



Effects of pharmaceutical pollutants and
their mixtures on aquatic organisms –
with particular focus on reproduction and
endocrine function in a fish model
species

A Thesis Submitted for the Degree of Doctor of
Philosophy

By

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Declaration

The work presented in this thesis was conducted between 2012 and 2016 at Brunel University London. All work was conducted in line with Home Office regulations (Under the Animals (Scientific Procedures Act) 1986, and ethical standards. The work was conducted independently by the author unless otherwise stated.

Abstract

A number of pharmaceuticals have been shown to have adverse effects on key biological processes of aquatic organisms at low concentrations (ng/l range). Key questions for chemical risk assessment are whether such pharmaceuticals can produce adverse effects on organisms when present in the environment in combination and at low concentrations, whether these can be classified as additive, and under what circumstances can they be predicted. The main purpose of this study was to assess the potential for combination effects of a multicomponent steroid pharmaceutical mixture of dissimilarly acting compounds on an ecologically relevant end point – reproduction, using the existing predictive toxicity models Concentration Addition (CA) and Independent Action (IA). Concentrations of steroids close to those reported in the environment were shown to produce adverse effects on reproduction when present in combination with other steroids. Clear combination effects significantly larger than the effects of the individual compounds were observed when each compound was present at a concentration below the detection limit of the assay, demonstrating a ‘something from nothing’ mixture effect. Furthermore, IA predicted more pronounced effects on egg production than CA, an observation previously unreported from the literature. Actual observed effects were closer to the IA prediction. Additional biomarker and molecular endpoints were examined in subsequent studies to establish the mechanisms of disrupted reproduction in pair-breeding FHMs exposed to the steroid mixture. Results from this study indicate that reproductive impairment of fish exposed to the steroid mixture is likely due to the masculinisation of female fish due to the androgenic activity of the mixture. These results have implications for chemical risk assessment, and in particular, highlight the need for caution when using CA as a worst-case approximation of mixture effects.

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Chapter 1. General Introduction

1.1. Pharmaceuticals in the Environment – An Overview

The presence of pharmaceuticals and active pharmaceutical ingredients (APIs) in the environment has become an emerging issue in recent decades (Heberer, 2002). Despite being first reported in the late 1970's (Jones et al, 2005), until the 1990s they received very little attention as an important environmental contaminant (Ågerstrand et al, 2015), in spite of growing usage worldwide. Pharmaceuticals are used extensively in human and veterinary medicine, agriculture and aquaculture (Dietrich et al, 2002) and it is estimated that there are over 3000 distinct pharmaceutical chemicals in use every day in the UK (Ternes et al, 2004; Sumpter, 2009). An increasing worldwide population and an ageing demographic combined with increasing scientific and technological developments have led to an upward trend in pharmaceutical usage. Due to growing consumption and demand for pharmaceuticals, discharge into the environment is inevitably increasing (Fent et al, 2006; Corcoran et al, 2010). As a consequence of the growing acknowledgement of the risk these contaminants might pose, combined with improvements in analytical chemistry, low levels of human and veterinary pharmaceuticals have become a focus of environmental concern, and as a result of increased research efforts, have been detected in the aquatic environment worldwide (Fent et al, 2006). The issue now is not whether these compounds are present in the environment, but more importantly, whether they pose a risk to aquatic organisms.

1.2. Pharmaceuticals and Active Pharmaceutical Ingredients (APIs)

Pharmaceuticals have a crucial role in treating, curing and preventing human and animal diseases. The discovery and advancement of pharmaceutical medicine has led to dramatic changes in human population health and welfare, and in animal-based industries such as agriculture. Human pharmaceuticals are defined as 'any substance or combination of substances which may be used in or administered to human beings either with a view to restoring, correcting or modifying physiological functions by exerting a

pharmacological, immunological or metabolic action, or to making a medical diagnosis' (EU, 2012; MHRA, 2016). Active Pharmaceutical Ingredients are defined as the active component in a drug, which can also be comprised of many other ingredients that are not biologically active. These are defined as excipients, and are included in many medications in order to aid absorption and enhance solubility.

Essentially, pharmaceuticals and APIs are substances with a known structure that produce a biological effect when administered (Rang et al, 2015). They are designed to initiate a change in the biological functions or processes within the human (or animal) body. They are typically organic molecules containing hydrocarbon groups. The molecular structure of most pharmaceuticals consist of both hydrophobic, or non-polar (water repelling) entities, and hydrophilic, or polar entities (those with an affinity for water). The overall lipophilicity of the substance depends on the proportion of these polar and non-polar entities, and their location within the molecular structure of the compound (Moynihan and Crean, 2009). Although many pharmaceuticals and API's are small organic molecules, some have much larger molecular weights. Some of the more recently-introduced API's are monoclonal antibodies, with molecular weights in the hundreds of thousands g/mol. However, antibody-based API's are typically of low environmental concern, since they are metabolised in human patients to very small compounds, such as peptides and free amino acids. The fundamental properties of an API affect the fate of the compound once it enters in the environment.

1.2.1. History of pharmaceutical usage

Products that cure, treat or alleviate symptoms of human illness and disease have been in use for many centuries. The beginnings of human therapeutics originated from the use of natural products to treat or cure common conditions. The earliest medicinal products came from plants, vines, roots and fungi (Jones, 2011). However over time therapeutic

products became more sophisticated, with the commercialisation of pharmaceuticals occurring in the late 19th century (Hughes et al 2013). With the discovery of chemical synthesis, the first synthetic pharmaceutical, Chloral hydrate, a sedative, was developed in 1869 (Jones, 2011). Since then, a wide range of therapeutic agents have been developed for all manner of diseases and ailments. For a more comprehensive review of the history and development of therapeutics, the reader is referred to the following publications; Katzung, 2006; Jones, 2011.

1.2.2. Pharmacology

Pharmaceuticals are inherently biologically active, designed with a specific molecular mechanism through which they exert a biological effect. In general there are four main drug binding targets; receptors, enzymes, transporters and ion channels, with most drugs acting via one (or more) of these protein types (Rang et al, 2015). In the field of pharmacology, there is often confusion in relation to the use of the term 'receptor'. In general, a pharmacological receptor is defined as 'a cellular macromolecule, or an assembly of macromolecules, concerned specifically with chemical signalling between and within cells' (Neubig et al, 2003). However, the term can denote a number of other meanings, for instance in cellular biology, a receptor can describe specific cell surface molecules such as T-cell receptors that respond to proteins or peptides, rather than signalling molecules (Rang et al, 2015). Within a pharmacological context, a receptor is essentially a molecule (or group of molecules) that responds to a chemical mediator (either endogenous or exogenous). Although most drugs act via specific 'primary' targets, no drug is completely specific. They often interact with secondary 'non-target' receptors, resulting in unwanted side effects. Although the specificity of more recently developed drugs is increasing.

Drugs interact with biological mechanisms through chemical processes, affecting regulatory molecules that inhibit or initiate biological processes in the body to achieve the desired therapeutic effect (Katzung, 2006). Some synthetic pharmaceuticals are designed to mimic endogenous molecules, such as in the case of synthetic steroids. Once administered, the compounds bind to their target receptors in place of the natural ligand. The binding of a molecule, ion or protein (either endogenous or xenobiotic) to a receptor changes the physical structure of the receptor itself, which in turn determines its function (fig. 1). If the pharmaceutical compound is a receptor agonist, the drug will activate the receptor and initiate processes downstream of the site of action, with the biological response varying with respect to the binding affinity and efficiency of the drug itself. If the compound is an antagonist, binding to the target site prevents natural ligands from binding to the receptor via competitive binding and therefore inhibits certain reactions and processes that would be initiated downstream by the action of these natural molecules. Antagonists effectively occupy receptors but do not activate them.

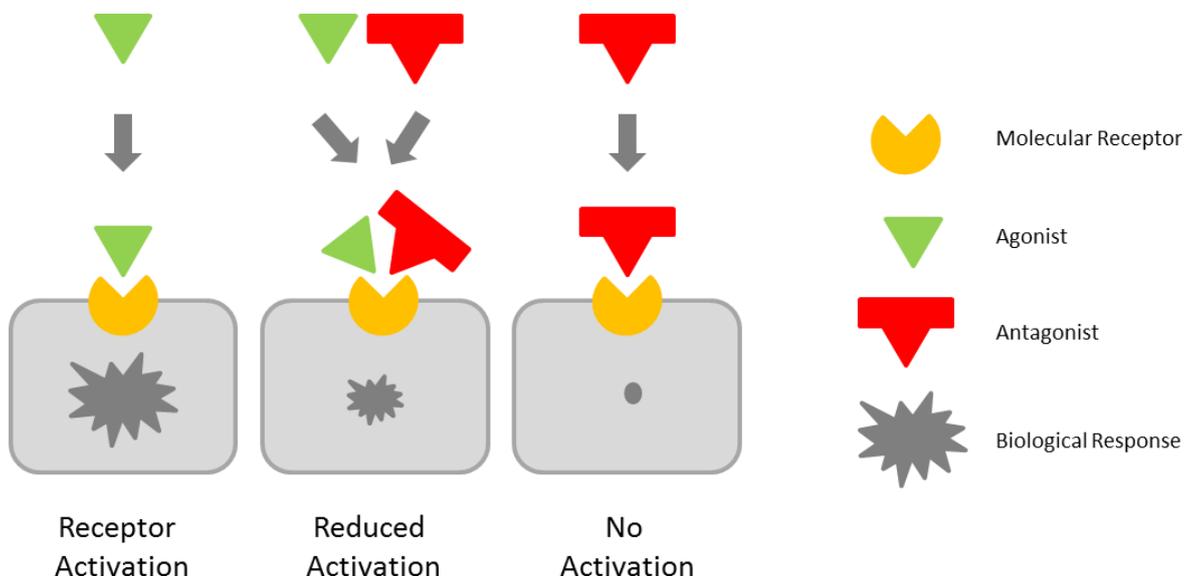


Figure 1. Agonistic and antagonistic behaviour of a drug on a target receptor

Whilst many pharmaceuticals act agonistically, several important drug groups are receptor antagonists, for example the beta blockers. These compounds bind to the beta adrenergic receptors (β_1 , β_2 and/or β_3) to block the catecholamines epinephrine, norepinephrine and dopamine, resulting in a decrease in renin production (a mechanism controlling vasoconstriction) and a subsequent decrease in cardiac output, decreased blood pressure and decreased vascular resistance (Katzung, 2006). However, biological effects of pharmaceuticals are not usually regulated in a simple linear process. Effects are often mediated through complex processes and feedback mechanisms.

Pharmaceuticals as a collective group possess very diverse chemical structures, a necessary requirement for addressing a wide range of biological ailments. Similar compounds can be grouped on the basis of their chemical structures, the compounds pharmacological activity (mode of action) or their physiological class (Williams, 2005). Pharmaceutical products can be administered to patients via different routes; enteral (orally) via tablet capsules or liquids; parenteral (directly into the blood stream) i.e. intravenously; intranasal (through the nose); topical application; inhalation; and rectally (Rang et al, 2015). Once in the human (or animal) body, the drug undergoes several key processes - absorption, distribution, metabolism and elimination (through excretion), collectively known as ADME. These processes are known as the pharmacokinetic properties of the compound, the biological processes of the body acting upon the drug. In addition to the pharmacokinetic properties, pharmaceuticals also exhibit an effect on the body (which they are designed to do), typically (but not always) via a target receptor or enzyme for example. These processes are known as the pharmacodynamic processes. The pharmacokinetics and pharmacodynamics can have important implications for the bioavailability of the drug, both within the human (or animal) body, and, once excreted, in the environment.

Many pharmaceutical groups have now been identified as being of environmental concern, and include some of the more widely used products such as steroid hormones, antianxiety drugs, antibiotics, anti-inflammatories and analgesics (Ågerstrand et al, 2015; Küster and Adler, 2014; Fent et al, 2006). For example, since pre marketing screening began in Germany in accordance with regulatory requirements, around 10% of pharmaceutical products have been identified as having the potential to cause risk to the environment (Küster and Adler, 2014). In addition, a review by the German Federal Environment Agency (UBA) found that 131 different pharmaceuticals and API's had been detected in surface waters around Germany, in concentrations ranging from 0.1-10µg/L (Bergmann et al, 2011; Küster and Adler, 2014; Umwelt Bundesamt, 2014).

1.2.3. Consumption of Pharmaceuticals

Pharmaceutical consumption has been steadily increasing in the UK and worldwide. In 2012/2013, the number of items prescribed in England exceeded 1 billion (HSCIC, 2013; BNF, 2014). Usage quantities vary considerably depending on pharmaceutical class, however the trend appears to be towards increasing usage for most compound groups. The drug group prescribed in the highest quantities in 2013/14 was the lipid regulating pharmaceuticals, such as those used to treat high cholesterol, with over 68 million items prescribed for that year (based on BNF data 2014). Some pharmaceuticals can be purchased 'over the counter', such as the analgesics paracetamol and aspirin, and as a result usage is likely to be much higher than prescription data suggests. However, prescription data does not necessarily suggest environmental risk. There are many other factors in addition to usage trends that determine environmental persistence, such as the nature and structure of the compound, and the uptake potential to organisms prior to eliciting any effect.

The use of pharmaceutical products has been growing since the development of the first synthetic product. Not only are novel drugs continuously being developed to treat a growing number of ailments, but newer versions of existing drugs are being continuously developed and improved, namely to increase specificity and reduce unwanted side effects, and to improve effectiveness. As a specific example, synthetic progestins (oral nortestosterone derivatives) have been in use since the early 1960's. They are used to treat conditions of the reproductive cycle, and to control embryogenesis, pregnancy and the mensural cycle in women. However, early synthetic progestins caused undesirable side effects, largely due to their affinity not only to the progesterone receptor (PR), but also to the androgen receptor (AR). Side effects were typically a result of this secondary mechanism, which led to masculinisation effects, including the growth of facial hair in women taking the drugs. Since the development of norethisterone, the first synthetic progestin used as an oral contraceptive, several more generations of progestins have been developed. Third and fourth generation progestins, such as desogestrel and drospirenone (respectively), are known to be 'purer' progesterone receptor agonists in that they are more similar to the natural ligand progesterone, and possess less androgenic activity. Thus newer progestins are more specific to the progesterone receptor, and therefore cause fewer side effects as a result of unspecific receptor binding. Mechanisms and therapeutic uses of synthetic progestins will be discussed further in chapter 3.

The growth and development of therapeutic agents in treating cancers is another area of continued drug development. Many of the first antineoplastic drugs possessed highly toxic side effects. The first chemotherapy drugs were developed in the 1940's. Studies on military personnel exposed to mustard gas during World War II found that the toxic gases designated for warfare had cytotoxic effects on bone marrow cells, precursors to blood cells (Colvin, 2003). As a result, nitrogen mustards (alkylating agents) were researched and developed for the treatment of cancers of the blood, such as lymphoma. The earliest cytotoxic drug chlormethine (HN2) is no longer in regular use due to high levels of toxicity,

however newer nitrogen mustards such as cyclophosphamide and ifosamide are commonly used in treatment of many cancers (Buerge et al, 2006). The therapeutics of antineoplastic pharmaceuticals will be further discussed in detail in Chapter 2.

1.3. Sources and pathways of Pharmaceuticals and Pharmaceutical Products to the Environment

It has been widely established that pharmaceuticals are present in wastewater effluent and surface waters (Petrie et al, 2013; Jones et al, 2001). Manufactured chemicals and anthropogenic substances enter the aquatic environment both directly and indirectly as a result of human activities. The main sources and pathways of pharmaceuticals and pharmaceutical products to the environment have been theorised, modelled and subsequently measured in monitoring studies around the world. The major pathway of these pollutants to the environment is via sewage treatment plant (STP) effluent (or municipal waste water treatment plants (WWTP) in the USA) after excretion as a result of patient use (Corcoran et al, 2010).

Since pharmaceutical products are in widespread use by human populations, pharmaceutically active compounds can be found in domestic waste, hospital effluents, and agricultural waste (since many veterinary pharmaceuticals are used routinely in agricultural practises) (figure 2.).

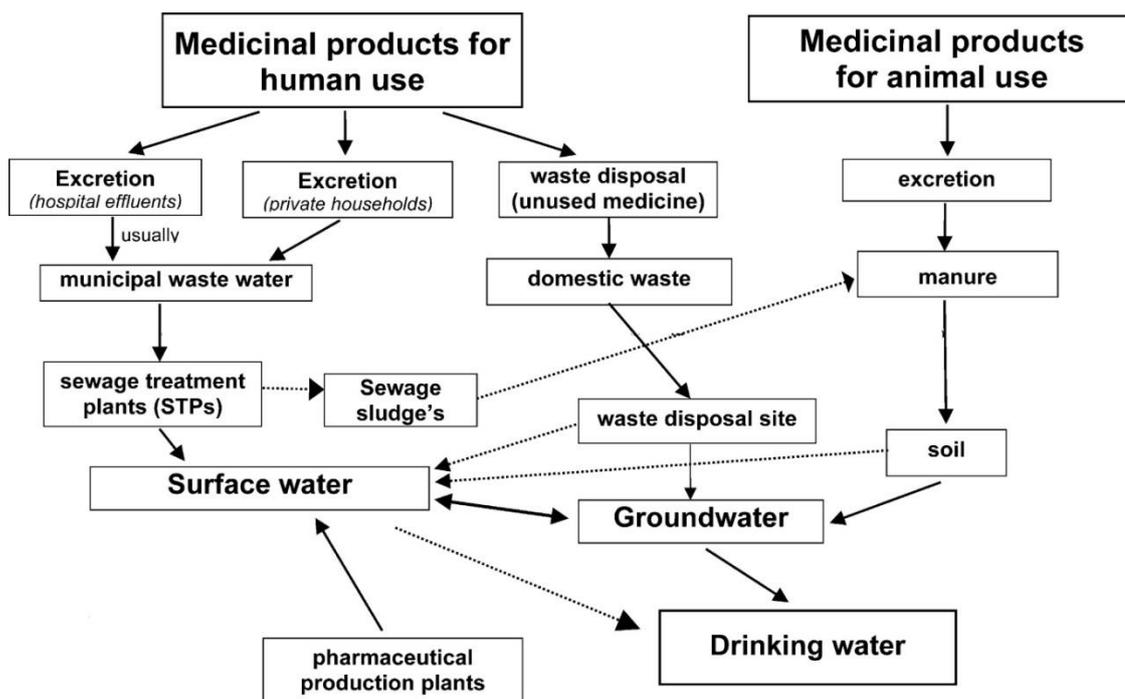


Figure 2. Major sources and pathways of pharmaceuticals and pharmaceutical products to the aquatic environment. Modified from Heberer et al, 2002.

To a lesser extent, input of pharmaceuticals to the environment can result from alternate pathways (fig. 2.). Non-routine events such as accidents or spills, discharge from manufacturing facilities and the supply chain, seepage from septic tanks and sewers (Mceneff et al, 2014), illegal disposal, and improper domestic disposal of unused pharmaceutical products all contribute to the presence of pharmaceuticals in the aquatic environment, although to a much lesser extent than routine human excretion from domestic and hospital waste. In the UK, pharmaceutical manufacturing is strictly controlled and regulated by Good Manufacturing Practise. However in developing countries, such as India, the industry is not well regulated and discharge of manufacturing waste to the environment is common (Larsson and Fick, 2009). API's have been detected in effluents in China (Cui et al, 2006; Li et al, 2008a; Li et al, 2008b; Lin and Tsai, 2009), India (Larsson et al, 2007) and the USA (Phillips et al, 2010) as a result of the disposal of

manufacturing waste. Illegal and unregulated release has been linked to major environmental issues such as widespread antibiotic resistance and pharmaceutical pollution in Asia.

1.3.1. Absorption, Distribution, Metabolism and Elimination (ADME)

In human (and animal) patients, pharmaceuticals and API's undergo biological processes that enable the drug to work effectively, and then to be excreted once the therapeutic function has been achieved. Drug disposition is separated into four main processes: absorption, distribution, metabolism and elimination (ADME) (fig. 3). These processes control how much of the compound is excreted, whether it is excreted in a biologically active form, how much of the excreted product is in the form of metabolites (active and/or inactive), and whether the compounds are in a conjugated form. These factors influence the compounds presence and persistence during waste water treatment and subsequently in effluent.

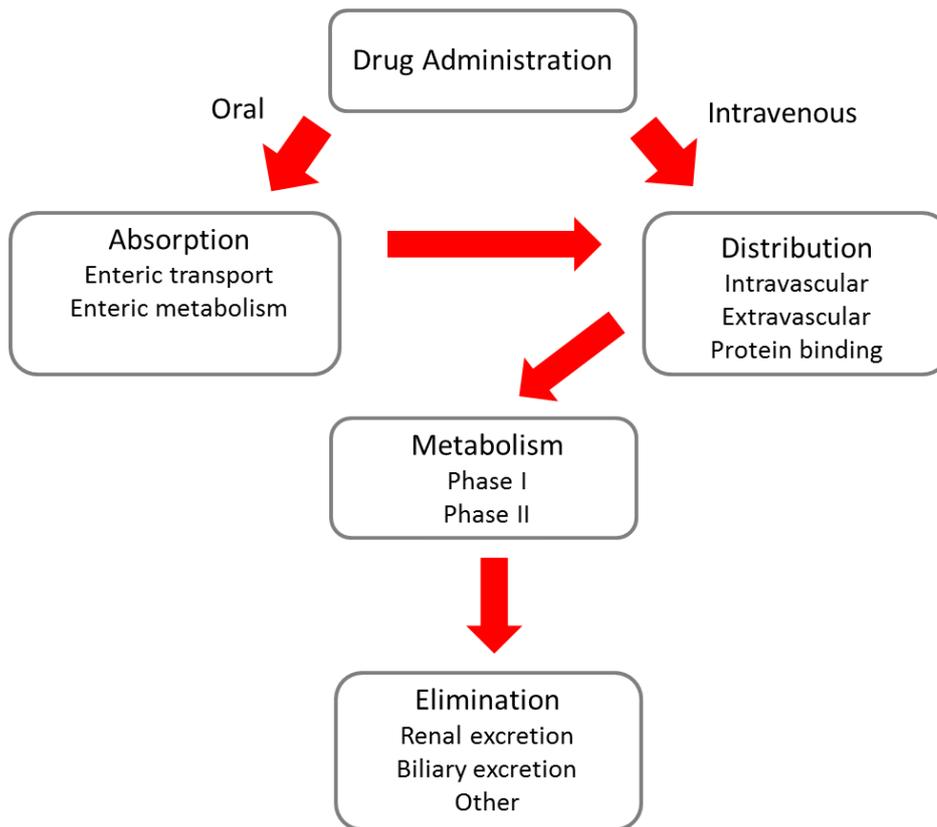


Figure 3. The processes of pharmaceutical disposition in human patients - absorption, distribution, metabolism and elimination (ADME). Modified from Undevia et al, 2005.

After being administered, pharmaceutical molecules are transported around the body in bulk flow (i.e. in the circulatory or lymphatic system), and by diffusion. The rate of diffusion depends on the molecules size and lipophilicity. Enzymatic biotransformation of exogenous compounds takes place in the kidneys, liver, intestines and the lungs, which form the body's main detoxification mechanisms. Enzymatic transformation typically results in the production of polar metabolites and the depletion of pharmacological activity (Wilkinson 2001; Williams 2005). However, some metabolites can also be pharmacologically active, such as clofibric acid, the active metabolite of the lipid lowering drug clofibrate. Furthermore, some pharmaceuticals are administered in the form of a 'prodrug', an inactive compound that requires enzymatic metabolism to become biologically active.

Drug metabolism is separated into two phases, which all compounds typically undergo. Phase I reactions are catabolic and typically take place in the liver. They involve the addition of a new or existing functional group, and include reactions such as oxidation, hydroxylation, reduction, and hydrolysis. Phase II reactions are anabolic, and involve the conjugation of molecules by the addition of acetyl, sulphate, glucuronic acid or other amino acids. Phase II reactions typically increase the polarity of a compound and aid excretion (Williams, 2005; Rang et al, 2015).

Elimination of the parent compound and/or metabolites is through excretion in urine or in bile via the faeces. To a lesser extent, drugs can be eliminated through other means such as via the lungs, perspiration, breast milk, or saliva. Excretion rates and the ratio of parent compound to metabolites during excretion vary depending on the compounds structure and mechanism of action. Some compounds are excreted from the human body entirely unchanged in its parent form. For example, the beta-blocker nadolol is not metabolised by the liver and is excreted in urine completely unchanged. Conversely, only 3% of the parent form of the analgesic carbamazepine is excreted unchanged, with most of the compound being excreted as conjugated and hydroxylated metabolites (Bound and Voulvoulis, 2005; www.drugbank.ca). The pharmacological activity of many compounds is removed by the biological processes discussed above. However, some pharmaceuticals do not completely lose their biological activity. This can be the case for compounds that are applied externally (i.e. topically), or those that are not fully absorbed (Roberts and Thomas, 2006). Furthermore, excreted conjugated metabolites can undergo de-conjugation during sewage treatment processes (Larsson et al, 1999), and it has been hypothesised that de-conjugation could lead to some pharmaceutically inactive molecules becoming once again active (Ternes, 1998; Heberer, 2002).

1.3.2. The Wastewater Treatment Process

Typical wastewater treatment in the UK involves the treatment of waste via the following processes: 1. initial removal of waste from domestic and industrial sources via an extensive sewage system, 2. upon arrival at a STP, screening process and grit removal system designed to remove large items and debris from the sewage influent, 3. primary settlement treatment, where heavier waste particles are removed and deposited at the bottom of sedimentation tanks as sewage sludge, 4. biological treatment of residual wastewater (after sludge removal) by bacterial (aerobic) digestion, e.g. filtration beds or aeration lanes, 5. final settlement treatment in order to remove any remaining particles i.e. in humus tanks, and 6. the return of treated water to rivers (or seas, in the case of overflow systems) (fig. 4). In addition, the solid sludge that is generated during the primary settlement treatment stage undergoes further stabilisation treatment in designated sludge treatment plants. The sludge is screened before being subjected to stabilisation processes (fig. 5) to reduce organic matter and water content, and to destroy harmful microorganisms commonly present in human and animal waste (i.e. *Clostridium* spp., *Salmonella* spp., *Escherichia coli*). This is achieved by either anaerobic digestion, aerobic digestion, lime stabilisation, composting or heat drying (Arthurson, 2008). The bacteria (if utilised) are subsequently killed after digestion and resultant dry solids are distributed to be used as agricultural fertiliser.

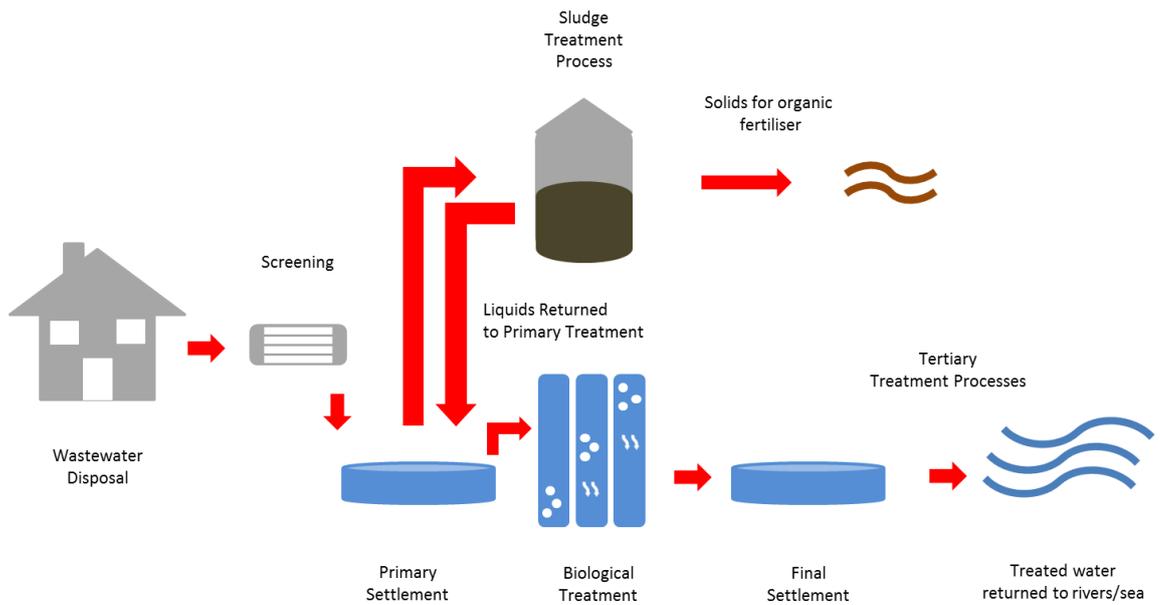


Figure 4. The fundamental principles of the wastewater treatment process in the UK. Modified from Southern Water, UK. <https://www.southernwater.co.uk/the-wastewater-process>

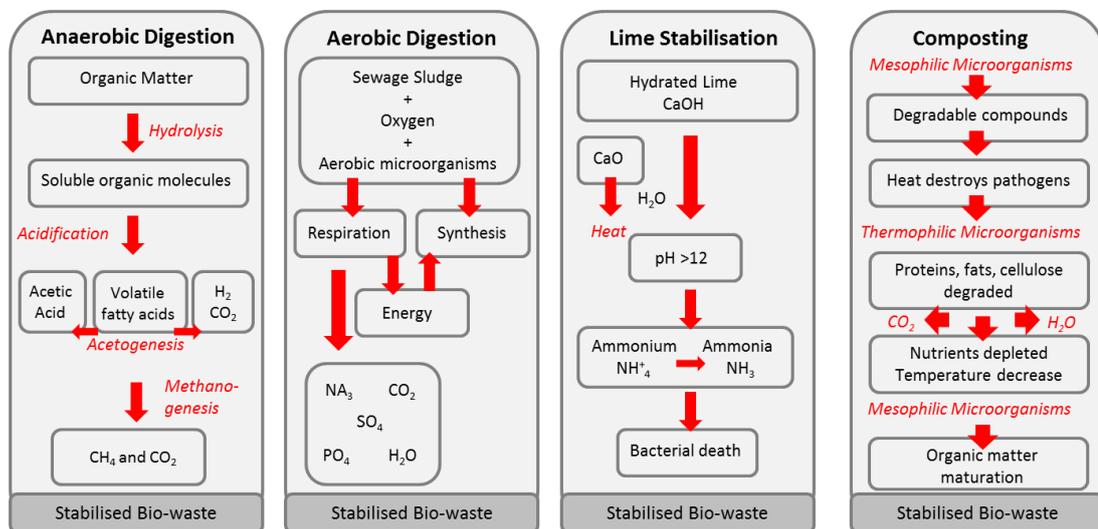


Figure 5. Common sewage sludge stabilisation treatment approaches prior to use in land applications. Adapted and modified from Arthurson, 2008.

There is growing concern that some pharmaceutical products are not effectively removed during this treatment process. Though the majority of APIs are efficiently eliminated (>90% removal rates for many pharmaceutical classes, (Watts et al, 2007)), removal rates of some pharmaceuticals have been calculated and have been found to be largely insufficient. For example, removal rates from wastewater of the drugs carbamazepine and diclofenac are around 8%, and 17% respectively (Heberer, 2002). Although monitoring of ammonia, metals and biochemical oxygen demand is routinely carried out in the UK, at present, water companies have no legal obligation to monitor the presence of any pharmaceutical in treated effluents. Recent changes in EU legislation, particularly with reference to the Water Framework Directive (2000/60/EC), have begun to encourage the inclusion of chemical pollutants in water quality assessments and regulation. Chemicals that are considered potentially harmful to aquatic organisms, or to human health via aquatic systems, are deemed as priority substances and are monitored and regulated at EU level. The recently published Priority Substances Directive (2013/39/EU) provides a revised list of high priority substances for monitoring and regulation, as an amendment of the Water Framework Directive (2000/60/EC) and the Directive on Environmental Quality Standards (2008/105/EC). The amendment, which includes the addition of the pharmaceuticals ethinyl estradiol (EE2) and diclofenac (Annex X, Water Framework Directive, 2000/60/EC (WFD)) also requires stricter Environmental Quality Standards (EQS) in relation to priority substances. In addition, EU member states are required to further identify regional priority substances, deemed 'River Basin Specific Pollutants', and to provide EQS's, monitoring programmes and appropriate regulation for these additional pollutants (JRC Technical Report, 2010). Initiatives from the UKWIR's Chemical Investigation Programme and the UK Technical Advisory Group (UKTAG) have already begun to develop monitoring programmes, recommendations and ecological standards for these additional priority substances for UK waters (Defra, 2014).

1.3.3. Fate and Behaviour of Pharmaceuticals in the Aquatic Environment

It is important to understand the fate and behaviour of pharmaceuticals and pharmaceutical products during both the wastewater treatment process, and in the aquatic environment, in order to assess their potential to cause harm to aquatic species and human health. In general, degradation potential of pharmaceuticals varies significantly. Some have high water solubility (relative to their molecular weight), and are not readily degradable (Ternes, 1998; Daughton and Ternes, 1999; Kümmerer, 2008), such as the analgesic, carbamazepine. However, many other pharmaceuticals and API's readily degrade during the treatment process. The behaviour of pharmaceutical compounds during the treatment process and in surface water depends on the specific chemical properties, and consequently the chemical properties of a given compound affect the degree of risk posed to wildlife.

1.3.4. During Wastewater Treatment (WWTP)

Since many pharmaceuticals are not completely metabolised in the human body, they are often excreted (either through urine or to a lesser degree, faeces) as unchanged parent compounds, metabolites (degradation products), or as conjugated polar molecules (Kümmerer, 2008), which subsequently enter WWTP. Depending on the structure of the compound, once in water treatment plants, drugs may undergo biodegradation, remain suspended or dissolved in water, or become bound to sewage sludge (Trudeau, et al, 2005; Corcoran et al, 2010). Generally speaking, pharmaceuticals undergo several processes during sewage treatment that determine their persistence in the aquatic environment.

1.3.5. Degradation

Degradation typically occurs, either in the form of biological degradation (by microbes such as bacteria), heat degradation, and/or photodegradation. However, many studies have found that certain pharmaceuticals are not effectively degraded by these processes and as a result these bioactive compounds, be that the parent compound or active metabolites, enter our aquatic systems, where they continue to resist degradation (Daughton and Ternes, 1999; Heberer, 2002). In a study investigating the degradation of antineoplastic drugs from hospital effluent during sewage treatment, Kümmerer and Al-Ahmad (1997) tested several antitumour agents using the closed bottle test system (OECD 301 D) and a modified Zahn-Wellens test (OECD 302 B). The pyrimidine analog 5-Fluorouracil was found not to degrade, whilst biodegradation of gemcitabine was 42% and 50% in two separate investigations. Other studies have reported several additional pharmaceuticals that do not readily degrade during treatment, such as clofibric acid, the active metabolite of the cholesterol lowering drug clofibrate, and methotrexate, which is used as a chemotherapeutic and in treating autoimmune disorders such as rheumatoid arthritis (Henschel et al, 1997).

The mechanistic properties of some types of pharmaceuticals are largely the cause of this degradation resistance. Resilience to degradation is as a function of design, since drugs are required to remain stable within the human body, for a sufficient amount of time, in order to exert their pharmacological action and ensure maximum efficiency on the therapeutic target. This design function can have implications for environmental concentrations, potentially leading to more stabilised concentrations in the environment, and therefore increasing the likelihood that aquatic wildlife will be exposed (Dietrich et al, 2002).

1.3.6. De-conjugation

As previously discussed, during drug elimination, many pharmaceuticals are conjugated through processes such as glucuronidation and sulphation, in order to increase their water solubility and therefore excretion efficiency from the body. During the waste water treatment process, conjugates are easily cleaved from these molecules, resulting in the release of the active compounds into outflowing discharge waters (Heberer, 2002). For example, organic steroid hormones conjugated as gluconorides and sulfates have been found to be easily de-conjugated during wastewater treatment, due in part to the amount of β -glucuronidase enzymes present, the products of bacterial processes (Jones et al, 2005). It is extremely likely, therefore, that the synthetic steroidal pharmaceuticals will be as rapidly de-conjugated during WWT processes, resulting in the increased persistence of these drugs in outflowing waters.

1.3.7. Partitioning Between Aqueous and Solid Phases

During primary treatment, separation of the aqueous and solid phase occurs. Compounds with a low K_{ow} (Octanol-Water Partition Coefficient) tend to remain dissolved in the aqueous phase, whilst compounds with a higher K_{ow} bind to solid sludge. Many pharmaceuticals are lipophilic (they have a low K_{ow}), and are designed as such so as to be able to freely pass through cell membranes (Halling-Sorenson, 1998). Since the majority of pharmaceuticals have a low K_{ow} , they typically remain in the aqueous phase. In other words, their hydrophilic nature allows these compounds to dissolve in water and become more freely available for uptake by aquatic wildlife.

A small minority of pharmaceutical compounds with higher K_{ow} values may become bound to solid sludge. Since treated sludge is often used as agricultural fertiliser, or stored as landfill, this becomes an additional potential route of pharmaceuticals and pharmaceutical

products to the aquatic environment (as a result of agricultural run-off and leaching from landfill) (Jones et al, 2005). Despite this representing a potential route of these compounds to aquatic systems, it should be noted that considerable data and knowledge gaps exist, and in reality, this route of entry is poorly understood.

1.4. Detection of Pharmaceuticals in the Aquatic Environment

Pharmaceuticals have been detected and measured in the aquatic environment, in STP effluent (Cui et al, 2006), surface waters, groundwater, coastal waters, soil and in drinking water (Fent et al 2006; Weber et al, 2014). Individual pharmaceuticals have been reported to be present in concentrations as high as in the $\mu\text{g/L}$ range, although the majority have been detected at much lower concentrations, in the ng/L range. Others that have not yet been detected in the environment, likely in some cases due to the limits of detection of analytical instrumentation, may be present in lower concentrations still, i.e. in the pg/L range. However, analytical chemistry has been continuously improving, allowing for the detection of compounds at much lower concentrations, thus facilitating a greater knowledge of what is present in the environment, and at what concentrations. The development of high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) in particular has significantly increased the ability to detect low levels of pharmaceuticals in environmental samples. In HPLC-MS/MS, high pressure liquid is used to force the sample through a column containing particles of a shape and size suitable for the separation of the desired compound. The instrument then measures the molecular mass of the separated particles, based on a mass to charge ratio after ionisation has occurred, in order to determine the structure and composition of the compound, and to quantify the amount in a given sample. The development of HPLC MS/MS has initiated a surge in the number of pharmaceuticals measured from environmental samples, owing to

increased specificity and the ability to handle complex samples such as effluents (Pitt, 2009).

In the early 1990's, clofibric acid, the active metabolite of the lipid lowering drug clofibrate (used in controlling cholesterol and triglyceride levels in the blood) was detected in drinking water samples in Berlin, Germany (Heberer and Stan, 1997; Heberer et al, 2002). Subsequent studies have detected over 150 pharmaceuticals or pharmaceutically active substances in the aquatic environment from a wide range of drug groups, including steroid hormones, antineoplastics, lipid-lowering compounds, antibiotics, analgesics and beta-blockers (Koplin et al, 2002; Fick et al, 2009; Loos et al 2013).

Since it is not currently feasible to measure accurately all compounds present in surface waters, modelling of predicted concentrations offers a useful tool in gaining a relative understanding of likely environmental concentrations. In predicting pharmaceutical concentrations, models provide predictions based upon a number of factors ranging from the chemicals mode of action (well understood for human pharmaceuticals), human therapeutic dose, usage, plasma bioconcentration factors, drug potency, physico-chemical properties and a host of other factors, although input parameters can vary a great deal between models (Fick et al, 2010). Using these models, scientists can predict environmental concentrations of pharmaceuticals in surface water, where data on measured concentrations is scarce (Bound and Voulvoulis, 2006; Johnson et al, 2008; Johnson et al, 2013_a, Johnson et al, 2013_b).

While predictions of concentrations are beneficial, it is important to understand the effects on organisms across a wide range of concentrations, thus allowing us to discern at what concentrations certain compounds cause adverse effects in wildlife, and how close to actual environmental concentrations these are. Predicted concentrations can help in determining starting concentrations for laboratory concentration-response studies; however, measured concentrations are preferable when designing laboratory-based

toxicology studies aimed at determining environmental risk. Quantification of concentrations from laboratory studies is also important in order to link adverse effects to concentrations of toxic substances.

Until relatively recently, analytical limitations in the detection of low concentrations prevented the quantification of these compounds in environmental samples. However, the recent technical improvements discussed previously have largely overcome these constraints, and actual environmental measurements can now be compared to toxicological data from laboratory studies. For example, synthetic steroids in the aquatic environment are of particular concern as they can mimic the effects of natural ligands by binding to the corresponding receptors and therefore influence important processes such as growth, metabolism, endocrine function and reproduction. They have been detected in the aquatic environment individually at relatively low concentrations (ng/L range) (Kugathas et al, 2013). More importantly, measured environmental concentrations have been reported to be similar to experimental exposure concentrations used in laboratory studies that are able to initiate responses on key biological processes in fish species (Nash et al, 2004). However, for most pharmaceuticals, concentrations present in the environment are considered to be too low to cause any significant direct adverse effects to aquatic organisms, particularly when considering chronic, rather than acute, exposure.

Given that a growing number of pharmaceuticals are now being detected at low levels in surface waters across the world (Weber, 2014) and since it has been shown from studies with synthetic estrogens that low levels, in the range of ng/L, can cause adverse effects on aquatic organisms, there is a growing research effort towards predicting and measuring potential adverse effects of pharmaceuticals and their products in a range of organisms (Fick et al, 2010).

1.5. Toxicological Effects in Wildlife

Toxic effects have been demonstrated for a number of pharmaceuticals and pharmaceutical derivatives. One of the best documented examples is the impact of the anti-inflammatory drug Diclofenac on several Asian vulture populations in India and Pakistan (Oaks et al, 2004; Shultz et al, 2004; Green et al, 2006). A decline of more than 95% of some Asian vulture populations in the 1990s led to the species reaching a 'critically endangered' status, and at serious risk of extinction. Subsequent studies found that residues of diclofenac from the carcasses of livestock (a large part of the birds main food source) treated with the veterinary drug caused extensive renal failure and visceral gout, leading to death in adult and sub-adult birds (Oaks et al, 2004). In many Asian countries, livestock are treated with this anti-inflammatory drug in order to treat pain and inflammation associated with disease and injury. Veterinary diclofenac is widely marketed and easily accessible, particularly in the developing world. Livestock that die as a result of disease or injury are typically left for the scavenging vulture populations, which unfortunately became a source of diclofenac poisoning to the birds. Experimental exposures and field sampling studies have attributed the decline of vulture populations to direct exposure to diclofenac, to which susceptibility is widespread throughout the *Gyps* genus (Fent et al, 2006a; Green et al, 2006).

Another well-documented example of adverse effects as a result of pharmaceutical exposure is the feminization of male fish from UK river waters and STP effluents (Purdom et al, 1994). A study by Desbrow et al (1998) identified the active component of the female contraceptive pill, ethinyl estradiol (EE2), as the main estrogenic contaminant of STP effluents in UK rivers contributing to endocrine disruption in fish. Jobling et al (1998) demonstrated high incidences of intersexuality in roach, *Rutilus rutilus*, associated with exposure to chemical contaminants from sewage treatment works. STP effluent is known to contain hormonally active substances, particularly estrogens and estrogen mimics,

including synthetic steroid pharmaceuticals. Roach living upstream and downstream of STPs were sampled across the UK. Large numbers of roach were found to be intersex, hence containing both male and female gonadal characteristics when examined by histological analysis. Intersexuality was significantly more prevalent in male fish at sites exposed to effluent from STPs. Furthermore, intersex incidence was demonstrated to be as high as 100% in two populations of roach sampled from downstream of sewage treatment works (Jobling et al, 1998). Subsequent studies by Jobling and colleagues attempted to correlate this observed endocrine disruption observed in wild river roach with predicted exposure to synthetic and endogenous steroid estrogens. Using a model developed by Johnson and Williams (2004), Jobling et al (2006) modelled concentrations of estrogens, including ethinyl estradiol (EE2), the synthetic estrogen, widely used in the female contraceptive pill, derived from predicted effluent discharge loads as a key model parameter. The model was developed in order to obtain predicted influent and effluent concentrations of steroid estrogens, since at the time, measured concentrations were not available. The model also accounted for additional parameters including excretion estimates, population cohorts of catchment areas, sewage treatment removal rate efficiencies, human and industrial population equivalent values (PE) and STW flow rates. The study found that the incidence and severity of intersex fish was significantly and positively correlated with the predicted concentrations of EE2 (Jobling et al, 2006).

Since the early 1990s, many pharmaceuticals have been tested in acute toxicity tests and found to have detrimental impacts to aquatic wildlife. These include several of the steroid hormones (estrogens, androgens, progestogens, glucocorticoids), non-steroidal anti-inflammatory substances (NSAID's), fibrates and statins, neuro-active substances (antidepressants and antiepileptics), antibiotics, and antineoplastics (cytotoxics and cytostatics). However many pharmaceuticals remain to be tested for their toxic effects in wildlife. Legislation now dictates that any new drug developed is required to undergo an environmental risk assessment (EU Directive 92/18/EEC; EU Directive 2001/83/EC).

However, for existing drugs that have been, and are still being, extensively used, no such assessment is required. For many of the pharmaceuticals in use, data on environmental toxicity remains limited, if not completely absent.

In most cases, effects will vary depending on the species and the concentration at which the compound is tested. It is for this reason that standard aquatic environmental testing for risk employs a 'model' species approach, employing organisms that are representative of numerous trophic levels. Typical toxicology assays are performed using model species from three major taxonomic groups; a green microalga, a crustacean (typically *Daphnia* sp.) and a fish species. For toxicology testing with model species, general standards are set by the OECD and employ a range of end points including growth inhibition (algae), immobilisation (daphnia), reproductive toxicity (daphnia and fish), early life stage development (fish), and multigenerational studies (fish), as well as sometimes incorporating more specific endpoints such as vitellogenin induction and secondary sexual characteristic assessment, for example when screening for estrogenic/androgenic activity (OECD, Guidelines for the Testing of Chemicals, Section 2).

As previously discussed, pharmaceuticals are biologically active compounds with specific targeted mechanisms, and are widely regarded as having the potential to cause biological responses in aquatic life. It is widely accepted that adverse effects in aquatic wildlife (i.e. non-target species) exposed to pharmaceuticals and pharmaceutical products are likely to arise due to high affinity interactions with conserved targets, rather than a more general toxic effect via unspecific modes of action, as occurs with many other chemical contaminants (Gunnarsson et al, 2008; Corcoran et al, 2010). Since many molecular targets and pathways that exist in humans also exist in certain aquatic species, in particular vertebrate species such as fish (Gunnarsson et al 2008), it is expected that drugs designed to act on these common targets in humans will also cause adverse effects to species with these well-conserved molecular sites. Gunnarsson et al, (2008) reported that the zebrafish (*Danio rerio*), a commonly used laboratory fish species, had orthologs to

86% of 1318 human drug targets tested, whilst *Daphnia magna* (a planktonic crustacean) and *Chlamydomonas reinhardtii* (a unicellular green alga) had 61% and 35% orthologs respectively. Furthermore, since organisms are typically exposed to low concentrations of these compounds over more chronic temporal scales, effects due to general toxicity are less likely than specific target-mediated effects. However, the presence of conserved receptors does not always ensure a receptor mediated effect, highlighting the requirement for additional research in this area (Rand-Weaver et al, 2013).

A key issue is whether or not a pharmaceutical present in the environment can reach a concentration in an aquatic organism, such as a fish, that will cause an effect. As stated above, most pharmaceuticals in the environment are present at very low concentrations, levels that would appear unlikely to cause effects in aquatic species. However, relatively little is known about the bioaccumulation potential of pharmaceuticals in non-target organisms. There is the possibility that some pharmaceuticals could bioaccumulate in aquatic species to a degree that their concentrations may become high enough to initiate an effect. Various approaches have been developed to predict whether a particular environmental concentration of a given pharmaceutical could present a risk to aquatic organisms (discussed below).

1.5.1. The Read-Across Approach

A useful tool in predicting risk to organisms from pharmaceutical exposure is the read-across approach. The read-across approach, first proposed by Huggett et al (2003), has been developed as a means of extrapolating biological and toxicological data from one species to another. This kind of cross-species extrapolation has been well demonstrated in the drug development field, whereby animal models, usually rodents, are used in initial drug testing phases, with pharmacology and toxicity data being later extrapolated to humans before clinical studies on human volunteers can begin. The read-across

hypothesis stipulates that a drug will cause a pharmacological response in a non-target organism before a general toxicity response, firstly, if the biological target (i.e. receptor or enzyme, etc.) is present, and secondly, if the internal level of the drug reaches that of the therapeutic levels required to initiate a response in humans. The read-across hypothesis has been investigated using the Fish Plasma Model (FPM), first developed by Fitzsimmons et al (2001), and further developed by Huggett et al (2003). The model compares measured human therapeutic plasma levels to the predicted steady state concentrations fish plasma to obtain an effect ratio. The ratio affords the prediction of the likelihood of a pharmacological effect in fish, whereby the lower the ratio value, the more likely a pharmacological effect is. The Read-Across Approach has been validated using the FPM in studies investigating the selective serotonin reuptake inhibitors (SSRI's) fluoxetine (Margiotta-Casaluci et al, 2014), and sertraline (Valenti et al, 2012). Theoretically, all pharmaceuticals present in the environment have the potential to cause a biological response in aquatic organisms if the molecular target of the drug is present in the organism, although the reality is much more complicated. Furthermore, many drugs have more than one target in human therapeutic use, either through design or due to secondary 'non-target' mechanisms. This artefact increases the risk of adverse effects in non-target species.

Although laboratory and predictive toxicity studies are important in determining risk to aquatic wildlife, assessing chronic exposure effects can be a challenge. Laboratory investigations are typically limited to short term acute tests with concentrations often many orders of magnitude higher than those that would typically be found in the environment. Higher concentrations are generally utilised to ensure that adverse effects on organisms can be observed and quantified, and so that concentrations can be determined analytically. Associating adverse effects to measured levels of compounds as opposed to nominal concentration values is of growing importance, given increased understanding of the variability of the fate and behaviour of compounds with even similar molecular

structures. Tested concentrations also need to be within detection ranges of analytical instruments and methodological capabilities. Furthermore, long-term and multigenerational studies are possible under laboratory conditions, particularly with certain well-chosen model species. However, given time and financial constraints, chronic studies are not always feasible, particularly in the case of long term studies, such as multigenerational studies with fish.

1.6. Human Health Risks

Risks to human health as a direct result of pharmaceutical products present in the environment are typically considered to be low. Concentrations present in drinking water, surface and ground waters (if present) are typically well below the minimum therapeutic dose (MTD), and in most cases more than 1000-fold lower, and are therefore highly unlikely to cause any biological response in humans or wildlife (Webb et al, 2003; Cunningham et al, 2009; de Jongh et al, 2012; WHO, 2012). The Drinking Water Inspectorate (DWI), the regulator of public water supplies in England and Wales, considers there to be no risk to human health from pharmaceuticals in potable water supplies, since advanced treatment is employed at drinking water treatment plants in the UK (Jones et al, 2005). Advanced treatment for direct and indirect drinking water use include technologies such as advanced oxidation (AO), ultraviolet radiation (UV), reverse osmosis and granular activated carbon (GAC) (Jones et al, 2005; Monteiro and Boxall, 2010). The opinion of the DWI has considerable validity, since advanced treatment in drinking water plants has been shown to efficiently remove many pharmaceuticals and API's. For example, in a pilot treatment study of 17 pharmaceuticals present in municipal STP effluent in Germany, all 17 were effectively removed by ozonation and UV disinfection (Ternes et al, 2003). In another study by Cunningham et al (2008), human health risks of 44 API's from across 22 pharmaceutical classes were assessed using

calculated ratio values of predicted environmental concentrations (PECs) compared with predicted no effect concentrations (PNECs). The study concluded that there was no risk to human health from any of the pharmaceuticals tested in the study. Similar conclusions have been drawn from a number of other studies (Webb et al, 2003; de Jongh et al, 2012). de Jongh et al (2012) conducted a study monitoring drinking and surface water samples in the Netherlands for seventeen widely used pharmaceuticals and nine transformation products. They sampled across a range of water treatment stages including pre-treated surface waters, bank filtrates, groundwater and drinking water. As predicted, concentrations of API's diminished with each stage of treatment, with none of the pharmaceuticals being detected in drinking water produced from surface water sources. Additional exposure routes of pharmaceuticals and API's to humans could include food consumption, and bathing/washing in contaminated water sources, although the risk from these are considered to be negligible (Cunningham et al, 2008).

Although advanced wastewater treatment technologies are now employed in most of the developed world, as previously discussed, these technologies are not always efficient at removing pharmaceuticals and pharmaceutical derivatives. Jones et al (2005) reviewed the available data on concentrations of pharmaceuticals detected in final drinking water samples worldwide. They noted a distinct lack of data and inefficient monitoring. The limited data that were available were restricted to a small number of northern European countries and the USA. Jones and colleagues reported based on a summary of the literature that eleven pharmaceuticals and API's from six drug classes had been detected in final drinking water samples. However, in reality this figure could be much greater, considering the lack of monitoring and measurement studies for most pharmaceuticals and API's currently in use.

Another key emerging issue from pharmaceutical pollution is antibiotic resistance. Despite the long history and extensive usage of these drugs in human and veterinary medicine, their presence in the environment has until recently been largely overlooked (Kümmerer,

2009a). On average, up to 70% of an antibiotic may be excreted from the human or animal body unchanged (Kümmerer and Henninger, 2003), which can lead to their presence in the aquatic environment. Antibiotic substances have been detected in various water sources including effluents (Hirsch et al, 1999) and surface waters (Christian et al, 2003) at concentrations of around 2.5µg/L and 300ng/L, respectively. In certain developing countries, concentrations in effluents linked to large scale manufacturing processes have been reported to be as high as 30mg/L (Larsson et al, 2007). However, it is important to note that these concentrations are reported for specific regional cases, and are unlikely to be representative of the global picture (at least not for the developed world). A recent study in China by Wang et al (2016) screened for twenty one commonly used antibiotics in drinking water supplies. The study detected two veterinary antibiotics, florfenicol and thiamphenicol, in tap water and bottled water samples, however concentrations were typically low (<0.007ng/L). The same study detected 19 commonly used antibiotics in the urine of school children from the same region, however the presence of antibiotics in urine could not be correlated to exposure via drinking water alone.

The widespread presence of antibiotic compounds entering the environment has greatly contributed to the emerging issue of antibiotic resistance in many bacterial strains. Though a certain degree of natural resistance is normal, the development of secondary resistance is becoming more widespread and occurring over much shorter time frames, most likely as a result of exposure of bacterial organisms to low levels of antibiotics, both in the environment and in medical usage. This has potentially worrying consequences for future bacterial infection treatment, since antibiotic use to treat a wide range of potentially deadly infections is common practise.

Despite the generally low risk to human health from the presence of pharmaceuticals in drinking water, long term chronic studies of exposure i.e. full life cycle exposure studies, particularly during critical stages such as pregnancy and neonatal development, are

largely lacking. Though current risk to human health may be low, the increased demand facing water resources from growing domestic, industrial and agricultural usage will inevitably increase pressure on obtaining clean potable water, and could lead to reduced water treatment efficiencies. Regulatory screening for pharmaceuticals in drinking water, surface water and ground water samples is largely absent (WHO, 2012). As previously discussed, there are currently no standardised regulatory risk measures to assess this group of pollutants. However with increasing research this situation is slowly changing.

1.7. Environmental Risk Assessment of Pharmaceuticals

Under EU legislation, Directive 2001/83/EC, EU, 2001, all new pharmaceutical products are required to undergo an environmental risk assessment during the drug development stage. An environmental risk assessment is required for both the drug manufacturing stage prior to development, and for post-consumer usage, prior to marketing approval. In 2006, in accordance with EU legislation (2001/83/EC), the European Medicines Agency (EMA) published guidelines for environmental risk assessment of medicinal products (EMA, CHMP. 2006). The EMA guidelines consist of a two phase approach. Phase I is conducted during the pre-screening stage, for the purpose of estimating environmental exposure to a drug. First stage assessment is conducted through the calculation of the predicted environmental concentration (PEC), where basic assumptions are made in relation to geographical and temporal usage, entry routes, biodegradation and metabolism. Where the PEC is above 0.01µg/L, the assessment for that particular drug proceeds to Phase II. During the first phase of assessment the octanol-water partition coefficient (K_{ow}) is also calculated. In accordance with the OSPAR Convention, products with a $\log K_{ow}$ of more than 4.5 should undergo additional investigation for persistence, bioaccumulation and toxicity (European Medicines Agency, 2006; European Chemicals Bureau, 2003). For products that do not meet satisfactory requirements in the first phase,

Phase II of the assessment consists of a basic investigation of the likely fate and potential environmental effects of the drug. Phase II consists of two tiers. Tier A involves an initial prediction of the environmental risk, and should consist of standard aquatic toxicity testing and the calculation of PEC/PNEC ratio. If the ratio value is greater than 1, further assessment is required (Tier B), whereby extended fate and effects investigations are carried out in order to refine the PEC and PNEC values (Ågerstrand et al, 2015; EMA, 2006). Although there is a legal requirement to perform an environmental risk assessment, the EU Directive also stipulates that an identified environmental risk does not serve as valid grounds for the refusal of market approval of a new drug (Ågerstrand et al, 2015; Tarazona et al, 2005).

1.7.1. Prioritisation of High Risk Pharmaceuticals

Since there are a few thousand pharmaceuticals and API's currently in use, and many of these are likely to be present in the environment, laborious laboratory-based studies to assess the risk of all substances is not feasible. New studies are now emerging that attempt to prioritise contaminants based on many different parameters. For example, in a study by Donnachie et al (2016), an unbiased multi-tiered ranking approach to the prioritisation of pharmaceuticals in terms of environmental risk was investigated. Using this approach, ethinyl estradiol, fluoxetine and propranolol were ranked of highest concern, however the authors note the inherent biases of literature-based assessments, due to the 'popularity' of certain compounds in toxicology assessments over others. In a similar study by Guo et al, (2016), risk scores were applied to pharmaceutical substances by comparing exposure predictions to potential hazard in organisms, both acute and chronic. Guo and colleagues reported the highest priority substances in acute exposures were from antibiotic classes, including amoxicillin and azithromycin, whilst the more important for chronic exposure were the NSAID diclofenac, the cholesterol lowering drugs atorvastatin, the proton pump inhibitor omeprazole, and the estrogen estradiol. The different approaches taken by these studies may account for the deviations in results, and

demonstrates how challenging ranking studies can be. Though fundamentally difficult and complex in nature, the emergence of prioritisation studies in environmental toxicology is of growing importance.

