs. None of the treatments significantly affected the whole body condition or organ indices, except for LSI (Table 4.2). There was a decreasing trend in the LSI with increasing concentrations of Beclomethasone dipropionate experiment (Figure 4.5, green bars). But it was not significant by non-parametric test. Male (Figure 4.5, blue bars) and female (Figure 4.5, pink bars) populations were analysed separately and this revealed that female LSI had a significant decreasing trend ($p < 0.001$), whereas the LSI of males was not affected by the GC. Since gonad weights are different from female and male, GSI data were analysed separately, but there were no significant differences due to the treatment.

Table 4-2. General body measurements from both experiments

Experiment	Treatment and number of fish	Condition factor	GSI	LSI
1	Control (5 male + 5 female)	1.55±0.17	M: 1.63±0.14 F: 13.42±1.83	M:1.53± 0.51 F: 3.24±0.90
	1 µg Prednisolone/L (5 male + 5 female)	1.62±0.17	M: 1.88±0.42 F: 13.02±2.84	M: 2.10±0.56 F: 3.11±0.60
	1 µg Beclomethasone dipropionate/L (5 male + 5 female)	1.53±0.26	M: 1.33±0.34 F: 12.22±4.89	M: 1.60±0.38 F: 3.63±3.43
2 (set-1+set-2)	Control (20 male + 20 female)	1.46±0.18	M: 2.03±0.81 F: 12.95±5.09	M:1.89± 0.37 F: 3.19±0.56
	100 ng beclomethasone dipropionate/L (20 male + 20 female)	1.47±0.27	M: 1.53±0.29 F: 11.63±3.53	M: 1.88±0.31 F: 2.68±0.45
	1 µg beclomethasone dipropionate /L (20 male + 20 female)	1.50±0.22	M: 1.57±0.56 F: 12.02±5.81	M: 1.88±0.38 F: 2.42±0.41
	10 µg beclomethasone dipropionate /L (20 male + 20 female)	1.48±0.17	M: 1.57±0.43 F: 13.23±5.04	M: 1.87±0.29 F: 2.23±0.57
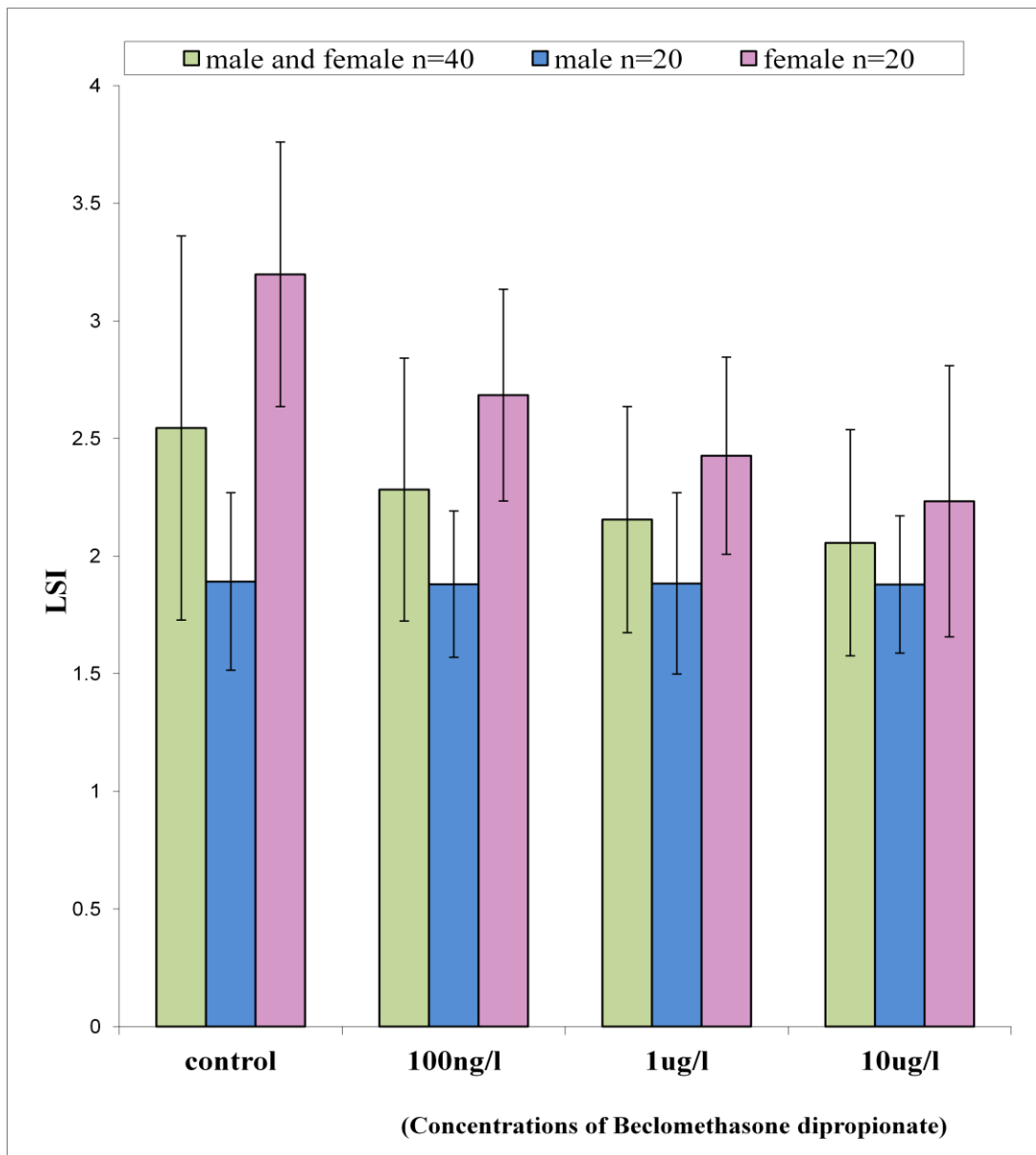
Figure 4-5. The liver-somatic index (LSI) of fish from experiment-2. Trend analysis revealed that the LSI of female fish had a significant decreasing trend with increasing concentration of Beclomethasone dipropionate (JT test; p < 0.001).

4.3.2 Plasma Glucose Concentrations

In experiment-1, plasma glucose concentrations were significantly (one way ANOVA; p < 0.001) increased in GC - exposed groups of fish compared to the control group. The effect of beclomethasone dipropionate was higher than that of prednisolone, but the difference was not significant (Figure 4.6, Table 4.3).
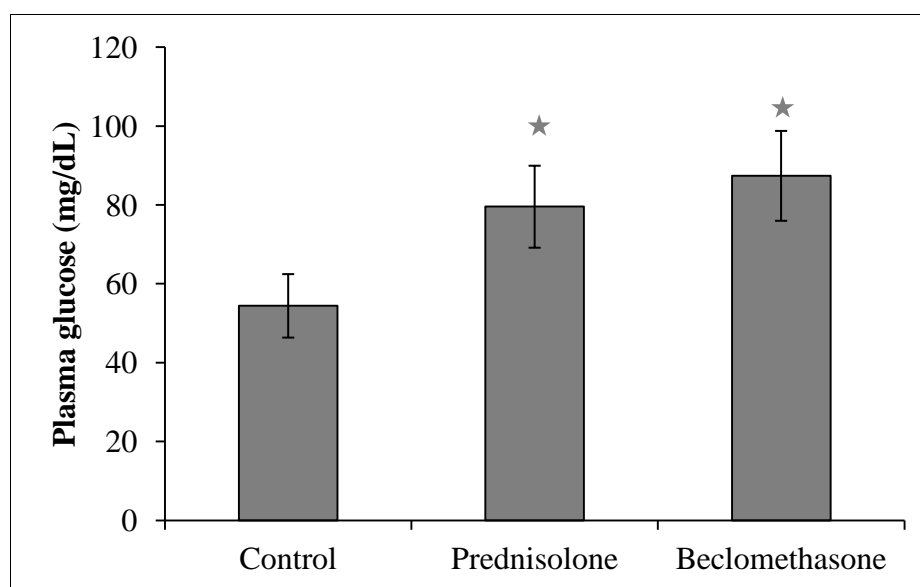
Figure 4-6. Plasma glucose concentrations (mean ± standard deviation) in control and treated (either 1µg prednisolone/L or 1µg Beclomethasone dipropionate/L) groups of fish. (n=10, mixed sex in each group). ★ Indicates a significant difference (one way ANOVA, Tukey test; p<0.001) from the control.

Plasma glucose concentrations from experiment-2 are summarised in Table 4.3. There was a dose-related increase (JT test; p < 0.001) of the plasma glucose concentrations in beclomethasone dipropionate-treated fish. The plasma concentrations in set-1 are presented in Figure 4.7. In set-1, glucose concentrations in fish exposed to 1µg/L and 10 µg/L were significantly higher than that of control fish, but the concentration in fish exposed to 100 ng/L was not significantly different (although weakly significant by Holm-Sidak test; p=0.035) from control (Tukey test; p=0.149). Similar statistical results were obtained when set-2 data were analysed (Figure 4.8). When data from set-1 and set-2 were pooled and analysed, both Holm-Sidak (p=0.001) and Tukey (p=0.006) tests revealed that the exposure to 100 ng/L had also elevated the plasma glucose concentration significantly (Figure 4.9).

Table 4-3. Plasma glucose concentrations of fish exposed to different GCs at various concentrations

Experiment	Treatment and number of fish		Mean glucose concentrations (mg/dL)	±SD	Significant difference from control
1	Control (n=10)		54.42	8.07	-
	1 µg Prednisolone/L (n=10)		79.56	10.39	Yes
	1 µg Beclomethasone dipropionate/L (n=10)		87.39	11.39	Yes
2 (Beclo-methasone dipropionate)	Set-1	Control (n=20)	54.146	10.382	-
		100 ng/L (n=20)	63.667	9.033	No
		1 µg/L (n=20)	92.178	13.628	Yes
		10 µg/L (n=20)	111.297	13.817	Yes
	Set-2	Control (n=20)	58.926	8.952	-
		100 ng/L (n=20)	65.303	7.285	No
		1 µg/L (n=20)	86.140	12.295	Yes
		10 µg/L (n=20)	107.028	8.334	Yes
	Pooled	Control (n=40)	56.536	9.870	-
		100 ng/L (n=40)	64.485	8.142	Yes
		1 µg/L (n=40)	*89.159*	13.171	Yes
		10 µg/L (n=40)	109.163	11.468	Yes
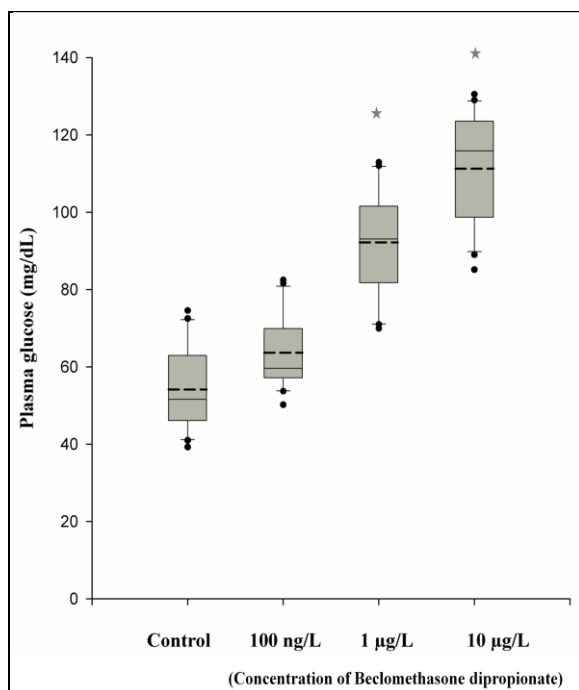
Figure 4-7. Plasma glucose concentrations of fish (n = 20) exposed to different concentrations of beclomethasone dipropionate (set-1). The box indicates the mid 50% of values. The straight line inside the box corresponds to the median and the dashed line corresponds to the mean. Whiskers indicate the 10th and 90th percentile values and circles indicate the outliers.★ Indicates a significant difference from control (ANOVA followed by Tukey test; p < 0.05).
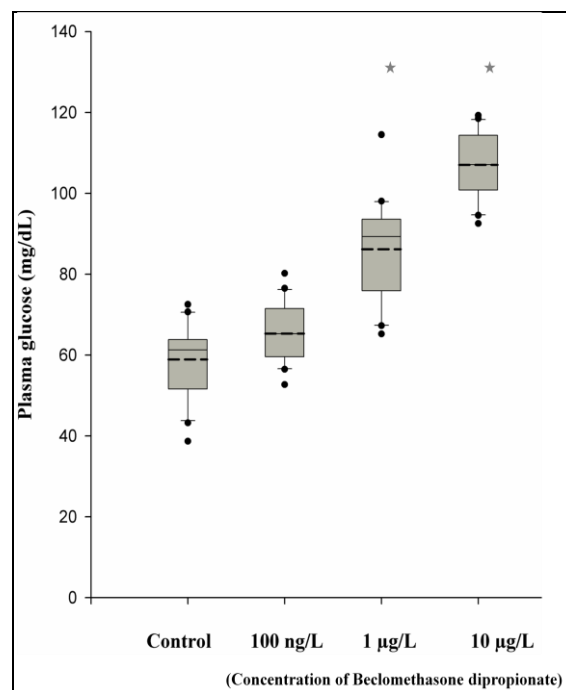
Figure 4-8. Plasma glucose concentrations of fish (n = 20) exposed to different concentrations of beclomethasone dipropionate (set-2). The box indicates the mid 50% of values. The straight line inside the box corresponds to the median and the dashed line corresponds to the mean. Whiskers indicate the 10th and 90th percentile values and circles indicate the outliers.★ Indicates a significant difference from control (ANOVA followed by Tukey test; p < 0.05).
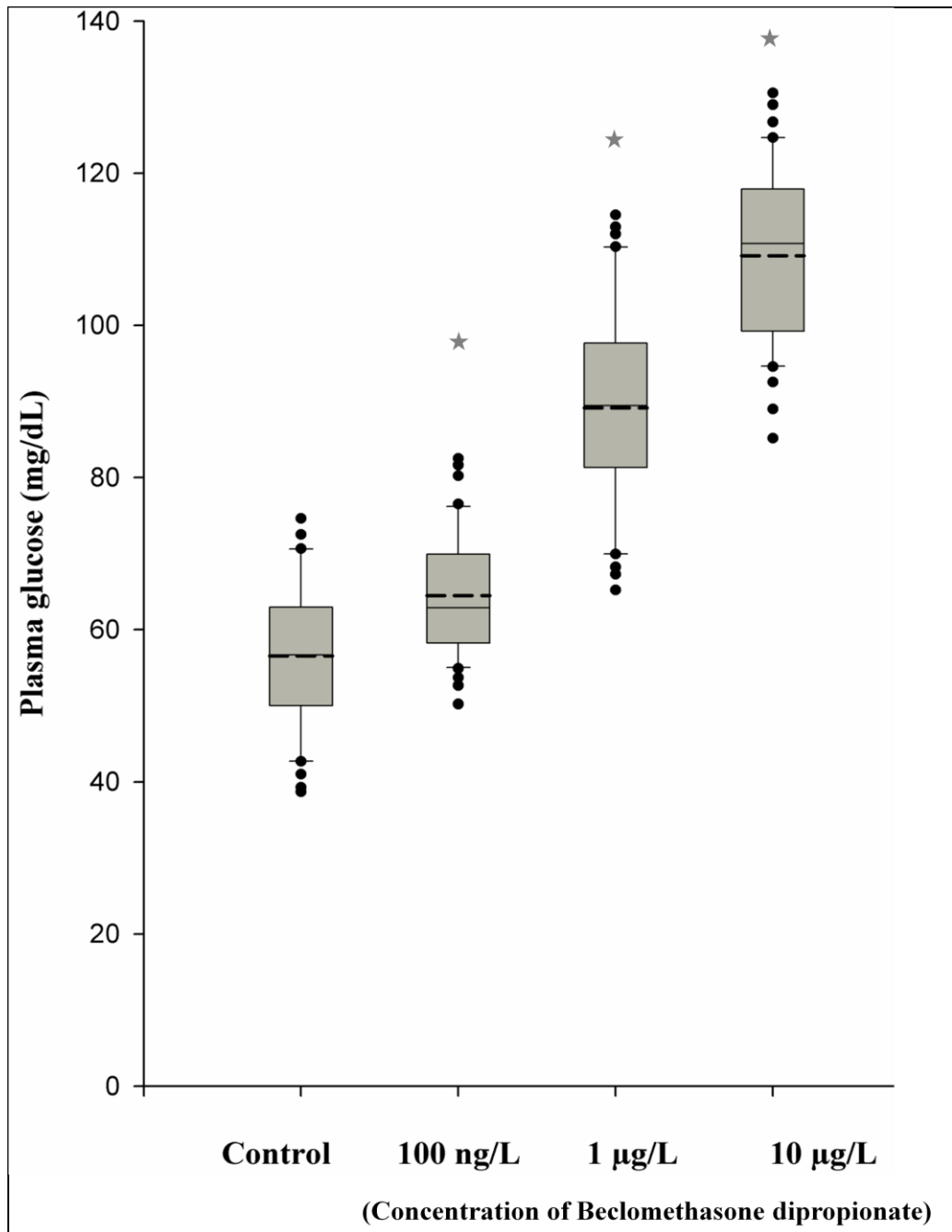
Figure 4-9. Plasma glucose concentrations of fish (n = 40 per treatment) exposed to different concentrations of beclomethasone dipropionate. Values from the same concentrations of each set were pooled for analysis. The box indicates the mid 50% of values. The straight line inside the box corresponds to the median and the dashed line corresponds to the mean. Whiskers indicate the 10th and 90th percentile values and circles indicate the outliers. ★ Indicates a significant difference from control (ANOVA followed by Tukey test; p < 0.05).

4.3.3 Leukocyte Counts

In experiment-1, the total leukocyte count was reduced significantly in both prednisolone and beclomethasone-treated groups (Figure 4.10). Total leukocytes were reduced significantly(one way ANOVA followed by Holm-Sidak pair wise comparison; $p < 0.001$) in GC exposed groups ($126.1 \pm 8.99 \times 10^3$ mm^{-3} and $119.1 \pm 7.21 \times 10^3$ mm^{-3} for prednisolone and beclomethasone, respectively) compared to the control group ($144.7 \pm 10.83 \times 10^3$ mm^{-3}).
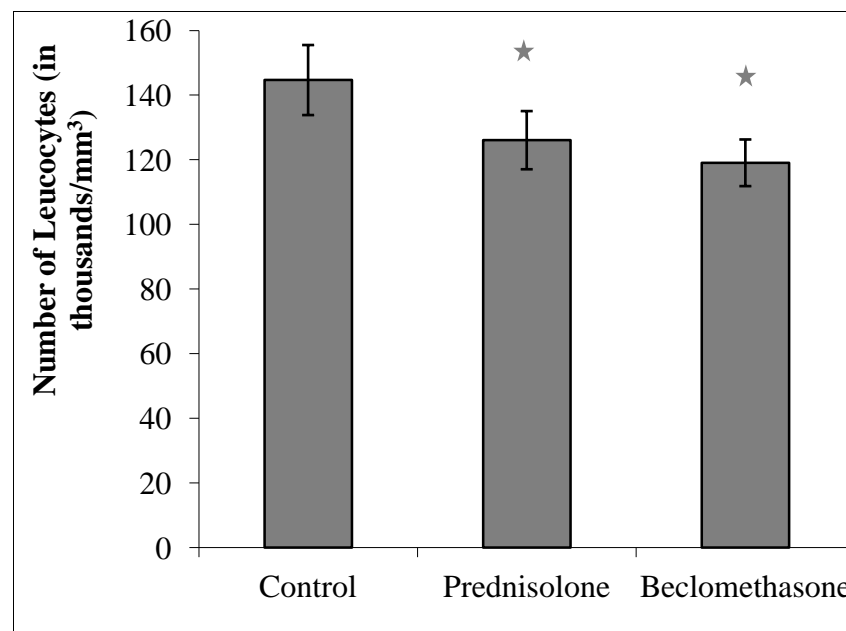
Figure 4-10. Blood leukocyte counts (mean ± standard deviation) in control and treated (either 1μg prednisolone/L or 1μg Beclomethasone dipropionate/L) groups of fish (n=10, mixed sex in each group). ★ Indicates a significant difference (one way ANOVA followed by Holm-Sidak pairwise comparision; p < 0.001) from control.

In experiment-2, although set-1, set-2 and the pooled data provided similar results, their statistical analyses differed slightly. In all cases, the combined lymphocyte and thrombocyte population expressed as a percentage of the total number of leukocytes had a decreasing trend. Set-1 passed the normality test and there were statistically significant differeneces between controls (90.71 ± 4.71 %) and the fish treated with 1 μg/L (71.71 ± 5.77 %) and 10 μg/L

(58.75±4.90%) concentrations (Figure 4.11). Fish treated with 100 ng/L (88.30±4.58 %) were not statistically different from control (one way ANOVA followed by Holm-Sidak pair wise comparison; $p < 0.001$).
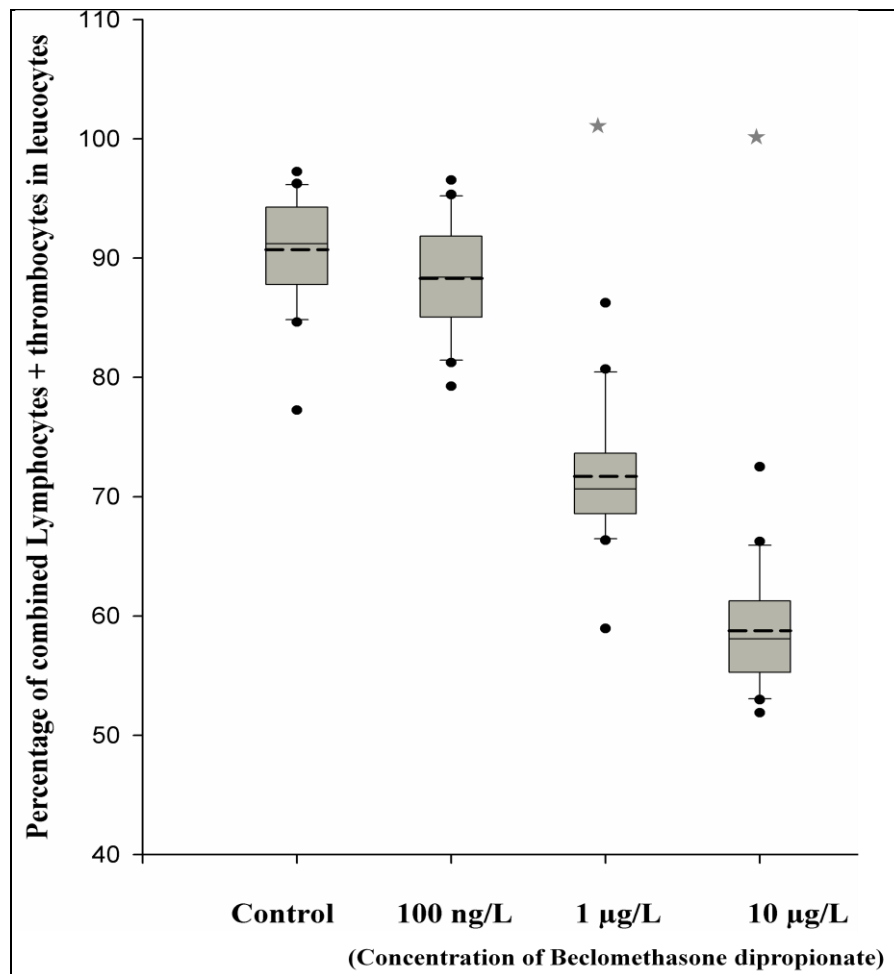
Figure 4-11. Combined lymphocyte and thrombocyte population expressed as a percentage of the total leukocytes (set-1, n = 20) in fish exposed to different concentrations of beclomethasone dipropionate. The box indicates the mid 50% of values. The straight line inside the box corresponds to the median and the dashed line corresponds to the mean. Whiskers indicate the 10th and 90th percentile values and circles indicate the outliers. ★ Indicates a significant difference from the control (one way ANOVA followed by Holm-Sidak pair wise comparison; p < 0.001).

Set-2 data failed the normality test, even after log transformation. However, one way ANOVA on ranks (Kruskal-Wallis non-parametric test followed by Tukey test; $p < 0.001$)

revealed similar results (Figure 4.12). There were statistically significant differeneces between controls (median, 90.415 %) and the fish treated with 1 µg/L (median 70.79 %) and 10 µg/L (median 59.44 %) concentrations (Figure 4.11). The difference between the fish treated with 1 µg/L and 10 µg/L concentrations was not statistically sinificant. Fish treated with 100 ng/L (median 84.95 %) were not statistically different from the control.
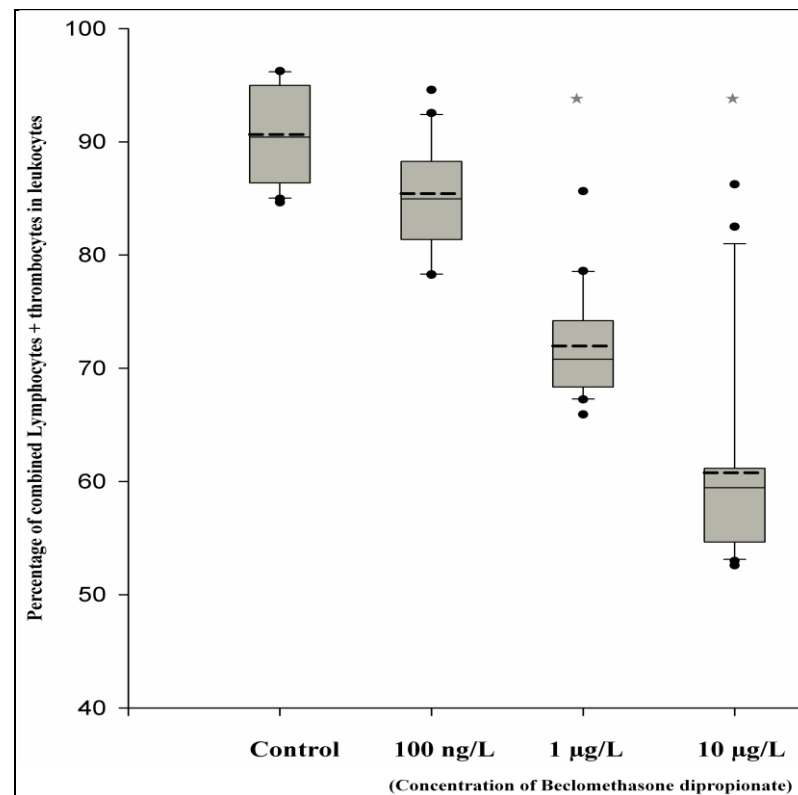
Figure 4-12. Combined lymphocyte and thrombocyte population expressed as a percentage of total leukocytes (set-2, n = 20) in fish exposed to different concentrations of beclomethasone dipropionate. The box indicates the mid 50% of values. The straight line inside the box corresponds to the median and the dashed line corresponds to the mean. Whiskers indicate the 10th and 90th percentile values and circles indicate the outliers. ★Indicates a significant difference from control (one way ANOVA on ranks followed by Tukey pair wise comparison; p < 0.001).

When the data from set-1 and set-2 were pooled, they failed the normality test, even after log transformation. Similar to set-2, one way ANOVA on ranks (Kruskal-Wallis non-parametric test followed by Tukey test; p < 0.001) revealed (Figure 4.12) the statistically significant

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differeneces between controls (median, 91.195 %) and the fish treated with 1 µg/L (median 70.65 %) and 10 µg/L (median 58.645 %) concentrations (Figure 4.11). In this case, the difference between the fish treated with 1 µg/L and 10 µg/L concentrations was also not statistically significant. Fish treated with 100 ng/L (median 84.275 %) were not statistically different from control.
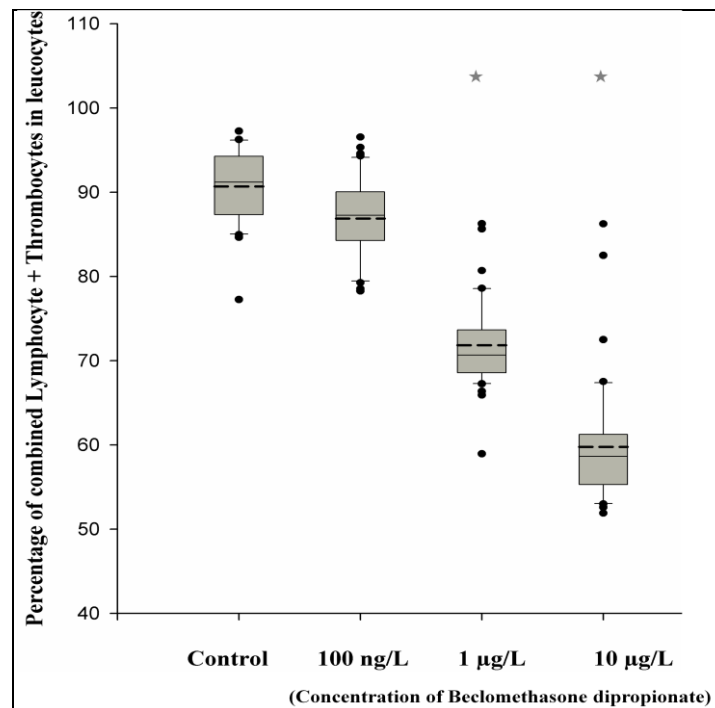
Figure 4-13. Combined lymphocyte and thrombocyte population expressed as a percentage of total leukocytes (set1 and set-2,n = 40) in fish exposed to different concentrations of beclomethasone dipropionate. The box indicates the mid 50% of values. The straight line inside the box corresponds to the median and the dashed line corresponds to the mean. Whiskers indicate the 10th and 90th percentile values and circles indicate the outliers. ★Indicates a significant difference from control (one way ANOVA on ranks followed by Tukey pair wise comparison; p < 0.001).

4.3.4 Plasma Cortisol Concentrations

Although there was a drop in the mean plasma concentration in GC-treated groups, there was no significant difference in mean plasma cortisol concentrations in control (53.108±13.63),

prednisolone-treated (45.02±12.85) and beclomethasone-treated (39.55±15.66) groups in

experiment-1 (Figure 4.14).
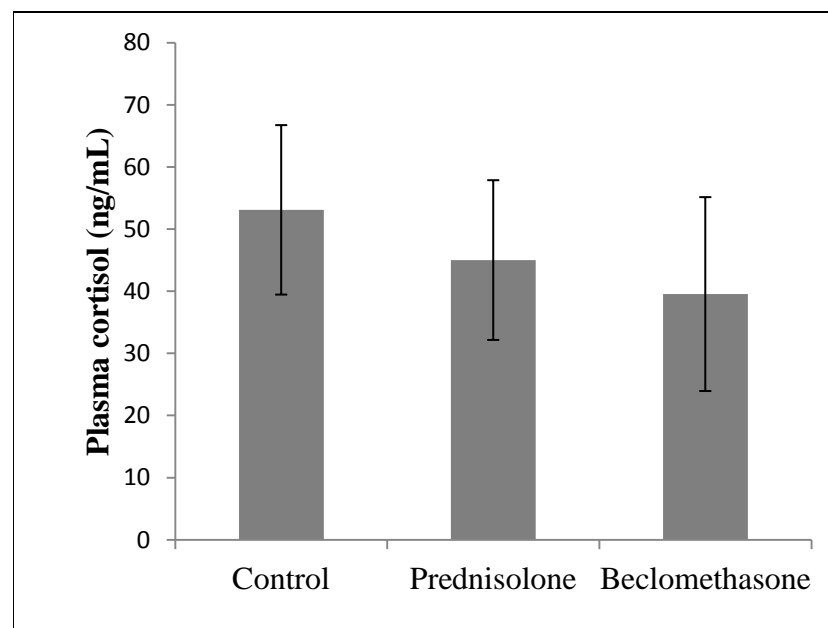
Figure 4-14. Plasma cortisol concentrations in experiment-1 (mean ± SD). There were no significant differences between either of the treatment groups and the control group (n=10, one way ANOVA; p = 0.178).

Plasma cortisol concentrations in experiment-2 did not change in a dose-response manner. In

both set-1 and set-2, fish exposed to 100 ng/L had slightly higher cortisol concentrations than

the controls (although not significantly so), while fish exposed to 1 µg/L and 10 µg/L had

lower cortisol concentrations (Figure 4.15). Set-1 data failed the normality test, even after log

transformation. However, one way ANOVA on ranks (Kruskal-Wallis non-parametric test

followed by Tukey test; $p < 0.001$) revealed (Figure 4.15a) that there were statistically

significant differeneces between controls (mean: 41.28±8.67 ng/mL; median: 40.65 ng/mL)

and the fish treated with 1 µg/L (mean: 29.36±9.69 ng/mL; median: 32.60 ng/mL) and 10

µg/L (mean: 21.34±4.49 ng/mL; median: 20.38 ng/mL) beclomethasone dipropionate. The

difference between fish treated with 1 µg/L and 10 µg/L concentrations was not statistically

significant. Fish treated with 100 ng/L (mean: 40.89±10.50 ng/mL; median: 40.26 ng/mL) were not statistically different from the control.

Set-2 data were found to be normally distributed, but they failed the equal variance test. Therefore ANOVA on ranks was performed and it revealed that neither fish exposed to 100 ng/L (mean: 37.68±11.34 ng/mL; median 38.26 ng/mL) nor 1 µg/L (mean: 27.66±8.58 ng/mL; median: 24.95 ng/mL) concentrations were statistically different from control(Figure 4.15b). However, fish exposed to 10 µg/L had a significant reduction of plasma cortisol concentration (mean: 22.21±5.08 ng/mL; median:20.98 ng/mL) compared to the control (mean: 34.97±5.61 ng/mL; median: 34.95 ng/mL).

(a)   (b)
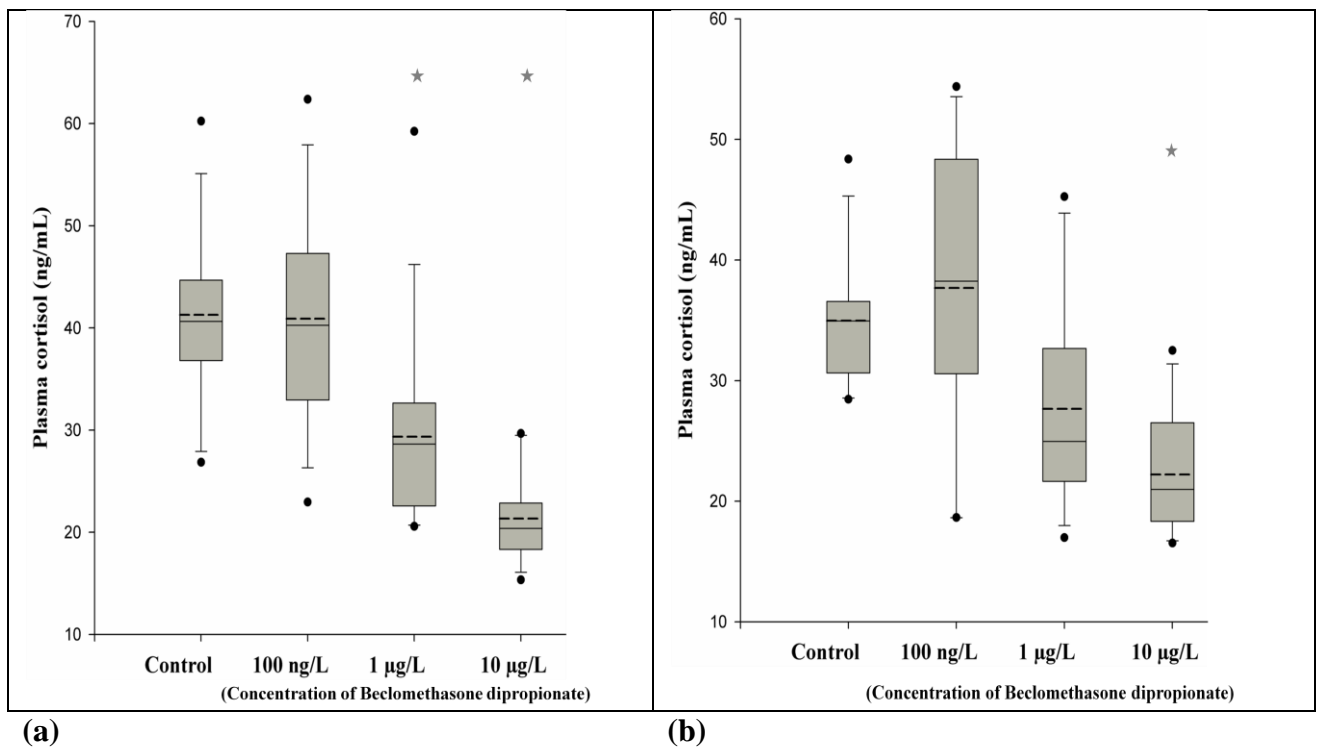
Figure 4-15. Plasma cortisol concentrations of fish (n = 15) exposed to different concentrations of beclomethasone dipropionate and controls in (a) set-1 and (b) set-2 of experiment-2. The box indicates the mid 50% of values. The straight line inside the box corresponds to the median and the dashed line corresponds to the mean. Whiskers indicate the 10th and 90th percentile values and circles indicate the outliers. ★ Indicates a significant difference from control (one way ANOVA on ranks followed by Tukey pair wise comparison; p < 0.001).

When set-1 and set-2 data were pooled together, again the data failed the normality test even after log transformation. However, one way ANOVA on ranks (Kruskal-Wallis non-parametric test followed by Tukey test; $p < 0.001$) revealed (Figure 4.16) that there were statistically significant reductions of plasma cortisol concentrations in fish exposed to 1 µg/L (mean: 28.51±9.04 ng/mL; median: 24.96 ng/mL) and 10 µg/L (mean: 21.77±4.73 ng/mL; median: 20.63 ng/mL) concentrations of beclomethasone dipropionate compared to controls (mean: 38.12±7.86 ng/mL; median: 36.69 ng/mL). The difference between the fish treated with 1 µg/L and 10 µg/L concentrations was also statistically significant. Fish treated with 100 ng/L (mean: 39.29±10.86 ng/mL; median: 40.06 ng/mL) were not statistically different from the control.
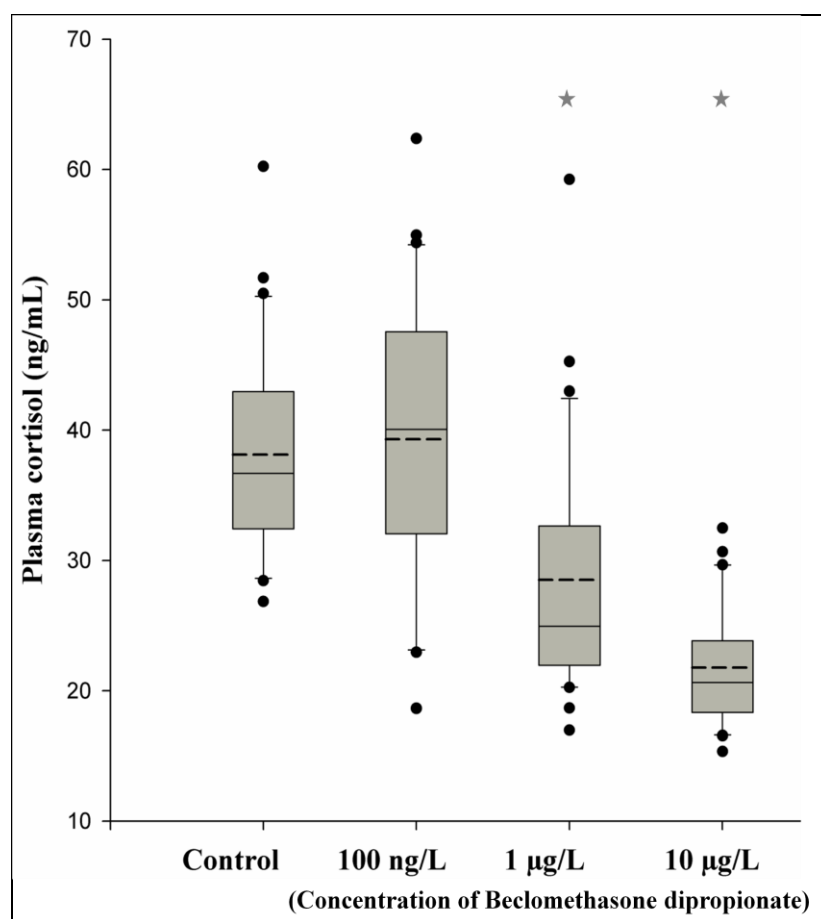
Figure 4-16. Plasma cortisol concentrations of fish (n = 30) exposed to different concentrations of beclomethasone dipropionate and control, in duplicate. The box indicates the mid 50% of values. The straight line inside the box corresponds to the median and the dashed line corresponds to the mean. Whiskers indicate the 10th and 90th percentile values and circles indicate the outliers. ★ Indicates a significant difference from control (one way ANOVA on ranks followed by Tukey test; p < 0.001.

4.3.5 Plasma Vtg Concentrations in Females

Set-1, set-2 and pooled plasma Vtg concentrations were found to be normally distributed with equal variance. One way ANOVA did not show any significant differences between control (14.15±5.24 mg/mL) and any of the treatments: 100 ng/L (11.97±4.80 mg/mL), 1 µg/L (11.98±4.69 mg/mL) and 10 µg/L (9.15±4.71 mg/mL) in set-1 (Figure 4.17a). In set-2, there

was a weakly significant reduction in plasma Vtg concentration in the 10 µg/L group (12.26±3.83 mg/mL) compared to the control (17.46±3.39 mg/mL; ANOVA followed by Holm-Sidak test; $p < 0.05$). Fish treated with 100 ng/L (17.22±4.84 mg/mL) and 1 µg/L (15.01±4.38 mg/mL) did not show any significant difference from control (Figure 4.17b).
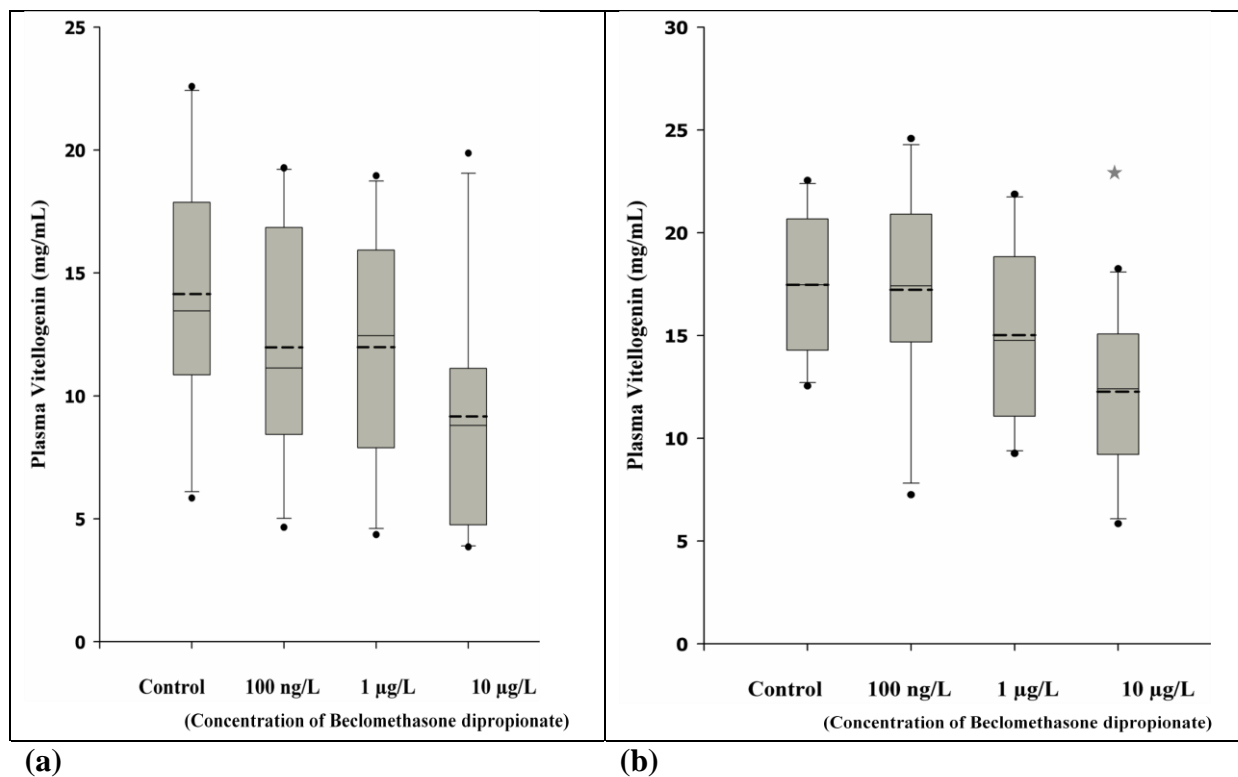
(a)   (b)

Figure 4-17. Plasma Vtg concentrations of female fish (n = 10) exposed to different concentrations of beclomethasone dipropionate and controls in (a) set-1 and (b) set-2 of experiment-2. The box indicates the mid 50% of values. The straight line inside the box corresponds to the median and the dashed line corresponds to the mean. Whiskers indicate the 10th and 90th percentile values and circles indicate the outliers.★Indicates a significant difference from the control (one way ANOVA followed by Holm-Sidak pair wise comparison; p < 0.05).

When set-1 and set-2 data were pooled, mean plasma concentrations of Vtg in control, 100 ng/L, 1 µg/L and 10 µg/L treatment groups were 15.80±4.62 mg/mL, 14.59±5.41 mg/mL, 13.49±4.69 mg/mL and 10.70±4.47 mg/mL, respectively (Figure 4.18). The difference between controls and the 10 µg/L group was significant (ANOVA followed by Holm-Sidak test; $p < 0.05$).
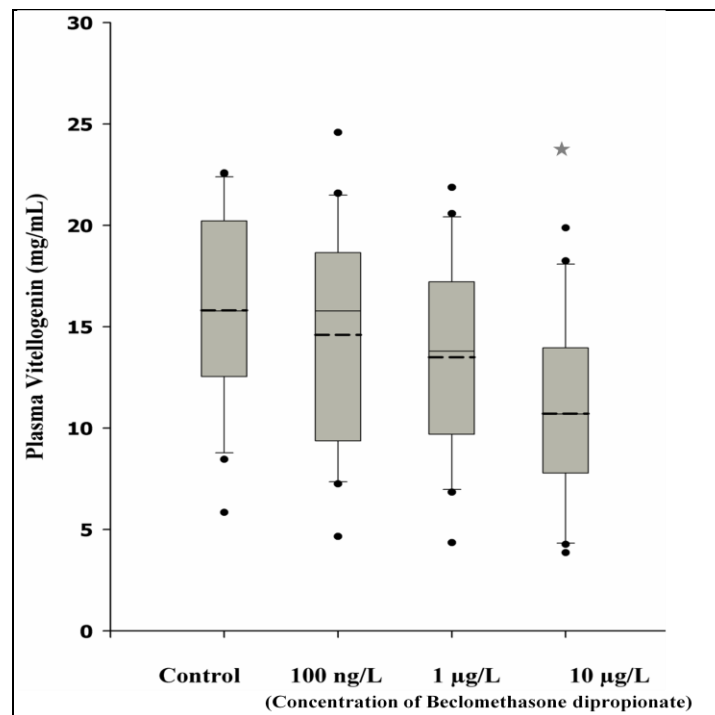
Figure 4-18. Plasma Vtg concentrations of female fish (n = 20) exposed to different concentrations of beclomethasone dipropionate and control, in duplicate. The box indicates the mid 50% of values. The straight line inside the box corresponds to the median and the dashed line corresponds to the mean. Whiskers indicate the 10th and 90th percentile values and circles indicate the outliers. ★ Indicates a significant difference from the control (one way ANOVA followed by Holm-Sidak test; p < 0.05).

4.3.6   Secondary Sexual Characters

Set-1 and set-2 datasets for male and females in experiment-2 were pooled separately for the analysis of secondary sexual characters. There was a significant increase in the mean number of nuptial tubercles of male fish in the 10 µg/L group (19.95±3.30) compared to the control group (15.1±3.99; ANOVA followed by Tukey test; $p < 0.05$), while fish treated with 100 ng/L (14.60±2.34) and 1 µg/L (17.45±3.08) did not show any significant difference from the control group (Figure 4.19).
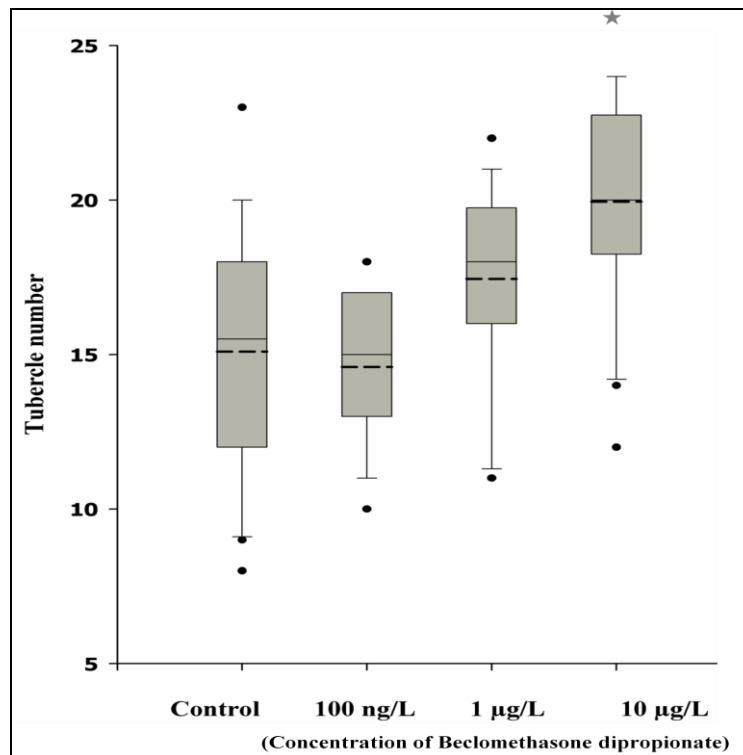
Figure 4-19. The number of nuptial tubercles in males (n = 20) exposed to different concentrations of beclomethasone dipropionate and control. The box indicates the mid 50% of values. The straight line inside the box corresponds to the median and the dashed line corresponds to the mean. Whiskers indicate the 10th and 90th percentile values and circles indicate the outliers. ★ Indicates a significant difference from control (one way ANOVA followed by Tukey test; p < 0.05).

Figure 4.20 shows that prominence of tubercles as measured by tubercle grade increases with increasing exposure concentrations, as the number of fish with grade-3 tubercles was highest in the 10 µg/L group.
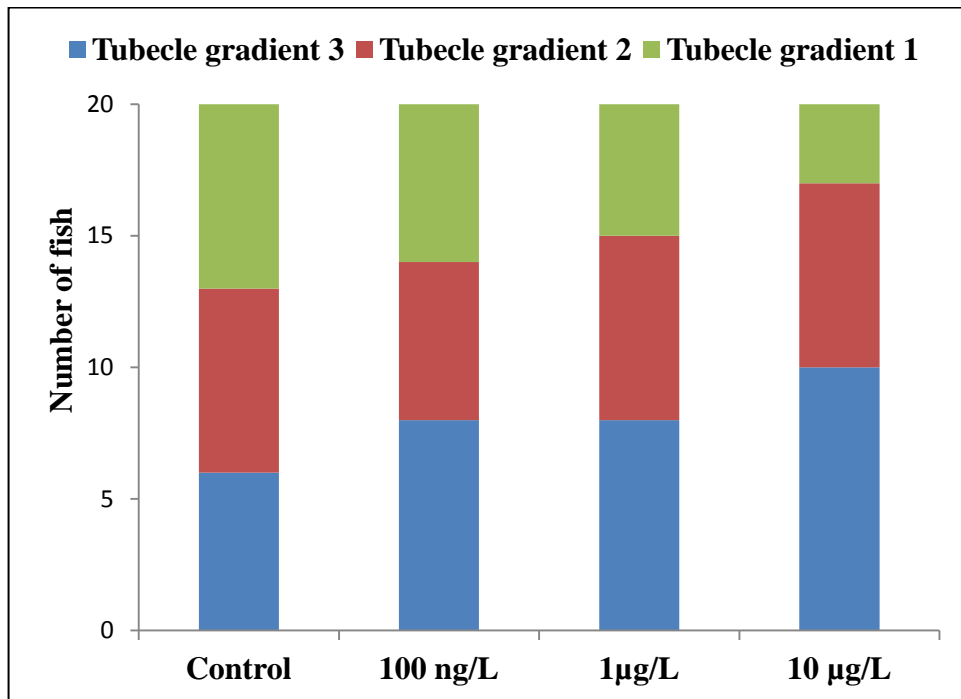
Figure 4-20. Prominence of tubercles in males (n=20) as measured by tubercle grade (scored from 1 to 3) in controls and fish exposed to different concentrations of beclomethasone dipropionate. The number of fish observed with each grade is illustrated.

Differences in mean fat pad height between the treatment groups and the control group were not significant (Figure 4.21a). Mean fat pad height in control, 100 ng/L, 1 µg/L and 10 µg/L treatments were 2.36±0.83 mm, 2.26±0.89 mm, 2.84±1.28 mm and 2.73±0.97 mm, respectively. Similarly, fat pad index, measured from the relationship between the fat pad weight and body weight, did not show any significant differences between any of the treatment groups and the control group (Figure 4.21b). Mean fat pad indices of control, 100 ng/L, 1 µg/L and 10 µg/L groups were 2.62±0.52, 2.75±0.59, 2.72±0.51 and 2.83±0.67, respectively.
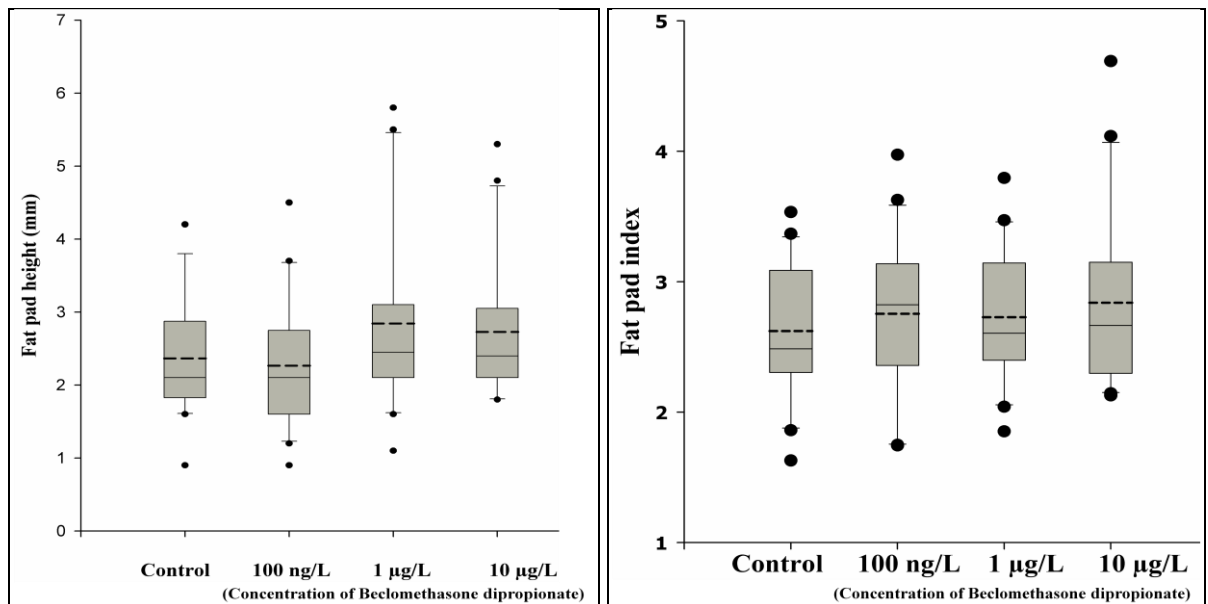
(a)   (b)

Figure 4-21. Fat pad height (a) and fat pad index (b) of male fish (n=20) in treatment and control groups. The box indicates the mid 50% of values. The straight line inside the box corresponds to the median and the dashed line corresponds to the mean. Whiskers indicate the 10th and 90th percentile values and circles indicate the outliers.

Many females in treatment groups exhibited male SSCs (Figure 4.22). None of the control fish (n=20) showed a fat pad and they were all given a fat pad score of 1. Fish from the treatment groups exhibited various degree of fat pad development and the mean fat pad scores indicate a significant masculinisation due to exposure to beclomethasone dipropionate (Table 4.4). There was also an increasing number of fish with a black spot on their dorsal fin (another male SSC) with an increasing concentration of beclomethasone dipropionate (Figure 4.23).

Quantitative measures of female SSC also showed an impact of the exposure to beclomethsone dipropionate. Data for the ovipositor length of females (n=20) failed the normality test. However, Kruskal-Wallis one way ANOVA on ranks followed by a Tukey test indicated that there was a significant reduction in ovipositor length in the groups exposed to 1 µg/L and 10 µg/L compared to the control group (Table 4.4; Figure 4.24).

141
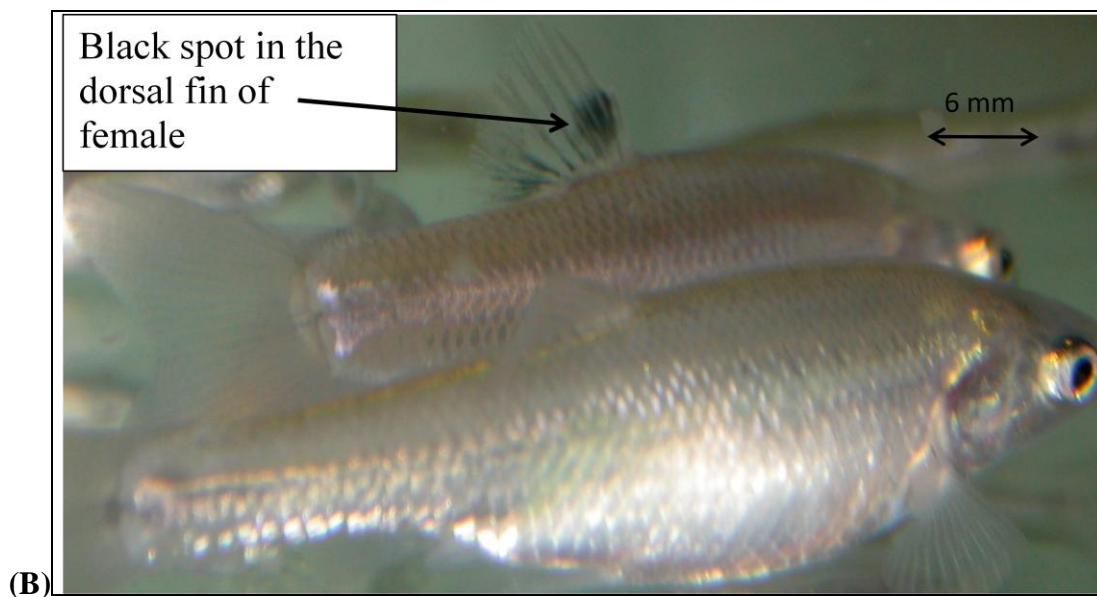
Figure 4-22. (A) A female fish exposed to 10 µg beclomethsone dipropionate/L, identified as a female by its ovipositor (b) and slender body, exhibiting a male SSC, a fat pad (a). (B) Two females exposed to 10 µg beclomethsone dipropionate/L, one of them showing a black spot on its dorsal fin (which is a male secondary sex character).
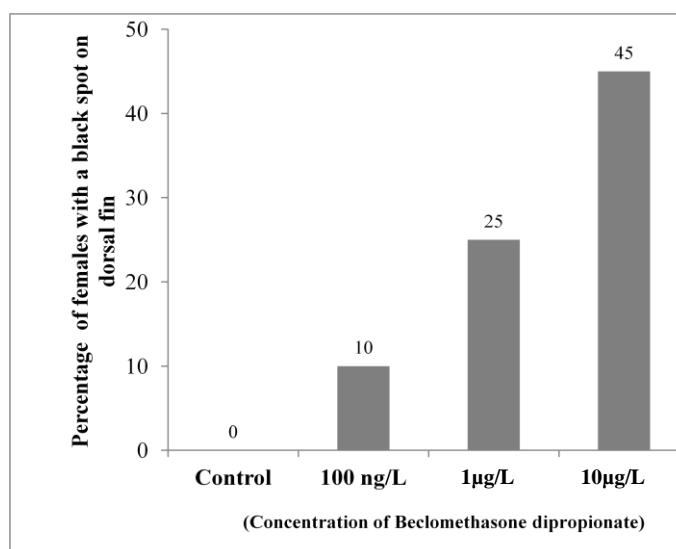
Figure 4-23. The percentage of female fish (n=20) in control and treatment groups exhibiting a black spot on their dorsal fin.

Table 4-4. Secondary sexual characteristics of female Fish (n = 20) of Experiment-2

	Number of fish					Mean fat pad score for the group	Number of fish with dorsal fin spot	Mean ovipositor length (mm)
Fat pad score	**1**	**2**	**3**	**4**	**5**			
Control	20	0	0	0	0	1	0	1.775±0.53
100 ng/mL	18	2	0	0	0	1.1	2	1.605±0.60
1 µg/L	11	6	2	1	0	1.65	5	1.185±0.48
10 µg/L	7	5	6	2	0	2.15	9	0.965±0.36
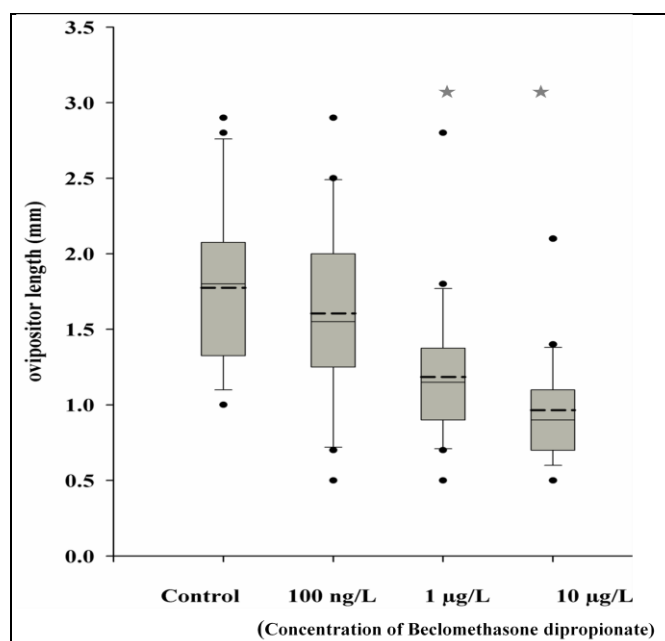
Figure 4-24. Ovipositor length of female fish (n = 20) exposed to different concentrations of beclomethasone dipropionate. The box indicates the mid 50% of values. The straight line inside the box corresponds to the median and the dashed line corresponds to the mean. Whiskers indicate the 10th and 90th percentile values and circles indicate the outliers. ★ Indicates a significant difference from control (one way ANOVA on ranks followed by Tukey pair wise comparison; p < 0.001).

4.3.7 Gonadal Histology

Two fish in exposed to beclomethasone dipropionate were found to be egg-bound and their ovaries were atretic. Apart from this, there were no signs of intersex characters in the ovarian cross sections (Figure 4.25).
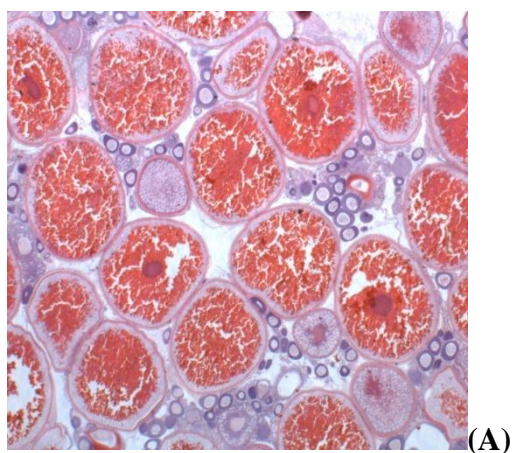
(A)
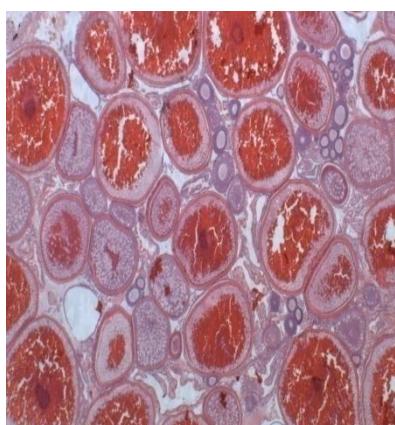
(B)

(C)
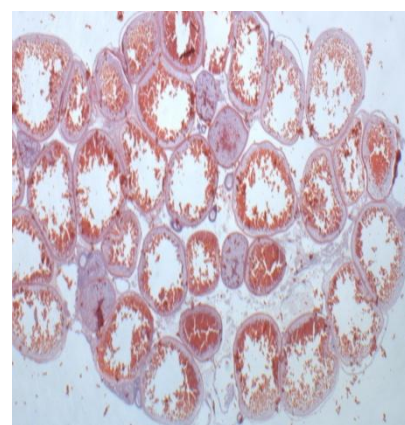
(D)

Figure 4-25. Photomicrographs of cross-sections of control (A) and treated ovaries (B - 100 ng/L, C - 1 µg/L and D - 10 µg/L beclomethasone dipropionate exposed) showing reduction in the number of perinucleolar follicles and follicles with balbiani bodies due to the treatment.

Overall there was a reduction in number of early developmental stages and an increased proportion of maturing follicles observed. The percentages of perinucleolar follicles in 1 µg/L (29.78±9.69 %) and 10 µg/L (21.40±9.10 %) treatments were significantly lower than that of the control (42.22±7.09%). Difference between control and 100 ng/L treatment (38.11±6.23 %) was not significant. (Figure 4.26a).
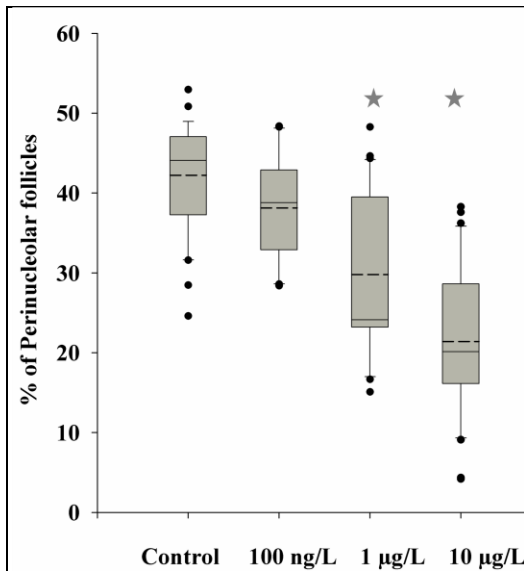
(a): Perinucleolar follicles
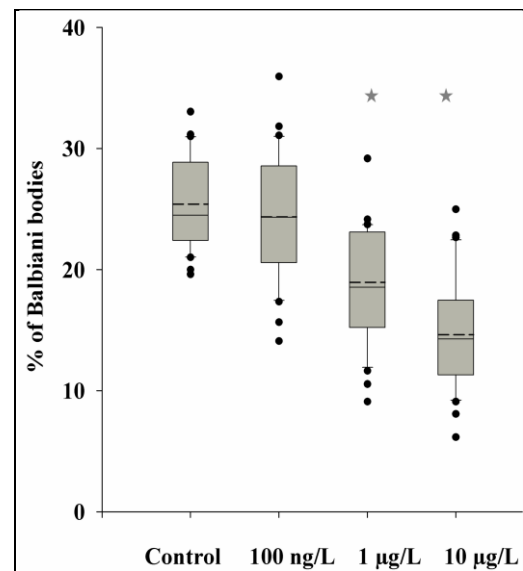
(b): Follicles with Balbiani bodies
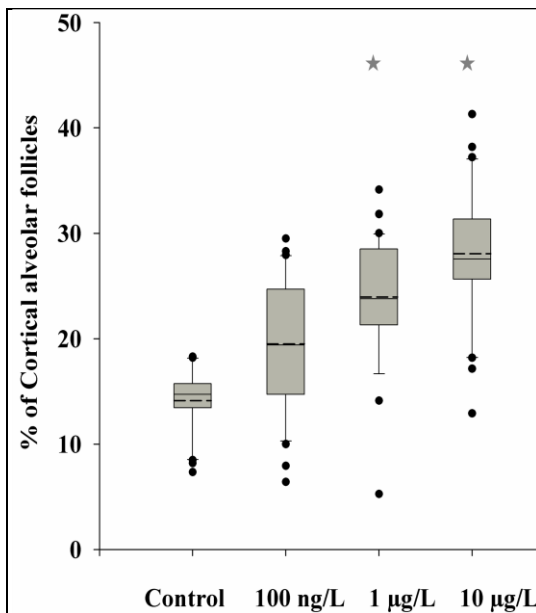
(c): Cortical alveolar follicles
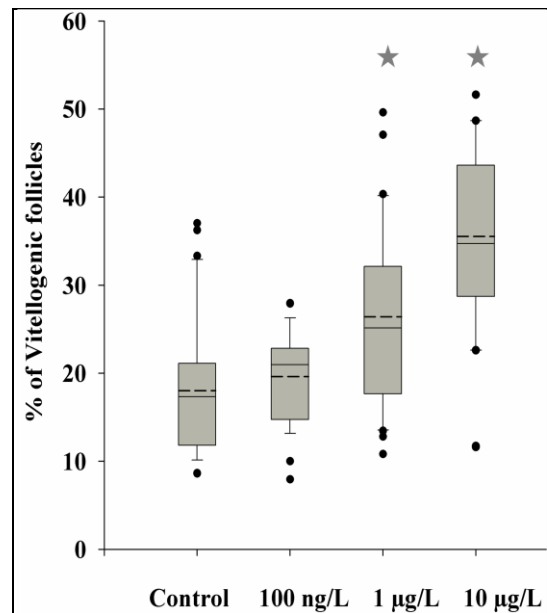
(d): Vitellogenic follicles

Figure 4-26. Numbers of the different stages of oogenesis in a cross section of an ovary as a percentage of the total number of cells. X axis represents the different concentrations of beclomethasone dipropionate.The box indicates the mid 50% of values. The straight line inside the box corresponds to the median and the dashed line corresponds to the mean. Whiskers indicate the 10th and 90th percentile values and circles indicate the outliers. ★ Indicates a significant difference from control (one way ANOVA on ranks followed by Tukey pair wise comparison; p < 0.001)

Similarly, the percentages of follicles containing balbiani bodies in 1 µg/L (18.95±4.6 %) and 10 µg/L (14.62±4.53 %) treatments were significantly lower than that of the control (25.39±3.83 %). The difference between control and 100 ng/L treatment (24.35±5.1 %) was not significant (Figure 4.26b). JT test (p<0.001) indicated that the decreasing trends in the number of both cell types were significant.

Cortical alveolar follicles and vitellogenic follicles showed a significant increasing trend (JT test; p<0.001) with treatment. Percentages of cortical alveolar follicles in 1 µg/L (23.96±5.99 %) and 10 µg/L (28.05±6.30 %) treatments were significantly higher than that of the control (14.12±3.00 %). The difference between control and 100 ng/L treatment (19.49±5.98 %) was not significant (Figure 4.26c). Similarly, the percentages of vitellogenic follicles in 1 µg/L (26.41±9.81 %) and 10 µg/L (35.52±10.45 %) treatments were significantly higher than that of the control (18.01±7.83 %). The difference between control and 100 ng/L treatment (19.06±5.36 %) was not significant (Figure 4.26d).

4.3.8 Water Chemistry

In experiment-1, measured prednisolone concentrations in tank water were not significantly different between sampling days (Figure 4.27). The average concentration of all 15 samples was 902.5 ± 124.6 ng/L, compared to the nominal concentration of 1000 ng/L. Prednisolone concentrations in the control tanks were below detection limit (less than 56 pg/mL).
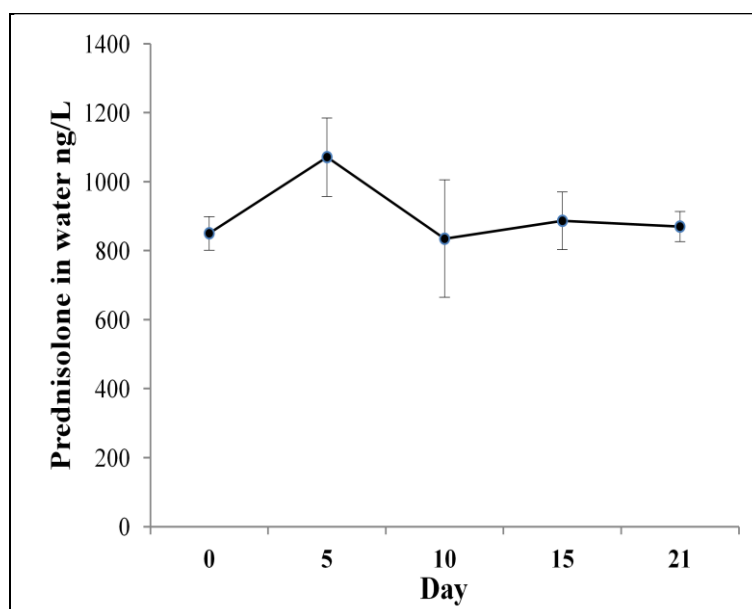
Figure 4-27. Concentrations of prednisolone in tank water of experiment 1, sampled on days 0, 5, 10, 15 and 21. Fish were transferred into experimental tank on day 0, but dosing of the tanks was started one week before day 0. Values are the average and standard deviation of triplicate samples per day.

4.4 Discussion

In this study, a range of endpoints, some of them are not routinely used in ecotoxicology experiments using fathead minnows, were used in order to assess the impacts of GCs as well as to suggest a reliable biomarker for risk assessment of GCs present in the environment. Baseline values of these endpoints are compared with previously reported measurements for fathead minnows (Ankley *et al*., 2001; Jensen *et al*., 2001; Leino *et al*., 2005; Watanabe *et al*., 2007; Dang *et al*., 2011) and the responses of fathead minnow to GCs are discussed based on their known mode of action.

Since the exposure concentrations were very low and there was no reported lethal toxicity in this concentration range, the present results showing no mortality were expected. Similarly, there were no impacts on length, weight and other organ indices due to the treatments, as would be expected. The decreasing trend in LSI of females exposed to GCs needs further verification. This effect may relate to Vtg production, as male and female fish vary on this

aspect of normal physiology. However, knowing that Vtg is not stored in the liver, the explanation for the LSI reduction in females due to the exposure is not clear.

It is well established that GCs induce a significant tendency to hyperglycaemia in humans. Gluconeogenesis, a major effect of GCs on glucose metabolism, has been well documented (David *et al.*, 1970; Rutkowski, 2001). More recently, some clinical trials report the increased risk of diabetes onset in patients with chronic use of topical corticosteroids (Van der Linden *et al.*, 2009). This effect of GCs also occurs in fish. Cortisol administration to fish elevated the plasma glucose concentration two to five-fold, depending on the concentration (reviewed by Martinez-Porchas, 2009; Table 4.5). The timing of peak plasma glucose concentrations following stress coincided with the timing of elevated cortisol concentrations, indicating a role for cortisol in mobilising glucose during stress in teleosts (Mommsen *et al.*, 1999). Both synthetic GCs tested caused an increase in the plasma glucose concentrations of about 50% in experiment-1. Higher dose in experiment-2 elevated it about 2-fold. This is a considerable impact as plasma glucose concentration of an animal is not a measurement that can change 10's or 100's of fold. In human a 2 to 3-fold increase is a sign of disease and a 4 to 5-fold increase could be fatal.