1.8. The Mixtures Issue

Chemical pollutants are conventionally assessed for risk on a chemical by chemical basis. However, in reality organisms (including humans) are exposed continuously to a multifaceted combination of chemicals simultaneously throughout their lives. Mixture compositions are highly variable in nature, both temporally and spatially, and are controlled by many factors including hydrological and climatic parameters, population densities and configurations, land and water use patterns, diffuse and point source input sources, water treatment processes (or lack of), along with many other factors. Such chemical contaminants can include pesticides, herbicides, personal care products, pharmaceuticals and API's metals, and industrial chemical pollutants. Most of these are present at low concentrations. Pharmaceuticals comprise a considerable component of these real-world mixtures. Since they are designed specifically to act on biological receptors and enzymes, many of which are well conserved across vertebrate taxa (as previously discussed), mixtures of these compounds could be of concern for aquatic wildlife, particularly for other vertebrates such as fish. Since individual pharmaceuticals do not exist in isolation in the environment, risk cannot be effectively assessed based on single exposure data. The key question is whether or not these chronic low concentrations present a risk to aquatic wildlife and human health when present as complex mixtures.

The need to consider mixture effects of chemicals present in the environment has been widely recognised (Daston et al, 2003; Kortenkamp, 2008). However, testing for adverse effects of every potential mixture combination on many different taxonomic groups in the

laboratory is, of course, unfeasible. There is also a growing demand towards observing the 3R's, the replacement, refinement and reduction of animal models in scientific research, further restricting the potential in pursuing large scale traditional animal model toxicity assessments.

As previously discussed, many pharmaceuticals are found in the aquatic environment in the low $\mu\text{g/L}$ - ng/L range. For drugs that have been investigated in toxicological assessments, *in vivo* and *in vitro* studies have reported LOECs (Lowest Observed Effect Concentrations), the concentration below which no effects can be observed, to be much higher than these reported environmental levels. A key question therefore arises as to the environmental relevance of pharmaceutical concentrations with respect to the associated risks. Theoretically, many pharmaceuticals that are present in the environment are present at concentrations well below those required to produce any adverse response in aquatic species, and therefore pose no risk. However the accuracy of this assumption should be (and is being) challenged.

The question therefore arises as to the combined toxicity potential of these chemical mixtures. Since an organism would be continually exposed to complex undefined mixtures (albeit at low levels) for most, if not all of its life cycle, there exists the potential for chemicals to act additively through common targets to cause adverse effect. Thus, despite the fact that many pharmaceuticals are found in the environment at concentrations well below their No Observed Effect Levels (NOEL), the concentration at which no effects in organisms can be observed, and therefore should cause no harmful effect to wildlife, the same compounds may have the potential to cause serious adverse effects when combined in a mixture. Additive toxicological effects have been demonstrated by recent studies (Brian et al, 2005; Brian et al, 2007). Chemicals with similar modes of action, for example several estrogenic compounds (those than have an agonistic effect on the estrogen receptor (ER)), each present at concentrations below their NOELs, could have

the potential to act in an additive manner and cause adverse effects mediated through the same molecular target i.e. the estrogen receptor. For example, a study by Brian et al (2005), investigated combination effects of five estrogenic compounds on vitellogenin induction in male fathead minnows. Vitellogenin (VTG) is an egg yolk precursor protein normally produced in the liver of female fish prior to ovulation. Vitellogenin synthesis is largely controlled by endogenous steroid estrogens, and has been established as a good indicator for estrogen exposure and estrogenicity of compounds. Brian and colleagues demonstrated that the five compounds acted additively and that effects could be accurately predicted according to the Concentration Addition (CA) additivity model. Another study by Pottinger et al (2013) reported additivity of four androgen receptor antagonists in the androgenised female stickleback screen assay, again according to CA model predictions. The CA model has been well demonstrated in a range of assays as a good predictor of mixture effects of similarly-acting compounds.

Understanding mixtures toxicology is a complex and relatively new sub field of human and environmental toxicology, and much remains to be addressed in terms of the potential risks associated with chemical mixtures. Since laboratory testing for risk for every possible mixture combination and potential species response is entirely unfeasible, predictive toxicology is becoming of increasing importance and relevance, particular in the case of environmental mixtures.

1.9. Predictive Mixture Modelling - Introduction to Model Principles

Several mixture models have been developed to assess potential combination effects of chemical mixtures, though most are based around two major concepts, Concentration Addition (CA) and Independent Action (IA) (or Response Addition) (Boedeker et al, 1993; Faust et al, 2003). The models are biomathematical and can be used to formulate mixture

effect predictions based on information of the single compounds. CA and IA can in theory be applied to any mixture composition, however they have fundamental underlying assumptions that many realistic mixtures will not meet. These assumptions are based largely on the behaviour of the individual compounds and their pharmacological properties.

CA assumes that all chemicals in a given mixture have similar modes of action, and that they act as dilutions of one another. In this scenario, one compound could therefore be easily substituted for another at a concentration corresponding to the same effect level and still produce the same overall mixture response. The CA model assumes that all chemicals in a mixture contribute to the overall toxic effect. In this concept, individual compounds can be present at concentrations that would cause no effect individually, but cause combination effects if the components sum up to produce a high enough dose (Kortenkamp and Altenburger, 2011). In contrast, the IA model assumes a dissimilar mode of action of the compounds in the mixture, assuming all chemicals act independently to create the overall effect. The IA model requires more detailed information about the effects of the single compounds in the mixture, particularly at low dose levels, and is therefore somewhat more data intensive than CA.

Chemicals are classified as 'similar' when they have the same mechanisms of action in an organism; for example, 5 synthetic progestagens, which all target primarily the PR receptor, and follow similar biological pathways in an organism. Conversely, compounds are said to be dissimilar when they possess different mechanisms of action, different target receptors, and/or different pathways in an organism; for example, a mixture comprising of an estrogen and an androgen.

For conducting low dose mixture experiments, several essential criteria must be met. Kortenkamp (2008) reviewed the risk and implications of potential low dose mixture effects from endocrine disruptors, and highlighted a number of key requirements for

conducting low dose mixture experiments. For a more detailed analysis of low dose mixture experiments, the reader is referred to the review by Kortenkamp (2008).

1.9.1. Interactions and Predictive Modelling

1.9.1.1. Concentration Addition (CA)

It has been well established that pharmaceuticals with the same mode-of-action (MOA) will act additively. For example, two or more estrogenic pharmaceuticals will act additively, and the effect of any mixture of these drugs would be predictable using the concept of concentration addition (Kortenkamp, 2007). Concentration addition (CA), or dose addition, is based on early work of (Frei, (1913), but was first proposed in 1926 by Loewe and Muischnek, and is represented by a mathematical equation. For a simple binary (two compound) mixture, this is expressed as:

$$\frac{c_1}{ECx_1} + \frac{c_2}{ECx_2} = 1,$$

Source: Faust et al, 2003

Where C_i is the individual substance concentration in the mixture (1), x is the elicited effect, and $EC x_i$ is the equivalent effect concentrations of the single substances. The equation can be modified for multicomponent (more than two compounds) to:

$$\sum_{i=1}^n \frac{c_i}{ECx_i} = 1.$$

Source: Faust et al, 2003; adapted from Berebaum 1985).

For further explanation of the mathematical basis of this model, the reader is referred to Altenberger et al, 2004.

The concept is founded around the idea that each chemical in a chemical mixture contributes to the overall effect outcome at a common site of action. Essentially, CA is said to occur if one chemical acts as a dilution of another, and so the overall mixture effect can be attained by replacing one chemical, or one part of a chemical, with an equi-effective dose of another (Altenberger et al, 2000; Backhaus et al, 2000).

1.9.1.2. Independent Action (IA)

Much less is known about how compounds with different MOAs interact, and whether or not the net effect can be predicted. For example, how would two different pharmaceuticals that both target the reproductive axis (e.g. a synthetic estrogen and a synthetic progestogen) interact? These compounds could be classed as similarly acting based upon their ability to affect the same biological process, and produce some degree of interaction at the effect level. Conversely, these two compounds could be classified as dissimilar, based upon their different specific molecular targets, in this case the nuclear receptors, and therefore lead to quite distinct sets of effects. It is for this reason that there is still a large degree of validation required for these predictive models, particularly in *in vivo* studies.

The concept of independent action (IA) was developed to aid in predicting the effects of mixtures of chemicals with different MOAs at the molecular site (Cedergreen et al, 2008), but it has rarely, if ever, been tested at the whole-organism level. It has, to a limited degree, been tested *in vitro*, and predictions of mixture effects from dissimilarly acting chemicals appear to align more to the model predictions of IA than to CA. The theory of IA was first proposed by Bliss (1939), and is mathematically expressed as:

$$E(c_{\text{mix}}) = 1 - \prod_{i=1}^n (1 - E(c_i))$$

Source: Faust et al, 2003

Where $E(C_{\text{mix}})$ is the total mixture effect, and $E(C_i)$ is the fractional effect caused by the individual compounds. The models will be discussed in more detail in Chapter 3.

To date, there are only a few ecotoxicological studies that have been able to demonstrate synergism or antagonism in mixtures studies, and provide a good mechanistic explanation of such interactions at various biological levels.

1.9.2. Limitations in Predictive Mixture Modelling

There are several limitations to mixture assessments based on predictive models. Since these are component-based approaches, detailed information of all individual compounds in any given mixture is required, especially in the case of IA. For *in vivo* chronic studies involving vertebrates such as fish, this can be a major limitation, since obtaining information on concentration-dependant effects for many substances can be extremely costly and timely. For this reason, the majority of mixture studies in the current literature involve *in vitro* investigations or the use of lower taxa that exhibit, for example, faster growth and lower maintenance demands in laboratory conditions.

Another key issue with the dependency on these models is that it is sometimes difficult to define 'similar' and 'dissimilar' modes of action. In addition to aforementioned synthetic reproductive steroids, many more pharmaceutical classes would be difficult to quantify strictly into either category. For example, when considering the anticancer pharmaceuticals, many of these are primarily cytotoxic, acting on DNA to disrupt the natural cell-cycle and prevent cell replication. The function of these drugs is essentially to kill the rapidly dividing cancerous cells, however different cytotoxic drug classes do this in very different ways. For example, Carboplatin and Cisplatin are derivatives from the alkylating agents, or platinum compounds, which bind to DNA, forming intra-strand cross-

links (crosslinks on the same strand on DNA), thereby blocking DNA replication and triggering apoptosis (programmed cell death). Another widely used anticancer agent, 5-fluorouracil (5-FU) belongs to the pyrimidine analog group. This drug also acts on DNA to prevent DNA synthesis, but acts by inhibiting the enzyme thymidylate synthase, thereby blocking the synthesis of thymidine, an important nucleoside essential for DNA replication, again leading to cell death. Both of these compounds act directly on DNA to initiate cell death, however they act in very different ways. Therefore, if an organism was to be exposed to these particular anticancer agents as part of a mixture, would these chemicals act additively and follow the Concentration Addition model (since the compounds could be classed as similar, i.e. both acting on DNA), or would the effects more closely resemble that of Independent Action (since the mechanisms of action for these drugs are not the same – they do not act on DNA in the same manner)? A crucial factor in addressing this issue is to consider the particular endpoint in question. Some studies have shown that the same mixture of compounds can act additively on a particular end point, whilst acting more independently on another in the same organism (Cedergreen and Streibig, 2005). Where this has been demonstrated, additive effects are typically at higher level endpoints which are more specific to drug mechanisms of action, for example VTG induction through modulation of the estrogen receptor in male fish. Studies demonstrating additive mixture effects on ecologically important end points such as reproduction and development are largely lacking. However recent studies have begun to focus research efforts in this area. For example, a study by Brian et al (2007) demonstrated additive effects of mixtures of estrogenic compounds on reduced fecundity in the fathead minnow, *Pimephales promelas*.

A further, somewhat important, limitation of these models is that they assume no interaction between the components in a mixture. In reality, chemicals most likely exert an effect on other chemicals. For example, one may alter the way another behaves by affecting its potency, its chemical composition or through competitive binding activity.

Unfortunately, this level of complexity is currently beyond the scope of these models. Despite the limitations, modelling approaches based on CA and IA offer the most useful tool in providing mixture toxicity assessments with sufficient accuracy for use in risk assessment and regulation.

1.9.3. Pharmaceuticals as a Focus of Predictive Mixture Studies

Pharmaceuticals as pollutants offer a unique opportunity to study the effects of 'real world' contaminant mixtures. Since pharmaceuticals are synthetic compounds designed with particular mechanisms of action, a great deal is known about the compounds molecular structure and chemical fingerprint. The biological effects of these chemicals (both desired and undesired) are also well established as a result of the stringent clinical trials the drugs are subjected to prior to their marketing, which typically employ a number of biological assays with a diverse array of organisms and *in vitro* systems. As such, component-based mixture studies using these compounds benefit from a significant initial advantage of a detailed knowledge of the individual chemicals in a given mixture. As previously discussed, for predictive mixture modelling, particularly in the case of Independent Action (IA), comprehensive information of the individual components in a mixture is required. For many other chemical contaminant classes, this information is obtained through many years of scientific studies on the chemical and its biological effects in the laboratory. For pharmaceuticals, this knowledge is inherent, and can thus save many years of laboratory studies on the individual chemicals themselves.

Furthermore, when an organism is exposed to a chemical, or mixture of chemicals, changes occur within an organism across multiple effect levels. Toxicological effects of a chemical act initially at the molecular level, acting upon genes and transcription factors, then at a biochemical level, causing changes within the organism, and finally at physiological levels, i.e. whole organism level. It is the aim of the project to study the

effects of steroidal pharmaceuticals across all of these effect levels, starting with changes at a molecular level.

1.10. General Aims and Objectives

The purpose of this study was to assess potential combination effects of mixtures of pharmaceuticals on ecologically important endpoints *in vivo*. Two principle aquatic model organisms were employed to assess potential mixture effects from two different pharmaceutical classes; the antineoplastic agents, and the synthetic steroids. Both pharmaceutical classes are widely used and have been detected in the aquatic environment at a range of concentrations. In line with the aims of this project, compounds selected from both groups display characteristics that could lead to their classification as both 'similar' and 'dissimilar' compounds.

Chapter 2 of this thesis will examine the effects of antineoplastic pharmaceuticals and their mixtures on growth inhibition in a microalga bioassay using a standardised toxicity testing approach with the green algae *Raphidocelis subcapitata*. The research basis for this chapter was formed primarily as a training exercise in mixtures toxicology and was conducted in line with a separate project (EU FP7 PHARMAS), in collaboration with a fellow PhD student Graham Harris (GH). The mixture experiments conducted in this chapter were conducted by GH for the PHARMAS project. The author of this thesis contributed in performing some of the single compound assays, serving as training in mixtures toxicology research. Chapter 3 will investigate potential mixture effects of five synthetic steroids on fecundity in a freshwater fish model species, *Pimephales promelas*, (Fathead minnow). Chapter 4 will further investigate endocrine related effects due to exposure to a steroidal pharmaceutical mixture, on the fathead minnow, by analysis of a range of biological end points. Mechanisms of disrupted reproduction and endocrine function at the level of pituitary gonadotropins will be further investigated in Chapter 5. Chapter 6 and 7 will be a general project discussion and conclusion, respectively.

**Chapter 2: Toxicity of anti-cancer
pharmaceuticals and their mixtures in the
green microalgae *Raphidocelis
subcapitata*.**

2.1. Introduction

2.1.1. Chemotherapeutic Anti-Cancer Agents

Antineoplastic pharmaceuticals are one of the major treatment processes offered to patients presenting with a malignancy. The wide range of cancer types and causations has led to the development of a large number of anticancer pharmaceuticals. Different forms of cancers require treatment with different antineoplastic agents, and in the case of most treatments, multiple drugs are administered in combination (combination therapy). Chemotherapy (administration of antineoplastic pharmaceuticals) can also be used in combination therapies alongside surgery (to resect tumours), and radiotherapy (the use of high-energy radiation to shrink tumours and kill cancerous cells) (Rafi, 2005). In cancer patients, the normal balance between proliferation, growth and death of cells is disrupted. Cell growth and proliferation accelerate to an uncontrolled rate and cells undergo malignant transformation, leading to the formation of tumours. Chemotherapeutic agents are used to promote cell death (particularly of rapidly dividing cancer cells) and return the normal regulatory balance between cell growth and cell death. Since cells are dependent on a good supply of blood, nutrition and oxygen, as the tumour grows these factors are reduced and growth slows. As a result, antineoplastic agents are most effective during the earlier, fast-growing stage of cell proliferation, becoming less effective as the tumour growth slows and cells proliferate more slowly (Rafi, 2005). Since the majority of these drugs target DNA, interrupting the cell cycle and/or disrupting DNA repair to promote apoptosis, they have the potential to damage other healthy cells in the body. Thus anticancer compounds can be genotoxic, carcinogenic, mutagenic, and/or teratogenic (Kummerer, 2001; Rowney et al, 2009; Lutterbeck et al, 2015.).

Since traditional anticancer pharmaceuticals target rapidly dividing cells, a fundamental characteristic of cancerous cells, rapidly dividing healthy cells can also be targeted. These compounds have no way of distinguishing between cancerous cells and healthy cells, thus many healthy cells are often destroyed or affected in the process, causing some of the

common side effects seen in patients undergoing treatment. Rapidly dividing healthy cells, such as hair follicle cells and those of the bone marrow and the digestive tract, can also become a target of traditional chemotherapeutic agents, resulting in the common side effects of treatment (i.e. hair loss, immunosuppression and mucositis) (Cancer Research UK). To achieve a more effective targeting of cancerous cells and to limit harmful effects to healthy cells, many anticancer agents are administered as pro-drugs, an inactive parent form that requires metabolisation in the body to exert its pharmacological effect. This means that the drug can be transported to the tumour site before being transformed into the active metabolite, and can therefore target cells displaying neoplasia more effectively.

Chemotherapeutic drugs are often classified into two main categories, antineoplastics and endocrine therapy agents (Besse et al, 2012). Within the antineoplastics, two further subclasses separate chemotherapeutic agents by mechanism of action; the cytotoxic compounds, which are those that act directly and/or indirectly on DNA to cause metabolic and morphological changes to the cell, eventually leading to its apoptosis; and the cytostatic compounds, which are those that do not act directly on DNA but work via other mechanisms, such as blocking cell growth factors (Besse et al, 2012). The latter group includes the alkylating agents, antimetabolites, antibiotics and the mitotic inhibitors. Cytotoxic agents are conventional drugs that work by promoting cell death through various mechanisms, whereas cytostatic drugs are molecular targeted therapies which do not kill the cell but halt their proliferation, stopping tumour growth and preventing metastases (Rixe and Fojo, 2007). In reality, cytotoxic and cytostatic agents are not strictly dissimilar in terms of their mechanisms of action. Many cytotoxic drugs also display cytostatic activity on the cell, and vice versa (Blagosklonny, 2004). Nevertheless, the differences between the mechanisms of action of cytotoxic and cytostatic agents result in different drug delivery regimes during therapeutic treatment. Cytostatics tend to be administered continuously while cytotoxics are usually administered as a high 'pulse' dosage at regular intervals. Compounds from each of the two classes are often administered together in

combination therapy, whereby the drugs work together - the cytostatic compound halts the cell cycle at a certain stage, allowing the cytotoxic compound to promote cell death before the cell cycle has been completed. Their administration regimes will significantly affect their presence and fate in the environment.

Enzyme inhibitors, drugs that inhibit certain enzymes that undertake crucial functions within the steroidogenesis pathways, are also useful in treating certain malignancies. Although not currently approved for the treatment of cancer, Finasteride acts by inhibiting 5 α -reductase, the enzyme responsible for converting testosterone to dihydrotestosterone (DHT). DHT is the main androgen that promotes prostatic growth, therefore a reduction in DHT in the prostate and plasma can lead to a reduction in prostatic growth. The drug is currently approved for the treatment of benign prostatic hyperplasia, and is currently under long term review for use against prostate cancer.

2.1.2. Antineoplastic Usage

There are over 50 antineoplastic pharmaceuticals routinely used across hospitals in the developed world (Johnson et al, 2008; Kosjek and Heath, 2011). The nature of usage of these drugs is also increasing, due to the increase in cancer incidence across the global population. Current projections suggest that the demand for anticancer treatments is rising by around 10% a year (Johnson et al, 2008; DoH, 2004). Therefore usage of these compounds will continue to increase, potentially leading to higher amounts of these compounds entering the environment. In the UK, as in many other developed countries, the use of these compounds is steadily increasing (table 1.)

Table 1. Trends in antineoplastic usage in the UK from 2006 to 2010. IMS Health

Drug	Trends in Usage in kilograms				
	2006*	2007*	2008*	2009*	2010*
Hydroxycarbamide	6219	6566	6839	7673	9836
Fluorouracil	1075	1373	1328	1335	1320
Imatinib	540	595	673	745	734
Cyclophosphamide	222	232	256	281	270
Methotrexate	153	160	177	184	202
Carboplatin	47	51	58	58	57

* Based on figures of community and hospital prescriptions

2.1.3. Anticancer pharmaceuticals in the environment

As discussed in Chapter 1, pharmaceuticals and pharmaceutical derivatives have been detected in the aquatic environment in recent years, and are becoming a topic of increased concern. Antineoplastic agents in the environment have up until recently been largely overlooked, however they are now considered as emerging contaminants, and are thought to be of key concern due to the nature of their mechanisms of action (Buerge et al, 2006; Elersek et al, 2016;) and increased global usage. They have the potential to be cytotoxic, genotoxic, mutagenic and tetragenic in non-target species. Despite this, there is still limited information on the environmental toxicities of these drugs, however they are believed to be of potential risk to aquatic organisms (Kümmerer 2001; Besse et al, 2012). Since antineoplastic agents are designed to target DNA, and DNA is common to all living organisms, there is a high likelihood that these drugs will cause adverse effects in any organism exposed to them at concentrations high enough to induce effects. In principle, due to their mode of action, it is supposed that all eukaryotes are vulnerable to damage

from antineoplastic agents (Kümmerer, 2001; Johnson et al, 2008; Kosjek and Heath, 2011; Besse et al, 2012).

Cytotoxic and cytostatic pharmaceuticals are administered intravenously, orally or topically, with dosage varying according to patient weight or body surface area (Rowney et al, 2009). Most agents are administered on hospital wards by doctors, although this is gradually changing, with a shift towards more home-based treatments. Due to the nature of administration, the route of transmission of these drugs into the environment differs somewhat from more regular, over the counter pharmaceuticals. Hospitals act as point source inputs of antineoplastic agents into the environment. As a consequence they appear in relatively high concentrations in hospital effluent and treatment plants, and therefore their distribution in the environment is likely to be more localised. Since many patients receiving treatment are outpatients, they typically return home soon after being administered with chemotherapeutic treatment, and therefore excretion of the drugs also occurs while the patient is away from the hospital. As a result, these compounds also appear in municipal waste water treatment plants, albeit at lower concentrations.

Attention has only recently been focused towards measuring environmental concentrations of antineoplastic agents in surface waters and effluents. Recent studies have begun to investigate the chemical properties of these drugs in relation to predicting their environmental persistence, including for example, the solubility, dissociation constant (pK_a) bioconcentration factors (BCF), octanol-water partition coefficient ($\log K_{ow}$), vapour pressure, organic carbon partition coefficient (K_{oc}), and atmospheric OH rate. For a detailed review of the physio-chemical properties of these chemicals in the environment, the reader is referred to Kosjek and Heath (2011).

Cytostatic agents are typically polar molecules. Given that water itself is a polar molecule, polar substances usually dissolve readily in water, suggesting that these compounds will become bioavailable to aquatic organisms. They are considered to be relatively persistent,

and likely to pass through WWTPs to dissipate widely in surface waters (Kosjek and Heath, 2011). The group include a wide range of drugs with vastly different modes of action and differences in the bioavailability of these substances in surface waters.

Most cytostatic drugs are not readily biodegradable; however, advanced oxidation processes, ozonation and chlorination are thought to be sufficient to remove many of these compounds during the wastewater treatment process (Kosjek and Heath, 2011). However, this belief is currently based on knowledge of the chemical structures and known modes of action, and has not yet been demonstrated in definitive tests. Further research is required to improve knowledge and understanding of the fate and behaviour of these drugs in the environment.

To further complicate the question of environmental relevance of these compounds, analytical chemistry techniques are often the limiting factor when determining environmental concentrations. Many analytical techniques employed to measure concentrations of these drugs in real world samples are not sensitive, specific or accurate enough to detect the compounds at the low concentrations usually present (Kosjek and Heath, 2011). Despite this, antineoplastic agents have been detected in the aquatic environment (Steger-Hartmann et al, 1996; Steger-Hartmann et al, 1997; Ternes, 1998; Buerge et al, 2006). For example, a study by Aherne et al (1985) detected the cytotoxic pharmaceutical methotrexate in hospital effluent of an oncology clinic at concentrations of 1µg/l, and in downstream river systems and potable water resources at around 6.25ng/l (Aherne et al, 1990). Modelling and predictive techniques have recently been applied to predict concentrations of antineoplastic agents in surface waters. Besse et al. (2012) calculated Predicted Environmental Concentration (PEC) values for French surface waters for a wide range of anticancer agents based on consumption trends (using national and local data sets), pharmacological properties, metabolism and excretion rates, dilution factors, and WWTP removal rates. Based on these criteria, the study predicted the compound hydroxyurea (hydroxycarbamide) to be present in the highest concentrations,

with a conservative PEC value of around 156.13ng/l, and a refined PEC value of 78.07ng/l (based on 2008 consumption data). Fluorouracil and Imatinib PEC values put them within the top 4 high ranking anticancer drugs, and cyclophosphamide, methotrexate and carboplatin were all within or close to the top 20, based on conservative PEC values. In a similar study by Brooker et al (2014), antineoplastic compounds were ranked and prioritised with regards to environmental risk. The study considered consumption rates, human metabolism and excretion, discharge sources, and the fate and behaviour of the compounds once in the environment. Cyclophosphamide was predicted to have the highest PEC, with concentrations of 4.1ng/L and 40.9ng/L in surface waters and effluent respectively. Hydroxyurea was predicted a lower concentration than that predicted by the Besse study, with 0.5ng/L and 4.7ng/L in surface waters and effluent respectively. The differences in predicted concentrations observed between these two studies highlights the potential for large disparities in predicted concentrations, most likely due to the parameters incorporated during the modelling, and regional data differences. Nevertheless, despite the differences, the predicted concentrations are within a similar range (i.e. ng/L), and therefore disparities between predictions remain relatively inconsequential.

Table 2. The Predicted Effect Concentrations of anticancer agents in French surface waters based on 2008 prescription data, before and after refinement from the model. Adapted from Besse et al, 2012.

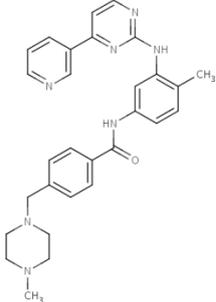
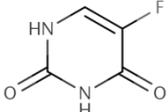
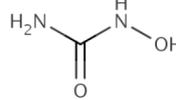
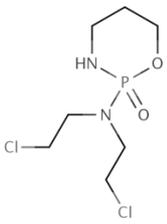
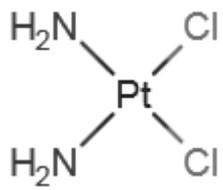
Compound	Class	Conservative PEC (ng/L)	Fraction Excreted (unchanged)	Refined PEC (ng/L)
Hydroxycarbamide	Enzyme inhibitor	156.13	0.50	78.07
5-FU	Antimetabolite	39.57	0.20	7.91
Imatinib	Tyrosine kinase (Tk) inhibitor	19.95	0.25	4.99
Cyclophosphamide	Alkylating agent	6.98	>0.25	>1.74
Methotrexate	Antimetabolite	1.71	0.90	1.54

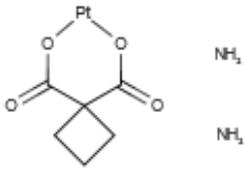
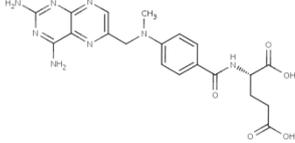
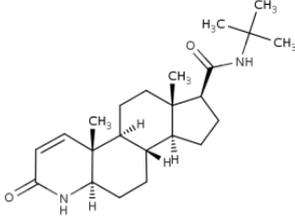
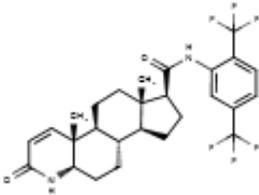
Since being recognised as a potentially important risk group to the environment (both wildlife and human health), an increasing amount of studies have been undertaken to establish toxicity responses in non-target organisms. *In vivo* and *in vitro* studies on a range of organisms from biofilms and microalga, to invertebrates and higher vertebrates have shown that this pharmaceutical group and their mixtures have the potential to cause adverse effects. Studies have demonstrated that aquatic organisms can be sensitive to antineoplastic compounds (Zounkova et al, 2007, Zounkova et al, 2010; Brezovšek et al, 2014). Though many antineoplastic compounds are administered as prodrugs, and it is known that not all aquatic species have the enzymes required to metabolise them into their active forms, there is still relatively little known about non-specific enzyme transformation, for example in aquatic plants and microbes. Furthermore, cytotoxic compounds directly targeting DNA without the need for biotransformation could be expected to affect all organisms.

2.1.4. Target drugs:

Target drugs were selected on the basis of their usage and likely presence in the environment. Additionally, compounds were also selected to encompass a range of mechanisms of action, in order to investigate effects of dissimilarly acting chemicals individually and in a mixture. All of the pharmaceuticals tested target and destroy cancer cells by interrupting DNA replication, though they act through a range of different mechanisms and pathways. The compounds selected for this study are shown in table 3.

Table 3. Anticancer pharmaceuticals utilised in the presented study.

Compound	Structure	Mechanism of Action
<p>Imatinib mesylate CAS: <u>152459-95-5</u> <u>Cayman Chemicals</u> Chemical Formula: $C_{29}H_{31}N_7O$</p>	 <p>The structure shows a central pyrimidopyrimidine ring system. One ring is substituted with a methyl group and a methoxy group. The other ring is substituted with a methyl group and a methoxy group. The central ring is linked to a piperazine ring via a methylene group.</p>	<p>Enzyme inhibitor - Tyrosine kinase</p>
<p>5 Fluorouracil (5-FU) CAS: <u>51-21-8</u> Sigma-Aldrich Chemical Formula: $C_4H_3FN_2O_2$</p>	 <p>The structure is a pyrimidine ring with a fluorine atom at the 5-position and carbonyl groups at the 2 and 4 positions.</p>	<p>Antimetabolite; inhibits DNA synthesis</p>
<p>Hydroxyurea (Hydroxycarbamide) CAS: <u>127-07-1</u> Sigma-Aldrich Chemical Formula: $CH_4N_2O_2$</p>	 <p>The structure shows a central carbon atom double-bonded to an oxygen atom and single-bonded to a nitrogen atom with a hydroxyl group. The carbon is also single-bonded to another nitrogen atom with a hydrogen atom and a methyl group.</p>	<p>Enzyme inhibitor - Ribonucleotide reductase</p>
<p>Cyclophosphamide CAS: <u>15-80-0</u> Sigma-Aldrich Chemical Formula: $C_7H_{15}Cl_2N_2O_2P$</p>	 <p>The structure shows a six-membered ring containing one phosphorus atom and one nitrogen atom. The phosphorus atom is double-bonded to an oxygen atom and single-bonded to a nitrogen atom. The nitrogen atom is single-bonded to a chlorine atom and a methyl group. The phosphorus atom is also single-bonded to a chlorine atom and a methyl group.</p>	<p>Alkylating agent; covalent binding to DNA</p>
<p>Cisplatin CAS: <u>15663-27-1</u> Sigma-Aldrich Chemical Formula: $Cl_2H_4N_2Pt$</p>	 <p>The structure shows a central platinum atom (Pt) coordinated to two ammonia (H₂N) groups and two chlorine (Cl) atoms in a cis configuration.</p>	<p>Alkylating agent; Platinum analogue</p>

<p>Carboplatin CAS: 41575-94-4 Sigma-Aldrich Chemical Formula: C₆H₁₂N₂O₄Pt</p>		<p>Alkylating agent; Platinum analogue</p>
<p>Methotrexate CAS: 59-05-2 Sigma-Aldrich Chemical Formula: C₂₀H₂₂N₈O₅</p>		<p>Antimetabolite; inhibits DNA synthesis</p>
<p>Finasteride CAS: 98319-26-7 Chemical Formula: C₂₃H₃₆N₂O₂</p>		<p>Enzyme inhibitor - 5 α reductase</p>
<p>Dutasteride CAS: 164656-23-9 Chemical Formula: C₂₇H₃₀F₆N₂O₂</p>		<p>Enzyme inhibitor - 5 α reductase</p>

2.1.4.1. Imatinib mesylate

Imatinib mesylate (Table 3) is a first generation Bcr-Abl tyrosine kinase inhibitor that inhibits Bcr-Abl tyrosine kinase enzyme activity to prevent phosphorylation by ATP. It is widely used for the treatment of chronic myelogenous leukaemia (CML), a cancer of the white blood cells. The drug is administered orally, with typical doses of around 400-600mg per patient per day. It is metabolised in the liver by the cytochrome P450 enzymes, and excreted primarily in faeces as metabolites. However, 25% of the drug is excreted unchanged (www.drugbank.ca).

In patients, the drug is well absorbed and well tolerated, with high bioavailability (98%), and less severe side effects when compared with other antineoplastic drugs. Cells that express Bcr-Abl are targeted and undergo inhibition of growth and cell death, whereas normal healthy cells remain unaffected (Marcucci et al, 2004). Elimination half-life of the parent compound in the human body is around 18 hours, and 40 hours for the active metabolite N-desmethylimatinib. Excretion is via the urine and the faeces over 7 days (BCCA Cancer Drug Manual). Approved in 2001 by the FDA, it is now widely used to treat CML, with high success rates. In up to 75% of patients CML progression is prevented and in up to 50% complete normal bone marrow function is resumed (Katzung 2006). However, since leukemic cells persist in patients indefinitely, continued treatment is required throughout the patient's lifetime (American Cancer Society).

2.1.4.2. 5-Fluorouracil (5-FU)

5 Fluorouracil (Table 3) is a pyrimidine analog of uracil, and acts primarily as an antagonist, inhibiting thymidylate synthesis, a crucial enzyme in DNA synthesis and repair (the nucleic base pairs cytosine, thymine and uracil are all pyrimidine derivatives) by causing a shortage of thymidine triphosphate (dTTP). It belongs to the antineoplastic antimetabolite drug group (structural analogs), which act on the metabolism of proliferating cancer cells by targeting the biochemical pathways and enzymes that lead to cell replication. It is a cell cycle-specific agent (CCS), only having an effect on the cell during the S-phase of the cycle. These compounds work by imitating purine or pyrimidine (the latter in the case of 5-FU), thereby preventing these substances from being incorporated into DNA during cell cycle S phase and hence preventing normal cell division (DrugBank). 5-FU is a prodrug, requiring transformation in the body to become pharmacologically active. It is metabolised in the liver to several active metabolites, namely fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP), and one inactive metabolite, dihydrofluorouracil. FdUMP competes with uracil, forming covalent bonds with the enzyme

thymidylate synthase and is responsible for the aforementioned thymineless death. The active metabolite FUTP is incorporated into RNA, interfering with mRNA translation, whilst FdUTP can be incorporated into cellular DNA, leading to the inhibition of DNA synthesis and function. The drug is administered intravenously, usually at a dose of 15 mg/kg/d for 5 days, following a 24 hour infusion schedule, followed by 15mg/kg weekly. In patients the drug has a short metabolic half-life of around 15 minutes. Excretion of the parent compound in urine has been found to be <10% (BC Cancer Agency, 2012), however around 57% of the active metabolite can be excreted from the body (Heggie et al, 1987; www.drugbank.ca). It is a widely used treatment for colorectal cancer, and is also prescribed for the treatment of cancers of the breast, ovary, prostate, stomach, skin, pancreas, bladder, oesophagus, liver, head and neck. It is one of a number of older chemotherapeutic agents that has been in continued use for over 40 years.

2.1.4.3. Hydroxycarbamide/Hydroxyurea

Hydroxycarbamide (table 3) is one of the most widely prescribed and heavily utilised antineoplastic pharmaceuticals. It is the most highly prescribed antineoplastic drug in the UK, with 9836 kilograms of the drug being administered in 2010 alone (IMS, 2011), and its usage is steadily increasing (table 1). It is a hydroxylated molecule of urea, and works by selectively inhibiting the synthesis of DNA in the S phase by inhibition of ribonucleoside reductase (RNR), the enzyme responsible for catalysing deoxyribonucleotides from ribonucleotides. The reduction in creation of deoxyribonucleotides prevents DNA synthesis by interrupting the conversion of the DNA bases. It also inhibits the incorporation of thymidine into DNA, causing direct DNA damage, and reducing DNA repair. This compound is often used in combination therapies with radiation and other chemical agents that cause DNA damage to cancerous cells.

It is usually administered orally, with typical doses of around 300mg/m² for 5 days, metabolised in the liver and excreted via the urine. Up to 50% is excreted as an unchanged form, and around 30% as the inactive metabolite urea. It has close to 100% bioavailability, and has a half-life of 3-4 hours in the human body. It is used in the treatment of melanoma, chronic myelocytic leukemia (CML), acute myeloid leukemia (AML) and recurrent or metastatic carcinoma of the ovary, as well as in cancers of the head and neck in combination with radiotherapy. In research trials, hydroxycarbamide has been shown to have dose-dependent synergistic activity with the antineoplastic agent cisplatin *in vitro*.

2.1.4.4. Cyclophosphamide

Cyclophosphamide (Table 3) is an alkylating agent used in the treatment of a variety of cancers and autoimmune disorders. It was developed in the 1950s as nitrogen mustard and is still in widespread use in chemotherapy treatments, particular in combination with other antineoplastic agents. It is a cell cycle nonspecific agent (CCNS), meaning it has antitumor effects at all/any of the cell cycle phases (Craig and Stitzel, 2004). It is used in combination therapies for the treatment of malignant lymphomas, leukaemia, multiple myeloma, neuroblastoma, retinoblastoma, and cancers of the breast and ovary.

Cyclophosphamide is a pro-drug that requires hepatic metabolism to its main active metabolites, phosphoramidate mustard and acrolein. Intermediate metabolites 4-hydroxycyclophosphamide and aldophosphamide are carried in circulation to the sites of tumours and undergo further metabolism to the final active metabolites at the site of action (SMPDB.ca). Once metabolised to its active form, the compound transfers an alkyl group to DNA, forming cross-links in the guanine base pairs (and to a lesser extent adenine and cytosine base pairs) of the DNA double helix, preventing the strands from uncoiling and separating (cross linking). Since this process is necessary for DNA replication, replication is prevented and tumour growth is halted (Chu and Sartorelli,

2007). Cyclophosphamide also suppresses T cell and B cell function, the mechanism that permits successful treatment of rheumatic conditions with this drug (Clements et al, 1974).

Metabolism and activation is carried out by various cytochrome P450 enzymes in the liver. The drug is usually administered orally at doses of 3.5-5mg/kg per day for 10 days, and/or 1g/m² as a single dose intravenously. It has a half-life of 3-12 hours in the human body and excretion is via urine and bile. 10-20% of the drug is excreted as unchanged compound.

2.1.4.5. Carboplatin

Carboplatin (table 3) is a second generation platinum analog, an alkylating agent that causes an inhibition of cell division. The drug works by forming crosslinks between the guanine base pairs, preventing the strands from undergoing separation during the normal replication process. The drug also acts via the addition of a methyl group (or other alkyl groups in similar drugs) to DNA bases, causing DNA fragmentation by repair enzymes trying to replace the alkylated bases. The addition of alkylated bases also causes mistakes in base pairing and miscoding of DNA by the base pairs, leading to mutations. As with other alkylating agents, the drug is non cell cycle specific, causing interruption of DNA function and cell death at all stages of the cell cycle (Katzung, 2006). It is similar in structure to its predecessor Cisplatin, and is now used in place of Cisplatin in combination therapies since it demonstrates significantly lower renal and gastrointestinal toxicity (Duffull and Robinson, 1997). Since the drug displays lower potencies than its predecessor, it can be less effective than cisplatin in successfully treating certain cancers. As a result, carboplatin tends to be administered in much higher doses than Cisplatin to achieve similar desired effects. Carboplatin is used in the initial treatment of advanced ovarian carcinoma, small cell and non-small cell lung cancers, cancers of the head and neck, oesophageal cancers, and solid tumours of genitourinary cancers (Katzung, 2006).

The drug has high bioavailability, a half-life of 1-2 hours in plasma and a clearance rate from the human body of around 4.4 L/hour (DrugBank). Excretion is via renal pathways. The drug is extremely stable, with up to 90% of the active compound being excreted unchanged in the urine. In clinical oncology, both carboplatin and cisplatin have been shown to interact with other antineoplastic agents to produce a synergistic effect.

2.1.4.6. Cisplatin

Cisplatin (table 3) is an alkylating platinum compound similar to carboplatin that prevents cell division by cross-linking of guanine bases, thus preventing the unwinding of DNA strands that precedes cell division and DNA replication. This compound, like carboplatin, also works by attaching alkyl groups to the bases to prevent DNA synthesis via fragmentation by repair enzymes (Drugbank.ca). Cisplatin is typically used in combination therapy with other cytotoxic agents for various forms of cancers, including the bladder, brain, cervix, lung, testicles, prostate, liver and kidneys. It has higher general toxicity than the second generation platinum analogue carboplatin, and for this reason, carboplatin is preferentially used over cisplatin in the treatment of many cancers.

2.1.4.7. Methotrexate

Methotrexate (table 3) is an antineoplastic antimetabolite that targets cancer cells through an inhibition of DNA and RNA synthesis, and the synthesis of thymidylates and certain proteins. It is a folic acid antagonist, causing the inhibition of dihydrofolate reductase (DHFR), the enzyme that converts dihydrofolic acid to tetrahydrofolate (or tetrahydrofolic acid). Methotrexate has a similar structure to folic acid and a high binding affinity to DHFR, thereby inhibiting the enzyme from binding folic acid and converting it to the much needed tetrahydrofolic acid. Tetrahydrofolate is required as a vital component of DNA synthesis (drugbank.ca). Methotrexate targets rapidly dividing cells, selectively affecting neoplastic and psoriatic cells. It is used as a treatment for gestational choriocarcinomas, chorioadenoma destruens, and non-cancerous molar pregnancies. It is used in

combination with other antineoplastics in the treatment of lymphocytic leukaemia, meningeal leukaemia, and advanced stage non-Hodgkin's lymphomas. It is also used alone or in combination for treatment of T-cell lymphoma, cancers of the breast, epidermoid cancers of the neck and head, and lung cancer. Additionally, methotrexate possesses immunosuppressive activity and is used successfully to treat autoimmune disorders such as rheumatoid arthritis, psoriatic arthritis, psoriasis, lupus, and sarcoidosis. The drug has around 60% bioavailability in the human body (decreasing at higher doses), and a half-life of between 3 and 15 hours depending on dosage. It is metabolised in the liver and excreted largely in the urine (drugbank.ca).

2.1.4.8. Finasteride

Finasteride (table 3) is a 4-azasteroid compound used in the treatment of benign prostatic hyperplasia, and androgenic alopecia. It inhibits Type II 5 α -reductase, an intracellular enzyme responsible for the conversion of testosterone into 5 α -dihydrotestosterone (DHT). By inhibiting the enzyme responsible for converting testosterone to DHT, the drug causes a significant decrease in tissue and plasma levels of DHT, and an increase in the levels of testosterone in the prostate and plasma. Since DHT is the main androgen responsible for prostatic growth, the drug is effective in treating enlarged prostate conditions. Although this drug has a much higher selectivity towards the type II form, it is also found to elicit an effect on type I 5 α -reductase form when administered in high doses (drugbank.ca). Finasteride is not currently approved for treatment of prostate cancers, despite being the focus of ongoing studies to determine the drug's usefulness in cancer treatment. The drug is still under consideration as to whether it could be effectively used as a treatment for prostate cancer.

2.1.4.9. Dutasteride

Dutasteride (table 3), like finasteride, is a competitive and specific 5-alpha reductase inhibitor, but unlike finasteride, it inhibits both isoforms of alpha-reductase. Type I 5-alpha-

reductase is pre-dominant in the sebaceous gland and is responsible for around a third of circulating DHT. It is used for the treatment of benign prostatic hyperplasia. However, studies have found that the dual inhibition of both isoforms by dutasteride could inhibit tumour growth in hormone refractory prostate cancer (Arena, 2008).

2.1.5. Mixture Toxicity of antineoplastic pharmaceuticals

As previously discussed, these compounds are often administered in combination therapy, since they have been shown clinically to cause synergistic and antagonistic effects (Tanaka et al, 2001). Recent studies have demonstrated the potential of antineoplastic pharmaceuticals to produce mixture effects in non-target species, including microalgae (Vannini et al, 2011; Brezovšek et al, 2014; Cesen et al, 2016; Elerseck et al, 2016).

2.1.6. Microalgae as model species in ecotoxicology

Planktonic microalgae form a vast group of photosynthetic, heterotrophic organisms and are crucially important in the aquatic environment, forming a central part of the food chain. They sustain the ecosystem, providing food for zooplankton which are subsequently fed upon by larger invertebrates and vertebrates. They are a substantial producer of atmospheric oxygen, and also play a key role in water quality (Willén, 2000). Algae are often used as sentinel species in environmental monitoring, both in the present as an indicator species, and from geological samples as indicators of past environmental conditions, suggesting that algae can be sensitive to environmental changes, including that caused from chemical pollutants. Changes in the composition and structure of microalga communities can cause significant changes to an ecosystem, both directly and

indirectly (Aruoja, 2011). Therefore in the context of environmental quality, microalga species are extremely useful model species.

Studies suggest that many toxins and chemicals present in the environment affect taxa and species differently. Thus risk assessment procedures for environmental water quality now often include toxicological testing for representative species across three main taxa, namely vertebrates, invertebrates and aquatic plants. A number of studies have also found that some microalga species have greater sensitivity to certain pollutant chemicals than higher organisms such as vascular plants (Juneau et al, 2003).

The majority of toxicity tests involving microalga species use inhibition of growth as the major toxic end point (Aruoja, 2011). This endpoint is easily monitored and quantified in artificial laboratory settings, and has been used as an established end point to assess a range of toxicants. Furthermore, toxicity screening using microalgae is rapid, and since microalgal cells are fast growing, such screens can be considered as chronic, multigenerational bioassays.

2.1.7. Aims

- To screen a range of antineoplastic pharmaceuticals with varying mechanisms of action in order to quantify their effect on growth of a freshwater microalga.
- To test a range of antineoplastic pharmaceuticals in a standardised assay to select a smaller number of compounds for use in a multicomponent mixture study to assess the effects of mixtures of antineoplastic agents with varying mechanisms of action on growth inhibition in a freshwater microalga.
- To obtain training in the theory and practise of mixture toxicology using a simple bioassay prior to undertaking larger, more complex *in vivo* experiments using higher organisms.

2.1.8. Hypotheses and Null Hypotheses

Hypotheses

- A range of antineoplastic pharmaceuticals with various mechanisms of action will produce an inhibitory effect on growth of a freshwater alga individually, most likely at concentrations higher than those present in the environment.
- A simplistic multicomponent mixture of antineoplastic compounds will cause an inhibitory effect on growth of a freshwater alga that is larger than that of any of the single compounds individually.

Null hypotheses

- Antineoplastic pharmaceuticals will not produce any inhibitory effect on growth of a freshwater alga.
- Antineoplastic pharmaceuticals will cause an inhibitory effect on growth of a freshwater alga at concentrations that are reported in the environment.
- A multicomponent mixture of antineoplastic pharmaceuticals will not produce an inhibition of growth that differs from any of the single compounds individually (i.e. no combined mixture effects).

2.2 Materials and Methodologies

2.2.1. Test species: *Raphidocelis subcapitata* (synonym *Pseudokirchneriella subcapitata*)

R. subcapitata is a microalga species commonly used in toxicological studies. The taxonomic history of the species is complex, since it has been reclassified several times. It was originally described in 1914 as *Selenastrum capricornatum*, later renamed *Kirchnerellia subcapitata*, *Pseudokirchneriella subcapitata* and most recently *Raphidocelis subcapitata* in 1990. Several of these taxonomic synonyms are still commonly used to describe the species. *Raphidocelis subcapitata* and *Pseudokirchneriella subcapitata* are considered valid for taxonomy (www.algaebase.org).

R. subcapitata is a planktonic freshwater species, found commonly in both oligotrophic and eutrophic environments. It's cells are uninucleate with a single chloroplast, helical in shape, and smooth cell walls. Cells vary in size between 4-11µm in diameter (www.algaebase.org). They have parietal chloroplasts (chloroplasts that lie against the cell wall), and contain no pyrenoids (sub cellular micro-compartments within the chloroplasts). Reproduction is asexually by division of cells into daughter autospores, 2-4 per sporangium (Nygaard et al, 1986; F.Hindák, 1990: www.algaebase.org).

R. subcapitata has been shown to have increased sensitivity to toxicants compared to other species of microalgae. It also has a high growth rate and is relatively easy to culture. For these reasons, it is widely used as a preferred test species in ecotoxicology, and is recommended by the OECD, EPA and ISO (Aruoja, 2011). Furthermore, its status as a preferred, commonly used species in conventional culture assays has led to its incorporation into, and development of, standardised toxicity testing kits.

2.2.2. Standardised Toxicity Assay: Algal ToxKit F™; 72hr Freshwater Toxicity Test (MicroBioTests Inc.) www.microbiotests.be

An algal growth inhibition assay was used to assess the toxicity of anti-cancer pharmaceuticals on the growth rate of a freshwater alga, *Raphidocelis subcapitata*. The standardised test (Algal ToxKit F™, MicroBio Tests), developed in line with OECD 201 guidelines, is issued as a comprehensive kit containing all the necessary materials, including the test organisms, culture media and a description of the standardised protocols required to run the assay. Using a commercial kit such as this has many benefits. The assay is relatively simple to perform, eliminates the need for continuous organism culturing, gives rapid results (72 hours completion), and is reproducible (standardised test procedure). The kit can also be stored for several months without affecting the survival and growth of the algae, making this assay an ideal alternative to laborious culturing and maintenance of laboratory strains.

2.2.3. Test organisms

R. subcapitata were supplied in kit form as algal beads (> one million cells per bead, MicroBio Tests) preserved and immobilized in a matrix solution. The algal beads were re-activated at the start of the assay by the addition of a matrix dissolving medium and cultured using an algal growth medium (pH 8.1±0.2), which was supplied with each assay kit. Once the algae were mobilised, a concentrated algal inoculum was prepared. A dilution factor was applied to the algal stock to achieve a starting concentration of 1×10^6 cells/ml suspension, which was calculated based upon the standard curve issued with each specific test kit.

2.2.4. Single compound assays

To first obtain information of the general toxicity range of each chemical compound, an initial range-finding test was performed. A concentration chemical stock solution was made up in algal medium, and a tenfold dilution series was prepared (0.01-100mg/l). The algae cells were added to each toxicant concentration to obtain an initial concentration of 1×10^4 algae/ml. Each toxicant concentration was then transferred to 25ml long cells (made of biologically inert material) provided in the kit. Samples were assayed in triplicates to increase the reliability of the results. The location of the long cells was randomised in the holding tray, which was subsequently incubated at $23^\circ\text{C} \pm 2^\circ\text{C}$ for the remainder of the assay (72 hours). Cell density was recorded every 24 hours (24, 48 and 72hrs) until test completion at 72 hours. Algal growth was determined from optical density (OD) readings of each replicate algal suspension cell using a Jenway 6300 spectrophotometer measuring at a wavelength of 670 nm. Growth inhibition for each toxicant concentration was calculated using the data treatment procedure program provided with the kit (ISO Standard 8692 – Section 9), based on regression modelling (REGTOX, Eric Vindimian, Algal ToxKit FTM, MicroBio Tests). EC₅₀, EC₂₀, and EC₁₀ values were derived using the provided data program.

Range finding assays for the majority of the compounds were repeated once to ensure repeatability of results. Subsequent to completion of range finding assays, definitive tests were carried out for some of the compounds. A dilution series spanning the range from the lowest concentration producing 90-100% inhibition, to the highest concentration producing 0-10% inhibition relative to the control in the range finding assay, was incorporated (Algal ToxKit FTM SOP). Three of the nine compounds screened required the use of a solvent carrier, due to low solubility of these compounds in water (methotrexate, finasteride, and dutasteride). After a comprehensive review of the literature, DMSO was chosen as an appropriate solvent for the compounds and test species in question. For compounds that required the use of DMSO, a solvent control was included each time,

replacing the lowest concentration (0.01mg/L) in the assay. Given that the assay has a standardised protocol with six fixed treatment groups (one control plus five concentrations), and the typically low potency of these compounds, the lowest concentration was excluded to accommodate the solvent control.

The assay described above was utilised to screen six antineoplastic compounds individually. A further three compounds were tested by Dr Graham Harris (Brunel University London) during his PhD research as part of a collaborative study (the EU project PHARMAS). Based on the results of the single compounds, a five component mixture study was undertaken using five of the compounds selected for inclusion, based on a number of parameters (these are discussed in subsequent sections).

In addition, a reference chemical was screened in the assay prior to commencement of the pharmaceutical study to ensure that the assay and assay conditions were functioning correctly and to ensure adequate sensitivity. Potassium dichromate ($K_2Cr_2O_7$) was employed as the reference chemical, as advised in the kit protocol. A dilution series was prepared as above but with concentrations ranging from 0.18-18mg/L. Mean growth inhibition in the form of the E_rC_{50} (reduction in growth rate) after 72 hours was compared to the figure issued on the specification sheet from the corresponding kit (specific for algae batch, culturing medium and matrix medium).

2.2.5. Organic Solvents in Bioassays

In laboratory bioassays, the use of carrier solvents is often required due to the low water solubility of many chemicals. To ensure that tested chemicals are bioavailable, hydrophobic chemicals require dissolution in solvents prior to organism exposure. Therefore toxicity and stress effects caused by solvents are of concern in such bioassays. Although such assays should always incorporate a solvent control group, it is important to select the most appropriate solvent carrier, taking into consideration the test organism,

test conditions (i.e. oxidative properties, pH), and the chemical to be tested. The US Environmental Protection agency recommends maximum solvent exposure limits of 0.05% for acute tests and 0.01% for chronic tests for all organisms. However, limits should be tailored to individual studies depending on test conditions, since certain organisms may have higher solvent tolerances than others. Additionally, some chemicals are known to interact with solvents, resulting in alteration of their toxicity. This is the case for many pesticides, and has also been found to be true for some cytotoxic pharmaceuticals, notably the platinum compounds - cisplatin and carboplatin (Fischer et al, 2008). For reasons discussed above, the use of a solvent carrier in toxicity studies should be considered thoroughly.

2.2.6. Statistical Treatment of Data

To assess the reproducibility of the toxicity data obtained from testing the single compounds in the AlgalTox assay, a number of compounds were screened twice, using the same concentrations and experimental parameters in two separate experiments. Where compounds were repeated, a two-tailed student's t-test was used for comparison of the means of two groups to assess whether there was any statistical difference between data from the two separate experiments, and where there was found to be no difference, results from each experiment were pooled and treated as comparable replicates. The mean values of each time point (24, 48 and 72 hours) and each concentration were compared between the two experiments. Growth inhibition quantified as optical density was assessed for normality using descriptive statistics and histogram analysis. Where data were found to be normally distributed, a traditional Levene's test was used to test for homogeneity of variance, and if variances were found to be equal, statistical significance of growth inhibition was analysed by one-way Analysis of Variance (ANOVA), followed by Tukey's post hoc analysis. Non-parametric data (data that was not normally distributed) were tested for homogeneity of variance using a non-parametric

Levene's test and/or the Brown Forsythe test. Kruskal-Wallis analysis was used to determine statistical significance of non-parametric data where equal variance was found. Post hoc pairwise comparison was subsequently performed to compare growth inhibition between individual treatment groups.

2.2.7. Toxicity of antineoplastic mixtures

A basic mixture study was undertaken to test the effects of multicomponent mixtures of antineoplastic pharmaceuticals on growth of the green algae *R. subcapitata*. Since only partial concentration response curves were generated for two of the compounds, more sophisticated mixture modelling of the data was not possible.

Five of the nine antineoplastic drugs tested in the assay were selected for the mixture experiment; 5-fluorouracil (5FU), imatinib mesylate (IM), cisplatin (CS), hydroxycarbamide (HY) and cyclophosphamide (CY). Selection criteria were based on the quality of individual compound data, the mechanism of action of the compound, and the environmental relevance. Dutasteride was excluded due to problems with solubility. Methotrexate and finasteride were also excluded from the mixture due to the requirement for a solvent carrier to aid solubility of both of these compounds. Since all other compounds were tested without the use of a solvent, their inclusion in the mixture was considered to be unsuitable. Carboplatin was also excluded due to its low potency (no observable effects were recorded up to the highest concentration tested individually, 100mg/L). Since an effect concentration (EC_{50}) was required for the mixture design, it was not possible to include this compound. Furthermore, it was deemed unfeasible to test higher concentrations due to both the high cost of the compound and questions of environmental relevance of such high concentrations.

The mixture experiment was based on a simple ratio design with equipotent concentrations of the individual components (Cleavers, 2003). Since the mixture chosen

was made up of five compounds, each chemical was included within the mixture at 1/5th of its EC₅₀, calculated by regression modelling of the dose-response curves generated from the individual compounds by software supplied with the Algal Tox Kit F assay. This basic design affords observable mixture effects at an effect range that can be easily quantified. Table 4 shows the concentrations of each compound in the mixture. Two replicates of the mixture were run in parallel. Alongside the mixture, each compound was tested individually at 1/5th of its EC₅₀ (i.e. the concentration present in the mixture) to assess the effects of the individual chemicals at this concentration.

Table 4. Antineoplastic compounds selected for the mixture assay. EC₅₀ values are shown, as is the concentration of each compound in the mixture

Compound	EC50 in AlgalTox assay (µg/L)	1/5th of EC50 (µg/L)
5-flurouracil	435	87
Imatinib mesylate	6 430	1 286
Cisplatin	7 400	1 480
Hydroxycarbamide	97 200	19 440
Cyclophosphamide	3 179 000	635 800

This research was carried out in collaboration with Dr Graham Harris (GH), a former PhD student also at the Institute of Environment, Health and Societies, Brunel University London. The assays testing the compounds 5-flurouracil, cisplatin and cyclophosphamide were run by GH, as was the final multicomponent mixture study. All other assays were conducted by the author.

2.3. Results:

2.3.1. Test organisms, kit validation and method development

The assay was validated twice using the reference chemical potassium dichromate ($K_2Cr_2O_7$). On both occasions, the observed E_rC_{50} was within the 95% confidence limits of the mean E_rC_{50} documented on the kit specification sheet. Therefore, the assay was deemed to be performing appropriately and concluded to be sufficiently sensitive and specific enough to continue with the experiment.

2.3.2. Single Compound Data

Both data collected by the author and those collected by Dr. Graham Harris will be presented. Data on the individual compounds tested by the author are presented in more detailed analysis.

All but one of the compounds tested by the author as part of this study were found to inhibit growth of *R. subcapitata* in a dose-dependent manner. At 72 hours (assay termination), four compounds were shown to cause inhibition of growth in *R. subcapitata* (fig. 6). Carboplatin was the only exception, which had no observed effect on growth at the highest concentration tested (100mg/L). The most potent compound adversely affecting growth was imatinib mesylate, with an EC_{50} of 6.2mg/L (fig. 6). The results for dutasteride are not presented due to significant solubility problems with this compound. At the commencement of the assay, DMSO was shown to be a suitable solvent carrier for dutasteride, however over the course of the assay, the compound was visually observed precipitating out of solution as a cloudy white aqueous precipitate. This caused interference with the spectrophotometer readings due to colouration and turbidity of the samples in question, and therefore optical density readings could not be reliably obtained for this compound.

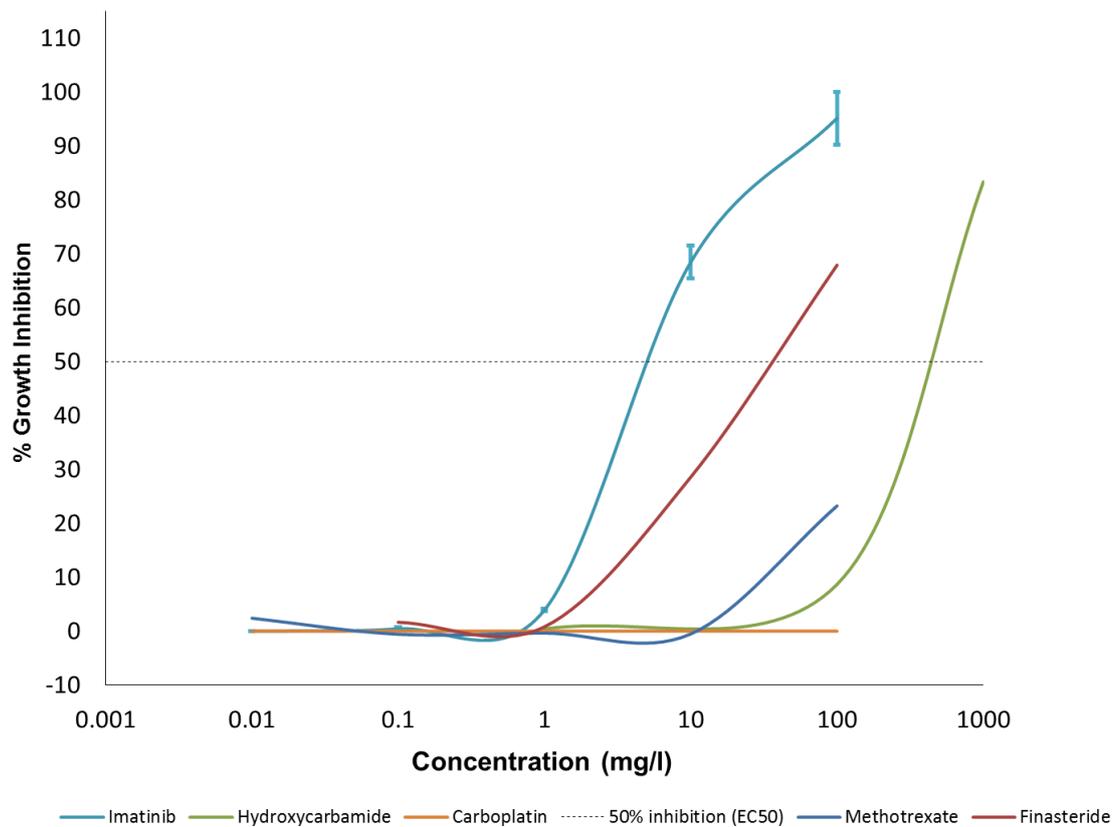


Figure 6. Percentage growth inhibition of *R. subcapitata* after 72 hours of exposure to five antineoplastic pharmaceuticals individually screened. SEM is included for one of the compounds (Imatinib mesylate) as this assay was performed twice and data from both experiments were found to be statistically similar, and hence combined.

2.3.3. Temporal trends in growth inhibition

Time series data were obtained since optical density readings were taken every 24 hours (up to 72 hours). The results demonstrate a temporal dose-response relationship for all four compounds that were shown to negatively affect growth. Growth inhibition was most markedly observed at the 72 hour time point. Rather than converting the raw optical density readings into percentage growth (as was done for the 72 hour concentration-effect data), the optical density values themselves were used to assess any temporal effects, in order to eliminate data conversion errors and for ease of statistical analysis. Data were assessed for normality by analysis of data distribution using histograms and descriptive statistics, and either parametric or non-parametric statistical tests were applied for comparison of differences between the means of treatment groups. Levene's Test for Homogeneity of Variances was performed on all data to test the assumption that the variances are equal and that chosen tests could be appropriately applied. For three of the compounds, namely imatinib mesylate (IMT), Hydroxycarbamide (HYD), and finasteride (FIN, the null hypothesis that the variances are the same in all treatment groups could be accepted ($p > 0.05$ for all compounds), and hence no transformation of the data was required. The Levene's Test for methotrexate (MET) revealed no homogeneity of variance in the data for two of the time points, therefore a Brown-Forsythe test was applied to ensure robustness (discussed below).

2.3.3.1. Imatinib mesylate

Imatinib mesylate caused an adverse effect on growth in a temporal dose-response manner (fig. 7a). Since IMT was tested in two separate repeated experiments, a two-tailed t-test was used to assess whether the data from the two separate experiments were similar and could therefore be combined to strengthen the statistical power of subsequent analysis. Mean temporal and concentration dependant data from both experiments were

found to be statistically similar (two-tailed t-test, -2.187 , $d.f=23$, $p=0.039$, $p<\text{critical value}$, 95% confidence level), and data was subsequently pooled to increase replicate number.

Data across all time points was found to be homogenous with no significant variation (Levene's Test ($p>0.05$) at 24, 48 and 72 hours; $F=0.632$, $d.f=5$, $d.f=30$, $p=0.677$; $F=1.107$, $d.f=5$, $d.f=30$, $p=0.377$; $F=2.349$, $d.f=5$, $d.f=30$, $p=0.065$; respectively). At 24 hours, there was no significant difference in growth between the treatments (one-way ANOVA, $F=2.242$, $d.f=5$, $p=0.076$). There was a statistically significant difference in growth between the control group and the three highest concentrations of 1mg/L, 10mg/L and 100mg/L at 48 hours (one-way ANOVA, $F=219.26$, $d.f=5$, $p<0.005$; Tukey's Post Hoc Test, $p<0.005$). The two highest concentrations, 10mg/L and 100mg/L, were not significantly different from one another (Tukey's Test, $p=0.998$), and the two lowest concentrations were not statistically different from the control (Tukey's Test, $p>0.05$). Growth at 1mg/L was found to be significantly different to all other treatment groups (Tukey's Test, $p<0.005$). As with the 48 hour time point, at 72 hours there was a significant difference in growth between treatments (one-way ANOVA, $F=2225.437$ $d.f=5$, $p<0.005$), with a dose-dependent relationship. The three highest treatments (1mg/L, 10mg/L and 100mg/L) were again statistically different from the control (Tukey's Test, $p<0.005$), and at the highest two concentrations, 10mg/L and 100mg/L, growth was statistically similar ($p>0.005$ Tukey's Test). Growth in the 1mg/L treatment was again statistically different to all other treatment groups (Tukey's Test, $p<0.005$).

2.3.3.2. Hydroxycarbamide

Hydroxycarbamide caused an adverse effect on growth in a temporal dose-response manner (fig. 7b). Homogeneity of variance was observed at all time points (Levene's Test, ($p>0.05$) at 24, 48 and 72 hours: $F=0.969$, $d.f=5$, $d.f=12$, $p=0.474$; $F=1.752$, $d.f=5$, $d.f=12$, $p=0.197$; $F=2.677$, $d.f=5$, $d.f=12$, $p=0.075$; respectively).

At 24 hours, no significant difference in growth of the algae between treatments was observed (one-way ANOVA, $F=1.992$, $d.f=5$, $p=0.152$). At 48 hours, there was a significant difference between treatment groups (one-way ANOVA, $F=34.745$, $d.f=5$, $p<0.005$). Post hoc analysis revealed there was no significant difference in growth between the control group and the three lowest concentrations of 0.01mg/L, 0.1mg/L and 1mg/L (Tukey's Post Hoc Test, $p>0.05$). A significant difference in growth was observed between the control group and the two highest concentrations, 10mg/L and 100mg/L (Tukey's Test, $p<0.05$ and $p<0.005$ respectively), with increasing inhibition at the higher concentrations. Furthermore, growth responses at the two highest concentrations were shown to be significantly different to each other and to all other groups (Tukey's Test, $p<0.05$ and $p<0.005$, respectively). Similarly, at 72 hours, growth was found to be significantly inhibited at higher concentrations (one-way ANOVA, $F=354.783$, $d.f=5$, $p<0.005$). The differences in inhibition were attributed to the two highest concentrations (10mg/L and 100mg/L), which were found to be significantly different to the control by post hoc analysis (Tukey's Test, $p<0.005$). As with the 48 hour time point, inhibition of growth in the 10mg/L and 100mg/L treatment groups were found to be statistically different from each other (Tukey's Test, $p<0.005$). Growth in the treatments 0.01mg/L, 0.1mg/L and 1mg/L was found to be statistically similar to the control group and to each other (Tukey's Test, $p>0.05$), therefore at the three lowest concentrations, the hydroxycarbamide did not inhibit growth.

2.3.3.3. Finasteride

Finasteride caused an adverse effect on growth in a temporal dose-response manner (fig. 8a). Homogeneity of variance of the data was observed at all time points ($F=2.384$, $d.f=5$, $d.f=12$, $p=0.101$; $F=2.364$, $d.f=5$, $d.f=12$, $p=0.103$; $F=2.889$, $d.f=5$, $d.f=12$, $p=0.061$); at 24,

48 and 72 hours respectively. Finasteride caused an inhibition of growth in a dose dependant manner, however this was only evident at the highest two concentrations.

In contrast to the other compounds analysed, finasteride caused a significant difference in growth between treatments at 24 hours ($F=3.448$, $d.f=5$, $p=0.037$). However, post hoc analysis revealed that the statistical difference was produced solely by the difference in inhibition between the 1mg/L and 10mg/L treatments (Tukey's Test, $p=0.022$). This was largely due to the reduced inhibition in the 1mg/L within the first 24 hours.

After 48 hours of exposure to finasteride, significant differences in growth were observed (one-way ANOVA, $F=32.893$, $d.f=5$, $p<0.005$). The two highest concentrations were found to have caused an inhibition of growth significantly different to that of the control (Tukey's Test, $p<0.005$). Further, these two groups were significantly different to all other treatment groups ($p<0.005$), but were found to be statistically similar to one another ($p=0.33$, $p>0.05$). Inhibition of growth for treatments 0.01mg/L, 0.1mg/L and 1mg/L, were statistically similar to one another, and to the control group ($p>0.05$), therefore no effect on growth was observed at these concentrations.

After 72 hours of exposure, in all treatment groups except 1mg/L, growth was found to be significantly different to the control (Tukey's Test, $p<0.05$). No significant difference in growth was found between the 0.01mg/L and 0.1mg/L treatment groups (Tukey's Test, $p>0.05$) and the 0.1mg/L and 1mg/L treatments (Tukey's Test, $p>0.05$). However, growth was statistically different between the 0.1mg/L and 10mg/L treatments ($p=0.02$, $p<0.05$). The two highest treatments 10mg/L and 100mg/L were statistically different to all other treatments and to each other (Tukey's Test, $p<0.005$).

2.3.3.4. Methotrexate

Methotrexate caused an adverse effect on growth in a temporal dose-response manner (fig. 8b). At the 24 hour time point, the data failed to fulfil the assumption of homogeneity of variance under a parametric classification (traditional Levene's Test, $F=3.183$, $d.f=5$, $d.f=12$, $p=0.027$ ($p<0.05$)), therefore a parametric one-way ANOVA could not be used to analyse the data, since this test assumes homogeneity. Data were subsequently transformed using log and square root transformations, and again failed the test for homogeneity under parametric assumptions. Data were then analysed for skewness and kurtosis using descriptive statistics and histogram analysis in SPSS, and found not to follow a normal Gaussian distribution. To account for the lack of statistical power and sensitivity to data variations of the traditional Levene's Test in analysing homogeneity of variance of data with considerable skew, the Brown-Forsythe test was utilised in its place. Instead of using the mean values, as in the Levene's test, the B-F test uses the median values for the point of comparison, and is better suited to data with distribution variations. Using this better suited method, the data variance was found to be homogeneous (B-F test, $F=0.619$, $p=0.688$, $p>0.05$).

Since transforming the data failed to normalise the distribution, analysis by Kruskal-Wallis (K-S), a non-parametric test, was adopted to compare data from the six treatment groups for this compound. Since Kruskal-Wallis also assumes homogeneity of variance, albeit in a non-parametric sense (i.e. data skew and kurtosis are similar in all groups), a non-parametric Levene's test was used to test for homogeneity of variance, whereby a one-way ANOVA of ranked data was used to compare the differences in mean ranks between treatment groups. Variance was shown to be homogeneous at the 24 hour time point (Non-Parametric Levene Test, $F=1.715$, $d.f=5$, $d.f=12$, $p=0.206$, $p>0.05$) and therefore the null hypothesis was accepted (that there was homogeneity of variance).

Since the assumption of homogeneity was satisfied, a Kruskal-Wallis test was employed to analyse differences between the ranked means. The K-S test found no significant difference of growth rate for treatments at 24 hours (Chi-Square, 7.097, $p=0.214$, $d.f=5$). Post hoc pairwise comparison was used to compare growth between treatment groups. Only the highest concentration, 100mg/L, was found to be significantly different from the control (Kruskal-Wallis, Chi Square =0.046, $p<0.05$). However, the difference was not significant enough to produce an overall statistically significant effect at this time point.

At 48 hours, data variance was found to be homogeneous in a traditional Levene's test ($p>0.05$) (based on the mean values) (Levene's Test, 1.063, $d.f=5$, $d.f=12$, $p=0.427$). Significant differences in growth were observed corresponding to treatment (one-way ANOVA, $F=12.320$, $d.f=5$, $p<0.005$). Post hoc analysis revealed the differences were largely attributed to the highest treatment (100mg/L), which was the only treatment to cause a statistically significant difference in growth when compared to all other treatments (Tukey's Test, $p\leq 0.005$).

As with the 24 hour time point, at 72 hours, data were found to be not normally distributed, and were therefore treated as non-parametric data. Skew and kurtosis were analysed using descriptive statistics and found to vary between groups. As discussed above, since the traditional Levene's Test is not suited to data where variation is not homogeneous, the Brown-Forsythe (B-F) test was used to assess homogeneity of variance using the median values for the point of comparison. The data were found to be homogenous (B-F test, $F=0.60$, $p=0.701$, $p>0.05$, rejection of null hypothesis), therefore allowing a K-S test to be performed. Growth was found to vary significantly due to treatment (Chi Square=12.322, $df=5$, $p<0.05$). At the highest concentration, 100mg/L, growth was significantly different to the control and all other treatment groups, including the solvent control. At 72 hours, growth in the solvent control group was found to be significantly different to the control group, and the other treatment groups (K-S test, Chi Squared=3.857, $df =1$, $p\leq 0.05$).

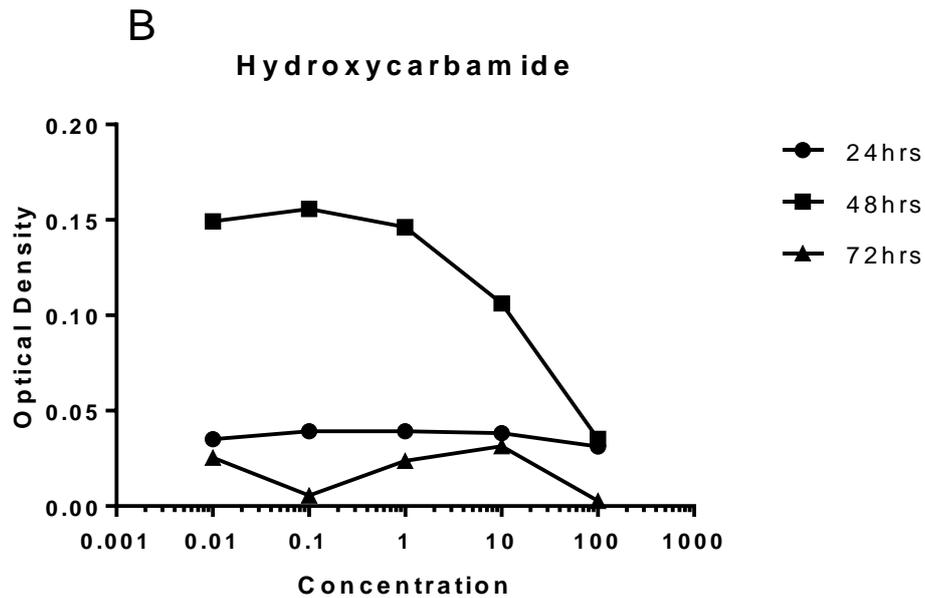
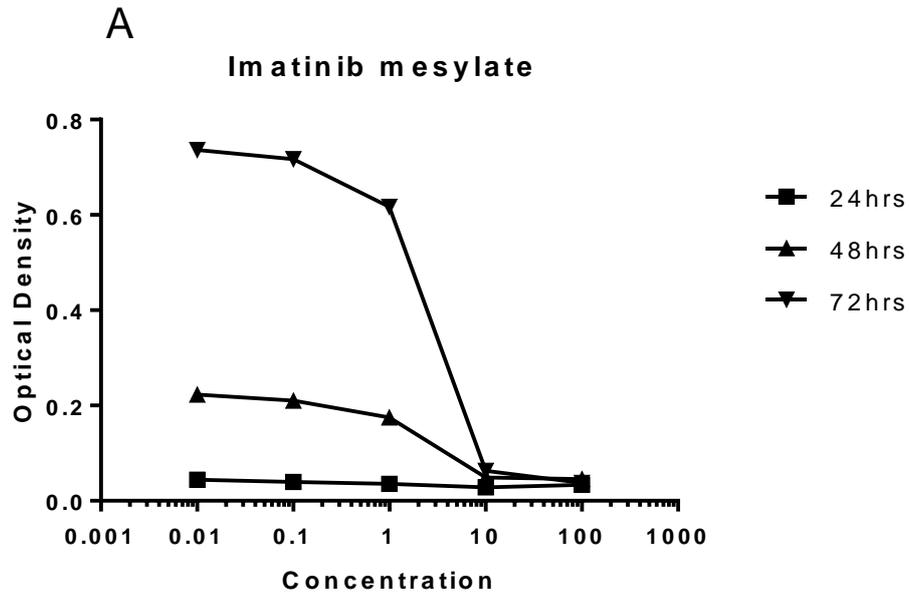


Figure 7. Temporal response of growth inhibition of *R. subcapitata* exposed to (a) imatinib mesylate, and (b) hydroxycarbamide. Growth is represented as optical density quantified from a spectrophotometer. *Optical Density (OD) represents a proxy for growth inhibition; higher OD values indicate a higher number of algae cells and therefore more growth.

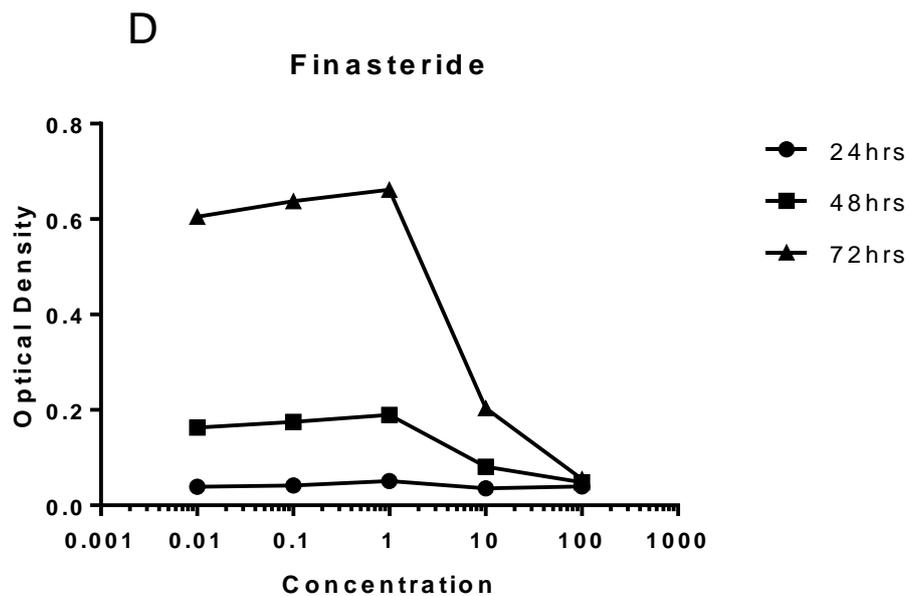
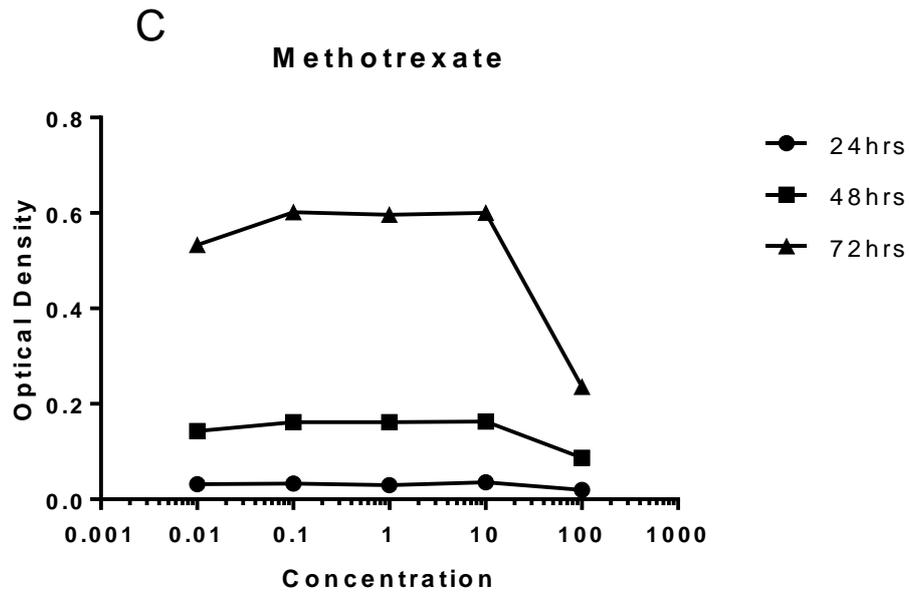


Figure 8. Temporal response of growth inhibition of *R. subcapitata* exposed to (a) methotrexate, and (b) finasteride. Growth is represented as optical density quantified from a spectrophotometer. *Optical Density (OD) represents a proxy for growth inhibition; higher OD values indicate a higher number of algae cells and therefore more growth.

For the purpose of addressing potential impacts of mixtures of antineoplastic pharmaceuticals on a freshwater microalga species, results from three additional compound tested by Dr Graham Harris have been included in the following analysis. Three compounds discussed in the introductory section of this chapter were tested in the algae toxicity screen assay by Dr Graham Harris. Figure 9 (below). is a reproduction of figure 6, with the inclusion of data from 5-Fluorouracil (5-FU), cyclophosphamide (CYL) and cisplatin (CIS), which was provided by Dr Graham Harris.

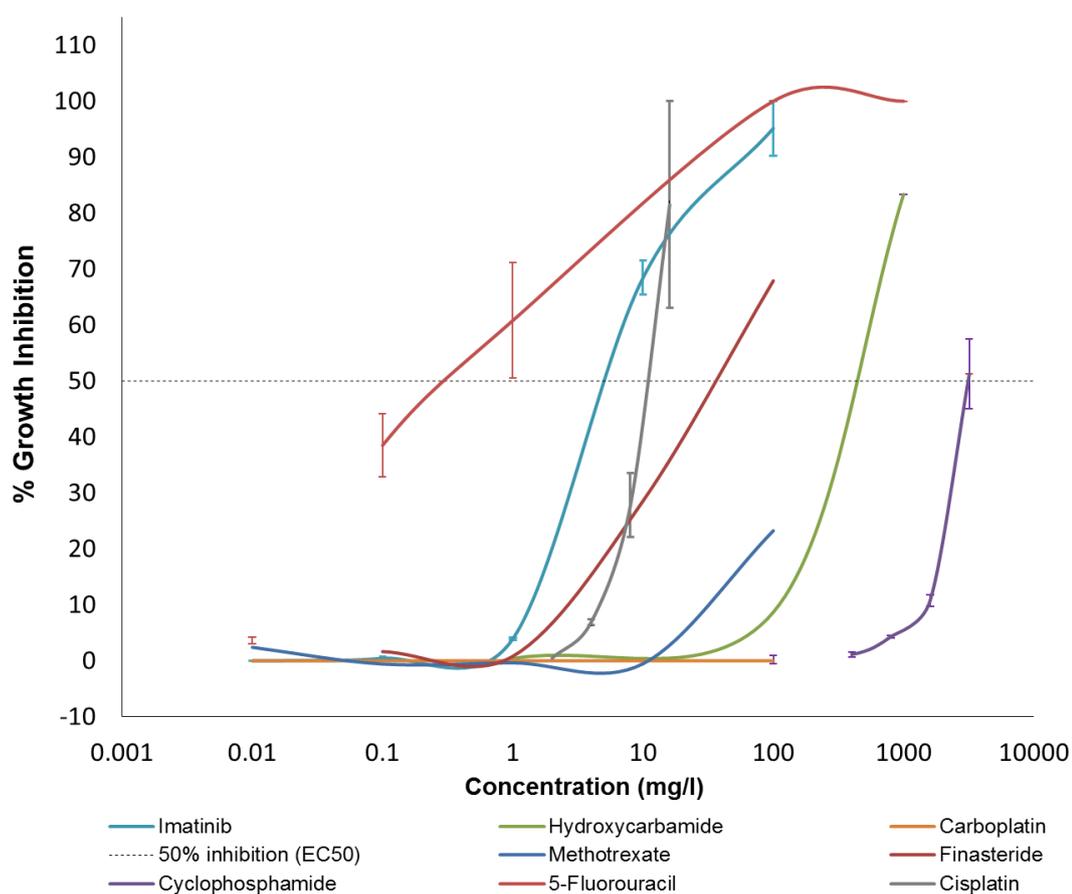


Figure 9. Percentage growth inhibition of *R. subcapitata* after 72 hours of exposure to eight antineoplastic pharmaceuticals individually screened. SEM are included for four of the eight compounds (IMT, 5FU, CYC, CIS) since these assays were performed twice, and hence combined, since data from both experiments were found to be statistically similar.

For all compounds with the exception of carboplatin (which produced no response at any concentration tested), effect concentrations (EC's) were found to vary by 5000-fold (table 5).

Table 5. EC₅₀, EC₂₀, and EC₁₀ values for each compound tested in the algal assay, including the experiment number and treatment of data. Compounds highlighted in grey were included in the mixture experiment. * Dutasteride EC₅₀, EC₂₀, and EC₁₀ was not recorded due to colour interference of the compound caused from ineffective solubility.

Compound	No. of Experiment's	Pooled?	ng/L		
			EC ₅₀	EC ₂₀	EC ₁₀
Imatinib mesylate	2	YES	6.21E+03	2.65E+03	1.62E+03
5-Flurouracil	2	YES	4.35E+02	4.50E+01	1.00E+01
Hydroxycarbamide	1	N/A	1.00E+05	9.57E+04	9.31E+04
Cyclophosphamide	2	NO	3.18E+06	1.99E+06	1.52E+06
Cisplatin	2	YES	1.09E+04	7.33E+03	5.89E+03
Carboplatin	2	N/A	0.0	0.0	0.0
Methotrexate	1	NO	1.03E+05	9.93E+04	9.71E+04
Finasteride	1	N/A	3.64E+04	6.40E+04	2.32E+03
Dutasteride	1	N/A	n/a*	n/a*	n/a*

For one compound tested in two separate repeated experiments, namely 5-flurouracil, data were found to be normally distributed, and mean temporal and concentration dependant data from both experiments were found to be statistically similar (two-tailed t-test, t=-2.545, d.f=23, p=0.018, p<0.05). However, data from the two individual studies for

cyclophosphamide were not statistically similar (two-tailed t-test, -0.279, d.f=23, p=0.783), and were therefore not pooled.

2.3.4. Toxicity of a mixture of antineoplastic drugs

To assess the potential mixture effects of these pharmaceuticals, single compound data reported above were analysed for suitability of inclusion in a basic mixtures study. Since the purpose of screening a larger number of antineoplastic compounds was to identify and select a smaller subset of suitable compounds to be included in a mixture study, some compounds were excluded from the mixture study for a number of reasons. Since carboplatin had no observable effects on growth inhibition up to the highest tested concentration, and it was deemed unfeasible to test higher concentrations due to cost, this compound was excluded from the mixture study. For successful analysis of mixture effects, each compound present in any given mixture should have information about its effect levels individually i.e. its EC₅₀, LOEC and/or NOEC. These data were not obtained for carboplatin and therefore the compound was omitted from the study.

Dutasteride was also omitted from the mixture study. As discussed previously, solubility problems were encountered with this compound within the initial 24 hours. Colour and turbidity interference caused by insufficient solubility (in spite of the use of a solvent carrier) resulted in unreliable and irregular optical density readings and therefore no reliable effect level data could be generated. Finasteride was also excluded from the study after analysis of the single compound data revealed a non-linear dose-response relationship, which was likely caused by solubility issues and interference on growth from the solvent carrier DMSO. Finally, methotrexate was omitted from the mixture study due to the fact that only a partial concentration response curve was obtained, which did not reach the EC₅₀ effect level. Given sufficient time and resources, this work could have been expanded in order to obtain a full dose response curve for this compound, however since

there was an adequate number of additional compounds that were suitable for inclusion into the mixture study, this was considered to be inconsequential, and the compound was excluded. Further, as the main aim of this mixture experiment was to provide sufficient training in mixture toxicology testing necessary to conduct a well-designed *in vivo* experiment using a fish model organism, it was not considered imperative to conduct a large-scale mixture experiment involving the use of large numbers of compounds, given time and feasibility constraints.

Four of the five compounds produced an effect individually, inhibiting growth at 1/5th of their EC₅₀ concentrations (fig. 10). Cisplatin produced no inhibitory effect on growth at 1/5th of its EC₅₀ (fig. 10). Hydroxycarbamide and 5-Flurouracil produced the largest inhibitory effect on the algal cells, with an inhibition of 26.36%. The simple arithmetic sum total of the inhibitory effects of all five compounds was 68.71%, 23.93% higher than the actual observed inhibitory effect caused by the mixture (44.78%).

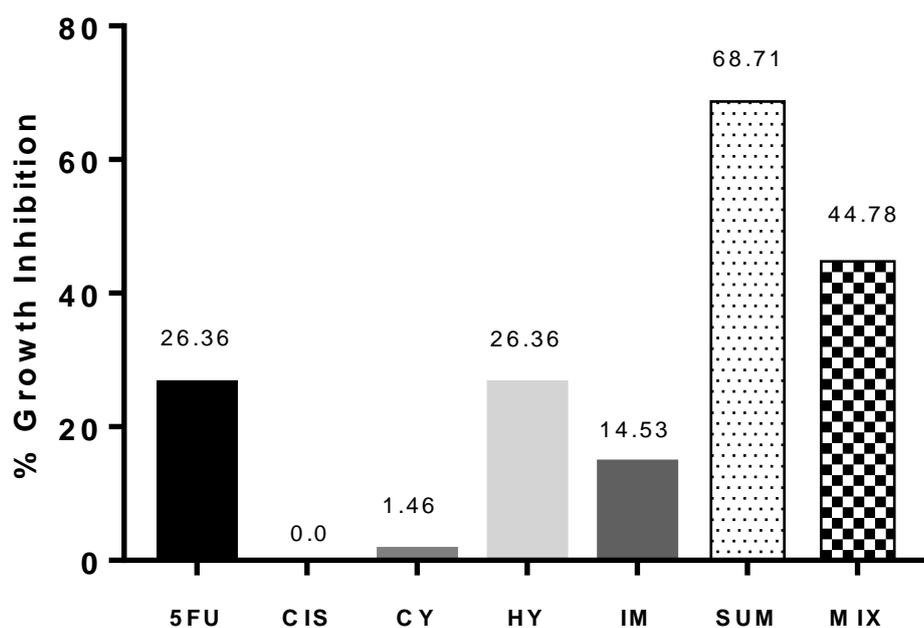


Figure 10. Percentage growth inhibition of five individual antineoplastic compounds at $1/5^{\text{th}}$ of their EC_{50} concentrations (based on dose-response data obtained from the effect of each compound on algal growth) and the predicted and observed mixture effects from an equipotent mixture (EC_{50} concentrations). 5-FU – 5-fluorouracil, CIS – cisplatin, CY – cyclophosphamide, HY - hydroxyurea, IM – imatinib mesylate, SUM – summation of the individual compounds, MIX – mean mixture effect of growth. Percentage growth inhibition calculated from optical density readings. Data courtesy of Dr. Graham Harris.

2.4. Discussion and Conclusions

The experiments presented in this chapter were conducted in collaboration with GH and the EU PHARMAS project as a training exercise in mixtures toxicity research. Before undertaking mixtures experiment on fish in semi chronic exposure conditions, it was deemed appropriate to contribute to other work on mixtures toxicology taking place at Brunel University London which included the use of rapid assays on a lower organism.

The algal species chosen for the experiments presented here is widely used in toxicity studies, since it has been shown to be one of the more sensitive species to chemical pollutants (Brezovšek et al, 2014). For example, Vannini et al (2011) subjected this species to a mixture of pharmaceuticals found in effluent and river waters in Italy, and subsequently measured traces of the tested pharmaceuticals in the exposed algae cells. The study demonstrated that this species is able to absorb pharmaceuticals from its surrounding environment, and is therefore a suitable test species for assessing the effects of pharmaceutical pollutants.

All but one of the antineoplastic compounds tested in the assay led to an inhibition of growth in *R. subcapitata*, albeit across a relatively wide range of concentrations. The most potent compound tested across this study (including the compounds tested by GH) was the antimetabolite (pyrimidine antagonist) 5-Fluorouracil (5FU), with an EC₅₀ of 435µg/L. This was followed by the tyrosine inhibitor imatinib mesylate (IMT), with an EC₅₀ of 6.2mg/L, and the platinum analog cisplatin (CIS), which had an EC₅₀ of 10.9mg/L. The other platinum analog tested in this study, carboplatin, showed no effects on growth up to concentrations of 100mg/L. The findings presented here are in line with those demonstrated elsewhere (table 6). Brezovšek, et al (2014) reported on a similar study testing three of the compounds also tested in the study presented here; 5FU, IMT and CIS. They reported comparable findings, that all three compounds produced an inhibition of growth in this species, at a range of different potencies. They also reported that 5FU was the most potent compound. The study observed an EC₅₀ value for 5FU of 0.13mg/L,

which is within the same range as the EC_{50} reported from the current study (0.43mg/L). While these findings are in line with the findings presented here, the Brezovšek study observed quite different EC_{50} values for cisplatin and imatinib (table 6). The reported EC_{50} for cisplatin was around seven-fold higher than the EC_{50} reported here, and for imatinib, the difference was almost three-fold. Furthermore, in contrast to the present study, Brezovšek and colleagues found that cisplatin caused higher inhibition of growth than imatinib.

Similarly, methotrexate was found to be more than two-fold more potent in this study than similar recently published studies. For example, Henschel et al (1997) reported an EC_{50} of 260mg/L in the green microalgae *Scenedesmus subspicatus*, compared with an EC_{50} of 103mg/L in the study presented here. Though it should be noted that only a partial concentration response curve was generated for this compound, and therefore the EC_{50} value is a predicted effect concentration.

Table 6. Comparison of EC₅₀ values for growth inhibition in aquatic primary producers between cited studies and the current study.

Compound	Species	EC ₅₀	EC ₅₀	Reference
		This Study (mg/L)	Literature (mg/L)	
5-Fluorouracil	<i>R. subcapitata</i>		0.13	Brezovšek et al, 2014
	<i>R. subcapitata</i>	0.43	0.11	Zouunkova et al, 2007
	<i>Desmodesmus subspicatus</i>		48.00	Zouunkova et al, 2010
Imatinib mesylate	<i>R. subcapitata</i>	6.2	2.29	Brezovšek et al, 2014
Cisplatin	<i>R. subcapitata</i>	10.9	1.52	Brezovšek et al, 2014
	<i>Lemna minor</i>		1.5	Supalkova et al 2008
Carboplatin		>100	no data	-
Hydroxycarbamide/ Hydroxyurea		100,000	no data	-
Methotrexate	<i>Scenedesmus subspicatus</i>	103.36	260	Henschel et al, 1997
Finasteride		36,400	>49	Venkataramani et al, 1994
Dutasteride		no data	no data	-
Cyclophosphamide	<i>R. subcapitata</i>		>100	Cesen et al, 2016
			3,180,000	Grung et al, 2008
			930	Zouunkova et al, 2007

There are a number of factors that could explain the differences between the findings of these studies. Firstly, variations in assay protocols can cause significant differences in observed results. The Brezovšek experiments were carried out using algae from a continuous culture source, whilst the current study used immobilised cells that were reactivated in a growth medium. Studies have highlighted the benefits of using immobilised cells from a single source as a means of reducing natural biological variability in growth rates between different laboratory strains (Daniel et al, 2004). However, assays such as the AlgalToxF kit used in this study are typically validated against traditional cultured algae methods across many different laboratories during the development stage, and in this case the kit developed was found to be comparable to more conventional cultured algae assays in terms of sensitivity. In spite of this, it could be expected that there may be some variation between results from studies using the immobilised cells, and those using conventional cultured cells, particularly since the cells in this study were exposed to an additional matrix, the dissolving medium, prior to culture.

Other potential factors that may lead to discrepancies in toxicity data across studies include: growth and culture medium volumes, choice of experimental vessel, light intensity, light exchange surface, CO₂ exchange, and choice of growth media. The choice of assay vessel, for example, can influence a number of other factors, such as the amount of light and CO₂ that reaches the cells, and the cell growth potential. OECD guidelines recommend a test vessel with a sufficient volume to produce a surface volume ratio of at least 0.15cm²/ml (OECD 201, 2002). As described in section 2.2. (materials and methods), the current study utilised 25cm long cells for the exposures, however, some studies use multiwell plates (6, 12, 24, 48 or 96 well format), or flasks. In the Brezovšek study for example, exposures were performed using Erlenmeyer glass flasks with a culture volume of 20ml, which may afford more light penetration and CO₂ exchange than the long cells used in this study. Another similar study by Zounkova et al (2010) utilised 24 well microplates with much lower volume capacity for their exposures of *D. subspicatus* to

three antineoplastic compounds and their metabolites. However, standard procedure (OECD 201) for any algal bioassay is to adjust the starting volume of cells to a baseline to allow for exponential growth throughout the duration of the experiment, with no nutrient depletion (OECD 201, 2011). Although standardised, starting volumes vary between test species, a further consideration when making direct species comparisons of toxicity data.

The Brezovšek study subjected the algae cultures to continuous shaking in order to keep the algae in suspension throughout the duration of the assay, a commonly employed practise in conventional algae toxicity studies. Another recent study (Zounkova et al, 2010) adopted discontinuous shaking, with 30 minute of shaking followed by 15 minute intervals of no shaking). This technique (either continuous or discontinuous shaking) was not undertaken as part of the standardised AlgalToxKit F assay employed here. However, during the validation stage, the assay developers found that re-suspending the algae once every 24 hours, immediately before taking the optical density measurement, was sufficient enough to meet the standard procedure requirements (AlgalToxKit F SOP, 2013). Although this protocol meets the requirements and has been considered adequate, the fact that the algae were not kept in continuous suspension whilst the Brezovšek and Zounkova study cells were, could partially account for the differences in effect concentrations.

Differences in the toxicity of some compounds observed in this study compared with the toxicities reported in other recent studies (Zounkova et al, 2010; Brezovšek et al, 2014) could also be partially attributed to the use of different growth media. The current study used a growth media supplied by the assay manufacturer, optimised for cell growth and experimental conditions; however, culture media choice has also been shown to effect results in similar studies. Millington et al (1988) studied the effects of different growth media on the toxicity of a number of chemicals on three commonly used microalga, and found that composition of growth medium significantly affected the toxicity of the compounds by altering species sensitivity. This finding highlights the limitations of direct

study comparisons, particularly given that many reported studies do not specify the composition of the growth media used. Furthermore, as previously discussed, the algae cells used in this study were also subjected to a matrix dissolving medium, in order to dissolve the immobilisation matrix that the algae beads are supplied in, and therefore the cells were not treated the same as in conventional assays.

The pH of the growth media is also of importance, since slight changes in pH can cause dissociation of some compounds, or changes in chemical species, thereby changing the toxic action of the compound in question (Altenburger et al, 2010). It has been well established in algae suspension culture studies that as algal cell density increases, the pH is altered (Scherholz and Curtis, 2013). The OECD guidelines address this effect by affording some degree of pH drift (1.5 units maximum), but cautions against direct comparison of results when using different media (i.e. OECD and Algae Assay Procedure (AAP)). The pH of the media used in this study was recorded at the start of the assay and adjusted suitably. However, pH measurements were not taken at any other time points throughout the assay. It is probable that the pH of the algae inoculum drifted during the assay as cell density increases and nutrients are being used up. However, since this assay has been standardised and validated in line with OECD 201 recommendations, a pH shift of more than 1.5 units is unlikely.

Inter- and intra- laboratory variation could also account for differences in toxicity data in apparently similar studies. Age and condition of equipment, laboratory conditions, and laboratory-specific protocols could all affect the results. However, as previously discussed, this kit has been validated across numerous laboratories globally (Daniel et al, 2004), so this is unlikely to be a major contributing factor. Furthermore, the validation step prior to this experiment was carried out successfully and the reference chemical (potassium dichromate) toxicity data were within the acceptable range. For the compounds that were repeated in the assay, there was generally good reproducibility, so intra-lab variability should not be considered an issue. Cisplatin was the only exception, with poor

reproducibility of results across two definitive experiments. However this could be attributed to the chemical properties of the compound itself, since the results of all other compounds were shown to be reproducible. No chemical analysis was undertaken on either the stock solutions or the media used in the experiment, therefore the possibility that one (or both) of the experimental exposures may not have been at the nominal concentrations cannot be fully ruled out.

Effective concentrations reported in this study vary somewhat compared to those reported in similar studies. For example, Zounkova et al (2010) reported an EC₅₀ value for 5FU of 48,000µg/L, whilst the present study observed a much lower EC₅₀ of 435µg/L. However, the Zounkova study utilised a different species of green microalgae (*D. subspicatus*) in their study. Similarly, Henschel et al, (1997) also utilised a different test species (*Scenedesmus subspicatus*), which could account for the differences on EC₅₀ values observed for methotrexate between these two studies. Typically in the field of environmental and regulatory toxicology, different model species can be employed in chemical testing. It is apparent from the literature that *R. subcapitata* is a universally used test species, however other similar species are commonly used, including *Scenedesmus subspicatus*, *Desmodesmus subspicatus*, *Chlorella vulgaris*, and *Chlamydomonas moewusii*. Extrapolation of toxicity data between species, even closely related species, should be undertaken with caution (Ma, 2005; Li and Randak, 2009, Hagenbuch and Pickney, 2012). Ma, (2005) reported significant variations in species sensitivity to pesticides between five species of microalgae, with sensitivities varying over three orders of magnitude (Ma et al, 2002_a; Ma et al, 2002_b; Ma et al, 2004_a; Ma et al, 2004_b). Though species sensitivity is very likely to be contributing to the differences in results between the studies undertaken by Henschel et al (1997), Zounkova et al (2010) and the study presented here, it is not clear how much of the difference could be attributed to this variable. Nevertheless, caution should be taken when using toxicity data to extrapolate to

other species, even species in similar taxonomic assemblages, since sensitivity differences across taxonomic groups is common.

The use of solvents in bioassays has the potential to affect the results. Toxicity of the solvent, together with potential interactions with the compounds being tested, both contribute to effects on toxicity results. For example, cisplatin and carboplatin have been shown to bind to Dimethyl sulfoxide (DMSO) in solution, forming an adducted compound with reduced cytotoxicity and neurotoxicity. The interaction causes a reduction in the ability of the drugs to cause double strand breaks, and has been shown to significantly reduce effectiveness on cancer cells in vitro (Fischer et al, 2008). This reduced toxicity could explain the low potency of carboplatin in this study. Furthermore, the results from the finasteride experiment indicated that the solvent, DMSO, was causing a significant effect on growth inhibition in this species. Growth inhibition in the solvent control was shown to be statistically greater than that of the control group. Some studies have reported significant toxic effects to microalga from solvents frequently used in chemical toxicity studies. For example, a study by Jay (1996) investigated the effects of four solvents, namely ethanol, methanol, dimethyl sulfoxide (DMSO) and dimethyl formamide (DMF), on two species of green algae, *Chlorella vulgaris* and *Selenastrum capricornutum* (subsequently reclassified *Raphidocelis subcapitata*). The study compared solvent effects on growth inhibition of both species across five concentrations: 0.05%, 0.1%, 0.2%, 0.5% and 1.0%. Ethanol was found to be the most toxic to both test species, with around 37% inhibition of growth at 0.05% and 95% inhibition at the highest concentration when tested on *C. vulgaris*. *R. subcapitata* was slightly less sensitive: 14% and 48% inhibition occurred at 0.05% and 1.0% ethanol, respectively. DMSO was found to induce the lowest toxicity to both species, with no significant effect on the stimulation or inhibition of growth, across the range of concentrations tested. The results of the Jay study show not only marked differences of toxicity between solvents, but also species-specific differences, highlighting the need to tailor choice of solvents and concentrations to bioassays. Based on the results

found by Jay, DMSO was selected as the solvent carrier for this assay. DMSO had previously been shown to have no significant effects on growth inhibition on the test species *R. subcapitata*, and reported to be compatible with all chemicals used in the study. Despite this, a significant inhibition was observed in the finasteride experiments. Significant inhibition of growth caused by DMSO when compared to the control was also observed in the methotrexate experiment. Although the use of a solvent is required for these compounds to remain in solution, it is apparent toxic effects may occur that are not caused by the compound in question. The use of a solvent control is always recommended, and to a certain extent can reduce the problem of unknown toxic potential of the solvent. Furthermore, good experimental design necessitates that the same concentration of solvent is present in every treatment group, therefore all groups would have the same added toxicity of the solvent, and concentration response data can still be used, although the exact effect concentration values should be taken as an indicator value only.

In risk assessment studies, mechanisms of toxicity may not be of overall importance, since regulators only require information on whether or not a compound poses a risk to a species, and at what concentrations it does so. However, in investigative toxicology studies, particularly with mixture assessments, understanding the mechanisms of growth inhibition in *R. subcapitata* as a result of exposure to these compounds is important. Since these compounds are largely designed to inhibit cell proliferation and induce apoptosis, effects on the reduction of algae cell growth were expected. As previously mentioned, since these compounds initiate effects on the cell cycle, specifically targeting DNA, inhibition of cell growth in *R. subcapitata* could be attributed to specific mechanism mediated toxic effects. However, since a more general toxicity would in essence produce the same outcome (i.e. lower cell growth and induced cell death), this mechanism cannot be ruled out without further analysis. Gene expression studies (where specific genes, receptors and pathways are assessed, for example DNA repair enzyme genes),

bioaccumulation rates, and/or analysis of detoxification mechanisms and pathways would aid in providing information on the mechanisms of induced toxicity caused by these compounds.

Some of the mechanisms that are targeted by these anti-cancer pharmaceuticals do not appear to exist in plants. For example, the classic tyrosine kinase enzymes that are targeted by imatinib mesylate are not present in plants. However, proteomic studies, largely with *Arabidopsis* sp., have shown that plants do possess phosphotyrosine (a phosphorylated form of tyrosine) and dual-specificity kinases that can act as both tyrosine kinases as well as serine and threonine kinases (Rudrabhatla et al, 2006). Plants typically have an extension of the receptor-like kinases (RLK) of the tyrosin kinase-like group (TKL), which have been shown to be similar to the TK group of enzymes, though typically lacking the TK-specific motifs. Given this evidence from the literature, it is not unreasonable to suggest that the inhibition of growth observed in the study presented could be mechanism mediated, and not the result of a more general toxic effect. Conversely, relatively high concentrations were used in this study, concentrations that have been aligned with general toxicity effects in similar studies (Zounkova et al, 2010; Brezovšek et al, 2014). However, this study made no attempt to investigate the mechanisms of disrupted growth of *R. subcapitata* as a result of exposure to this, or any other compound, and therefore only speculative conclusions can be made.

2.4.1. Mixture effects of antineoplastic pharmaceuticals

Mixture effects were observed on growth of *R. subcapitata* as a result of exposure to five antineoplastic pharmaceuticals. Inhibitory effects of the mixture on algae growth were larger than those of any of the single compounds when tested individually, though less than the sum total of the effects produced individually.

Similar studies of the effects of pharmaceutical mixtures of growth of *R. subcapitata* or similar species have also reported what appeared to be additive effects (Cleuvers, 2003; Cleuvers, 2004; Eguchi et al, 2004; Yang et al, 2008). In a recent study by Brezovšek et al (2014), binary mixtures of four antineoplastic pharmaceuticals exhibited mixture effects. The study found that the effects could not be accurately predicted by Concentration Addition (CA) or Independent Action (IA). Although it is important to note that the study focus was on binary mixtures composed of only two compounds, therefore the CA and IA model predictions were not that dissimilar to one another, since the mathematical basis of these models requires higher numbers of compounds (i.e. multicomponent mixtures) to increase the strength of the predictions. Although the two predictions were too close together to be able to determine which predictive model best described the observed effects, the authors further reported what they considered to be synergistic and antagonistic effects of binary mixtures. At all effective concentrations, CA and IA could not accurately predict the observed mixture effects. For example, CA and IA underestimated the effects on growth of *R. subcapitata* from exposure to a binary mixture of 5-FU and cisplatin. Conversely, for a binary mixture of cisplatin and etoposide, both models overestimated the mixture effect. The authors of the study suggest the disparities in the magnitude of the observed and predicted effects may be due to the mechanisms of action of these compounds. Anticancer pharmaceuticals are commonly used in combination therapies, exploiting the potential additive or synergistic effects one compound has on another. For example, treatment with 5FU can enhance cytotoxicity and retention of cisplatin in human patients by suppressing the repair of cisplatin induced cross-links and adducts (Tanaka et al, 2001). Though this explanation is quite plausible, caution should be taken in drawing synergistic and antagonistic conclusions from studies of mixtures with only two components. In the Brezovšek study, though the observed effects were visibly different from either model prediction, the strength of the predictions compared with each other and with the observed effects is relatively weak. A multicomponent study with more

compounds would provide stronger evidence for additive, synergistic, or antagonistic mixture effects.

A recent study expanding the work of Brezovšek et al (2014) addressed this issue by investigating the effects of a three component mixture of 5-Fluorouracil, imatinib mesylate and etoposide on growth inhibition in *R. subcapitata*. Again, they reported observed mixture effects higher than that predicted by Concentration Addition and Independent Action (Elser et al, 2016). Furthermore, they reported that the observed effects were significantly more at lower effect concentrations (EC_5-EC_{50}). The later study strengthens the findings of Brezovšek et al (2014), in that additive or synergistic effects are likely as a consequence of exposure to antineoplastic mixtures, due largely to their complementary mechanisms of action.

The approach used in the currently presented study was a basic component-based mixture design using the EC_{50} values of the individual components. Though designs based on the EC_{50} values are not typically environmentally relevant, due to the high concentrations required to cause effects at this level (usually in the mg/L range), EC_{50} based mixture studies are appropriate for conceptual mixture studies, and can allow for direct comparison of mixture effects with the effects of the individual mixture components at known effect levels (Vasquez et al, 2014). Mixture effects observed in the current study were lower than the sum of the individual effects. This could be attributed to the highly variable effects from the individual compounds at 1/5 of the EC_{50} . For example, cisplatin produced no observable effect on algal growth at 1/5 of its EC_{50} , whilst 5-FU and hydroxycarbamide produced an effect of over 26% inhibition. The disparity of effects produced by each compound at a concentration 20% of its EC_{50} is largely due to the shape of the concentration-response curves of the individual compounds. For example, cisplatin and cyclophosphamide, the compounds that displayed no, or limited effect at concentrations 1/5 of their EC_{50} 's have steeper concentration response curves, indicating a high potency change per unit dose. The compounds that produced higher inhibitory

effects at concentrations of 1/5 of their EC_{50} 's were the compounds with much shallower slopes, such as 5-FU.

Despite the popularity of using this approach in mixtures assessment studies, anticipated combination effects cannot be accurately predicted using simple arithmetic summation of the individual compound effects (Kortenkamp, 2007). Kortenkamp and Altenburger (1998) highlight the errors in assessing mixture effects based upon 'effect summation'. For example, the concept is based around the presumption that the effect of a mixture of chemical A and chemical B will equal the sum of the effects of chemical A and B individually. However, this scenario could only occur where the dose-response relationship of each individual compound was linear and passed through the origin of the dose-response plot (fig. 11), (Berenbaum, 1989; Kortenkamp and Altenburger, 1998). Sigmoidal dose-response curves are more typical in toxicology. Sigmoidal curves can be steep or relatively shallow, however it is obvious when viewing these curves that each arbitrary unit increase in dose does not produce one arbitrary unit increase in effects (fig. 12). In the case of the study presented, dose-response relationships of all chemicals were not linear, but rather, sigmoidal. Since linear dose-response curves are extremely rare, the concept of effect summation cannot be successfully applied to most mixture prediction assessments.

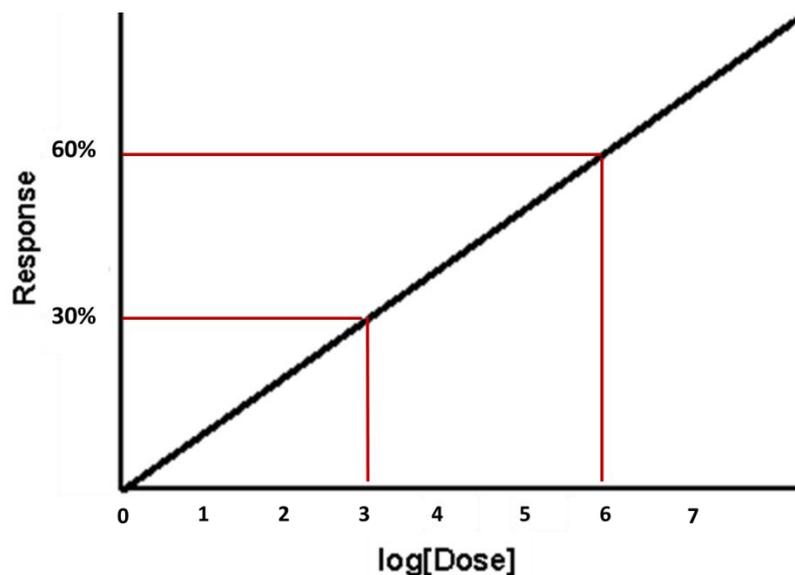


Figure. 11. Schematic illustration representing the application of 'effect summation' to mixture predictions where compounds have linear dose-response relationships (non-typical). A theoretical compound with a linear dose-response curve (shown in bold black line) causes 30% effect at an arbitrary unit dose of 3. A two-fold increase in dosage to 6 (double the original dose) causes 60% effect, in agreement with the principle of 'effect summation'.

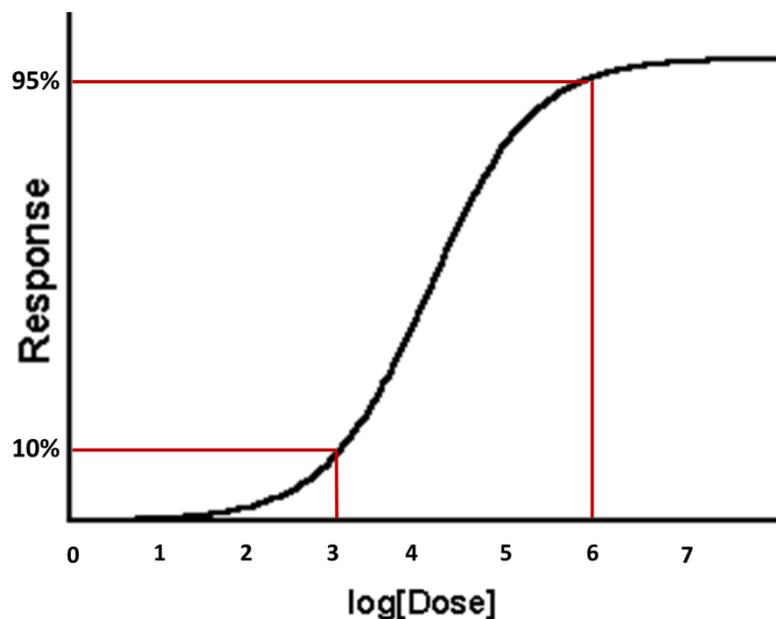


Figure. 12. Schematic illustration representing the problematic application of 'effect summation' to mixture predictions where compounds have non-linear dose-response relationships. A theoretical compound with a typical sigmoidal dose-response curve (shown) causes 10% effect at an arbitrary unit dose of 3. A two-fold increase in dosage to 6 (double the original dose) causes 95% effect. If effect summation was applied, a two-fold increase in the dosage to 6 would suggest a 20% effect. This highlights the impractical application of 'effect summation' to real-world compounds that do not have a linear dose-response relationship.

The problems illustrated above with the 'effect summation' concept as used in this chapter and in many other studies in the literature highlights the invalidity in concluding additive or synergistic effects when using this concept to predict interactions. Predictive tools that are not affected by the shape of the dose-response curves of the individual compounds have been increasingly used in the field of mixture toxicology. The additivity model 'concentration addition' (Loewe and Muischnek, 1926), is based around the concept of comparing doses that produce equipotent effects, and has successfully been applied to mixture predictions with real-world sigmoidal dose-response curves. Furthermore, the concept can also be applied to mixtures where individual components have curves of different shapes and steepness, more typical of real-world mixture scenarios. The CA model was utilised in subsequent mixture experiments as part of this PhD research, and will be investigated further in chapter 3.