In fish, it is evident that the change in plasma glucose concentration due to up-regulation of cortisol depends on the species (Table 4.5). Since the previous studies induced stress effects by either implantation of cortisol, handling and confinement, or cortisol administration via food, it is not possible to compare them directly with the present study. It may also be relevant that cortisol is a natural GC in fish and its concentration is elevated in response to stress, but it is less potent than the GCs used in the present exposure study.

Plasma glucose measurement seems to be a reliable endpoint in GC exposure studies, at least those using fathead minnows, as they responded to concentrations as low as 100 ng

beclomethasone dipropionate per litre. However, when assessed individually, set-1 and set-2 did not produce significant results for the lowest concentration tested. Therefore, the number of replicates used may be important for this endpoint; the higher the number, the greater the chance of detecting a significant increase.

Table 4-5. Previously reported plasma glucose changes due to stressors (Table taken from Martine-Porchas *et al*., 2009). These data shows that a 2 to 5-fold increase of plasma glucose concentrations is typical, and that the change probably varies from species to species.

Species	Stressor	Glucose (mmol/l)		Exposure
		Prestress	Poststress	
Atlantic cod *Gadus morhua*	Nitrite exposure	0.17	0.23	Chronic
Bald notothen *Pagothenia borchgrevinki*	Temperature	4.5	10	Chronic
Channel catfish *Ictalurus punctatus*	Handling	1.7	2.8	Acute
Coral trout	Capture and handling			Chronic
Plectropomus maculatus		1.6	7.9	
Plectropomus leopardus		1.9	7.4	
Emerald rockcod *Trematomus bernacchii*	Temperature	1.5	7.5	Chronic
Matrinxã *Brycon amazonicus*	Handling and transportation	2.8	10	Acute
Nile tilapia *Oreochromis niloticus*	Electroshock	2.2	6.4	Acute
Nile tilapia	Social stressor	1.9	6.7	Acute
Rainbow trout *Oncorhynchus mykiss*	Pollutant	4.2	9	Acute
Rainbow trout	Copper and air exposure	5.1	7.2	Chronic/Acute
Sunshine bass *Morone chrysops x saxatilis*	Temperature and confinement	6.1	10.5	Chronic/Acute
White sturgeon *Acipenser transmontanus*	Air exposure	1.6	1.7	Acute

GCs are mainly used clinically for their anti-inflammatory and immuno-suppressive properties. A number of human trials have revealed the reduction of leukocyte counts after treatment with synthetic GCs. Similarly, dexamethasone treatment in fish significantly reduced the leukocyte count (Pickering *et al*., 1987). Lymphocytopenia is a consequence of acute and chronic stress in trout (Pickering 1984; Pickering and Pottinger 1989; Morgan *et al*., 1993), and is a direct effect of the elevation of plasma cortisol concentrations. However in another study, Pickering and Pottinger (1985) found that cortisol can increase the susceptibility of brown trout to disease without affecting the white blood cell count. In the present study, both traditional leukocyte counts as well as more precise differential counts were employed. Since GCs have a tendency to induce lymphocytopenia in human, differential counting targeting the lymphocytes is a more useful endpoint. However, it was not possible to differentiate lymphocytes and thrombocytes. Therefore they were grouped together. The leukocyte number in the controls fell within the reported range for fathead minnows (Thomas *et al*., 1999). Lymphocytopenia in fish is associated with increased susceptibility to disease (Pickering and Pottinger, 1989), and hence fish chronically exposed to synthetic GCs in their environment may be more susceptible to disease.

Another endpoint tested, plasma cortisol level, seems an inconsistent one. Plasma cortisol was targeted as it is a standard measure of GC potency in human. Due to the negative feedback mechanism via the hypothalamus-pituitary-adrenal axis, plasma cortisol concentration in human is down regulated by GC treatment, and it is usually measured in morning salivary or urinary samples. It can be an accurate measure in humans, as it is measured after rest and sleep, so that a reduction of plasma cortisol in response to GC can be measured. But in fish, obtaining a blood sample without stressing the fish is challenging. Stress immediately elevates the plasma cortisol concentration and this rise could mask any reduction due to the GC's impact. This may be the reason for the wide variation in plasma cortisol concentrations in the

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present result. However, in the high dose treatment group, the reduction in plasma cortisol concentration was significant. Plasma cortisol concentrations also appear to vary depending on species (reviewed by Pankhurst, 2011) and the concentration measured in laboratory fish (even without any treatment or stressors) is often higher than that of wild fish. Several stress-related studies have reported plasma concentrations of cortisol (reviewed in Martine-Porchas *et al*., 2009) in fish, pre-stress concentrations of which varies from 2-315nmol/L (~ 0.8 – 120 ng/mL). These baseline levels tend to increase over 100-fold due to handling stress (Table 4.6). Cortisol concentrations in control fish of the present study was about 40 ng/mL. There are not many reports of measured cortisol concentrations for fathead minnows. One laboratory study with fathead minnows (control fish average) reports 147 -153 ng/mL (Richards *et al*., 2007). Although there was a significant impact revealed in the present study, due to the varied nature of the results, plasma cortisol concentrations is probably not a promising endpoint in GC-related studies. However, any reduction in cortisol means that hypothalamic controlled endocrine activities such as gonodal sex steroids and growth may also be impacted in GC-exposed fish. Therefore, reproductive endpoints come into the picture.

Results from previous studies investigating the effect of cortisol on reproduction are of considerable interest (reviewed in Letherland and Barkataki, 2010). A reduction in the number of perinucleolar follicles in ovaries could mean that GCs arrest development of primary oocytes. This could be explained via the HPG negative feedback loop, leading to reduced concentrations of gonodotophins in plasma and hence reduced stimulation of oogenesis.

Table 4-6. Basal plasma cortisol concentrations and their changes due to stressors in different species of fish (Table taken from Martine-Porchas, 2009)

Species	Stressor	Cortisol (nmol/l)		Exposure
		Prestress	Poststress	
Atlantic char *Salvelinus alpinus*	Handling	5	449	Acute
Atlantic salmon *Salmo salar*	Sea lice challenge	99	339	Chronic
Atlantic salmon (diploid) *Salmo salar*	Confinement	27	151	Acute
Atlantic salmon (triploid) *Salmo salar*	Confinement	27	124	Acute
Brook trout (diploid) *Salvelinus fontinalis*	Handling and confinement	19	242	Acute
Brook trout (triploid) *Salvelinus fontinalis*	Handling and confinement	2	146	Acute
Common carp *Cyprinus carpio*	Density	19	206	Acute
Pallid sturgeon Scaphirhynchus albus	Confinement	5	16	Acute
Pallid sturgeon *Scaphirhynchus albus*	Handling	5	8	Acute
Rainbow trout *Oncorhynchus mykiss*	Chemical exposure	49	110	Chronic
Rainbow trout (diploid) *Oncorhynchus mykiss*	Handling and confinement	77	698	Acute
Rainbow trout male *Oncorhynchus mykiss*	Trapping	16	380	Acute
Rainbow trout female *Oncorhynchus mykiss*	Trapping	57	764	Acute
Sea bream *Sparus aurata*	Crowding	13	358	Chronic
Walleyes *Stizostedion vitreum*	Capture and transport	33-315	380-480	Acute

Plasma Vtg concentrations were found to be lowered in sexually maturing trout implanted with cortisol (Carragher *et al*., 1989). A similar reduction was also found (with elevated cortisol concentrations) in trout following 2 weeks confinement stress (Campbell *et al*., 1994). Female brook trout subjected to acid stress had lower Vtg concentrations (Roy *et al*., 1990). In contrast, cortisol implants in juvenile arctic charr elevated plasma Vtg concentrations (Berg *et al*., 2004). However, in that study, Vtg elevation was not associated with Vtg gene expression and it was concluded that cortisol acts on post-translational aspects of vitellogenesis. In another study, there was no effect on Vtg concentrations by cortisol alone, but a dose-dependent stimulation effect of cortisol on oestrogen-induced plasma Vtg concentrations was found (Brodeur *et al*., 2005). Several studies report that cortisol directly

inhibited the production of oestrogen in fish (Carragher and Sumpter, 1990; Foo and Lam, 1993; Reddy *et al*., 1999; Pankhurst and Van Der Kraak, 2000). These observations are supported by a recent report showing *in vitro* suppression of estradiol synthesis by ACTH in ovarian follicles (Alsop *et al*., 2009). It is well known that vitellogenesis is oestrogen-dependent. Collectively, these works suggest that GCs may exert inhibitory effects on vitellogenesis. In the present study, a reduction in the Vtg concentrations was only evident at the highest exposure concentration. Plasma Vtg concentrations in the control fish were in agreement with the reported range of Vtg concentrations for unexposed, sexually mature, female fathead minnow (17.4±8.4 mg/mL; Watanabe *et al*., 2007).

In the fathead minnow, SSCs are under endocrine control. Nuptial tubercles are located on the head (dorsal pad) of reproductively active male fathead minnows, and are usually arranged in a bilaterally symmetric pattern. Normal mature females and juveniles of both sexes exhibit no tubercle development (Jensen *et al*., 2001). An increasing number of nuptial tubercles in the treatment groups suggest masculinisation effects of GCs. But fat pad index or fat pad height of treated males did not change towards any increased masculinisation. In contrast, females did show more masculinisation characters. Although no female fish exhibited a fat pad with score of 5 (which is usually given to sexually active large males with well developed fat pads), overall an average fad pad score of 2.15 for GC exposed females means a considerable number of female fish had male characteristics. This was also indicated by the presence of a black spot on the dorsal fin and a significant decrease in ovipositor length.

Control of sexual determination and differentiation varies greatly among teleost fish. There are two main types, genetic sex determination and temperature-dependant sex determination, and in both groups, sex reversal has been reported. Environmental factors such as temperature, pH, stocking density, and social interactions have been found to determine the

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proportion of male and female fish (Nakamura *et al*., 1998; Baroiller *et al*., 2009). In teleosts, sex steroids affect the development of germ cells and organs involved in sexual differentiation (Devlin and Nagahama 2002). The sex steroid 17β-estradiol is considered to be responsible for the development of the ovaries and female SSCs, and its concentration is considerably higher in females than in males. Testis development and male SSCs are mainly regulated by the androgen 11-ketotestosterone (11KT), not by testosterone as in mammals. Testosterone acts as precursor of 11-KT and 17β-estradiol (Nakamura *et al*., 1998; Baroiller *et al*., 1999). According to Bogart (1987), sexual differentiation depends on the balance between 11KT and 17β-estradiol; in this way excess 11KT induces masculine differentiation, while excess 17β-estradiol induces feminine differentiation. A similar hypothesis has been applied to sex reversal of mature fish, as in the case of feminisation with exogenous estradiol.

Elevated concentrations of cortisol due to stress has been shown to induce masculinisation in fish, but the mechanism by which cortisol masculinises fish has not been reported (Yamaguchi *et al*., 2010; Hayashi *et al*., 2010). It is not clear if the synthetic GCs used in the present study bio-concentrated in fish and mimicked the situation of elevated cortisol concentrations of fish, nor is it known if the synthetic GCs bind more tightly to the fish GR and thus have a greater half-life. Evidence from rodents and humans has shown that GCs oppose the action of oestrogens (Sahlin, 1995; Rhen *et al*., 2003). A recent study has demonstrated that activation of GR by dexamethasone induced the expression and activity of oestrogen sulfotransferase, an enzyme important for the metabolic deactivation of oestrogens, because sulfonated oestrogens fail to activate the oestrogen receptor. Treatment with dexamathasone lowered circulating oestrogens, compromised uterine oestrogen responses, and inhibited oestrogen-dependent breast cancer growth *in vitro* (Gong *et al*., 2008). This study also confirmed that the mouse and human oestrogen sulfotransferase gene is the transcriptional targets of GR and deletion of this gene in mice abolished the dexamethasone

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effect on oestrogen responses. A similar mechanism in fish could reduce the concentration of active estradiol and may affect the balance of 11KT and estradiol in fish to alter the SSCs.

In human, GCs are used to treat androgen-dependant prostate cancer and it is known that GCs reduce the plasma testosterone levels in males via a negative feedback loop of the HPG axis. This contradicts the present finding in males, where the number of nuptial tubercles was increased with GC treatment. This needs further verification.

It has also been questioned whether GCs act as ligands for androgen receptors (AR) and induce the masculinisation. In fish, two ARs, called AR1 and AR2, have been characterised. AR1 has a high affinity for testosterone. AR2, which is equivalent to human AR, has affinity for a variety of natural and synthetic androgens, including DHT. AR2 has been found to be expressed in ovaries (Sperry and Thomas, 1999). SSC are thought to be controlled via AR2, and possibly cross activation of AR2 by GCs could masculinise the fish. It has been shown that some corticosteroids are also able to bind to gonadal progestogen receptors. 11-deoxycorticosterone (DOC), the teleost mineralocorticoid, and to a lesser extent 11-deoxycortisol, bound to a membrane progestogen receptor (receptor for 17α-20β-dihydroxyprogesterone, which is known as maturation inducing hormone, MIH) in spotted sea trout *Cynoscion nebulosus*. But, cortisol did not bind to these receptors (Pinter and Thomas, 1995). In male Japanese eel, DOC did show high affinity for MIH receptor in the testis but cortisol did not bind to it (Todo *et al*., 2000). In yellowtail (*Seriola quinqueradiata*), 11-deoxycortisol bound to the MIH receptor (Rahman *et al*., 2002). Interestingly, Fathead minnows exposed to low concentrations of progestins have been shown to be masculinised, in research conducted in the same lab as the present study (per.com: Dr Tamsin Runnalls). This strongly suggests the possible cross activation of different steroid receptors by GCs.

Overall, the effects on the reproductive endpoints assessed in the present study suggest that low concentrations of GCs have impacts on every endpoint studied. What is not known is whether the effects are reversible or not. However, reproductive impacts could pose a threat to wild populations. Sex reversal towards masculinisation has a more severe impact than vice-versa. Therefore the next logical step in the environmental risk assessment of GCs would be to establish concentration-response relationships in a reproductive performance assay, and to compare these with reported environmental concentrations of GCs, in order to ascertain whether or not environmental concentrations of GCs are high enough to challenge reproduction in wild fish.

All the endpoints studied in experiment-2 did show a concentration-related impact of GC on fathead minnows. If not, there was a concentration-related trend or no effect. There were no signs of 'U' shaped dose-response curves, or any other unusual shaped curves. This make the results look reasonable and strongly suggest that they will be repeatable. Measured tank water concentrations of the exposure chemical are only presented for prednisolone in experiment-1. Results are presented with nominal concentrations for experiment-2. However, the concentration-related effects in Experiment-2 confirm the presence of beclomethasone dipropionate at the expected concentrations.

It was not possible to suggest a NOEC for beclomethasone dipropionate from the present study, as it produced one or more effects at all tested concentrations. The LOEC was 100 ng/L, which may not be environmentally-relevant. However, it should be noted that the present experiments involved relatively short exposure periods. Perhaps longer exposure would lead to more pronounced effects, and/or effects at lower concentrations. Whole lifetime exposure studies with EE2 have shown that extremely low concentrations (less than 1 ng/L) cause dramatic effects (Lange *et al*., 2001). The same concentrations do little, or nothing, in

short term tests. Therefore, in the case of GCs, the NOEC must be below 100 ng/L and may well be in the low ng/L range, which could be environmentally-relevant.

Because many different GCs are in widespread clinical use, it seems likely that, concurrently, many different GCs will be present in the aquatic environment. As our binding studies demonstrate, all of the GCs tested here can bind to the fish GR (as expected), and therefore it can be argued that the total concentration of GC in the environment, rather than the concentration of each individual GC, is of most relevance to the risk assessment of GCs on aquatic organisms. Nevertheless, it is very likely that the effects of different GCs will be additive, as has been shown for oestrogenic chemicals (Brian *et al*., 2005). Interestingly, the present study reveals a masculinising tendency of GCs in fish, which is in the opposite direction of the effects of oestrogenic chemicals in the environment. Therefore, assessment of the impacts of mixtures of these steroids (for example, a GC plus an oestrogen) on fish reproduction is needed before a complete picture of their potential to affect wild population of fish is obtained.

4.5   Conclusions

The present chapter describes experiments conducted to test the null hypothesis, which was that environmentally-relevant concentrations of GCs do not have adverse impacts on fish. Results from the experiments indicate that the null hypothesis cannot be accepted, because low concentrations of GCs via water exposure had significant impacts on fathead minnows. Increased plasma glucose concentration may affect their normal physiology and immuno-suppression could make them more susceptible to disease. Adverse impacts on reproduction could result in population-level changes in wild fish. Reproductive impacts, which are in the opposite direction to the reported effect of oestrogens in wild, suggest a need for the studies of mixtures of synthetic steroids on reproduction and other physiological processes of fish.

Chapter 5.0

Glucocorticoid Exposure And Gene Expression

5.1   Introduction

'Omic' technologies, including metabolomics, genomics and proteomics, have advanced considerably in recent years and have many applications in environmental toxicology. Although a significant amount of basic research and validation is needed before 'omic' endpoints are incorporated in routine environmental risk assessments, these tools and associated endpoints are already significantly improving our understanding of how individual chemicals and mixtures affect organisms and are influencing risk assessment (Snape *et al*., 2004; Van Aggelen *et al*., 2010). It has already proved possible to identify signatures of oestrogen exposure (Larkin *et al*., 2007; Santos *et al*., 2007), the stress response (Aluru and Vijayan, 2009), stress and reproduction (Alsop *et al*., 2009) and metal signalling pathways (Zheng *et al*., 2008). The high cost of 'omic' techniques, however, imposes restrictions on the number of doses, replicates, and time points assessed after chemical exposure *in vivo* and *in vitro*. Therefore 'omics' technologies can be viewed as complementary testing procedures that can improve understanding of mechanisms of toxicity, differential species sensitivity, and classification of chemical-specific biological responses. This approach also provides leads for identification of novel biomarkers of exposure and can lead to the development of simpler individual assays with defined end points. This chapter investigates the expression of selected genes in the fathead minnow in response to GC exposure and aims to identify a possible signature gene. Along with this, the aim is to understand the mechanism of action of GCs in fathead minnows.

5.1.1   Gene Expression and Real-Time PCR

In molecular biological terminology, gene expression is the transcription of DNA into messenger RNA (mRNA) by RNA polymerase. mRNA is the template from which proteins are synthesized. Briefly, an mRNA strand is specific for a certain protein/enzyme, and each

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one is transcribed directly from a specific DNA-sequence, or gene. Therefore, in gene expression analysis, the expression level is directly proportional to the amount of mRNA detected in a sample. Polymerase Chain Reaction (PCR) is the amplification of a single or few copies of a specific region of DNA of interest using thermal cycling. After amplification, the product can be verified by visualising it on agarose gel. Absolute quantification of this product is not possible. It is more suited for determining the presence/ absence of a gene.

Use of a one step quantitative real-time PCR (qRT-PCR) kit enables the reverse transcription of mRNA into complementary DNA (cDNA) and subsequent PCR in the same tube. The use of these kits in modern thermo-cyclers enables the measurement of PCR product in real time and subsequent quantification of mRNA in the sample. qRT-PCR is a very sensitive technique that requires good quality RNA. It is the most accurate, advanced and partially automated method for the quantification (as the reaction progresses) of gene expression. Although initially there were some problems caused by variability of RNA templates, assay designs and protocols, as well as inappropriate data normalization and inconsistent data analysis reported, qRT-PCR is now widely accepted as the method of choice for the quantification of mRNA in molecular medicine, biotechnology, microbiology and diagnostics fields (Nolan *et al*., 2006).

The qRT-PCR consists of a fluorescence-based assay, which detects, amplifies and simultaneously quantifies the amount of messenger RNA in a sample. This real-time detection of PCR products is achieved by the presence of a fluorescent molecule (SYBR Green in this study) in the reaction that binds to double-stranded DNA fragments, and hence as the amount of DNA increases, there is a proportional increase in the fluorescent signal. Specific primers target the specific cDNA fragments which are derived from the gene of interest. These fragments are copied using an enzyme (Taq polymerase) which enzymatically assembles a new strand of DNA from a single strand of DNA (template) and primers, which are needed

for initiation of DNA synthesis. The number of target fragments increases exponentially during every amplification cycle. Therefore the amount of fluorescence in a sample is relative to the amount of the cDNA fragments produced in each amplification cycle. By comparing the number of cycles needed to produce a certain amount of fluorescence in control and treated samples (Figure 5.1), gene expression can be assessed and quantified (Bustin, 2004).
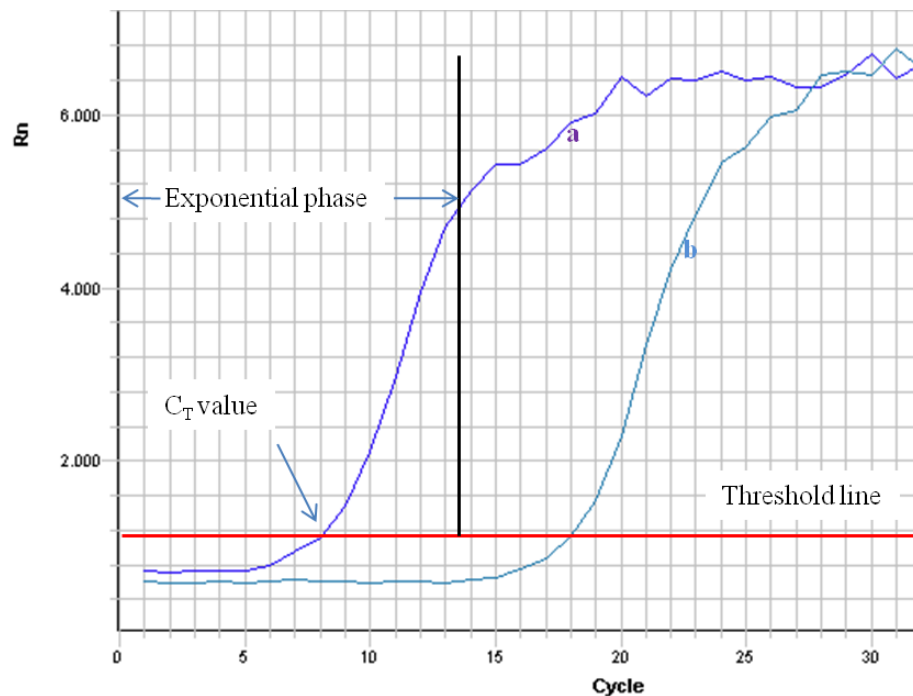
Figure 5-1. Typical amplification plots of two samples a and b. Threshold line is set above the noise and C_T value corresponds to the number of cycles required for the particular amplification. If sample-a is control and sample-b is treated fish, then this particular gene is said to be down regulated by the treatment. But the quantitative differences needs normalisation with another reference gene or with a standard curve, as explained in 5.2.1.

The qRT-PCR technique can be used for both relative and absolute quantification of a gene. Both strategies involve gene expression quantification using a small amount of RNA template and are equally sensitive. Usually absolute quantification, which derives the copy number with the help of a standard curve of known mRNA concentration, is used in medical diagnostic purposes. In order to compare the level of gene expression between control and treated fish, relative quantification was used in this study (See Section 5.2.1). Relative

quantification determines the changes in RNA level of a gene and expresses it relative to the level of an internal control "housekeeping" gene. A gene that is transcribed at a relatively constant level across many or all known conditions is called a housekeeping gene. The housekeeping gene's products are typically needed for maintenance of the cell. It is generally assumed that their expression is unaffected by experimental conditions. Examples include beta-actin (β-actin), GAPDH and ubiquitin. It is possible to directly compare the level of expression of the gene in question from different treatment groups with the internal housekeeping gene. Some of the known housekeeping genes have recently been reported to be unsuitable in endocrine disrupting studies. For example, Runnalls *et al*. (2005) found that expression of the β-actin gene of fathead minnows was affected by the lipid lowering drug clofibric acid. Similarly, Vtg levels were over estimated in an oestrogen exposure experiment using β-actin as the internal control (Filby and Tyler, 2007). Therefore two housekeeping genes were selected (β-actin, 18sRNA) and were tested for their suitability in the present study (see Section 5.2.1).

5.1.2   Gene Expression and Ecotoxicology.

Gene expression endpoints are frequently used in mammalian toxicology, where the human and mouse genomes are fully available. Microarray techniques can study the expression profile of a large number of genes at one time. However, its application in aquatic toxicology is considerably limited because full genome sequencing for ecologically relevant species is still in the early stages. Completed genomes are available for green spotted puffer fish (*Tetraodon nigroviridis*), the Japanese puffer fish (*Takifugu rubripes*) and the zebrafish (*Danio rerio*), and commercial microarrays are available for zebrafish. Several studies have used these arrays to understand physiological responses, to determine mechanisms of action for toxicants and other natural stressors, and to define modes of action for new chemicals (reviewed by Denslow *et al*., 2007).

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Although whole genome data are often not available, lots of individual genes have been identified and sequenced from many fish and other aquatic species. Using these data, changes in gene expression profiles, in response to aquatic pollutants and climatic factors, have been studied (reviewed in Scholz and Mayer, 2008). For example, the up-regulation of P450 aromatase mRNA expression in the liver and brain in response to environmental EDCs has been reported in the Atlantic salmon (Lyssimachou *et al*., 2006). Lange *et al*. (2008) exposed roach to various concentrations of EE2 and observed that effects on gonadal development were associated with alterations in expression of the oestrogen receptor and aromatase genes. Werner *et al*. (2010) studied the expression of six genes responsive to endocrine-disrupting compounds, stress and metals after exposing fathead minnows to kraft and paper mill effluents, and found sex-specific changes in gene expression, suggesting that the effluent had androgenic activity. Several studies used the metallothionin gene as a biomarker for metal exposure and hypoxia-related stress (reviewed by Zheng *et al*., 2008). Gene expression profiling (using microarrays) of fish exposed to sewage effluent showed that significant changes occurred in the gonads of fish held below, compared to above, the treatment plant (and to laboratory control fish). Among the biological processes affected were the innate immune response, tthe stress response, control of homeostasis, control of transcription, metabolism, and cell communication. This work suggested that fish are impacted by exposure to sewage treatment effluents and showed that effects can be detected rapidly by gene expression profiling (Garcia-Reyero *et al*., 2008). All over the world, molecular biology techniques are increasingly used and hence more reports are appearing in the literature containing gene sequences and microarray details, which could mean that gene expression may be a useful endpoint in regulatory ecotoxicology in the future.

5.1.3 Genes of Interest.

There have been several studies published which are relevant to the present study, involving measuring gene expression in fish in response to acute stress and exogenous cortisol exposure. Some of these genes are summarised in Table 5.1. GCs play several roles in the animal's physiology and they are known to act on almost every organ (Chapter 1). Thousands of genes could therefore be affected by GC exposure. As *in vivo* exposure had an impact on glucose levels (Chapter 4), it was decided that it would be important to investigate a gene that is involved in glucose metabolism. It is also known that GCs increase the plasma glucose concentration by inducing gluconeogenesis, which is the process of synthesising glucose from non-carbohydrate sources such as lactate, glycerol and glucogenic amino acids (Figure 5.2). Phosphoenolpyruvate carboxykinase (PEPCK; EC: 4.1.1.32) is an enzyme that controls the rate limiting step in this process (Matte *et al*., 1997). PEPCK has been reported in several species and it has been shown that two forms (mitochondrial and cytosolic) exist. The gene sequence for PEPCK is not available for the fathead minnow. However, it is available for other fish species, including rainbow trout (see Section 5.2.4), which makes constructing primers possible. The trout PEPCK sequence has been shown to have 67% similarity with the human PEPCK sequence (Matte *et al*., 1997).

The second gene investigated in this study was the glucocorticoid receptor (GR). The negative feedback loop of the HPI axis has been reported to be controlled via GR down-regulation in stressed fish. Two GR genes have been found in rainbow trout (Bury *et al*., 2003), *Haplochromis burtoni* (Greenwood *et al*., 2003), European sea bass *(D. labrax*; GR1-Terova *et al*. (2005), GR2- Vizzini *et al*. (2007)), *T. rubripes* and *T. nigroviridis* (Stolte *et al*., 2006), and *O. latipes* and *G. aculeatus* (Alsop and Vijayan, 2008). In contrast, only a single GR was identified in zebrafish, which is homologous to GR2 in other teleosts (Alsop and Vijayan, 2008). Stolte *et al*. (2008) also identified two GRs in carp. Recently, the sequence of the GR

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in fathead minnow (Gene Bank: AY533141) has been reported (Filby and Tyler (2007) which is also homologous to GR2 in other teleosts. The present study used this sequence for GR-primer construction.

The third gene investigated in this study was the Vtg gene. Vtg is a well known biomarker for oestrogenic chemical exposure and it has been characterised and sequenced in several fish species, including the fathead minnow (Gene bank: AF130354; Korte *et al*., 2000). The expression of the Vtg gene in fish liver (up-regulation due to oestrogenic chemicals) has been reported in several studies (reviewed in Rotchell and Ostrander, 2003; Larkin *et al*., 2003). Vtg gene expression has been shown to be down-regulated by exposure to androgenic substances in the environment (Dorts *et al*., 2009; Ekman *et al*., 2011). Cortisol has been shown to inhibit vitellogenesis by down-regulation of ER and Vtg expression in salmonids (Lethimonier *et al*., 2000). Vitelline envelope protein subunits, regulated by ER signalling, were also down-regulated with cortisol treatment and acute handling stressors (Aluru and Vijayan, 2007), which confirms a role for cortisol in affecting oestrogen-responsive gene expression in the liver. It was also noted that exposure to GC resulted in the development of male SSCs in female fathead minnows (Chapter 4). Thesrfore, it was decided to measure Vtg gene expression in females.

Filby and Tyler (2007) have identified several genes that are appropriate as housekeeping genes for use in oestrogenic endocrine disruption studies. β-actin has been used as a housekeeping gene in several studies. However, β-actin was reported to be affected by a pharmaceutical, clofibric acid (Runnalls, 2005), and also by thermal exposure (Brian *et al*., 2008). As none of the housekeeping genes have previously been reported to be suitable in studies involving beclomethasone dipropionate exposure (the present study), it was decided to test the suitability of both β-actin and 18s ribosomal RNA (18s rRNA) as housekeeping genes

in this study. For the fathead minnow, a partial sequence of the 18s rRNA gene (Gene Bank: AY855359) has been reported (Filby and Tyler, 2005). A partial sequence of the fathead minnow β-actin gene (Gene Bank: EU195887) is also available online via the NCBI web site.

Table 5-1. Fish genes known to respond to acute stressors and exogenous cortisol treatment.

Function	Gene name	Treatment	Reference
Metabolism-related genes	PEPCK, Pyruvate kinase, Glucokinase, Arginase, Ubiquitin, Cathepsin D, GR, Glutamine synthetase-2 (GS-2). Matrix metalloproteinase-2, Glucose transporter-2, Lipoprotein lipase, Glucose-6-phosphatase	Acute stressor	Wiseman *et al.*, (2007)
	Glucose-6-phosphatase	Acute stressor	Momoda *et al.*, (2007)
	Glyceraldehyde-3-phosphate dehydrogenase Fructose-bisphosphate aldolase A Serine pyruvate aminotransferase Glutamate carboxy peptidase like protein, Transaldolase	Acute stressor	Krasnov *et al.*, (2005)
	PEPCK, GS-1, GS-2, GS-4, Arginase, Cathepsin D, GR, Glutamate decarboxylase 65, Lipoprotein receptor	Cortisol *in vitro*	Aluru and Vijayan, (2007)
	Fructose-1,6-bisphoshatase, Ornithine decarboxylase, Sodium–potassium ATPase	Cortisol *in vivo*	Sarropoulou *et al.*, (2005)
	PEPCK, heat shock protein-90 (hsp-90), hsp-70	Cortisol *in vivo*	Vijayan *et al.*, (2003)
Immunity-related genes	Major histocompatibility complex-2 (MHC-2), Tumor necrosis factor-α, Interleukin-1b Natural resistance associated macrophage-a, Transcription facor Nupr1.	Acute stressor	Wiseman *et al.*, (2007)
	Protooncogene JunB, MHC-1, Complement receptor, Complement factor H, Class I helical cytokine receptor number 21, Interferon inducible proteins Tumor necrosis decoy factor receptor	Acute stressor	Momoda *et al.*, (2007)
	Immunoglobulin epsilon receptor alpha Lysozyme C precursor, Transposon like protein, ATP-dependent CLP protease, Nuclear factor-kB) inhibitor, Stress activated protein kinase-4	Acute stressor	Krasnov *et al.*, (2005)
	Complement factor H, Anti-trypsin, b-Fibrinogen	Acute stressor	Cairns *et al.*, (2008)

Function	Gene name	Treatment	Reference
	Transferrin receptor, Ferritin heavy and light subunits	Cortisol *in vivo*	Sarropoulou *et al.*, (2005)
Reproduction-related genes	Vitellogenin envelope protein-b (VEP-b), Oestrogen receptor,	Cortisol *in vitro*	Aluru and Vijayan, (2007)
	VEP-c, VEP-b, Androgen receptor	Acute stressor	Wiseman *et al.*, (2007)
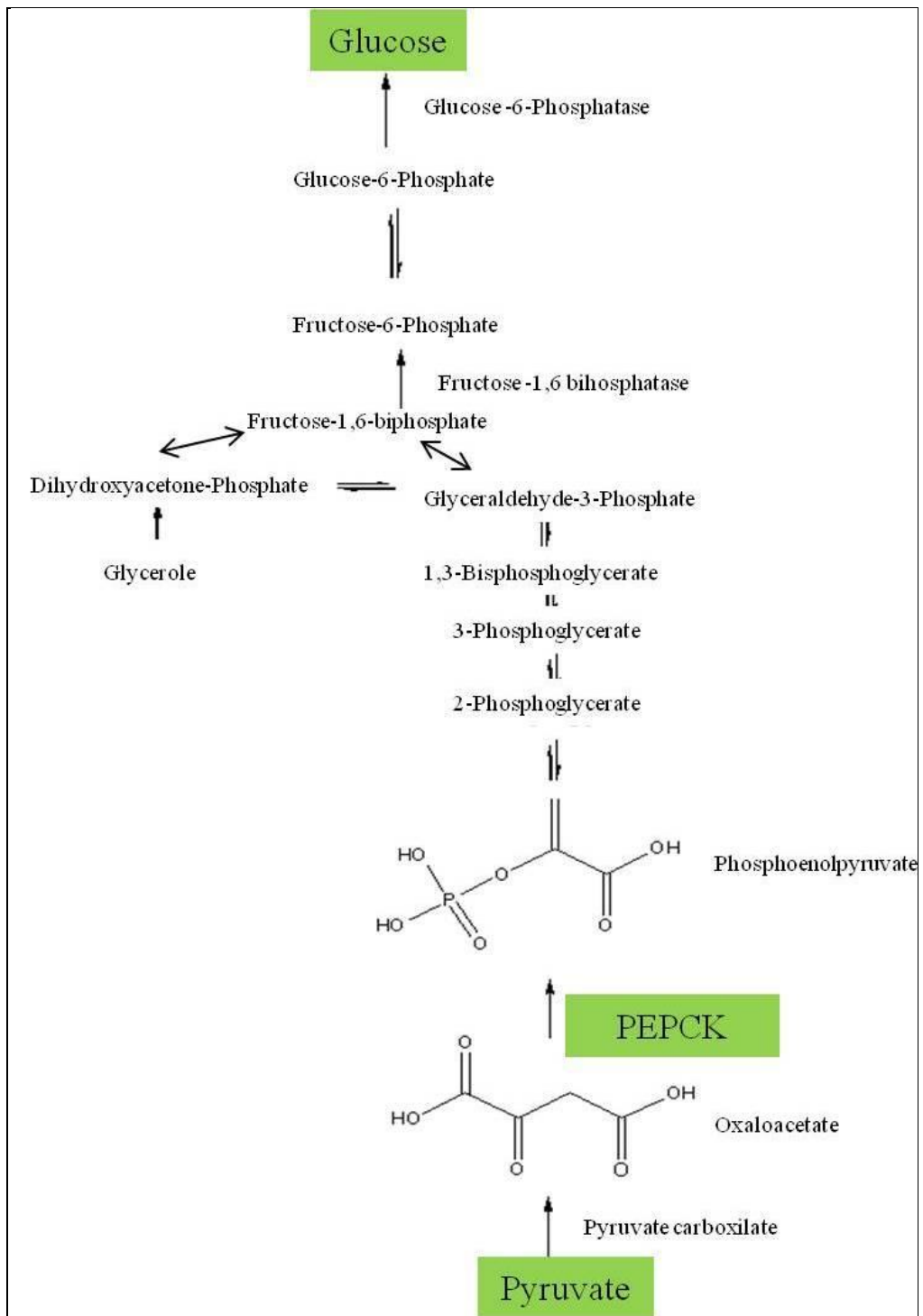
Figure 5-2. The metabolic pathway of gluconeogenesis, where glucose is synthesised from non-carbohydrate sources, in which PEPCK has a rate limiting role.

5.1.4   Objectives

The objective of the study reported in this chapter was to assess the effects of beclomethasone dipropionate on the expression of selected genes in fathead minnows.

Specific objectives were

- Assess the suitability of β-actin and 18s rRNA as housekeeping genes.
- Quantify PEPCK and GR gene expression in the liver of both sexes and Vtg gene expression in female fathead minnows exposed to different concentrations of beclomethasone dipropionate.

5.2   Materials and Methods

5.2.1   Exposure and Sampling

Exposure conditions and the experimental set up are described in Chapter 4.2. Liver samples were taken from beclomethasone-exposed fish of experiment-1(n=10), set-2 of experiment-2 (n=60) and their respective control fish (n=30). For Vtg expression, only females were used. All the work surfaces and dissection tools were prepared for molecular work – they were previously autoclaved and wiped with 100% ethanol and RNase away solution between samples, to avoid cross-contamination. Liver samples were placed in RNA-free tubes, snap-frozen and stored in a -80° C freezer until RNA extraction.