2.4.2. Environmental Relevance

Concentrations at which effects were observed on growth of *R. subcapitata* were several orders of magnitude higher than those recorded in the environment. A number of studies have reviewed the existing literature on the occurrence of anticancer pharmaceuticals in the environment, and suggests that individually, anticancer pharmaceuticals pose limited risk to human health and aquatic organisms (e.g. Kosjek and Heath, 2011; Besse et al, 2012). Though concentrations in effluent and surface waters are typically much lower than those tested in this study, and studies have concluded limited risk from these compounds individually (Straub, 2010), the importance of mixture effects has to be considered (Johnson et al, 2008). Mixture effects on growth of microalgae have been demonstrated from across many pharmaceuticals classes (Richards et al, 2004; Yang et al, 2008; Vannini et al, 2011) including antineoplastic mixtures (Brezovšek et al, 2014; Elerseck et al, 2016; Česen et al, 2016, and the study presented here). However, although the results from both the literature and the current study demonstrate that combination effects from antineoplastic mixtures can occur, the concentrations of anticancer pharmaceuticals

measured and predicted in the environment are several orders of magnitude lower than those required to initiate a response in organisms in laboratory tests. Even accounting for potential mixture effects, levels in the environment are extremely unlikely to contribute such that adverse effects in wildlife or human health could be observed.

2.5. Conclusion

The research presented in this chapter was undertaken to assess the effects of pharmaceuticals and their mixture on a simple easily quantifiable end point, in an assay that can be completed rapidly and at relatively low cost, therefore allowing a large number of pharmaceuticals to be investigated. Nine compounds were screened in the assay (six by the author and three by Dr Graham Harris), allowing a selective mixture experiment to be designed from a number of suitable compounds. The majority of antineoplastic compounds investigated caused an inhibition of growth in *R. subcapitata* in a dose-dependent manner.

The subsequent mixture study was performed to obtain a basic training in mixture toxicity, both in theory and in practice, and to complete a relatively simplistic mixture experiment investigating the potential for mixture effects of antineoplastic pharmaceuticals on growth in a commonly used species of microalga. Mixture effects were observed, however true additivity or independency cannot be determined from this study. The research presented in this chapter provided a practical lesson in the issues surrounding the basic mixture design of 'effect summation', and served to reinforce the concepts, theory and fundamental training in designing effective mixture experiments that would be utilised as part of the research presented in subsequent chapters. The research presented here will inform the work of the next chapter, to undertaken more complex and informative mixture modelling studies in a more advanced organism, on more complex and ethically important endpoints.

Chapter 3: Effects of mixtures of steroidal pharmaceuticals with diverse mechanisms of action, on the reproductive capacity of the Fathead minnow, *Pimephales promelas*.

Part 1: Assessments of mixture effects on an ecologically relevant end point – egg production

3.1 Introduction

As discussed in previous chapters, a wide range of pharmaceuticals and their transformation products have been detected in the aquatic environment, likely due to their increased usage, improvements in analytical technologies, and the increasing attention afforded to their status as an emerging group of contaminants. Concentrations of individual pharmaceuticals and API's are typically low (in the ng/L range); however, until recently, relatively little is known with regards to potential effects of low levels of these compounds. Furthermore, compounds are present in the environment chronically and simultaneously as complex cocktails of mixtures, not just with other pharmaceuticals but with a wide range of other contaminants. Although mixture effects are widely recognised as being important in environmental risk assessment, tools for assessing the potential for mixture effects are not well established. It is currently (and most likely will always be) unfeasible to test every potential mixture combination to determine possible effects. Predictive toxicology models offer a practical solution, particularly for the purpose of risk assessment. Given the nature of predictive modelling (discussed in subsequent sections), most studies investigating mixture effects have been conducted using *in vitro* endpoints. Difficulties in linking observed effects *in vitro* to an effect at the whole organism level (phenotypic anchoring) can be difficult. For example, a two-fold change in the expression of a reproductively responsive gene after exposure to an endocrine disruptor (or mixture) can be difficult to anchor to a specific level of effect observed at the level of reproduction. This chapter will address the application of the predictive models Concentration Addition (CA) and Independent Action (IA) in assessing combination effects from a multicomponent mixture on an ecologically relevant endpoint – egg production in fish.

3.1.1. Synthetic Steroids as Endocrine Disruptors

Chemical pollutants that have the potential to disrupt the endocrine system of humans and wildlife are classed as endocrine disruptors, and have emerged as an important

contaminant 'group' on global watch lists. An endocrine disruptor is defined by the World Health Organisation (WHO) as 'any exogenous substance or mixture (of substances) that alters the function(s) of the endocrine system and consequently causes health effects in an intact organism, or its progeny, or subpopulations' (WHO, State of the Science Report on Endocrine Disruptors, 2012). Disruption to endocrine function can result in adverse effects on reproduction, development, growth, metabolism, neurological function and/or immunity (Gore et al, 2014). In recent decades, adverse effects linked to EDC's have been observed in aquatic wildlife such as fish, amphibians and birds. There has also been an observed increase in the recorded number of endocrine diseases in humans, which has been attributed to the increased EDC exposure of human populations (IPCS, WHO, 2016). Since synthetic steroidal pharmaceuticals are designed to act on endocrine tissues in humans to modify hormone signalling and biological function, their presence in the environment affords them the status of an environmental endocrine disruptor.

3.1.2. Endogenous Steroid Hormones, Hormone Receptors and Mechanisms of Hormone Action in Humans

Steroid hormones are substances produced within certain endocrine tissues, and transported to the desired site of action elsewhere in the body. They are therefore chemical messengers, produced by certain cells to initiate transcriptional changes in other (target) cells. They are lipophilic in nature, allowing them to cross the plasma membrane of cells easily. Steroids are synthesised from cholesterol, obtained either through the diet or synthesised from acetate via hydroxymethylglutaryl-coenzyme A, a CoA reductase enzyme (Nussey and Whitehead, 2001). They are produced in endocrine tissues (ovaries, testes, adrenal glands etc.), synthesised in the smooth endoplasmic reticulum and mitochondria of cells in response to external signals, in a processes known as steroidogenesis (fig. 13). In the case of the sex steroids estrogens, androgens and progestogens, the stimulus is in the form of gonadotropins from the pituitary gland

(discussed in detail in subsequent sections). Synthesised steroid hormones are then secreted into interstitial fluid and/or blood and transported to target cells bound to carrier proteins, such as albumin, sex-hormone binding globulin (SHBG), corticosteroid-binding globulin (CBG) and α_1 -acid glycoprotein. Only around 1% of circulating sex steroids (estrogens and androgens) remains free in circulation, unbound to proteins.

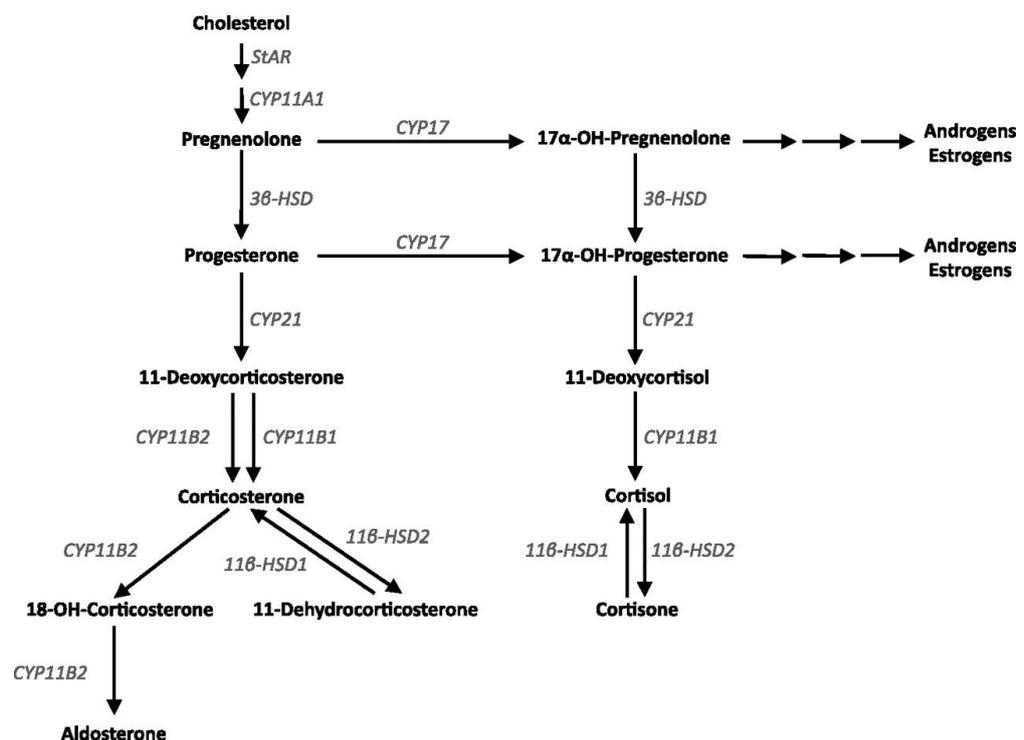


Figure 13. Pathways of steroidogenesis (StAR - steroidogenic acute regulatory protein; CYP11A1 - P450 side-chain cleavage; 3 β -HSD - 3 β -hydroxysteroid dehydrogenase/ Δ 5- Δ 4 isomerase; CYP17 - 17 α -hydroxylase/17,20-lyase; CYP21 - 21-hydroxylase; CYP11B1 - 11 β -hydroxylase; CYP11B2 - aldosterone synthase; 11 β -HSD types 1 and 2. From Taves et al, 2011.

Once they reach the target cell, the hormones become disassociated from their protein carriers and diffuse through the cell membranes, where they bind to their corresponding receptor. These are intracellular proteins typically located in the cell nucleus or cytosol. In these classic steroid receptor pathways, the hormone binds to the ligand receptor in the cytosol to form a hormone-receptor complex (HRC) (fig. 14). The formation of the HRC

leads to a conformational change in the receptor molecule, necessary for translocation from the cytosol into the cell nucleus. For some hormones such as the estrogens, progestogens, androgens, and glucocorticoids, formation of the hormone-receptor complex causes the release of certain proteins bound to the receptor in the cytosol, such as heat shock proteins (HSP90). Release of these proteins allows the formation of receptor-dimers (where two receptors join), exposure of the nuclear localisation sequence by receptor phosphorylation, and subsequent translocation into the nucleus through nuclear pores. Once in the nucleus, hormone-receptor complexes bind to specific sequences of DNA via association with transcriptional coactivators known as hormone-response elements (HRE), which then act as transcription factors to initiate messenger RNA (mRNA) transcription, followed by protein translation and target tissue response. Steroid hormone receptors are generally specific to one type or class of messenger hormone, though some hormone ligands can bind weakly to other receptors to initiate transcription factors, or to block transcription of endogenous hormones.

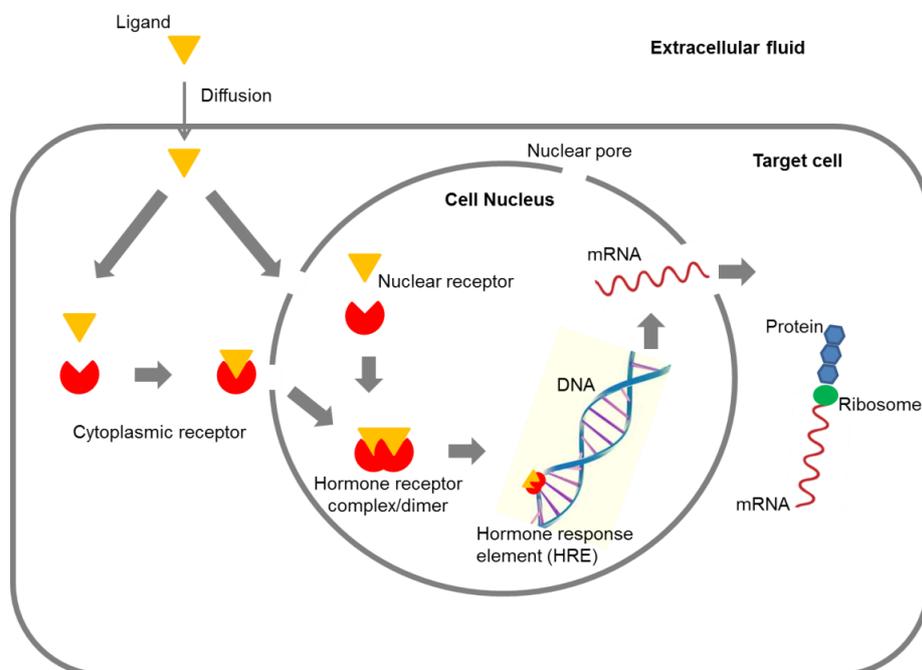


Figure 14. Signal transduction mechanisms in intracellular receptors in the cytosol and the nucleus of cells.

More recently, so-called non-classical mechanisms of steroid action have been established, first described by Hans Selye in 1942 (Selye, 1942; Singh et al, 2013), whereby some hormones also bind to receptors located on the plasma membrane of cells. This non-classical mechanism is much more rapid than classical steroid signalling pathways, occurring over a much shorter time than DNA binding by receptors. Recent research using transfected mice and gene knock-out techniques demonstrated the presence of distinct steroid receptors on cell membranes (Razandi et al, 1999). Endogenous and exogenous steroid ligands can also bind to these membrane receptors, mediating their effect through cell signalling pathways via intracellular signalling cascades (i.e. non genomic mediation) (Levin et al, 2009; Soltysik and Czekaj, 2013).

3.1.3. The Nuclear Receptor Super-Family

Nuclear receptors are responsible for controlling a wide range of biological processes. They have been extensively studied due to their importance in regulating and controlling critically important biological functions, and the range of diseases their dysregulation can cause. Diabetes, obesity, autoimmune diseases and many types of cancer can result from dysfunctional regulation of nuclear receptors (Zhang et al, 2015). Thus the nuclear receptor super family is a key target for drug development in human diseases, with drugs that target the NR family accounting for around 13% of all drugs approved by the FDA (Overington et al, 2006; Zhang et al, 2015). There are several main subgroups of the nuclear receptor family. The estrogen, progesterone, glucocorticoid and androgen receptors all belong to the Nuclear Receptor Sub-Family 3, Type I. Nuclear receptors are ligand-regulated transcription factors located in the nucleus and cytoplasm of cells. They regulate expression of target genes through the formation of hormone-binding complexes and subsequent binding to HREs (discussed above), regulating biological processes such as homeostasis, metabolism, development, growth and reproduction. Studies on the human genome have identified forty eight nuclear receptors in humans (Magelsdorf et al,

1995; Sever and Glass, 2013; McEwan and Kumar, 2015). Some of the more well-known nuclear receptors are shown in table 7. Unlike many other cell receptors, the ligands for nuclear receptors can directly cross the cell membrane and bind to receptors inside the cell, rather than having to act via a cell-surface receptor (Sever and Glass, 2013). Members of the nuclear receptor family have a commonly shared structure of five domains that vary in their conservation; a variable A/B N-terminal transactivation domain, a highly conserved DNA-binding domain (DBD) (C domain), a short variable hinge region (D domain), a conserved ligand binding domain (LBD) and a variable carboxyl-terminal domain (F domain), (figure 15). A key characteristic of the nuclear receptor family is their capacity to modulate a range of genes in a range of different tissues, leading to tissue specific responses (Sever and Glass, 2013).

Receptor	Abbreviation	Ligand
Androgen receptor	AR	Testosterone
Estrogen receptor	ER	Estrogen
Estrogen-related receptor	ERR	?
Glucocorticoid receptor	GR	Cortisol
Mineralocorticoid receptor	MR	Aldosterone
Progesterone receptor	PR	Progesterone
Retinoic acid receptor	RAR	Retinoic acid
Retinoid orphan receptor	ROR	?
Retinoic acid-related receptor	RXR	Rexinoids
Liver X receptor	LXR	Oxysterols
Peroxisome proliferator-activated receptor γ	PPAR γ	Fatty acid metabolites
Thyroid hormone receptor	TR	Thyroid hormone
Vitamin D ₃ receptor	VDR	Vitamin D ₃

Table 7. Common nuclear receptors and their corresponding ligands. **From Sever and Glass, 2013.**

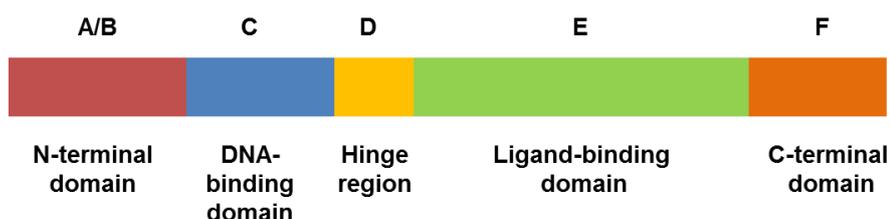


Figure 15. The structural organisation of nuclear receptors.

3.1.4. Human Steroid Receptors and their Ligands

Steroids can be classified into groups according to the number of carbons they contain. The estrogens for example contain 18 carbons (C18 steroids), androgens contain 19 (C19 steroids), and progesterone and corticosteroids contain 21 (C21 steroids). They typically have similar functional groups, such as alcohol, hydroxyl and methyl groups, though estrogens are unique in that their synthesis involves conversion of the A-ring to an aromatic ring.

3.1.4.1. Estrogen receptors (ER α and ER β) and their ligands

Estrogens regulate growth, development and reproduction, and have significant involvement in the regulation of cell proliferation. They are also involved in skeletal, neuroendocrine, adipogenesis and cardiovascular development and function (Lee et al, 2012), and are key sex hormones. They, along with androgens, are synthesised in the gonads, and are responsible for the development and regulation of the female and male reproductive systems, respectively. Estrogens are mainly produced in the granulosa cells of the ovarian follicle, but are also produced by the corpus luteum, a temporary endocrine gland that develops in the ovary after discharge of an ovum in preparation for pregnancy. Natural estrogens include estrone (E1), 17 β -estradiol (E2), and estriol (E3). Estrogens and their metabolites are mainly excreted in bile and reabsorbed through the intestine (Katzung et al, 2006).

In humans, there are two estrogen receptors, er α and er β , which are each encoded by different genes, ESR1 and ESR2, on chromosome 6 and 14 respectively. Expression of each form can be tissue specific, or can be co-expressed in the same cells at various levels (Li et al, 2004). The two receptors have been found to have high sequence homology. Classic estrogen receptors are located in the cell cytosol. The formation of estrogen-receptor complexes in the cytosol leads to hetero- ($\alpha\beta$) and homodimerization

(α , or β) due to the co-expression of both isoforms. Dimerization of the ER results in nucleus translocation and the regulation of transcription through estrogen-response elements (ERE) of the DNA. Dysregulation of estrogen pathways can lead to a number of serious diseases. Endocrine cancers (breast, ovarian, endometrial), osteoporosis, cardiovascular diseases and obesity have all been linked to disrupted estrogen regulation (Deroo and Korach, 2006).

3.1.4.2. Androgen receptors and their ligands

Androgens are C19 steroids (because they have 19 carbons) and are synthesised in the testis, ovaries and adrenal glands. Natural ligands of the androgen receptor include testosterone (T) and dihydrotestosterone (DHT). Testosterone acts directly on the androgen receptor (AR) in certain cells, however in other cells, enzymatic action of 5 α -reductase in the testis and prostate converts testosterone to DHT, a more potent agonist of the AR. Around 10% of testosterone produced in males is converted to DHT (Society for Endocrinology). The two androgen ligands have been found to have tissue specific activity (Randall, 1994). Studies have shown that testosterone is primarily responsible for the development and regulation of male reproductive organs such as the testis and prostate. Dihydrotestosterone (DHT) has a key role in the development of the male reproductive organs, and (along with testosterone) is responsible for the development of male secondary sexual characteristics, traits that are sex specific but are not directly involved in reproduction, such as androgen-responsive facial and body hair and muscle development. Testosterone is the main male sex hormone in humans and other mammals, and also acts as an anabolic steroid. It is derived from cholesterol (as with all steroid hormones) and synthesised by the Leydig cells in the testis in the presence of LH, by conversion to pregnenolone, progesterone, 17 α -hydroxyprogesterone, androstenedione and finally testosterone. During early foetal development, testosterone is

responsible for the development of internal and external reproductive organs. In mature males, testosterone is essential for the production of spermatozoa. The control of testosterone and inherently DHT is through feedback mechanisms to the hypothalamic-pituitary-gonadal (HPG) axis. Decreasing amounts of testosterone in the testis leads to negative feedback to the hypothalamus, initiating the release of GnRH, which in turn stimulates the production and release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Testosterone is also synthesised in females (though at much lower concentrations than in males) largely in peripheral tissues such as skin and adipose cells, through conversion from androstenedione. It is also produced in the thecal cells of ovarian tissue, the adrenal cortex, and in placental cells (Sowers et al, 2001). Its function in females is largely related to the development of bone strength and muscle mass, and has also been linked to energy levels and general well-being (Davis and Tran, 2001).

Unlike the estrogen receptor, in mammals there is only one androgen receptor, known as AR, or *ar*. As with all nuclear receptors, it acts as a ligand-dependant transcription factor, in this case for action of natural (and synthetic) androgens. The structure of the AR ligand-binding domain has been shown to be similar to other steroid receptors, in that it selectively binds androgenic ligands, altering the conformation and allowing nucleus translocation and binding to androgen response elements (ARE) on DNA. Dysregulation of androgen production and secretion can lead to a number of serious medical conditions, such as reproductive impairment, cardiovascular diseases and diabetes (Kapoor et al, 2005). Unusually high testosterone levels in human females have been linked certain diseases such as polycystic ovary syndrome and hirsutism (Speroff et al, 1999).

3.1.4.3. Progesterone receptors and their ligands

Natural progesterone receptor (PR) agonists include progesterone (P4), 17 α -hydroxyprogesterone, 20 α -dihydroprogesterone, 5 α -dihydroprogesterone, 5 α -dihydrodeoxycorticosterone and 21-hydroxyprogesterone. Progesterone (P4) is the principal endogenous ligand of the progesterone receptor. P4, like all other steroid hormones, is derived from cholesterol, and synthesised from pregnenolone via enzymatic activity of 3 β -hydroxysteroid dehydrogenase (3 β -HSD). P4, like estradiol, is a key ovarian steroid. It is a potent agonist of the PR, and is involved in the regulation of the menstrual cycle, ovulation, development of mammary glands, pregnancy and embryogenesis. It is a crucial hormone involved in preparing the uterus for, and in maintaining pregnancy. It is also the intermediate metabolite in the synthesis of both male and female sex steroids (estrogens and androgens), and the corticosteroids from cholesterol. In females, P4 is synthesised in the ovary by granulosa cells, and the corpus luteum after ovulation. It is also secreted by the placenta during pregnancy. P4 is also produced in males, in the adrenal cortex and the testes as a precursor to testosterone (Tait et al, 2008).

As with other steroid receptors, the progesterone receptor (PR) is a cytoplasmic nuclear receptor, which, after translocation to the nucleus, binds to the DNA promoter via progesterone response elements. Though only one progesterone receptor exist in mammals, two isoforms have been identified, PR-A and PR-B. Both are encoded by a single gene, and have arisen from differences in transcriptional start sites on that gene (i.e. the isoforms have two different promoter regions). The two isoforms have been shown to have functional distinct transcriptional profiles, and are differentially expressed depending on the cell type and target gene promoter. Effects of progesterone can be mediated or enhanced when present in combination with estrogens. For example, the estrogen and progesterone receptors can regulate the expression of each other. The ER α has been found to positively regulate the expression of the PR (DeMayo et al, 2006). In fact, the natural synergistic action of both progesterone and endogenous estrogens

affords the successful control of many biological functions, allowing synthetic estrogens and progestagens to be effectively used in combination as birth control in women.

Since progesterone is a key intermediate in the steroidogenesis pathway, and an important hormone in its own right, dysfunction of its production and regulation can contribute to the risk of a number of diseases. Since estrogens, androgens and corticosteroids are all synthesised from progesterone, its dysregulation has implications for a wide range of functions controlled by all steroid hormones.

3.1.4.4. Corticosteroid receptors and their ligands

Corticosteroids are produced in the adrenal cortex of mammals. There are two types of corticosteroids; the glucocorticoids and the mineralocorticoids, which are involved in regulating a wide range of biological processes including nutrient metabolism (including the synthesis of glucose and the mobilisation of fats), immune response, electrolyte and water homeostasis, stress response and inflammation. There are three endogenous corticosteroids, corticosterone, cortisol and aldosterone, which are synthesised from cholesterol by a series of enzymatic reactions, via the following pathway; cholesterol – pregnenolone – 11-deoxycorticosterone/17 α -hydroxyprogesterone – corticosterone /cortisol/aldosterone. Cortisol is a glucocorticoid, and the primary corticosteroid in humans, and an important catabolic hormone (Christiansen et al, 2009). It is produced in the zona fasciculata of the adrenal cortex, and released as a response to physical and mental stress stimuli and low levels of blood glucose. The synthesis and release of cortisol is controlled by the hypothalamus. Despite being a major corticosteroid in other vertebrates, corticosterone, another glucocorticoid, is only a weak agonist of both the GR and MR in humans. Its main function is as an intermediate metabolite from the conversion from pregnenolone to the main mineralocorticoid, aldosterone.

Glucocorticoids and mineralocorticoids share two nuclear receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR), respectively. The GR is present in almost every cell type in the human and is highly selective to glucocorticoids (mineralocorticoids do not bind to the GR with much affinity). There are two highly homologous isoforms of the human (h)GR, GR α and GR β , which are encoded by the same gene, arising as the result of alternative splicing. In humans, the mineralocorticoid receptor (MR) binds aldosterone, the mineralocorticoid responsible for electrolyte and water balance. Given the key role of corticosteroids in regulating the immune system, and in maintaining homeostasis, a whole host of diseases are associated with their dysregulation. Autoimmune diseases such as rheumatoid arthritis, Crohn's disease, inflammatory bowel disease, multiple sclerosis, and colitis have all be linked to impaired function of the hypothalamic-pituitary-adrenal axis and glucocorticoid dysregulation (Fasano, 2012). Furthermore, allergy conditions (dermatitis, asthma), inflammatory diseases and neurological disorders, (Silverman and Sternberg, 2012), Addison's disease, Chushing's syndrome, Chronic fatigue syndrome, fibromyalgia and a range of psychiatric conditions have also been linked to the disruption of the corticosteroid pathways (Tomas et al, 2013).

3.1.4.5. Receptor/Ligand specificity and non-specific binding

Though each steroid ligand binds to their corresponding nuclear receptors, they can also bind non-specifically to other nuclear receptors. This is largely due to the fact that steroid hormone receptors have highly conserved DNA-binding sites. For example, the glucocorticoid cortisol has a similar affinity to the MR as aldosterone, a mineralocorticoid (Savory et al, 2001). Progesterone (P4) has known androgenic activity, acting as an agonist of the AR, and also has a strong binding affinity to the MR, but as an antagonist, blocking the binding of mineralocorticoids and preventing their action. P4 is also a partial agonist of the GR (weak binding affinity), as well as several other nuclear receptors including the sigma-1 receptor (σ_1 R), a modulator of ion channels (Waterhouse et al,

2007; Johannessen et al, 2007), and the pregnane x receptor (PXR) involved in detoxification mechanisms (Staudinger et al, 2006). This non-specific receptor binding can also happen in the case of synthetic steroids. For example, progestins used in oral contraceptives can cause a range of androgen-related side effects through agonistic affinity to the AR (discussed in detail in subsequent sections).

3.1.5. Steroidal Pharmaceuticals

Over 4 and a half million items of synthetic progestins and over 2 million of estrogens were prescribed in England alone 2014 (BNF 2014) (fig. 16). BNF data reports that over 44 million items of corticosteroids were dispensed in 2014, making the drug class one of the three highest prescribed classes of pharmaceuticals (fig. 16). This includes topical, local and respiratory applications of corticosteroids (BNF, 2014). Dexamethasone, an anti-inflammatory corticosteroid, is amongst the top selling pharmaceuticals (Zhang et al, 2015). Synthetic androgens are somewhat less commonly prescribed, with around 865,000 dispensed items in 2014 (BNF, 2014). (fig.16). However, illicit usage of these drugs as growth and strength enhancers in athletes and within the male population for their masculinisation and virilising properties suggests that this group of compounds have a higher usage than official figures report.

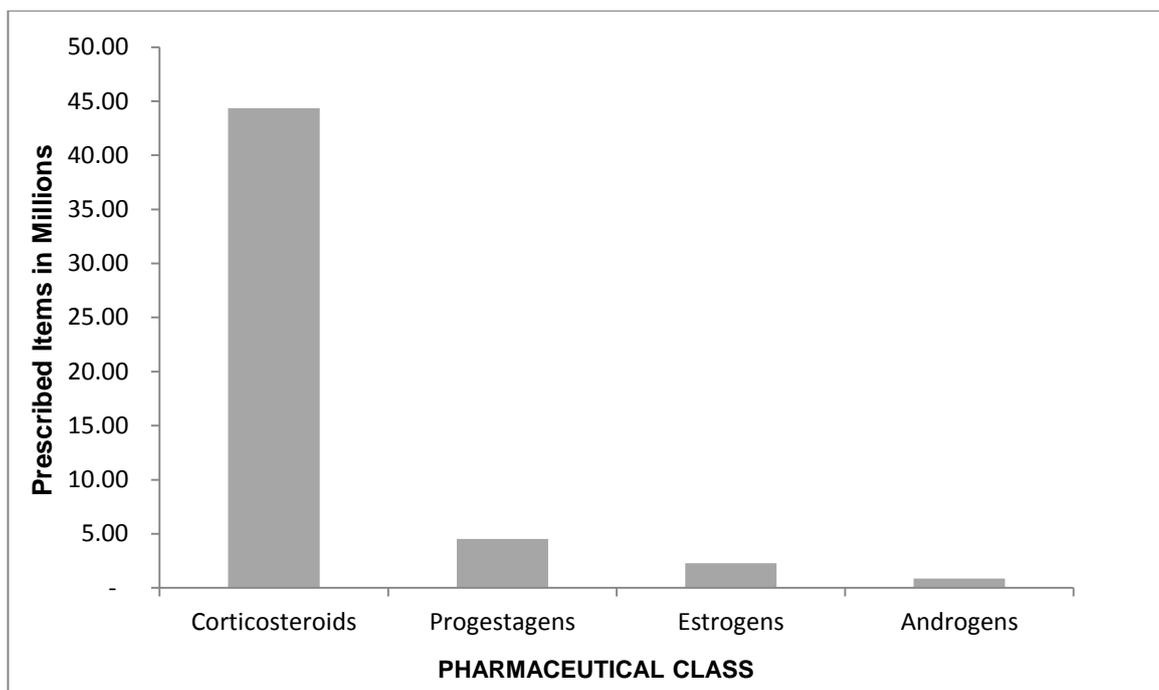


Figure. 16. Quantity of items containing steroid pharmaceuticals prescribed in England during 2014, based on British National Formulary data.

3.1.6. Therapeutics of Steroidal Pharmaceuticals

Synthetic steroids are used widely in pharmaceutical medicine to treat conditions related to the endocrine system; reproductive function, diseases of the endocrine system, metabolism, inflammation, immune function and osmoregulation. They act as receptor ligands by mimicking endogenous hormones, binding to their respective target receptors and triggering a biological process in the organism, acting agonistically or antagonistically to mediate endocrine function. The synthetic steroids can be classified further into five sub-groups according to their target receptor: estrogens, progestogens, androgens, glucocorticoids and mineralocorticoids. Steroid pharmaceuticals are used widely for a range of medical conditions regulated by the endocrine system. They are prescribed to act in place of natural steroids (derived from cholesterol), and help to control and regulate metabolism, growth and development, reproduction, immune function and inflammation.

They exert a wide range of effects, both from primary and secondary sites of action. Steroidal pharmaceuticals are designed with a similar molecular structure to natural ligands, making them able to mimic the body's endogenous hormones, resulting in competitive binding at the desired receptor with the natural ligand (where present). They bind to nuclear receptors in the cell with high binding affinity, sometimes equalling that of natural ligands (Liao et al, 1973; Blair et al, 2000).

Five compounds from the synthetic steroid group were selected for this study due to their pharmacological action, and the potential to cause adverse effects in fish (Table 8). They are relatively potent drugs, having been shown to cause effects on fish in the low ng/L range. These effects can be ecologically important, i.e. effects on reproduction and sexual development can lead to population-level effects. These compounds, or very structurally similar compounds, have also been detected in the environment, at concentrations relatively close to those shown to cause effects on wildlife. The target compounds in this study were also chosen because they represent a unique means of testing predictive mixture toxicity models. The selected compounds do not strictly follow the pharmacological assumptions of either of the models (described in Chapter 1), i.e. they are neither similar nor dissimilar. They are all known endocrine modulators, and have been shown to affect fish reproduction adversely, but they are thought to do so via different receptors and biological pathways.

3.1.6.1. 17 α -Ethinyl Estradiol (EE2)

17 α -ethinyl estradiol (EE2) is a 17 β -estradiol (E2) derivative. It is a synthetic alkylated estradiol with a 17 α ethinyl substitute (Pharmgkb.org), and is one of the most widely used synthetic estrogens. It is prescribed, typically in combination with a synthetic progestin, as a female oral contraceptive. Its mechanism of action in its use as a female contraceptive is via the increase in secretion of serum proteins such as of sex hormone binding globulin

(SHBG) in the liver, and the suppression of follicle stimulating hormone (FSH) from the anterior pituitary, therefor inhibiting follicle maturation and preventing pregnancy. The combination of EE2 with a synthetic progestin leads to a decrease in the production of gonadotropin releasing hormone (GnRH) via suppression of the HPG axis (drugbank.ca). EE2 is also used in postmenopausal hormone therapy to treat symptoms related to the onset of the menopause (caused by a natural decline in endogenous estrogen production), and in replacement therapy in treating female hypogonadism (caused by deficiency of natural estrogens) (Katzung, 2006). EE2 has a high binding affinity to the estrogen receptor, higher than all three endogenous estrogens. A study of 188 natural and synthetic estrogens in an estrogen receptor competitive binding assays found that EE2 had 190% relative binding affinity (RBA) to the ER, compared with 100% for 17β estradiol (E2), the most potent natural estrogen (to which RBA of all tested compounds was normalised) (Blair et al, 2000). Average daily therapeutic doses of EE2 in combined oral contraceptives are around $35\mu\text{g}$, taken daily for 21 days out of a 28 day cycle (de Mes et al, 2005; Katzung, 2006). Studies have shown that up to 80% of the compound can be excreted as un-metabolised conjugates (Ranney, 1977) and up to 16.5% as unconjugated free EE2 (Reed et al, 1972). It is a moderately hydrophobic compound with a $\text{Log } K_{ow}$ of 4.15 (Lai et al, 2000).

3.1.6.2. Levonorgestrel

Levonorgestrel is a synthetic progestin. Though often structurally different (some are C19, as opposed to C21 steroids), synthetic progestins have a similar mechanism of action to the endogenous progestogen progesterone (P4), and therefore have an inhibitory effect on ovulation. Increased plasma concentrations of progestogens prevent the release of another ovum, (which protects the developing pregnancy under natural conditions). Furthermore, progestogens cause a thickening of the uterine lining, preventing fertilisation

by sperm and making embryo implantation difficult. P4's inhibitory effect on ovulation has led to the development and use of synthetic progestins as a means of female contraception. Furthermore, synthetic progestins have a longer biological half-life than P4, affording slower metabolism and more effective use as a contraceptive (Africander et al, 2011). Synthetic progestins as a group differ structurally and pharmacologically to P4 and to each other, however some of the more recently introduced compounds have a structure relatively similar to P4. They are used as a female oral contraceptive, both in combination with synthetic estrogens (typically EE2) and on their own. They are also used in emergency birth control, again either individually or in combination with an estrogen, due to their ability to inhibit ovulation. The use of progestins is also approved in hormone replacement therapy, and in treating endometriosis and dysmenorrhea (Katzung, 2006).

Levonorgestrel is a second generation progestin. Early progestins were found to be associated with androgenic secondary effects, due in part to their affinity for the androgen receptor. Newer (third and fourth) generation progestins have since been developed that do not possess the androgenic and metabolic side-effects of earlier first and second generation progestins. Despite this, levonorgestrel is still used in monophasic and triphasic combined oral contraceptives, in progestogen-only oral contraceptives, and in emergency contraceptive pills (ECPs). It is also widely used as the active progestin in the hormone-releasing coil, or intrauterine system (IUS), as an alternative contraception method to daily oral contraceptives. It is typically administered at doses of around 1.5mg in ECPs, and 50-250µg in monophasic and triphasic combination pills (drugbank.ca). Target tissues include the ovaries, and indirectly, the hypothalamus and the pituitary. As with other progestins, it prevents ovulation by maintaining high levels of the progesterone mimic in circulation, and by inhibiting the release of GnRH from the hypothalamus, thereby inhibiting the secretion of LH (the hormone responsible for stimulating ovulation). Levonorgestrel is 100% bioavailable in the human body. 45% of the compound and its active metabolites are excreted in the urine and around 32% in the faeces as conjugates

(Drugbank.ca). It has a Log K_{ow} of 3.72 (Runnalls et al, 2015) and is therefore moderately hydrophobic.

3.1.6.3. Desogestrel

Desogestrel is a third generation progestin used as a female oral contraceptive. It has the same mechanism of action as Levonorgestrel, but has a lower binding affinity to the AR, and therefore produces fewer side effects caused by androgenicity. Desogestrel is an inactive prodrug, which is metabolised in the intestinal mucosa by hydroxylation to its active compound etonogestrel (3-ketadesogestrel). It has shown binding affinity for the PR, the AR and the GR *in vitro* (Fuhrmann et al, 1995). Therapeutic doses are around 0.15mg in the combined oral contraceptives. It is metabolised in the intestinal mucosa and the liver to the metabolites 3 α -hydroxydesogestrel and 3 β -hydroxydesogestrel before being oxidised to the active metabolite 3-ketodesogestrel (Drugbank.ca). Excretion is comparable with that of levonorgestrel; around 60% is excreted in the urine, whilst around 35% is excreted in the faeces (Verhoeven et al, 2001). Desogestrel has a Log K_{ow} of 5.65 (Medicines and Healthcare Products Regulatory Agency) and is therefore the most hydrophobic compound used in this study.

3.1.6.4. Beclomethasone Dipropionate

Beclomethasone dipropionate is a synthetic halogenated glucocorticoid used to treat inflammatory conditions. It is prescribed as a topical anti-inflammatory to treat a range of skin conditions such as eczema, dermatitis, and idiopathic rashes, and as an aerosol for the prophylactic treatment of asthma and allergic rhinitis. Daily doses vary between 200-800 μ g taken two to three times a day (BNF). Beclomethasone is administered as a pro-drug, which is metabolised to beclomethasone 17-monopropionate (17-BMP) via esterase enzymes in tissues throughout the body. Its binding to the GR inhibits leucocyte infiltration at localised inflammation sites, and suppresses humoral immunity (drugbank.ca). It is one

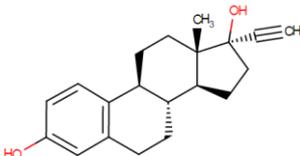
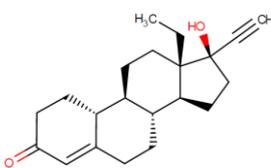
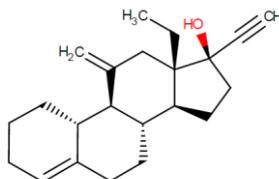
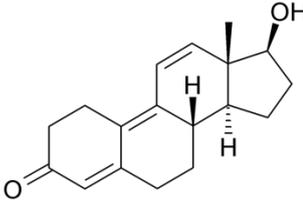
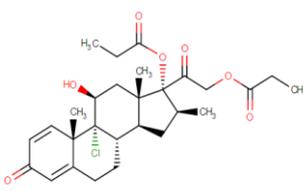
of the most potent synthetic glucocorticoids, with a half-life of 2.8 hours. Beclomethasone dipropionate and its metabolites are mostly excreted in the faeces, with less than 10% excreted in the urine (Drugbank.ca). It is moderately hydrophobic, with a Log K_{ow} of 3.69 (Margiotta-Casaluci et al, 2016). The acute toxicity of beclomethasone is relatively low, however, prolonged use has been linked to suppression of the adrenal system, with the potential to lead to the development of diseases such as Addison's disease and congenial adrenal hyperplasia.

3.1.6.5. 17 β -Trenbolone

17 β -Trenbolone is an androgenic anabolic steroid, first synthesised in 1963 and used, together with synthetic estrogens, as a veterinary pharmaceutical, to promote growth in livestock. Although no longer used in the UK, trenbolone is widely used across North America in beef cattle to increase muscle mass, efficiency of cattle feed and to increase the absorption of minerals (Song and Choi, 2001). Trenbolone is also misused illegally by athletes as a performance enhancer, promoting muscle growth and strength, which is known in the industry as doping. As a veterinary pharmaceutical, it is administered to cattle as the pro-drug trenbolone acetate (TBA), typically in the form of a controlled-release implant (Ankey et al, 2003). Trenbolone acetate is metabolised to its active form 17 β -trenbolone through hydrolysis (Khan et al, 2008; Parker et al, 2012), and to the active metabolites triendione and 17 α -trenbolone (Wilson et al 2002). Once metabolised, trenbolone increases the production of insulin growth factor 1 (IGF-1), a protein with important growth and anabolic functions. It is moderately hydrophobic with a Log K_{ow} of 3.08.

Unlike the endogenous androgen testosterone, it is thought that trenbolone cannot be aromatised to estradiol. For this reason, it proves a popular anabolic steroid amongst athletes. It also increases male secondary sexual characteristics. Trenbolone has a higher binding affinity to the AR than the endogenous androgen testosterone, and also has an affinity for the PR and GR as an agonist and antagonist, respectively.

Table. 8. Synthetic steroidal pharmaceuticals selected for investigation in the presented study.

Compound	Structure	Primary Mechanism of Action (MOA)	Non-specific MOA
<p>Ethinyl Estradiol CAS: 57-63-6 Sigma-Aldrich Chemical Formula: $C_{20}H_{24}O_2$</p>		<p>Estrogen receptor agonist. Suppresses release of GnRH from hypothalamus</p>	
<p>Levonorgestrel CAS: 797-63-7 Sigma-Aldrich Chemical Formula: $C_{21}H_{28}O_2$</p>		<p>Progesterone receptor agonist. Suppresses GnRH and the subsequent release of LH to prevent ovulation</p>	AR agonist
<p>Desogestrel CAS: 54024-22-5 Sigma-Aldrich Chemical Formula: $C_{22}H_{30}O$</p>		<p>Progesterone receptor agonist. Suppresses GnRH and the subsequent release of LH to prevent ovulation</p>	AR agonist GR (weak) ER (weak)
<p>17β-Trenbolone CAS: 10161-33-8 Sigma-Aldrich Chemical Formula: $C_{18}H_{22}O_2$</p>		<p>Androgen receptor agonist. Active metabolite of trenbolone acetate. Increases IGF-1, protein synthesis and resultant increased muscle mass and strength</p>	AR agonist GR (weak) ER (weak)
<p>Beclomethasone dipropionate CAS: 5534-09-8 Sigma-Aldrich Chemical Formula: $C_{28}H_{37}ClO_7$</p>		<p>Pro-drug, agonist of the GR. Metabolised to beclomethasone monopropionate. Anti-inflammatory – inhibits leukocyte infiltration at site of action</p>	AR agonist PR (weak)

3.1.7. Steroidal pharmaceuticals in the Environment

Steroidal pharmaceuticals are known environmental contaminants, having been detected in surface waters, waste water and ground water (Vulliet and Cren-Olivé, 2011; Kostich et al. 2014). Though present in trace amounts in the environment, synthetic steroids are highly potent, reflected in the low doses prescribed to human patients. Due to their high potency, effects of synthetic steroids on ecologically-important processes such as reproduction, development, growth and metabolism have been demonstrated. Furthermore, adverse effects on aquatic organisms can occur when those organisms are exposed to concentrations similar to those detected in the environment (Jobling et al, 1998; Jobling et al, 2002; Länge et al, 2001; Nash et al, 2004). Some studies have even linked environmentally-relevant exposure-related adverse effects to significant population level consequences (Kidd et al, 2007).

3.1.7.1. Estrogens

Synthetic estrogens have been detected in the aquatic environment, typically (but frequently) at relatively low concentrations (ng/L) (Baronti et al, 2000; Koplín et al, 2002). Endocrine effects as a result of exposure to synthetic estrogens are relatively well studied. The first documented cases of adverse effects in fish as a result of exposure to estrogenic effluent were in the early 1990's (Purdom et al, 1994; Folmar et al, 1996). Male roach (*Rutilus rutilus*) exposed to sewage effluents were found to be highly feminised, with a high rate of intersex observed in males. Further studies investigating the mechanisms of endocrine disruption in roach exposed to effluent found that intersex fish had significantly impaired reproduction (Jobling et al, 1998; Jobling et al, 2002a; Jobling et al, 2002b). Further studies elucidated the synthetic estrogen EE2 as one of the causative agents in effluent leading to intersex and repaired reproduction (Jobling et al, 2006). Although EE2 exerts a significant contribution, the effects observed on fish in rivers are likely to be a consequence of exposure to mixtures of estrogens. Since then, adverse effects as a result of synthetic steroidal estrogens in wildlife have been extensively studied. EE2 in particular

has been demonstrated to be a potent endocrine disruptor in fish, causing disruption to reproductive and endocrine pathways via disruption of estrogen mediated pathways (Länge et al, 2001; Folmar et al, 2002; Brion et al, 2004; Nash et al, 2004; Fenske et al, 2005; Armstrong et al, 2016). Concentrations reported to induce adverse effects on the reproductive axis in fish are consistently reported in the ng/L range, similar to concentrations thought to cause adverse effects in some aquatic organisms. For example, Armstrong et al, (2016) demonstrated significantly reduced fecundity of fathead minnows (*P. promelas*) exposed to environmentally-relevant concentrations of EE2 at 0.47, 1.54 and 3.92ng/L. In another study, Caldwell et al (2012) used existing long-term fish reproductive studies to develop species sensitivity distribution (SSD) and derive a predicted no effect concentration (PNEC) for EE2. They derived a PNEC of 0.1ng/L based on an extensive review of no-observed-effect concentrations, compared with a PNEC of 2ng/L for estradiol (E2), the most potent natural estrogen.

Many studies have utilised biomarkers to study estrogen-related endocrine disruption in fish. Induction of vitellogenin (vtg) (an estrogen-dependant process) in male fish is used extensively as a biomarker of estrogen exposure. Vitellogenin is an egg yolk precursor protein expressed only in the female sex in oviparous species. Vitellogenesis is the synthesis of vtg and its uptake into a developing oocyte. In fish it is synthesised in the liver and transported to the follicle cells surrounding the maturing ovum for nourishment of the developing embryo. In reproductively active females, plasma vtg levels are highly regulated by endogenous estrogens. In male fish, vtg is usually present in plasma at very low background concentrations (Routledge et al, 1998; Smeets et al, 1999). It has been extensively demonstrated that exposure to estrogens can stimulate vitellogenesis in male and juvenile fish (Tyler et al, 1996; Harries et al, 1997; Routledge et al, 1998; Denslow et al, 1999; Harries et al, 1999; Tyler et al, 1999; Panter et al, 2002; Rose et al, 2002), and hence shown to be an extremely sensitive biomarker.

Perhaps somewhat concerning, chronic exposure to EE2 has been shown to cause significant effects on fish populations. Kidd et al (2007) utilised an experimental lake system to expose wild fish populations to environmentally-relevant concentrations of EE2 over a seven year period. Fathead minnows (*Pimephales promelas*) were collected by live trapping twice a year and assessed for a number of estrogen biomarkers, including vtg induction, VTG mRNA expression, gonadosomatic index (GSI), and histopathology of the gonads. A population census was carried out using index trap netting. The authors of the study reported significant feminisation of male fish (through upregulation of VTG mRNA and induction of vitellogenesis), impaired gonadal development, intersex of male fish, and altered oogenesis in females. Furthermore, the authors reported the almost complete collapse of the fathead minnow population over the study period, due to EE2 exposure, and attributed the collapse to the loss of juveniles and the impaired breeding ability of adult fish. Though the population-level effects reported in this study are concerning, it is important to note that due to the nature of the experiment (experimental lake system), it is impossible to control for a number of factors that one could with laboratory experiments, such as temperature, pH, predation, other chemical contaminants, and additional stressors that may be present, that may also be contributing to population declines in this species. Nevertheless, no such effects were observed in the control lake system from the same study, suggesting that some level of endocrine disruption, and possibly population-level effects, are occurring as a result of chronic exposure to EE2. Although present in the environment, concentrations of synthetic estrogens are relatively low. EE2 measured in surface waters and effluent waters has been found to be lower than the endogenous hormone 17 β estradiol (Huang and Sedlak, 2001; Belfroid et al, 1999). Furthermore, concentrations of EE2 in drinking water have been found to be below analytical detection limits (Conley et al, 2016). Nevertheless, although present at low concentrations when compared with many other chemical contaminants, the high potencies of these compounds, in addition to their designed biological action, affords their consideration as high-risk chemical pollutants.

3.1.7.2. Progestins

Although relatively little is known about the effects of synthetic progestins on aquatic organisms when compared with the knowledge of estrogenic effects, recent studies have begun to focus on the effects of progestins in the environment. Synthetic progestins were first detected in environmental river samples in the 1980's by radioimmunoassay (Aherne, 1985). Since then, many other studies have detected progestins in the environment (Kuch and Ballschmitter, 2001; Vulliet et al, 2007; Pu et al, 2008; Viglino et al, 2008; Al-Odaini et al, 2010), at concentrations in the low ng/L range (table 9). Progestins are typically prescribed in higher doses than estrogens, for example in combined oral contraceptives, and therefore they may be expected to be present in the environmental at similar, or not higher concentrations.

A number of synthetic progestins have been shown to cause adverse effects on the reproductive axis in fish (Zeilinger et al, 2009; Paulos et al, 2010; Zucchi et al, 2012; Runnalls et al, 2013; Overturf et al, 2014; Runnalls et al, 2015). Concentrations capable of causing reproductive effects on fish have been demonstrated to be similar to those found in the aquatic environment. For example, Zeilinger et al (2009) exposed adult fathead minnows to two synthetic progestins, levonorgestrel and drospirenone, in a 21-day fish reproduction assay. The study demonstrated impaired reproduction ability at concentrations of <1ng/L, in addition to the masculinisation of females, quantified by the development of nuptial tubercles (male secondary sexual characteristics) in females, most likely due to the androgenic properties of the progestins. In another study by Runnalls et al (2013), fathead minnow were exposed to a range of concentrations of two progestins, desogestrel and gestodene, to investigate their effect on reproductive capabilities. The authors observed impaired reproduction which varied across a range of potencies for the different progestins, with gestodene being the most potent, affecting reproduction and a range of *in vitro* endpoints at concentrations as low as 1ng/L, whilst desogestrel was shown to be much less potent. Since some synthetic progestins are known agonists of the

androgen receptor, the androgenic potential of progestins led to further cause for concern in their ability to affect endocrine function in fish. Svensson et al (2013) reported significant induction of spiggin in female sticklebacks exposed to >40ng/L of levonorgestrel, demonstrating the potent androgenicity of this progestin. Furthermore, pronounced effects of progestins at low concentrations could be due to their highly potent nature, and their potential for bioaccumulation. For example, levonorgestrel has been shown to bioconcentrate in rainbow trout, with measured fish plasma concentrations of 8.5-12µg/L, in fish exposed to effluent concentrations as low as <1ng/L (Fick et al, 2010).

3.1.7.3. Androgens

Studies regarding the presence and persistence of synthetic androgenic steroids in the environment have been somewhat limited when compared to those on the synthetic estrogens. Nevertheless, androgenic effects have been observed in aquatic organisms as a direct result of exposure to synthetic androgens. Androgenic effects were first demonstrated in fish as a result of exposure to pulp and paper mill effluent (Karels et al, 2001; Parks et al, 2001; Ellis et al, 2003; Orlando et al, 2007). Since these early studies, androgenic effects in fish have been observed as a result of exposure to other types of effluents, particularly feedlot effluent and agricultural runoff (Soto et al, 2004; Jensen et al, 2006; Wilson et al, 2002), due to the use of androgenic veterinary pharmaceuticals on livestock. For example, Orlando et al, (2004) reported significant de-masculinisation of male, and de-feminisation of female, wild fathead minnows exposed to feedlot effluent. The study confirmed the androgenic activity of the effluent through *in vitro* cell line experiments based on cells transfected with the human androgen receptor.

As previously discussed, synthetic androgens are used extensively in agriculture. Trenbolone acetate is widely administered to livestock across North America, either as controlled-release implants or in feed. The widespread use and application suggest that there may be a significant likelihood that trenbolone and its metabolites will be present in

agricultural run-off from feedlots and land fertilised with treated animal manure. Schiffer et al, (2001) demonstrated that both 17α and 17β trenbolone metabolites were persistent in manure, with half lives of around 260 days. Furthermore, in the same study, trenbolone was detected in soil samples eight weeks after its fertilisation with trenbolone-contaminated manure, highlighting its persistence in the environment. Since it has been shown that trenbolone residues appear in fertilised agricultural land and in animal manure, it is likely that trenbolone metabolites are also entering rivers through agricultural run-off. In fact, trenbolone and its metabolites have been detected in environmental samples. Parker et al, (2012) measured concentrations of 31 and 52ng/L of 17β -trenbolone and trendione, respectively, in receiving waters and soil samples associated with agricultural water runoff.

Recent studies on fish exposed to synthetic androgens have demonstrated endocrine disrupting potential of androgens at relatively low concentrations (ng/L range). Trenbolone has been shown to cause masculinisation of female fish and reduced fecundity in laboratory studies (Ankley et al, 2003; Jensen et al, 2006). For example, Collette et al, (2010) observed masculinisation of female fathead minnows exposed to nominal concentrations of trenbolone of 500ng/L, demonstrated by the development of nuptial tubercles, an androgen-controlled male secondary sexual characteristic. In another recent study, adult fathead minnows exposed to 17β -trenbolone displayed significant reduction in fecundity at a concentration of 27ng/L (Ankley et al, 2003). Furthermore, in line with findings from similar studies, females appeared masculinised, demonstrated by the development of nuptial tubercles. Plasma concentrations of the endogenous steroids estradiol and testosterone were significantly reduced in females, as was the production of vitellogenin. Conversely, in male fathead minnows exposed as part of the same study, plasma 11KT levels were depressed, whereas estradiol levels were significantly higher, and the induction of vitellogenesis was also observed. This study highlights the complexity

of the HPG axis and the regulation of reproduction in fish, with the potential for synthetic androgens to interfere with feedback mechanisms and hormone signalling.

The nature of reproductive regulation in fish is further complicated due to the diverse strategies employed throughout the fish taxa. The regulation of steroid hormones and their feedback mechanisms can be vastly different between species. In salmonids for example, studies have demonstrated evidence for an aromatase-dependant positive feedback of androgens on gonadotropin hormone II, analogous to luteinizing hormone (LH) (Swanson and Dickhoff, 1988; Antonopoulou et al, 1999). Further studies have also demonstrated negative feedback control on LH in castrated adult coho salmon (*Oncorhynchus kisutch*), outlining the complex nature of feedback control of steroids on the regulation of gonadotropin. In several species, gonadotropin beta sub-units are expressed differentially at different stages of the reproductive cycle (Melamed et al, 1998).

Agonists of the androgen receptor are not the only compounds that have been linked to adverse effects in wildlife. Androgen antagonists, or anti-androgens, have also been demonstrated to cause adverse effects to reproductive capacity in fish (Sebire et al, 2011; Katsiadaki et al, 2012). Field studies using androgen-specific biomarkers have revealed the presence of anti-androgenic activity in STP effluent in the UK. Katsiadaki et al, (2012) demonstrated that exposure to effluent after treatment by granular activated carbon (GAC) caused an increase in spiggin production in female sticklebacks. This finding was correlated to increasing spiggin production in females with increased distance from pre-GAC treated effluent, elucidating to the presence of anti-androgenic compounds in GAC effluent, which are effectively removed by GAC treatment. Furthermore, other recent studies have demonstrated impaired reproduction of male sticklebacks exposed to the anti-androgenic effluent, highlighting the importance of assessing antagonistic behaviour of compounds in effluents (Sebire et al, 2011).

3.1.7.4. Glucocorticoids

Synthetic glucocorticoids have been detected in the aquatic environment in the ng/L range. Some synthetic glucocorticoids have a substitution of hydrogen (H) on the ninth carbon by a halogen. This substitution increases the bio-stability of the compound in human patients, extending the half-life of the drug in the body. It is hypothesised that this property may also increase the stability of such compounds and reduce their degradability once in the environment (Kugathas and Sumpter, 2011).

Reported concentrations of the synthetic glucocorticoid dexamethasone in China are 1.2ng/L in influent and <0.02-0.09ng/L in effluent (Chang et al, 2007). Schricks et al (2010) reported concentrations of dexamethasone in industrial wastewater in The Netherlands of up to 90ng/L; however the compound was not detected in any samples of hospital effluent or domestic effluent in the same study. Natural corticosteroids have been detected in surface waters and effluents, and have been shown to produce effects in fish at low concentrations. . Van der Linden et al (2008) utilised a human cell reporter gene assay (CALUX) to test a range of environmental samples (industrial, hospital, paper mill, and domestic sewage effluent, surface water and drinking water) for steroid hormone activity. Glucocorticoid activity was observed in all samples except drinking water, at relatively high activity levels of 11-243ng/L (dexamethasone equivalents) for effluents, and 0.39-1.3ng/L (dexamethasone equivalents) for surface waters.