5.2.2   Total RNA Extraction

Total RNA from each liver sample was extracted using RNeasy midi kit (Qiagen, UK) according to the manufacturer's instructions. Although DNase treatment (to exclude any DNA contamination) was not necessary according to the protocol, extraction of spare samples with and without DNase treatment (Figure 5.3) revealed the need for DNAse treatment. Therefore all the samples were treated with RNase-free DNase (Qiagen, UK). Any cross-contamination

was avoided by using trigene surface disinfectant and RNase away. Before starting the procedure, 10 µL of β-mercaptoethanol (Sigma) was added to each mL of buffer RLT (supplied with the kit). Buffer RPE (supplied with the kit) was diluted with four volume of ethanol. 70% ethanol was prepared using milliQ water.
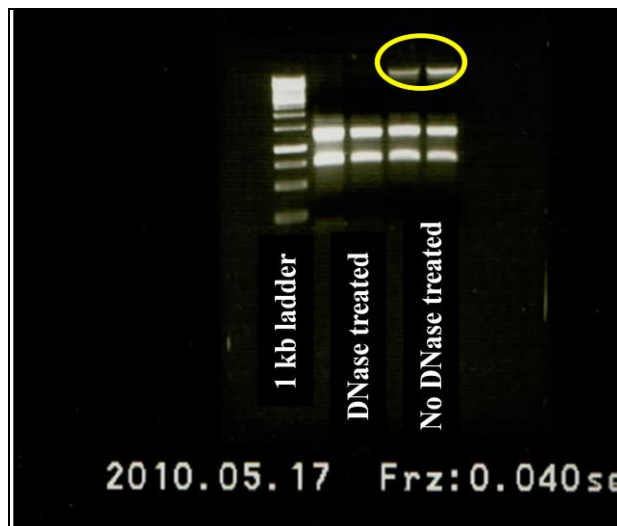
Figure 5.3. An example of RNA bands on agarose gel, under UV illumination. RNA samples extracted with and without DNase treatment were run in 1.2% agarose gel with ethidium bromide for 45 minutes. Band inside yellow circle indicates DNA contamination in non-treated samples.

Liver samples were taken from the freezer on dry ice and 200 µL buffer RLT was added before the samples were immediately homogenised using a rotor-stator homogenizer (Fisher Scientific Ltd). The homogenised sample was then transferred into a 15mL falcon tube (Fisher) and another 1800 µL of buffer RLT was added. This was centrifuged at 25 °C at 4000 g for 10 minutes. The supernatant was collected (avoiding the fatty layer and the sediment) and transferred into another 15mL tube and 2 mL of 70% ethanol was added and the tube was immediately shaken. This mixture was transferred into a midi column which was placed in a 15mL tube and was centrifuged for 5 minutes at 4000 g. The flow-through was discarded and 4 mL buffer RW1 (supplied with the kit) was added to the column and centrifuged at 4000 g

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for 5 minutes. Again the flow-through was discarded and 2.5 mL RPE buffer was added and centrifuged at 4000 g for 3 minutes. This step was repeated with another 2.5 mL RPE buffer for 5 minutes. Then the column was transferred into a clean RNA-free tube and the RNA was eluted using 150 μL RNAse-free water. Elution was repeated into another tube with 150 μL RNAse-free water.

Extracted RNA was quantified using a Nanodrop N-1000 spectrophotometer (Fisher Scientific, Loughborough) in ng/μL and the quality (integrity) of each sample was assessed by the 260/280 ratio of OD values. All the 260/280 ratios were between 1.90 and 2.10, and most of them were close to 2.00.

5.2.3   Gel Electrophoresis

All the extracted RNA was checked for possible DNA contamination and for integrity using gel electrophoresis. Gel electrophoresis is a technique for separating molecules based on their charge. RNA is negatively charged. When added to an agarose gel matrix and exposed to an electrical current, RNA products migrate towards the positive anode; smaller products will move further and faster than large products, thereby separating RNA according to product size in a series of bands. A DNA ladder, which indicates the size of bands, is used to estimate the size of the products. Ethidium bromide is a florescent tag and visualisation is achieved by subsequent ultraviolet (UV) illumination of the gel (Figure 5.3).

Tris borate EDTA buffer (TBE) of ten times concentrate was prepared with 109 g Tris base, 7.3 g EDTA and 55 g boric acid dissolved and make up to 1 litre in distilled water. This buffer was autoclaved and diluted ten times (1×TBE) for bench use. 1.2 g agarose (Biorad, molecular grade) was dissolved in 100 mL 1×TBE and warmed in a microwave oven for 2 minutes. Once the temperature of the solution was lowered to about 50 °C, 1μL of ethidium bromide (Sigma) was added and swirled to mix it before it was poured into the prefixed cavity

with comb and allowed to set. Once the gel was set, the comb was carefully removed and 1×TBE was poured in to fill the wells. Then the gel was placed in the reservoir filled with 1×TBE and connected to an electric source.

1 µg RNA was made up in 10 µL solution and 2 µL loading dye was added before it was loaded into a well. 8 µL of 1 kb DNA ladder was also run in parallel for 45 minutes. After 45 minutes, the gel was viewed under UV illumination and photographed (Figure 5.4).

All the RNA samples were found to be without DNA contamination. RNA samples were placed in a -80 °C freezer until analysed by qRT-PCR.
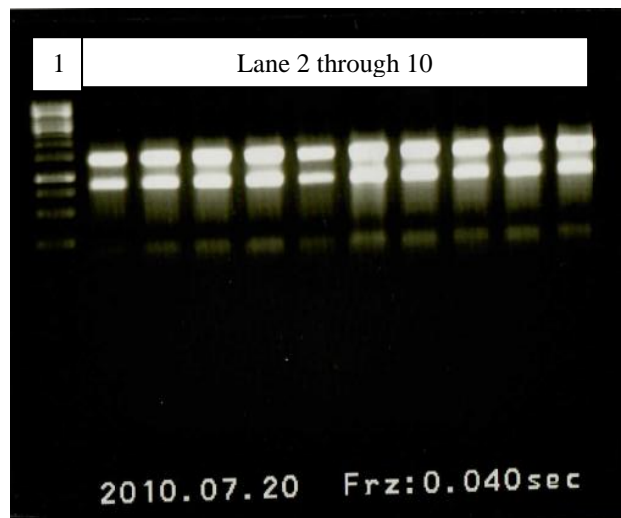
Figure 5-4. Gel electrophoresis image under UV illumination. RNA samples were run in 1.2% agarose gel with ethidium bromide for 45 minutes and show no sign of DNA contamination. 1 kb DNA ladder was run on the lane 1. Lane 2 through 10 were different RNA samples

5.2.4 Primer Construction

For every gene of interest, two sets (for Vtg and β-actin-3 sets) of forward and reverse primers were tested. When designing primers, the following criteria were considered.

- Design primers with a G, C content of 50 - 60%.
- Maintain a melting temperature (Tm) between 50 °C and 65 °C.

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- Avoid secondary structure.

- Avoid repeat of G or C longer than three bases.

- Place G or C on the end of primer.

- Check forward and reverse primers to ensure no 3' complementarity (to avoid primer-dimer formation).

- Try and design primers between 16 and 24 bp length.

- Try overlapping an intron-exon boundary.

- Design a primer set for an amplicon size of 75 to 200 bp lengths.

Primers were designed according to one of the following methods:

1. Sequence taken from previously published studies (see Table 5.2).

2. If the gene sequence for fathead minnow was available, then the software Primer 3 was used to design the primer sequence (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). The chosen primer sequence was then checked for specificity for the gene by running the NCBI Basic Local Alignment Search Tool (BLAST).

3. In the case of PEPCK, there was no gene sequence available for the fathead minnow. Therefore, a search was carried out using entries on the NCBI website. PEPCK sequences of three fish species were found; *Danio rerio* (Gene Bank: GI31418741), *Cyprinus carpio* (partial sequence; Gene Bank: GI24637091) and *Oncorhynchus mykiss* (Gene Bank: GI13506885) and were aligned by using the alignment tool ClutalW (Appendix 2). Two sets of primers were designed taking into account the criteria specified above, from the most similar regions within the alignment.

Table 5.2 summarises information about the primers designed for use in this study. All of the primers were purchased from Sigma (UK), and diluted to 100 µM according to the manufacturer's instructions. Two aliquots of 10 µM primers were also made up by adding 10 µL of above primer solution into 90 µL RNAse-free water, clearly labelled and stored in a -20°C freezer.

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Table 5-2. Primer sequence information, annealing temperature, G,C perentage, product length and the sources of the sequences used to design the primers

Primer name	Position in its sequence and length (bp)	Primer sequence: 5' -3'	Exon/intron boundary	Tm	GC%	Secondary structure	Product length	Source
Cβ-actin F	336-353 (18)	GAATCCCAAAGCCAACAG	NO	60.7	50	None	148	Filby and Tyler, 2007 Gene Bank:BC165331
Cβ-actin R	483-466(18)	AACACCATCACCAGAGTC	NO	55.5	50	None		
Tβ-actin F	247-265(19)	GATATGGAGAAGATCTGGC	NO	56.1	47.3	V week	105	Runnalls, 2005 Gene Bank: AF025305
Tβ-actinR	351-331(21)	GTTGGCCTTGGGGTTCAGGGG	NO	74.4	66.6	Week		
‡Sβ-actinDF	824-842(19)	CCTTCCTTCCTGGGTATGG	836,838	63.2	57.8	None	158	Manual Gene Bank:BC165331
‡Sβ-actinR	981-962(20)	TCCTTCTGCATACGGTCAGC	NO	65.3	55	Week		
‡JVTGF	1278-1298(21)	TGGCCTCTGCAGCAATATCAT	NO	70.9	57.1	Week	128	Brian *et al.*, 2008 Gene Bank: AF130354
‡JVTGR	1405-1385(21)	TGGCCTCTGCAGCAATATCAT	NO	66.7	47.6	None		
DVTGF	1393-1414(22)	GCTGCAGAGGCCATTTCTAAGA	NO	66.6	50	V week	69	Dorts *et al.*, 2008 Gene Bank: AF130354
DVTGR	1461-1441(21)	AGCATTGCCCAGAACTTTCAG	NO	65.4	47.6	None		
3VTGF	1677-1696(20)	CGAGGCCAAGCCCTCAGTGG	NO	73.5	70	Strong	147	Primer 3 software Gene Bank: AF130354
3VTGR	1823-1804(20)	GCACCCGCAACAGGTGCCAT	1809,14	74.4	65	Strong		
PEPCKAF	*827-847(21)	CTGCTGGGGAAGAAGTGCTTC	NO	67.3	57.1	Mode	89	*Danio rerio* (Gene Bank: GI31418741), *Cyprinus carpio* (partial sequence; Gene Bank: GI24637091) and *Oncorhynchus mykiss* (Gene Bank: GI13506885)
PEPCKAR	*915-896(20)	CCCAGAATCAGCATGTGTTC	NO	63.2	50	V week		
‡PEPCKBF	*1508-1529(22)	GCTGCTGAACACAAAGGTAAAGTG	1522,26	65.7	45.8	Week	153	
‡PEPCKBR	*1660-1640(21)	GAACCAGTTGACGTGGAAGAT	NO	62.9	47.6	Week		
FGRF	2164-2184 (21)	GAAAGTCCTTCTGCTCCTGAG	NO	62.1	52.3	V week	125	Filby and Tyler, 2007 Gene Bank: AY533141
FGRR	2288-2267(22)	AGTTCTCCTCTCTCTTCACAATG	NO	60.5	43.4	None		
‡3GRF	1737-1756(20)	CCATGCCTCAGCTGGTGCCC	NO	74.6	70	Week	134	Primer 3 software Gene Bank: AY533141
‡3GRR	1870-1851(20)	GCCGCCCAGCCTGTTCAGAG	NO	73.6	70	None		
‡C18SF	19-40(22)	AATGTCTGCCCTATCAACTTTC	NO	60.9	40.9	None	117	Filby and Tyler, 2007 GenBank: AY855349)
‡C18SR	135-117(19)	TGGATGTGGTAGCCGTTTC	NO	63.6	52.6	None		
318SF	513-532(20)	CGTCGCCGCTGAATACCGCA	NO	75.4	65	None	160	Primer 3 software GenBank: AY855349
318SR	672-653(20)	CTCTCGTCCGTCTTGCGCCG	662	74.7	70	none		

‡ Primers used in qRT-PCR after validation as described in 5.2.5.   * Position in the *Danio rerio* PEPCK sequence (Gene Bank: GI31418741)

5.2.5   Primer Validation with Taq PCR

A single RNA sample (highly concentrated and showing clear banding and no degradation or DNA contamination from the gel electrophorosis) was chosen to check the best primers to use. RNA was converted to cDNA using the Omniscript reverse transcription Kit (Qiagen Cat. No: 205110). Total reaction volume was 20 μL, using the components from the kit as shown in Table 5.3. Reaction time was one hour at 37°C. This cDNA was diluted 5 times (by adding 80 μL RNase free water from the Omniscript kit) and stored in a -20 °C freezer.

Table 5-3. Reverse transcription reaction components

Component	Volume (μL) / reaction	Final concentration
10× buffer RT	2	1×
dNTP mix (5 mM each dNTP)	2	0.5 mM each dNTP
Oligo dT primer: random hexamer (25μM)	0.8	1μM
RNase inhibitor (40 units/μL)	0.25	10 units
Omniscript reverse transcriptase	1	4 units
RNase free water	12.45	
Template RNA	1.5	Up to 2 μg

All designed primers were validated using the above cDNA in a Taq PCR reaction on the iCycler PCR instrument (Bio-Rad Laboratories Inc). The Taq PCR master mix kit was purchased from Qiagen (Cat NO: 201443) and the PCR method followed according to the instructions supplied with the kit. Instead of a 100 μL reaction volume as suggested in the kit, 20 μL reactions were set up (Table 5.3). Each sample was set up in a sterile, thin-walled PCR tube on ice, according to the information in Table 5.4.

Table 5-4. Composition of Taq PCR reactions.

Component	Volume (µL) / reaction	Final concentration
Taq PCR master mix	10	2.5 units Taq DNA polymerase 1× Qiagen PCR buffer 200 µM of dNTP
Forward primer	0.5	0.1 – 0.5 µM
Reverse primer	0.5	0.1 – 0.5 µM
RNase free water	7	
Template DNA	2	< 1 µg/reaction

DNA amplification was conducted on the iCycler using the following thermal program:

Initial denaturation: 94°C for 3 minutes

Denaturation: 94°C for 30 seconds

Annealing: Temperature gradient from 50°C to 65°C for 30 seconds (35 cycles)

Extension: 72°C for 1 minute

Final extension: 72°C for 10 minutes.

For each primer pair, amplification of DNA was carried out over a temperature gradient to determine the best temperature for each pair. Following amplification, a 1.2 % agarose gel was prepared as described in section 5.2.3. The PCR products were then mixed with 2 µl loading buffer, and 12 µl of each sample was pipetted into the appropriate well of the gel alongside a 1 kb ladder, and run in an electrophoresis chamber at 80V. After 45 minutes, the gel was analyzed under UV light to check that the products of the bands were the right length, as indicted in Table 5.2. Figure 5.5 shows a photograph of a gel with different bands for the PCR products corresponding to different temperatures (difference in intensity) and different primers (difference in sizes). Primer pairs that produced a clear band at the right product length for all four temperatures were chosen for the qRT-PCR study.
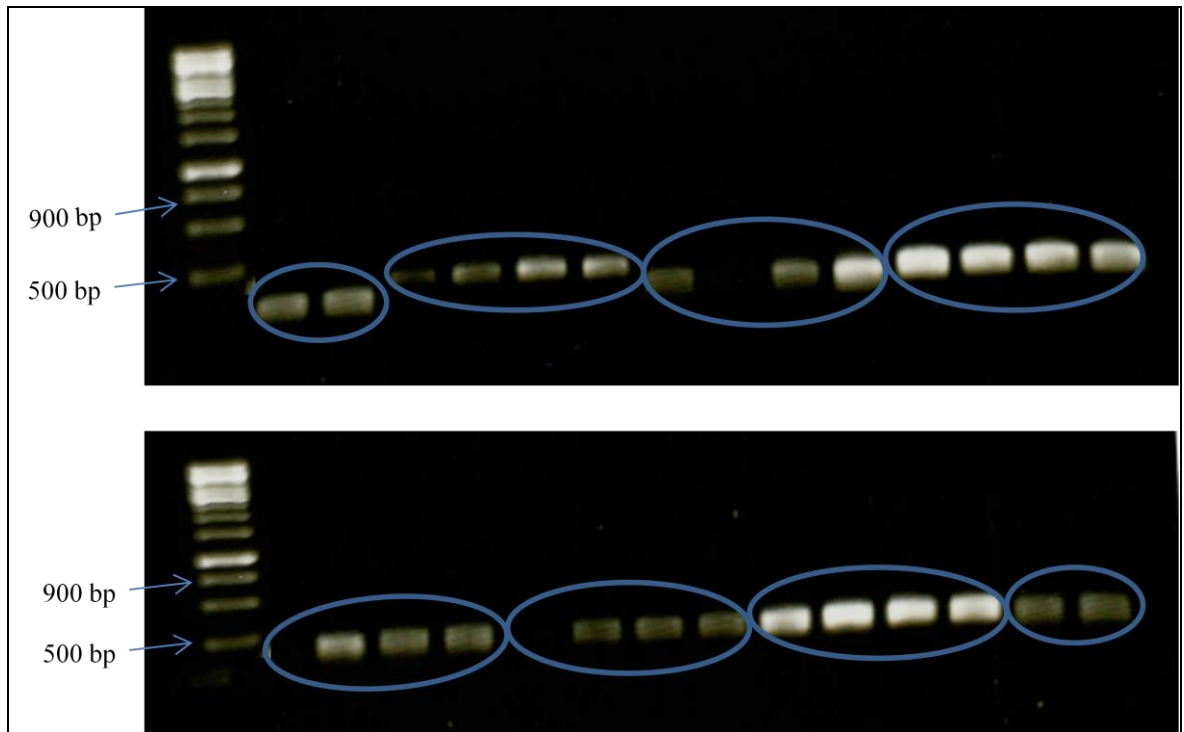
Figure 5-5. Photograph of an agarose gel under UV light, loaded with PCR products from different primer pairs at 4 different temperatures. Each circle corresponds to a different primer pair used in the PCR and each band inside the circle corresponds to four different annealing temperatures (except for the small circles with 2 bands; both from same primer pair, half was loaded on to one gel and hald on to the other gel). Primer pairs that produced a clear band of the right product length for all four temperatures were chosen for the qRT-PCR study.

5.2.6   Real-Time RT-PCR: Optimization

All the assays were conducted using the QuantiFast SYBR Green qRT-PCR kit (Qiagen cat no: 214154). This is a fast, single-step procedure that uses RNA as starting material (because it comes with the RT step in one tube) and a reaction volume as low as 10 µL.

Each kit contains:

1. QuantiFast SYBR Green qRT-PCR Master Mix (a mixture of HotStarTaq DNA Polymerase, QuantiFast SYBR Green qRT-PCR Buffer, dNTP mix (dATP, dCTP, dGTP, dTTP) and ROX passive reference dye).

2. QuantiFast RT Mix (a mixture of Omniscript Reverse Transcriptase and Sensiscript Reverse Transcriptase).

3. RNase-Free Water.

96-well plates (MicroAmp Optical 96-well Reaction Plate, Applied Biosystems Inc.) were also purchased, as they were compatible with the ABI prism.

Although a standard curve is not mandatory for quantification of relative gene expression, in order to determine the efficiency of qRT-PCR, standard curves were included on every plate. This was done so that the expression of the different genes could be compared directly between samples using the Pfaffl method of quantification (Pfaffl, 2001). Efficiency curves were determined by running serially diluted RNA (50, 25, 12.5, 6.25, 3.125, 1.56 ng/µL) with each primer set in the qRT-PCR reaction (in duplicate). This plate was run also to optimise the PCR, for example the amount of RNA in the reaction

The qRT-PCR was run as follows:

10 µL reactions were carried out in each case, using the following protocol.

Table 5-5. qRT-PCR reaction composition in 10 µL reaction

Component	Volume (µL) / reaction	Final concentration
SYBR green qRT-PCR master mix	5	1×
Forward primer	1	1 µM
Reverse primer	1	1 µM
RNase free water	1.9	
Template RNA	1	< 100 ng/reaction
QuantiFast RT mix	0.1	

Diluted primers, SYBR green qRT-PCR master mix and RNase-free water were thawed on ice. RT mix was taken out of the freezer just prior to use. Each RNA sample was thawed on ice and serially diluted from 50 to 1.56 ng/µL concentrations. Master Mixes were prepared for

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each primer sets by adding SYBR green qRT-PCR master mix, forward and reverse primer,

Quantifast RT mix and RNase-free water (according to Table 5.5). This master mix was

mixed by reverse pipeting and brief spining and was then pipetted into the appropriate wells

of 96-well plate in duplicate for each concentration of template RNA. Wells without RT mix

(-RT) were also included in all cases. Template RNA was added to each well except for the

non-template controls (NTC). The plate was sealed and briefly centrifuged (13000 g at 4 °C)

and loaded into a qRT-PCR machine (ABI Prism, 7900HT fast real-time PCR system;

Applied Biosystems). The specific cycling conditions are given in Figure 5.6.
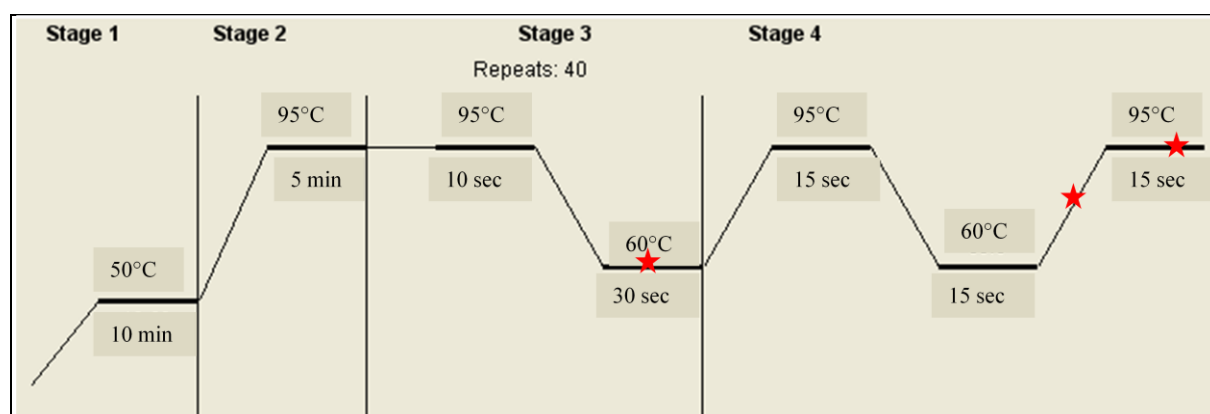
Figure 5-6. qRT-PCR cycling conditions and thermal profile. Stage 1 is reverse transcription, stage 2 is PCR initial activation, stage 3 is 2-step cycling of denaturation, annealing and extension, and stage 4 is dissociation/melting curve. Red stars indicate the florescence data collection points.

The Ct values are the cycle numbers at which the fluorescence (Rn) meets a threshold, a value

arbitrarily set by the computer program (SDS 2.3). Each Ct value is inversely correlated to the

amount of DNA. As stated previously, the amount of fluorescence of the SYBR Green, which

is dependent on the amount of double-stranded DNA, increased with each cycle. ΔRn is

calculated by subtracting the signal baseline from the real time data and it represents the

magnitude of the signal. When the ΔRn is plotted against the Ct, the results of a qRT-PCR,

called an amplification plot, appear as in Figure 5.7.

The software itself produces the standard curve plot for Ct values corresponding to the nominal concentrations of template RNA. An example of a standard curve is presented in Figure 5.8. This is a straight line; R^2 equals 1 is ideal, but above 0.95 is acceptable. The slope of this curve is used to calculate the efficiency of the qRT-PCR amplification (as described in 5.2.8). Ideally, the efficiency of qRT-PCR should be 2, so that every time a double-stranded cDNA fragment is replicated, two double-stranded copies result. However, in reality primers are not 100% efficient, and thus the number of copies is often less than 2.
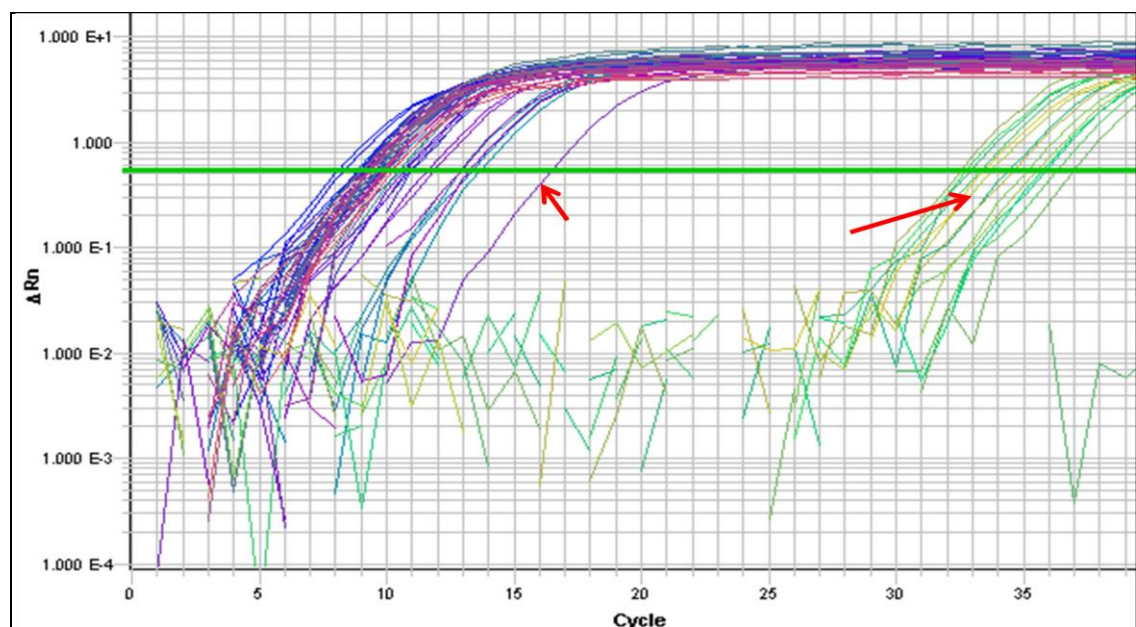
Figure 5-7. An example of amplification plot. The green horizontal line is the automatically generated Ct threshold line. ΔRn is calculated from subtracting the signal baseline from the initial stages of PCR from the real time data and it represents the magnitude of the signal. Plots indicated by long red arrow are from NTC and –RT wells. Plot indicated by short red arrow is one of the samples which did not fit into the standard curve and so this was repeated at a different dilution.

As part of the qRT-PCR run, a dissociation (or melting) curve was conducted. This involved heating the plate to 95°C for 15 seconds at a 100% ramp rate, then to 60°C (15 seconds at a 100% ramp rate), and again to 95°C (15 seconds at a 2% ramp rate). The dissociation curve measures the fluorescence of each well (by a first-order derivative) as the temperature is

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slowly increased. When the temperature reaches the specific melting temperature of a

product, the product is indicated by a peak. If several peaks which melt at different

temperatures are present, this indicates the presence of other products in the sample, such as

primer-dimers and contamination products (eg. amplified bacterial DNA). An example of the

results of an acceptable dissociation curves are shown in the Figure 5.9.
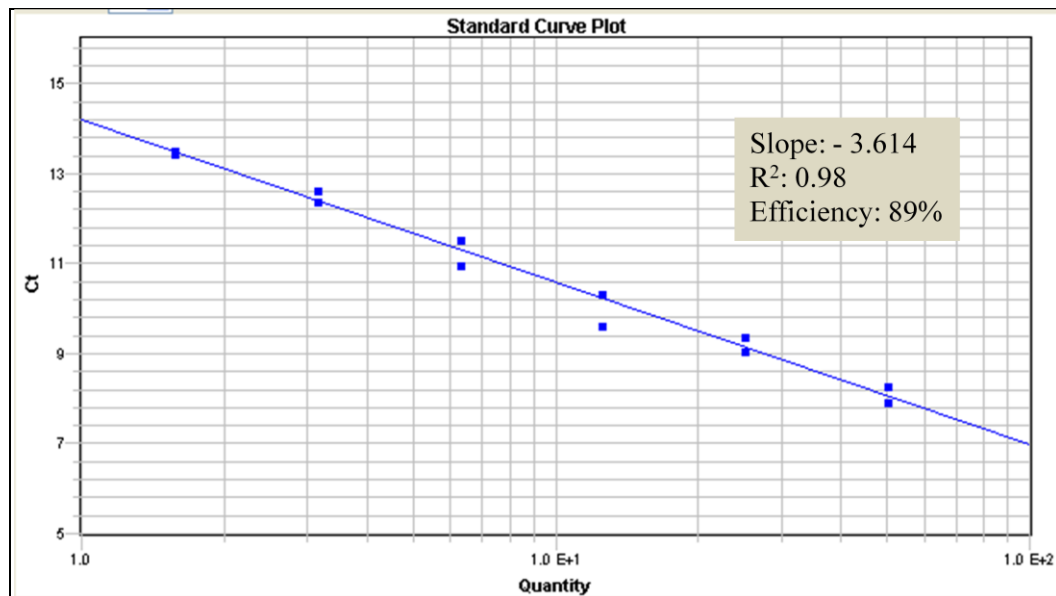
Figure 5-8. An example standard curve obtained with Vtg gene amplification. Efficiency was calculated from the slope as explained in 5.2.8.

5.2.7   Experiment 1

RNA extracted from liver samples from control and beclomethasone dipropionate-treated fish

of Experiment-1 (Chapter 4) were used. There were 5 males and 5 females in the control and

treatment groups. For the Vtg gene, only female samples were used, as male fish do not

produce Vtg normally. For each gene, a separate 96-well plate containing controls, treatment

samples, NTC and –RT samples was prepared, and qRT-PCR was carried out as described in

Section 5.2.6.

From this experiment, both β-actin and 18s rRNA were found not to be affected by treatment. However, 18s rRNA was found to be highly abundant (mean Ct value is lower than that of β-actin). Therefore 18s rRNA was chosen as the housekeeping gene for subsequent analyses.
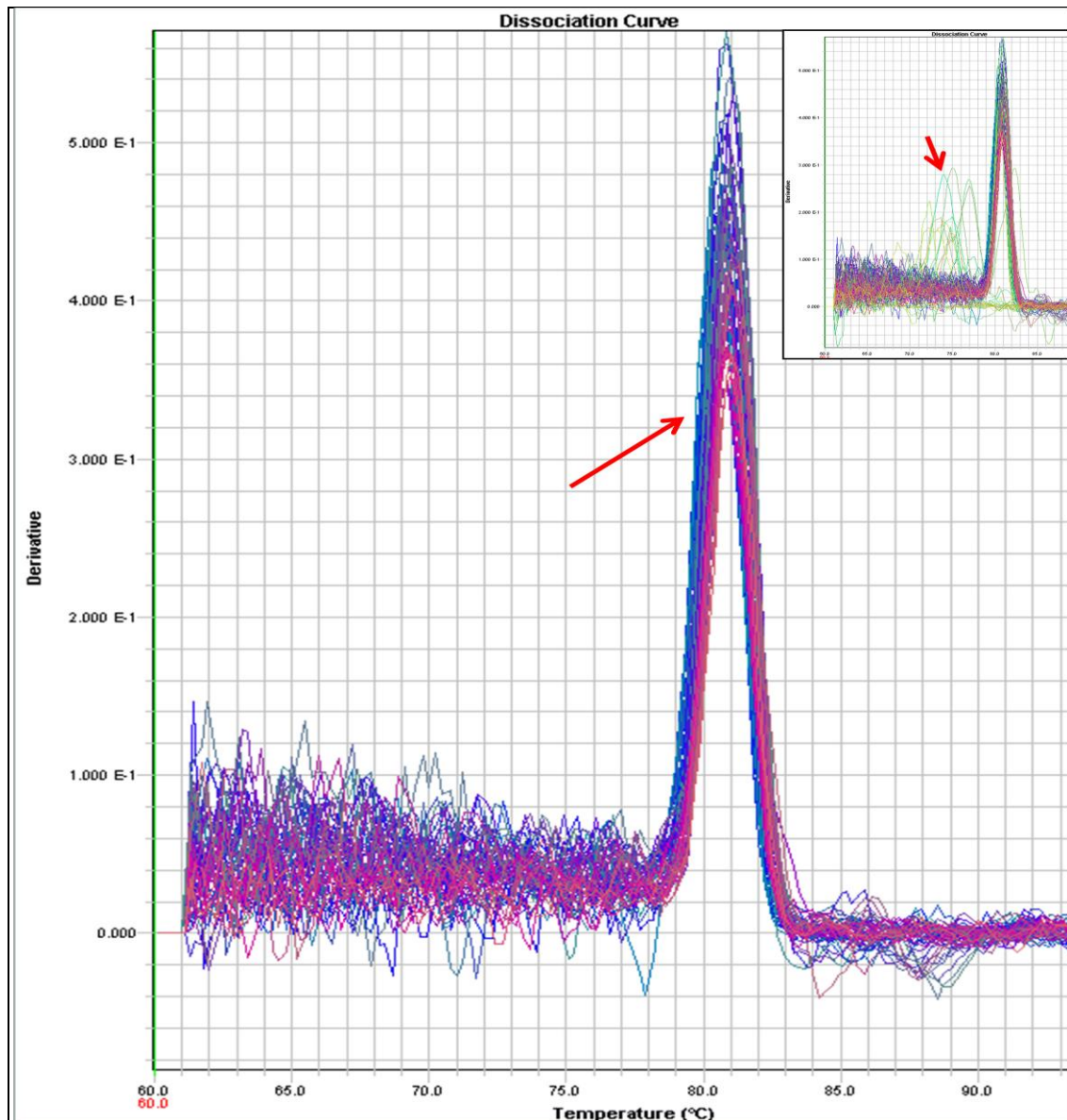
Figure 5-9. An example of the SDS 2.3 program's dissociation curve of GR gene amplification using Primer 3 software designed primers, plotting temperature against the fluorescence. All sample peaks are shown at a single melting temperature, and thus, a single PCR product was present (long red arrow). Inset is the same dissociation curve, but NTC and –RT wells also included. Primer dimmers are indicated by the short red arrow.

5.2.8 Experiment 2

RNA extracted from liver samples of fish from set-2 of experiment-2 (Chapter 4) were used. There were 20 fish (mixed sex) in control, 100 ng/L, 1 µg/L and 10 µg/L beclomethasone dipropionate treatment groups. For Vtg expression, only female fish were used. 18srRNA was used as the housekeeping gene. The primer pairs used for the qRT-PCR are shown (‡) in Table 5.2.

5.2.9 Interpretation of Results

The efficiency of the PCR(E) was calculated using the following equation:

$$\overline{}$$

Efficiency in percentage (%) was calculated from the following equation.

$$\overline{}$$

To enable us to compare gene expression levels between control and treated groups, the relative quantification using 18s rRNA as the housekeeping gene was calculated. The Pfaffl method was employed to analyse the results by comparing the mean Ct value of the control group with that of each individual sample, using the following equation:

$$\overline{}$$

Where 'E target gene' is the efficiency of the target gene (PEPCK, GR or Vtg) primers and E18s rRNA is the efficiency of the housekeeping gene primers and 'average control' is the

average Ct value of all of the samples from the control group for that gene (target or reference) (Pfaffl 2001).

5.2.10 Statistical Analysis

All experimental results are presented as means ± standard deviations. All statistical analyses were carried out using SigmaStat 3.5. As appropriate, normality tests and equal variance tests were performed and statistical significances were tested with a t-test or ANOVA. If datasets passed the equal variance test, one way ANOVA followed by a pair wise comparison (with Holm-Sidak test or Tukey test) was carried out. If the data failed the equal variance test, then non-parametric one way ANOVA on ranks (Kruskal-Wallis) followed by a Tukey test was carried out. In this case, median values are indicated in addition to means. $p < 0.05$ was considered to be significant.

For some dose-response effects in the second experiment, in addition to ANOVA, trend analysis (using SPSS: Jonckheere-Terpstra (JT) test) was carried out. JT test is a non-parametric test for ordered differences among classes. It tests the null hypothesis that the distribution of the response variable does not differ among classes. It is designed to detect alternatives of ordered class differences. For such ordered alternatives, the Jonckheere-Terpstra test can be preferable to tests of more general class difference alternatives, such as the Kruskal-Wallis test.

5.3 Results

5.3.1 PEPCK and GR

The results indicate that both PEPCK and GR genes were up-regulated by treatment with beclomethasone dipropionate. In Experiment-1, both PEPCK and GR mRNA were significantly upregulated (slightly more than 2-fold; Figure 5.10). Mean PEPCK mRNA expression (normalised to 18srRNA) in liver of control (n=10) fish was 1.22±0.44 and that of the treated group (n=10) was 2.78±1.69. Means of GR mRNA expression levels in control and treatment groups were 1.1±0.68 and 2.7±1.1, respectively.
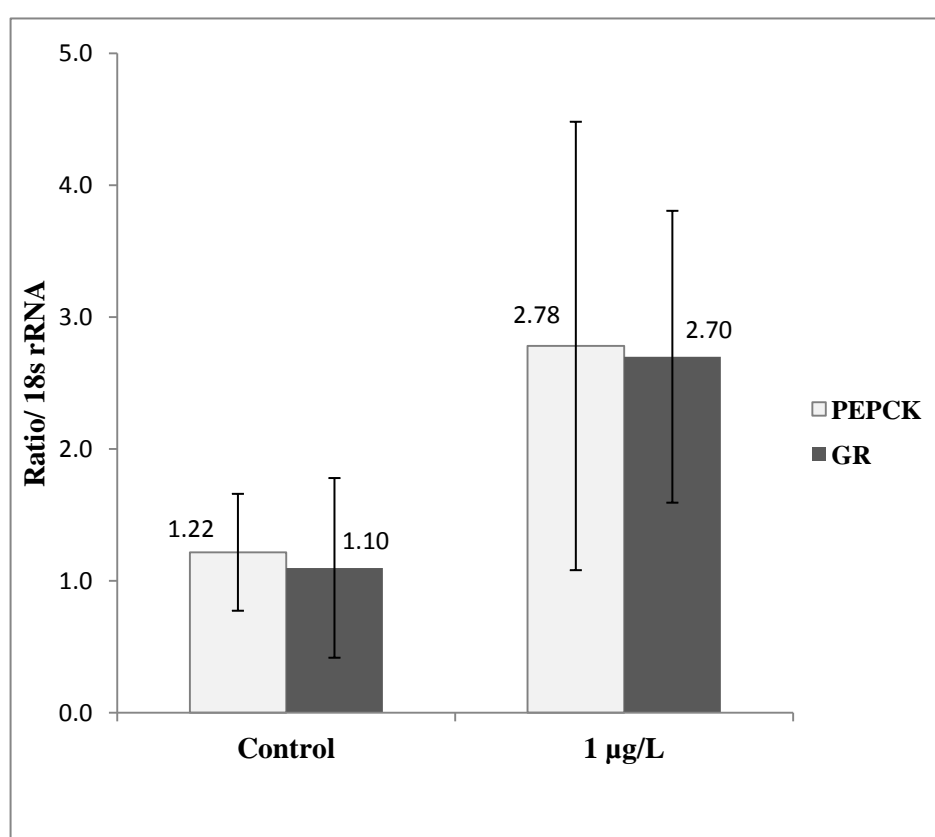
Figure 5-10. Mean relative levels of PEPCK and GR mRNA (normalised to 18srRNA) in the livers of control fish (n=10) and 1µg beclomethasone/L-treated fish (n=10). Both genes were significantly up-regulated over two fold due to the treatment (student t-test p< 0.05).

There was a concentration-related increase in the level of mRNA expression in both PEPCK and GR in Experiment-2. Figure 5.11 shows the dose-dependent increase in the PEPCK

mRNA expression normalised to 18s rRNA. Mean PEPCK mRNA expression ratios in controls, 100 ng/L, 1µg/L and 10 µg/L treated fish were 1.06±0.35, 2.54±0.78, 3.56±1.63 and 5.25±2.6, respectively. This indicates about a 4-fold increase in PEPCK mRNA expression in the high dose group. Each treatment group was significantly different from the control group (one way ANOVA on ranks followed by Tukey pair wise comparison; p < 0.001). Although values within the treatment groups were statistically not significant from each other, JT test revealed that there is a significant increasing trend(p<0.001) in gene expression due to the treatment.
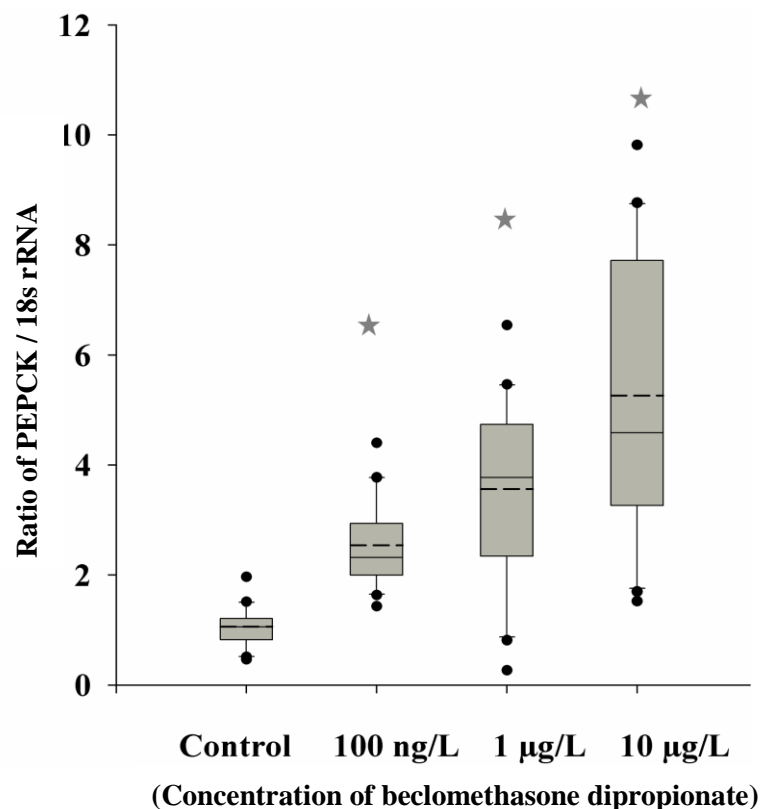
Figure 5-11. PEPCK mRNA expression normalised to 18s rRNA from the livers of control (n=20, mixed sex) and three different concentrations of beclomethasone dipropionate treated fish (n=20, mixed sex). The box indicates the mid 50% of values. The straight line inside the box corresponds to the median and the dashed line corresponds to the mean. Whiskers indicate the 10th and 90th percentile values and the circles indicate the outliers. ★ Indicates significant difference from control (one way ANOVA on ranks followed by Tukey pair wise comparison; p < 0.001).

There was a similar dose-related increase in the GR mRNA expression level normalised to 18s rRNA (Figure 5.12). The mean GR mRNA expression level from livers of control, 100 ng/L, 1µg/L and 10 µg/L treated-fish were 1.10±0.52, 3.1±1.32, 4.2±2.5 and 5.08±1.5, respectively. This shows about a 4-fold increase in GR mRNA expression level in the high dose group. Each treatment group was significantly different from control group (one way ANOVA on ranks followed by Tukey pair wise comparison; $p < 0.001$). Although values within the treatment groups were statistically not significant from each other, JT test ($p<0.001$) revealed that there is a significant increasing trend in gene expression due to the treatment.
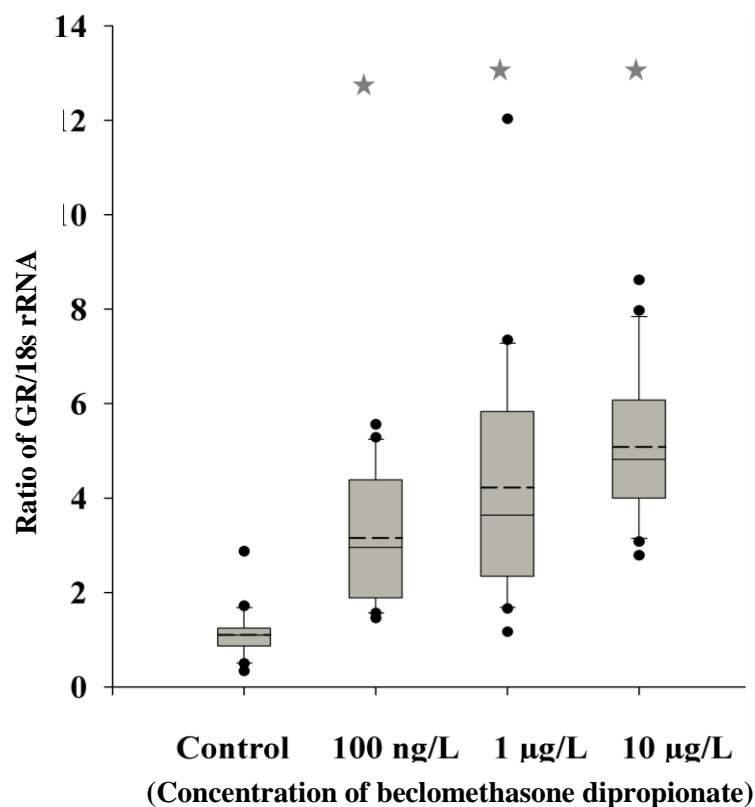
Figure 5-12. GR mRNA expression relative to 18s rRNA in livers from control (n=20, mixed sex) and three different concentrations of beclomethasone dipropionate treated fish (n=20, mixed sex). The box indicates the mid 50% of values. The straight line inside the box corresponds to the median and the dashed line corresponds to the mean. Whiskers indicate the 10th and 90th percentile values and the circles indicate the outliers. ★ Indicates significant difference from control (one way ANOVA on ranks followed by Tukey pair wise comparison; p < 0.001).

Figure 5.13 shows relative fold increase in PEPCK and GR mRNA expression compared to the controls in experiment-2.
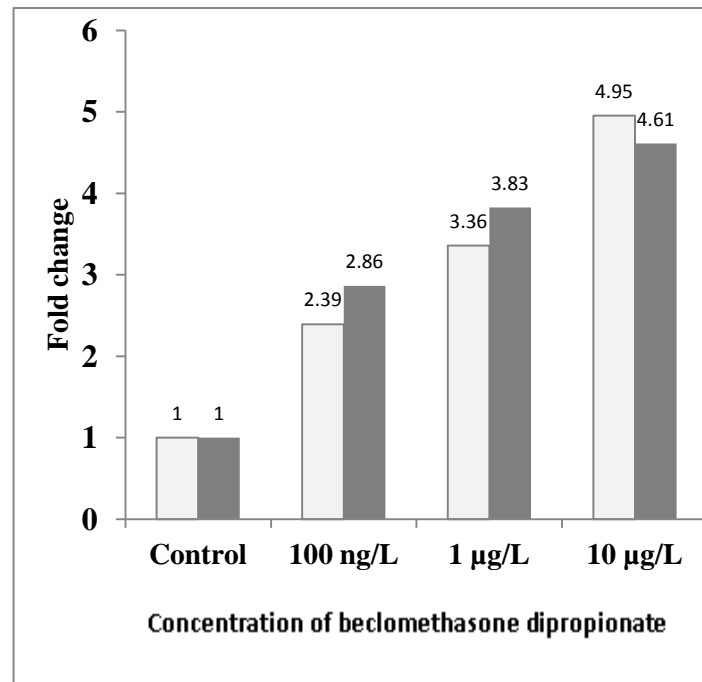
Figure 5-13. Relative fold changes of PEPCK (light coloured bars) and GR (dark coloured bars) mRNA expression level in Experiment-2. Mean values are plotted relative to the control group.

5.3.2   Vtg Gene Expression

Vtg mRNA levels appeared to be down-regulated by beclomethasone dipropionate treatment. In Experiment-1, the Vtg mRNA level was significantly ($p < 0.05$) reduced by slightly less than 2-fold (Figure 5.14). Mean Vtg mRNA expression level normalised to 18srRNA, relative to control fish, was 1.04 ± 0.15 and that of treated fish was 0.66 ± 0.10.
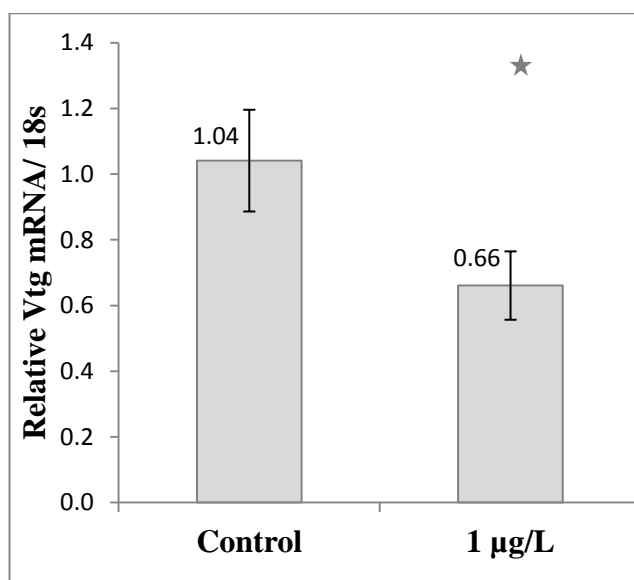
Figure 5-14. Vtg mRNA expression level normalised to 18s rRNA from livers of control fish (n=5 females) and 1µg beclomethasone/L-treated fish (n=5 females). Vtg mRNA expression was significantly down-regulated (slightly less than two fold) with treatment (student t-test p< 0.05).

There was a concentration-related decrease in the level of Vtg mRNA expression in Experiment-2. Figure 5.15 shows the concentration-related down-regulation in Vtg mRNA expression level normalised to 18s rRNA. Mean Vtg mRNA expression from livers of control, 100 ng/L, 1µg/L and 10 µg/L treated fish (n=10 females in each case) were 1.00 ± 0.17, 0.70 ± 0.17, 0.67 ± 0.11 and 0.31 ± 0.24, respectively. This indicates a decrease of about 3-fold occurred in the level of Vtg mRNA expression in the high dose group. Mean Vtg expression levels in the 1µg/L and 10 µg/L-treated fish were significantly different from control group, as was also found in Experiment-1 (one way ANOVA on ranks followed by Tukey pair wise comparison; $p < 0.001$). Although the treatment groups were statistically not significantly different from each other, JT test ($p<0.001$) revealed a statistically significant decreasing trend in the level of Vtg mRNA expression due to the treatment.
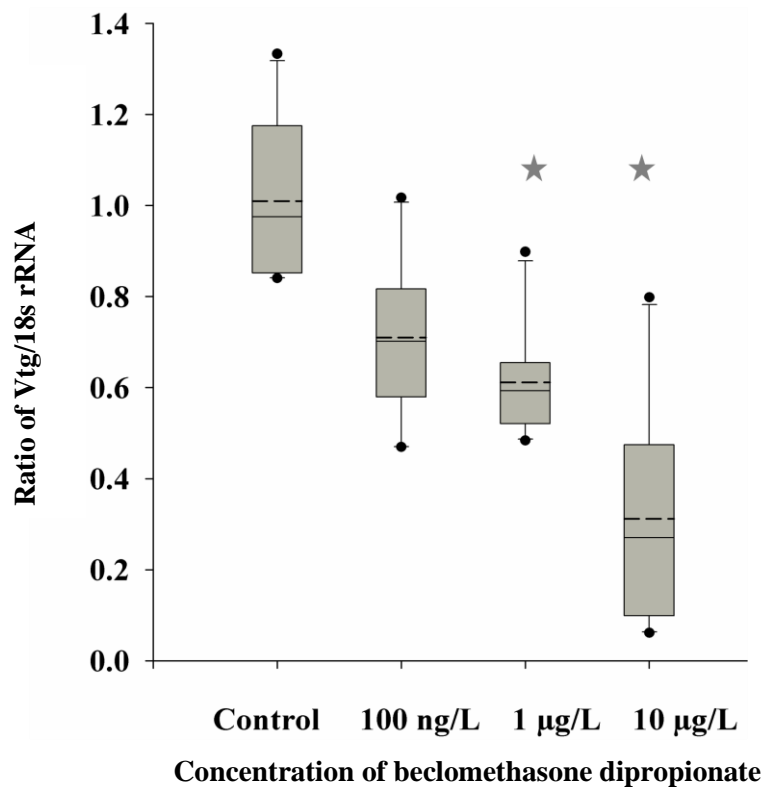
Figure 5-15. Vtg mRNA expression normalised to 18s rRNA from livers of control (n=10, females) and three different concentrations of beclomethasone dipropionate-treated fish (n=10, females). The box indicates the mid 50% of values. The straight line inside the box corresponds to the median and the dashed line corresponds to the mean. Whiskers indicate the 10th and 90th percentile values and the circles indicate the outliers. ★ Indicates significant difference from control (one way ANOVA on ranks followed by Tukey pair wise comparison; p < 0.001)

Figure 5.16 shows the relative fold changes compared to the controls in Vtg mRNA

expression levels in the dose-response experiment. It shows a concentration related down –

regulation (3-fold in the highest dose) in gene expression level.
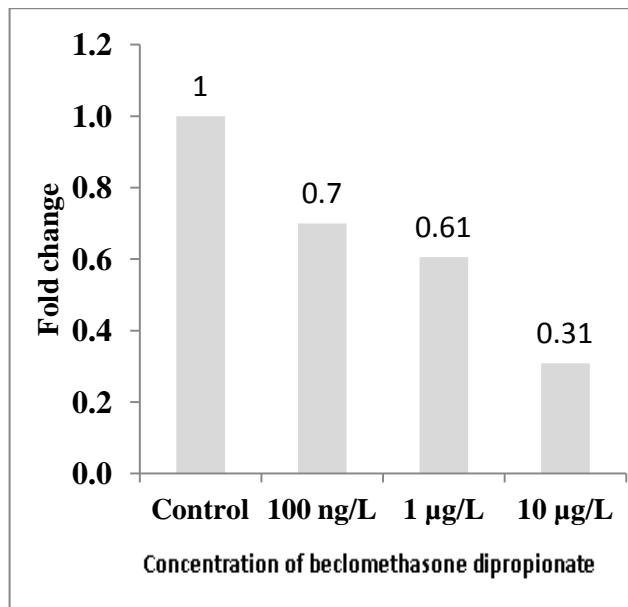
Figure 5-16. Relative fold change of Vtg mRNA expressions in beclomethasone dipropionate dose-related experiment. Mean values are plotted for comparison relative to the control group.

5.4   Discussion

The most important finding from this study was that concentrations of beclomethasone dipropionate as low as 100 ng/L can have significant effects on the expression of selected genes in the liver of fathead minnows. These genes control the proteins that play important roles in metabolism as well as reproduction in fish. Therefore possible negative consequences of chronic exposure on individual fish, as well as on a population of fish, cannot be exluded.

A well established metabolic response to stress is the elevation of plasma glucose concentration (both in mammals and in fish). Glucose is an important fuel that is oxidized to meet the increased energy demand during stress in fish. The liver has the ability to synthesise glucose from non-carbohydrate sources, in order to provide glucose for essential organs, including the brain, gills and the heart during periods of stress (Mommsen *et al*., 1999). Several genes that are responsible for proteins involved in glycolysis and gluconeogenesis were elevated after an acute stress in fish (Table 5.1). This is in agreement with studies that

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have reported higher activities of glycolytic enzymes after exposure of fish to an acute stressor, which need more energy to re-establish homeostasis (Mommsen *et al*., 1999). Several studies support the process of up-regulation of enzymes involved in gluconeogenesis as a key aspect of stress recovery (reviewed in Iwama, 1998). Plasma glucose levels and PEPCK gene expression levels were found to increase with increasing salinity (Singer *et al*., 2007). Some other molecular biological studies also support PEPCK up-regulation, as we found. For example, an increase in liver PEPCK mRNA levels was observed both *in vivo* as well as *in vitro* in trout hepatocytes when stimulated with cortisol (Sathiyaa and Vijayan, 2003; Vijayan *et al*., 2003; Aluru and Vijayan, 2007).

The observed up-regulation of PEPCK gene expression in the liver explains the increase in the plasma glucose levels observed *in vivo* (Chapter 4). However, plasma glucose levels in the 100 ng beclomethasone/L exposed fish were not statistically higher than those in the controls, whereas the PEPCK gene expression level in these two groups were statistically different. There are several possible reasons for these differences. Concentrations of a pollutant that affect the expression of a gene do not necessarily affect the levels of the protein controlled by that gene. In some cases the exposure period can cause this difference. Plasma glucose clearance rate is also another factor that could contribute to this difference. Nevertheless, PEPCK gene expression and plasma glucose levels show an increasing trend (JT test) which was found to be significant at all concentrations tested. This finding might be of significant interest, as a recent study involving exposure to a mixture of pollutants resulted in obesity in female zebrafish (Lyche *et al*., 2010). In mammals, particularly humans, obesity is also associated with up-regulation of PEPCK gene expression. Therefore the sensitivity of PEPCK of the fathead minnow to low concentrations of beclomethasone, as observed in the present study, could help to explain obesity in zebrafish.

GR mRNA levels have been shown to be lowered in sea bass (*Dicentrarchus labrax*) liver in response to chronic crowding stress (Terova *et al*., 2005). Some of the previous stress-related studies also reported GR down-regulation in response to endogenous cortisol, which is contradictory to the present finding. In the present study, GR up-regulation in response to synthetic GC has been reproduced in both experiments, and is also present in a concentration-dependent manner. In a recent study (Arterbery *et al*., 2010), high cortisol levels corresponded to up-regulation of the GR gene in plainfin midshipman fish (*Porichthys notatus*). This species has actively reproducing type-1 males and more reproductively less active type-2 males. Absolute quantitative real-time PCR subsequently revealed higher levels of GR in the central nervous system (CNS) of type-2 males than type-1 males and plasma levels of cortisol were 2 to 3-fold higher in type-2 males compared to type-1 males.

Vijayan *et al*. (2003) have also reported an up-regulation of GR mRNA and a down-regulation of GR protein in cortisol-treated fish. In an *in vitro* study, Aluru and Vijayan (2007) reported an up-regulation of GR gene expression in hepatocytes of trout in response to exogenous cortisol treatment. However, in the same study they also found GR protein down-regulation. Therefore, there is still uncertainty about GR gene expression in response to GC exposure and it has been suggested that the stress effect on GR gene expression may be either species-specific and/or dependent on the type, intensity and duration of the stressor (Wiseman *et al*., 2007). A reasonable explanation for these results has been proposed by Sathiyaa and Vijayan (2003) with the hypotheis of GR autoregulation. In this hypothesis, increasing cortisol levels tend to decrease the levels of GR proteins via a negative feedback loop and in order to compensate, GR mRNA levels are up-regulated. This could explain the present results; up-regulation of the GR gene in response to exposure to synthetic GCs. GR gene expression in response to external stimuli has been reported to be tissue specific. For example, rainbow trout exposed to high salinity showed a significant increase of GR gene expression in

the gill but not in liver. In that study, also GR gene expression did not correspond to GR protein expression (Singer *et al.*, 2007).

Any impact on the level of GR gene expression can have many consequences downstream, as all the GR-mediated physiology could be affected. This is particularly important in osmoregulation, which is an important physiological process completely under the control of the GR. Confounding factors such as environmental stress and disease outbreak could also be affected, and this may also have a population-level impact.

It is well established that stress affects all aspects of an animal's performance, including reproduction. Several studies have shown that stress-induced elevation in cortisol levels reduces the plasma concentrations of sex steroids in a variety of fish species (Pickering *et al.*, 1987; Schreck *et al.*, 2001). Deleterious effects of stress on vitellogenesis have also been reported (Teitsma *et al.*, 1998), and stressor-mediated cortisol elevation has been reported to affect reproductive performance (Carragher *et al.*, 1989; Campbell *et al.*, 1994; Schreck *et al.*, 2001). However, the molecular mechanisms of cortisol and/or stress-mediated reproductive dysfunction are not clear.

In teleosts, cortisol has been shown to mimic the effects of stress on reproduction by delaying gonadal development, reducing pituitary gonadotrophin production and lowering plasma steroid and vitellogenin levels, leading to a lower gamete quality (Teitsma *et al.*, 1998; Pankhurst and Van der Kraak, 2000; Consten *et al.*, 2001). Cortisol has been shown to inhibit vitellogenesis by down-regulation of ER and Vtg expression in salmonids (Lethimonier *et al.*, 2000). Vitelline envelope protein subunits, regulated by ER signalling, were also down-regulated with cortisol treatment and acute handling stressors (Aluru and Vijayan, 2007), which confirms a role for cortisol in affecting oestrogen-responsive gene expression in the liver. My present results on Vtg gene expression are in agreement with previous results. It

should be noted that there have not been any studies where synthetic GCs have been reported to affect vitellogenesis in the past. The cortisol levels reported in previous studies are not easily comparable to the present study, as these did not report Vtg levels as a consequence of water exposure. The Vtg gene down-regulation observed in this study can be compard to the plasma vitellogenin levels also measured in this *in vivo* experiment (chapter 4). The down-regulation of Vtg gene expression may have negative consequences at the population level, as female fish may be adversely affected. Low vitellogenin levels could also severely affect the hatching and survival rates.

The present study investigated only three selected genes that are important in metabolism and reproduction. There are, however, thousands of genes that could be affected if a fish is exposed to synthetic GCs. However, the fact that gene expression in fish is affected at such low concentrations will hopefully stimulate further studies. Studying more of the genome at different time points during exposure using microarrays could give a clearer picture of impacts. There have been some microarray assays already developed for use with the fathead minnow (reviewed by Denslow *et al*., 2007). For example, Marger *et al*., 2008 have developed a fathead array and have assessed the consequences of lead exposure. The first genomic studies in the field of ecotoxicology were used to identify a limited number of genes related to environmental stress or exposure to pollutants. These were then analyzed using array technology. Thus, using 110 fragments of stress-related genes from flounder (*Platichthys flesus*), Williams *et al*. (2003) compared the hepatic expression of these genes in fish reared in polluted and relatively non-polluted estuaries. In another study, in the context of endocrine disruptive chemicals in the aquatic environment, identification of oestrogen-responsive genes in zebrafish and sheepshead minnow (*Cyprinodon variegatus*) was carried out and they confirmed that oestrogenic effects of various compounds can be observed even at low concentrations (Larkin *et al*., 2002; Hoyt *et al*., 2003). Based on their first study, Larkin

et al. (2003) created a gene array containing 30 genes from sheepshead minnow that were previously identified as oestrogen-responsive, and used this system to screen endocrine-disrupting compounds that mimic oestrogens. Functional genomic technology has been used to both (a) identify novel genes to serve as biomarkers and (b) to understand the molecular mechanisms corresponding to toxicity. Similar studies targeting GC-responsive elements could be useful in order to determine the environmental impacts of GCs.

5.5   Conclusions

A 21-day exposure to beclomethasone dipropionate at low concentrations (as low as 100 ng/L) had impacts on the expression of some selected genes in fathead minnows. The results are reproducible and concentration-related. More studies involving GC transcriptomics are recommended in order to investigate the whole gemone impact of exposure to GCs and the mechanisms behind activity of synthetic GCs in fish.

Chapter 6.0

General Discussion
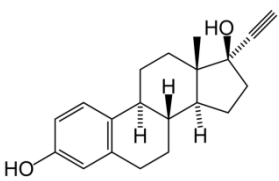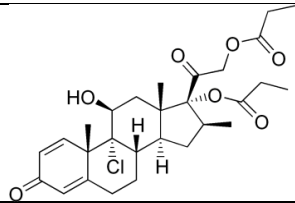
6.1 General Discussions

In order to satisfy the therapeutic needs of the human population, the increasing production of pharmaceuticals and the subsequent presence of pharmaceuticals in the environment are unavoidable. However, many pharmaceuticals are probably present in the environment at concentrations too low to cause any effects to fauna and flora (Sumpter, 2007). The presence of pharmaceuticals in the environment mainly depends on their metabolism and excretion from the human body, STP removal and then degradation in the environment. Possible effects on fauna and flora probably depend on the environmental concentrations of pharmaceuticals and the availability of drug targets (e.g. receptors and enzymes) in the organism of interest. There will possibly be some pharmaceuticals that pose a greater potential threat to aquatic organisms than others, because some are heavily used, some could be more potent, and some could be poorly degraded and have the ability to bioaccumulate in aquatic organisms. The ultimate aim of the risk assessment of pharmaceuticals in the environment is to identify those potential drug groups and to advise the regulatory authorities on the likely consequences of their impacts in the environment.

A total of about 4000 kg of GCs are used annually in the UK, which based on the present study indicate possible environmental concentrations in the ng/L range. However, the issue of oestrogens and feminisation of fish 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). As a steroid pharmaceutical, GCs share many common features with oestrogens that have a proven record of causing endocrine disruption (Table 6.1). Therefore, the results reported in the present study are probably not surprising.

In humans, GCs are normally applied discontinuously to avoid any serious side effects. As an example, beclomethasone dipropionate is an inhaler drug intended to have local anti-inflammatory effects. It aims to reduce inflammation in the upper respiratory system and the plasma concentrations are very minimal. Therefore the side effects when the drug is used for longer periods or the side effects of very high plasma concentrations are not well known in humans. But in the present study fish were chronically exposed to GCs via water for 21 days, so that some effects that are not seen in humans could occur.

Table 6-1. Comparison of EE2 and beclomethasone dipropionate with regard to some important parameters used in environment risk assessment.

Properties	Ethinylestradiol (EE2)	Beclomethasone dipropionate
Molecular structure		
Log P	4.52	4.59
BCF	1601	1808
Transport	SHBG, albumen	Transcortin, albumen
Receptors	ERα , ERβ	GR1, GR2, MR
Metabolism	Liver – CYP enzymes	Liver – 11β-HSD2
Excretion	As conjugates of glucuronide and sulfate	
Detected in rivers	Yes (0.5 -1 ng/L)	Not yet (other GCs 1-50 ng/L)
Usage in the UK (2006)	About 25 kg	275 kg (total GC 4000 kg)

There is a lack of knowledge about the possible 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. Recent research has shown that other factors besides degree of hydrophobicity and size affect the rate of uptake of chemicals, especially steroids, from the water into fish. Scott *et al.* (2005) were the first to show that the rate of uptake of sex steroids (oestrogens and androgens) from the water into fish was dramatically affected by their affinity for sex steroid binding protein (SHBG). The rate of uptake of sex steroids can be extremely fast (Maunder *et al.* 2007). The mechanism responsible for this very rapid uptake of sex steroids was recently elucidated: fish gills contain very high amounts of SHBG (Miguel-Queralt and Hammond, 2008). That latter study also demonstrated that fish SHBG also has a high affinity for synthetic sex steroids, such as EE2 and some progestogens. Collectively, the results in these papers probably explain why sex steroids, including synthetic ones, are so potent, and affect fish physiology at extremely low environmental concentrations (Länge *et al.* 2001; Purdom *et al.* 1994; Paulos *et al.* 2010; Zeilinger *et al.* 2009). Endogenous glucocorticoids and some synthetic corticoids have high affinity to the protein transcortin (also called CBG, corticosteroid-binding globulin), whereas all of them bind albumin. This means that GCs present in the water will be effectively taken up into fish and the plasma concentrations may become high enough to cause effects.

Once the drug is inside an organism, its distribution and consequently its effects will be dependent on factors such the octanol-water partition (known from log P) 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 extra energy demand, 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 human therapeutic level (Huggett *et al.*, 2004). Since beclomethasone dipropionate has a log P of 4.59 with a calculated BCF of 1808, plasma

concentrations of exposed fish could reach therapeutic concentrations even if the concentrations in the surrounding water are relatively low.

It is likely that some unique effects that do not occur in people taking the drug will manifest in aquatic organisms: vitellogenin synthesis in response to oestrogens is an example. Others will undoubtedly occur. Many pharmaceuticals have multiple modes of action: for example, some synthetic progestogens are also androgenic, and hence may induce androgenic effects as well as progestogenic effects (Paulos *et al.,* 2010). Drugs can reach non-target animals through unexpected routes (as diclofenac does in Indian vulture), and once inside an animal may act unexpectedly based on what is known for the drug, leading sometimes to unexpected outcomes. In the present study, plasma glucose concentrations, cortisol concentrations and Lymphocyte counts were changed in GC-exposed fish in agreement with the known mechanism of action of GCs, namely hyperglycaemic, HPI suppressive and immunosuppressive, respectively. However, the androgenic tendency observed in both males and females cannot be explained by the known main mechanisms of action of GCs. However, GC treatment in human females results in facial hair growth (hirsutism), which is linked with high plasma androgen concentrations, obesity and high insulin concentrations. There are no reports of androgenic activity of GCs in human males. In the present study, GCs did show some degree of androgenic activity in both males and female fish, at least at the higher exposure concentrations tested.

The results reported here demonstrate that relatively low concentration of synthetic GC (100 ng/L to 10 µg/L) can cause effects on fish. These effects are potentially important. For example, lymphocytopenia in fish is associated with increased susceptibility to disease (Pickering and Pottinger, 1989). If immunosuppression occurred in wild fish, then they would likely be more susceptible to disease. Different groups of steroids and steroid antagonists will

produce different effects. Some of these effects might be more serious than others. For example, preventing reproduction would undoubtedly be considered a catastrophic effect, whereas reducing inflammation would probably not be considered a serious, adverse effect. Thus it is possible to argue that pharmaceuticals such as oestrogens and progestogens, which can prevent fish reproducing at low environmental concentrations, are of more concern, and merit greater attention from environmental scientists, especially ecotoxicologists, than do GCs, which primarily target the immune system. However, the results reported in this thesis also suggest possible impacts on reproduction, as the androgenic tendency of GCs in fish has shown. These reproductive impacts may have population-level consequences.

It is very likely that pharmaceuticals with similar MOA will act in an additive manner, as has already been demonstrated for a mixture of oestrogenic chemicals (Brian *et al.*, 2005). However, it is much more difficult to predict whether or not antagonists might attenuate the effects of agonists when both groups are present simultaneously, or whether androgen agonists might neutralise the effects of oestrogen agonists. Because many different GCs are in widespread clinical use, it seems likely that, concurrently, many different GCs will be present in the aquatic environment. As Chapter 3 demonstrates, all of the GCs tested here can bind to the fish GR (as expected), and therefore it can be argued that the total concentration of GC in the environment, rather than the concentration of each individual GC, is of most relevance to the risk assessment of GCs on aquatic organisms. However, currently not enough is known to provide a full picture of GCs in the aquatic environment. Nevertheless, it is very likely that the effects of different GCs will be additive.

The EMEA guidelines for the risk assessment of pharmaceuticals in the environment is a tiered process. The first phase estimates the PEC in surface water. If the PEC value is equal or above 0.01µg/L, phase 2 Tier A is carried out. In some cases, if the drug substances may

affect reproduction of vertebrates 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 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.

A general indicator of the degree of risk is the risk characterisation ratio, which is the ratio between the PEC in surface waters and the predicted no effect concentrations (PNEC) derived from the toxicological tests. The PEC is derived from the data on usage, and the physical and chemical properties of the drug, STW effluent flows and surface water flows. The 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 PEC : PNEC ratio greater than 1 need more attention and further risk assessment steps will be involved. However, these ratios depend heavily on the data available for the estimation of PEC and the accuracy of the toxicological tests that derive the PNEC.

There have been some criticisms of the EMEA guidelines. 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. 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 oestrogens and xenoestrogens are undoubtedly present in the aquatic 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 oestrogen and a progesterone, and the question here would be whether these would act synergistically or independently of each other. The situation becomes even more complicated when the mixure of pharmaceuticals present in water is together with other pollutants, such as metals and industrial wastes.

Information on GCs currently available in the open literature is clearly not sufficient for the risk assessment of GCs present in the aquatic environment. A variety of GCs, some far more potent than endogenous cortisol, have been created for therapeutic use, for different age groups of patients and to treat different diseases. They differ in their pharmacokinetics (absorption factor, half-life, volume of distribution, clearance) and in their pharmacodynamics (for example, retention of sodium and water). More than 90 percent of them bind to a number of different plasma proteins, however with different binding specificities. In the liver, they are quickly metabolised by conjugation with a sulfate or glucuronic acid, and are secreted in the urine.

As discussed in Chapter 2, reported surface water concentrations for a few GCs are in the range between 1 and 50 ng/L. These reports are for individual GCs (prednisolone, triamcinolone, cortisone, etc). Therefore concentrations of total GCs could be more than this. The present study has not found the PNEC because significant effects occurred at all concentrations tested. However, the LOEC from this study was 100 ng/L, which is not very much higher than the reported surface water concentrations. Moreover, effects from different individual GCs reaching the surface water could be additive. Therefore, PEC calculations are more relevant when combining all the individual GCs in the market. The availability of about 30 different GCs in the market and their unknown STP removal rates and wide range of the percentage excreted make the predicted surface water concentrations cover a wider range, up

to a maximum of about 800 ng/L. This is higher than the LOEC of the present study. Therefore, GCs in the environment could pose a potential threat to aquatic organisms.

Further studies are recommended to improve the calculation of the PEC, especially determining the STP removal rate, which is not clear for many GCs, and the few reported removal rates are contradictory. Dose-response exposure studies that include the concentration range below 100 ng/L should be carried out to obtain the PNEC for GCs. If field studies to investigate possible effects of GCs on wild fish are conducted, they should be focused on the reported 'hot spots', where the highest concentrations of GCs are predicted to occur. More studies related to GC transcriptomics are recommended in order to investigate the whole gemone impact and the mechanism behind the activity of GCs in fish. Reproductive impacts, which are the opposite of those reported for oestrogens, suggest a need for studies of mixtures of synthetic steroids on reproduction and other physiological processes of fish.

6.2   Conclusions

The range of concentrations of total GCs along the river Thames, predicted by the LF2000-WQX model, is likely to be between 0 and 850 ng/L. The present study reveals that fish GRs respond to synthetic GCs and transcribe GR responsive genes that can produce significant effects in fish. Fish GR2 is more sensitive to all the tested GCs compared to GR1. A 21-day exposure of fish to beclomethasone dipropionate at low concentrations (as low as 100 ng/L) impacted on the plasma glucose concentration, cortisol concentration, blood lymphocyte count, vitellogenin concentration and the expression of some selected genes in fathead minnows. The results are reproducible and dose-related. Experiments were conducted to test the null hypothesis, which was that environmentally-relevant concentrations of GCs do not have adverse impacts on fish. Results from the experiments indicate that the null hypothesis cannot be accepted, because low concentrations of GCs via water exposure had significant

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impacts on fathead minnows. An increased plasma glucose concentration may affect their normal physiology, and immunosuppression could make them more susceptible to disease. Adverse impacts on reproduction could result in population-level changes in wild fish. It is very likely that the effects of different GCs will be additive, as has been shown for oestrogenic chemicals. Therefore, this study warrants further environmental risk assessment of GCs, especially in mixture scenarios.

Chapter 7.0

References

Aden, P., Goverud, I., Liestøl, K., Løberg, E.M., Paulsen, R.E., Mæhlen, J. & Lømo, J. (2008). Low-potency glucocorticoid hydrocortisone has similar neurotoxic effects as high-potency glucocorticoid dexamethasone on neurons in the immature chicken cerebellum. *Brain research,* **1236(C):** 39-48.