Fish exposed to both natural and synthetic glucocorticoids have demonstrated impaired reproduction. Adverse effects of elevated endogenous corticosteroids in relation to stress response have been consistently demonstrated (Pickering et al, 1987; Carragher et al, 1989; Carragher and Sumpter, 1990; Campbell et al, 1994; Pankhurst and Kraak, 2000; Schreck et al, 2001; Schreck, 2010). Studies investigating the effects of corticosteroids on reproduction and the HPG axis in fish have further demonstrated negative effects. Physiological levels of cortisol have been found to inhibit steroidogenesis on rainbow trout

ovarian follicle cells *in vitro* (Carragher and Sumpter, 1990), highlighting possible mechanisms of disturbed reproduction during stress response in many fish species. LaLone et al (2012), observed a reduction in fecundity (egg production) in fathead minnows exposed to 500µg/L of dexamethasone in a fish reproduction assay. Furthermore, dexamethasone depressed plasma estradiol levels, increased plasma vtg in females, and caused abnormalities in hatched fry. Though adverse effects observed in fish in these studies could be ecologically relevant, the concentrations required to produce effects were high, thousands of orders of magnitudes higher than those that would be expected in the environment. Nevertheless, studies using much lower concentrations of synthetic glucocorticoids have also demonstrated adverse effects in fish. For example, Kugathas et al (2013) exposed adult fathead minnows to 0.1, 1 and 10µg/L of beclomethasone dipropionate and observed a dose-dependent increase in plasma glucose, a decrease in plasma cortisol, and a decrease in lymphocyte and thrombocyte production. Furthermore, analysis of reproductive endpoints revealed a decrease in plasma vtg, a reduction in ovipositor length, and an induction of male secondary sexual characteristics (nuptial tubercles and fin spots in females). Currently, little is known about the concentrations of synthetic glucocorticoids in the environment. However, recent studies are beginning to contribute to knowledge in this field. A recent study detected six out of nine tested glucocorticoids at concentrations in the low ng/L range in sewage effluent samples from Japan (Nakayama et al, 2016). Furthermore, given the high amounts of glucocorticoids prescribed, their relatively high potencies, and the potential to interact with not only the glucocorticoid receptor, but also the androgen and progesterone receptors, adverse effects due to their environmental presence could be possible.

In light of the evidence for the potential for adverse effects in fish due to exposure to relatively low levels of steroidal pharmaceuticals, they could be considered as important chemical contaminants for risk assessment. Some studies have attempted to predict risk to aquatic species from exposure to these compounds, based upon current information on

their pharmacological action and environmental characteristics. Fick et al (2010) estimated the surface water concentrations required to cause pharmacological effects in fish (assuming equivalent pharmacological activity) for 500 pharmaceuticals, by calculating the predicted critical environmental concentrations (CECs) for each compound. CECs for three of the compounds used in the current study were predicted as follows; EE2 0.37ng/L, Beclomethasone, 26ng/L, and Levonorgestrel 52ng/L. The work of Fick et al (2010) suggests that relatively low concentrations of these compounds in the environment are sufficient to cause pharmacological effects in fish.

Table 9. Environmental concentrations of the synthetic steroids used in this study

Compound	Class	Environmental Matrix	Environmental Concentration ng/L	Reference
Ethinyl Estradiol	Synthetic Estrogen	Effluent	<0.2-7.0	Desbrow et al, 1998
		Effluent	1.0-9.0	Ternes et al, 1999
		Effluent	>5.0	Kopling et al 2002
		Effluent	<0.1-8.9	Kuch and Ballschmitter 2001
		Surface Water	0.1-5.1	Kuch and Ballschmitter 2001
Levonorgestrel	Synthetic Progestin	Effluent	<0.2-4.0	Petrovic et al, 2002
		Effluent	0.9-17.9	Vulliet et al, 2007
		Surface Water	5.3-7.0	Vulliet et al, 2008
		Ground Water	7.4-11.0	Vulliet et al, 2008
Desogestrel	Synthetic Progestin	-	No information	-
17α/β-Trenbolone	Synthetic Androgen	Effluent	< detection limit	Liu et al, 2011
Beclomethasone dipropionate	Synthetic Glucocorticoid	-	No information	-

3.1.8. Fish as model species in Ecotoxicology

In toxicological assessment studies, model species are typically employed as a representative of a certain taxonomic group or groups. Standards for aquatic toxicology testing involve the use of organisms from three taxa, namely a microalga, an invertebrate and a vertebrate species. Using this standardised approach, species sensitivity differences are minimised, and results can be integrated into risk and hazard assessment, and usually extrapolated to other species (although this can sometimes be difficult, as previously discussed in Chapter 2).

Fish have been used in toxicity testing for over 100 years, however it has been only since the 1940's that their use in this field of research has become more widespread (Ankley and Villeneuve, 2006). Small fish species such as freshwater cyprinids are considered good model vertebrate species for assessing ecological impacts of chemicals in the environment. Commonly-used species include the zebrafish (*Danio rerio*), the fathead minnow (*Pimephales promelas*), the three-spined stickleback (*Gasterosteus aculeatus*), and the Japanese medaka (*Oryzias latipes*). Small fish models are easily reared and maintained under laboratory conditions, develop and mature rapidly, and are relatively inexpensive to maintain. Experimental exposure routes can be via numerous pathways, modified to suit the experimental questions, for example through dosed tank water, injection or in feed (Norrgren, 2012). Fish model species are employed for a range of scientific purposes, and are often utilised in developmental studies, evolutionary studies, human toxicology, risk assessment, and human diseases, in addition to ecotoxicology. Since fish are high up in the food chain, typically feeding on other fish and invertebrates, they are known to bioaccumulate toxins (Streit, 1998), and therefore provide a sensitive model species in toxicological studies. Furthermore, fish are exposed directly to chemical contaminants throughout their lives through their natural environment. With regards to pharmaceutical pollutants, fish share many highly conserved molecular targets as humans, suggesting that these contaminants have the potential to cause pharmacological

effects. Thus fish prove an ideal model species for assessing the effects of pharmaceuticals and their mixtures on non-target species.

3.1.9. Reproduction in Teleost Fish

3.1.9.1. The Hypothalamic-Pituitary-Gonadal (HPG) Axis

Teleost fish possess the same fundamental reproductive and endocrine components as humans. Reproduction in fish is regulated by the hypothalamic-pituitary-gonadal (HPG) axis. The HPG axis in teleost fish serves the same function as in mammals, and the basic aspects of the system are well conserved across vertebrates. It regulates the development and function of the reproductive organs and reproductive cycles, and controls reproductive behaviour. It consists of multiple tissues, the hypothalamus, the pituitary, and the gonads, which are connected via neurone signalling and hormone secretion. Neuroendocrine neurons are triggered in the hypothalamus in response to external (environmental) and internal (feedback mechanisms) stimuli, resulting in the production of gonadotrophin releasing hormone (GnRH). GnRH released from the hypothalamus stimulates pituitary gland production and release of the gonadotropins GtH-I and GtH-II, analogous to mammalian FSH and LH, respectively. LH and FSH are released into the circulatory system, where they subsequently bind to their corresponding receptors in the gonads, regulating the production of the sex steroids, and stimulating gametogenesis (spermatogenesis and oogenesis) through a complex pathway of steroidogenic enzymes (Chong et al, 2005). In response, the ovaries of females start producing estradiol (E2), stimulating the production and transportation into oocytes of the glycolipoprotein vitellogenin, an egg yolk precursor that serves as nourishment in early egg development.

In essence, similar to the situation in humans, in fish estrogens regulate the gonadotropins LH and FSH, oogenesis, vitellogenesis and gonadal development in females, whilst androgens modulate LH and FSH, spermatogenesis, gonadal development and the development of secondary sexual characteristics in males (Nelson and Habibi, 2013). The main androgen synthesised in male fish is 11-ketotestosterone (11-KT), unlike in mammals where the main androgen is testosterone, although testosterone is produced in both sexes and plays an important role in regulating HPG feedback. To date, progesterone (P4) has no known function in fish, however, the progestagens $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP) and $17\alpha,20\beta,21$ -trihydroxy-4-pregnan-3-one (THP) are synthesised by fish, and control maturation of oocytes and ovulation in females (Kime et al, 1993) and spermiation and sperm motility in males. Furthermore, the sex steroids also play a role in sexual behaviour and in some species also act as sex pheromones (Thomas et al, 2012; Tokarz et al, 2013).

As in mammals, fish corticosteroids are important regulators of immune function, osmoregulation, respiration, metabolism, reproduction and growth (Prunet et al, 2006). Unlike in mammals however, fish do not possess discrete corticosteroid producing glands. Instead, adrenal tissue (termed interrenal in fish) is distributed throughout the anterior kidney. The main corticosteroid in teleost fish is cortisol, though fish also synthesise cortisone, 11-deoxycortisol and corticosterone.

3.1.9.2. Steroids and Steroid Receptors in Teleost Fish

As in humans, steroids are key regulators of the HPG axis. Since pharmaceuticals are designed to act on specific molecular targets (receptors, enzymes etc.) to elicit a biological response, it is possible that pharmacological effects could be observed in non-target species exposed to the drugs, given that these same molecular targets are present. Furthermore, pharmaceuticals are designed to do this at relatively low therapeutic

concentrations, so as not to illicit an overall toxic effect in human patients. Across the vertebrate taxa, many of these evolutionary targets are highly conserved. Gunnarsson et al (2008) investigated 1318 orthologs of human pharmaceutical targets across 16 species commonly used in testing. The study found that the zebrafish (*Danio rerio*) and the three-spined stickleback (*Gasterosteus aculeatus*) possess high conservation of these human drug targets, with over 86% ortholog conservation (1136 and 1160 respectively; similarity >60%). Conserved orthologs included the steroid receptors. Since these drug targets are present in fish, and relatively well conserved, it seems probable that effects of pharmaceuticals in fish reported from the literature suggest mechanism mediated effects, rather than a general toxicity effect.

Teleost fish have two estrogen receptors, $er\alpha$ and $er\beta$, encoded by different genes, similar to the situation in humans. Recently it has become apparent that some fish, notably the ray-finned fish, also have a distinct duplicate copy of the beta receptor, $er\beta$ -I (or $er\beta$ -1) and $er\beta$ -II (or $er\beta$ -2). Where present, fish $er\beta$ -II has the most similarity to mammalian $er\beta$, whilst $er\beta$ -I is the most dissimilar, and is thought to have arisen as a result of gene duplication. In order to standardised the nomenclature, in fish, $er\alpha$ is now classified as *Esr1*, $er\beta$ -1 is designated *Esr2b*, and $er\beta$ -2 is *Esr2a*, in order to bring them in line with the human estrogen receptor nomenclature (Filby and Tyler, 2005). A second $er\alpha$ form, $er\alpha$ -2, has also been reported in rainbow trout, and is thought to have arisen from a recent whole genome duplication event, after their divergence from the lobe-finned fish, (Amores et al, 1998; Nagler et al, 2007). Furthermore, though expressed ubiquitously in rainbow trout, the four subtypes were differentially expressed in different tissues, suggesting a complex regulation of estrogen receptor signalling in fish, and suggesting that, as in humans, transcriptional activity, protein sequence, and ligand binding differs across the receptor subtypes (Nagler et al, 2007).

In the fathead minnow, three distinct estrogen receptors have been described, namely *Esr1*, *Esr2b* and *Esr2a* (Filby and Tyler, 2005). In line with other reported findings, the

study found high homology between fathead minnow Esr1 and human Esr1, and fhm Esr2b and hErs2, suggesting similar binding affinities to estrogen ligands and estrogen response elements (EREs) as in humans. Characterising steroid receptors in model fish species is important, as it facilitates more detailed understanding of the mechanisms of endocrine disruption in these species. As in mammals, estrogen pathways in fish are modulated by two distinct transduction pathways, one via classical estrogen pathways (genomic modulation) and one via rapid non-genomic signalling by membrane estrogen receptors (mERs), belonging to the G protein-coupled receptor family (Thomas et al, 2005; Nagler et al, 2007). Fish genomes also encode two aromatase enzymes, CYP19a and CYP19b, that synthesise estrogens (and other steroids) from cholesterol (Kuhl et al, 2005).

Until relatively recently, only one androgen receptor has been reported from teleost fish. However, studies have revealed two androgen receptor forms from Japanese eel, *Anguilla japonica* (Ikeuchi et al, 1999; Ikeuchi et al, 2001), rainbow trout, *Oncorhynchus mykiss* (Takeo and Yamashita, 1999), and the Atlantic croaker, *Micropogonias undulatus* (Sperry and Thomas, 1999). However, it is currently not clear whether these are separate androgen receptor forms, or subtypes encoded by the same gene. Currently, only one androgen receptor has been identified in zebrafish and in fathead minnow, which is homologous to the mammalian AR. The variation in androgen receptor number and form in fishes reflect the nature of the diverse reproductive strategies differentially evolved by fish species. Based on current evolutionary gene characterisation, it is supposed that a common AR exists in all jawed vertebrates, and that two functionally different AR's have likely arisen as the result of a genome duplication event, namely the teleost-specific genome duplication (TSGD) that occurred after the split from the ray-finned fish (Ogino et al, 2009). As discussed previously, the main androgen in fish is 11KT, though testosterone (T) is also synthesised, albeit at lower levels. Furthermore, recent studies have demonstrated that DHT can be a highly potent androgen in fish (Margiotta-Casaluci

and Sumpter, 2011), despite the fact that teleosts do not naturally produce the hormone. Furthermore, early work on androgenic control of pituitary gonadotropins in species such as eel and trout demonstrated important species variations in the mechanisms of androgen-dependant regulation of gonadotropins (Huang et al, 1997).

Two main progestogens have been identified in fish, $17\alpha,20\beta$ -dihydroxy-4-pregnan-3-one (DHP) and $17\alpha,20\beta,21$ -trihydroxy-4-pregnan-3-one (THP). Although the role and regulation of progestogens differ in fish compared with mammals, all progestogens bind to the progesterone receptor (PR), although with varying affinities. Similar to human progesterone, fish progestogens also have binding affinity for other steroid receptors (Blüthgen et al, 2013). To date, only one PR has been identified in teleost fish.

The synthesis of corticosteroids in fish is distinct from that of mammals. The first fish glucocorticoid receptor (GR) was cloned from rainbow trout (Ducouret et al, 1995). Since then, multiple GR's have been reported from teleost fish (Bury et al, 2003; Greenwood et al, 2003; Schaff et al, 2008). The identification of two separate GR forms, termed GR1 and GR2, encoded by separate genes has been described, followed by a further observation of a splice variant of GR2, producing GR2a and GR2b, in the cichlid fish *Haplochromis burtoni* (Greenwood et al, 2003). The zebrafish glucocorticoid receptor 2 (zfGR2) has been found to be homologous to the human glucocorticoid receptor beta isoform (hGR β). The two forms have similar transcription activities as they do in humans (high mRNA expression for GR1 and low mRNA for GR2), and, as in humans, zfGR2 acts as a dominant-negative inhibitor of zfGR1 *in vitro* (Schaff et al, 2008). Furthermore, fish do not appear to produce aldosterone, the main mineralocorticoid present in humans (Gilmour, 2005). It has been shown that the glucocorticoid cortisol plays an important role in osmoregulation (McCormick, 2001; McCormick et al, 2008). It had previously been thought that cortisol carried out both glucocorticoid and mineralocorticoid functions in fish, acting through a single receptor, the GR. Given the overlap in binding affinities of the

endogenous human ligands to both these receptors (discussed previously), this assumption would be reasonable. However, recent studies have demonstrated that teleost fish do possess a mineralocorticoid-like receptor (Colombe et al, 2000). Furthermore, Greenwood et al (2003) demonstrated that the MR of the cichlid fish *Haplochromis burtoni* was similar in sensitivity to cortisol and aldosterone, just as the human MR, and that this MR was more sensitive to the ligands than the GR. Fish GR forms and MRs have been found to have high sequence identity within the teleost and across vertebrate taxa, and alternative splicing GR forms support the genome duplications reported in other genes (Prunet et al, 2006). Currently, mechanisms and signalling pathways of corticosteroids in fish are not well understood.

3.1.10. Fish Reproduction Assays for Investigating Endocrine Disruption

Fish reproduction is thought to be one of the most sensitive endpoints for exposure to synthetic steroids (Zeilinger et al, 2009). As previously discussed, the steroid receptors are highly conserved across vertebrates, and fish reproduction is controlled by many of the same mechanisms as in humans. Fish reproduction assays have relatively robust endpoints that are easily quantifiable (i.e. fecundity), and that have proven sensitive to such compounds. In addition, they are highly relevant from an ecological stance, since the reproductive ability controls population-level effects.

3.1.11. Test species: *Pimephales promelas*, Fathead minnow

The fathead minnow, *Pimephales promelas* (Rafinesque, 1820) is a freshwater cyprinid species native to temperate water regions of North America. It inhabits muddy pools, creeks, ponds, small rivers and lakes (Page and Burr, 1991), and can tolerate a range of temperature and pH conditions, (10-28°C). Females can grow to reach 1.5-3.0g in weight, whereas males can grow to 3.0-5.0g (pers. obs.), and maximum lengths of around 8cm

(fishbase.org). They typically live for 3 years, while the maximum recorded age is 5 years (Danylchuk and Tonn, 2006).

Fathead minnows, like most teleosts, are oviparous spawners. Females release eggs to the external environment to be fertilised by the male, and development subsequently occurs outside of the maternal body (Coward et al, 2002). They are asynchronous ovulators, whereby oocytes of all stages of development can be present in the ovary at a given time. Females produce yolky eggs that are externally fertilised by the males, and subsequently large clutches of fertilised eggs are deposited onto a hard substrate. Males guard and care for the nest site until the larvae have hatched, typically within 4-5 days post fertilisation. The males use their fleshy dorsal fat pad to keep the eggs aerated and clean, and to dislodge dead and diseased eggs to ensure the health of the remaining clutch. In their natural environment, fathead minnows are fractional spawners, spawning only when the temperature rises above 18°C during the summer months. They sustain spawning, producing clutches of eggs every few days until temperatures drop back below 18°C at the end of the summer (Encyclopaedia of Life, 2016). Since reproduction in this species is largely controlled by temperature and light, conditions that can be easily replicated in a laboratory, reproductive and endocrine studies using this species can be performed continually throughout the year, without the need to wait for seasonal reproductive cycles that occur in other fish species such as roach (*Rutilus rutilus*) and the salmonids.

The fathead minnow, *Pimephales promelas*, are one of the more commonly used fish model species in environmental toxicology, particularly in the study of endocrine disrupting compounds (EDCs). It is a commonly used species for assessing effects of environmental estrogens and androgens due in large part to the visual manifestation of secondary sexual characteristics in this species. Males develop androgen-dependant visually observable secondary sexual characteristics as they become reproductively active that are not exhibited in females. Dark bands of colouration on the body, dorsal fin spots, nuptial

tubercles (raised epidermal structures protruding from the head and snout) and fleshy fat pads (a dorsal pad of mucus-secreting cells used for mating courtship and in cleaning and defending egg nests) are androgen-controlled male traits (Cole and Smith, 1987). Conversely, females possess a protruding ovipositor, a typically more rounded abdomen, and are lighter in colour, traits that are largely controlled by estrogens. These physical characteristics can be quantified easily in both sexes. The secondary sexual characteristics are largely controlled by the endocrine system and are known to be highly sensitive to endocrine disrupting chemicals, making the fathead minnow an ideal species to use in toxicology tests involving chemicals that disrupt the endocrine system. This species has been used extensively in early life-stage and full life cycle studies in support of chemical regulatory programs by both the US EPA and the OECD in Europe (Jensen et al, 2001). In addition, fathead minnows are somewhat larger than other commonly used model fish species such as zebrafish (*Danio rerio*) and medaka, (*Oryzias latipes*), affording the potential to collect larger blood samples for plasma steroid analysis, larger tissue samples for analysis of gene expression, and increasing general ease of sampling over their smaller relatives. Furthermore, since the fundamental aspects of the Hypothalamic-Pituitary-Gonadal (HPG) axis are generally well conserved in all vertebrate species, the effects of EDCs on this model species can be extrapolated to other vertebrates (Ankley and Johnson, 2004). Early development of fathead minnow is relatively similar to that of other cyprinids such as the zebrafish. They have transparent embryos, a short gestation time (5 days from fertilisation to hatch; 3-4 months from hatch to reach full sexual maturity) and can be induced to spawn in a laboratory environment, whilst sustaining breeding for long periods of time under optimal conditions. Though the FHM genome has not been completely mapped, sequence data for key genes associated with the production and regulation of steroids are publically available.

Although the fathead minnow is an ideal model species in investigating the effects of endocrine disrupting chemicals, it is important to note the limitations in existing toxicity

testing. The three main species currently used in chemical testing, the zebrafish, the medaka and the fathead minnow, are fractional spawners, short-lived, and start spawning early in life. However, many species, particularly the wild populations that are the focus of required protection, are periodic annual spawners with a delayed spawning onset. Thus the wide-range of reproductive strategies employed by other fish are not well represented in existing toxicity testing (Hutchinson et al, 2006). Profiles of endogenous and exogenous steroids can differ substantially between species with varying reproductive physiologies, a factor which is not currently accounted for in chemical testing. Furthermore, certain well-established endocrine-related biomarkers cannot be applied to all species, for example, in some fish, the egg shell proteins zona radiata (ZR) and zona pellucida (ZP) are synthesised in the liver of female fish, under the control of endogenous estrogens, secreted into circulation by hepatocytes and integrated into developing oocytes. However, in two of the commonly used model species, the zebrafish and the fathead minnow, the proteins are only synthesised in maturing oocytes, and are therefore not detectable in the plasma in zebrafish and fathead minnows, making them unsuitable biomarkers for estrogenic exposure in these species (Carvan et al, 2007).

Physiology and reproductive strategy of fish can have substantial effects on their sensitivity to chemical pollutants, particularly in the case of endocrine disruptors. Since commonly-used endpoints for testing endocrine disruption are related to steroid hormone profiles and feedback mechanisms, sexual differentiation, gonadal maturation, and VTG levels for example, important differences would be expected between species. Due to the considerable differences in physiology within the fish taxa, the currently used fish models could be considered as not well representative.

3.1.12. Evidence for Effects of Steroidal Pharmaceutical Mixtures

Combination effects as a result of exposure to steroid or steroid mimics have been demonstrated throughout the scientific literature, both *in vitro* and *in vivo*. Synergistic effects from mixtures of estrogenic compounds were first reported in the 1990's. Arnold et al (1996) reported synergistic mixture effects on the human estrogen receptor (hER) *in vitro*, whereby the mixture was more toxic than expected based on the toxicity of the individual compounds, suggesting that similarly acting compounds could act synergistically in the environment when present in combination. However, in a similar study by Ashby et al (1997), the results were found to be un-reproducible, and no synergism was reported, subsequently leading to the Arnold paper being retracted. This highlights the difficulty of conducting and interpreting mixture assessments. Nevertheless, additivity of similarly acting compounds has been observed in aquatic species (Brian et al, 2005; Brian et al, 2007). Antagonistic mixture effects are also possible, although these are rarely reported in the literature. Given that the mixture models currently available fail to account for interactions of the individual compounds (i.e. competition for binding sites, changes to behaviour and potency of compounds due to other compounds in the mixture), synergistic and antagonist effects can be difficult to predict.

Experiments assessing mixture effects of pharmaceuticals using *in vitro* end points are perhaps the most well-represented in the literature. Since toxicity data on the same endpoint are required from each individual compound in a mixture before one is able to assess mixture effects, *in vitro* assays can offer rapid screening, robust endpoints and easily interpretable data. Mixture effects *in vitro* have been reported from similarly (Payne et al, 2000; Rajapaske et al, 2002; Thorpe et al, 2001; Brion et al, 2012) and dissimilarly (Pomati et al, 2006; Blake et al, 2010) acting pharmaceutical compounds. Although mixture effects on *in vitro* end points can suggest possible effects at higher biological levels, assessing mixture effects on *in vivo* endpoints at the whole organism level are

crucial from an ecological and environmental risk perspective. Recent studies have demonstrated the potential for combination effects *in vivo* as a result of exposures to similar (Altenburger et al, 2000; Brian et al, 2005; Jukosky et al 2008; Pottinger et al, 2013; Runnalls et al, 2013) and dissimilar (Faust et al, 2003; Velasco-Santamaria et al, 2013; Runnalls et al, 2015) mixtures, using the CA and IA models, respectively. Recent studies have also assessed mixture effects from similar compounds (i.e. affinity for the same receptor) that act in an opposing manner. For example, Sun et al, (2009) tested the effects of a binary mixture of an estrogen and an anti-estrogen on reproductive output and endocrine biomarkers in Japanese medaka (*Oryzias latipes*) in a 21-day reproduction assay. They authors found that estrogenic activity as indicated by selected biomarkers, such as plasma vitellogenin, could be counteracted to some extent by anti-estrogens. However at the highest biological level, reproduction, estrogenic effects were found to be more severe than those caused by each individual compound. This study highlights the potential for interpretation differences of mixture effects across different endpoints and biological levels.

As discussed in Chapter 1, CA typically predicts more accurately the mixture effects of similarly acting compounds, whilst IA more accurately predicts effects from dissimilar compounds. CA commonly predicts more pronounced combination effects than IA, and therefore, in the case of similarly acting compounds, IA generally underestimates the observed effects. CA is considered a 'worst-case' scenario, as it generally predicts the most pronounced effects, and can be used in risk assessment as a conservative risk approach.

3.1.13. Chapter Specific Aims and Objectives

The majority of mixture studies in ecotoxicology have focused on binary mixtures (i.e. two compounds), in simple bioassays (bacterial, algae, daphnia) (European Commission, 2009). The current study aimed to investigate the use of a fish reproduction assay for

assessing the combined effect of steroidal pharmaceuticals in a mixture, on an ecologically relevant endpoint. Some studies (reviewed in previous sections) have already demonstrated mixture effects from either similarly or dissimilarly acting compounds on ecologically relevant end points. The study presented here will focus on compounds that do not strictly fit these definitions. In 'real world' mixtures, chemicals are not likely to fall within these definitive categories, but are more likely to consist of similar and dissimilar chemicals, with many compounds acting as both similar and dissimilar. Since pharmaceuticals have well established mechanisms of action, and a great deal is known about the primary, secondary and indirect targets within biological systems (typically from human and rodent drug trial data), these compounds offer a valuable platform for investigating mixture effects using predictive modelling. The study will examine the effects of mixtures of steroids that in theory affect different physiological systems, however may be expected to interact at various levels via 'cross-talk'. For example many dissimilarly acting steroid compounds often share co-factors with others. In addition, many are also not completely specific to their target receptor, gene or protein, for example many synthetic progestogens are also strongly androgenic, as previously discussed.

3.1.13.1. Key Aims

- To demonstrate mixture effects of a five-compound steroidal pharmaceutical mixture on an ecologically relevant endpoint – egg production of pair breeding fathead minnows (*Pimephales promelas*).
- To assess whether the predictive mixture models Concentration Addition (CA) and Independent Action (IA) are able to accurately predict combination effects of a five steroidal compound mixture, and if so, which model predicts the observed effects more accurately?

3.1.13.2. Key Questions

- Can five steroidal pharmaceuticals with dissimilar modes of action produce combination effects on fecundity in the fathead minnow (*Pimephales promelas*)?
- Can five steroidal pharmaceuticals with dissimilar modes of action produced combination effects on fecundity in the fathead minnow (*Pimephales promelas*) when present at low concentrations individually, i.e. each is present in the mixture below their Lowest Observed Effect Concentration (LOEC)?
- Can we use the predictive mixture models CA and IA to accurately predict these effects?

3.2. Materials and Methodology: Reproductive Performance Assessment of Pair-Breeding Fathead minnows exposed to a five compound steroid mixture

Reproductive assays investigating the effects of the individual steroid pharmaceuticals were performed over a five year period at Brunel University London, by Dr Tamsin Runnalls and Dr Kugathas Subramaniam. The data collected from the single compounds was used to design two mixture studies that were completed by the author. For the focus of this chapter, the purpose of the study was to formulate predictions of mixture effects based on the individual compound data, and to subsequently compare predictions with observed mixture effects in the pair-breeding assay.

Furthermore, since previous reproductive assessment of beclomethasone dipropionate (undertaken by Dr Kugathas Subramaniam) produced higher than expected effects on fish reproduction, a single compound exposure experiment using this compound was run alongside the multicomponent mixture experiment in order to obtain a more complete dose-response curve for this chemical.

The assay used for the single compound and the mixture experiments was the fathead minnow pair-breeding reproduction assay (Harries et al, 2000) and is described in detail below. The assays were conducted in a similar manner (for the individual compound and the mixture experiments), and the same protocol was used to determine the effects on reproduction of the single compounds (undertaken at Brunel University London prior to this project) and the multi-compound mixtures (new research undertaken as part of the project presented here). Though not undertaken by the author, methodologies and results of the individual compounds will also be presented. Since this study was based on the effects of the individual compounds, it is important to include the results of these studies.

3.2.1. Environmental Conditions of the Assay

Throughout the experiment, fish pairs were held in 8 litre glass tanks under a continuous flow-through system of dechlorinated carbon filtered tap water (5µm and 10µm filters),

with a flow rate of 60 L/hour (per 8 replicate tanks), resulting in a complete change of water at least every 2 hours. Each tank was equipped with a tile, grid and dish as a spawning substrate. Fish were fed four times daily, twice with Tetramin dry flake food (Tetra, Southampton, UK) and twice with defrosted brine shrimp (Tropical Marine Centre, Gamma irradiated), with approximately 3 hours between feeds. On weekends, fish were fed three times daily, twice with dry flake and once with brine shrimp. Fish were not fed on sampling days to prevent dissection difficulties as a result of food in the digestive tracts of the fish. The photoperiod was maintained throughout the entire experiment at 16 hour light, 8 hour dark cycle, with 20 minute dawn and dusk periods. Dissolved oxygen and temperature was monitored daily and maintained at $25\pm 1^{\circ}\text{C}$ and 8 ± 1 mg/L, respectively. Nitrite, nitrate, pH and ammonia levels were checked once per week. Tanks were syphoned and cleaned weekly to remove uneaten food and faeces, and chemical mixing vessels were cleaned on a regular basis.

3.2.2. Chemical Exposure

3.2.2.1. Five steroid mixture experiment

Aqueous stock solutions of EE2 (Sigma-Aldrich, UK. CAS: 57-63-6, purity, $\geq 98\%$), Levonorgestrel (Sigma-Aldrich, UK. CAS: 797-63-7, purity, $\geq 99\%$), Desogestrel (Sigma-Aldrich, UK. CAS: 54024-22-5, purity, analytical standard), Trenbolone (Sigma-Aldrich, UK. CAS: 10161-33-8, purity, $\geq 93\%$), and Beclomethasone dipropionate (Sigma-Aldrich, UK. CAS: 5534—09-8, purity, $\geq 99\%$) were prepared weekly using 2.5L Winchester amber glass bottles and double distilled water. Dosing stock solutions were made at 5000 times concentrate to achieve desired tank concentrations (pump rate 0.2ml/min; flow rate 60L/hour). Master stocks were made up in ethanol and stored at 4°C throughout the experimental period. The highest concentration master stock was made up initially, and diluted to make the medium and low master concentration stocks (appendix 1). The same

master stocks were used throughout the experiment to make up all corresponding dosing stocks, to avoid variability. Stocks were made up so that ethanol concentrations in the experimental tanks were <0.00001%.

3.2.2.2. Beclomethasone dipropionate single exposure experiment

Beclomethasone dipropionate (Sigma-Aldrich, UK. CAS: 5534—09-8, purity, ≥99%) was prepared weekly using 2.5L Winchester amber glass bottles and double distilled water. As described above, dosing stocks were made at 5000 times concentrate to achieve the desired tank concentrations. Nominal tank concentrations of beclomethasone dipropionate were 25, 100 and 1000ng/L. Stocks were made up so that ethanol concentrations in the experimental tanks were <0.00001%.

3.2.2.3. All experiments

During the exposure period, medical grade silicone tubing delivered the chemical stock to glass mixing vessels, where the stock was mixed, diluted with water and delivered to the tanks (8 replicate tanks per mixing vessel). Flow rates were controlled using a multichannel peristaltic pump set at 0.2ml/minute (Watson Marlow, Cornwall, UK), equating to a flow of 12ml/hour. The flow rate (1000ml/min) was divided by 0.2, giving a dilution factor of 5000. Flow meters were set at 2x30L/hr per mixing vessel (i.e. per 8 tanks). Flow rates and dosing efficiency were monitored on a daily basis, and adjusted if necessary. Dosing stocks were changed every 4-5days throughout the exposure period.

3.2.3. Fathead minnow 21-day Pair Breeding Assay Protocol

Sexually mature fathead minnows, *Pimephales promelas*, were selected from a mixed sex breeding stock maintained at Brunel University London. The experiment was conducted in a sealed laboratory under temperature and light controlled conditions in the Biological Facility at Brunel University. Seven days prior to the start of the experimental pairing

period, fish were sexed and separated from mixed sex tanks into male and female only tanks to initiate breeding behaviour.

3.2.3.1. 14 day Pairing Period

A 14 day pairing period was performed prior the start of the exposure study to ensure compatibility and success of breeding pairs. Fish were randomly selected from mixed stocks and transferred into 8 litre glass tanks, with one male and one female per tank. In each assay, eight replicate pairs per treatment group were allocated. A spawning substrate was added to each tank in the form of a rounded tile. Tiles were checked daily for eggs and, if present, tiles were removed and replaced with a fresh tile. Tank floors, outflows and walls were also checked daily for eggs, and if present, these eggs were syphoned out and included in the breeding assessment results. Pairs were assessed daily for breeding compatibility. Fish were left undisturbed (with exception of the early morning feed) until 11am when the spawning substrate was checked and eggs were collected. Every day, the spawning substrate was disturbed in every tank, irrespective of whether the pair had spawned, to minimise bias in the results due to unequal disturbance. Reproductive capability of individual breeding pairs is of key importance in the utilisation of the pair-breeding assay. Without pre-study assessment into the reproductive potential of breeding pairs, results could be skewed due to biological variability, introducing an element of chance. Since previous experiments had shown that not all males will spawn with all females, and vice versa, individual pairs were continuously assessed over the two week pairing period based on condition and behaviour. Although it can be difficult to determine whether the lack of breeding success is due to the male, the female, or both, by visual assessment alone, close examination of their behaviour and physical condition can give an indication. For example, some males were extremely boisterous, and unsuccessful mating was due to the male's tendency to stress and injure his female mate. On the other hand, some males were found to have reduced secondary sexual characteristics (i.e. small fatpad, lighter body colour, reduced tubercle count/prevalence),

and failed to show any interest in breeding. In group spawning situations, these males would typically be the non-dominant, submissive males. However in a breeding pair scenario, many of the less dominant males begin to display enhanced male secondary sexual characteristics in the absence of other, more dominant males. In addition, the body shape of female fish can give an indication of potential breeding success. A female that appears more streamlined with a body shape more characteristic of male fish might have reduced breeding potential. Behaviour of both males and females were used as an indicator of potential breeding success. Males that did not try to entice females to the spawning substrate, and in some cases actively drove them away, were returned to the general holding stock. Females that spent all of their time at the top of the tank, away from the spawning substrate and the male, were also returned to stock.

After collection, spawned batches of eggs were graded in relation to egg number, and records were kept of each pairs breeding performance. Pairs that had not spawned after the first 6 days were separated, and either a new male or a new female was introduced to the tank, depending upon condition and behavioural assessments. On some occasions, the male or female of the pair was switched directly with those in another tank that had also been unsuccessful, if both the male and female of the pair appeared physically healthy and in good breeding condition. Pairs were continually assessed over 14 days and reallocated a new mate until breeding successfully. Males and females that failed to spawn over this two week period were transferred back to the general stock and not utilised in the experiment. After the 14 day pairing stage, the breeding success of each pair was evaluated based upon the number of batches of eggs produced, the number of eggs per batch, and the chosen spawning substrate (i.e., the tile, outflow, wall etc.). Since substitutions were occurring throughout the two week period, time with final mate was also accounted for in the assessment.

At day zero, the day before the start of the experiment, final breeding pairs were allocated to treatment groups and moved to their new tank (fig's 17 and 18). All pairs were moved to

new tanks to reduce variability and bias due to disturbance. Each treatment group received fish pairs from each of the five graded categories (appendix. 2) to ensure a good baseline and an even distribution of biological variability across treatments.

All pairs were moved once only on the same day, to limit any effect of stress due to disturbance. Once in their permanent tanks, each tank received a fresh tile with the addition of a glass dish and metal grid covering, in order to capture any eggs that did not adhere to the spawning substrate (i.e. the tile). After the allocation and movement to the new permanent tanks the experiment was initiated the following day.

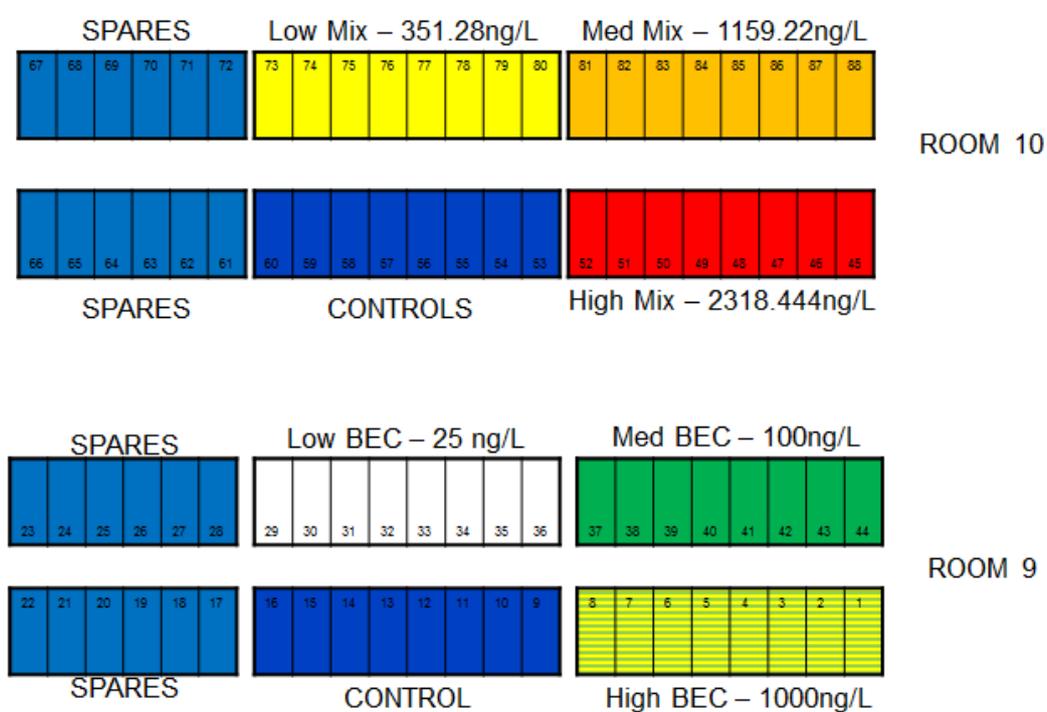


Figure. 17. Layout of experimental fish tanks (one male and one female per tank); 21-day fathead minnow pair breeding assay. Room 9 contained the single exposure experiment with beclomethasone dipropionate. Room 10 contained the five compound mixture experiment. Experiments were run in parallel.



Figure. 18. Wet lab experimental set up; 21-day fathead minnow pair breeding assay

3.2.3.2. Main Experimental Procedure

After the pairing assessment period, a 2 x 21-day experiment was carried out. Fish were subjected to a 21-day pre-exposure period, in which none of the groups were exposed to any test chemical. Fecundity in the form of egg production was quantified daily by recording the spawning frequency and the total egg counts at each spawning event. A 3 day acclimation period followed, when chemical dosing began on the day after day 21 of the pre-exposure period. During this 3 day period it was expected that the concentrations of the test chemicals would reach their desired concentrations. After 3 days acclimation, a 21-day 'exposure' period was initiated, during which fish were exposed to the appropriate concentrations of the test compounds (fig.19). Reproductive capacity of fish pairs was assessed daily during this period, as it was during the pre-exposure period.

Throughout the experiment, the fish were left undisturbed (with the exception of an early morning feed) until 11:00am, when the spawning substrate was checked and eggs were collected. Tanks were checked daily for eggs. Dishes, tiles and grids were removed from each tank, checked and replaced with a fresh set if eggs were present. The tank walls, floor and outflow units were also checked for the presence of eggs, and if any were observed, these were syphoned out into a collection dish. If no eggs were present, spawning substrates were rinsed and returned to the tank. Any tiles, grids, and/or dishes

that contained eggs were transferred to a holding vessel containing fresh aquarium water, enough to keep the eggs submerged, and labelled with the parent tank number. Once all egg batches had been collected, eggs were counted manually by eye with a handheld tally counter, and recorded on to a datasheet. The number of dead eggs was also recorded, as was the location of the spawned eggs i.e. tile, dish, on the outflow etc. Fecundity was quantified daily throughout both the pre-exposure and the exposure periods. The condition, health and behaviour of the fish were continually assessed over the experimental period. Fish were euthanized on day 46 of the experiment and sampled for a range of biological endpoints (discussed in Chapter 4).

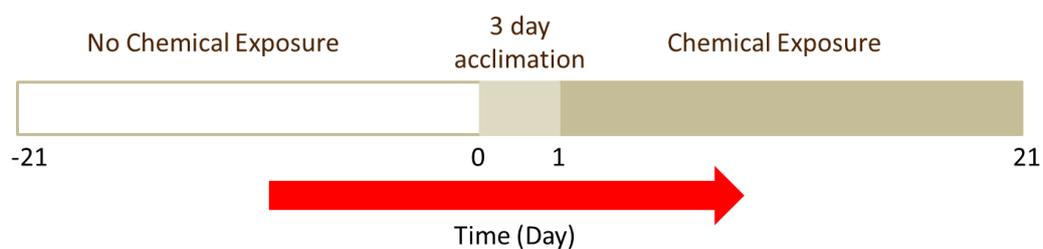


Figure 19: The experimental design of the 21-day fathead minnow pair-breeding assay.

3.2.3.3. Data Representation and Treatment

Fecundity was determined by changes in egg production between the pre-exposure period and the exposure period for each breeding pair, and is expressed as a ratio of cumulative egg production, based on the approach developed by Runnalls et al (2015):

$$R = \frac{\hat{N}_{post}}{\hat{N}_{pre}},$$

Equation 1. Egg production as a ratio of pre and post exposure

where (R) is defined as the ratio between the cumulative number of eggs produced, estimated in the exposure period (\hat{N}_{post}) compared with the number produced in the pre-exposure 21-day period (\hat{N}_{pre}).

Reproductive performance based on the above equation is represented as percentage change. For additional information on the approach used for the representation of pair breeding data, the reader is referred to Runnalls et al, 2015.

3.2.4. Mixture Design and Predictive Modelling

For the comparative assessment of mixture effects on an ecologically relevant end point, the concentration response data from the single compounds was used to design a multicomponent mixture study, using a mixture of the five steroidal pharmaceuticals previously assessed in the 21-day fathead minnow pair breeding assay. For mixture effect assessments on endpoints at the whole organism level, the robustness and interpretation ability of the end point can lead to problematic assessment. However, fish reproduction assays have been shown to offer a quantifiable end point, at organism level, that is also highly ecologically relevant. A novel means of data representation developed by Runnalls

et al (2015) was utilised for the analysis of mixture effects from the five steroidal pharmaceutical mixture. Mixture ratios, the fraction of each compound included in the mixture, and mixture concentrations, as well as the concentration of the whole mixture, were selected based on analysis of the single compound effect data (fig. 20). A fixed equipotent mixture ratio was used, whereby each compound was present at an equipotent concentration. The EC10 (the concentration producing 10% effect) was derived for each individual compound (table 10) and selected as the equipotent ratio after assessing fixed mixture ratio designs computationally (fig. 21). Three mixture concentrations were chosen in order to assess mixture effects at a range of concentrations (table 11). The mixture ratio was maintained across all concentrations according to the EC10 values. The lowest concentration was chosen so that the effects of the individual compounds were all expected to be below the statistical detection limit of the assay (which was a 20% reduction in egg production), and therefore each chemical on its own would be expected to have no significant effect on reproductive performance. Two other concentrations of the mixture were chosen whereby the model predictions were easily differentiated from one another and observed effects could be easily aligned to one of the models.

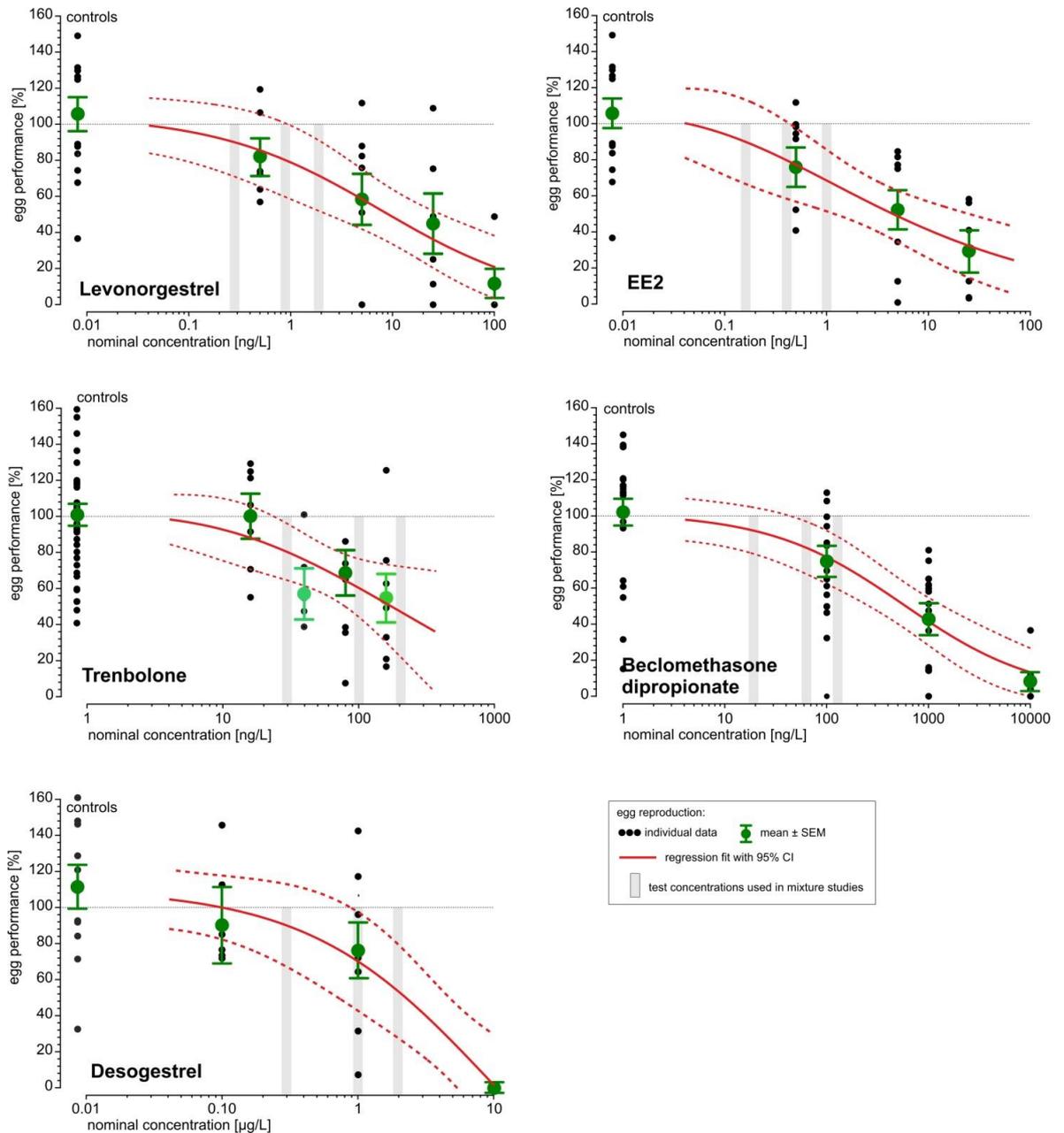


Figure 20. Fecundity (egg production) of pair-breeding fathead minnows exposed to five synthetic steroidal pharmaceuticals, in five independent exposure studies. Individual black dots represent each individual fish pair. Green dots represent the mean \pm standard error. Grey bars are the concentrations of the compound used in each of the three mixture concentrations. Results for the compound beclomethasone dipropionate represent data from the historic experiment only and does not include data collected in the second experiment by the author. Data from experiment 2 (beclomethasone only) is not presented here since it was not used to design the mixture study, which was run in parallel with the second beclomethasone only experiment. Produced by Martin Scholze for Runnalls et al (2015) (EE2 and Levonorgestrel) and unpublished (Trenbolone, Desogestrel and Beclomethasone).

Table.10. The fraction of each compound included in the mixture, based on a ratio design of the EC10 values. The fraction (or %) of each compound was maintained in each mixture concentration

Compound	EC10 ng/L	P [fraction] %
EE2	0.16	0.00005
Levonorgestrel	0.28	0.00009
Beclomethasone	19.24	0.00641
Trenbolone	31.62	0.01054
Desogestrel	299.98	99.9829
Mixture	351.28	100%

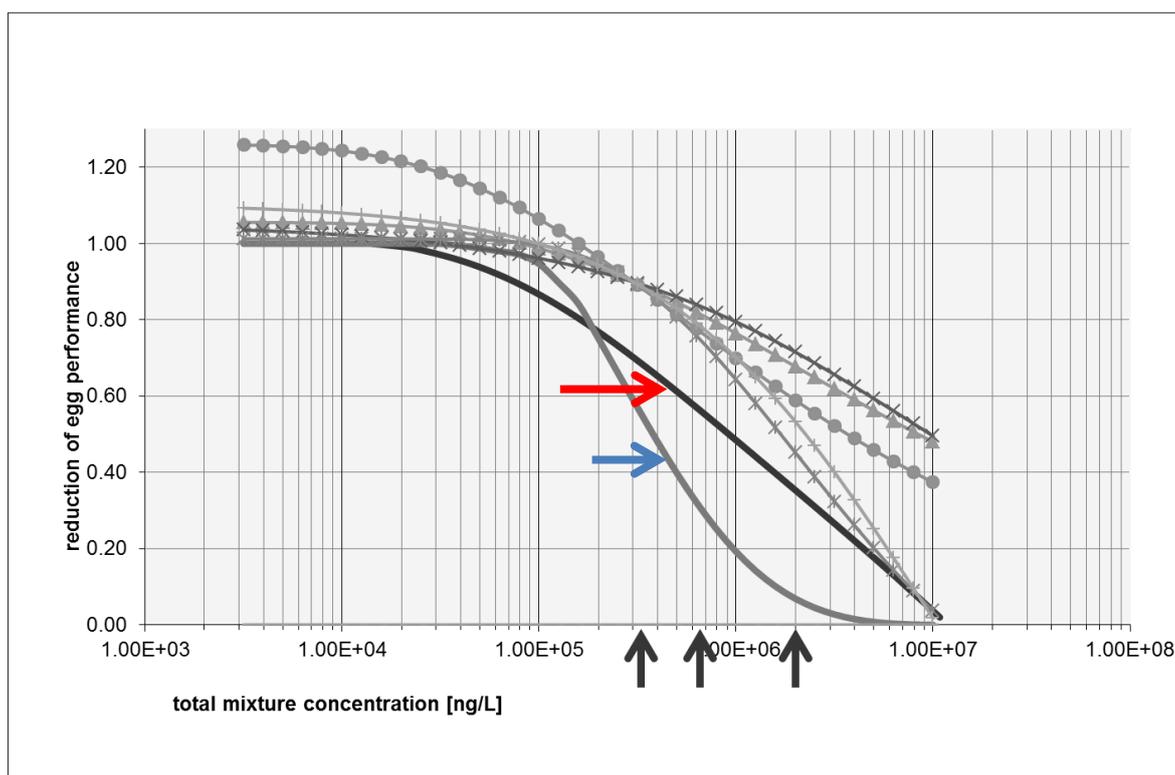


Figure 21. Mixture predictions CA (red arrow) and IA (blue arrow) based on an equipotent mixture ratio of each individual compounds EC10 (i.e. the concentration at which 10% reduction in fecundity can be observed). Individual compounds are indicated by the shape-marked lines, centring the single chemical response curves on the EC10 of each compound (therefore the lines intersect at 0.90). The hatched horizontal line is the detection limit of the assay (20%). Vertical black arrows indicate the whole mixture concentrations selected for the experiment.

Table 11. Composition of the three mixture concentrations to 2 d.p. Mixture ratios remain consistent at an equipotent concentration at each compound's EC10.

Pharmaceutical Compound	Mixture Composition ng/L		
	Low Mix	Medium Mix	High Mix
EE2	0.16	0.52	1.05
Levonorgestrel	0.28	0.93	1.86
Beclomethasone dipropionate	19.24	63.49	126.97
Trenbolone	31.62	104.35	208.70
Desogestrel	299.98	989.93	1979.87
Total	251.28	1159.22	2318.45

3.2.4.1. Statistical Data Treatment

All analysis of the data for the individual chemicals was performed by Martin Scholze, prior to the start of the project presented in this thesis. Single compound fecundity data were analysed for normality and homogeneity of variance, and subsequently examined by a generalised linear model (Poisson or logit) (see Runnalls et al (2015)). Statistical significance between control and treated groups was assessed using multiple contrast tests (Dunnett contrasts, global error rate $\alpha=5\%$, two-sided) (Bretz et al, 2005). Different regression models were applied independently to the individual data sets using a best-fit approach based on the criteria outlined in Scholze et al (2001).

3.2.4.2. Mixture Prediction and Assessment

For predicting joint mixture effects, the Concentration Addition (CA) and Independent Action (IA) predictive models were utilised (discussed in detail in Chapter 1). According to Bliss (1989), the CA prediction was made based on a given effect concentration. In this case, the EC10 of the individual substances was used, based on the criteria discussed

previously. As described by Faust *et al.* (2001), under the assumption of CA, a mixture concentration producing an effect X was calculated as:

$$ECx (mixture) = \left(\frac{P_{EE2}}{ECx (EE2)} + \frac{P_{Levonorgestrel}}{ECx (Levonorgestrel)} + \frac{P_{trenbolone}}{ECx (Trenbolone)} + \frac{P_{desogestrel}}{ECx (Desogestrel)} + \frac{P_{beclomethasone}}{ECx (Beclomethasone)} \right)^{-1},$$

Equation 2. Concentration Addition mixture predication

whereby $EC_x(\text{mixture})$ is the mixture concentration that produces the effect x (i.e. 50%) for a combination of C_{EE2} , $C_{Levonorgestrel}$, $C_{Trenbolone}$, $C_{Desogestrel}$, and $C_{Beclomethasone}$ (the concentration of the respective substances in the mixture). P (fraction of compound) is the ratio of the compound in the mixture, the sum of which equates to 1. $EC_x(\text{compound})$ is the effect concentration of the compound producing the same effect level x (i.e. 50%). The effect concentrations are derived from the non-linear regression models applied to each data set (performed by M.S prior to the start of this project) (table 12).

Table 12. Results of regression models applied to the data of each individual compound, including the EC10 and EC50 values, and the no observed effect concentrations (NOECs). Table modified by that produced by Martin Scholze for Thrupp et al, (in prep), based on modelling of data undertaken prior to the start of this project.

	Regression Model	EC10* [ng/l]	EC50* [ng/l]	NOEC* [ng/l]
EE2	weibull	0.16	4.44	<0.5
Levonorgestrel	logit	0.28	9.11	0.5
Trenbolone	weibull	13.4	176	16
Beclomethasone	logit	25.7	596	<100
Desogestrel	logit	300	2253	<10
Mixture	logit	n.d.	283	

Due to the complex nature of the IA prediction, the mathematical aspect of the modelling was modified based on Faust et al (2003) and undertaken by M.S. The IA prediction was formulated under the assumption that combination effects can be calculated from the individual compound responses by following the statistical concept of independently occurring events (Bliss, 1939; Ermler et al, 2013).

For a binary compound mixture, this is commonly defined by the equation as:

$$E(c_{mixture}) = E(c_1) + E(c_2) - E(c_1) \cdot E(c_2) \quad \text{Ermler et al (2013)}$$

Equation 3. Independent Action Mixture Prediction

where $E(c_1)$ and $E(c_2)$ are the effects produced by the individual compounds (compounds c_1 and c_2) and $E(c_{mixture})$ is the total mixture effect. The formula can be extended for n number of compounds (refer to Ermler et al, 2013).

To account for the statistical uncertainty in the CA and IA prediction, the bootstrap method (Efron and Tibshirani, 1993) was used to produce approximate 95% confidence limits around the mean predicted effect. Differences between predicted and observed effect concentrations were deemed statistically significant when the 95% confidence belts of the prediction did not overlap with those of the experimentally observed mixture effects.

3.2.4.3. Statistical Treatment of Data

To assess whether observed mixture effects were significantly different across treatments, data were analysed using descriptive statistics. Fecundity data were assessed for homogeneity of variance using the Pitman-Morgan test (Morgan, 1939; Pitman, 1939). Mean cumulative egg production was tested using a series of paired-sample t-tests.

3.4.5. Quantification of Exposure Concentrations

In any fish exposure experiment, quantification of actual concentrations is important. In the past, many studies presented data based on nominal concentrations (the concentrations that were added to the exposure tanks). However, since the fate and behaviour of these compounds in aqueous will significantly affect the bioavailability of the compound, and thus its ability to produce a pharmacological effect, quantification of the compounds during the exposure period is deemed a valuable experimental parameter, allowing observed effects on the organism in question to be related to the concentration present in the water that is actually causing that given effect. Although analytical techniques such as HPLC MS/MS are now the gold standard for measuring pharmaceuticals and pharmaceutical products in samples, they have their limitations. Concentrations are often below detection limits of current analytical techniques, with some techniques not affording enough sensitivity. Laborious method development is sometimes required to be able to measure low concentrations of pharmaceuticals from laboratory (as well as environmental) samples.

There are, however, alternative techniques commonly used to quantify measured concentrations in toxicological and pharmacokinetic studies. Assays involving the use of enzyme-linked antibodies or antigens such as enzyme linked immunosorbent assays (ELISA) and enzyme immunoassays (EIA), and assays that use radioisotopes such as Radioimmunoassay's (RIA) can also be used to detect and quantify compounds in a sample matrix. The RIA was first developed by Yalow and Berson (1960), as a means of improving sensitivity and specificity in measuring insulin concentrations in the plasma of human patients. It was subsequently adapted and developed to measure small quantities of a wide range of molecules including peptides and protein hormones (Gan and Patel, 2013). The assay uses a radioactive form of the substance of focus (the label or tracer) in a competitive binding assay, whereby the radioactive isotope competes with the non-radioactive form (i.e. in the sample of interest) for antibody binding sites (Perkin Elmer,

Copyright 1998-2015). Since a known fixed amount of tracer is used in every replicate in the assay, the un-labelled (sample) substance can be measured and quantified and therefore the concentration of a substance in a sample can be determined. Although RIA's can quantify substances in a sample with high sensitivity and are highly specific, their use of radioactive material has led to the development of less hazardous techniques based on similar principles, such as ELISAs.