Alder, A.C., Schaffner, C., Majewsky, M., Klasmeier, J. & Fenner, K. (2010). Fate of β-blocker human pharmaceuticals in surface water: Comparison of measured and simulated concentrations in the Glatt Valley Watershed, Switzerland. *Water research,* **44(3):** 936-948.

Alsop, D. & Vijayan, M.M. (2008). Development of the corticosteroid stress axis and receptor expression in zebrafish. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology,* **294(3):** 711-719.

Alsop, D., Ings, J.S. & Vijayan, M.M. (2009). Adrenocorticotropic hormone suppresses gonadotropin-stimulated estradiol release from zebrafish ovarian follicles. *PLoS ONE,* **4(7):** e6463. doi:10.1371/journal.pone.0006463

Aluru, N. & Vijayan, M.M. (2007). Hepatic transcriptome response to glucocorticoid receptor activation in rainbow trout. *Physiological Genomics,* **31(3):** 483-491.

Aluru, N. & Vijayan, M.M. (2009). Stress transcriptomics in fish: A role for genomic cortisol signaling. *General and comparative endocrinology,* **164(2-3):** 142-150.

Andersen, D.E. (1991). Metabolic Effects Associated with Chronically Elevated Cortisol in Rainbow Trout (*Oncorhynchus mykiss*). *Canadian Journal of Fisheries and Aquatic Sciences,* **48(9):** 1811-1817.

Anderson, P.D., D'Aco, V.J., Shanahan, P., Chapra, S.C., Buzby, M.E., Cunningham, V.L., Duplessie, B.M., Hayes, E.P., Mastrocco, F.J., Parke, N.J., Rader, J.C., Samuelian, J.H. & Schwab, B.W. (2004). Screening Analysis of Human Pharmaceutical Compounds in U.S. Surface Waters. *Environmental Science and Technology,* **38(3):** 838-849.

Ankley, G.T. & Johnson, R.D. (2004). Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. *ILAR journal / National Research Council, Institute of Laboratory Animal Resources,* **45(4):** 469-483.

Ankley, G.T. & Villeneuve, D.L. (2006). The fathead minnow in aquatic toxicology: Past, present and future. *Aquatic Toxicology,* **78(1):** 91-102.

Ankley, G.T., Brooks, B.W., Huggett, D.B. & Sumpter, J.P. (2007). Repeating history: Pharmaceuticals in the environment. *Environmental Science and Technology,* **41(24):** 8211-8217.

Ankley, G.T., Daston, G.P., Degitz, S.J., Denslow, N.D., Hoke, R.A., Kennedy, S.W., Miracle, A.L., Perkins, E.J., Snape, J., Tillitt, D.E., Tyler, C.R. & Versteeg, D. (2006). Toxicogenomics in regulatory ecotoxicology. *Environmental Science and Technology,* **40(13):** 4055-4065.

Ankley, G.T., Defoe, D.L., Kahl, M.D., Jensen, K.M., Makynen, E.A., Miracle, A., Hartig, P., Gray, L.E., Cardon, M. & Wilson, V. (2004). Evaluation of the model anti-androgen flutamide for assessing the mechanistic basis of responses to an androgen in the fathead minnow (*Pimephales promelas*). *Environmental Science and Technology,* **38(23):** 6322-6327.

Ankley, G.T., Jensen, K.M., Kahl, M.D., Korte, J.J. & Makynen, E.A. (2001). Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry,* **20(6):** 1276-1290.

Ankley, G.T., Jensen, K.M., Makynen, E.A., Kahl, M.D., Korte, J.J., Hornung, M.W., Henry, T.R., Denny, J.S., Leino, R.L., Wilson, V.S., Cardon, M.C., Hartig, P.C. & Gray, L.E. (2003). Effects of the androgenic growth promoter 17-β-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. *Environmental Toxicology and Chemistry,* **22(6):** 1350-1360.

Arterbery, A.S., Deitcher, D.L. & Bass, A.H. (2010). Corticosteroid receptor expression in a teleost fish that displays alternative male reproductive tactics. *General and comparative endocrinology,* **165(1):** 83-90.

Arterbery, A.S., Fergus, D.J., Fogarty, E.A., Mayberry, J., Deitcher, D.L., Kraus, W.L. & Bass, A.H. (2011). Evolution of ligand specificity in vertebrate corticosteroid receptors. *BMC Evolutionary Biology,* **11(1):**14; doi:10.1186/1471-2148-11-14.

Ashton, D., Hilton, M. & Thomas, K.V. (2004). Investigating the environmental transport of human pharmaceuticals to streams in the United Kingdom. *Science of the Total Environment,* **333(1-3):** 167-184.

Bahr, N.I., Palme, R., Möhle, U., Hodges, J.K. & Heistermann, M. (2000). Comparative aspects of the metabolism and excretion of cortisol in three individual nonhuman primates. *General and comparative endocrinology,* **117(3):** 427-438.

Balaam, J.L., Grover, D., Johnson, A.C., Jürgens, M., Readman, J., Smith, A.J., White, S., Williams, R. & Zhou, J.L. (2010). The use of modelling to predict levels of oestrogens in a river catchment: How does modelled data compare with chemical analysis and *in vitro* yeast assay results? *Science of the Total Environment,* **408(20):** 4826-4832.

Baroiller, J.F., D'Cotta, H. & Saillant, E. (2009). Environmental effects on fish sex determination and differentiation. *Sexual Development,* **3(2-3):** 118-135.

Barry, T.P., Riebe, J.D., Parrish, J.J. & Malison, J.A. (1997). Effects of 17α,20β-dihydroxy-4-pregnen-3-one on cortisol production by rainbow trout interrenal tissue *in vitro*. *General and comparative endocrinology,* **107(2):** 172-181.

Barton, B.A. & Iwama, G.K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases,* **1(C):** 3-26.

Becker, H., Sturm, A., Bron, J.E., Schirmer, K. & Bury, N.R. (2008). The A/B domain of the teleost glucocorticoid receptors influences partial nuclear localization in the absence of hormone. *Endocrinology,* **149(9):** 4567-4576.

, N.A., Ginn, T.R. & Loge, F.J. (2005). Occurrence and fate of pharmaceutically active compounds in the environment, a case study: Hoje River in Sweden. *Journal of hazardous materials,* **122(3):** 195-204.

Bernanke, J. & Köhler, H. (2009). The impact of environmental chemicals on wildlife vertebrates.*Reviews of Environmental Contamination and Toxicology,* **198**: 1-47.

Besse, J. & Garric, J. (2008). Human pharmaceuticals in surface waters. Implementation of a prioritization methodology and application to the French situation. *Toxicology letters,* **176(2):** 104-123.

BNF (2006) *British National Formulary. British Medical association and Royal Pharmaceutical Society of Great Britain.* p947.

Bogart, M.H. (1987). Sex determination: A hypothesis based on steroid ratios. *Journal of theoretical biology,* **128(3):** 349-357.

Boobis, A.R. (1998). Comparative physicochemical and pharmacokinetic profiles of inhaled beclomethasone dipropionate and budesonide. *Respiratory medicine,* **92(SUPPL. B):** 2-6.

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

Bound, J.P. & Voulvoulis, N. (2005). Household disposal of pharmaceuticals as a pathway for aquatic contamination in the United Kingdom. *Environmental health perspectives,* **113(12):** 1705-1711.

Boxall, A.B.A., Kolpin, D.W., Halling-Sørensen, B. & Tolls, J. (2003). Are veterinary medicines causing environmental risks? *Environmental Science and Technology,* **37(15):** 286A-294A.

Boyd, G.R., Reemtsma, H., Grimm, D.A. & Mitra, S. (2003). Pharmaceuticals and personal care products (PPCPs) in surface and treated waters of Louisiana, USA and Ontario, Canada. *Science of the Total Environment,* **311(1-3):** 135-149.

Brian, J.V., Harris, C.A., Runnalls, T.J., Fantinati, A., Pojana, G., Marcomini, A., Booy, P., Lamoree, M., Kortenkamp, A. & Sumpter, J.P. (2008). Evidence of temperature-dependent effects on the oestrogenic response of fish: Implications with regard to climate change. *Science of the Total Environment,* **397(1-3):** 72-81.

Brian, J.V., Harris, C.A., Scholze, M., Backhaus, T., Booy, P., Lamoree, M., Pojana, G., Jonkers, N., Runnalls, T., Bonfà, A., Marcomini, A. & Sumpter, J.P. (2005). Accurate prediction of the response of freshwater fish to a mixture of oestrogenic chemicals. *Environmental health perspectives,* **113(6):** 721-728.

Brian, J.V., Harris, C.A., Scholze, M., Kortenkamp, A., Booy, P., Lamoree, M., Pojana, G., Jonkers, N., Marcomini, A. & Sumpter, J.P. (2007). Evidence of oestrogenic mixture effects on the reproductive performance of fish. *Environmental Science and Technology,* **41(1):** 337-344.

Brion, F., Tyler, C.R., Palazzi, X., Laillet, B., Porcher, J.M., Garric, J. & Flammarion, P. (2004). Impacts of 17β-estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo-larval-, juvenile- and adult-life stages in zebrafish (Danio rerio). *Aquatic Toxicology,* **68(3):** 193-217.

Brodeur, J.C., Woodburn, K.B. & Klecka, G.M. (2005). Potentiation of the vitellogenic response to 17α-ethinylestradiol by cortisol in the fathead minnow *Pimephales promelas*. *Environmental Toxicology and Chemistry,* **24(5):** 1125-1132.

Brooks, B.W., Foran, C.M., Richards, S.M., Weston, J., Turner, P.K., Stanley, J.K., Solomon, K.R., Slattery, M. & La Point, T.W. (2003). Aquatic ecotoxicology of fluoxetine. *Toxicology letters,* **142(3):** 169-183.

Buchwald, P. (2008). Glucocorticoid receptor binding: A biphasic dependence on molecular size as revealed by the bilinear LinBiExp model. *Steroids,* **73(2):** 193-208.

Burkhardt-Holm, P. (2010). Endocrine disruptors and water quality: A state-of-the-art review. *International Journal of Water Resources Development,* **26(3):** 477-493.

Bury, N.R., Sturm, A., Le Rouzic, P., Lethimonier, C., Ducouret, B., Guiguen, Y., Robinson-Rechavi, M., Laudet, V., Rafestin-Oblin, M.E. & Prunet, P. (2003). Evidence for two distinct functional glucocorticoid receptors in teleost fish. *Journal of Molecular Endocrinology,* **31(1):** 141-156.

Bustin, S.A. (2004). A-Z of quantitative PCR. *International University line*, La Jolla, CA. p882.

Caldwell, D.J., Mastrocco, F., Hutchinson, T.H., Länge, R., Heijerick, D., Janssen, C., Anderson, P.D. & Sumpter, J.P. (2008). Derivation of an aquatic predicted no-effect concentration for the synthetic hormone, 17α-ethinyl estradiol. *Environmental Science and Technology,* **42(19):** 7046-7054.

Campbell, P.M., Pottinger, T.G. & Sumpter, J.P. (1992). Stress reduces the quality of gametes produced by rainbow trout. *Biology of reproduction,* **47(6):** 1140-1150.

Campbell, P.M., Pottinger, T.G. & Sumpter, J.P. (1994). Preliminary evidence that chronic confinement stress reduces the quality of gametes produced by brown and rainbow trout. *Aquaculture,* **120(1-2):** 151-169.

Carpenter, D.O. (2006). Polychlorinated biphenyls (PCBs): Routes of exposure and effects on human health. *Reviews on environmental health,* **21(1):** 1-23.

Carragher, J.F. & Sumpter, J.P. (1990). The effect of cortisol on the secretion of sex steroids from cultured ovarian follicles of rainbow trout. *General and comparative endocrinology,* **77(3):** 403-407.

Carragher, J.F., Sumpter, J.P., Pottinger, T.G. & Pickering, A.D. (1989). The deleterious effects of cortisol implantation on reproductive function in two species of trout, Salmo trutta L. and Salmo gairdneri Richardson. *General and comparative endocrinology,* **76(2):** 310-321.

Castiglioni, S., Fanelli, R., Calamari, D., Bagnati, R. & Zuccato, E. (2004). Methodological approaches for studying pharmaceuticals in the environment by comparing predicted and measured concentrations in River Po, Italy. *Regulatory Toxicology and Pharmacology,* **39(1):** 25-32.

Chang, H., Hu, J. & Shao, B. (2007). Occurrence of natural and synthetic glucocorticoids in sewage treatment plants and receiving river waters. *Environmental Science and Technology,* **41(10):** 3462-3468.

Chang, H., Wan, Y. & Hu, J. (2009). Determination and source apportionment of five classes of steroid hormones in urban rivers. *Environmental Science and Technology,* **43(20):** 7691-7698.

Chapman, P.M. (2000). Whole effluent toxicity testing - Usefulness, level of protection, and risk assessment. *Environmental Toxicology and Chemistry,* **19(1):** 3-13.

Clearwater, S.J. & Pankhurst, N.W. (1997). The response to capture and confinement stress of plasma cortisol, plasma sex steroids and vitellogenic oocytes in the marine teleost, red gurnard. *Journal of fish biology,* **50(2):** 429-441.

Cleuvers, M. (2005). Initial risk assessment for three β-blockers found in the aquatic environment. *Chemosphere,* **59(2):** 199-205.

Colborn, T., Vom Saal, F.S. & Soto, A.M. (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environmental health perspectives,* **101(5):** 378-384.

Colombe, L., Fostier, A., Bury, N., Pakdel, F. & Guiguen, Y. (2000). A mineralocorticoid-like receptor in the rainbow trout, *Oncorhynchus mykiss*: Cloning and characterization of its steroid binding domain. *Steroids,* **65(6):** 319-328.

Consten, D., Bogerd, J., Komen, J., Lambert, J.G.D. & Goos, H.J.T. (2001). Long-term cortisol treatment inhibits pubertal development in male common carp, *Cyprinus carpio* L. *Biology of reproduction,* **64(4):** 1063-1071.

Corcoran, J., Winter, M.J. & Tyler, C.R. (2010). Pharmaceuticals in the aquatic environment: A critical review of the evidence for health effects in fish. *Critical reviews in toxicology,* **40(4):** 287-304.

Culig, Z. & Bartsch, G. (2006). Androgen axis in prostate cancer. *Journal of cellular biochemistry,* **99(2):** 373-381.

Cunningham, V.L., Binks, S.P. & Olson, M.J. (2009). Human health risk assessment from the presence of human pharmaceuticals in the aquatic environment. *Regulatory Toxicology and Pharmacology,* **53(1):** 39-45.

Dai, A. (2010). Drought under global warming: A review. *Climate change,* DOI: 10.1002/wcc.81.

Dang, Z., Li, K., Yin, H., Hakkert, B. & Vermeire, T. (2011). Endpoint sensitivity in fish endocrine disruption assays: Regulatory implications. *Toxicology letters,* **202(1):** 36-46.

Daughton, C.G. & Ternes, T.A. (1999). Pharmaceuticals and personal care products in the environment: Agents of subtle change? *Environmental health perspectives,* **107(SUPPL. 6):** 907-938.

David, D.S., Grieco, M.H. & Cushman Jr., P. (1970). Adrenal glucocorticoids after twenty years. A review of their clinically relevant consequences. *Journal of chronic diseases,* **22(10):** 637-711.

De Lange, H.J., Noordoven, W., Murk, A.J., Lürling, M. & Peeters, E.T.H.M. (2006). Behavioural responses of Gammarus pulex (Crustacea, Amphipoda) to low concentrations of pharmaceuticals. *Aquatic Toxicology,* **78(3):** 209-216.

Denslow, N.D., Garcia-Reyero, N. & Barber, D.S. (2007). Fish 'n' chips: The use of microarrays for aquatic toxicology. *Molecular BioSystems,* **3(3):** 172-177.

Desbrow, C., Routledge, E.J., Brighty, G.C., Sumpter, J.P. & Waldock, M. (1998). Identification of oestrogenic chemicals in STW effluent. 1. Chemical fractionation and *in vitro* biological screening. *Environmental Science and Technology,* **32(11):** 1549-1558.

Devlin, R.H. & Nagahama, Y. (2002). Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. *Aquaculture,* **208(3-4):** 191-364.

Dhabhar, F.S., Miller, A.H., McEwen, B.S. & Spencer, R.L. (1996). Stress-Induced Changes in Blood Leukocyte Distribution: Role of Adrenal Steroid Hormones. *Journal of Immunology,* **157(4):** 1638-1644.

Diaz-Nieto, J. & Wilby, R.L. (2005). A comparison of statistical downscaling and climate change factor methods: Impacts on low flows in the River Thames, United Kingdom. *Climatic Change,* **69(2-3):** 245-268.

Dorne, J.L.C.M., Ragas, A.M.J., Frampton, G.K., Spurgeon, D.S. & Lewis, D.F. (2007). Trends in human risk assessment of pharmaceuticals. *Analytical and Bioanalytical Chemistry,* **387(4):** 1167-1172.

Dorts, J., Richter, C.A., Wright-Osment, M.K., Ellersieck, M.R., Carter, B.J. & Tillitt, D.E. (2009). The genomic transcriptional response of female fathead minnows (*Pimephales promelas*) to an acute exposure to the androgen, 17β-trenbolone. *Aquatic Toxicology,* **91(1):** 44-53.

Drake, A.J., Tang, J.I. & Nyirenda, M.J. (2007). Mechanisms underlying the role of glucocorticoids in the early life programming of adult disease. *Clinical science,* **113(5-6):** 219-232.

Ducouret, B., Tujague, M., Ashraf, J., Mouchel, N., Servel, N., Valotaire, Y. & Thompson, E.B. (1995). Cloning of a teleost fish glucocorticoid receptor shows that it contains a deoxyribonucleic acid-binding domain different from that of mammals. *Endocrinology,* **136(9):** 3774-3783.

Edsbacker, S., Andersson, P. & Lindberg, C. (1987). Liver metabolism of budesonide in rat, mouse, and man. Comparative aspects. *Drug Metabolism and Disposition,* **15(3):** 403-411.

Eidem, J.K., Kleivdal, H., Kroll, K., Denslow, N., van Aerle, R., Tyler, C., Panter, G., Hutchinson, T. & Goksøyr, A. (2006). Development and validation of a direct homologous quantitative sandwich ELISA for fathead minnow (*Pimephales promelas*) vitellogenin. *Aquatic Toxicology,* **78(2):** 202-206.

Ekman, D.R., Villeneuve, D.L., Teng, Q., Ralston-Hooper, K.J., Martinović-Weigelt, D., Kahl, M.D., Jensen, K.M., Durhan, E.J., Makynen, E.A., Ankley, G.T. & Collette, T.W. (2011). Use of gene expression, biochemical and metabolite profiles to enhance exposure and effects assessment of the model androgen 17β-trenbolone in fish. *Environmental Toxicology and Chemistry,* **30(2):** 319-329.

EMEA (2006) *European Medicines Agency: Guideline of the Environmental Risk assessment of Medicinal Products for Human Use*. EMEA/CHMP/SWP/4447/00.

EPA, 2002. A short-term test method for assessing the reproductive toxicity of endocrine-disrupting chemicals using the fathead minnow (*Pimephales promelas*). U.S. Environmental Protection Agency, Duluth, MN, pp. 1-170.

Feher, T., Koref, O. & Tanko, A. (1975). Metabolism of a prednisolone compound in human under normal and pathological conditions. *Arzneimittel-Forschung/Drug Research,* **25(8):** 1314-1317.

Feijtel, T., Boeije, G., Matthies, M., Young, A., Morris, G., Gandolfi, C., Hansen, B., Fox, K., Holt, M., Koch, V., Schroder, R., Cassani, G., Schowanek, D., Rosenblom, J. & Niessen, H. (1997). Development of a geography-referenced regional exposure assessment tool for European rivers - GREAT-ER Contribution to GREAT-ER 1. *Chemosphere,* **34(11):** 2351-2373.

Fent, K., Weston, A.A. & Caminada, D. (2006). Ecotoxicology of human pharmaceuticals. *Aquatic Toxicology,* **76(2):** 122-159.

Fick, J., Lindberg, R.H., Parkkonen, J., Arvidsson, B., Tysklind, M. & Joakim Larsson, D.G. (2010). Therapeutic levels of levonorgestrel detected in blood plasma of fish: results from screening rainbow trout exposed to treated sewage effluents. *Environmental Science and Technology,* **44(7):** 2661-2666.

Filby, A.L. & Tyler, C.R. (2007). Appropriate 'housekeeping' genes for use in expression profiling the effects of environmental oestrogens in fish. *BMC Molecular Biology,* **8**: 10. doi: 10.1186/1471-2199-8-10.

Flippin, J.L., Huggett, D. & Foran, C.M. (2007). Changes in the timing of reproduction following chronic exposure to ibuprofen in Japanese medaka, *Oryzias latipes*. *Aquatic Toxicology,* **81(1):** 73-78.

Foo, J.T.W. & Lam, T.J. (1993). Retardation of ovarian growth and depression of serum steroid levels in the tilapia, *Oreochromis mossambicus*, by cortisol implantation. *Aquaculture,* **115(1-2):** 133-143.

Funder, J.W., Pearce, P.T., Smith, R. & Smith, A.I. (1988). Mineralocorticoid action: Target tissue specificity is enzyme, not receptor, mediated. *Science,* **242(4878):** 583-585.

Garcia-Reyero, N., Adelman, I., Liu, L. & Denslow, N. (2008). Gene expression profiles of fathead minnows exposed to surface waters above and below a sewage treatment plant in Minnesota. *Marine environmental research,* **66(1):** 134-136.

Ge, R.-., Dong, Q., Sottas, C.M., Latif, S.A., Morris, D.J. & Hardy, M.P. (2005). Stimulation of testosterone production in rat Leydig cells by aldosterone is mineralocorticoid receptor mediated. *Molecular and cellular endocrinology,* **243(1-2):** 35-42.

Giltrow, E., Eccles, P.D., Winter, M.J., McCormack, P.J., Rand-Weaver, M., Hutchinson, T.H. & Sumpter, J.P. (2009). Chronic effects assessment and plasma concentrations of the β-blocker propranolol in fathead minnows (*Pimephales promelas*). *Aquatic Toxicology,* **95(3):** 195-202.

Gong, H., Jarzynka, M.J., Cole, T.J., Jung, H.L., Wada, T., Zhang, B., Gao, J., Song, W.-., DeFranco, D.B., Cheng, S.-. & Xie, W. (2008). Glucocorticoids antagonize oestrogens by glucocorticoid receptor-mediated activation of oestrogen sulfotransferase. *Cancer research,* **68(18):** 7386-7393.

Goyal, R.N. & Bishnoi, S. (2009). Simultaneous voltammetric determination of prednisone and prednisolone in human body fluids. *Talanta,* **79(3):** 768-774.

Greenwood, A.K., Butler, P.C., White, R.B., Demarco, U., Pearce, D. & Fernald, R.D. (2003). Multiple corticosteroid receptors in a teleost fish: Distinct sequences, expression patterns, and transcriptional activities. *Endocrinology,* **144(10):** 4226-4236.

Gros, M., Petrović, M. & Barceló, D. (2009). Tracing pharmaceutical residues of different therapeutic classes in environmental waters by using liquid chromatography/quadrupole-linear ion trap mass spectrometry and automated library searching. *Analytical Chemistry,* **81(3):** 898-912.

Gros, M., Petrović, M., Ginebreda, A. & Barceló, D. (2010). Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes. *Environment international,* **36(1):** 15-26.

Gros, M., Petrovié, M. & Barceló, D. (2006). Multi-residue analytical methods using LC-tandem MS for the determination of pharmaceuticals in environmental and wastewater samples: A review. *Analytical and Bioanalytical Chemistry,* **386(4):** 941-952.

Guillette Jr., L.J., Gross, T.S., Masson, G.R., Matter, J.M., Percival, H.F. & Woodward, A.R. (1994). Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environmental health perspectives,* **102(8):** 680-688.

Gunnarsson, L., Jauhiainen, A., Kristiansson, E., Nerman, O. & Larsson, D.G.J. (2008). Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. *Environmental Science and Technology,* **42(15):** 5807-5813.

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten Lützhøft, H.C. & Jørgensen, S.E. (1998). Occurrence, fate and effects of pharmaceutical substances in the environment- A review. *Chemosphere,* **36(2):** 357-393.

Hannah, R., D'Aco, V.J., Anderson, P.D., Buzby, M.E., Caldwell, D.J., Cunningham, V.L., Ericson, J.F., Johnson, A.C., Parke, N.J., Samuelian, J.H. & Sumpter, J.P. (2009). Exposure assessment of 17α-ethinylestradiol in surface waters of the United States and Europe. *Environmental Toxicology and Chemistry,* **28(12):** 2725-2732.

Harper, J.M. & Austad, S.N. (2000). Fecal glucocorticoids: A noninvasive method of measuring adrenal activity in wild and captive rodents. *Physiological and Biochemical Zoology,* **73(1):** 12-22.

Harries, J.E., Runnalls, T., Hill, E., Harris, C.A., Maddix, S., Sumpter, J.P. & Tyler, C.R. (2000). Development of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (*Pimephales promelas*). *Environmental Science and Technology,* **34(14):** 3003-3011.

Harris, C.A., Hamilton, P.B., Runnalls, T.J., Vinciotti, V., Henshaw, A., Hodgson, D., Coe, T.S., Jobling, S., Tyler, C.R. & Sumpter, J.P. (2011). The consequences of feminization in Breeding groups of wild fish. *Environmental health perspectives,* **119(3):** 306-311.

Hayashi, Y., Kobira, H., Yamaguchi, T., Shiraishi, E., Yazawa, T., Hirai, T., Kamei, Y. & Kitano, T. (2010). High temperature causes masculinization of genetically female medaka by elevation of cortisol. *Molecular reproduction and development,* **77(8):** 679-686.

Heberer, T. (2002). Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: A review of recent research data. *Toxicology letters,* **131(1-2):** 5-17.

Henry, T.B. & Black, M.C. (2008). Acute and chronic toxicity of fluoxetine (selective serotonin reuptake inhibitor) in western Mosquitofish. *Archives of Environmental Contamination and Toxicology,* **54(2):** 325-330.

Hilton, M.J. & Thomas, K.V. (2003). Determination of selected human pharmaceutical compounds in effluent and surface water samples by high-performance liquid chromatography-electrospray tandem mass spectrometry. *Journal of Chromatography A,* **1015(1-2):** 129-141.

Hoeger, B., Köllner, B., Dietrich, D.R. & Hitzfeld, B. (2005). Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (Salmo trutta f. fario). *Aquatic Toxicology,* **75(1):** 53-64.

Holgate, S.T. & Polosa, R. (2008). Treatment strategies for allergy and asthma. *Nature Reviews Immunology,* **8(3):** 218-230.

Howell, W.M., Black, D.A. & Bortone, S.A. (1980). Abnormal expression of secondary sex characters in a population of mosquitofish, *Gambusia affinis holbrooki :* evidence for environmentally induced masculinization. Copeia **4**: 43-51.

Hoyt, P.R., Doktycz, M.J., Beattie, K.L. & Greeley Jr., M.S. (2003). DNA Microarrays Detect 4-Nonylphenol-induced Alterations in Gene Expression during Zebrafish Early Development. *Ecotoxicology,* **12(6):** 469-474.

Huggett, D.B., Brooks, B.W., Peterson, B., Foran, C.M. & Schlenk, D. (2002). Toxicity of select beta adrenergic receptor-blocking pharmaceuticals (B-blockers) on aquatic organisms. *Archives of Environmental Contamination and Toxicology,* **43(2):** 229-235.

Huggett, D.B., Ericson, J.F., Cook, J.C. & Williams, R.T. (2004). Plasma concentrations of human pharmaceuticals as predictors of pharmacological responses in fish. *Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks,* 373-386.

Ingerslev, F., Vaclavik, E. & Halling-Sørensen, B. (2003). Pharmaceuticals and personal care products: A source of endocrine disruption in the environment? *Pure and Applied Chemistry,* **75(11-12):** 1881-1893.

Inoue, T., Moritomo, T., Tamura, Y., Mamiya, S., Fujino, H. & Nakanishi, T. (2002). A new method for fish leucocyte counting and partial differentiation by flow cytometry. *Fish and Shellfish Immunology,* **13(5):** 379-390.

Iwama, G.K. (1998). Stress in fish.*Annals of the New York Academy of Sciences,* **85(1):**304-310.

Janssens, P.A. & Waterman, J. (1988). Hormonal regulation of gluconeogenesis and glycogenolysis in carp (*Cyprinus carpio*) liver pieces cultured *in vitro*. *Comparative Biochemistry and Physiology - A Physiology,* **91(3):** 451-455.

Jensen, K.M., Kahl, M.D., Makynen, E.A., Korte, J.J., Leino, R.L., Butterworth, B.C. & Ankley, G.T. (2004). Characterization of responses to the antiandrogen flutamide in a short-term reproduction assay with the fathead minnow. *Aquatic Toxicology,* **70(2):** 99-110.

Jensen, K.M., Korte, J.J., Kahl, M.D., Pasha, M.S. & Ankley, G.T. (2001). Aspects of basic reproductive biology and endocrinology in the fathead minnow (*Pimephales promelas*). *Comparative Biochemistry and Physiology - C Pharmacology Toxicology and Endocrinology,* **128(1):** 127-141.

Jjemba, P.K. (2006). Excretion and ecotoxicity of pharmaceutical and personal care products in the environment. *Ecotoxicology and environmental safety,* **63(1):** 113-130.

Jobling, S., Burn, R.W., Thorpe, K., Williams, R. & Tyler, C. (2009). Statistical modeling suggests that antiandrogens in effluents from wastewater treatment works contribute to widespread sexual disruption in fish living in english rivers. *Environmental health perspectives,* **117(5):** 797-802.

Jobling, S., Coey, S., Whitmore, J.G., Kime, D.E., Van Look, K.J.W., McAllister, B.G., Beresford, N., Henshaw, A.C., Brighty, G., Tyler, C.R. & Sumpter, J.P. (2002). Wild intersex roach (Rutilus rutilus) have reduced fertility. *Biology of reproduction,* **67(2):** 515-524.

Jobling, S., Nolan, M., Tyler, C.R., Brighty, G. & Sumpter, J.P. (1998). Widespread sexual disruption in wild fish. *Environmental Science and Technology,* **32(17):** 2498-2506.

Jobling, S., Williams, R., Johnson, A., Taylor, A., Gross-Sorokin, M., Nolan, M., Tyler, C.R., van Aerle, R., Santos, E. & Brighty, G. (2006). Predicted exposures to steroid oestrogens in U.K. rivers correlate with widespread sexual disruption in wild fish populations. *Environmental health perspectives,* **114 (Suppl 1)** 32-39.

Johansson, S.A., Andersson, K.E. & Brattsand, R. (1982). Topical and systemic glucocorticoid potencies of budesonide, beclomethasone dipropionate and prednisolone in man. *European journal of respiratory diseases,* **63(Suppl. 122):** 74-82.

Johnson, A.C. & Sumpter, J.P. (2001). Removal of endocrine-disrupting chemicals in activated sludge treatment works. *Environmental Science and Technology,* **35(24):** 4697-4703.

Johnson, A.C. (2010). Natural variations in flow are critical in determining concentrations of point source contaminants in rivers: An oestrogen example. *Environmental Science and Technology,* **44(20):** 7865-7870.

Johnson, A.C., Aerni, H.-., Gerritsen, A., Gibert, M., Giger, W., Hylland, K., Jürgens, M., Nakari, T., Pickering, A., Suter, M.J.-., Svenson, A. & Wettstein, F.E. (2005). Comparing steroid oestrogen, and nonylphenol content across a range of European sewage plants with different treatment and management practices. *Water research,* **39(1):** 47-58.

Johnson, A.C., Jürgens, M.D., Williams, R.J., Kümmerer, K., Kortenkamp, A. & Sumpter, J.P. (2008). Do cytotoxic chemotherapy drugs discharged into rivers pose a risk to the environment and human health? An overview and UK case study. *Journal of Hydrology,* **348(1-2):** 167-175.

Johnson, A.C., Keller, V., Williams, R.J. & Young, A. (2007). A practical demonstration in modelling diclofenac and propranolol river water concentrations using a GIS hydrology model in a rural UK catchment. *Environmental Pollution,* **146(1):** 155-165.

Johnson, A.C., Ternes, T., Williams, R.J. & Sumpter, J.P. (2008a). Assessing the concentrations of polar organic microcontaminants from point sources in the aquatic environment: Measure or model? *Environmental Science and Technology,* **42(15):** 5390-5399.

Johnson, A.C., Williams, R.J. & Matthiessen, P. (2006). The potential steroid hormone contribution of farm animals to freshwaters, the United Kingdom as a case study. *Science of the Total Environment,* **362(1-3):** 166-178.

Johnson, A.C., Williams, R.J., Simpson, P. & Kanda, R. (2007). What difference might sewage treatment performance make to endocrine disruption in rivers? *Environmental Pollution,* **147(1):** 194-202.

Jones, O.A., Lester, J.N. & Voulvoulis, N. (2005). Pharmaceuticals: A threat to drinking water? *Trends in biotechnology,* **23(4):** 163-167.

Jones, O.A.H., Voulvoulis, N. & Lester, J.N. (2001). Human pharmaceuticals in the aquatic environment a review. *Environmental technology,* **22(12):** 1383-1394.

Jones, O.A.H., Voulvoulis, N. & Lester, J.N. (2002). Aquatic environmental assessment of the top 25 English prescription pharmaceuticals. *Water research,* **36(20):** 5013-5022.

Kanda, R. & Churchley, J. (2008). Removal of endocrine disrupting compounds during conventional wastewater treatment. *Environmental technology,* **29(3):** 315-323.

Katsiadaki, I., Sanders, M., Sebire, M., Nagae, M., Soyano, K. & Scott, A.P. (2007). Three-spined stickleback: an emerging model in environmental endocrine disruption. *Environmental sciences : an international journal of environmental physiology and toxicology,* **14(5):** 263-283.

Keay, J., Bridgham, J.T. & Thornton, J.W. (2006). The Octopus vulgaris oestrogen receptor is a constitutive transcriptional activator: Evolutionary and functional implications. *Endocrinology,* **147(8):** 3861-3869.

Keller, V.D.J. & Young, A.R. (2004). Development of the IntegratedWater Resources and Water Quality Modelling System. Science Report P2-248/SR. Environment Agency. Bristal, UK. .

Khetan, S.K. & Collins, T.J. (2007). Human pharmaceuticals in the aquatic environment: A challenge to green chemisty. *Chemical reviews,* **107(6):** 2319-2364.

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M. & Flick, R.W. (2007). Collapse of a fish population after exposure to a synthetic oestrogen. *Proceedings of the National Academy of Sciences of the United States of America,* **104(21):** 8897-8901.

Kim, S.D., Cho, J., Kim, I.S., Vanderford, B.J. & Snyder, S.A. (2007). Occurrence and removal of pharmaceuticals and endocrine disruptors in South Korean surface, drinking, and waste waters. *Water research,* **41(5):** 1013-1021.

Kolodziej, E.P., Gray, J.L. & Sedlak, D.L. (2003). Quantification of steroid hormones with pheromonal properties in municipal wastewater effluent. *Environmental Toxicology and Chemistry,* **22(11):** 2622-2629.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B. & Buxton, H.T. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: A national reconnaissance. *Environmental Science and Technology,* **36(6):** 1202-1211.

Korte, J.J., Kahl, M.D., Jensen, K.M., Pasha, M.S., Parks, L.G., LeBlanc, G.A. & Ankley, G.T. (2000). Fathead minnow vitellogenin: Complementary DNA sequence and messenger RNA and protein expression after 17β-estradiol treatment. *Environmental Toxicology and Chemistry,* **19(4):** 972-981.

Kota, B.P., Huang, T.H.-. & Roufogalis, B.D. (2005). An overview on biological mechanisms of PPARs. *Pharmacological Research,* **51(2):** 85-94.

Kummerer, K. (2001). Drugs in the environment: Emission of drugs, diagnostic aids and disinfectants into wastewater by hospitals in relation to other sources - A review. *Chemosphere,* **45(6-7):** 957-969.

Kümmerer, K. (2009). The presence of pharmaceuticals in the environment due to human use - present knowledge and future challenges. *Journal of environmental management,* **90(8):** 2354-2366.

Lange, A., Katsu, Y., Ichikawa, R., Paull, G.C., Chidgey, L.L., Coe, T.S., Iguchi, T. & Tyler, C.R. (2008). Altered sexual development in roach (Rutilus rutilus) exposed to environmental concentrations of the pharmaceutical 17α-Ethinylestradiol and associated expression dynamics of aromatases and oestrogen receptors. *Toxicological Sciences,* **106(1):** 113-123.

Länge, R., Hutchinson, T.H., Croudace, C.P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G.H. & Sumpter, J.P. (2001). Effects of the synthetic oestrogen 17α-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry,* **20(6):** 1216-1227.

Larkin, P., Knoebl, I. & Denslow, N.D. (2003). Differential gene expression analysis in fish exposed to endocrine disrupting compounds. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology,* **136(2):** 149-161.

Larkin, P., Sabo-Attwood, T., Kelso, J. & Denslow, N.D. (2002). Gene expression analysis of largemouth bass exposed to estradiol, nonylphenol, and p,p′-DDE. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology,* **133(4):** 543-557.

Larkin, P., Villeneuve, D.L., Knoebl, I., Miracle, A.L., Carter, B.J., Liu, L., Denslow, N.D. & Ankley, G.T. (2007). Development and validation of a 2,000-gene microarray for the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry,* **26(7):** 1497-1506.

Larsson, D.G.J. & Förlin, L. (2002). Male-biased sex ratios of fish embryos near a pulp mill: Temporary recovery after a short-term shutdown. *Environmental health perspectives,* **110(8):** 739-742.

Larsson, D.G.J., Adolfsson-Erici, M. & Thomas, P. (2006). Characterization of putative ligands for a fish gonadal androgen receptor in a pulp mill effluent. *Environmental Toxicology and Chemistry,* **25(2):** 419-427.

Larsson, D.G.J., Adolfsson-Erici, M., Parkkonen, J., Pettersson, M., Berg, A.H., Olsson, P.-. & Förlin, L. (1999). Ethinyloestradiol - An undesired fish contraceptive? *Aquatic Toxicology,* **45(2-3):** 91-97.

Larsson, D.G.J., de Pedro, C. & Paxeus, N. (2007). Effluent from drug manufactures contains extremely high levels of pharmaceuticals. *Journal of hazardous materials,* **148(3):** 751-755.

Legler, J., Dennekamp, M., Vethaak, A.D., Brouwera, A., Koeman, J.H., Van der Burg, B. & Murk, A.J. (2002). Detection of oestrogenic activity in sediment-associated compounds using *in vitro* reporter gene assays. *Science of the Total Environment,* **293(1-3):** 69-83.

Leino, R.L., Jensen, K.M. & Ankley, G.T. (2005). Gonadal histology and characteristic histopathology associated with endocrine disruption in the adult fathead minnow (*Pimephales promelas*). *Environmental toxicology and pharmacology,* **19(1):** 85-98.

Lethimonier, C., Flouriot, G., Valotaire, Y., Kah, O. & Ducouret, B. (2000). Transcriptional interference between glucocorticoid receptor and estradiol receptor mediates the inhibitory effect of cortisol on fish vitellogenesis. *Biology of reproduction,* **62(6):** 1763-1771.

Lim-Tio, S.S., Keightley, M.-. & Fuller, P.J. (1997). Determinants of specificity of transactivation by the mineralocorticoid or glucocorticoid receptor. *Endocrinology,* **138(6):** 2537-2543.

Lister, A.L. & Van Der Kraak, G. (2008). An investigation into the role of prostaglandins in zebrafish oocyte maturation and ovulation. *General and comparative endocrinology,* **159(1):** 46-57.

Liu, Z.-., Kanjo, Y. & Mizutani, S. (2009). Urinary excretion rates of natural oestrogens and androgens from humans, and their occurrence and fate in the environment: A review. *Science of the Total Environment,* **407(18):** 4975-4985.

López-Serna, R., Pérez, S., Ginebreda, A., Petrović, M. & Barceló, D. (2010). Fully automated determination of 74 pharmaceuticals in environmental and waste waters by online solid phase extraction–liquid chromatography-electrospray–tandem mass spectrometry. *Talanta,* **83(2):** 410-424.

Lyche, J.L., Nourizadeh-Lillabadi, R., Almaas, C., Stavik, B., Berg, V., Skare, J.U., Alestrøm, P. & Ropstad, E. (2010). Natural mixtures of persistent organic pollutants (POP) increase weight gain, advance puberty, and induce changes in gene expression associated with steroid hormones and obesity in female zebrafish. *Journal of Toxicology and Environmental Health - Part A: Current Issues,* **73(15):** 1032-1057.

Lyssimachou, A., Munro Jenssen, B. & Arukwe, A. (2006). Brain cytochrome P450 aromatase gene isoforms and activity levels in atlantic salmon after waterborne exposure to nominal environmental concentrations of the pharmaceutical ethynylestradiol and antifoulant tributyltin. *Toxicological Sciences,* **91(1):** 82-92.

Madsen, S.S. (1990). The role of cortisol and growth hormone in seawater adaptation and development of hypoosmoregulatory mechanisms in sea trout parr (Salmo trutta trutta). *General and comparative endocrinology,* **79(1):** 1-11.

Mahajan, A. & Tandon, V.R. (2005). Corticosteroids in rheumatology: Friends or foes. *Journal, Indian Academy of Clinical Medicine,* **6(4):** 275-280.

Martinelli, E., Ferrari, P. & Ripamonti, A. (1979). Metabolism of deflazacort in the rat, dog and man. *Drug Metabolism and Disposition,* **7(5):** 335-339.

Martínez-Porchas, M., Martínez-Córdova, L.R. & Ramos-Enriquez, R. (2009). Cortisol and glucose: Reliable indicators of fish stress? *Pan-American Journal of Aquatic Sciences,* **4(2):** 158-178.

Matte, A., Tari, L.W., Goldie, H. & Delbaere, L.T.J. (1997). Structure and mechanism of phosphoenolpyruvate carboxykinase. *Journal of Biological Chemistry,* **272(13):** 8105-8108.

Matthiessen, P. (2003). Historical perspective on endocrine disruption in wildlife. *Pure and Applied Chemistry,* **75(11-12):** 2197-2206.

Maunder, R.J., Matthiessen, P., Sumpter, J.P. & Pottinger, T.G. (2007). Rapid bioconcentration of steroids in the plasma of three-spined stickleback Gasterosteus aculeatus exposed to waterborne testosterone and 17β-oestradiol. *Journal of fish biology,* **70(3):** 678-690.

McCormick, S.D. (1996). Effects of growth hormone and insulin-like growth factor I on salinity tolerance and gill Na+, K+-ATPase in Atlantic salmon (Salmo salar): Interaction with cortisol. *General and comparative endocrinology,* **101(1):** 3-11.

McKim, J., Schmieder, P. & Veith, G. (1985). Absorption dynamics of organic chemical transport across trout gills as related to octanol-water partition coefficient. *Toxicology and applied pharmacology,* **77(1):** 1-10.

McQuillan, H.J., Lokman, P.M. & Young, G. (2003). Effects of sex steroids, sex, and sexual maturity on cortisol production: An *in vitro* comparison of chinook salmon and rainbow trout interrenals. *General and comparative endocrinology,* **133(1):** 154-163.

Mehinto, A.C., Hill, E.M. & Tyler, C.R. (2010). Uptake and biological effects of environmentally relevant concentrations of the nonsteroidal anti-inflammatory pharmaceutical diclofenac in rainbow trout (*oncorhynchus mykiss*). *Environmental Science and Technology,* **44(6):** 2176-2182.

Melby, J.C. (1977). Clinical pharmacology of systemic corticosteroids. *Annual Review of Pharmacology and Toxicology,* **17**: 511-527.

Miguel-Queralt, S. & Hammond, G.L. (2008). Sex hormone-binding globulin in fish gills is a portal for sex steroids breached by xenobiotics. *Endocrinology,* **149(9):** 4269-4275.

Miles-Richardson, S.R., Kramer, V.J., Fitzgerald, S.D., Render, J.A., Yamini, B., Barbee, S.J. & Giesy, J.P. (1999). Effects of waterborne exposure of 17 β-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). *Aquatic Toxicology,* **47(2):** 129-145.

Milla, S., Terrien, X., Sturm, A., Ibrahim, F., Giton, F., Fiet, J., Prunet, P. & Le Gac, F. (2008). Plasma 11-deoxycorticosterone (DOC) and mineralocorticoid receptor testicular expression during rainbow trout *Oncorhynchus mykiss* spermiation: Implication with 17alpha, 20beta-dihydroxyprogesterone on the milt fluidity? *Reproductive Biology and Endocrinology,* **6(1):** 19

Milla, S., Wang, N., Mandiki, S.N.M. & Kestemont, P. (2009). Corticosteroids: Friends or foes of teleost fish reproduction? *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology,* **153(3):** 242-251.

Mimeault, C., Woodhouse, A.J., Miao, X.-., Metcalfe, C.D., Moon, T.W. & Trudeau, V.L. (2005). The human lipid regulator, gemfibrozil bioconcentrates and reduces testosterone in the goldfish, Carassius auratus. *Aquatic Toxicology, **73(1):** 44-54.

Mocarelli, P., Gerthoux, P.M., Patterson Jr., D.G., Milani, S., Limonta, G., Bertona, M., Signorini, S., Tramacere, P., Colombo, L., Crespi, C., Brambilla, P., Sarto, C., Carreri, V., Sampson, E.J., Turner, W.E. & Needham, L.L. (2008). Dioxin exposure, from infancy through puberty, produces endocrine disruption and affects human semen quality. *Environmental health perspectives, **116(1):** 70-77.

Mommsen, T.P., Vijayan, M.M. & Moon, T.W. (1999). Cortisol in teleosts: Dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries, **9(3):** 211-268.

Momoda, T.S., Schwindt, A.R., Feist, G.W., Gerwick, L., Bayne, C.J. & Schreck, C.B. (2007). Gene expression in the liver of rainbow trout, *Oncorhynchus mykiss*, during the stress response. *Comparative Biochemistry and Physiology - Part D: Genomics and Proteomics, **2(4):** 303-315.

Morgan, J.A.W., Pottinger, T.G. & Rippon, P. (1993). Evaluation of flow cytometry as a method for quantification of circulating blood cell populations in salmonid fish. *Journal of fish biology, **42(1):** 131-141.

Möstl, E., Messmann, S., Bagu, E., Robia, C. & Palme, R. (1999). Measurement of Glucocorticoid Metabolite Concentrations in Faeces of Domestic Livestock. *Journal of Veterinary Medicine Series A: Physiology Pathology Clinical Medicine, **46(10):** 621-631.

Munck, A., Guyre, P.M. & Holbrook, N.J. (1984). Physilogical functions of Glucocorticoids in stress and their relations to pharmacological actions. *Endocrine reviews, **5(1):** 25-44.

Nagahama, Y., Yoshikuni, M., Yamashita, M. & Tanaka, M. (1994). 13 Regulation of Oocyte Maturation in Fish.*Fish Physiology.***13**:393-439.

Nagel, R. (2002). DarT: The embryo test with the Zebrafish Danio rerio--a general model in ecotoxicology and toxicology. *ALTEX : Alternativen zu Tierexperimenten, **19 Suppl 1** 38-48.

Nakamura, M., Kobayashi, T., Chang, X.-. & Nagahama, Y. (1998). Gonadal sex differentiation in teleost fish. *Journal of Experimental Zoology, **281(5):** 362-372.

Nash, J.P., Kime, D.E., Van der Ven, L.T.M., Wester, P.W., Brion, F., Maack, G., Stahlschmidt-Allner, P. & Tyler, C.R. (2004). Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. *Environmental health perspectives, **112(17):** 1725-1733.

Nentwig, G. (2007). Effects of pharmaceuticals on aquatic invertebrates. Part II: The antidepressant drug fluoxetine. *Archives of Environmental Contamination and Toxicology, **52(2):** 163-170.

Newnham, J.P. (2001). Is prenatal glucocorticoid administration another origin of adult disease? *Clinical and Experimental Pharmacology and Physiology, **28(11):** 957-961.

Nickerson, J.G., Dugan, S.G., Drouin, G. & Moon, T.W. (2001). A putative β2-adrenoceptor from the rainbow trout (Oncorhynuchus mykiss): Molecular characterization and pharmacology. *European Journal of Biochemistry,* **268(24):** 6465-6472.

Nishizuka, M., Heitaku, S., Maekawa, S., Nishikawa, J.-. & Imagawa, M. (2004). Development of standardized *in vitro* assay system for oestrogen receptors and species specificity of binding ability of 4-nonylphenol and p-octylphenol. *Journal of Health Science,* **50(5):** 511-517.

Nolan, T., Hands, R.E. & Bustin, S.A. (2006). Quantification of mRNA using real-time RT-PCR. *Nature Protocols,* **1(3):** 1559-1582.

Oaks, J.L., Gilbert, M., Virani, M.Z., Watson, R.T., Meteyer, C.U., Rideout, B.A., Shivaprasad, H.L., Ahmed, S., Chaudhry, M.J.I., Arshad, M., Mahmood, S., Ali, A. & Khan, A.A. (2004). Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature,* **427(6975):** 630-633.

Odermatt, A. & Nashev, L.G. (2010). The glucocorticoid-activating enzyme 11β-hydroxysteroid dehydrogenase type 1 has broad substrate specificity: Physiological and toxicological considerations. *Journal of Steroid Biochemistry and Molecular Biology,* **119(1-2):** 1-13.

Orlando, E.F., Kolok, A.S., Binzcik, G.A., Gates, J.L., Horton, M.K., Lambright, C.S., Gray Jr., L.E., Soto, A.M. & Guillette Jr., L.J. (2004). Endocrine-disrupting effects of cattle feedlot effluent on an aquatic sentinel species, the fathead minnow. *Environmental health perspectives,* **112(3):** 353-358.

Örn, S., Holbech, H., Madsen, T.H., Norrgren, L. & Petersen, G.I. (2003). Gonad development and vitellogenin production in zebrafish (Danio rerio) exposed to ethinylestradiol and methyltestosterone. *Aquatic Toxicology,* **65(4):** 397-411.

Owen, S.F., Giltrow, E., Huggett, D.B., Hutchinson, T.H., Saye, J., Winter, M.J. & Sumpter, J.P. (2007). Comparative physiology, pharmacology and toxicology of β-blockers: Mammals versus fish. *Aquatic Toxicology,* **82(3):** 145-162.

Owen, S.F., Huggett, D.B., Hutchinson, T.H., Hetheridge, M.J., Kinter, L.B., Ericson, J.F. & Sumpter, J.P. (2009). Uptake of propranolol, a cardiovascular pharmaceutical, from water into fish plasma and its effects on growth and organ biometry. *Aquatic Toxicology,* **93(4):** 217-224.

Palermo, F.A., Mosconi, G., Angeletti, M. & Polzonetti-Magni, A.M. (2008). Assessment of water pollution in the Tronto River (Italy) by applying useful biomarkers in the fish model Carassius auratus. *Archives of Environmental Contamination and Toxicology,* **55(2):** 295-304.

Pankhurst, N.W. & Van Der Kraak, G. (2000). Evidence that acute stress inhibits ovarian steroidogenesis in rainbow trout *in vivo*, through the action of cortisol. *General and comparative endocrinology,* **117(2):** 225-237.

Pankhurst, N.W. (2011). The endocrinology of stress in fish: An environmental perspective. *General and comparative endocrinology,* **170(2):** 265-275.

Panter, G.H., Hutchinson, T.H., Hurd, K.S., Sherren, A., Stanley, R.D. & Tyler, C.R. (2004). Successful detection of (anti-)androgenic and aromatase inhibitors in pre-spawning adult fathead minnows (*Pimephales promelas*) using easily measured endpoints of sexual development. *Aquatic Toxicology,* **70(1):** 11-21.

Panter, G.H., Hutchinson, T.H., Lange, R., Lye, C.M., Sumpter, J.P., Zerulla, M. & Tyler, C.R. (2002). Environmental toxicology: Utility of a juvenile fathead minnow screening assay for detecting (anti-)oestrogenic substances. *Environmental Toxicology and Chemistry,* **21(2):** 319-326.

Parrott, J.L. & Blunt, B.R. (2005). Life-cycle exposure of fathead minnows (*Pimephales promelas*) to an ethinylestradiol concentration below 1 ng/L reduces egg fertilization success and demasculinizes males. *Environmental toxicology,* **20(2):** 131-141.

Paulos, P., Runnalls, T.J., Nallani, G., La Point, T., Scott, A.P., Sumpter, J.P. & Huggett, D.B. (2010). Reproductive responses in fathead minnow and Japanese medaka following exposure to a synthetic progestin, Norethindrone. *Aquatic Toxicology,* **99**:256-262

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research,* **29(9):** e45

Pickering, A.D. & Christie, P. (1981). Changes in the concentrations of plasma cortisol and thyroxine during sexual maturation of the hatchery-reared brown trout, Salmo trutta L. *General and comparative endocrinology,* **44(4):** 487-496.

Pickering, A.D. & Pottinger, T.G. (1985). Cortisol can increase the susceptibility of brown trout, Salmo trutta L., to disease without reducing the white blood cell count. *Journal of fish biology,* **27(5):** 611-619.

Pickering, A.D. & Pottinger, T.G. (1989). Stress responses and disease resistance in salmonid fish: Effects of chronic elevation of plasma cortisol. *Fish Physiology and Biochemistry,* **7(1-6):** 253-258.

Pickering, A.D. (1984). Cortisol-induced lymphocytopenia in brown trout, Salmo trutta L. *General and comparative endocrinology,* **53(2):** 252-259.

Pickering, A.D., Pottinger, T.G., Carragher, J. & Sumpter, J.P. (1987). The effects of acute and chronic stress on the levels of reproductive hormones in the plasma of mature male brown trout, Salmo trutta L. *General and comparative endocrinology,* **68(2):** 249-259.

Pinter, J. & Thomas, P. (1995). Characterization of a progestogen receptor in the ovary of the spotted seatrout, Cynoscion nebulosus. *Biology of reproduction,* **52(3):** 667-675.

Piram, A., Salvador, A., Gauvrit, J., Lanteri, P. & Faure, R. (2008). Development and optimisation of a single extraction procedure for the LC/MS/MS analysis of two pharmaceutical classes residues in sewage treatment plant. *Talanta,* **74(5):** 1463-1475.

Pomati, F., Castiglioni, S., Zuccato, E., Fanelli, R., Vigetti, D., Rossetti, C. & Calamari, D. (2006). Effects of a complex mixture of therapeutic drugs at environmental levels on human embryonic cells. *Environmental Science and Technology,* **40(7):** 2442-2447.

Porsbring, T., Blanck, H., Tjellström, H. & Backhaus, T. (2009). Toxicity of the pharmaceutical clotrimazole to marine microalgal communities. *Aquatic Toxicology,* **91(3):** 203-211.

Pottinger, T.G. & Carrick, T.R. (1999). A comparison of plasma glucose and plasma cortisol as selection markers for high and low stress-responsiveness in female rainbow trout. *Aquaculture,* **175(3-4):** 351-363.

Pottinger, T.G. & Pickering, A.D. (1990). The effect of cortisol administration on hepatic and plasma estradiol-binding capacity in immature female rainbow trout (*Oncorhynchus mykiss*). *General and comparative endocrinology,* **80(2):** 264-273.

Pottinger, T.G., Balm, P.H.M. & Pickering, A.D. (1995). Sexual maturity modifies the responsiveness of the pituitary-interrenal axis to stress in male rainbow trout. *General and comparative endocrinology,* **98(3):** 311-320.

Pottinger, T.G., Carrick, T.R., Hughes, S.E. & Balm, P.H.M. (1996). Testosterone, 11-ketotestosterone, and estradiol-17β modify baseline and stress-induced interrenal and corticotropic activity in trout. *General and comparative endocrinology,* **104(3):** 284-295.

Pozo, O.J., Ventura, R., Monfort, N., Segura, J. & Delbeke, F.T. (2009). Evaluation of different scan methods for the urinary detection of corticosteroid metabolites by liquid chromatography tandem mass spectrometry. *Journal of Mass Spectrometry,* **44(6):** 929-944.

Price, O.R., Williams, R.J., van Egmond, R., Wilkinson, M.J. & Whelan, M.J. (2010). Predicting accurate and ecologically relevant regional scale concentrations of triclosan in rivers for use in higher-tier aquatic risk assessments. *Environment international,* **36(6):** 521-526.

Price, O.R., Williams, R.J., Zhang, Z. & van Egmond, R. (2010a). Modelling concentrations of decamethylcyclopentasiloxane in two UK rivers using LF2000-WQX. *Environmental Pollution,* **158(2):** 356-360.

Prunet, P., Sturm, A. & Milla, S. (2006). Multiple corticosteroid receptors in fish: From old ideas to new concepts. *General and comparative endocrinology,* **147(1):** 17-23.

Purdom, C.E., Hardiman, P.A., Bye, V.J., Eno, N.C., Tyler, C.R. & Sumpter, J.P. (1994). Oestrogenic effects of effluents from sewage treatment works. *Chemical Ecology,* **8**:275-285.

Rahman, M.A., Ohta, K., Yoshikuni, M., Nagahama, Y., Chuda, H. & Matsuyama, M. (2002). Characterization of ovarian membrane receptor for 17,20β-dihydroxy-4-pregnen-3-one, a maturation-inducing hormone in yellowtail, Seriola quinqueradiata. *General and comparative endocrinology,* **127(1):** 71-79.

Rahman, M.F., Yanful, E.K. & Jasim, S.Y. (2009). Endocrine disrupting compounds (EDCs) and pharmaceuticals and personal care products (PPCPs) in the aquatic environment: Implications for

the drinking water industry and global environmental health. *Journal of Water and Health,* **7(2):** 224-243.

Ramirez, A.J., Brain, R.A., Usenko, S., Mottaleb, M.A., O'Donnell, J.G., Stahl, L.L., Wathen, J.B., Snyder, B.D., Pitt, J.L., Perez-Hurtado, P., Dobbins, L.L., Brooks, B.W. & Chambliss, C.K. (2009). Occurrence of pharmaceuticals and personal care products in fish: Results of a national pilot study in the United States. *Environmental Toxicology and Chemistry,* **28(12):** 2587-2597.

Reddy, P.K., Renaud, R. & Leatherland, J.F. (1999). Effects of cortisol and triiodo-L-thyronine on the steroidogenic capacity of rainbow trout ovarian follicles at two stages of oocyte maturation. *Fish Physiology and Biochemistry,* **21(2):** 129-140.

Rhen, T. & Cidlowski, J.A. (2006). Oestrogens and glucocorticoids have opposing effects on the amount and latent activity of complement proteins in the rat uterus. *Biology of reproduction,* **74(2):** 265-274.

Rhen, T., Grissom, S., Afshari, C. & Cidlowski, J.A. (2003). Dexamethasone blocks the rapid biological effects of 17β-estradiol in the rat uterus without antagonizing its global genomic actions. *FASEB Journal,* **17(13):** 1849-1870.

Richards, N.S., Chipps, S.R. & Brown, M.L. (2007). Stress response and avoidance behavior of fishes as influenced by high-frequency strobe lights. *North American Journal of Fisheries Management,* **27(4):** 1310-1315.

Roberts, P.H. & Thomas, K.V. (2006). The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment. *Science of the Total Environment,* **356(1-3):** 143-153.

Rotchell, J.M. & Ostrander, G.K. (2003). Molecular markers of endocrine disruption in aquatic organisms. *Journal of Toxicology and Environmental Health - Part B: Critical Reviews,* **6(5):** 453-495.

Routledge, E.J. & Sumpter, J.P. (1996). Oestrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environmental Toxicology and Chemistry,* **15(3):** 241-248.

Routledge, E.J., Sheahan, D., Desbrow, C., Brighty, G.C., Waldock, M. & Sumpter, J.P. (1998). Identification of oestrogenic chemicals in STW effluent. 2. *In vivo* responses in trout and roach. *Environmental Science and Technology,* **32(11):** 1559-1565.

Rowney, N.C., Johnson, A.C. & Williams, R.J. (2009). Cytotoxic drugs in drinking water: A prediction and risk assessment exercise for the Thames catchment in the United Kingdom. *Environmental Toxicology and Chemistry,* **28(12):** 2733-2743.

Roy, R.L., Ruby, S.M., Idler, D.R. & So, Y. (1990). Plasma vitellogenin levels in pre-spawning rainbow trout, *Oncorhynchus mykiss*, during acid exposure. *Archives of Environmental Contamination and Toxicology,* **19(6):** 803-806.

Runnalls, T.J. (2005). Pharmaceuticals in the environment: effects of Clofibric acid on fish. PhD thesis submitted to Brunel University, UK. p230

Runnalls, T.J., Hala, D.N. & Sumpter, J.P. (2007). Preliminary studies into the effects of the human pharmaceutical Clofibric acid on sperm parameters in adult Fathead minnow. *Aquatic Toxicology,* **84(1):** 111-118.

Runnalls, T.J., Margiotta-Casaluci, L., Kugathas, S. & Sumpter, J.P. (2010). Pharmaceuticals in the aquatic environment: Steroids and anti-steroids as high priorities for research. *Human and Ecological Risk Assessment,* **16(6):** 1318-1338.

Rupprecht, R., Reul, J.M.H.M., Van Steensel, B., Spengler, D., Soder, M.I., Berning, B., Holsboer, F. & Damm, K. (1993). Pharmacological and functional characterization of human mineralocorticoid and glucocorticoid receptor ligands. *European Journal of Pharmacology - Molecular Pharmacology Section,* **247(2):** 145-154.

Rutkowski, S. (2001). Oral corticosteroids-friend or foe? Taking a closer look at an often poorly understood medication. *Asthma Magazine,* **6(4):** 10-12.

Ruyter, B., Andersen, O., Dehli, A., Östlund Farrants, A., Gjøen, T. & Thomassen, M.S. (1997). Peroxisome proliferator activated receptors in Atlantic salmon (Salmo salar): Effects on PPAR transcription and acyl-CoA oxidase activity in hepatocytes by peroxisome proliferators and fatty acids. *Biochimica et Biophysica Acta - Lipids and Lipid Metabolism,* **1348(3):** 331-338.

Sahlin, L. (1995). Dexamethasone attenuates the estradiol-induced increase of IGF-I mRNA in the rat uterus. *Journal of Steroid Biochemistry and Molecular Biology,* **55(1):** 9-15.

Salassa, R.M., Bennett, W.A., Keating Jr., F.R. & Sprague, R.G. (1953). Postoperative adrenal cortical insufficiency; occurrence in patients. *Journal of the American Medical Association,* **152(16):** 1509-1515.

Sanderson, H., Johnson, D.J., Reitsma, T., Brain, R.A., Wilson, C.J. & Solomon, K.R. (2004). Ranking and prioritization of environmental risks of pharmaceuticals in surface waters. *Regulatory Toxicology and Pharmacology,* **39(2):** 158-183.

Santos, E.M., Paull, G.C., Van Look, K.J.W., Workman, V.L., Holt, W.V., van Aerle, R., Kille, P. & Tyler, C.R. (2007). Gonadal transcriptome responses and physiological consequences of exposure to ooestrogen in breeding zebrafish (Danio rerio). *Aquatic Toxicology,* **83(2):** 134-142.

Sapolsky, R.M., Romero, L.M. & Munck, A.U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine reviews,* **21(1):** 55-89.

Sarropoulou, E., Kotoulas, G., Power, D.M. & Geisler, R. (2005). Gene expression profiling of gilthead sea bream during early development and detection of stress-related genes by the application of cDNA microarray technology. *Physiological Genomics,* **23(2):** 182-191.

Sathiyaa, R. & Vijayan, M.M. (2003). Autoregulation of glucocorticoid receptor by cortisol in rainbow trout hepatocytes. *American Journal of Physiology - Cell Physiology,* **284(6 53-6):** C1508-C1515.

Schäcke, H., Döcke, W.-. & Asadullah, K. (2002). Mechanisms involved in the side effects of glucocorticoids. *Pharmacology and Therapeutics,* **96(1):** 23-43.

Scholz, S. & Mayer, I. (2008). Molecular biomarkers of endocrine disruption in small model fish. *Molecular and cellular endocrinology,* **293(1-2):** 57-70.

Schowanek, D., Fox, K., Holt, M., Schroeder, F.R., Koch, V., Cassani, G., Matthies, M., Boeije, G., Vanrolleghem, P., Young, A., Morris, G., Gandolfi, C. & Feijtel, T.C.J. (2001). GREAT-ER: A new tool for management and risk assessment of chemicals in river basins contribution to GREAT-ER #10.*Water Science and Technology,* **43(2):**179-185.

Schriks, M., Van Leerdam, J.A., Van Der Linden, S.C., Van Der Burg, B., Van Wezel, A.P. & De Voogt, P. (2010). High-resolution mass spectrometric identification and quantification of glucocorticoid compounds in various wastewaters in the Netherlands. *Environmental Science and Technology,* **44(12):** 4766-4774.

Schultz, M.M., Furlong, E.T., Kolpin, D.W., Werner, S.L., Schoenfuss, H.L., Barber, L.B., Blazer, V.S., Norris, D.O. & Vajda, A.M. (2010). Antidepressant pharmaceuticals in two U.S. effluent-impacted streams: Occurrence and fate in water and sediment and selective uptake in fish neural tissue. *Environmental Science and Technology,* **44(6):** 1918-1925.

Scott, A.P., Pinillos, M.L. & Huertas, M. (2005). The rate of uptake of sex steroids from water by Tinca tinca is influenced by their affinity for sex steroid binding protein in plasma. *Journal of fish biology,* **67(1):** 182-200.

Sebastine, I.M. & Wakeman, R.J. (2003). Consumption and environmental hazards of pharmaceutical substances in the UK. *Process Safety and Environmental Protection: Transactions of the Institution of Chemical Engineers, Part B,* **81(4):** 229-235.

Sebire, M., Allen, Y., Bersuder, P. & Katsiadaki, I. (2008). The model anti-androgen flutamide suppresses the expression of typical male stickleback reproductive behaviour. *Aquatic Toxicology,* **90(1):** 37-47.

Seitz, W., Weber, W.H., Jiang, J.-., Lloyd, B.J., Maier, M., Maier, D. & Schulz, W. (2006). Monitoring of iodinated X-ray contrast media in surface water. *Chemosphere,* **64(8):** 1318-1324.

Seki, M., Fujishima, S., Nozaka, T., Maeda, M. & Kobayashi, K. (2006). Comparison of response to 17β-estradiol and 17β-trenbolone among three small fish species. *Environmental Toxicology and Chemistry,* **25(10):** 2742-2752.

Sharpe, R.L., MacLatchy, D.L., Courtenay, S.C. & Van Der Kraak, G.J. (2004). Effects of a model androgen (methyl testosterone) and a model anti-androgen (cyproterone acetate) on reproductive endocrine endpoints in a short-term adult mummichog (Fundulus heteroclitus) bioassay. *Aquatic Toxicology,* **67(3):** 203-215.

Sherer, J.T. (2006). Pharmaceuticals in the environment. *American Journal of Health-System Pharmacy,* **63(2):** 174-178.

Sheridan, M.A. (1986). Effects of thyroxin, cortisol, growth hormone, and prolactin on lipid metabolism of coho salmon, Oncorhynchus kisutch, during smoltification. *General and comparative endocrinology,* **64(2):** 220-238.

Siebe, H., Baude, G., Lichtenstein, I., Wang, D., Buhler, H., Hoyer, G.-. & Hierholzer, K. (1993). Metabolism of dexamethasone: Sites and activity in mammalian tissues. *Renal physiology and biochemistry,* **16(1-2):** 79-88.

Singer, T.D., Raptis, S., Sathiyaa, R., Nichols, J.W., Playle, R.C. & Vijayan, M.M. (2007). Tissue-specific modulation of glucocorticoid receptor expression in response to salinity acclimation in rainbow trout. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology,* **146(2):** 271-278.

Smith, B.S. (1981). Tributyltin compounds induce male characteristics on female mud snails Nassarius obsoletus = Ilyanassa obsoleta. *Journal of Applied Toxicology,* **1(3):** 141-144.

Smith, R.J.F. (1974). Effects of 17α methyltestosterone on the dorsal pad and tubercles of fathead minnows (*Pimephales promelas*). *Canadian journal of zoology,* **52(8):** 1031-1038.

Snape, J.R., Maund, S.J., Pickford, D.B. & Hutchinson, T.H. (2004). Ecotoxicogenomics: The challenge of integrating genomics into aquatic and terrestrial ecotoxicology. *Aquatic Toxicology,* **67(2):** 143-154.

Sonneveld, E., Jansen, H.J., Riteco, J.A.C., Brouwer, A. & van der Burg, B. (2005). Development of androgen- and oestrogen-responsive bioassays members of a panel of human cell line-based highly selective steroid-responsive bioassays. *Toxicological Sciences,* **83(1):** 136-148.

Sparagana, M., Kucera, L., Phillips, G. & Hoffmann, C. (1970). Automated determination of urinary corticosteroids. *Steroids,* **15(3):** 353-371.

Sperry, T.S. & Thomas, P. (1999). Characterization of two nuclear androgen receptors in Atlantic croaker: Comparison of their biochemical properties and binding specificities. *Endocrinology,* **140(4):** 1602-1611.

Spika, I., Hammer, S., Kleuser, B., Korting, H.C. & Schäfer-Korting, M. (2003). Transcriptional activity of potent glucocorticoids: Relevance of glucocorticoid receptor isoforms and drug metabolites. *Skin pharmacology and applied skin physiology,* **16(3):** 143-150.

Stolte, E.H., de Mazon, A.F., Leon-Koosterziel, K.M., Jesiak, M., Bury, N.R., Sturm, A., Savelkoul, H.F.J., van Kemenade, B.M.L.V. & Flik, G. (2008). Corticosteroid receptors involved in stress regulation in common carp, *Cyprinus carpio*. *Journal of Endocrinology,* **198(2):** 403-417.

Stolte, E.H., Verburg van Kemenade, B.M.L., Savelkoul, H.F.J. & Flik, G. (2006). Evolution of glucocorticoid receptors with different glucocorticoid sensitivity. *Journal of Endocrinology,* **190(1):** 17-28.

Stouthart, X.J.H.X., Huijbregts, M.A.J., Balm, P.H.M., Lock, R.A.C. & Wendelaar Bonga, S.E. (1998). Endocrine stress response and abnormal development in carp (*Cyprinus carpio*) larvae after exposure of the embryos to PCB 126. *Fish Physiology and Biochemistry,* **18(4):** 321-329.

Struijs, J. (1996). *SimpleTreat 3.0: A Model to Predict the Distribution and Elimination of Chemicals by Sewage Treatment Plants*. National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, the Netherlands. Report no. 719101025

Sturm, A., Bury, N., Dengreville, L., Fagart, J., Flouriot, G., Rafestin-Oblin, M.E. & Prunet, P. (2005). 11-Deoxycorticosterone is a potent agonist of the rainbow trout (*Oncorhynchus mykiss*) mineralocorticoid receptor. *Endocrinology,* **146(1):** 47-55.

Sturm, A., Colliar, L., Leaver, M.J. & Bury, N.R. (2011). Molecular determinants of hormone sensitivity in rainbow trout glucocorticoid receptors 1 and 2. *Molecular and cellular endocrinology,* **333(2):** 181-189.

Sumpter, J.P. & Jobling, S. (1995). Vitellogenesis as a biomarker for oestrogenic contamination of the aquatic environment. *Environmental health perspectives,* **103(SUPPL. 7):** 173-178.

Sumpter, J.P. & Johnson, A.C. (2005). Lessons from endocrine disruption and their application to other issues concerning trace organics in the aquatic environment. *Environmental Science and Technology,* **39(12):** 4321-4332.

Sumpter, J.P. & Johnson, A.C. (2008). 10th Anniversary Perspective: Reflections on endocrine disruption in the aquatic environment: From known knowns to unknown unknowns (and many things in between). *Journal of Environmental Monitoring,* **10(12):** 1476-1485.

Sumpter, J.P. (2005). Endocrine disrupters in the aquatic environment: An overview. *Acta Hydrochimica et Hydrobiologica,* **33(1):** 9-16.

Sumpter, J.P. (2007). Environmental effects of human pharmaceuticals. *Drug information journal,* **41(2):** 143-147.

Sumpter, J.P., Johnson, A.C., Williams, R.J., Kortenkamp, A. & Scholze, M. (2006). Modeling effects of mixtures of endocrine disrupting chemicals at the river catchment scale. *Environmental Science and Technology,* **40(17):** 5478-5489.

Sun, L., Zha, J., Spear, P.A. & Wang, Z. (2007). Toxicity of the aromatase inhibitor letrozole to Japanese medaka (*Oryzias latipes*) eggs, larvae and breeding adults. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology,* **145(4):** 533-541.

Svenson, A. & Allard, A.-. (2004). Occurrence and Some Properties of the Androgenic Activity in Municipal Sewage Effluents. *Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering,* **39(3):** 693-701.

Tagawa, M., Hagiwara, H., Takemura, A., Hirose, S. & Hirano, T. (1997). Partial cloning of the hormone-binding domain of the cortisol receptor in tilapia, *Oreochromis mossambicus*, and

chages in the mRNA level during embryonic development. *General and comparative endocrinology,* **108(1):** 132-140.

Teitsma, C., Lethimonier, C., Tujague, M., Anglade, I., Saligaut, D., Bailhache, T., Pakdel, F., Kah, O. & Ducouret, B. (1998). Identification of potential sites of cortisol actions on the reproductive axis in rainbow trout. *Comparative Biochemistry and Physiology - C Pharmacology Toxicology and Endocrinology,* **119(3):** 243-249.

Ternes, T. (2001). Pharmaceuticals and metabolites as contaminants of the aquatic environment. *ACS Symposium Series,* **791:**39-54.

Ternes, T.A. (1998). Occurrence of drugs in German sewage treatment plants and rivers. *Water research,* **32(11):** 3245-3260.

Ternes, T.A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R.-. & Servos, M. (1999). Behavior and occurrence of oestrogens in municipal sewage treatment plants - I. Investigations in Germany, Canada and Brazil. *Science of the Total Environment,* **225(1-2):** 81-90.

Terova, G., Gornati, R., Rimoldi, S., Bernardini, G. & Saroglia, M. (2005). Quantification of a glucocorticoid receptor in sea bass (*Dicentrarchus labrax*, L.) reared at high stocking density. *Gene,* **357(2):** 144-151.

Thacker, P.D. (2005). Pharmaceutical data elude researchers. *Environmental science & technology,* **39(9):** 193-194.

Thomas, K.V. & Hilton, M.J. (2004). The occurrence of selected human pharmaceutical compounds in UK estuaries. *Marine pollution bulletin,* **49(5-6):** 436-444.

Thomas, M.B., Thomas, W., Hornstein, T.M. & Hedman, S.C. (1999). Seasonal leukocyte and erythrocyte counts in fathead minnows. *Journal of fish biology,* **54(5):** 1116-1118.

Thornton, J.W. (2001). Evolution of vertebrate steroid receptors from an ancestral oestrogen receptor by ligand exploitation and serial genome expansions. *Proceedings of the National Academy of Sciences of the United States of America,* **98(10):** 5671-5676.

Thornton, J.W., Need, E. & Crews, D. (2003). Resurrecting the ancestral steroid receptor: Ancient origin of oestrogen signaling. *Science,* **301(5640):** 1714-1717.

Thorpe, K.L., Benstead, R., Hutchinson, T.H., Cummings, R.I. & Tyler, C.R. (2003). Reproductive effects of exposure to oestrone in the fathead minnow. *Fish Physiology and Biochemistry,* **28(1-4):** 451-452.