ELISA's can also be used to quantify the amount of substance in a sample by using antibodies as reagents (Crowther, 2010). The ELISA is a robust assay used to quantify biological and synthetic molecules, and is used in diagnostic medicine as well as pharmacokinetic studies. The assays are based upon the same principle of molecule binding as in RIAs, however they do not require the use of radioactive substances. ELISA's employ well plates, typically 96 well microtiter plates, coated with an enzyme-labelled antibody specific to the substance to be quantified. Reagents are added to a solid phase bound substance, incubated and subsequently washed with a wash buffer to separate bound and free molecules. An enzyme-labelled reactant is then added and further incubated to initiate the development of a colour substrate for result quantification. After incubation, an acidic solution is added to terminate colour development and results are quantified immediately using a microtiter plate reader by measuring absorbance. There are three main ELISA systems; direct, indirect, and sandwich ELISA's, all of which are classed as competition or inhibition ELISAs (Crowther, 2010), and choice depends upon intended use. ELISA's have become commercialised, and are now readily available in kit form, with all of the necessary components for running an assay included. Since they can be used for measuring a whole host of molecules and substances at relatively low cost in a quick and easy bioassay, they are a popular choice in toxicological studies, where basic rapid quantification of substance concentration is required.

3.2.5.1. Individual Compounds

Actual exposure concentrations of the individual compounds were quantified during each experiment. Measured concentrations for all compounds were found to be within an acceptable range of the nominal concentrations (Runnalls et al, 2013, Runnalls et al, 2015; Runnalls, unpublished, Kugathas, unpublished).

3.2.5.2. Five Compound Mixture Experiment

Actual concentrations of three of the five compounds included in the mixture were measured for comparison to their nominal concentrations. Measuring all five compounds was considered unfeasible, due to the fact that it was not possible to extract all five compounds over the course of the experiment given the time, cost, and resources available. Furthermore, at present, to the author's knowledge, there are no reliable published procedures for measuring concentrations of desogestrel and beclomethasone dipropionate in this context, and thus timely development and validation of these methods would be required prior to use. Since this was outside of the remit of the current project, only validated methods were used (i.e. ELISAs and RIAs) to quantify a number of compounds in the mixture. Three compounds were selected, EE2, trenbolone and levonorgestrel, based upon the availability of sensitive enzyme-linked assays and radioimmunoassay's for rapid quantification. Water samples (2L) were collected from every other tank (4 out of 8 tanks) weekly during the exposure period on days 1, 7, 14 and 21, on the same day each week. 2L samples were collected in two separate 1L amber glass bottles (Fisher Scientific) from the same tank each week, weighed for accuracy and stored at 4°C until extracted. An additional set of samples was collected before the start of the chemical exposure period at week 0, as an initial control blank. Samples were extracted immediately over the following two days. Individual tank samples were split, with 1L extracted on the day of collection and 1L extracted the following day. Solid Phase

Extraction was used for sample clean up and pre-concentration. Two separate methods were used for the extraction of 3 compounds: 17 α -ethinyl estradiol, 17 β -trenbolone, and levonorgestrel.

3.2.5.2.1. EE2 and 17 β Trenbolone - Extraction Protocol

1L samples were extracted and eluted for quantification of EE2 and 17 β -trenbolone concentrations in exposure water. MilliQ water samples spiked with known concentrations of the mixture were also extracted alongside the samples each week, in order to quantify extraction efficiencies and recoveries. Samples were extracted by Solid Phase Extraction using a Visiprep SPE vacuum manifold (Supelco). Cartridges were primed with 5ml methanol (MeOH) followed by 5ml MilliQ H₂O. 1L samples were extracted onto Sep Pak C18 cartridges (Waters, UK), under a flow rate of 5ml/minute. Once the samples had finished flowing, cartridges were dried under a low vacuum for 30 minutes, and stored at -20°C until further analysis.

Prior to elution of samples, cartridges were allowed to warm to room temperature for approximately one hour. Extracts were eluted on a Visiprep SPE vacuum manifold (Supelco) from the cartridges with 2x5ml MeOH into 15ml polypropylene tubes. Samples were dried in a centrifugal vacuum concentrator (miVac Quattro and miVac SpeedTrap) at 30°C for 2 hour intervals until completely dry. Dried samples were stored at 4°C until analysed. A few days before the quantification assays were run, samples were re-suspended in ethanol at various dilutions to achieve final concentrations within the range of the standard curve of each assay (tables 13 and 14), vortexed, and stored at 4°C.

3.2.5.2.2. Trenbolone Quantification

Concentrations of 17 β -Trenbolone were measured using a commercially available competitive enzyme immunoassay (Trenbolone ELISA; 5081TREN, EuroProxima), following the kit protocol. All reagents and standards were supplied, including a 96-well

microtiter plate (12 strips, 8 wells per strip, coated with sheep anti-rabbit IgG), substrate chromogen (tetramethylbenzidine, TMB), reaction stop solution (sulphuric acid), specific antibodies solution (rabbit anti-Trenbolone), conjugated solution (enzyme-labelled Trenbolone), and trenbolone standards. Dilution and rinsing buffers were also provided. Phosphate buffer was made up in advance according to the assay protocol. The buffer pH was measured and adjusted where required. Bovine Serum Albumin (BSA) was added on the morning of the assay, dissolved using a heated magnetic stirrer, and left at room temperature to equilibrate. Samples from each treatment group were run in every assay, as were the spiked samples and the blank controls.

On the day of the assay, samples were removed from the fridge and allowed to warm to room temperature for approximately one hour. Samples were vortexed, and either 50µl or 100µl was removed and aliquoted to a polypropylene tube (LP3P; Luckham), depending upon initial nominal concentration (table 13). Samples were dried in a centrifugal vacuum concentrator (miVac Quattro and miVac SpeedTrap) at 30°C for 30 minutes. Phosphate buffer was added to the dried samples to achieve a starting concentration of approximately 2ng/ml, to bring the concentrations within range of the standard curve, vortexed and left at room temperature for 30 minutes.

Table 13. Volumes and concentration/dilution factors used for the quantification of 17β-Trenbolone from mixture exposure samples using a commercial ELISA. *denotes final nominal concentration.

Treatment	Start conc (ng/L)	Start vol (ml)	Vol etOH added (ml)	Conc factor	Final conc (ng/ml)	Start vol. (ul)	End vol. (ul)	Dilution factor	Overall conc factor	Final conc (ng/ml)*
Control	0	1000	2.5	400	0	100	500	5	80.0	0.0
Low Mix	31.62	1000	2.5	400	13	100	500	5	80.0	2.5
Medium Mix	104.35	1000	2.5	400	42	50	1000	20	20.0	2.1
High Mix	208.7	1000	5.0	200	42	50	1000	20	10.0	2.1

Reagents were made up on the day of the assay according to the protocol. Standards and samples were pipetted to the ELISA microtiter plate (50µl, 96 well format), followed by 25µl of conjugated (enzyme-labelled) trenbolone, and 25µl of antibody solution (rabbit anti-Trenbolone), sealed and shaken on an orbital shaker for five seconds. The plate was then incubated in the dark at room temperature for one hour. After incubation, the plate was washed three times with rinsing buffer using a Versa Microplate Washer, (Wellwash™, Thermo Scientific™). 100µl of substrate chromogen solution (tetramethylbenzidine, TMB) was added to each well and the plate was incubated in the dark at room temperature for a further 30 minutes. After incubation, 100µl of the stop solution (sulphuric acid) was added to each well and the plate was read immediately using a Spectra Max 340 Microplate Reader, (Molecular Devices) at an absorbance of 450nm. Data were analysed using SoftMax Pro Data Acquisition and Analysis Software (Molecular Devices).

3.2.5.2.3. 17α-ethinyl estradiol

Concentrations of 17α-ethinyl estradiol were measured using a commercially available competitive reaction ELISA kit (L22000405, Ethinyl estradiol EIA, Biosense). All reagents were provided in kit form, and included a 96-well microtiter plate coated with EE2 monoclonal antibody, a non-coated microtiter plate, EE2 standards, antigen-enzyme conjugate (EE2 labelled with horseradish peroxidase, HRP), buffer solution, wash solution, substrate chromogen (tetramethylbenzidine, TMB) and reaction stop solution (citric acid). Samples from each concentration group were run in every assay, as were the spiked samples and the blank controls.

Eluted samples were removed from 4°C storage and allowed to warm to room temperature. Samples were vortex mixed before removing 1ml and aliquoting this to a fresh polypropylene tube (LP3P; Luckham). Samples were dried in a centrifugal vacuum concentrator (miVac Quattro and miVac SpeedTrap) at 30°C at 30 minute intervals until

dry. Samples were reconstituted in 10% MeOH and 90% MilliQ water according to the desired final concentration (table 14).

Table 14. Volumes and concentration/dilution factors used for the quantification of 17 α ethinyl estradiol from mixture exposure samples using a commercial ELISA. *denotes final nominal concentration.

Start conc (ng/L)	Start vol (ml)	Vol etOH added (ml)	Conc factor	Final conc (ng/L)	Start vol (ul)	End vol (ul)	10% Vol MeOH (ul)	90% Vol MilliQ (ul)	Dilution factor	Overall conc factor	Final conc (ng/L) *
0	1000	2.5	400	0	1000	250	25	225	0.25	1600.0	0.0
0.16	1000	2.5	400	64	1000	250	25	225	0.25	1600.0	256.0
0.52	1000	2.5	400	208	1000	800	80	720	0.8	500.0	260.0
1.046	1000	5.0	200	209	1000	800	80	720	0.8	250.0	261.5

Kit components were reconstituted according to the assay protocol. 100 μ l of conjugate solution was pipetted to each well of the uncoated plate, followed by 100 μ l of either standards or samples, and mixed by pipetting. 100 μ l of the conjugate-sample/standard mixture was subsequently transferred to the antibody-coated microtiter plate and covered with an adhesive plate cover. The plates were incubated in the dark at room temperature for 60 minutes. After the incubation step, plates were washed three times with the wash buffer using a Versa Microplate Washer. 100 μ l of the substrate chromogen was added to each well and the plate incubated in the dark at room temperature for a further 30 minutes. After incubation, stop solution was added to each well and the plate was read immediately using a Spectra Max 340 Microplate Reader, at an absorbance of 450nm. As with the trenbolone ELISA, data were analysed using SoftMax Pro Data Acquisition and Analysis Software.

3.2.5.2.4. Levonorgestrel

1L samples were extracted and eluted for quantification of levonorgestrel concentrations in tank water. MilliQ water samples spiked with known concentrations of the mixture were

also extracted alongside the samples each week, in order to quantify extraction efficiencies and recoveries. Samples were extracted by Solid Phase Extraction using a Visiprep SPE vacuum manifold (Supelco), using the method described above but with some modifications. Cartridges were primed with 2.5ml of a 90% Methyl tert-butyl ether (MTBE):10% MeOH solution, followed by 2.5ml ethyl acetate, 5ml MeOH and 5ml MilliQ H₂O. 1L samples were extracted onto Sep Pak C18 cartridges according to the method described above, and stored at -20°C until further analysis. Samples were eluted as per the method described above, with some modifications. Extracts were eluted with 2.5ml 90% MTBE:10% MeOH, 2.5ml ethyl acetate and 5ml MeOH. Samples were dried in a centrifugal vacuum concentrator (miVac Quattro and miVac SpeedTrap) at 30°C for 2 hour intervals until completely dry, and stored at 4°C until analysed. Samples were subsequently re-suspended in ethanol at various dilutions according to the standard curve (table 15), vortexed, and stored at 4°C.

Table 15. Volumes and concentration/dilution factors used for the quantification of Levonorgestrel from mixture exposure samples using a commercial radioimmunoassay. *denotes final nominal concentration.

Start conc (ng/L)	Start vol (ml)	Vol etOH added (ml)	Conc factor	Final conc (ng/L)	Start vol (ul)	End vol (ul)	Dilution factor	Overall conc factor	Final conc (ng/L) *
0	1000	1.0	1000	0	100	200	2	500.0	0.0
0.28	1000	1.0	1000	280	100	200	2	500.0	140.0
0.93	1000	1.5	667	620	50	200	4	166.8	155.1
1.861	1000	3.0	333	620	50	200	4	83.3	154.9

Levonorgestrel concentrations were quantified using a commercially available radioimmunoassay (Levonorgestrel Radioimmunoassay (LG RIA), Immunometrics UK Ltd), adapted for use with aqueous samples. The conventional competitive binding assay

uses tritiated levonorgestrel as a label, and charcoal for separation of free levonorgestrel from antibody-bound levonorgestrel. Required reagents and components were provided in the kit, including the levonorgestrel antiserum, levonorgestrel standard (750nmol/L), tracer (tritiated levonorgestrel, 0.93MBq/mL of ethanol), charcoal, gelatine, and dextran. Reagents, buffers and standards were prepared according to the assay protocol. Tracer stock solution was prepared prior to the start of the assay. 50µl aliquot was tested for activity using a Liquid Scintillation Counter (Packard Bioscience Co.) to ensure the stock was prepared correctly.

Each assay was performed over two days, and all samples were run as two replicates. Samples from each concentration group were run in every assay, as were the spiked samples and the blank controls. The entire assay was performed on ice in a designated radioactive laboratory at Brunel University. Either 100µl or 50µl of sample was pipetted into polypropylene tubes (10x75mm) according to the standard curve calculations (table 15), and dried in a centrifugal vacuum concentrator (miVac Quattro and miVac SpeedTrap) with no heating, for 30 minutes. Buffer S, tracer solution and antibody solution was added to assay tubes according to the protocol (table 16).

Table 16. Volumes of reagents used in the levonorgestrel radioimmunoassay, according to the kit protocol.

	Volume (µl)			
	Buffer S	Standard or Sample or Blank	Tracer Solution	Antiserum
Total Counts	750	-	50	-
Non Specific Binding	250	-	50	-
Maximum Binding	200	-	50	50
Standards or Sample or Blank	200	200*	50	50

* evaporated samples were re-dissolved in 200 µl with Buffer S

Samples were incubated for 24 hours at 4°C. After 24 hours of incubation, 500µl of charcoal reagent was added to all tubes, which were then vortexed and incubated on ice for 30 minutes. After incubation, tubes were spun in a cooled centrifuge at 1000g for 15 minutes to precipitate the charcoal. The supernatant was decanted to scintillation vials filled with Ultima Gold liquid scintillation cocktail. Vials were shaken to mix and their contents allowed to equilibrate before counts were read using a Liquid Scintillation Counter (Packard Bioscience Co.). The amount of radiolabel that binds to the antibody is expressed as a percentage of Maximum Binding. Recovery was calculated according to the following calculation:

$$\frac{\text{dpm - Non Specific Binding}}{\text{Maximum Binding - Non Specific Binding}} \times 100$$

Equation 4. Recovery of measured concentrations of the mixture. (*Brunel University Radioimmunoassay Protocol, 2014*)

3.3. Results:

3.3.1 Fecundity - Single Compound Data

Exposure to each compound individually caused a reduction in fecundity in *P. promelas* in a dose-dependent manner (data presented in fig. 20, materials and methods). Egg production decreased in all treatment groups relative to the control (Runnalls et al, 2013; Runnalls et al, 2015, Runnalls, unpublished data, Kugathas, unpublished data). Mortalities during single compound exposures did not exceed 4%, and no abnormal behavioural or physiological characteristics were observed in any exposure study. Data from previous studies assessing effects of levonorgestrel and ethinyl estradiol (Runnalls et al, 2015) and desogestrel (Runnalls et al, 2013) on egg performance have already been reported in the literature. The reader is referred to these publications for more information on the effects of the single compounds on fecundity of the FHM. Effects of the remaining compounds trenbolone and beclomethasone on FHM fecundity have not yet been reported in the literature, and therefore will be described in more detail here. Potencies of the five compounds varied 500-fold. EE2 was the most potent compound, reducing egg performance at an estimated EC50 of 4.4ng/L. The synthetic progestin desogestrel was the least potent compound in this assay, with an EC50 of approximately 2.2µg/L. The FHM pair-breeding assay was repeated for trenbolone (both experiments undertaken by Dr T. Runnalls prior to the start of the project presented here), since the original concentrations were shown to be somewhat less potent than data from the previous literature suggested (Ankley et al, 2003). In two repeated studies, concentrations of trenbolone inhibited egg performance in a comparable manner, and therefore data from both studies were pooled for analysis. Beclomethasone dipropionate inhibited egg production but did not do so in a dose-dependent manner in the single compound exposure undertaken as part of the presented research.

3.3.1.1. Effects of beclomethasone dipropionate on fecundity in pair-breeding

Pimephales promelas

Exposure to beclomethasone dipropionate (BDP) caused a decline in fecundity of breeding pairs. The effect was most pronounced at concentrations of 25ng/L and 1000ng/L (fig. 22 and 23). Data were not normally distributed (Shapiro-Wilks test, 0.458, $df=32$, $p<0.001$) and variances were found to be equal (non-parametric Levene's test, $f=0.686$, $df=3$, $p=0.568$). No statistically significant difference was observed between treatment groups (Kruskal Wallis, Chi-Square 7.158, $df=3$, $p=0.067$). Furthermore, post hoc analysis revealed that there was no statistically significant difference between any of the treatment groups and the control.

Fecundity during the pre-exposure stage was compared with that of the chemical exposure stage by comparing cumulative egg production at the end of each respective experimental stage (fig. 23). At the lowest concentration (25ng/L), there was the largest inhibition of egg production quantified at the end of the exposure period compare with the pre-exposure period, however this was just outside of being statistically significant (Kruskal Wallis, Chi-Square=3.574, $df=1$, $p=0.059$), (data was assessed for normality using Shapiro-Wilks test, 0.885, $df=16$, $p<0.05$, and homogeneity of variance, non-parametric Levene's test, $f=1.150$, $df=1$, $p=0.302$ prior to analysis for significance).

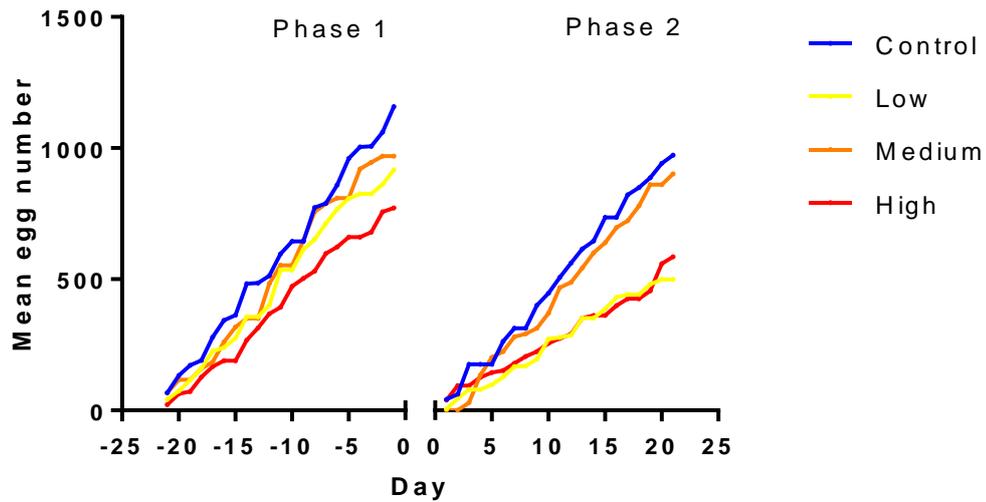


Figure 22. Mean total egg count over 2x21 day periods of phase 1. no exposure, and phase 2. exposure to beclomethasone dipropionate. Note the non-linear dose-response relationship.

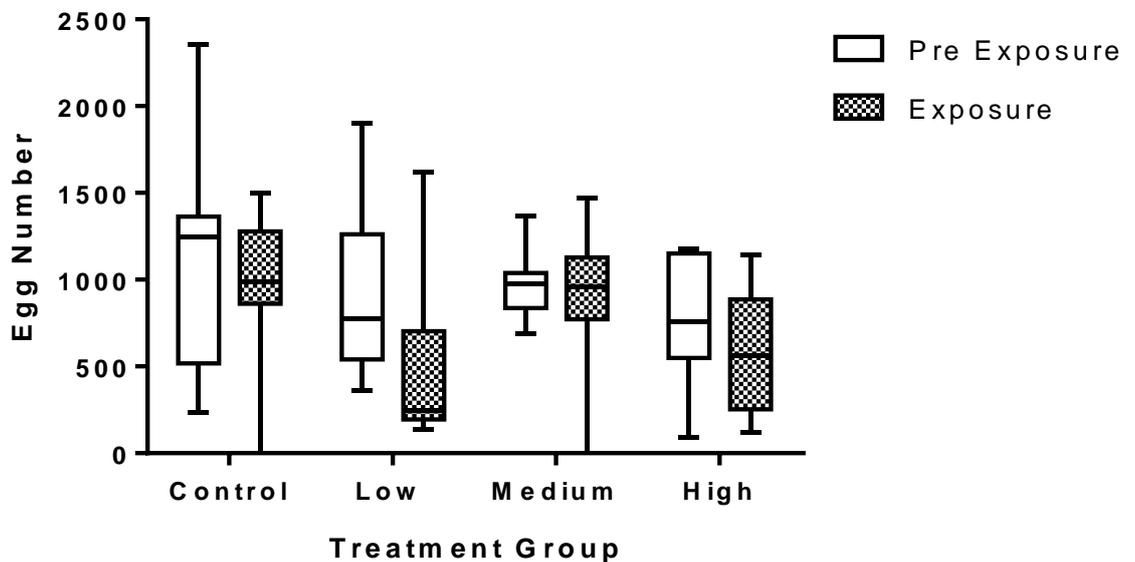


Figure 23. Mean total egg number in the pre-exposure and exposure phases of the experiment. n=8 for each treatment group, represented as box and whiskers plot. The box represents the lower and upper quartile (25th and 75th percentile), mid line represents the median. The whiskers represent the highest and lowest observations. No statistically significant changes in fecundity were observed as a result of exposure to BDP.

Data was relatively well-correlated with the pair-breeding experiment undertaken by Dr Subramaniam Kugathas (fig. 24). However, in the experiment undertaken as part of the research presented here, the lowest concentration (25ng/L) appeared to reduce egg production by around 60%, a more pronounced inhibition than that observed at 100ng/L in either experiment. Variations in the data across the two experiments were further investigated using a two-way ANOVA using the Type III univariate model (the unweighted means approach). Data failed to meet the assumption of equal variances (Levene's test, $f=3.392$, $df=5$, $df=42$, $p=0.012$). Since the assumption of homogeneity of variance was not met, and sample sizes were unequal, an alpha level of $p<0.001$ was adopted in order to safeguard against Type I errors (false positives). There was no statistically significant difference in fecundity in the chemical treatment groups between the two experiments (two-way ANOVA, $f=0.382$, $df=1$, $p=0.540$), suggesting that the differences observed in fecundity across treatment groups were not affected by whether they were in experiment 1 or experiment 2. However, fecundity in the control groups varied significantly between the two experiments. Data was normally distributed (Shapiro-Wilks test, 0.965 , $df=14$, $p=0.807$) and had equal variances (Levene's test, 0.296 , $df=1$, $df=12$, $p=0.596$). There was a statistically significant difference between fecundity in the control groups between the two experiments ($f=22.135$, $df=1$, $p\leq 0.001$).

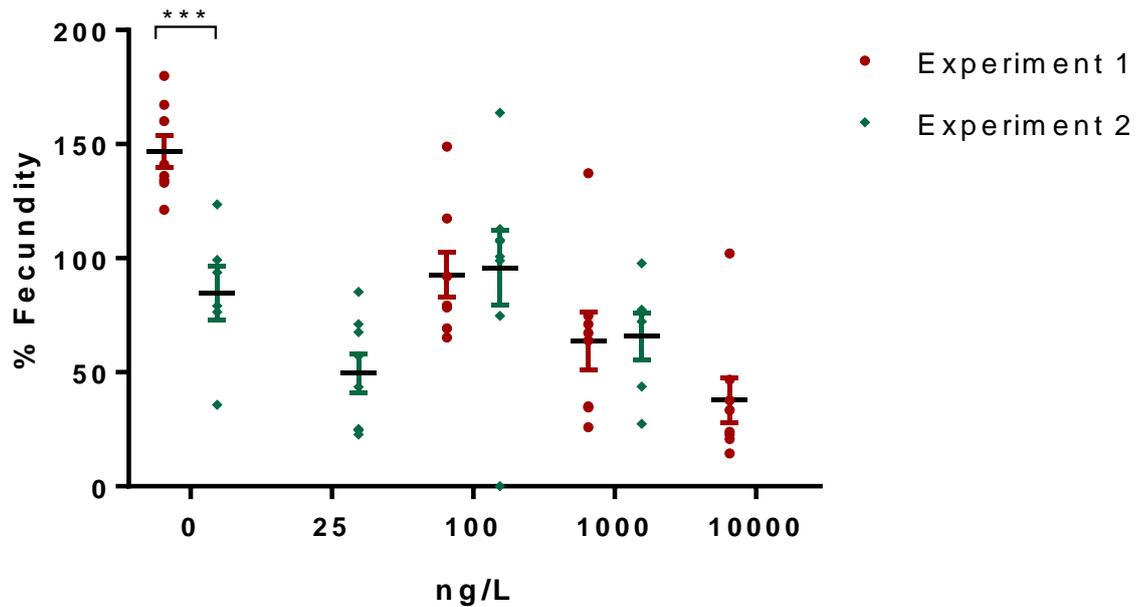


Figure. 24. Fecundity of pair-breeding fathead minnows exposed to four concentrations of beclomethasone dipropionate in two separate experiments. Individual points (circles/diamonds) represent individual fish pairs. Horizontal black lines represent the corresponding means. Error bars denote SEM. Experiment 1 performed by Dr Subramaniam Kugathas, Experiment 2 performed by the author. * denotes statistical significance.

All mixture predictions and assessments were conducted based on nominal values. The decision to use nominal, rather than measured, concentrations was due in large to the limitations generated from the pharmacological parameters of beclomethasone. The compound has poor water-solubility, and is unstable in aqueous (Margiotta-Casaluci et al, 2016). Furthermore, beclomethasone dipropionate is a prodrug, which requires biological transformation to beclomethasone monopropionate, its potent active form. Therefore, the use measured concentrations of the parent-compound are not particularly appropriate. For consistency, nominal concentrations were also used for all compounds in the mixture assessment. Based on previous studies carried out by TR and KS, recoveries of compounds were reproducible across studies.

3.3.2. Mixture Experiment

3.3.2.1. Quantification of Measured Concentrations

Average measured concentrations quantified by RIA and ELISA were reasonably similar to nominal values. Measured concentrations of EE2 from the mixture study were between 72 and 87% of nominal concentrations (table 17). The spiked MilliQ recoveries were also within this range (79-82%), suggesting that concentrations in the exposure tanks were close to nominal values. Furthermore, measured concentrations of EE2 from the mixture correlate well with concentrations measured from the single exposure studies carried out previously at Brunel University (data not shown).

Average measured concentrations of 17 β -trenbolone were between 93 and 110.5% of nominal concentrations (table 18). Spike recoveries were between 83-99% of expected concentrations. The results were within an acceptable range, with some deviations from nominal expected, likely due to inherent variations in factors such as flow rates, dosing rates, time of water sampling each week and errors involved in the quantification assay.

In the radioimmunoassay, average measured concentrations of Levonorgestrel were 158-167% of nominal concentrations (table 19). Recoveries from spiked samples were between 208-245% of nominal. However it is not possible to determine whether actually concentrations were higher than expected, or whether the difference between nominal and measured concentrations was actually an artefact generated during the measurement itself.

Table 17. Measured water concentrations of 17 α -Ethinyl Estradiol from the mixture experiment, quantified using ELISA. <DL denotes concentrations were below the detection limit of the assays

Nominal	17 α -Ethinyl Estradiol						% of nominal
	Week 0	Week 1	Week 2	Week 3	Week 4	Mean	
Control	<DL	<DL <DL <DL <DL	<DL <DL <DL <DL	<DL <DL <DL <DL	<DL <DL <DL <DL	<DL	-
0.16ng/L	<DL	0.10 0.10 0.11 0.19	0.13 0.18 0.14 0.08	0.16 0.15 0.15 0.10	0.17 0.21 0.16 0.09	0.14	87.9%
0.52ng/L	<DL	0.36 0.30 0.31 0.23	0.44 0.45 0.48 0.30	0.45 0.53 0.62 0.33	0.54 0.67 0.28 0.34	0.41	79.9%
1.046ng/L	<DL	0.68 0.72 0.59 0.45	0.86 0.97 0.60 0.50	1.04 1.06 1.20 0.61	1.06 0.05 0.84 0.82	0.75	72.1%
		Spiked Samples					
0.16ng/L		0.11	0.13	0.15	0.12	0.13	82.5%
0.52ng/L		0.40	0.42	0.46	0.42	0.42	82.5%
1.046ng/L		0.77	0.90	0.81	0.83	0.83	79.3%

Table. 18. Measured water concentrations of 17 β -Trenbolone from the mixture experiment, quantified using ELISA. <DL denotes concentrations were below the detection limit of the assays

Nominal	17 β Trenbolone						% of nominal
	Week 0	Week 1	Week 2	Week 3	Week 4	Mean	
Control	<DL	<DL <DL <DL <DL	<DL <DL <DL <DL	<DL <DL <DL <DL	<DL <DL <DL <DL	<DL	
31.62ng/L	<DL	17.53 19.99 23.89 12.21	31.48 44.43 43.74 34.73	33.43 44.78 42.41 42.88	43.08 42.71 30.83 31.41	33.72	106.6%
104.4ng/L	<DL	91.78 44.91 50.73 68.14	139.95 132.05 137.24 100.56	113.48 132.05 144.23 125.61	130.95 135.44 148.86 147.88	115.24	110.4%
208.7ng/L	<DL	110.53 107.45 130.11 178.89	233.67 290.41 141.54 160.81	278.10 180.84 273.96 225.65	219.09 101.96 267.56 217.60	194.87	93.4%
		Spiked Samples					
31.62ng/L		9.86	47.2	36.1	34.4	31.8	99%
104.4ng/L		54.0	104.5	112.4	107.6	94.6	90%
208.7ng/L		126.7	183.0	179.8	203.3	173.2	83%

Table 19. Measured water concentrations of levonorgestrel from the mixture experiment, quantified using a radioimmunoassay. <DL denotes concentrations were below the detection limit of the assay.

Nominal	Levonorgestrel						% of nominal
	Week 0	Week 1	Week 2	Week 3	Week 4	Mean	
Control	<DL	<DL <DL <DL <DL	<DL <DL <DL <DL	<DL <DL <DL <DL	<DL <DL <DL <DL	<DL	-
0.28ng/L	<DL	0.31 0.30 0.29 0.25	0.46 0.46 0.46 0.43	0.46 0.46 0.43 0.45	0.59 0.60 0.59 0.57	0.44	158%
0.93ng/L	<DL	1.09 1.08 1.17 1.67	1.70 1.61 1.59 1.64	1.59 1.56 1.63 1.06	1.88 1.91 1.87 1.90	1.55	167%
1.86ng/L	<DL	1.99 2.27 0.98 1.56	3.35 3.15 3.28 3.31	4.54 3.49 3.46 3.62	3.63 3.57 3.53 3.70	3.11	167%
Spiked Samples							
0.28ng/L		0.75	0.70	0.67	0.63	0.69	245%
0.93ng/L		2.19	2.11	2.0	1.94	2.06	221%
1.86ng/L		1.97	4.17	4.33	5.03	3.87	208%

3.3.2.2. Fecundity: Pre-Exposure versus Exposure

No acute toxicity effects were observed during the mixture experiment. Mortality was below 1.5% and well within the range of normal survival rates for this species, assessed from records of fish stock health held at Brunel University's aquatic facilities. No abnormal behavioural or physiological characteristics were observed, and there was no evidence of disease or infection in any of the experimental fish.

Fecundity of paired FHM exposed to the mixture was inhibited in a dose-dependent manner (fig. 25). Mean cumulative egg production was significantly reduced during the exposure period compared with the pre-exposure period for all chemical treatment groups. At the lowest mixture concentration, egg production was significantly inhibited during the exposure period compared with the pre-exposure ($t=2.682$, $d.f=7$, $p<0.05$). There was also a positive correlation between paired samples (0.667 , $p=0.07$), suggesting that the individual pairs with the lowest cumulative egg production in the pre exposure period were the same fish with the lowest cumulative egg production at the end of the exposure period, although this was just outside of being statistically significant. The medium concentration caused a significant inhibition of egg production in the exposure period compared with the pre-exposure period ($t=12.124$, $d.f=7$, $p<0.001$). There was also a positive correlation between paired samples (0.604 , $p=0.113$), although this was not statistically significant. Egg production during exposure to the highest mixture concentration was significantly reduced ($t=7.716$, $d.f=7$, $p<0.001$). Furthermore, the data showed a positive paired sample correlation (0.785 , $p<0.05$), suggesting that the individual pairs with the lowest cumulative egg production during the pre-exposure period also had the lowest cumulative egg production during the exposure period. In the control group, mean cumulative egg production in the exposure period was not significantly different from the pre-exposure period ($t=1.220$, $d.f=7$, $p=0.262$). There was a positive paired sample correlation between the two exposure periods ($p=0.525$), however the correlation was not statistically significant due to the fact that one pair ceased producing eggs shortly after the initiation of the second stage of the experiment. The reason for the cessation remains unknown.

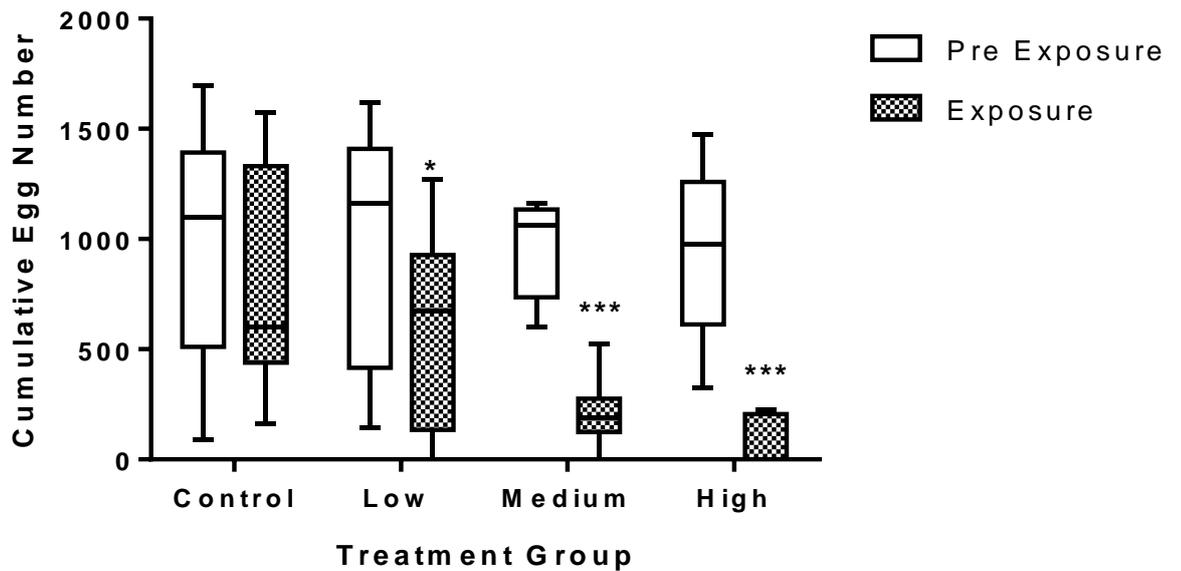


Figure. 25. Mean cumulative number of eggs produced during the pre-exposure and the exposure period for each treatment group. n=8 for each treatment group, represented as box and whiskers plot. The box represents the lower and upper quartile (25th and 75th percentile), mid line represents the median. The whiskers represent the highest and lowest observations. * denotes significance level (* p<0.05; *** p<0.001).

A dose-dependent reduction in egg production was observed with increasing exposure to the mixture (fig.26). Data were not normally distributed (Shapiro-Wilks test, 0.645, df=33, p<0.001) and had equal variances (non-parametric Levene's test, f=2.825, df=3, p=0.056), albeit were just outside of the threshold that defines a significant difference in variances. Fecundity was statistically different between treatment groups, Kruskal Wallis, Chi-Square=19.293, df=3, p<0.001). The lowest mixture concentration showed no significant difference in fecundity compared with the control (Chi-Square=1.817, df=1, p=0.178). There was a statistically significant reduction in the fecundity of breeding pairs in the medium concentration group compared with the control (Chi-Square=11.343, df=1, p<0.001), however there was no statistical difference in fecundity between the medium

and low concentration groups (Chi-Square=3.206, df=1, p=0.073). The highest mixture treatment caused a statistically significant reduction in fecundity compared with the control (Chi-Square=12.302, df=1, p<0.001), the lowest mixture treatment (Chi Square=5.300, df=1, p<0.05) and the medium concentration (Chi-Square=5.887, df=1, p<0.05).

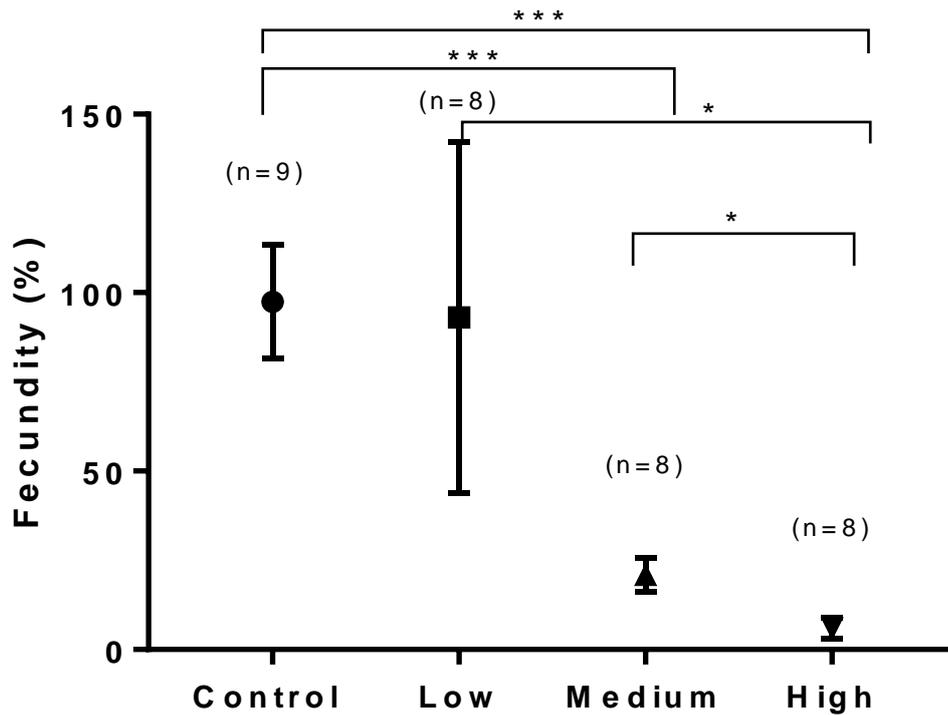


Figure 26. Relative change in fecundity observed in breeding pairs after exposure to three concentrations of the mixture and a control, expressed as a percentage egg performance. Percentage performance refers to cumulative egg production at the end of the exposure period compared to cumulative egg production at the end of the pre-exposure period. 100% equals no change in relative egg performance between the two treatment periods. Error bars represent SEM. Significant difference denoting by *. n=sample size.

3.3.3. Mixture Predictions and Observed Effects

3.3.3.1. Model Predictions

According to the CA model, exposure to the lowest mixture concentration was predicted to inhibit egg production by approximately 30% (fig. 27), whilst the highest concentration was predicted to inhibit egg production by more than 70%.

A key significant finding of this study was that the IA model predicted greater reductions in egg production than the CA model at all three concentrations of the mixture. For example, the IA model predicted a 60% reduction in egg production at the lowest mixture concentration, and almost complete reproductive failure at the highest concentration, whilst CA predicted a 30% reduction in egg production at the lowest concentration and just over 75% reduction at the highest concentration (fig.27).

3.3.3.2. Observed Effects and Accuracy of Model Predictions

The lowest mixture concentration reduced egg production by almost 60%, close to the effect predicted by the IA model (fig. 27). The highest concentration of the mixture caused complete reproductive failure, with all fish pairs ceasing egg production by day 4 of the 21-day exposure period. Overall, the IA model predicted the mixture effect more accurately than the CA model, which appeared to underestimate the mixture effects (figure 27).

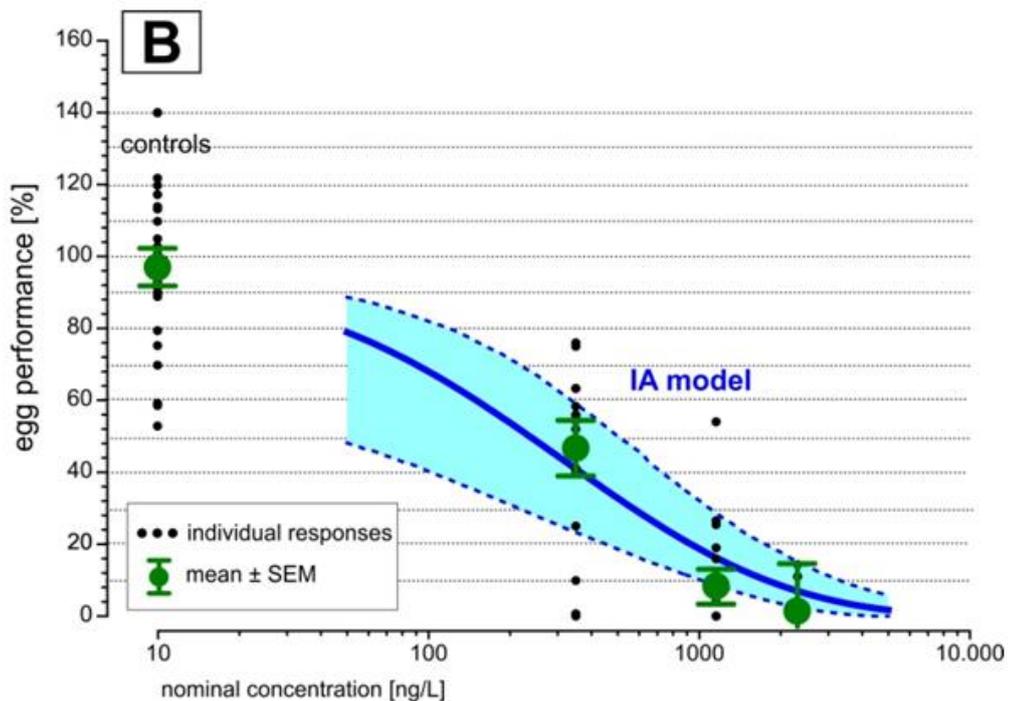
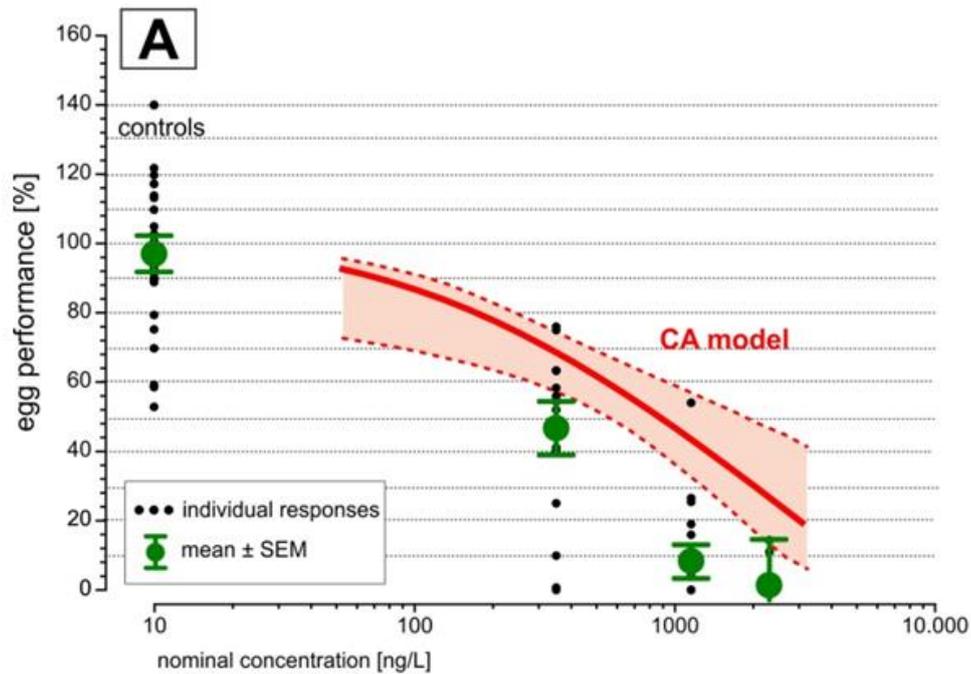


Figure 27. Observed and predicted egg performance in pair-breeding FHMs exposed to a five compound mixture, expressed as a percentage ratio, calculated by comparing each pair's cumulative egg production during the exposure period with that in the pre-exposure period. Data are represented as means (large green dot) with standard error (error bars=SEM). Small black dots represent the fecundity of individual fish pairs. The CA (A) and IA (B) model predictions are shown as means \pm 95% confidence belt. Data represented according to Runnalls et al, (2015). Figures produced by Martin Scholze for Thrupp et al, in prep.

3.3.3.3. 'Something from Nothing'

The results presented from this experiment demonstrated an effect known as 'something from nothing'. For instance, when the five steroid compounds were included in the mixture at a concentration corresponding to their EC10 (concentration causing 10% reduction in egg production), the mixture produced a significant inhibition of reproduction at almost 60% inhibition of egg production. However, each chemical individually would have been expected to produce a 10% reduction (verified from the single compound exposure experiments), which was below the statistical detection limit of the assay (20% inhibition). Thus, the five individual compounds produced a significant effect ('something') when combined, at concentrations producing no effect ('nothing') (defined by the parameters of the assay). To the author's knowledge, this is the first documented example demonstrating the 'something from nothing' effect *in vivo*.

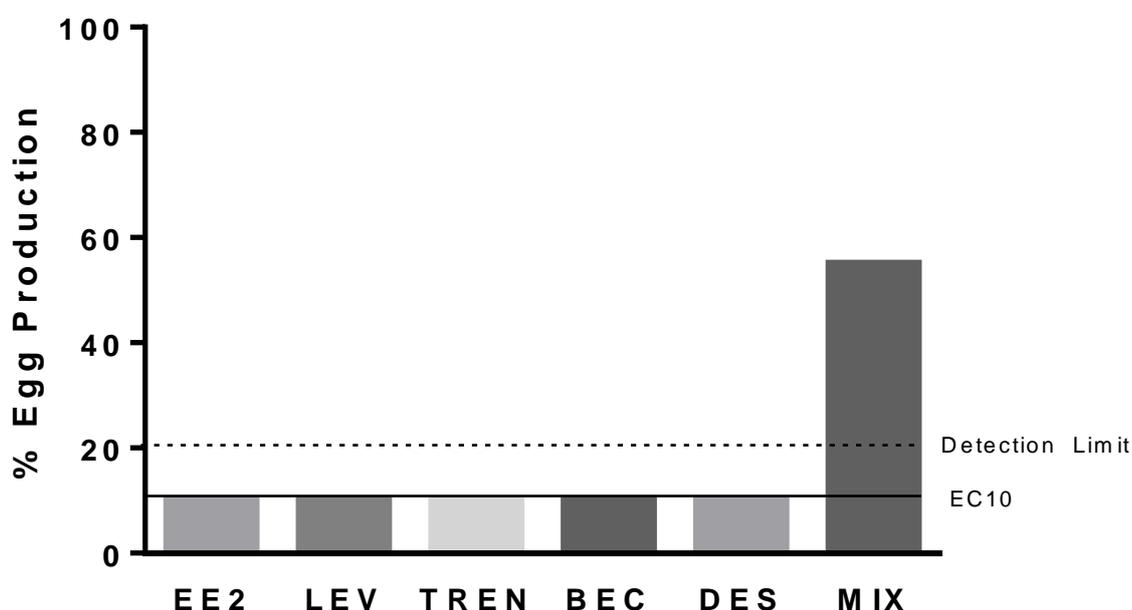


Figure 28. 'Something from nothing'. Combination effects of a five synthetic steroid mixture with each compound present at their respective EC10's (the concentration causing 10% reduction in egg production) (solid line). Each compound individually would be classed as having no effect on egg production since the effect is below the detection limit of the assay (20% - hatched line). The effect of the mixture on egg production was observed in the mixture experiment at each compounds EC10 concentrations.

3.4. Discussion

3.4.1. Single Compounds

The results obtained by T.R and K.S in the single compound reproductive experiments, and those demonstrated by the author for the single compound beclomethasone dipropionate experiment are similar to those reported in the literature (Ankley et al, 2003; Zeilinger et al, 2009). The results further demonstrated the usefulness of the FHM 21-day pair breeding assay (Runnalls et al, 2015, Runnalls, unpublished data, Kugathas, unpublished data). Since reproduction is a critically important ecologically relevant process, the pair-breeding assay serves as a validated, robust toxicity test that has proven to be sensitive to low concentrations of endocrine disrupting compounds, such as the steroidal pharmaceuticals (Arcand-Hoy and Benson, 1998). Furthermore, the robustness of the endpoint (egg production) affords the tests applicability to mixture assessment studies. Since assessments of mixture effects on ecologically important whole organism endpoints are largely lacking, this assay represents a unique application for use in mixture assessment studies.

3.4.1.1. Effect of beclomethasone dipropionate on reproductive capacity of pair-breeding *P. promelas*

Exposure to three concentrations of the synthetic glucocorticoid beclomethasone dipropionate caused a decline in egg production. Results at the 100 and 1000ng/L concentrations were comparable to the experiment undertaken as part of earlier research at Brunel University by K.S. Although effects were observed at a similar range, there were somewhat less-pronounced effects at both the 100 and 1000ng/L concentrations in this study compared with the K.S study, although statistical analysis demonstrated that there was no significant difference between the two experiments. Slight deviations are most likely due to natural variability in reproductive potential of fish pairs and the physical parameters of the assay, such as chemical dosing and flow rate fluctuations. Furthermore,

although both experiments were carried out in a similar fashion, this experiment presented here undertook a longer 'pairing period', whereby fish pairs are assessed for compatibility. The standard pairing period time adopted by the experiment conducted by the author was 14 days (the same as that for all other tested compounds; EE2, levonorgestrel, trenbolone and desogestrel). However, the pairing period undertaken by K.S during the initial beclomethasone pair-breeding experiment was shortened to 7 days. Although this artefact may have had no contribution to the result differences, since it was an additional variable between the experiments, it cannot be ruled out. Further reproductive assays using pair-breeding fathead minnows at Brunel University has demonstrated that the 14 day pairing period increases the robustness of the assay by ensuring optimum breeding potential between pairs at the commencement of the assay, thus demonstrating the imperativeness of the full 14 day pairing period.

The effects on egg production of the lowest concentration 25ng/L of beclomethasone were more pronounced than at the higher concentrations. 25ng/L inhibited egg production by around 60%, compared with 15% inhibition and 50% inhibition for 100ng/L and 1000ng/L respectively. The bell-shaped dose-response curve, along with known problems surrounding the stability of the compound, suggest that the effects concentrations observed may not truly reflect the actual effect concentrations if this compound remained at nominal tank levels. Bell-shaped dose response curves have been demonstrated, most notably from *in vitro* studies involving tumorigenesis (reviewed in Reynolds, 2009). One current theory is that bell-shaped curves, also known as a hormetic response, a biphasic dose-response characterised by pronounced effects at low doses (Mattson, 2008), are caused by overcompensation of an organisms biological processes in order to maintain homeostasis upon exposure to a low dose toxicant (Calabrese and Baldwin, 2001). However, in the case of the results demonstrated here, this explanation is unlikely. Effects are more likely to be attributed to the properties of the chemical and the design of the experiment as opposed to biological responses. In the case of this particular compound,

it's stability in aqueous is likely to be a contributing factor. The complications surrounding the stability of the compound were described by Margiotta-Casaluci et al (2016). In a similar experiment using *P. promelas* under flow-through exposure system, the authors compared two experimental designs for a 21-day exposure study. In the first experiment, nominal concentrations of beclomethasone dipropionate were administered via flow-through from a chemical dosing stock every 4-5 days as in the study presented here. In the second experiment, fish were exposed to continuously sustained concentrations of the compound throughout the experiment. The results of the two experiments demonstrated that, although peak concentrations obtained across the experiments were similar, the flow-through dosing system employed in the current study (and in experiment 1 of the Margiotta-Casaluci study) led to an oscillatory exposure, whereby the compound reached peak concentration only when the dosing stock was replaced with a freshly prepared stock, and maintained for only a few hours. Thus in their experiment (1) the fish were only exposed to nominal concentrations for 10 hours out of 504 total experiment hours (Margiotta-Casaluci et al, 2016), which is most likely also to be the case for the experiment presented in this chapter. Margiotta-Casaluci et al (2016) also found large variations in a number of endpoints between the oscillatory and sustained concentrations, including plasma levels of the compound and its primary metabolite (beclomethasone monopropionate), percentage lymphocytes and percentage granulocytes. The authors also reported significant phenotypic effects (lymphocytopenia) as a result to sustained exposure that did not occur with exposure to the same concentrations in an oscillatory exposure dynamic. Since it has become apparent from the literature on this compound that the concentrations in experimental tanks were likely not sustained at nominal concentrations for much of the experiment, it could be that the true effects on egg production at 100ng/L and 1000ng/L are much more pronounced than those observed.

3.4.2. Five Compound Mixture Experiment

3.4.2.1. Quantification of Exposure Concentrations

Measured concentrations of the three compounds quantified from the mixture were within expected range. EE2 recoveries from tank water samples were within the same range as the spiked MilliQ sample recoveries, suggesting that the EE2 ELISA was effective in quantifying EE2 from the mixture, and that actual concentrations in exposure tanks were similar to nominal concentrations across all samples taken in weeks 1 to 4. Recoveries from the mixture were also within similar range to recoveries observed during the single exposure experiments (Runnalls et al, 2015). Runnalls et al (2015) reported measured concentrations of EE2 from tank water samples of between 52% and 74% in a single exposure study. Although slightly lower recoveries were reported in the Runnalls et al. study compared with those presented here, the concentrations used were higher than those of the current study (0.5, 5 and 25ng/L). Since recovery rates from the presented study were slightly lower at the highest concentration compared with the lowest, it may be that the assay's efficiency decreases slightly with increasing concentrations, which may explain the slight differences in recoveries between the two studies. The results support the evidence, based upon the pharmacodynamics of the compound, in that it is stable in water. The concentration of EE2 available to the fish for uptake appears to be similar to nominal values. Therefore, the degree of inhibition of egg production caused by this compound at any of the tested concentrations is likely to be reliable and repeatable.

Measured concentrations of 17 β -trenbolone from the mixture were within the expected range. Recoveries were comparable with spiked MilliQ sample recoveries, suggesting that the ELISA was efficient and sensitive enough to detect trenbolone in samples within the exposure range. At the lowest and medium mixture concentrations, average percentage recoveries were slightly more than 100%. This is likely due to the timing of sampling, and natural fluctuations in flow rates and dosing rates during the experiment. Although flow-through systems and chemical pump delivery are efficient at regulating water flow and

dosing rates, it is difficult to ensure that the rates are exact and completely consistent across three weeks of exposure. In addition, each mixing chamber and dosing stock is distributed to eight replicate tanks, which also have their own individual flow-rate regulator, therefore keeping the flow and dosing rate exactly the same in each is difficult. Furthermore, since dosing stocks were replaced once every five days, and water sampling occurred once every seven days, variations may arise depending upon time of sampling with respect to when the dosing stock was last changed. For example, if water was collected the day after a dosing stock change, there is the possibility that concentrations in the stock and exposure tanks would be more stable than if a water sample was taken on the fifth day of a particular stock. However, since this was not tested for, this is purely speculative, and cannot be definitively demonstrated.

Measured trenbolone concentrations from the mixture were within the expected range, and found to be similar to that of the single compound experiments (91-135% of nominal), (Runnalls et al, unpublished). Similar to the EE2 ELISA, higher recoveries were observed in the lowest concentrations for both the samples and the spiked MilliQ positive controls.

Average measured concentrations of Levonorgestrel from tank water were higher than nominal values (158-167%). The spiked sample recoveries were also higher than nominal, with measured concentrations of between 208-245% of nominal. Although the radioimmunoassay has been validated and proven both sensitive and selective to levonorgestrel, it is possible that the quantification results from the RIA are due to an interaction with another compound in the mixture. Since there was another progestin (desogestrel) included in the mixture, it may be the case that desogestrel is interfering in the assay, since it has a relatively similar molecular structure to levonorgestrel. Since the spiked MilliQ samples used in the presented study were spiked with the concentrated mixture stock, not with levonorgestrel only stocks, this is reflected in the recoveries of the spikes, which suggests that there is no external factors due to the experimental design that would account for this result. The interference by the other progestin could have

produced the enhanced concentrations observed in the assay. To the author's knowledge, desogestrel has not been tested for activity in the RIA used in this study, so this possibility cannot be ruled out. Runnalls et al (2015) reported measured Levonorgestrel concentrations of between 63-105% from a single exposure experiment. During the Runnalls et al study, water samples were only collected once, on week 4 of the experiment, and every tank was sampled (instead of 4 from 8). The fact that all tanks were sampled, and that samples were collected at the same time point, highlights the variability in recoveries from individual samples. Variability in recovery rates may arise as a result of numerous parameters, such as flow and dosing rate variability, SPE extraction efficiency, etc. Nevertheless, since all techniques are associated with a degree of inaccuracy, the variability in the measured concentrations of all three compounds most likely arose as a result of experimental error, rather than specific factors (discussed).

For all compounds, measured concentrations from week 0 (control blank) were below the detection limit of the respective assays. Furthermore, all control groups in weeks 1-4 for all of the tested compounds were below the detection limit.

3.4.2.2. Fecundity of fathead minnows exposed to a five compound steroid mixture

Fecundity of adult fathead minnows exposed to an equipotent mixture of five steroidal pharmaceuticals was inhibited in a dose-dependent manner, demonstrating that combination effects from steroidal pharmaceuticals with different molecular targets are possible. Inhibition of reproductive performance due to exposure was more accurately described by Independent Action (IA), whereas effects were more pronounced than predicted by Concentration Addition (CA), with the lowest concentration of the mixture causing a significant inhibition of egg production by almost 60%. To the author's knowledge, this is the first instance whereby IA has predicted more pronounced mixture effects than CA. The CA model, working on the basis of additivity, predicts additive effects

of mixtures consisting of compounds with the same mode of action, on a given endpoint, assuming that the endpoint is sensitive to the mode of action of the compounds. In risk assessment, CA mixture predictions are typically employed as the conservative 'worst-case' approximation, since predicted effects by CA are typically greater (i.e. more toxic effects are predicted by the model) than effects predicted by IA (Broderius et al, 1995; Alternburger et al, 2000; Backhaus et al, 2000; Faust et al, 2003), and are deemed as sufficiently protective and conservative (Angerer et al, 2012). Due to the large data requirements of the IA model, which requires detailed information of individual compounds at low-dose effect levels, CA is also considered more feasible and applicable to risk assessment processes (State of the Art Report on Mixture Toxicology, 2009). The results of this study highlight the risk in using CA as a 'worst-case' approximation in risk assessment, since this study has shown that CA does not always predict the highest potential mixture effects. However, as demonstrated by this study, and many others in the literature, differences between the two model predictions are typically small, and therefore observed mixture effects more enhanced than those predicted by CA would not usually cause considerable problems for regulatory risk assessment of chemical mixtures.