Thorpe, K.L., Cummings, R.I., Hutchinson, T.H., Scholze, M., Brighty, G., Sumpter, J.P. & Tyler, C.R. (2003a). Relative potencies and combination effects of steroidal oestrogens in fish. *Environmental Science and Technology,* **37(6):** 1142-1149.

Tisler, T. & Kozuh Erzen, N. (2006). Abamectin in the aquatic environment. *Ecotoxicology,* **15(6):** 495-502.

Todo, T., Ikeuchi, T., Kobayashi, T., Kajiura-Kobayashi, H., Suzuki, K., Yoshikuni, M., Yamauchi, K. & Nagahama, Y. (2000). Characterization of a testicular 17α,20β-dihydroxy-4-pregnen-3-one (a spermiation-inducing steroid in fish) receptor from a teleost, Japanese eel (*Anguilla japonica*). *FEBS letters,* **465(1):** 12-17.

Tokuda, Y., Touhata, K., Kinoshita, M., Toyohara, H., Sakaguchi, M., Yokoyama, Y., Ichikawa, T. & Yamashita, S. (1999). Sequence and expression of a cDNA encoding Japanese flounder glucocorticoid receptor. *Fisheries Science,* **65(3):** 466-471.

Tölgyesi, A., Verebey, Z., Sharma, V.K., Kovacsics, L. & Fekete, J. (2010). Simultaneous determination of corticosteroids, androgens, and progesterone in river water by liquid chromatography-tandem mass spectrometry. *Chemosphere,* **78(8):** 972-979.

Tong, A.Y.C., Peake, B.M. & Braund, R. Disposal practices for unused medications around the world. *Environment international,* **37(1):** 292-298.

Triebskorn, R., Casper, H., Heyd, A., Eikemper, R., Köhler, H.-. & Schwaiger, J. (2004). Toxic effects of the non-steroidal anti-inflammatory drug diclofenac: Part II. Cytological effects in liver, kidney, gills and intestine of rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology,* **68(2):** 151-166.

Tyler, C.R., Filby, A.L., Bickley, L.K., Cumming, R.I., Gibson, R., Labadie, P., Katsu, Y., Liney, K.E., Shears, J.A., Silva-Castro, V., Urushitani, H., Lange, A., Winter, M.J., Iguchi, T. & Hill, E.M. (2009). Environmental health impacts of equine oestrogens derived from hormone replacement therapy. *Environmental Science and Technology,* **43(10):** 3897-3904.

Tyler, C.R., Jobling, S., Sumpter, J.P. (1998). Endocrine disruption in wildlife: A criticalreview of the evidence. *Critical Reviews in Toxicology* **28:** 319-361.

USEPA (2002). Fish short-term reproduction test protocol - Fathead Minnow. Fish Screening Assay Protocol. U.S.EPA, p29.

Van Aggelen, G., Ankley, G.T., Baldwin, W.S., Bearden, D.W., Benson, W.H., Chipman, J.K., Collette, T.W., Craft, J.A., Denslow, N.D., Embry, M.R., Falciani, F., George, S.G., Helbing, C.C., Hoekstra, P.F., Iguchi, T., Kagami, Y., Katsiadaki, I., Kille, P., Liu, L., Lord, P.G., McIntyre, T., O'Neill, A., Osachoff, H., Perkins, E.J., Santos, E.M., Skirrow, R.C., Snape, J.R., Tyler, C.R., Versteeg, D., Viant, M.R., Volz, D.C., Williams, T.D. & Yu, L. (2010). Integrating omic technologies into aquatic ecological risk assessment and environmental monitoring: Hurdles, achievements, and future outlook. *Environmental health perspectives,* **118(1):** 1-5.

Van Der Linden, M.W., Penning-Van Beest, F.J.A., Nijsten, T. & Herings, R.M.C. (2009). Topical corticosteroids and the risk of diabetes mellitus: A nested case-control study in the Netherlands. *Drug Safety,* **32(6):** 527-537.

Van Der Linden, S.C., Heringa, M.B., Man, H., Sonneveld, E., Puijker, L.M., Brouwer, A. & Van Der Burg, B. (2008). Detection of multiple hormonal activities in wastewater effluents and surface water, using a panel of steroid receptor CALUX bioassays. *Environmental Science and Technology,* **42(15):** 5814-5820.

Vane, J.R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature: New biology,* **231(25):** 232-235.

Vannini, C., Domingo, G., Marsoni, M., De Mattia, F., Labra, M., Castiglioni, S. & Bracale, M. (2011). Effects of a complex mixture of therapeutic drugs on unicellular algae Pseudokirchneriella subcapitata. *Aquatic Toxicology,* **101(2):** 459-465.

Vijayan, M.M., Mommsen, T.P., Glémet, H.C. & Moon, T.W. (1996). Metabolic effects of cortisol treatment in a marine teleost, the sea raven. *Journal of Experimental Biology,* **199(7):** 1509-1514.

Vijayan, M.M., Raptis, S. & Sathiyaa, R. (2003). Cortisol treatment affects glucocorticoid receptor and glucocorticoid-responsive genes in the liver of rainbow trout. *General and comparative endocrinology,* **132(2):** 256-263.

Vizzini, A., Vazzana, M., Cammarata, M. & Parrinello, N. (2007). Peritoneal cavity phagocytes from the teleost sea bass express a glucocorticoid receptor (cloned and sequenced) involved in genomic modulation of the in vitro chemiluminescence response to zymosan. *General and comparative endocrinology,* **150(1):** 114-123.

Vulliet, E., Baugros, J., Flament-Waton, M. & Grenier-Loustalot, M. (2007). Analytical methods for the determination of selected steroid sex hormones and corticosteriods in wastewater. *Analytical and Bioanalytical Chemistry,* **387(6):** 2143-2151.

Vulliet, E. Cren-Olivé, C. & Grenier-Loustalot, M. (2009). Occurrence of pharmaceuticals and hormones in drinking water treated from surface waters. *Environmental Chemistry Letters,* 1-12.

Watanabe, K.H., Jensen, K.M., Orlando, E.F. & Ankley, G.T. (2007). What is normal? A characterization of the values and variability in reproductive endpoints of the fathead minnow, *Pimephales promelas. Comparative Biochemistry and Physiology - C Toxicology and Pharmacology,* **146(3):** 348-356.

Weisbart, M., Chakraborti, P.K., Gallivan, G. & Eales, J.G. (1987). Dynamics of cortisol receptor activity in the gills of the brook trout, *Salvelinus fontinalis*, during seawater adaptation. *General and comparative endocrinology,* **68(3):** 440-448.

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

Werner, J., Ouellet, J.D., Cheng, C.S., Ju, Y.-. & Law, R.D. (2010). Pulp and paper mill effluents induce distinct gene expression changes linked to androgenic and oestrogenic responses in the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry,* **29(2):** 430-439.

Westlake, S. & Cooper, N. (2008). Cancer incidence and mortality: trends in the United Kingdom and constituent countries, 1993 to 2004. *Health statistics quarterly / Office for National Statistics,* **(38):** 33-46.

Williams, R.J., Johnson, A.C., Smith, J.J.L. & Kanda, R. (2003). Steroid oestrogens profiles along river stretches arising from sewage treatment works discharges. *Environmental Science and Technology,* **37(9):** 1744-1750.

Williams, R.J., Keller, V.D.J., Johnson, A.C., Young, A.R., Holmes, M.G.R., Wells, C., Gross-Sorokin, M. & Benstead, R. (2009). A national risk assessment for intersex in fish arising from steroid oestrogens. *Environmental Toxicology and Chemistry,* **28(1):** 220-230.

Williams, T.D., Caunter, J.E., Lillicrap, A.D., Hutchinson, T.H., Gillings, E.G. & Duffell, S. (2007). Evaluation of the reproductive effects of tamoxifen citrate in partial and full life-cycle studies using fathead minnows (*Pimephales promelas*). *Environmental Toxicology and Chemistry,* **26(4):** 695-707.

Winberg, S., Winberg, Y. & Fernald, R.D. (1997). Effect of Social Rank on Brain Monoaminergic Activity in a Cichlid Fish. *Brain, behavior and evolution,* **49(4):** 230-236.

Winter, M.J., Lillicrap, A.D., Caunter, J.E., Schaffner, C., Alder, A.C., Ramil, M., Ternes, T.A., Giltrow, E., Sumpter, J.P. & Hutchinson, T.H. (2008). Defining the chronic impacts of atenolol on embryo-larval development and reproduction in the fathead minnow (*Pimephales promelas*). *Aquatic Toxicology,* **86(3):** 361-369.

Wiseman, S., Osachoff, H., Bassett, E., Malhotra, J., Bruno, J., VanAggelen, G., Mommsen, T.P. & Vijayan, M.M. (2007). Gene expression pattern in the liver during recovery from an acute stressor in rainbow trout. *Comparative Biochemistry and Physiology - Part D: Genomics and Proteomics,* **2(3):** 234-244.

Wolf, J.C., Dietrich, D.R., Friederich, U., Caunter, J. & Brown, A.R. (2004). Qualitative and quantitative histomorphologic assessment of fathead minnow *Pimephales promelas* gonads as an endpoint for evaluating endocrine-active compounds: A pilot methodology study. *Toxicologic pathology,* **32(5):** 600-612.

Woodling, J.D., Lopez, E.M., Maldonado, T.A., Norris, D.O. & Vajda, A.M. (2006). Intersex and other reproductive disruption of fish in wastewater effluent dominated Colorado streams. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology,* **144(1):** 10-15.

Yamaguchi, F. & Brenner, S. (1997). Molecular cloning of 5-hydroxytryptamine (5-HT) type 1 receptor genes from the Japanese puffer fish, Fugu rubripes. *Gene,* **191(2):** 219-223.

Yamaguchi, T., Yoshinaga, N., Yazawa, T., Gen, K. & Kitano, T. (2010). Cortisol is involved in temperature-dependent sex determination in the Japanese flounder. *Endocrinology,* **151(8):** 3900-3908.

Yoon, Y., Ryu, J., Oh, J., Choi, B.-. & Snyder, S.A. (2010). Occurrence of endocrine disrupting compounds, pharmaceuticals, and personal care products in the Han River (Seoul, South Korea). *Science of the Total Environment,* **408(3):** 636-643.

Young, A.R., Grew, R. & Holmes, M.G.R. (2003). Low flows 2000: A national water resources assessment and decision support tool. *Water Science and Technology,* **48(10):**119-126.

Young, G., Thorarensen, H. & Davie, P.S. (1996). 11-Ketotestosterone suppresses interrenal activity in rainbow trout (*Oncorhynchus mykiss*). *General and comparative endocrinology,* **103(3):** 301-307.

Zeilinger, J., Steger-Hartmann, T., Maser, E., Goller, S., Vonk, R. & Länge, R. (2009). Effects of synthetic gestagens on fish reproduction. *Environmental Toxicology and Chemistry,* **28(12):** 2663-2670.

Zheng, D., Feeney, G.P., Kille, P. & Hogstrand, C. (2008). Regulation of ZIP and ZnT zinc transporters in zebrafish gill: Zinc repression of ZIP10 transcription by an intronic MRE cluster. *Physiological Genomics,* **34(2):** 205-214.

Zitko, V. & Hutzinger, O. (1976). Uptake of chloro and bromobiphenyls, hexachloro and hexabromobenzene by fish. *Bulletin of environmental contamination and toxicology,* **16(6):** 665-673.

Zuccato, E., Calamari, D., Natangelo, M. & Fanelli, R. (2000). Presence of therapeutic drugs in the environment. *Lancet,* **355(9217):** 1789-1790.

Zuccato, E., Castiglioni, S., Fanelli, R., Reitano, G., Bagnati, R., Chiabrando, C., Pomati, F., Rossetti, C. & Calamari, D. (2006). Pharmaceuticals in the environment in Italy: Causes, occurrence, effects and control. *Environmental Science and Pollution Research,* **13(1):** 15-21.

Concentrations of human pharmaceuticals in surface waters from different countries.

Pharmaceutical group	Active principal ingredient	Concentration range/ maximum(ng/L)	Country	Reference
Analgesics and non-steroidal anti-inflammatories	Diclofenac	150-1200	Germany	Ternes, 1998
		8-195	UK	Hilton and Thomas, 2003
		60	Croatia	Gros *et al*., 2006
		42-359	Canada	Metcalfe *et al*., 2003
		10-568	UK	Ashton *et al*., 2004
		1-7	S.Korea	Kim *et al*., 2007
		10-120	Sweden	Bendz *et al*., 2005
		1-30	S.Korea	Yoon *et al*., 2010
		219	Spain	Gros *et al*., 2009
		176	Spain	Lopez-Serna *et al*., 2010
	Ibuprofen	70-530	Germany	Ternes, 1998
		8-5044	UK	Ashton *et al*., 2004
		150	Croatia	Gros *et al*., 2006
		8-755	UK	Thomas and Hilton, 2004
		170-2324	UK	Roberts and Thomas, 2006
		4-78	Italy	Calamari *et al*., 2003
		11-38	S.Korea	Kim *et al*., 2007
		80-220	Sweden	Bendz *et al*., 2005
		93-1885	Canada	Metcalfe *et al*., 2003
		61-84	Romania	Moldovan, 2006
		129	Spain	Gros *et al*., 2009
		1-51	S.Korea	Yoon *et al*., 2010
		134	Spain	Lopez-Serna *et al*., 2010
	Mefenamic acid	3	Croatia	Gros *et al*., 2006
		20-196	UK	Thomas and Hilton, 2004
		50-366	UK	Ashton *et al*., 2004
		6	Spain	Lopez-Serna *et al*., 2010
	Dextropropoxyphene	8-682	UK	Ashton *et al*., 2004
		8-80	UK	Thomas and Hilton, 2004
	Naproxen	70-390	Germany	Terns, 1998
		94-551	Canada	Metcalfe *et al*., 2003
		50	Croatia	Gros *et al*., 2006
		68	USA	Boyd *et al*., 2003
		1-18	S.Korea	Kim *et al*., 2003
		90-250	Sweden	Bendz *et al*., 2005
		90	Spain	Gros *et al*., 2009
		5-100	S.Korea	Yoon *et al*., 2010
		67	Spain	Lopez-Serna *et al*., 2010
	Indomethacine	10	Croatia	Gros *et al*., 2006
		30	Spain	Lopez-Serna *et al*., 2010
		37		
	Ketoprofen	17-50	Canada	Metcalfe *et al*., 2003
		0-120	Germany	Ternes, 1998
		3.18	Spain	Lopez-Serna *et al*., 2010

Pharmaceutical group	Active principal ingredient	Concentration range/ maximum(ng/L)	Country	Reference
	Acetaminophen	250 4-73 146	Croatia S.Korea Spain	Gros *et al*., 2006 Kim *et al*., 2007 Lopez-Serna *et al*., 2010
	Aspirin (Salicylic acid)	17000 25-4100 28-37 333	Canada Germany Romania Spain	Brun *et al*., 2006 Ternes. 1998 Moldovan, 2006 Lopez-Serna *et al*., 2010
	Hydrocodeine	1-3	S.Korea	Kim *et al*., 2007
	Phenazone	24-950 40	Germany Spain	Ternes, 1998 Lopez-Serna *et al*., 2010
	Codeine	109	Spain	Lopez-Serna *et al*., 2010
Antibiotics	Erythromycin	1000 4-70 30 24-1842 10-1022 1-16 4 174	UK UK Croatia UK UK Italy S.Korea Spain	Hilton and Thomas, 2003 Roberts and Thomas, 2006 Gros *et al*., 2006 Senta *et al*., 2005 Aston *et al*, 2004 Calamari *et al*., 2003 Kim *et al*., 2007 Lopez-Serna *et al*., 2010
	Trimethoprim	9-194 4-569 4-19 20 10-42 3-5 10-20 1-17 33	Canada UK UK Croatia UK S.Korea Sweden S.Korea Spain	Metcalfe *et al*., 2003 Thomas and Hilton, 2004 Roberts and Thomas, 2006 Gros *et al*., 2006 Ashton *et al*., 2004 Kim *et al*., 2007 Bendz *et al*., 2005 Yoon *et al*., 2010 Lopez-Serna *et al*., 2010
	Sulphamethoxazole	50-132 1-36 1-61 78	UK S.Korea Germany Spain	Ashton *et al*., 2004 Yoon *et al*., 2010 Ternes, 1998 Lopez-Serna *et al*., 2010
	Sulphadiazene	13	Spain	Lopez-Serna *et al*., 2010
	Sulphamethazine	112	Spain	Lopez-Serna *et al*., 2010
	Lincomycin	80 3-248	Italy Italy	Castiglioni *et al*., 2004 Calamari *et al*., 2003
	Azythromycin	20 71	Croatia Spain	Gros *et al*., 2006 Lopez-Serna *et al*., 2010
	Ciprofloxacin	14-26 1-25 109 13	Italy Finland Spain Spain	Calamari *et al*., 2003 Vieno *et al*., 2006 Gros *et al*., 2009 Lopez-Serna *et al*., 2010
	Ofloxacin	75	Spain	Lopez-Serna *et al*., 2010
	Enrofloxacin	40	Spain	Lopez-Serna *et al*., 2010
	Norfloxacine	15	Spain	Lopez-Serna *et al*., 2010
	Clarithromycin	1-21 88	Italy Spain	Calamari *et al*., 2003 Lopez-Serna *et al*., 2010

Pharmaceutical group	Active principal ingredient	Concentration range/maximum(ng/L)	Country	Reference
	Spiramycin	43-74	Italy	Calamari *et al*., 2003
		68	Spain	Lopez-Serna *et al*., 2010
	Oxyteracycline	14-19	Italy	Calamari *et al*., 2003
	Clotrimazole	6-34	UK	Roberts and Thomas, 2006
	Tetracycline	29	Spain	Lopez-Serna *et al*., 2010
	Metronidazole	44	Spain	Lopez-Serna *et al*., 2010
β-blockers	Atenolol	27	Spain	Gros *et al*., 2009
		250	Croatia	Gros *et al*., 2006
		3-241	Italy	Calamari *et al*., 2003
		10-60	Sweden	Bendz *et al*., 2005
		2-150	S.Korea	Yoon *et al*., 2010
		11-25	Finland	Vieno *et al*., 2006
		38	Spain	Lopez-Serna *et al*., 2010
	Metroprolol	45-2200	Germany	Ternes, 1998
		3-116	Finland	Vieno *et al*., 2007
		60-70	Sweden	Bendz *et al*., 2005
		327	Spain	Lopez-Serna *et al*., 2010
	Sotalol	70	Croatia	Gros *et al*., 2006
		3-52	Finland	Vieno *et al*., 2006
		44	Spain	Lopez-Serna *et al*., 2010
	Acebutolol	1-8	Finland	Vieno *et al*., 2006
	Propranolol	12-590	Germany	Ternes, 1998
		10-215	UK	Ashton *et al*., 2004
		4-56	UK	Thomas and Hilton, 2004
		10	Sweden	Bendz *et al*., 2005
		35-107	UK	Roberts and Thomas, 2006
		14	Spain	Lopez-Serna *et al*., 2010
Lipid regulators	Bezafibrate	350-3100	Germany	Ternes, 1998
		10	Croatia	Gros *et al*., 2006
		1-57	Italy	Calamari t al., 2003
		65-259	Canada	Metcalfe *et al*., 2003
		67	Spain	Lopez-Serna *et al*., 2010
	Gemfibrozil	52-510	Germany	Ternes, 1998
		60	Croatia	Gros *et al*., 2006
		67-1493	Canada	Metcalfe *et al*., 2003
		1-9	S.Korea	Kim *et al*., 2007
		1-170	Sweden	Bendz *et al*., 2005
		1-17	S.Korea	Yoon *et al*., 2010
		2.14	Sapin	Lopez-Serna *et al*., 2010
	Astrovastatin	19-44	Canada	Metcalfe *et al*., 2003
		1-5	S.Korea	Yoon *et al*., 2010
		2.39	Sapin	Lopez-Serna *et al*., 2010
	Fenofibrate	45-280	Germany	Ternes, 1998
		82	Spain	Lopez-Serna *et al*., 2010

Pharmaceutical group	Active principal ingredient	Concentration range/ maximum(ng/L)	Country	Reference
	Clofibric acid	66-550	Germany	Ternes, 1998
		20	Croatia	Gros *et al.*, 2006
		20-111	UK	Thomas and Hilton, 2004
		1-6	Italy	Calamari *et al.*, 2003
		15-177	Canada	Metclafe *et al.*, 2003
		24	Spain	Lopez-Serna *et al.*, 2010
Psychiatric drugs	Fluoxetine	0.5-1.5	S.Korea	Yoon *et al.*, 2010
		50-99	Canada	Metcalfe *et al.*, 2003
	Carbamazepine	250-1100	Germany	Ternes, 1998
		110	Croatia	Gros *et al.*, 2006
		4-61	S.Korea	Kim *et al.*, 2007
		100-500	Sweden	Bendz *et al.*, 2005
		1-66	Finland	Vieno *et al.*, 2006
		20-650	Canada	Metcalfe *et al.*, 2003
		65-75	Romania	Moldovan, 2006
		8-68	S.Korea	Yoon *et al.*, 2010
		58	Spain	Lopez-Serna *et al.*, 2010
	Diazepam	27-33	Romania	Moldovan, 2006
		6.5	Spain	Lopez-Serna *et al.*, 2010
	Lorazepam	41	Spain	Lopez-Serna *et al.*, 2010
Anti-histamine	Loratidine	20	Croatia	Gros *et al.*, 2006
		3	Spain	Lopez-Serna *et al.*, 2010
	Ranitidine	10	Croatia	Gros *et al.*, 2006
		1-38	Italy	Calamari *et al.*, 2003
		61	Spain	Lopez-Serna *et al.*, 2010
	Cetirizine	9	Finland	Kosonen & Kronberg, 2009
	Fexofenadine	11	Finland	Kosonen & Kronberg, 2009
Anti-cancer drug	Tamoxifen	10	UK	Ashton., 2004
		4-71	UK	Thomas and Hilton., 2004
		27-212	UK	Roberts and Thomas, 2006
	Bleomycin	5-17	UK	Aherne *et al.*, 1990
	Cyclophosphamide	0.05-0.17	Switzerland	Buerge *et al.*, 2006
		2-10	Italy	Zuccato *et al.*, 2000
Diuretic	Furosemide	2-255	Italy	Calamari *et al.*, 2003
		168	Spain	Gros *et al.*, 2009
	Hydrochlorothiazide	4-255	Italy	Calamari *et al.*, 2003
		106	Spain	Gros *et al.*, 2009
X-ray contrast media	Iopromide	20-361	S.Korea	Kim *et al.*, 2007
		33-1800	S.Korea	Yoon *et al.*, 2010
		100-220	Germany	Seitz *et al.*, 2006
	Diatrizoic acid	155-580	Germany	Seitz *et al.*, 2006
	Iomeprol	100-480	Germany	Seitz *et al.*, 2006
	Iohexol	86-360	Germany	Seitz *et al.*, 2006
	Iopamidol	210-500	Germany	Seitz *et al.*, 2006

Appendix 2.

Alignment of PEPCK gene sequences of three fish species using ClustalW software

Green highlight area indicates areas of similarity. Red and blue highlights indicate the primer
sequence designed by eye.

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Danio sp            1 --------ca--tcatcatcatc-accacagactgatgaagagc------tgctaaaccttcactc-aaggctc
Onchorhynchus sp    1 gaaagcagttcttcctcatagaccaatcagatatgagttgtagttggtgctaaagggcacacgacgctc

Danio sp           57 tctccctctctctctctctcggtcagcatctctccatccctccgctcatcatgcctcctcagctgcagtc
Onchorhynchus sp   70 ttattctctctctctatatatattt---attttttcagacttttat---tttcattaccccatttt—agtattt

Danio sp          127 tcaggaccggtcatgcccgc------------------------gggtcctgcagggcgatctg----gcgtc
Onchorhynchus sp  140 ttacaagatgtcgtgccttttgcttggacttatcagaagacggggtggagtggggacatccgtgggcgtc

Danio sp          172 t-----ctgagc----------------------gccagcg-tgcgggagttcatcgacagcagtgtgagtctgt
Onchorhynchus sp  210 cgctccttagcctcgatcccctccctgc  cgccagcggtggctgactttgtgaagagggccgtggatgagt

Danio sp          219 gccagccggacgctctgcacatctgtgacggctc-cgagcaggagaacagcaccatcctcagcctgc-tg
Onchorhynchus sp  280 gcaagcctgccaatgtgcatgt-ggtgacggggagcgcggaggagtccgctcacatcct-agctggcctg

Danio sp          287 gaggagcagggcgccatcaagaggctgcgcaagtacagcaactgctggctggcgcgcactgacccacgtg
Cyprinus sp         1 ---------------------------------tatgataactgctggctggcacgtactgacccccaaag
Onchorhynchus sp  348 gagaaagacggcatggtgaagaggctacccaagtatgagaactgctggctggcacgtacagacccccaagg

Danio sp          357 atgtggcccgtgtgggagagtaaaacggtgatcgtgacggcggagcagcgggacacggttcccacaccgac
Cyprinus sp        38 atgtggctcgtgtgggagagtaaaactgtgattgtcactaaggaccaaagagacactattcccattcccac
Onchorhynchus sp  418 acgtggctcggggtggagagtaagactgtgatcgtcaccaagaaccagaggggacaccatccctatcccga

Danio sp          427 tggaggaggagtcagtcagctggccgctggatgtgtcctgaggagtgggacaaagccatgaacctgcgc
Cyprinus sp       108 cggaggtgccaagtcccagctgggcagctggatgagtgaggaaccctttcagaaagccagagaggaccgc
Onchorhynchus sp  488 tgggggggctaagagccagctgggcagctggatgagtgagggtgacttccagaaggccagaaggaccgc

Danio sp          497 ttccctggctgcatgaaaggggcgtgtgatgtacgtgatcccctt cagcatggggccggtgggctctccgc
Cyprinus sp       178 tttcctggctgcatggcgggacgcactatgtatgtgatcccccttcagtatgggccctgtgaactcttctc
Onchorhynchus sp  558 ttcccaggctgcatgtcaggtcgaaccatgtatgtgatcccccttcagcatggggcccggtgggctctccgc

Danio sp          567 tctccaagatagggtggagctgacggactcgccgtatgtggtggccagcatgcggatcatgacgcggat
Cyprinus sp       248 ttgctaagtttggtgttcaggtgacagattctccatatgtggtggctagcatgggcatcatgacacgcat
Onchorhynchus sp  628 tgtctaagtttggcgtgcaggtgacagactcaccctacgtggtggccagtatgggcattatgacgcgcat
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Danio sp            637  gggggaaaactgtgctgagcgcgctgggaaacgg---agagttcgtccggtgtctgcactcagtcggctgc
Cyprinus sp         318  ggggactcctgtgctggaaaaactagccgagggtgcggagtttgtgcgctgccagcactcttgggcaga
Onchorhynchus sp    698  gggcacccccgtcatggacaaactggcacaggggcagagtttgtacgctgccagcactccctcggtcgg

Danio sp            704  cctctgccactcaagaagccgctggtcaacaactggccctgtaacccggagctgacgctggtggcccaca
Cyprinus sp         388  cctttaccactcaaagctccttagtagacagctggccttgtaacccggacaaggtgttgatctcacatc
Onchorhynchus sp    768  cccctcccactgaaagctcccctggtcaactcgtgggccgtgtaacccagagaaggtgctgatctcccacc

Danio sp            774  tcccggatcagaggaagatcgtgtcgttcggcagcggatacggaggaaactcactgctggggaagaagtg
Cyprinus sp         458  ttcctgacaccaga-cagatcctgtccttcggcagcgg-ttacggtggaaactcgctccttggaaagaaatg
Onchorhynchus sp    838  tgccagacaccaggcagatcctgtcgttcggcagtggctacggaggcaactccctgctggggaagaagtg

Danio sp            844  cttcgctctgcgcatcgcatcacgcatcgctaaagag-gagggctggctggcagaacacatgctgattctg
Cyprinus sp         528  ttttgctcttcgtatcgcctcacgcattgccaaagacgaa-----ggctggttggctgaacacatgctgattctg
Onchorhynchus sp    908  cttcgccctgaggatcgcctcgcgcatcgccaaggacgagggctggctggccgaacacatgctgatcctg

Danio sp            914  ggcatcaccaaccoggcgggacagaagaagtatttcgcagcggcgttccccagcgcctgcgggaagacca
Cyprinus sp         598  ggaatcacaaatcctcagggtgtaaaacggtacattgcagcagcgttcccgagcgcttgtgggaaaacta
Onchorhynchus sp    978  ggcatcaccaatcctcagggagtgaagcgctacgtggcggcggcgtttcccagtgcctgtgggaaaacta

Danio sp            984  acctggccatgctgaagccgtcgctgcccggctggaaggtggagtgtgtgggagacgacatcgcatggat
Cyprinus sp         668  acctggccatgatgaaaccatcactgccaggctggacggttgagtgtgtgggcgatgacatcgcctggat
Onchorhynchus sp   1048  acctggccatgatgaagccagcgctgcctggctggactgtggagtgtgtaggagacgacatcgcctggat

Danio sp           1054  gaagttcgacaaagaagggaatctgagagccatcaacccagagaacggcttcttcggtgtggctccgggа
Cyprinus sp         738  gaaatttgacagtcagggtaaactcagagccattaatccagagaatgggtttttttggagtcgccccgggg
Onchorhynchus sp   1118  gaagttcgacagtcagggtaaactcaggggcaatcaacccagagaacggcttttttcggtgtggctcccggc

Danio sp           1124  acctccagcaaaaccaaccccaacgccatgagcaccatcagctgcaacacactcttcaccaacgtggcgg
Cyprinus sp         808  acatccctaaagacaaaaccctcatgccatggcaaccatctccaggaacacagtgttcactaacgtgggag
Onchorhynchus sp   1188  acgtccctgaagaccaaccctcatgccatggcgaccatcgccaaaacactgtgttcaccaatgtgggtg

Danio sp           1194  agagcagtgacggagggggtgttctgggagggcatggatgaggatctgcctgaaggagtgacgctgacgtc
Cyprinus sp         878  agaccagcgatggtggagtttggtgggagggtctgga--accacctg-cacctggaatcaaactcacaga
Onchorhynchus sp   1258  agaccagtgacggagggggtgtggtgggagggactgga--cccccctg-ccgcaggggtctccctgaccga

Danio sp           1264  ctggaagaaccagccctggacaccagaggacggtgaaccgtgtgctcacccgaactcccgcttctgtacg
Cyprinus sp         945  ctggcatggaaaatcctggaagtatggtgattctacactgtgtgctcacccgaactccaggttttgtgcc
Onchorhynchus sp   1325  ctggcacggcaaatcctggaaagcaggagactctggccccgtgtgctcatcccaactccaggttctgtacc

Danio sp           1334  ccggccgctcagtgtcccatcatcgacccgcagtgggagtctcctgaaggcgtccccatcgaggccatca
Cyprinus sp        1015  ccagctggccagtgccccatcatagacccactctgggaaagtgatgagggcgtccccattgatgccattg
Onchorhynchus sp   1395  ccggcggcccagtgccccatcatcgacccccagtgggagagtgacgagggtgtgcccatcgatgccatca

Danio sp           1404  tcttcggcgggcggcgtccgcagggcgtcccgctggtgtacgaggccttcgactgggcgcacggagtgtt
Cyprinus sp        1085  tattggtggaagaagaccagaaggtgtgccttggtgtacgagtcatttaactggcgtcacggtgtgtt
Onchorhynchus sp   1465  tcttcggggggcaggaggccagagggagtccctctggtgtacgagtcgtttaactggcgccacggtgtgtt

Danio sp           1474  tgtagggcgtccatgaggtcagaggccacgcgctgctgaacacaaaggtaaagtgatcatgcatgac
Cyprinus sp        1155  tgtgggtgcagccatgagatctgaatccacagctgctgctgaacataagggtaaagtaatcatgcatgac
Onchorhynchus sp   1535  tgtaggagcctcaatgaggtctgaggccacagcgctgctgagtacaaaggcaaggttatcatgcacgac

Danio sp           1544  ccgttcgccatgcgtccgttcttcggctataacttcggtcagtatctctccactggctgagcatggagc
Cyprinus sp        1225  cccttcgccatgcgtcctttctttggctacaacttcgg------------------------------
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Onchorhynchus sp 1605 cccttcgccatgcgcccccttcttcggctacaacttcggtgactacctagcccactggctgagcatggaga

Danio sp   1614 agcggcccggcgcta----agctgcccaaaatcttccacgtcaactggttcggcaggagctcctcggggcg
Onchorhynchus sp 1675 cccgcaagggcgccacccacctgcccaagatcttccacgtcaactggttccggaaggacccacgtcggg

Danio sp   1681 ct---tcctgtggccgggctttggggagaacatccgcgtactggagtggatgttcg-------gcaggctg
Onchorhynchus sp 1745 ctcttcctctggccgggtttcggtgacaacgcccgcgttctggagtggatcttcaagcgcctgcagccgc

Danio sp   1742 agcggaggggccgaggcg---aggaccactgcggtggggctggtgcctgcagacggtgccctgaacctgc
Onchorhynchus sp 815gagagggaggacgaggcggccaagaagagcatggtgggctgggtgccactggagggagccatcaacctgc

Danio sp   1809 acgggct---cccggatgtggagccgctggagctcttcagggtctcgcaggagttctggatgcaggagct
Onchorhynchus sp 885 agggactgggcagcaaggtggacatgggtgccctcttcgacctgcccaaggccttctgggagaaggagac

Danio sp   1876 gcaggagatcagagagagtattcagccgtgaactgaaccgagacctgccgcaggagatgcagagacagctg
Onchorhynchus sp1955 ccaggagctgagggcgtactttacccagcaggtgggagccgacctccccaacaggtggagggagagctg

Danio sp   1946 gagctgctggagcacaggctcacacacacacacgtgagcagtaaacacggctgaagacacgcaacacaca
Onchorhynchus sp2025 aaggctctggaggacaggatc---------------aggaattgaga--ggtggagggac--------ca

Danio sp   2016 cgttaatctgcattacgcacacttaaagggacggtgcactcgaatattaaaatatgtcatcgtttgctct
Onchorhynchus sp2070 gatgattcag--------------agagagaaagt---attgactattggacattatactacgstg----

Danio sp   2086 caagtgcttcaaaaccgttctttagtttccttctgctgaacacagaagaagatattttgaaaaatgctga
Onchorhynchus sp 2119 ---atgctgctaaatgatatggtagt-------------actgacaaaaattact----aacaagctma

Danio sp   2156 agattgttcacttccacagtatttaattttccctcttttgtttactttttattttgtgtttttttcttt
Onchorhynchus sp 2169 ---------------------------------------tttcaattttgtggaagctgcttt

Danio sp   2226 gtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtttactgagaaaactcatttaaa----gagacaga
Onchorhynchus sp2193 gcctatatgtgggtagatgtaaaccaaagtttctaaattgtaagaaaatacaatgagatgatgtgactgc

Danio sp   2292 acacacacaataatcagcatttatgcacagttaaagggacggtgctctctaaaatgaaaaatatgtcact
Onchorhynchus sp2263 at-cactgaagaat---------------gcagttca------------tgctatc---------acacactgtcagt

Danio sp   2362 atttgtttta--accctgaacacaaagaagatatttagagaaatgctgatgatcattggcgtcttcagg
Onchorhynchus sp2304 ggctatatcagtaggttgtaggtgatggagcagaatcggtgtttagcagtaccttactgacatctt--gc

Danio sp   2430 aaggactgttt----gttatttaaccttataaaattattgatgcgcacatttatttctgctggagatgtta
Onchorhynchus sp 2372 aaaga-agtataaaggtattggatgtt-tacaagcttta---gcccaaataatct---------aatcta

Danio sp   2497 tggcggaaacctttaaacaaccctct-catttttaaatgtggtaatttattttttctatgtattctatgt
Onchorhynchus sp2428 atcaggacaagcttaaatatccatattcatttaaacatttttaaacagcattttttttgtcactttgta

Danio sp   2566 cagtcttccatgatattaataa--agtta---tttaaatataaaaaaaaaaaaaaaaaaaaaaaaaaaaa
Onchorhynchus sp2498 ttgttccaataaaatgattatgagctaatgtttacacatgagatatgtaacatgttggaccaataaaa

Danio sp   2631 aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa--
Onchorhynchus sp 2568 atgatttaatgtcaaggttttfattacagtgcaattaataaacaacctcaaattcaaaaaaaaaaaaaaa

Appendix 3.

List of publications from this research

Papers

Kugathas S and Sumpter JP. (2011). Synthetic glucocorticoids in the environment: First results on their potential impacts on fish. *Environmental Science and Technology*. **45(6)**: 2377-2383.

Runnalls TJ, Margiotta-Casaluci L, **Kugathas S** and Sumpter JP. (2010). Pharmaceuticals in the aquatic environment: steroids and anti-steroids as high priorities for research. *Human and Ecological Risk Assessment* **16(6)**: 1318-1338.

Kugathas S, Williams RJ and Sumpter JP. Prediction of environmental concentrations of glucocorticosteroids: the River Thames, UK, as an example. *Environment International.* (Accepted: EI2304)

Another substantial paper on the exposure study is in preparation to be submitted to *Environmental Science and Technology*.

Abstracts

Kugathas S Runnalls TJ and Sumpter JP. Dose-response effects of chronic exposure of fathead minnows to low concentrations of a synthetic glucocorticoid. Poster presented at the SETAC-Europe conference. Milan, Italy, 16-19 May 2011.

Kugathas S and Sumpter JP. Use of corticosteroid pharmaceuticals in the UK and their *In-vitro* potencies with fish corticosteroid receptors. Poster presented at the SETAC-Europe conference. Seville, Spain, 23-27 May 2010.

Kugathas S and Sumpter JP. *In-vitro* potencies of ten of the most used glucocorticoids in the UK. Platform presentation at the SETAC-UK Young Scientists Conference. Lancaster 2009.