Since real-world mixtures are not likely to be classified as strictly similar or strictly dissimilar, the observed effects from 'realistic' mixtures would be expected to be somewhere between the CA and IA model predictions. Given that the compounds in this study could be classed as similar, i.e. they all inhibit egg production, and also 'dissimilar', i.e. they act via modulation of different receptors, the observed effects were expected to be between the model predictions. Although this is true of the data, the observed effects appeared to be more accurately predicted by IA. This may suggest that the compounds are behaving more dissimilarly than similarly. However, there is a degree of uncertainty behind both predictions, and therefore drawing conclusive assumptions based on the more accurate model is difficult. Since mean effects (in this case mean inhibition of egg production) and effect concentrations of the single compounds are subject to stochastic

variability, there is an inherent degree of uncertainty around the predictions made by CA and IA, which are represented by the 95% confidence belts of both models. Furthermore, uncertainty behind the IA prediction is enhanced at low effect concentrations, due to limitations behind the model, which becomes increasingly difficult with decreasing concentrations (Faust et al, 2003).

In general, the use of the 21-day pair breeding assay as a tool to assess the combination effects of mixtures of steroidal pharmaceuticals on an ecologically important endpoint is highly suitable. There are however several limitations, arising from the models themselves (discussed above), and the reproductive assay's use in mixture assessments. Although biological variability due to natural variations in reproductive performance of individual fish is controlled for to a certain degree, the assay cannot control for variability over time. Since each breeding pairs reproductive performance is being compared to itself over two 21 day periods, breeding fatigue may be a possibility. Although fathead minnows are easily manipulated under laboratory conditions to produce eggs every few days for an extended period of time, reproductive performance during the latter part of the experiment may be slightly decreased compared with the earlier stage in some pairs (pers. obs). However, in both the mixture experiment undertaken in the presented study, and in the individual compound studies, the difference in the control groups reproductive capacity between the two experimental periods was not found to be significant, and therefore the temporal variability in breeding capacity is not considered a significant limitation to this assay. In general, the assay, and particularly the quantified endpoint, was proven as a robust method in assessing the effects of 'realistic' mixtures on ecologically important functions in aquatic species.

3.4.2.3. Something from Nothing

The presented study demonstrated combination effects from five steroid compounds present below their NOEC's on an important ecological function – reproduction. At the lowest mixture concentration, each compound was present at a concentration producing a 10% effect, i.e. a 10% reduction in egg performance. The detection limit of the reproductive assay is 20%, therefore each compound was present below the level that would cause a significant inhibitory effect on reproduction. The observed effect produced by the mixture at this concentration was an almost 60% reduction in egg performance, demonstrating that combination effects of steroidal pharmaceuticals present below their NOEC's can result in much higher toxic effects. Existing studies have demonstrated enhanced mixture effects from individual compounds at low doses (below their NOECs) present as mixtures (Silva et al, 2002; Rajapakse et al, 2002). Silva et al (2002), demonstrated enhanced effects at the level of the estrogen receptor ($er\alpha$) in a yeast reporter assay of a mixture of eight xenoestrogens individually at 50% of their NOECs. The so-called 'something from nothing' effect has been well demonstrated *in vitro* for mixtures consisting of compounds with similar mechanisms of action. However, to the author's knowledge, studies demonstrating mixture effects *in vivo* of compounds with dissimilar modes of action, present below their NOECs are not reported in the literature. This study represents a good example of the potential for 'something from nothing' effects on ecologically important endpoints at the whole organism level. Furthermore the individual compounds tested in this mixture within the range of environmental relevance (ng/L). As discussed previously, synthetic steroids have been detected in effluent and in some cases surface water in the ng/L range. The study presented demonstrates the potential for adverse effects to aquatic organisms posed by multicomponent mixtures when compounds are present at environmental concentrations.

3.4.3. Chemical Interactions

The nature of chemical interactions in any mixture is complex. A key limitation of the CA and IA model predictions is that they assume no interaction between the compounds in the mixture. In reality, compounds in any given mixture are likely to have an effect on the fate and behaviour of others, such as their uptake, transport, receptor affinity, metabolism, and excretion potential. For example, where two compounds with the same target receptor are present in a mixture, there may be competitive binding at the receptor level, particularly if one compound has higher binding affinity than another. In this case, is it still reasonable to assume that two synthetic progestins for example, can act according to dose additivity at the level of the progesterone receptor, when both compounds may not be able to regulate transcription at the receptor level as they would if they were present alone? This is likely to contribute to the reason why observed effects of most realistic mixtures (i.e. consisting of both similar and dissimilar compounds) are between the predictions of CA and IA. In the case of the presented study, five compounds with primary affinity to four different target receptors, and secondary binding affinity with other receptors, will likely be interacting to affect the fate and behaviour of one another. Furthermore, reproduction is regulated by complex positive and negative feedback mechanisms (discussed previously), so combined exposure to estrogens, progestogens, androgens and glucocorticoids will inevitably cause a succession of regulation and feedback mechanisms on the reproductive axis of fish. In addition, it is also possible that different chemicals in the mixture are acting in different ways, for example chemical X and chemical Y act additively according to CA, whilst chemical X and chemical Z act independently (Sumpter et al, 2006), and the type of mixture interaction/effect is likely to depend on the endpoint in question. Furthermore, exogenous compounds in the mixture are likely to also affect and be affected by endogenous hormones, but the complexity of these interactions is not well understood.

The binding affinity of both endogenous and exogenous ligands to steroid receptors has been shown to be species-specific, with certain estrogenic compounds demonstrating species-dependant binding preferences for $er\alpha$ and $er\beta$ (Matthews et al, 2000; Harris et al, 2002). Harris et al, (2002) investigated ligand binding profiles of estrogen receptors $er\alpha$ and $er\beta$ in human, rat and mouse, and found that the binding affinity of a number of tested estrogens had differential affinities across the three species, highlighting the complex nature of ligand-mediated transcription and endocrine feedback mechanisms.

3.5. Conclusions

The results described here demonstrate that combination effects larger than those caused by the individual compounds can occur in fish at the whole organism level, even when individual compounds are acting via different molecular targets. Furthermore, combination effects as a result of exposure to mixtures of compounds at environmental concentrations can occur on ecologically crucial biological processes, such as reproduction. The results additionally demonstrate that significant effects on reproduction can occur as a result of exposure to steroidal pharmaceuticals, when present at low concentrations as mixtures.

Furthermore, the results presented suggest that observed mixture responses can be underestimated by CA, highlighting the caution in assuming that CA can always be used as a worst-case expectation of mixture toxicity, and that using the CA prediction as a worst-case scenario could lead to the underestimation of the risk. This finding is particularly important because of the ecological relevance of the endpoint investigated in this study.

Further endocrine disruption biomarkers as well as the mechanisms of disrupted reproduction as a result of exposure to five steroidal pharmaceuticals will be investigated further in chapters 4 and 5, respectively.

Chapter 4: Effects of mixtures of steroidal pharmaceuticals with diverse mechanisms of action, on the reproductive capacity of the Fathead minnow, *Pimephales promelas*.

Part 2: Assessment of biomarkers and indicators of endocrine disruption

4.1 Introduction

The successful use of biomarkers as tools in assessing and quantifying exposure to environmental endocrine disruptors has been widely reported, particularly in the case of estrogens (Sumpter and Jobling, 1995; Hansen et al, 1998), and androgens (Björkblom et al, 2013; Margiotta-Casaluci and Sumpter, 2011). Biomarkers are classified as any change in a biological function or response that can be directly correlated to exposure (Peakall, 1994; Hutchinson et al, 2006) and can at any level of biological organisation (Ankley et al, 2009). Biomarkers can be highly complementary to the use of other endpoints in determining the risk of a compound to an organism. Furthermore, since the use of apical endpoints only (such as reproduction or growth) does not always reveal a great deal about the mechanism of action of a compound (or groups of compounds), collecting additional biomarker data can supplement effect data with more mechanistic knowledge on a particular compound. The most robust studies typically employ a range of endpoints using biomarkers from across many biological levels (i.e. molecular, cell, biochemical and apical (whole organism)). Moreover, biomarkers can be useful in providing an association between effects observed under laboratory conditions to those observed in the field (Hutchinson et al, 2006).

Robust biomarkers exist for a number of steroidal compounds. For example, the assessment of vitellogenesis, the synthesis of the egg yolk precursor protein vitellogenin (VTG) produced in the liver of female fish, has been widely used as an indicator of exposure to environmental estrogens (Sumpter and Jobling, 1995; Tyler et al, 1996). VTG induction has been shown to be a reliable and reproducible indicator particularly in fish (Kime et al, 1999), and has been validated to estrogen-related effects at the population level (Kidd et al, 2007; Miller et al, 2007). Assessment of plasma VTG and vtg mRNA levels (the gene coding for VTG synthesis) have been widely used in *in vivo* experiments with a range of aquatic animals, and have also been shown to be robust indicators of

estrogenicity *in vitro*, having been validated against *in vivo* endpoints. Thus in line with recommendation to reduce, refine and replace the use of animals in scientific research, the use of this biomarker has led to the reduction in fish required for *in vivo* studies in assessing potential estrogenicity of certain compounds. The occurrence of intersex in fish has also been successfully used as a biomarker of estrogen exposure in a range of species (Jobling et al, 1998; Metcalfe et al, 2001; Jobling et al, 2005; Jobling et al, 2006; Williams et al, 2009). Together with the use of VTG induction, the application of this biomarker revealed the estrogenicity of sewage effluents across the UK and Europe. The application of VTG has also revealed the presence of anti-androgens in sewage effluent (Katsiadaki et al, 2012), further demonstrating its usefulness as a robust biomarker of exposure to environmental endocrine disruptors.

Though estrogen biomarkers were the first markers of endocrine disruption in wildlife, sensitive biomarkers for androgenicity (and anti-androgenicity) have also been developed and have proven robust indicators of exposure. The induction of nuptial tubercles in pre-spawning fathead minnows (Panter et al, 2004; Margiotta-Casaluci and Sumpter, 2011), somatic growth (Margiotta-Casaluci and Sumpter, 2011), gonopodial development in mosquito fish (*Gambusia affinis*) (Ellis et al, 2003), and the formation of papillary processes in the anal fin of female medaka (Okada and Yamashita, 1944; Hishida and Kawamoto, 1970), have all been proposed as biomarkers for androgen (or antiandrogen) exposure. Spiggin, a glycoprotein produced in the kidneys of male sticklebacks that is used to construct their nests, has been recently discovered as a robust and sensitive biomarker to androgen (and antiandrogen exposure) (Katsiadaki et al, 2002a; Katsiadaki et al, 2002b; Hahlbeck et al, 2004; Katsiadaki et al, 2006). Moreover, spiggin induction in female and juvenile sticklebacks has been found to be a sensitive biomarker to androgen exposure. Furthermore, the biomarker has also been successfully used to determine androgenic properties of some synthetic progestins in fish. For example, Svensson et al, (2013) exposed female three-spined stickleback (*Gasterosteus aculeatus*) to the synthetic

progesterone levonorgestrel, and reported a significant induction in spiggin at exposure concentrations of >40ng/L. Since female (and juvenile) sticklebacks do not normally produce the protein, the study demonstrated the potent androgenicity of the compound in fish.

Although well-defined biomarkers exist for assessing estrogenicity and androgenicity, no real distinct biomarkers of exposure to corticosteroids have been established. However, some studies have suggested several biological indicators for assessing exposure to corticosteroids. For example, results demonstrated by Kugathas et al (2013) indicate several potential markers, such as the measurement of plasma glucose, the quantification of hepatic phosphoenolpyruvate carboxykinase (PEPCK) mRNA levels, and the quantification of lymphocyte/thrombocytes, however, since many compounds and environmental conditions have the potential to alter these biological parameters (such as natural or experimentally induced stress), further investigation and validation is required.

Biomarkers for androgen/estrogen exposure as discussed serve as robust and sensitive indicators of exposure to a particular type of compound because they are subject to significant changes (inhibition or induction) when exposed to low concentrations of the compounds in question, allowing ease of quantification and determination of variability in the given endpoint. Furthermore, the use of sensitive and specific biomarkers in mixture studies can prove even more valuable, particularly when assessing mixture effects from a combination of dissimilarly acting compounds. In assessing mixture effects from multicomponent mixtures comprised of similarly acting compounds, a robust biomarker can allow for accurate effect predictions based around the concept of concentration addition (CA). For example, spiggin production in androgenised female three-spined sticklebacks (in the androgenised female stickleback screen, AFSS) has been successfully used to predict mixture effects from exposure to anti-androgens using the principle of concentration addition (Pottinger et al, 2013). In assessing combination effects from mixtures containing dissimilarly acting compounds, biomarkers can be useful

determining the mechanisms of action of a particular compound. For example, Runnalls et al (2015) exposed breeding pairs of adult fathead minnow (*Pimephales promelas*) to fixed concentrations of the synthetic estrogen ethinyl estradiol (EE2), and the synthetic progestin levonorgestrel individually and as a mixture. They demonstrated conclusively that some biomarkers closely mimic the response of the individual compound, for example plasma vitellogenin levels in the fish in the mixture experiment (exposed to both an estrogen and an androgen) were similar to those observed in fish exposed only to EE2, suggesting that androgens have no effect on vitellogenesis. In the same study however, fish exposed to the mixture demonstrated combination effects larger than those that occurred upon exposure to the compounds individually, on another endpoint, fecundity (quantified as egg production per breeding pair). The effects of the mixture on pair-breeding *P. promelas* caused a more pronounced reduction in egg performance compared with the individual compounds. The results from the Runnalls et al (2015) study and similar studies reported in the literature (Zucchi et al, 2014; Säfholm et al, 2015; Zhao et al, 2015) demonstrate that additive mixture effects can occur at some biological levels whilst simultaneously not occurring at others.

4.1.1. Aims and Objectives

At present, the process of reproduction in teleost fish is complex and not completely understood. As a general taxonomic group, teleosts undertake a range of different reproductive strategies (discussed in detail Chapter 3). Reproductive endpoints offer a valuable tool for the assessment of endocrine-related effects of chemical pollutants. As part of the presented study, fully mature (12 months of age) adult fathead minnows, *Pimephales promelas*, were exposed to a equipotent mixture of five steroidal pharmaceuticals; 17 α -ethinyl estradiol, levonorgestrel, desogestrel, 17- β trenbolone and beclomethasone dipropionate, at close to environmentally relevant concentrations. Fecundity of breeding pairs was found to be significantly inhibited by exposure to the five

compound steroid mixture in a concentration-dependant manner. At the highest mixture concentration, reproductive failure was observed in all study subjects. Several biomarkers indicative of reproductive disruption were investigated to further elude the mechanisms of disrupted reproductive output from exposure to the multicomponent mixture. Secondary sexual characteristics, Condition Factor (CF), Hepatosomatic Index (HSI), Gonadal Somatic Index (GSI), and general physiological endpoints were analysed to assess for physiological changes. Histopathological analysis of the gonads was undertaken to investigate pathological changes in the ovaries and testis of fish that may have been contributing to reproductive decline. A hatchability study of selected embryos was undertaken to investigate adverse effects on the hatching potential of embryos produced under exposure conditions. Fecundity of experimental fish was investigated in Chapter 3, and therefore will not be discussed here.

4.1.2. Hypotheses

1. There will be a concentration-related effect on biomarkers indicative of endocrine disruption in breeding pairs of adult (12 month old) *P. promelas* exposed to a five compound mixture of synthetic steroidal pharmaceuticals
2. There will be a masculinisation of female *P. promelas* exposed to a five compound mixture of synthetic steroidal pharmaceuticals
3. There will be an enhancement of male secondary sexual characteristics in both male and female *P. promelas* exposed to a five compound mixture of synthetic steroidal pharmaceuticals

4.1.3. Null Hypothesis

1. There will be no effect on additional biochemical endpoints associated with reproduction and endocrine function in male or female *P. promelas* exposed to a five compound mixture of synthetic steroidal pharmaceuticals

4.2. Materials and Methodologies

4.2.1. Fish Reproduction Assay – 21 Day Pair Breeding (Harries et al, 2000)

The 21 day pair breeding assay was utilised for the assessment of endocrine related effects in breeding pairs of fathead minnow (*Pimephales promelas*). The assay, described in detail in Chapter 3, was undertaken for comparison of predicted and observed mixture effects on an ecologically relevant endpoint. A number of additional endpoints were quantified at the end of the experiment in order to assess the effects of the mixture of synthetic steroids on a number of biological functions associated with reproduction and endocrine function in fish. The detailed methodologies of the pair breeding assay will not be discussed again here, but the reader is asked to refer to Chapter 3 for full details.

4.2.2. Quantification of Exposure Concentrations

Measured concentrations of the mixture were quantified based on the measurement of three of the five compounds. 17α -ethinyl estradiol, 17β -trenbolone, and levonorgestrel were analysed using commercial enzyme-linked immunosorbent assays (ELISA) and a radioimmunoassay. The methods are described in detail in Chapter 3.

4.2.3. Sampling Procedure

On day 46 of the experiment (the day following day 21 of the exposure period), fish were randomised using a random number generator (RANDOM.org) and allocated a new sampling number. All fish were sampled on the same day. Fish pairs were sampled blindly according to their sampling number. The male and female of each pair were sampled in succession, with no knowledge of their exposure group. Treatment groups were not sampled in succession, eliminating sampling biases due to treatment group knowledge, and potential for variability due to the time of day. On the day of final sampling, fish were not fed, to ensure no food remained in the digestive tract to avoid interference with tissue

dissection. Fish were transported from their tanks to holding boxes, one pair at a time. Each fish was euthanized with tricaine methanesulfonate (MS-222) (Sigma-Aldrich, UK).

4.2.4. General physical characteristics

Wet weight (g) and fork length (mm) were quantified in both sexes, and were used to calculate the Fulton's Condition Factor (CF) (Nash et al, 2006); expressed as:

$$K = \frac{W}{L^3} \times 100 ,$$

where W is the wet weight (mg), and L is the fork length (mm). Blood was collected through caudal puncture, spun in a cooled centrifuge, and the plasma subsequently collected. Plasma volume was quantified and aliquoted into 1.5ml Eppendorf tubes and stored at -80°C. Liver tissues were dissected, weighed and rapidly frozen in liquid nitrogen before being stored at -80°C. Gonads were dissected, weighed, and divided into two sections. One section was immediately frozen in liquid nitrogen before being transported to storage at -80°C. Liver and gonad weights were used to calculate the hepatosomatic index (HSI = liver weight(g)/total body weight(g) x 100); and the gonadosomatic index (GSI = gonad weight(g)/total body weight(g) x 100), respectively.

4.2.5. Secondary Sexual Characteristics

Sex specific characteristics were assessed by visual grading of fin spots and nuptial tubercles. Male fatpads were measured with callipers, dissected and weighed, and allocated a grading with respect to prominence (appendix 3). In females, fatpad (where present) presence or absence was noted. Tubercles of both males and females were assessed by immersion of the head of the fish into liquid nitrogen (10 seconds) followed by visual quantification of the number of tubercles and the prominence grade (appendix 3). Length of the ovipositor and the abdominal girth was recorded in females.

4.2.6. Histopathology of the gonads

A section of the gonad dissected from each individual fish was fixed in Bouin's solution for 24 hours and subsequently transferred to 70% ethanol until processing for histopathology. Time was recorded on the data sheets every 30 minutes so that individual samples could be transferred from Bouin's to ethanol at the appropriate times (24 hours after sampling).

4.2.6.1. Preparation, processing and embedding of tissues

The dissected gonadal tissue was removed from ethanol with fine tweezers and further dissected into smaller sections with a surgical blade. Larger tissue sections were cut into three individual pieces, whilst smaller tissues (typically male testis) were dissected into two. Dissected tissues were carefully transferred to a histology cassette lined with a fine mesh to prevent small sections of tissue from sliding out of the cassette. The cassettes were placed in a beaker containing 70% ethanol until each cassette was filled. The cassettes were then loaded into a tissue processor (TP1020 benchtop tissue processor, Leica, UK) for subsequent dehydration, clearing and infiltration of tissues. Tissues were processed in solutions of 2 litres (IMS solutions were diluted in distilled water where necessary) using the following standardised protocol of 12 cycles:

CYCLE NUMBER	TREATMENT	TIME (hours)
1	70% IMS	3
2	90% IMS	2.5
3	95% IMS	1.5
4	100% IMS	1.5
5	100% IMS	1.5
6	100% IMS	1.5
7	100% IMS	1.5
8	HISTOCLEAR	1.5
9	HISTOCLEAR	1.5
10	HISTOCLEAR	1.5
11	WAX	1.25
12	WAX	1.25
		20 HOURS TOTAL

4.2.6.2. Wax embedding

Metal embedding cassettes were heated on a hot plate for approximately one hour before embedding. Plastic processing cassettes were removed from the final wax cylinder of the tissue processor and transferred to a heated wax container, making sure that all cassettes were fully immersed. Processed tissues were removed from plastic cassettes using fine tweezers and transferred to a metal embedding cassette pre-filled with liquid paraffin wax. Each tissue section was oriented longitudinally so that the transverse section through the tissue could be sectioned by the microtome.

The corresponding empty plastic cassette was subsequently placed on top of the metal cassette containing the sample and topped up with liquid wax. The wax block was

transferred to a cooled plate and left to set for a few hours. Once completely set, the wax blocks were removed from the metal cassettes and stored at 4°C until further processing.

4.2.6.3. Sectioning

Prior to sectioning, wax blocks were removed from the fridge and held on ice to ensure they remained cooled and the wax remained solid, in order to obtain high-quality sections.

Sections were cut using an Anglia Scientific microtome (fig 29).

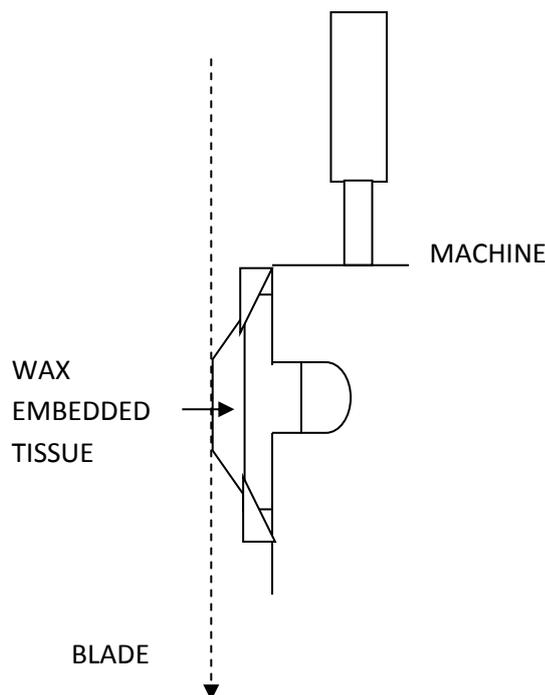


Figure 29. Microtome and sectioning procedure represented schematically. Modified from Laboratory Histology Protocol, Brunel University London. 1999.

Thicker sections were initially cut at 10µm in order to reach sufficient depth of tissue within the block. Subsequently, tissues were sectioned at 3µm, immersed in methanol to allow the section to expand, and then transferred to a warm water bath containing purified water heated to 40°C. The section was then transferred and mounted on a Superfrost glass microscope slide (Polysine, 25 x 75 x 1.0mm, ThermoFisher Scientific) and placed on a heated plate set at 45°C for approximately 24 hours. Six sections were cut for each sample, with three sections obtained initially, followed by deeper sectioning of tissue

before obtaining a further three sections. Where tissues were difficult to section, for example due to the presence of solid oocytes or slight over-fixation of tissue, more sections were cut to increase the potential for analysis.

4.2.6.4. Staining - Haemotoxylin and Eosin (H&E)

Staining of sections was undertaken using haemotoxylin and eosin (H&E) stain using an automatic stainer (StainMate, Thermo Fisher Scientific) following the protocol described in table 20, mounted with a protective cover slip using Histomount (Natural Diagnostics), and left to dry for approximately 24 hours in a fume hood.

Table 20. Haemotoxylin and eosin (H&E) staining procedure, based on the protocol developed by Brunel University London.

Stain no.	STAIN	Purpose	Time (mins/seconds)
1	HISTOCLEAR	Dissolves wax	15 m
2	100% IMS	Step-wise hydration	2 m
3	90% IMS	Step-wise hydration	2 m
4	70% IMS	Step-wise hydration	2 m
5	TAP WATER (RUNNING)	Rinse	2 m
6	HAE (Haemotoxylin eosin)	One part binds to basic components of tissue, one part to acid components (NUCLEUS)	10-15 m
7	TAP WATER (RUNNING)	Remove excess stain	10-15 m
8	ACID / IMS	Dechlorination	20 s
9	TAP WATER (RUNNING)	Rinse	20 s
10	LiCO ₃	Salt	20 s
11	TAP WATER (RUNNING)	Rinse	20 s
12	EOSIN	Colour binds to cytoplasm	5-10 s
13	TAP WATER (RUNNING)	Remove excess stain	5 m
14	70% IMS	Step-wise dehydration	2 m
15	90% IMS	Step-wise dehydration	2 m
16	100% IMS	Step-wise dehydration	2 m
17	HISTOCLEAR	Remove IMS, binding agent	2 m
18	RESIN (Histomount) AND COVERSIP	Protective layer	n/a

4.2.6.5. Analysis

Slides from fish exposed to the highest mixture concentration and the control groups were analysed for pathological changes in the gonads. All slides were blinded prior to analysis to avoid biasing the results. Histological sections were analysed using an Olympus BX51 compound light microscope and images were captured using QCapture Pro. Initial examination of slides was carried out at a low magnification (x10 magnification) and assessed visually for any abnormalities. Detailed analysis was performed using a x40 magnification. Sections were examined for abnormal changes including cell atresia, apoptotic cells, degeneration and vacuolisation. Semi-quantitative analysis of histopathological sections were carried out based on the US Biomonitoring of Environmental Status and Trends (BEST) morphological criteria, modified by Wolf et al, (2004). The grading system was further modified by the author for the purpose of further statistical analysis (table 21 and 22). Grading criteria were based on the visual assessment of gametogenic precursors in comparison with mature gametocytes. In males, number of spermatogonia, spermatocyte, spermatid and spermatozoa were quantified and graded (table 21). In females, oocyte atresia, edema, atretic follicles, and number and stage of vitellogenic oocytes were quantified (table 22).

Table 21. Morphological grading criteria for analysis of fathead minnow testis, based on assessment of the presence and abundance of mature and immature sperm stages. The criteria based on that developed by the Biomonitoring Environmental Status and Trends (BEST) system (McDonald et al, 2000), and subsequently modified by Wolf et al (2004), were further modified by the author for the purpose of further analysis. Modification was made based on the splitting on the stage 3 category (previously stage 3.a) and 3.b)) into separate categories (3 and 4).

Stage	Description
0	Undeveloped, immature phases – spermatogonia and spermatids, no spermatozoa
1	Early spermatogenic - predominantly immature phases, some spermatozoa may be observed
2	Mid-spermatogenic – spermatocytes, spermatids, and spermatozoa present in roughly equal proportions
3	Late spermatogenic – All stages of sperm maturation observed, mature sperm predominate, but immature spermatogenic phases still observed throughout testis
4	Late spermatogenic – All stages of sperm maturation observed mature sperm predominate, immature spermatogenic phases absent or restricted to small scattered regions
5	Spent – loose connective tissue with some remnant sperm

Table 22. Morphological grading criteria for staging ovaries. From BEST (McDonald et al, 2000).

Stage	Description
0	Undeveloped - entirely immature phases (oogonia to perinucleolar oocytes, no cortical alveoli)
1	Early development - >90% pre-vitellogenic follicles, predominantly perinucleolar through to cortical alveolar
2	Mid-development - at least half of observed follicles are early to mid vitellogenic
3	Late-development - majority of developing follicles are late vitellogenic
4	Late development/Hydrated - majority are late vitellogenic and mature/spawning follicles. Follicles are larger compared with stage 3
5	Post-ovulatory – predominantly spent follicles, remnants of theca externa and granulosa

4.2.7. Embryo Hatchability

A small-scale embryo hatchability study was conducted on eggs collected from randomly selected fish pairs from each treatment group, selected using a random number generator, in order to investigate whether chemical exposure affected the hatching success of the developing embryo. Two pairs from each treatment group (from a total of eight) were selected for inclusion in the study. Every batch of eggs produced by selected pairs during the exposure period were counted (for assessment of mixture effects on fecundity) and subsequently transferred to a glass petri dish in clean aquarium water. 48 embryos were randomly collected from the batch and transferred to 12-well microtiter plates (one embryo per well, four plates per egg batch) filled with 4ml of clean (chemical-free) aquarium water and kept in an incubator (Sanyo MIR152) at 25.6±0.3°C until

hatched. Culture water was renewed by removing and replacing three-quarter volumes every 48 hours. Embryos were checked every 24 hours. Dead embryos were recorded and removed each day. After 120 hours post fertilisation the study was terminated by emersion of larvae and unhatched embryos in MS222. Total hatching success per plate was recorded daily, and quantified at day 3 and day 5. Abnormalities in hatched larvae were assessed and recorded as either present or absent.

4.2.8. Statistical Data Treatment

Data were statistically analysed for normal distribution using the Shapiro-Wilk Test and histogram analysis. Assessment of homogeneity of variance was performed using either traditional or non-parametric Levene's Test (where appropriate). The non-parametric Levene's test uses the ranked mean values for comparison, and has been found to be a more robust test for assessing homogeneity of variance for non-normally disturbed data. Normally distributed data with equal variance were analysed using a one-way ANOVA and post-hoc Tukey's test for determination of where the significant differences lay (where appropriate). The Kruskal Wallis test was used to analyse non-parametric data with equal variances. Where data were non-parametric and variances were not equal, the Mood's Median test was utilised to compare median values across treatments groups. The Mood's Median test is suggested as the most appropriate test for nonparametric data where the variances are not equal.

4.3. Results

4.3.1. Quantification of exposure concentrations

Quantification of exposure concentrations over the experimental period is described and discussed in Chapter 3.

4.3.2. General physiological characteristics

Fork length of male fish across the treatment groups ranged from 52 to 70mm, and females between 45 and 57mm, (fig.30a) both considered a normal size range for this species. Length was found to be normally distributed (Shapiro-Wilks test, 0.972, df=32, p=0.566) and have equal variances (Levene's test, 0.463, df=28, p=0.710). Length was found to remain unaffected by exposure to the steroid mixture (one-way ANOVA, f=0.659, p=0.584). Length of female fish was normally distributed (Shapiro-Wilks test, 0.971, df=31, p=0.539), with unequal variance (Levene's test, 4.825, df=3, df=27, p<0.005). Data were subsequently analysed using the Welch test. Length of females was found to be unaffected by exposure to the five compound steroidal mixture (Welch test, 4.816, df=3, df=14, p=0.16). Condition Factor of male fish was found to be normally distributed (Shapiro-Wilks test, 0.936, df=32, p>0.05), and have equal variance (Levene's test, 0.657, df=28, p=0.585). CF was statistically similar across all treatment groups (one-way ANOVA, f=0.642, df=3, p=0.595) (fig.30b).

Weight of male and female fish was between 2.7 and 6.0g, and 1.2 and 2.9g, respectively. Weight of both males and females was found to be normally distributed (Shapiro-Wilks test, p>0.05, df=32) and have homogeneity of variance (Levene's Test, p>0.05). Weight of both male and female fish was found to be unaffected by exposure to the five compound steroid mixture (fig 31a) (one-way ANOVA, f=14.813, df=31, p>0.05).

Abdominal girth of males varied relatively little, and was within the range of 5.1-6.2mm. In females, abdominal girth variation was slightly higher, between 4.8 and 6.8mm (fig 31b). Both male and female abdominal girth was normally distributed (Shapiro-Wilks test,

$p > 0.05$) and had equal variances (Levene's test, $p > 0.05$). Abdominal girth of male fish was not significantly affected by exposure to the five steroid compound, (one-way ANOVA, $f=2.387$, $df=31$, $p > 0.05$). In female fish, abdominal girth was statistically different between treatments (one-way ANOVA, $f=8.774$, $df=30$, $p=0.028$). Post hoc analysis revealed that there was a statistical difference between the lowest and highest treatment concentration (Tukey's post hoc test, $p=0.029$). However, abdominal girth was not found to be significantly different in comparisons between any other group. Furthermore, abdominal girth in the control group was found to be statistically similar to that of all the other treatment groups.

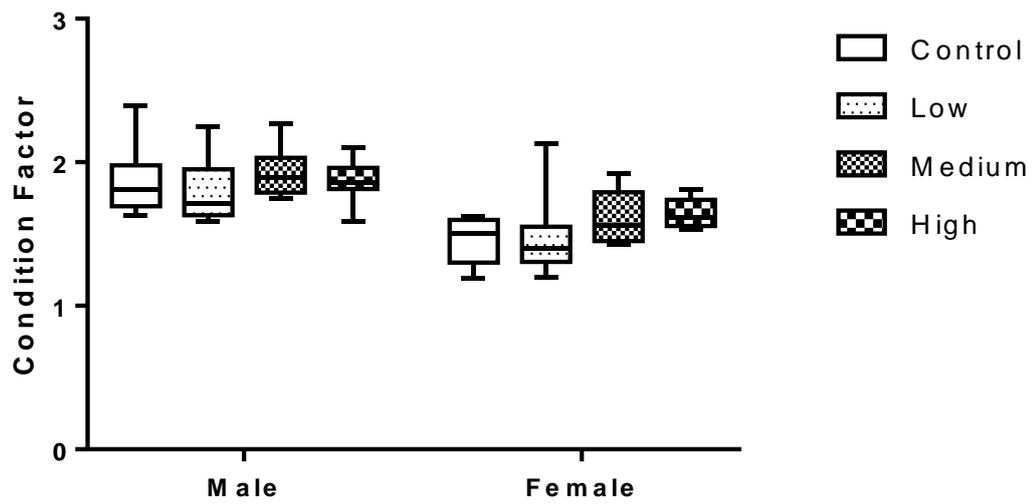
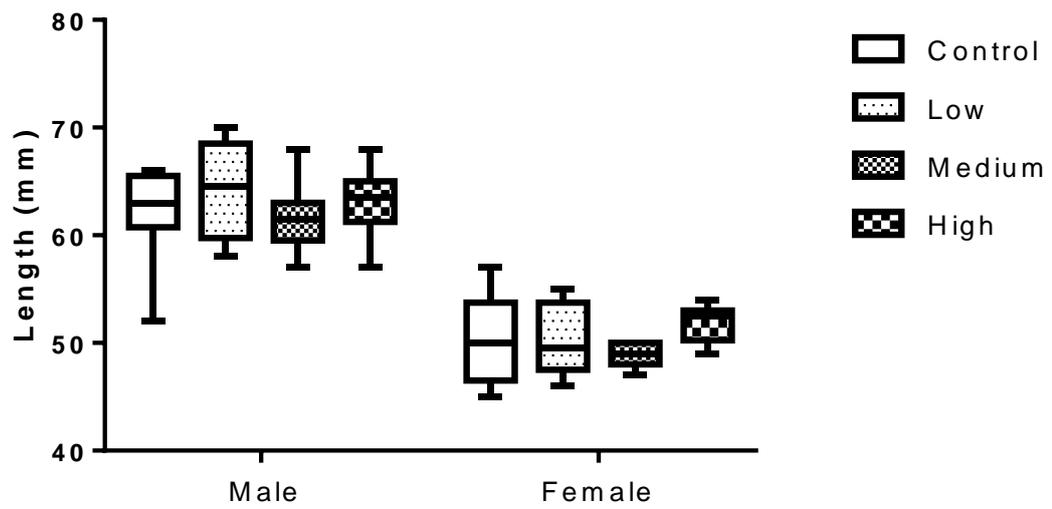


Fig 30. Length (a) and condition factor (b) of male and female *P. promelas* exposed to a five compound steroid mixture, quantified at study termination. Box extends from 25th to 75th percentiles (the interquartile range), horizontal line represents the median. Whiskers represent the minimum and maximum observations. n=8 for all treatment groups.

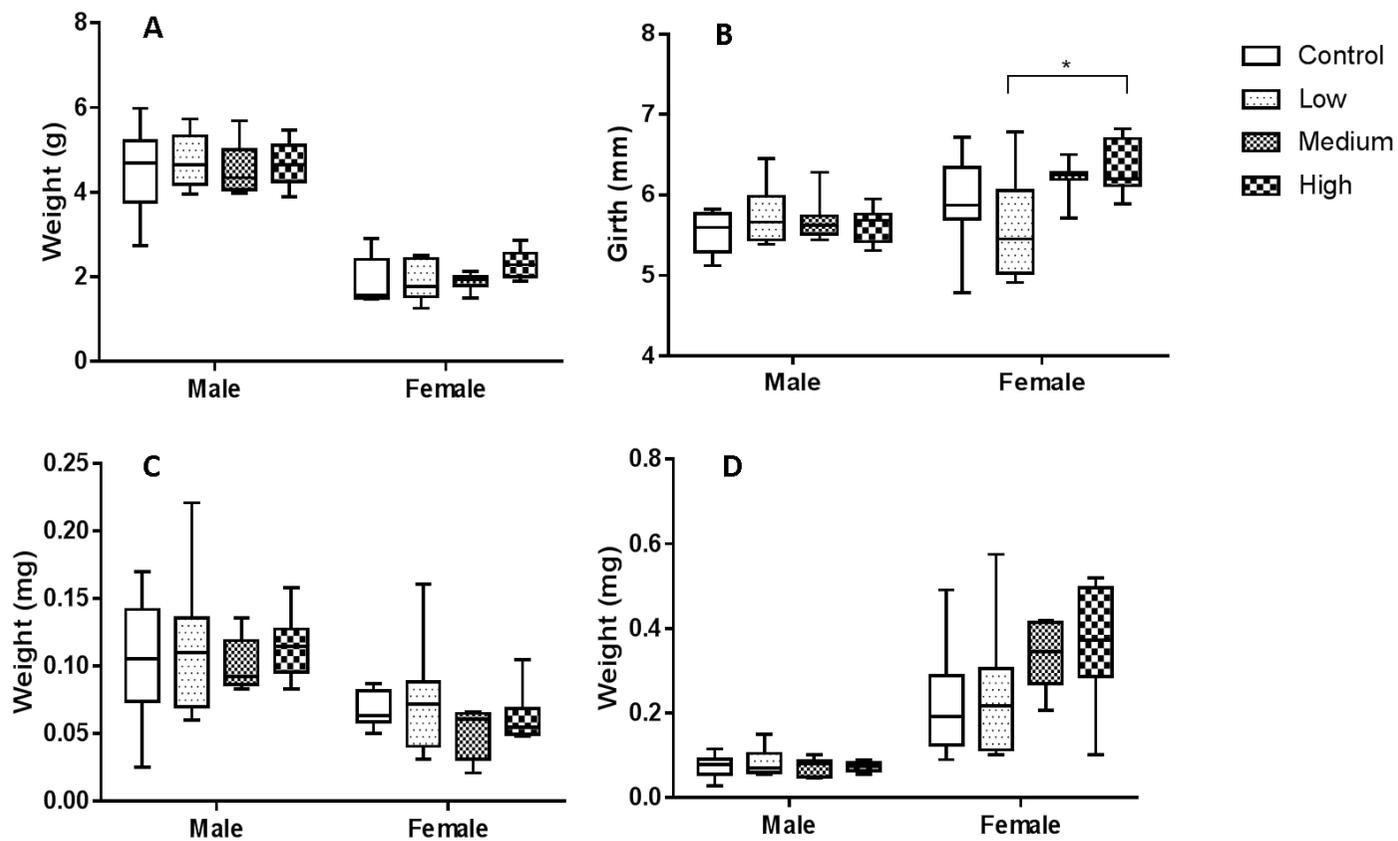


Figure 31. Physiological characteristics of male and female *P. promelas* exposed to a five steroidal compound mixture, assessed at study termination: A. wet weight, B. abdominal girth, C. liver weight, and D. gonad weight. Box extends from 25th to 75th percentiles (the interquartile range), horizontal line represents the median. Whiskers represent the minimum and maximum observations. n=8 for all treatment groups. * denotes statistical significance.

The GSI for both males and females were found to be within the normal expected range (1-2.6 and 3.6-21, respectively). The GSI of males and females was normally distributed (Shapiro-Wilks test, $p > 0.05$), and data were found to have homogeneity of variance across all treatments (Levene's test, $p > 0.05$). The GSI of male fish was found to remain unaffected by treatment dose (fig 32a, one-way ANOVA, $f = 0.2081$, $p = 0.89$). The GSI of female fish appeared to increase with increasing concentration to the mixture, however the trend was found not to be statistically significant (fig. 32b, one-way ANOVA, $f = 2.163$, $p = 0.11$).

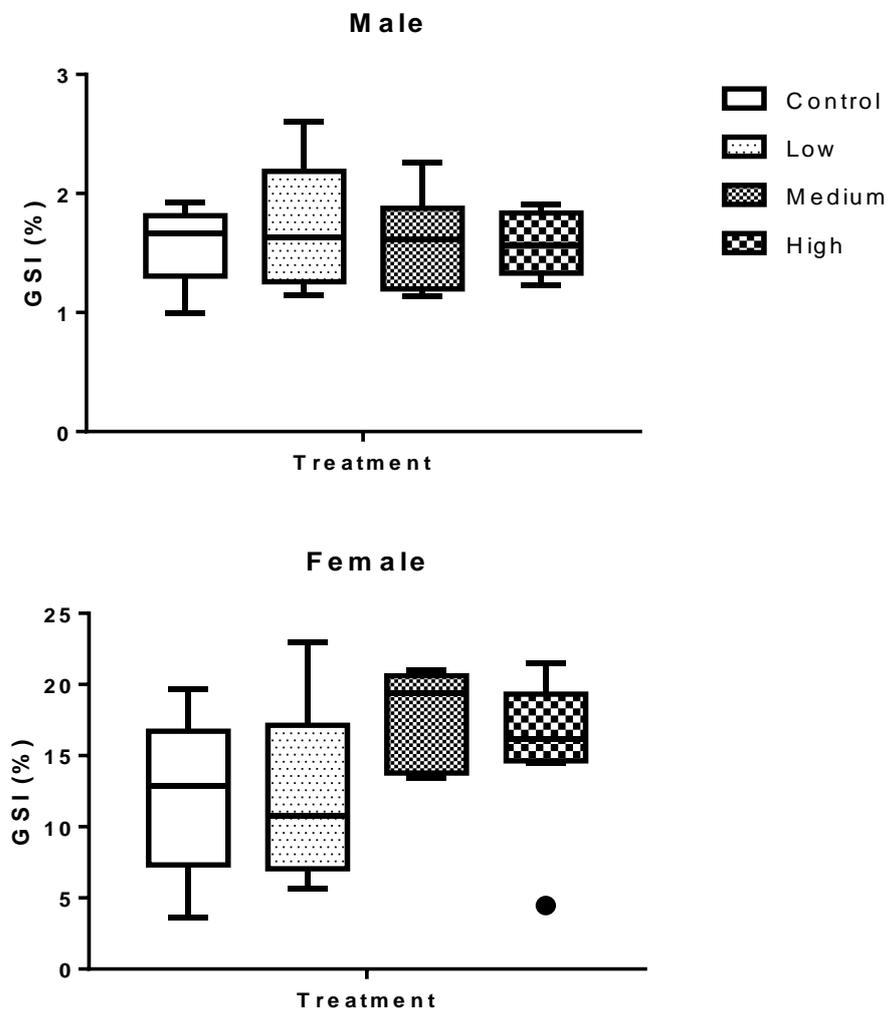


Figure. 32. Mean Gonadal Somatic Index of A. male and B. female fish, represented as box and whiskers plots showing the medium value, 25th and 75th percentiles (interquartile range), and the highest/lowest values (whiskers). Outlier represented by black dot. $n = 8$ for all treatment groups.

The Hepatic Somatic Index (HSI) of male fish was considered to be within the normal range (0.9-3.8). Data were found to be normally distributed (Shapiro-Wilks test, $p > 0.05$) and have homogeneity of variances (Levene's test, $p > 0.05$). Male HSI was unaffected by exposure to the steroidal mixture (fig. 33a) (one-way ANOVA, $f = 0.267$, $p = 0.85$). Female HSI was also within the normal expected range (1.4-10.9). Data were found to have a non-parametric distribution (Shapiro-Wilks test, $p < 0.000$, rejection of null hypothesis), and therefore a non-parametric Levene's test was used to assess for homogeneity of variance, whereby ranked data were analysed against mean ranked values for each group. Data were found to fail the assumption of homogeneity of variance, therefore accepting the null hypothesis of non-equal data deviations. Data were subsequently analysed by Mood's Median test, which showed that the HSI of females was found to decrease with exposure to the five compound steroid mixture (fig. 33b). However, this apparent effect was shown not to be statistically significant (Chi-Squared = 7.118, $df = 3$, $p = 0.068$).

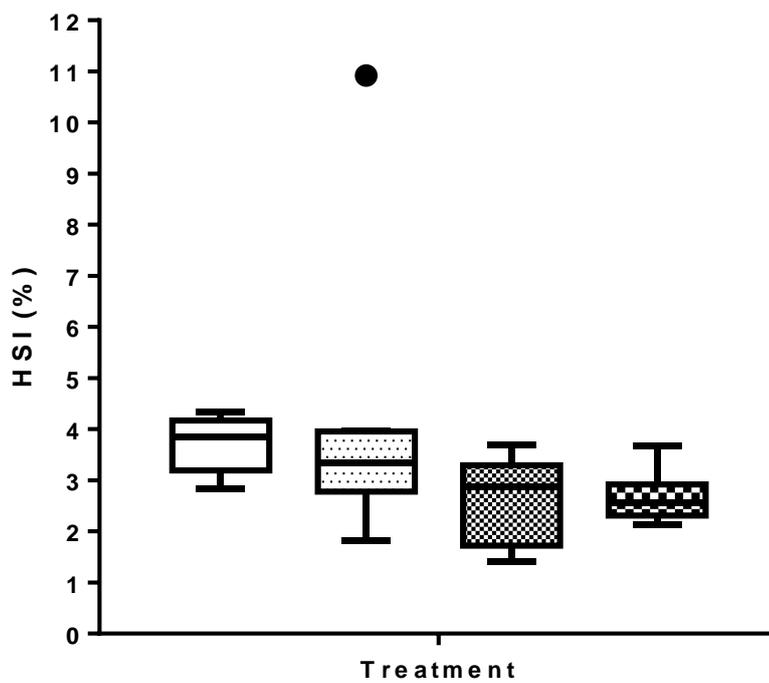
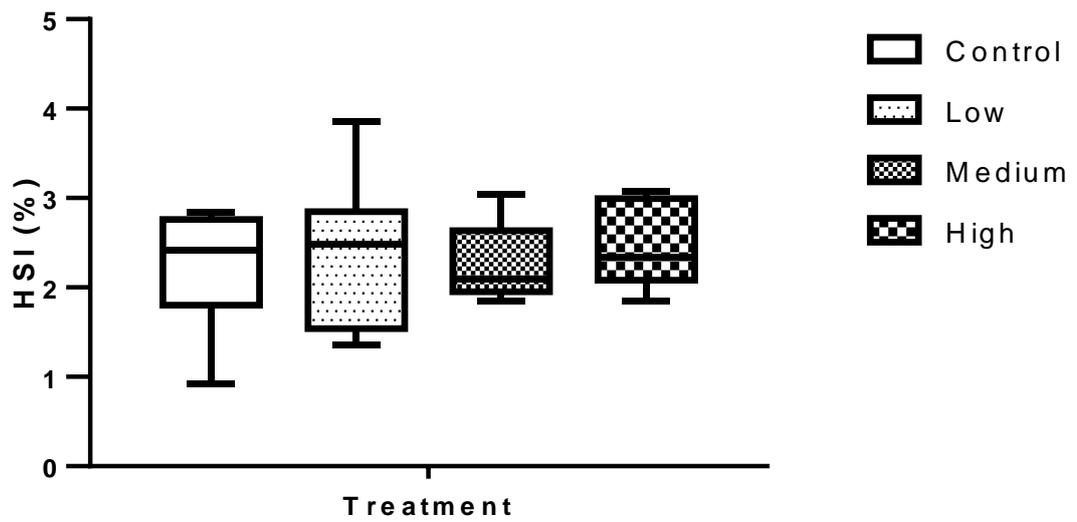


Figure. 33. Mean Hepatosomatic Index of A. male and B. female fish, represented as box and whiskers plot showing the medium value, 25th and 75th percentiles (interquartile range), and the highest/lowest values (whiskers). Outlier represented by black dot. n=8 for all treatment groups.

Ovipositor length in females varied slightly across all groups, from 1.6 to 2.2mm. Data were not normally distributed (Shapiro-Wilks test, $p < 0.00$), so a non-parametric Levene's test was used to assess equal distribution of variances. Data were found to have equal variances, satisfying the assumption of homogeneity, therefore a Kruskal-Wallis test was used to compare group mean ranks. Ovipositor length in females was not significantly different between any treatment group (Kruskal-Wallis test, Chi-Square = 3.448, $df=3$, $p=0.328$) (fig. 34). Furthermore, higher variation in ovipositor length was observed in fish in the lowest mixture concentration and the control group.

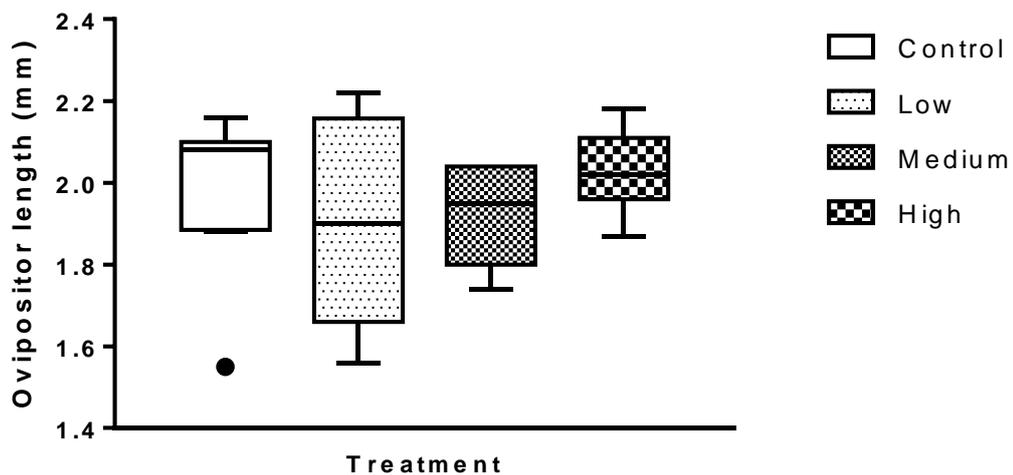


Figure. 34. Length (in mm) of the ovipositor of female fish exposed to a five compound steroidal mixture at study termination, represented as box and whiskers plot showing the median value, 25th and 75th percentiles (interquartile range), and the highest/lowest values (whiskers). Outlier represented by black dot. $n=8$ for all treatment groups.

4.3.3. Secondary sexual characteristics

The presence of nuptial tubercles in males was observed in all male fish across all treatment groups. The number of nuptial tubercles in male fish was normally distributed (Shapiro-Wilks test, $p > 0.05$), and found to have equal variances (Levene's test, $p > 0.05$). The number of tubercles in male fish was statistically similar across all treatment groups (fig. 35a), (one-way ANOVA, $f = 0.9$, $df = 31$, $p = 0.454$). Tubercle grade was also recorded in males, based on the shape and prominence of the tubercles. Tubercle grade data were found to not follow a normal Gaussian distribution, and were therefore analysed for equal variance using a non-parametric Levene's test. Data did not have equal variances ($f = 3.326$, $df = 3$, $p = 0.34$), therefore the Mood's Median test was used to compare medians across treatment groups. In contrast to tubercle number in male fish, tubercle grade was found to be statistically different across groups (Mood's Median test, Pearson's Chi-Square, 8.408 , $df = 3$, $p = 0.038$), decreasing at higher concentrations (fig. 35c). However, post hoc analysis revealed no statistically significant difference between any of the groups, and analysis of plotted standard errors revealed no statistical difference between treatments, suggesting that the Mood's Median Test is perhaps not a robust enough test for this data. To ensure more robustness when using this test, useful when applying an alpha level of $p < 0.005$

In females, the presence of tubercles was observed in the medium and highest treatment groups (fig. 35b). In the highest treatment group, tubercles were present and extremely prominent in all females (fig 36). Tubercle presence in females was not normally distributed (Shapiro-Wilks test, $p < 0.00$), and therefore a non-parametric Levene's test was used to test for homogeneity of variance. Data were found to have unequal variances (non-parametric Levene's test, $f = 6.847$, $p = 0.01$), and therefore a Mood's Median test was used to analyse the data. The number of tubercles in female fish was found to increase in a concentration-dependant manner (fig. 35b). The concentration-dependant increase in

tubercles was found to be statistically significant (Mood's Median test, Pearson's Chi-Square, 23.512, df = 3, $p < 0.000$).

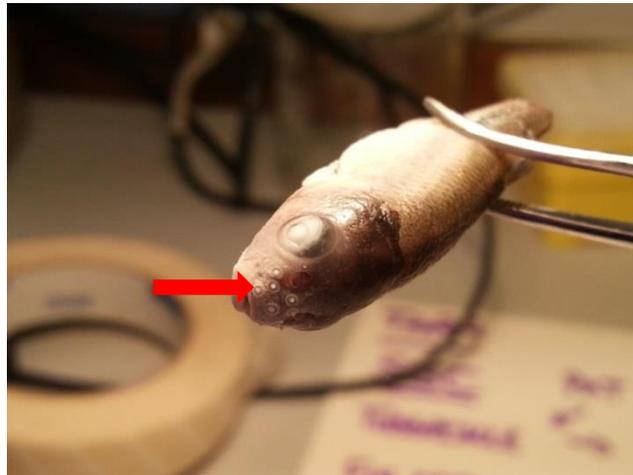


Fig.36. Tubercle formation (red arrow) in a female *P. promelas* exposed to the highest mixture concentration. Image taken after emersion in liquid nitrogen for ease of quantification of tubercle number and prominence.

Fatpads (fig. 37) were observed in all males in the study prior to the start of the experiment, and were present at termination of the experiment.

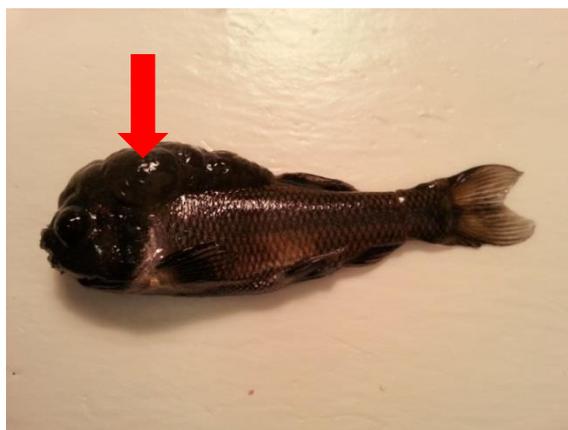


Fig. 37. Fatpad (red arrow) of a control male *P. promelas* taken at the end of the study period.

Since natural biological variability exists in fatpad size in males, the results are not deemed to be a sensitive indicator of exposure to steroids, whereas they are in female fish (where fatpads are not present under normal conditions). The height of the fatpad of male fish was not normally distributed (Shapiro-Wilks test, $p < 0.05$), and data were therefore analysed for homogeneity of variance using a non-parametric Levene's test. Data were found to have equal variance, and were subsequently analysed by Kruskal-Wallis. Fatpad height in males was not significantly different between any treatment groups (fig. 38a), (Pearson's Chi-Square statistic, 5.844, $df=3$, $p=0.119$). Fatpad weight of male fish was not normally distributed (Shapiro-Wilks test, $f=0.747$, $df=2$, $p < 0.000$), and was found to have equal variance (non-parametric Levene's test, $f=0.542$, $df=3$, $p=0.658$). A Kruskal-Wallis test revealed no significant difference in fatpad weight of male fish across treatment groups (Pearson's Chi-Square 1.724, $df=3$, $p=0.632$) (fig 38b). Fatpad grade was also assessed for male fish based on prominence. Average fatpad grade was highest in the control and the lowest treatment group, with a decrease in average grade at the medium and highest concentrations (fig. 38c). Data were analysed for normality by Shapiro-Wilks test and were found to be not normally distributed ($f=0.334$, $df=32$, $p < 0.000$). Data were subsequently analysed for homogeneity of variance using a non-parametric Levene's test, and found to have equal variances ($f=1.187$, $df=3$, $p=0.332$). Data were subsequently analysed using the Kruskal-Wallis test. Fatpad grade in male fish was found to be statistically similar across treatment groups (Pearson's Chi-Square 3.920, $df=3$, $p=0.270$).

In females, fatpad induction was observed in all females in the high treatment group, and in two out of eight females in the medium treatment group (fig. 38d). Fatpad grade (but not height or weight) was quantified, due to the difficulty in measuring height and weight of female fatpads. Fatpad grade in female fish was found to be not normally distributed (Shapiro-Wilks test, $p < 0.000$), and was therefore subsequently analysed for homogeneity of variance using a non-parametric Levene's test. Data were found to have unequal

variances, and were therefore analysed using the Mood's Median test. Fatpad grade in females was found to be statistically different across treatments (Pearson's Chi-Square=12.539, df=3, p=0.006), with a concentration dependant increase (fig. 38d). Post hoc analysis revealed a statistically significant difference in fatpad grade between the control and the highest concentration (Pearson's Chi Square=6.349, df=1, p<0.05), however fatpad grade between other treatment groups were not statistically different.

The presence and number of fin spots were quantified in female fish only, due to the natural presence of fin spots in males. Fin spots were present in all females in the medium and highest concentrations, and in all but one individual in the lowest concentration of the mixture (fig 39). Furthermore, two fin spots were observed in all but one individual in the medium and high concentrations, and in one individual in the low treatment group. Single fin spots were also, however, observed in two individuals in the control group. Fin spot data were not normally distributed (Shapiro-Wilks test, p<0.000), and had unequal variances (non-parametric Levene's test, f=19.089, df=3, p<0.000). A Mood's Median test was subsequently utilised to compare median values across treatment groups. There was a statistically significant increase in the number of fin spots with increasing concentration of the mixture (Mood's Median test, Chi-Square statistic, 19.597, df=3, p<0.000). Post hoc analysis revealed that the presence of fin spots was significantly higher in the medium and highest concentration groups relative to the control (Pearson's Chi Square=11.429, df=1, p≤0.001; and Pearson's Chi Square=12.444, df=1, p<0.001, respectively).

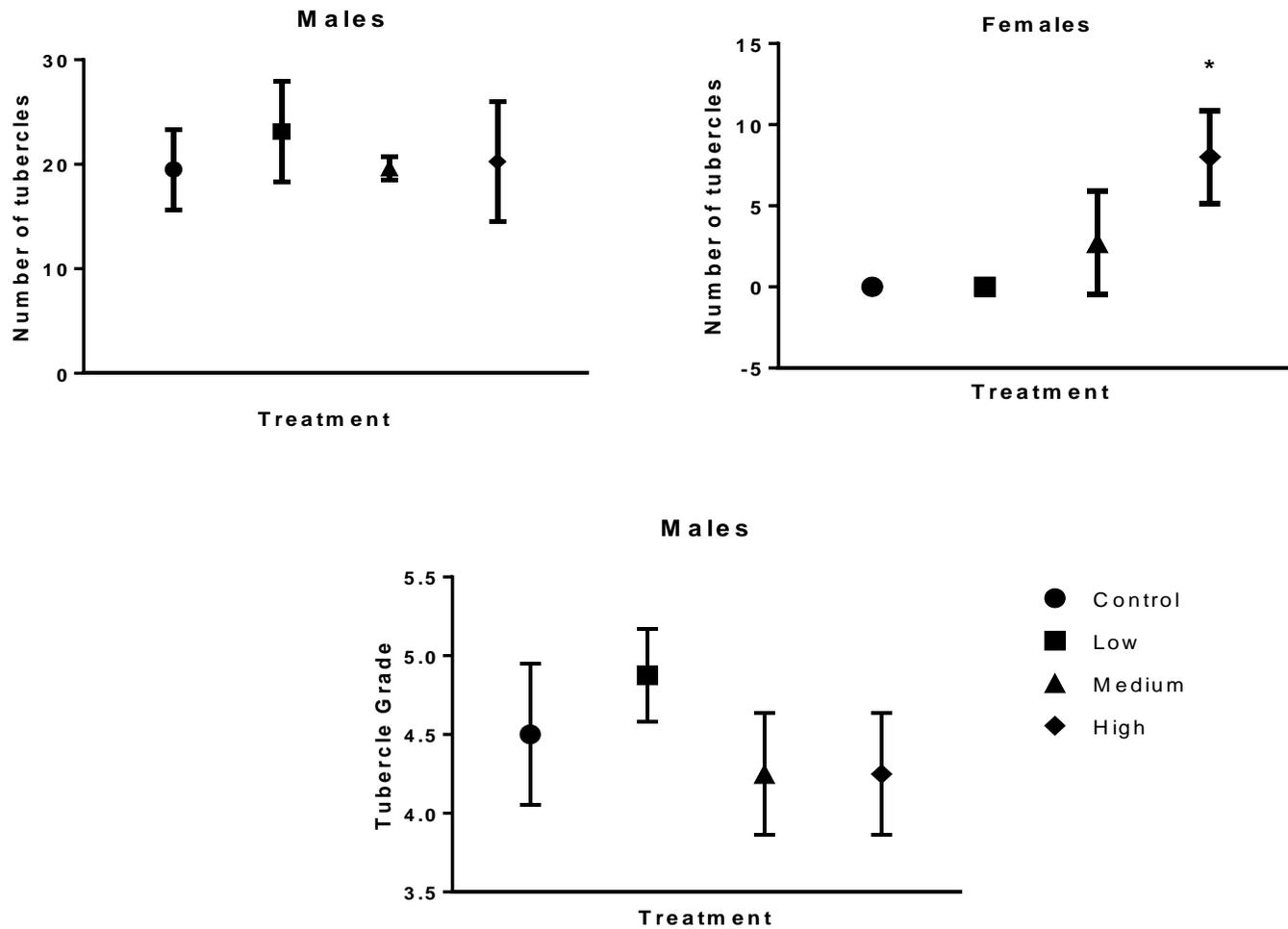


Fig. 35. Mean number of tubercles in A.) male and B). female *P. promelas* exposed to the steroidal mixture, and C). tubercle prominence grade in males. Error bars represent 95% confidence intervals. * denotes statistical significance. n=8 for all treatment groups.

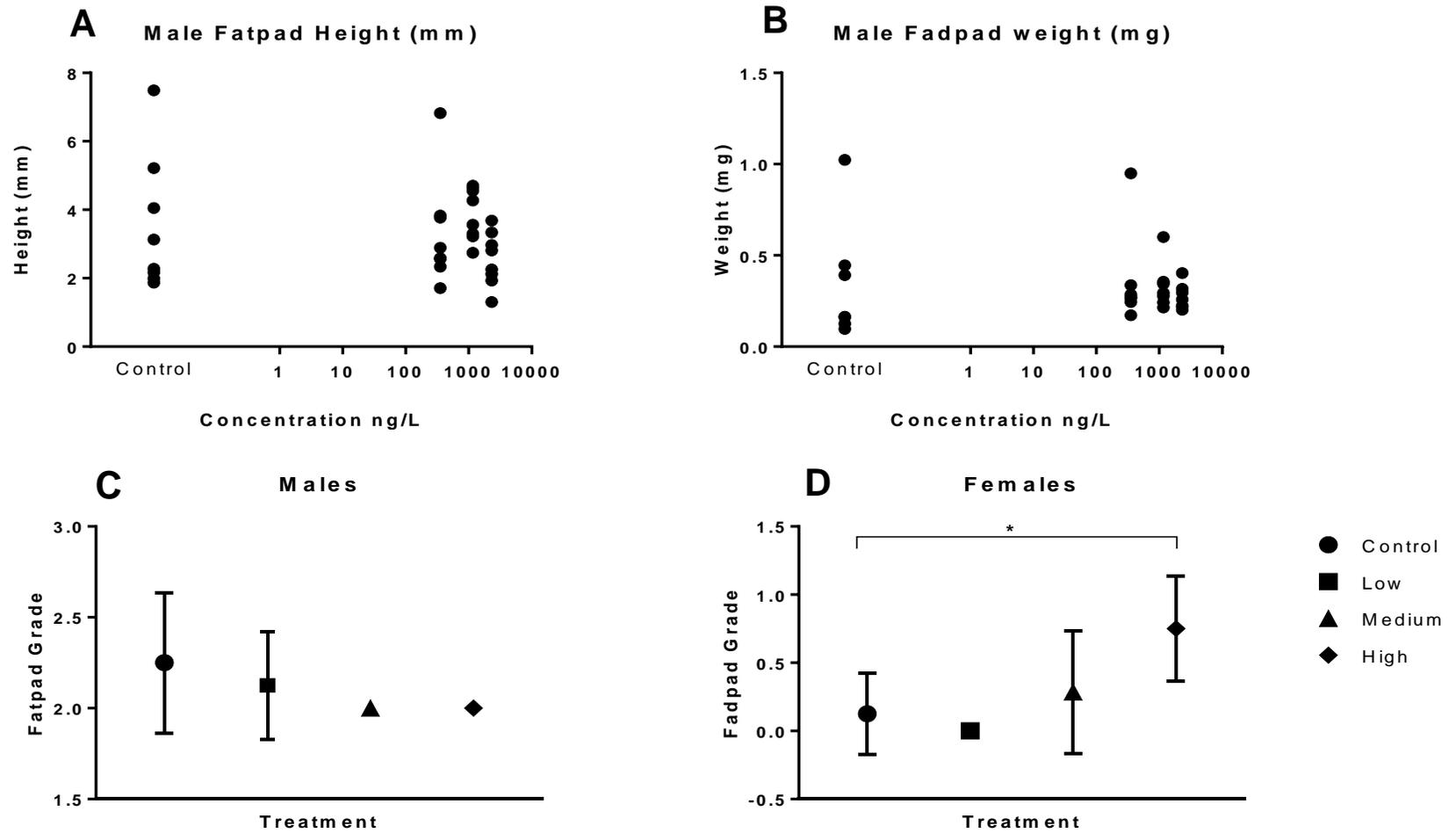


Figure. 38. Fatpad height A). and weight B). in male *P. promelas*, represented as individual data points on a logarithmic scale. Average fatpad grade in C). male and D). female *P. promelas* exposed to the steroid mixture. Graphs C and D; data presented as mean fatpad grade, error bars represent 95% confidence intervals. n=8 for each treatment group. * denotes statistical significance.

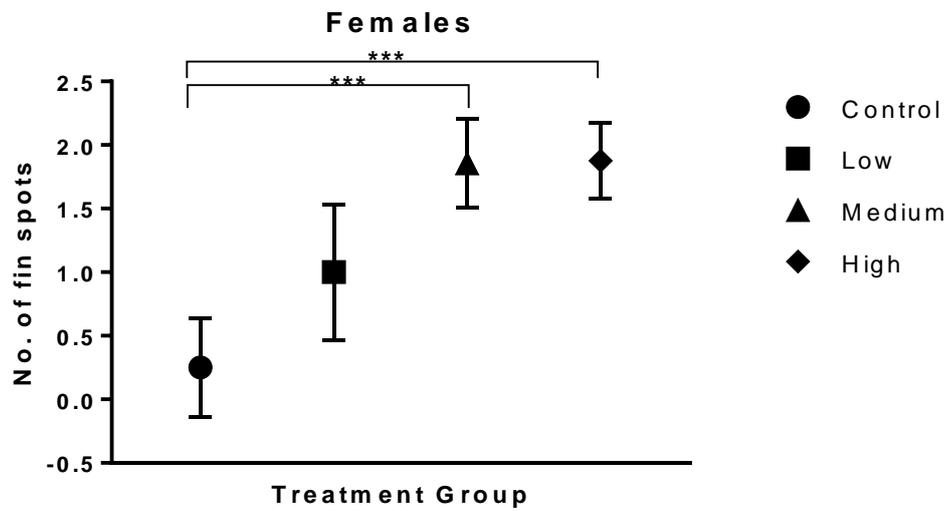


Figure 39. Mean number of fin spots observed on female fish exposed to a five steroid compound mixture, observed at study termination. The medium and highest mixture concentrations produced a statistically significant increase in the presence of fin spots. Error bars represent 95% confidence intervals. n=8 for each treatment group. * denotes statistical significance

4.3.4. Histopathological analysis of gonadal tissue

4.3.4.1. Male Testis

Initial analysis under a low magnification found no gonadal abnormalities in the testis of any male fish included in the study. The presence of intersex was not observed in any of the fish. All male testis appeared to be developing as healthy asynchronous testis (i.e. testis possessed a range of sperm cells in apparently healthy proportions). No degeneration or cell atresia was observed in the testis of any male fish.

Most testis were observed at the mid-to-mature sperm stages (fig's. 40 and 41).

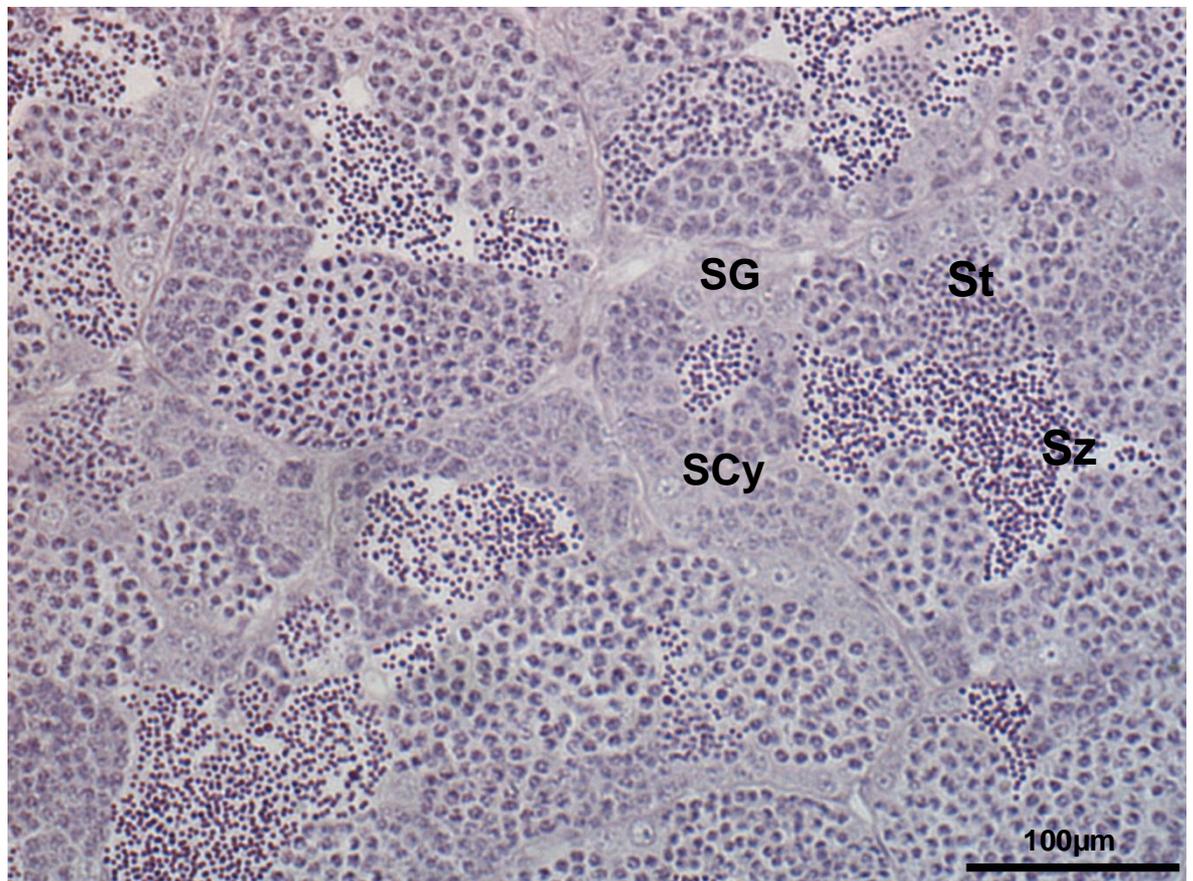


Figure. 40. A section of a healthy testis from a control male, showing the presence of spermatogonia (SG), spermatocytes (SCy), spermatid (St) and spermatozoa (Sz) at x40 magnification. (5µm sections, H&E staining). Scale bar = 100µm.

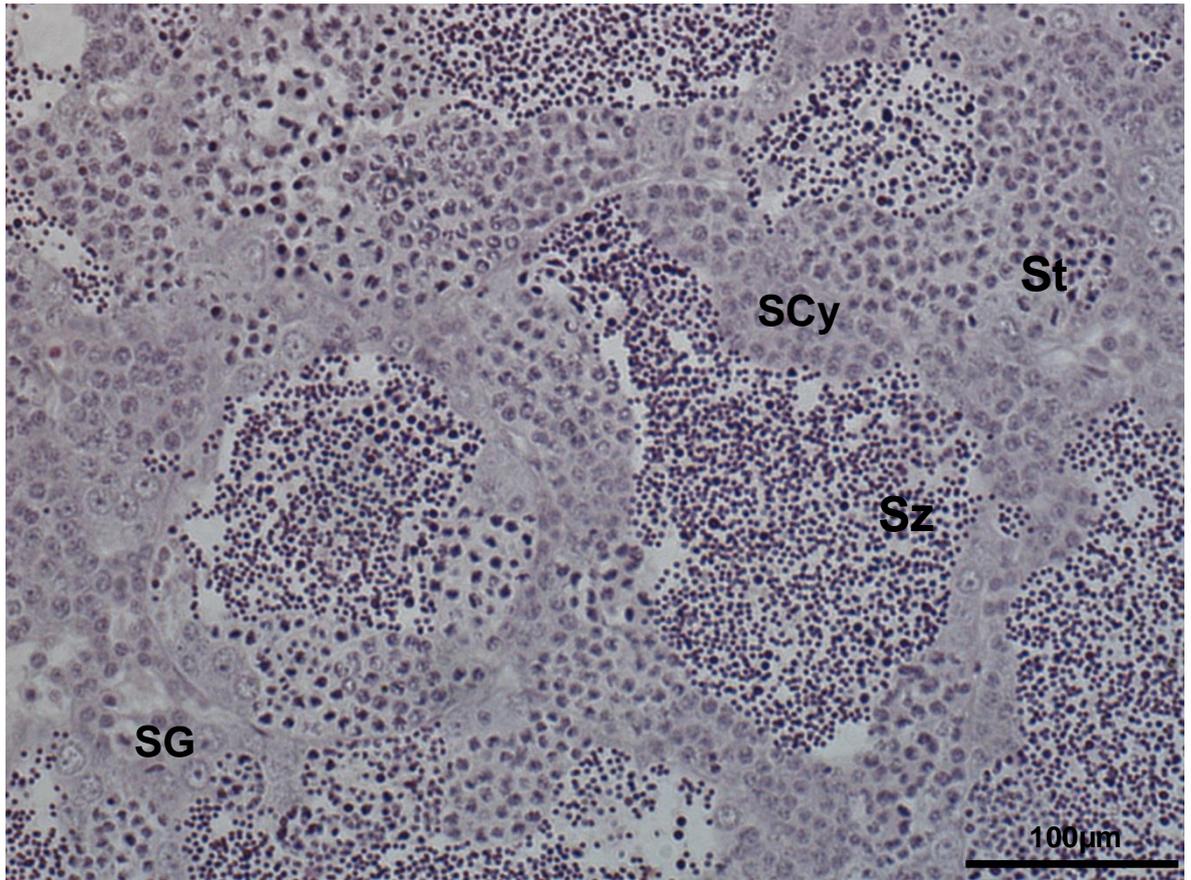


Figure. 41. A section of a testis at x40 magnification from a male *P. promelas* exposed to the highest concentration of a five compound steroidal mixture. Note the presence of all stages of sperm maturation. Scale bar = 100μm.

Sperm maturation stage in the testis was not normally distributed (Shapiro-Wilks test, 0.785, df=16, $p < 0.05$). Data were subsequently assessed for homogeneity of variance using a non-parametric Levene's test, and were found to have equal variance ($f = 0.226$, df=1, $p = 0.642$). A Kruskal-Wallis test was therefore utilised for comparison of the group means. Sperm maturation stage was found to be statistically unaffected by exposure to the five compound steroid mixture (fig. 42), (Pearson's Chi-Square, 0.529, df=1, $p = 0.467$).

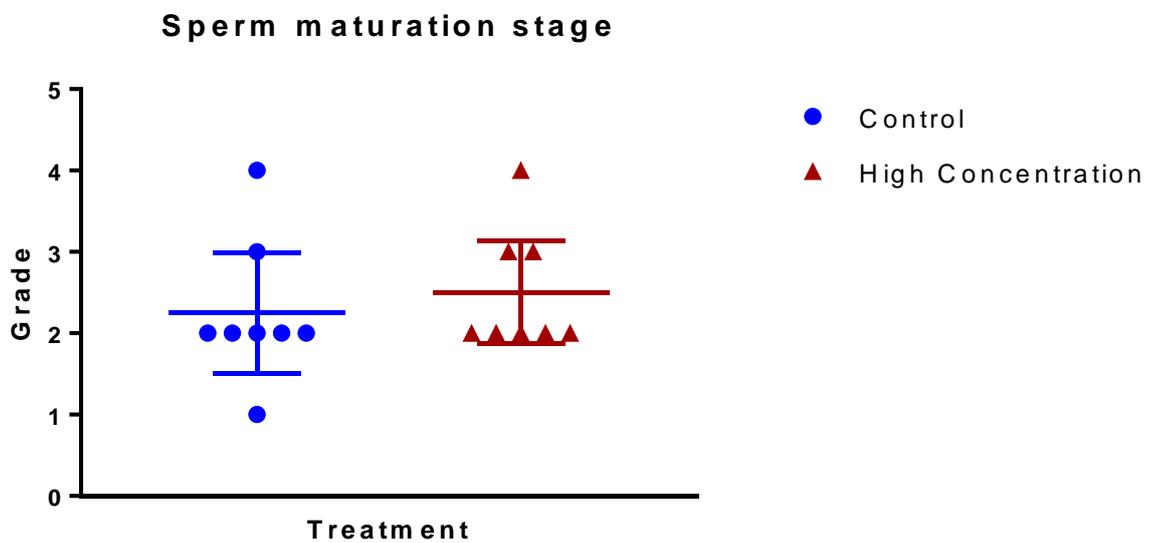


Figure. 42. Sperm maturation stage of male fish from the control and high mixture concentration treatments, as determined by comparison of sperm cell stages in the testis (based on the criteria developed by BEST (McDonald et al, 2002), and Wolf et al (2004), with some modification by the author (described in materials and methods section). Data are represented by plotting the individual values, horizontal lines represent the means, error bars represent 95% confidence intervals. $n = 8$ for both treatment groups.

4.3.4.2. Female Ovaries

Initial analysis under a low magnification found no gonadal abnormalities in the ovaries of any female fish included in the study. All female ovaries appeared to be healthy, with the

majority exhibiting all oocyte stages (fig 43 a & b). No degeneration or cell atresia was observed.

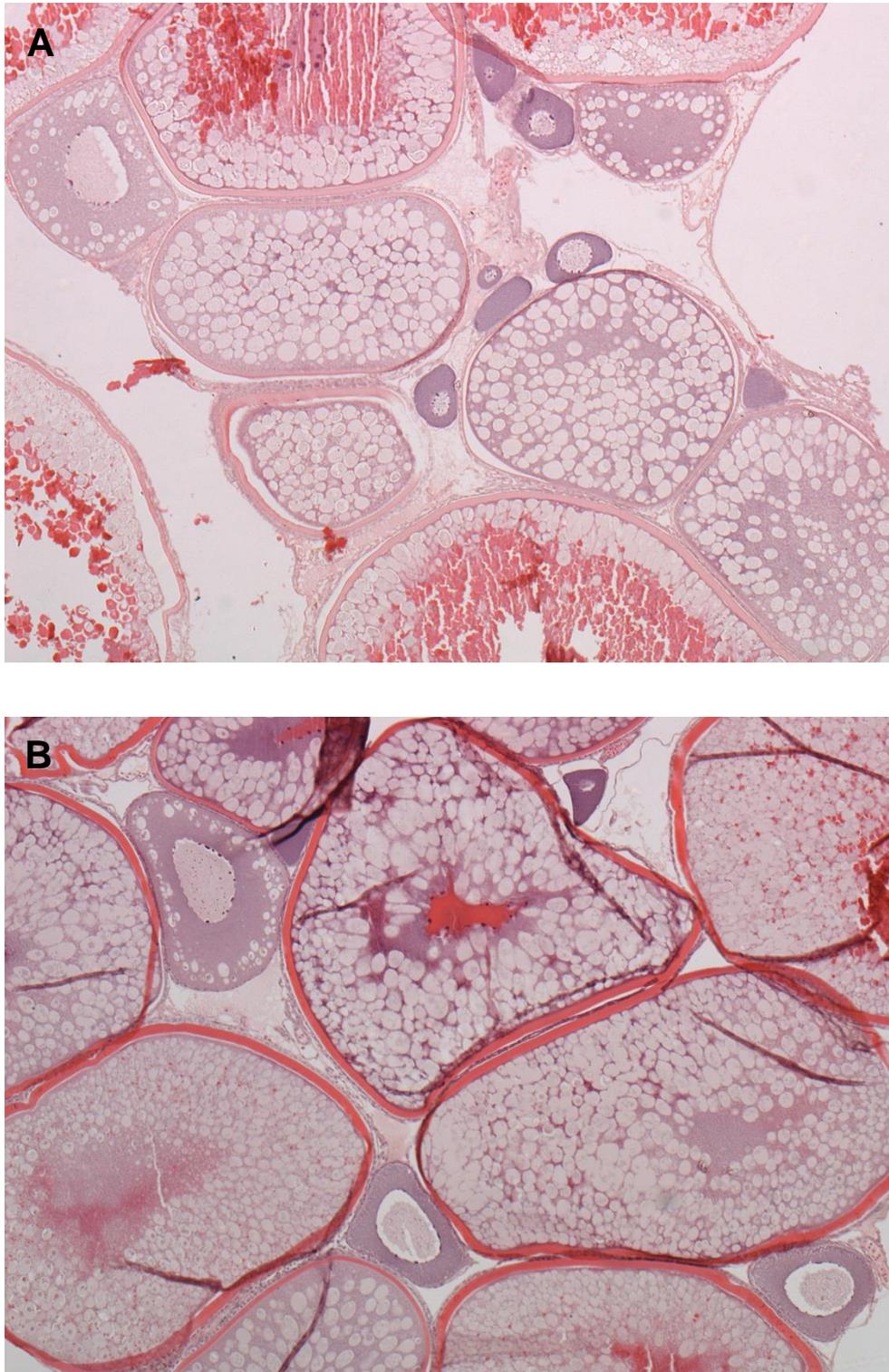


Figure. 43. Histological section from the ovary of A). a control female, and B). a female exposed to the highest concentration of the mixture. Note both sections exhibit a range of early and late stage developing oocytes. (5 μ m sections, H&E staining).

More mature (stage 3 and 4) oocytes appeared to be more prevalent in the highest concentration treatment. Data were found to not follow a Gaussian distribution (Shapiro-Wilks test, 0.817, $df=15$, $p<0.005$). Data were found to have equal variances (nonparametric Levene's test, $f=0.576$, $df=1$, $p=0.461$), and data were subsequently analysed using a Kruskal Wallis test. Despite the apparent trend in increasingly mature oocytes at the highest concentration, maturation stage of oocytes was not significantly different between the high concentration and the control groups (Chi-Square 1.563, $df=1$, $p=0.211$) (fig. 44).

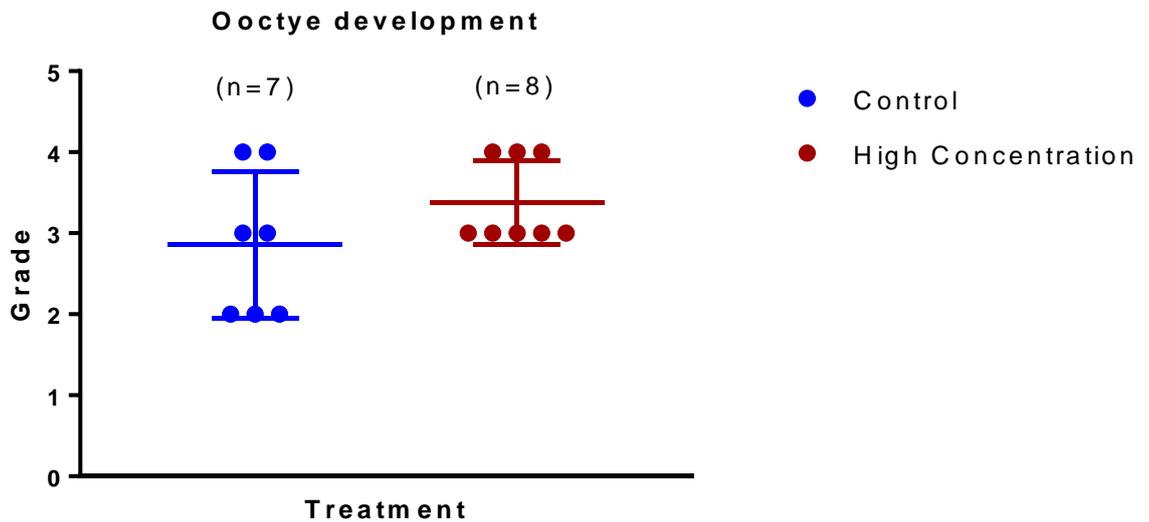


Figure. 44. Maturation stage of oocytes in female *P. promelas* exposed to a five compound steroid mixture, quantified at study termination. Data are represented by plotting the individual values, horizontal lines represent the means, error bars represent 95% confidence intervals. n=sample number.

4.3.5. Embryo hatchability study

Across the study exposure period (21 days), 21 batches of spawn and 1137 embryos were quantified. In the highest mixture treatment, only one and two spawning events were observed in the two selected fish pairs, since the mixture concentration in this group caused a complete cessation in egg production in all exposed fish after 5 days. Exposure to the multicomponent steroid mixture appeared to cause an increase in embryo death at both three and five days post fertilisation (dpf) (fig. 45 and fig. 46). At 5dpf, exposure to the mixture also appeared to lead to a decrease in hatching success of embryos (fig. 46). Although exposure to the mixture appeared to reduce hatching success at 5dpf, this was not significant (one-way ANOVA, $f=0.172$, $df=3$, $p=0.910$). Early hatching at 3dpf was not recorded in any of the treatment groups, with the exception of the lowest treatment group, where a single embryo across the entire study had hatched by day 3. Survival in the form of percentage of dead embryos was low at 3dpf, and appeared to decrease due to increasing exposure to the mixture, (fig. 45). However, the decrease in survival was not significant at 3dpf (Mood's Median test, Chi Square = 0.00, $df=3$, $p>0.05$) or 5dpf (Mood's Median test, Chi Square = 0.00, $df=3$, $p>0.05$). Minor abnormalities were observed on a small scale, in <1% of hatched larvae, and were therefore considered of low relevance.

In light of the findings presented, it is important to note that although the observations recorded in this study indicate no significant effects on embryo hatchability or embryo survival due to exposure to the steroid mixture, a small samples size ($n=2$) was utilised for each treatment groups due to time and feasibility limitations. Therefore, definitive conclusions as to the effect of the mixture on these endpoints cannot be drawn based on this study alone. A more comprehensive study, solely focusing on embryo hatching time and survivability, would be required to further strengthen the preliminary findings observed in the present study.

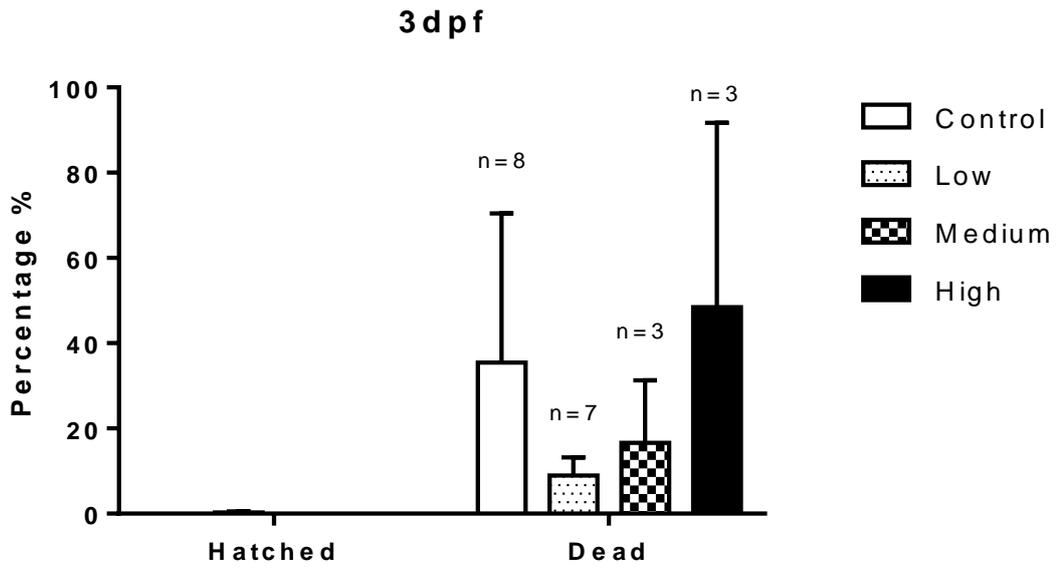


Figure 45. Percentage of embryos hatched and percentage dead embryos observed at 3dpf. Data presented as means with standard error (SEM), based on data collected from 2 breeding pairs per treatment group. n=sample size (number of batches per group).

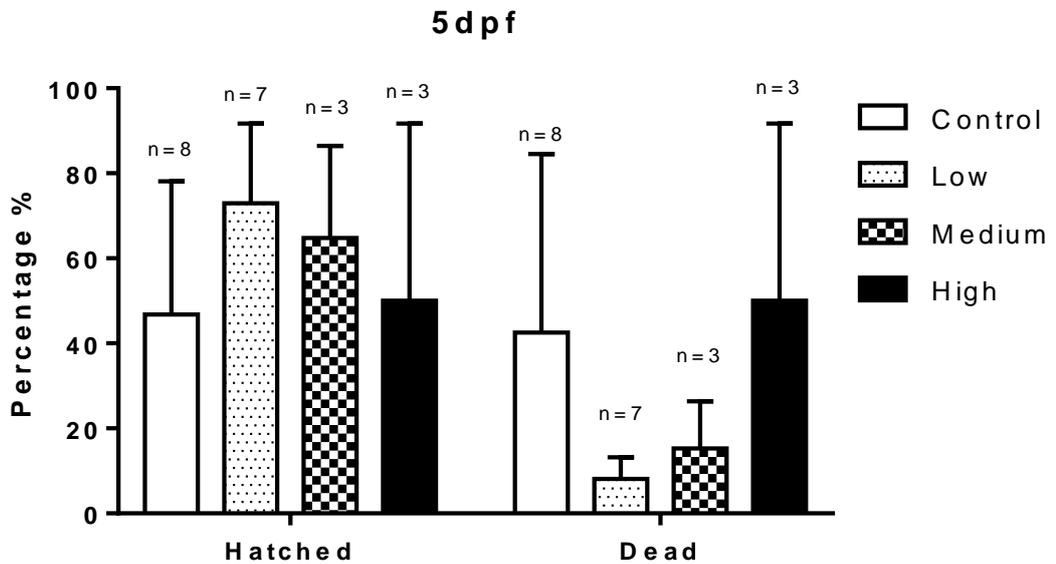


Figure 46. Percentage of embryos hatched and percentage dead embryos observed at 5dpf. Data presented as means with standard error (SEM), based on data collected from 2 breeding pairs per treatment group. n=sample size (number of batches per group).

4.4. Discussion

General physiological characteristics and condition of experimental fish were largely unaffected by exposure to the steroidal mixture. Length, weight, condition factor, and HSI were not affected by exposure. Some synthetic steroids such as the corticosteroids are associated with effects on growth and metabolism, and therefore have the potential to affect the weight of fish, and also weight of organs such as the liver and the gonads. Morphological characteristics have been shown to be affected by exposure to synthetic steroids elsewhere. Runnalls et al (2015) reported an increase in the length, weight, condition factor and abdominal girth of adult *P. promelas* exposed to the synthetic progestin levonorgestrel at a concentration of 25ng/L. However, the same study found that none of these parameters were affected by exposure to EE2 at concentrations up to 25ng/L. Since the concentrations of individual compounds used in this mixture study were relatively low, each compound acting individually may have been present at a concentration too low to cause an effect, particularly if each compound was acting via a different mechanism and pathway. Furthermore, the steroids used in the mixture study have individually been shown elsewhere to have opposing effects. Some of these compounds have been found to cause a measureable increase in these endpoints (Magiotta-Casaluci and Sumpter, 2011; Runnalls et al, 2015), others have been found to produce a decrease (Ankley et al, 2003), and others have been found to have no effect at all, at concentrations higher than those tested in the presented study (Runnalls et al, 2015). Androgens have been found to affect physiological characteristics in small fish models. Margiotta-Casaluci and Sumpter (2011) found that *P. promelas* exposed to concentrations as low as 20ng/L of the androgens KT and DHT caused a concentration-dependant increase in fork length and weight. In another study, Ankley et al (2003) found that exposure to 17 β -trenbolone caused a significant reduction in the weight of adult male *P. promelas*, whilst causing an increase in weight in females. However, similarly to other affected endpoints recorded in the literature, the concentrations required to induce these

changes were much higher than those investigated in the current study (50µg/L and 0.5µg/L, respectively).

The GSI and HSI indices have been shown to be useful indicators of exposure to endocrine disrupting compounds. Recent studies have reported similar findings to those presented here; for example, Jukosky et al (2008) found no effect on the GSI of the Japanese medaka *Oryzias latipes* exposed to mixtures of environmental estrogens. In contrast, some studies have reported significant decreases in GSI after exposure to estrogens (Kang et al, 2002; Tilton et al, 2005), although this was only found to be the case at higher concentrations of more than 400ng/L. Furthermore, Kugathas et al (2013) reported a decrease in the HSI of adult *P. promelas* exposed to the synthetic glucocorticoid beclomethasone dipropionate, however the concentrations used in that study were much higher than those of the presented study (in the µg/L range).

Ovipositor length in females was also unaffected by exposure to the mixture in the present study. In contrast to the results presented here, Kugathas et al (2013) reported a significant concentration-dependant decrease in ovipositor length in female *P. promelas* exposed to Beclomethasone dipropionate, however that study used higher concentrations than those presented here, and the results was only significant at concentrations of more than 1µg/L. Although many studies have demonstrated effects on ovipositor length at high concentrations, chronic exposures using low concentrations have also demonstrated effects. Parrott and Blunt (2005) exposed fertilised *P. promelas* embryos to environmentally relevant concentrations of EE2 and raised them to maturity. At 60dph, a significant increase in ovipositor index was observed at concentrations as low as 3.5ng/L. Although the concentrations used in the aforementioned study are within a similar range to those used in the present mixture study, the longer life cycle studies of the Parrott and Blunt study would likely induce a different profile of endocrine disruption than exposure of breeding adults only.

In review of the results from the current study and the evidence presented in the literature, it is reasonable to conclude that, although these compounds have the potential to induce effects on the endpoints discussed, the concentrations of the single compounds used in the present study are much lower than those that are required to initiate changes. These parameters are therefore not sensitive biomarkers of exposure to these compounds, particularly for exposure to environmentally relevant concentrations. Furthermore, in light of the evidence that some of the compounds in the mixture have been shown to affect these endpoints, whilst some do not, together with the significant reduction in fecundity of adult fish investigated in Chapter 3, the observed effects are most likely due to interactions between the compounds in the mixture, and cannot be aligned to specific chemical signatures.

4.4.1. Secondary sexual characteristics

Secondary sexual characteristics have been shown to be extremely sensitive biomarkers of exposure to steroidal compounds and steroid mimics. Exposure to environmental estrogens has also been shown to cause adverse effects in fish endocrine function. For example, Länge et al (2001) found that male *P.promelas* exposed to 4ng/L of EE2 during development resulted in male fish that developed no secondary sexual characteristics. However, whether the future reproductive capacity of the fish was affected was not within the remit of that study. Tubercle formation in female fathead minnows, and increased tubercle growth in males, has been shown to be a sensitive biomarker of masculinisation as a result of exposure to androgens, progestagens and anti-estrogens (Ankley et al, 2003; Collette et al, 2010; Margiotta-Casaluci and Sumpter, 2011; Kugathas et al, 2013, Runnalls et al, 2013, Runnalls et al, 2015). In the present study, tubercle formation in females was induced at relatively low concentrations (medium mixture treatment group, <1.2µg/L total mixture concentration). At the highest mixture concentration (2.3µg/L), tubercle growth was observed in all exposed females, with high prominence grades (grade 4). Runnalls et al (2015) reported a significant induction in tubercle appearance in

female *P. promelas* exposed to concentrations of 25ng/L of levonorgestrel, similar to the concentrations of levonorgestrel used in the present study. The same study found that exposures to EE2 alone at concentrations of up to 25ng/L had no stimulatory effects on tubercle growth in females, and significantly reduced tubercle number and prominence in males. Tubercle growth has been shown to be an extremely sensitive biomarker of exposure to environmental androgens and progestagens (Ankley et al, 2003). The results presented here reflect what is presented elsewhere in the literature, since very low concentrations of two progestagens and an androgen induced tubercle formation in females. The appearance of prominent male secondary sexual characteristics in female fish in the present study suggest that the steroid mixture may be causing reduced fecundity via masculinisation of females rather than the feminisation of male fish. Since the mixture composition involved a potent androgen, in addition to a glucocorticoid and two progestagens (that have been both been shown to possess androgenic properties), this is a reasonable assumption to conclude.

4.4.2. Histopathology

Histopathological analysis of the gonads revealed no significant effects on sperm and oocyte development stages in male and female fish, respectively. A number of earlier studies have revealed significant adverse effects of steroid compounds on fish gonad pathology. Intersex, the development of oocytes in male testis, has been widely demonstrated as a result of exposure to environmental estrogens (Jobling et al, 1998; Krisfalusi and Nagler, 2000; Vajda et al, 2008) and antiandrogens (Kiparissis et al, 2003; Green et al, 2015). However, concentrations required to initiate such gonadal changes are typically much higher than those used in the presented study. For example, concentrations used in a study by Kiparissis et al (2003), who investigated induction of intersex in male rainbow trout (*Oncorhynchus mykiss*) exposed to EE2, were much higher, in the range of 250µg/L. Similarly, in the present study, female gonads appeared normal, with oocytes of all stages observed across all treatment groups. Other studies reported

elsewhere in the literature have observed histopathological changes in the ovaries as a result of exposure to steroids at concentrations similar to those used in the present study (Weber et al, 2003; Zeilinger et al, 2009). For example, Weber et al (2003) reported a concentration-dependant suppression of the development of mature oocytes in zebrafish (*D. rerio*) exposed to EE2 at concentrations as low as 10ng/L, whilst Zeilinger et al (2009) reported large amounts of degenerating vitellogenic oocytes, a decrease in oogonia, and an increase in atretic follicles in fish exposed to 20ng/L and 100ng/L of the progestin levonorgestrel. The conflicting results between the study presented here and that of Weber et al (2003) and Zeilinger et al (2009) could be accounted for by the action of the other compounds in the mixture. Since the mixture included an estrogen, an androgen, and two progestagens, the effects of EE2 and levonorgestrel (individually) observed elsewhere could have been suppressed, or even neutralised, by the action of these additional compounds. Furthermore, since the mixture was composed of equipotent concentrations (based on fecundity effect concentrations) each compound was at a concentration enabling an effect, thus reducing the likelihood that higher potencies of some compounds are controlling the majority of the effect.

Despite the reported findings throughout the literature, the lack of changes observed histologically in this study could be attributed to variances in the exposure times, in particular the time between exposure and sampling. For example, in the Weber study, fish were exposed for much longer periods (60 days). Since morphological changes and tissue reorganisation in the gonads typically require longer time frames to become visually evident, it is likely that the tissue was sampled and fixed much earlier than physiological changes in the gonads would have manifested.

4.4.3. Hatching success and survival

Exposure to the steroidal mixture caused no significant difference in the hatching success of *P. promelas* embryos. Similar findings have been reported elsewhere (Ankley et al, 2003; Jukosky et al, 2014). Exposure at early development stages (from fertilisation to a few weeks post hatch) and multigenerational exposure have been shown to produce pronounced effects on embryonic development and hatching success. Sex steroids, and in particular maternal steroids, play an important role in early development of vertebrates, including teleost fish (Tanaka et al, 1995, McCormick et al, 1999), particularly at the early stages before the embryo begins to produce its own hormones (Hwang et al, 1992). Cortisol and testosterone have been shown to be particularly important during the early development stages. For example, in some species of birds and mammals, prenatal exposure to elevated levels of testosterone has been shown to affect growth rate, social status, aggressiveness, and reproductive success (Shwabl et al, 1993; Clark and Galef, 1995; Shwabl et al, 1996). It has been suggested that similar effects could occur in fishes, since reproduction and endocrine function is governed by similar hormone action. Indeed, in agreement with findings in other vertebrates, recent studies in fish have shown that exposure to elevated levels of steroids can affect fish. For example, McCormick et al (1999) reported that elevated levels of cortisol post-fertilisation resulted in smaller larvae of the tropical damselfish *Pomacentrus amboinensis*. The study also reported that elevated levels of testosterone resulted in increased yolk sac size and yolk utilisation rate.

Sexual differentiation in fish occurs after hatching, and is largely governed by steroid hormones. In a study by van Aerle et al (2002), newly fertilised *P. promelas* embryos were exposed to environmentally relevant concentrations of EE2 (10ng/L) for varying periods of time up to 20 days post hatch (dph). Exposure to EE2 was found to disrupt duct development, alter sex cell development patterns, reduce the number of spermatozoa, and induce vitellogenin synthesis in males, producing an overall 'feminisation' effect. 'Egg quality' is a key factor affecting the hatching success of developing embryos. Egg quality

(sometimes interchangeable with gamete quality) has been defined in terms of pre-hatching mortality rates, larval deformities, time of hatching and independent feeding, egg shape, transparency, and appearance of the zona pellucida, although there is no definitive consensus on what classifies as 'good quality'. Quality of spawned eggs varies naturally due to a variety of factors, including genetic factors, diet and condition, endocrine status of parental fish and the physiochemical conditions of the environment (Brooks et al, 1997).

Egg quality, in particular hatching success and time to hatch, can be significantly affected by exposure to steroids. Schubert et al, (2014) reported that exposure to estradiol (E2) caused premature hatching in brown trout (*Salmo trutta fario*), and significantly increased the amount of time taken from hatching initiation until completion. Similarly, Hashimoto et al (2009) reported that exposure of adult Japanese medaka (*Oryzias latipes*) to 60ng/L of EE2 caused a decrease in hatchability of spawned embryos. Tilton et al (2005) found that exposure of Japanese medaka to 500ng/L of EE2 had reduced hatching ability. It is also supposed that cortisol plays an important role in hatching ability and motion during early development (Wilson et al, 2015).

In the present study, the steroidal mixture was found to have no effect on hatching success or time to hatch. Similar findings have been reported from other studies investigating the effects of single compounds (Kang et al, 2002; Ankley et al, 2003; Jukosky et al, 2008). DeQuattro et al (2012) found no significant effects on hatching success in developing *P. promelas* embryos exposed to the natural progestogen progesterone (P4) up to concentrations of 1000ng/L. Jukosky et al (2008) reported no effect of estrogenic mixtures on the hatching success of Japanese medaka eggs. The findings of previously reported studies could explain the results of the present study. In studies where effects on hatching success and embryo survival were observed, much higher concentrations were tested. Since much lower concentrations of the individual compounds were used in this study, similar to concentrations used in other studies where no effects were observed, concentrations utilised in the presented study were probably

too low to induce an effect on the hatching success of embryos spawned from exposed parents.

It is important to note that in the presented study, one set of breeding pairs from the control group continued to produce eggs that had high mortality rates throughout the entire experiment. By 3dpf mean mortality rates were 68%, and by 5dpf over 85% were typically dead embryos. Since this was consistent throughout the experiment and not effected by exposure or environmental conditions, and given that the sample size for this supplementary study was low (n=2 breeding pairs), the results from the embryo hatchability study were undoubtedly effected by this pairs inability to produce viable eggs. Therefore, although the results suggest that exposure caused an increase in the amount of dead and unhatched embryos by 5dpf, no significant declines were noted due to the variability of the data surrounding this control pair, causing a large skewness of the data. Before any substantial conclusions could be drawn on the effect of the mixture of the survival and hatching success of embryos produced by exposed breeding pairs, this study should be repeated and most likely also expanded to include more pairs, in order to control for erroneous results such as those observed here.

4.4.4. The use of biomarkers in mixture studies

In line with similar findings from the literature, the results of the study presented here suggest that biomarkers can be a useful tool in elucidating mechanisms of exposure-mediated effects in *in vivo* studies. Although some of the additional biomarker endpoints quantified in this study exhibited no changes due to exposure (i.e. HSI, condition factor, GSI, ovipositor length, and gonadal histopathology), others proved sensitive biomarkers. Notably the androgen-regulated secondary sexual characteristics such as the induction of nuptial tubercles, dorsal fatpads and fin spots all proved as sensitive to the mixture as they do to androgenic compounds individually, and suggested that one of the main mechanisms behind reduced fecundity in pair-breeding fathead minnows was masculinisation of females due to the androgenic properties of the compounds in the

mixture. Although not within the remit of this project, it would be hugely beneficial to extend this research further to quantify a number of other biomarkers such as plasma VTG, plasma steroid levels (estradiol, testosterone and 11-KT), and additional markers of corticosteroid exposure such as leukocyte counts and mRNA PEPCK levels. Although the biomarkers investigated here suggest that androgenicity is largely driving reproductive failure in fish exposed to the five compound mixture, having additional endpoints that are specific to estrogen and corticosteroid exposure could further strengthen (or dispute) this theory.

4.5. Conclusion

In review of the results of the effect of the steroidal mixture on morphological biomarkers investigated as part of this chapter, key findings suggest that the mixture may be causing an inhibition of egg production in *P. promelas* via the androgenic properties of the compounds included. This conclusion is supported by the significant induction of male secondary sexual characteristics in female fish. However, the situation is likely to be more complicated, since the chemicals in the mixture may be having opposing effects on biomarkers at the biological levels presented here. Evidence from the literature has shown that the chemicals tested here in combination can have opposite effects when investigated individually, and therefore attributing the overall effect to specific compounds is inherently difficult. Furthermore, although the single chemical concentrations used in the mixture were sufficient to cause a substantial decrease in overall fecundity, they were much lower than those generally observed to cause effects on the biomarker endpoints investigated in this chapter, which may further explain the lack of significant effects observed on these endpoints.

Chapter 5: Effects of mixtures of steroidal pharmaceuticals with diverse mechanisms of action on the reproductive capacity of the Fathead minnow, *Pimephales promelas*.

Part 3: Analysis of gene expression changes in key pituitary genes involved in reproduction

5.1. Introduction

5.1.1. Gonadotropins in teleost fish

The effects of synthetic steroids on the reproductive system in fish have been well documented, particularly with respect to biochemical and histopathological endpoints. There is also growing evidence for the effects of steroids and steroid mimics on expression of key genes involved in reproduction and homeostasis (Filby et al, 2007; Cheshenko et al, 2008; Kroupova et al, 2014; Overturf et al, 2014). As discussed in detail in Chapter 3, Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) are the key hormones controlling reproduction in vertebrates, regulating gametogenesis and spermatogenesis. FSH largely controls the initiation of gametogenesis, steroidogenesis and the regulation of gonadal growth, whilst LH regulates gonadal maturation, as well as spermiation in males and ovulation in females. Gonadotropin secretion is controlled by gonadotropin-releasing hormone (GnRH) in the brain and complex steroid feedback mechanisms (Mateos et al, 2002). LH and FSH levels are crucial in regulating reproduction. For example, in a study by Zhang et al (2015), TALEN, a genome editing technique, was utilised to independently delete the FSH β and LH β genes in the zebrafish. LH β deficient females subsequently proved to be infertile, failing to spawn entirely.

Steroid hormones (including synthetic hormones) exert positive and/or negative feedback on neuroendocrine systems that control the synthesis and release of gonadotropins (Zohar et al, 2010). Synthetic steroids known to cause adverse effects to reproduction and endocrine function have been well documented (Jukosky et al, 2008; Sun et al, 2009; Zeilinger et al, 2009; LaLone et al, 2012; Runnalls et al, 2013), however specific mechanisms of disruption to the reproductive axis have been less well explored.

Since reproduction in teleost fish (like most vertebrates) is controlled largely by the HPG axis and regulated by a complex cycle of feedback mechanisms via the endocrine tissues with natural hormones as the messengers, synthetic steroids are extremely likely to effect

the HPG axis and cause dysregulation of the system. Since FSH and LH are key components in the regulation of reproduction and secondary sexual characteristics via this axis, and are largely regulated by feedback from gonadal steroids (Harding et al, 2016), their mRNA levels in the pituitary gland are likely to be affected by exposure to steroid compounds. In some higher vertebrates for example, estradiol has been shown to increase sensitivity to GnRH by increasing the synthesis and insertion of additional GnRH receptors (Nett et al, 2002) in the pituitary.

Although studies of the effects of estrogens and androgens on pituitary gonadotropin mRNA levels are relatively widespread, to the authors' knowledge, no studies have been conducted on the effects of synthetic glucocorticoids or progestins on FSH β and LH β expression. Furthermore, the effects of multicomponent mixtures of synthetic steroids on pituitary gonadotropin mRNA levels in fish *in vivo* have not been documented. The aim of the research presented in this chapter was to investigate further the mechanisms of reproductive disruption observed in breeding pairs of *P. promelas* exposed to a five-compound steroid mixture, to determine if disruption to reproductive output is initiated by changes at the level of the pituitary gonadotropins and the corresponding feedback mechanisms.

5.1.2. Transcriptomics uses in ecotoxicology - toxicogenomics

The use of transcriptomics, or gene expression profiling, has been successfully adopted in ecotoxicology in recent years. Since all toxic responses at phenotypic level are accompanied by changes to expression of certain (and in most cases several) genes, analysing genes that become actively up or down regulated due to exposure to a chemical can be a useful means of assessing changes that take place inside an organisms due to exposure. Furthermore, in most cases, gene expression changes are more sensitive, occurring at concentrations below those required to initiate a phenotypic response, and

therefore could offer an early warning prediction of likely phenotypic changes where exposure concentrations were higher (Daston, 2008). As a result, the field of toxicogenomics (the study of gene and protein activity in response to a substance in order to identify potential toxic risk), has emerged and is typically employed either in place of, or complementary to, traditional toxicological endpoints. Despite being only recently adopted in ecotoxicology, this approach has been used in human toxicology studies for much longer. Tools such as the Comparative Toxicogenomics Database (ctdbase.org) are well populated with information with regards to the effect of a wide range of compounds on a wide suite of genes relating to human health.

The use of gene expression profiling has been successfully used to study toxicological effects of a wide range of environmental pollutants on an increasing number of organisms. DNA-based array technologies such as microarrays, a chip that allows the analysis of the expression of thousands of genes simultaneously, have been successfully used in the application of ecotoxicological studies (Denslow et al, 2007), including synthetic steroids (Dorts et al, 2009; Katsiadaki et al, 2010). For example, DNA array technologies have been successfully used to examine the effects of environmental estrogens on hepatic mRNA transcripts of estrogen-responsive genes in male and female sticklebacks (*Gasterosteus aculeatus*) (Katsiadaki et al, 2010). Array technologies have the benefit of allowing high-throughput analysis of gene expression changes in thousands of genes simultaneously. The technology has also been used to define mechanisms of toxicity in compounds where nothing is known about the MOA causing toxic effects (Neumann and Galvez, 2002).

Sequencing of the genomes of model species used in aquatic ecotoxicology such as the zebrafish (*Danio rerio*) the Japanese pufferfish (*Takifugu rubripes*), and the three-spined stickleback (*Gasterosteus aculeatus*), and the available genomic information in many other species including the medaka (*Oryzias latipes*) and the fathead minnow (*Pimephales promelas*), has further facilitated the usefulness of genomic studies in

environmental toxicology. To date, full or partial genome information is available for 80 fish species (<http://www.ncbi.nlm.nih.gov/genome/browse/>). There are many benefits in applying these technologies to ecotoxicological studies. They are typically rapid and data rich, for example, microarrays generate information on thousands of genes simultaneously. Furthermore, toxicogenomic technologies are particularly useful when addressing the toxicity of mixtures, due in large to the unfeasible nature of testing all compounds individually on whole organism endpoints. The use of real-time Polymerase Chain Reaction (qPCR) has also become widespread in ecotoxicology, enabling simple targeted transcript expression analysis of genes or gene clusters from small starting amounts of DNA, when changes to specific genes in particular tissues are the focus of investigation.

5.1.3. Principles of PCR

Traditional polymerase chain reaction (PCR) is a molecular technique used for a wide array of purposes. It is the process of amplifying DNA from a small starting amount to an amount sufficient for quantification, using a reaction mixture and a series of heating and cooling steps. PCR can be applied, for example, to verify the presence and abundance of a particular species in a sample, such as the presence of a pathogen in a human blood sample to indicate infection. PCR has also been widely used to study how factors, both internal and external, effect the expression of certain genes. For this application, total RNA is extracted from a sample (rather than DNA), since the focus of interest is only in the DNA that is being actively transcribed from messenger RNA (mRNA) at that moment in time. Since PCR amplifies DNA only, total RNA needs to be reverse transcribed into DNA before PCR. This is typically undertaken using reverse transcriptase, an enzyme isolated from a retrovirus that is used to transcribe its own RNA into a host cell DNA, and is now routinely and commercially used in gene transcription studies to produce DNA from mRNA. Reverse transcriptase transcribes RNA into single stranded DNA (ssDNA), otherwise known as complimentary DNA (cDNA). This is undertaken in a reaction using a

thermocycler, following a series of heating and cooling steps. The RNA is then degraded with the enzyme ribonuclease (RNase), and removed from the sample to leave only the newly produced cDNA.

DNA, or cDNA in gene transcription studies, is amplified by PCR in reactions with the aid of a number of components. Where a specific DNA sequence is the target, for example in identifying the presence of a particular species in a sample, or quantifying gene expression, sequence-specific oligonucleotide primers are used to amplify only the target region in question. Primers are identifiers of specific sections of the DNA, such as a gene sequence. They are short sequences of nucleotides that are complementary (i.e. they contain the complementary base pair) to a section of the gene sequence in question. During the reaction, the primer locates its complementary sequence, anneals to it, and is then able to begin the process of DNA 'copying' to form dsDNA. The newly formed dsDNA is denatured using heat and each strand is used in a subsequent cycle as a template to produce a new strand of dsDNA. Primers are utilised in pairs, the forward primer and the reverse primer, each with a complementary sequence to the 5' and 3' end of the sequence respectively. Primer design is crucial in successful amplification of the target sequence. They should be 16-30 nucleotides in length, contain around 50% GT content and approximately equal numbers of each nucleotide, have no repetitive sequences, and have similar melting temperature to its paired primer (McPherson and Møller, 2006).

The PCR reaction also requires deoxyribose nucleoside triphosphates (dNTPs) (the building blocks of nucleic acids), used during the process to form the new DNA strands, thermostable DNA polymerase (a naturally occurring enzyme that synthesises DNA, modified to increase stability under high temperatures), and specific thermocycling conditions. Thermocycling involves a series of heating and cooling steps, repeated in cycles to achieve the following processes:

- DNA denaturation – DNA is heated at a high temperature (usually 95°C) until the double strands separate to single strands
- Primer annealing – reaction is cooled to a specific temperature ($T_m - 5^\circ\text{C}$) to allow oligonucleotide primers to anneal to the complementary sequence on the forward and reverse strands
- Extension – Heated to optimum temperature for extension of DNA sequence by DNA polymerase activity (typically around 70°C).

The process is repeated for n number of cycles, but typically a cycle number of between 30 and 40 is used. With each repeated cycle, the amount of DNA doubles (fig. 47). In general, after 40 cycles, the reaction reaches a critical level whereby the amount of dNTP's, primers and DNA polymerase has been depleted to levels such that exponential increase is not possible, and this therefore limits the amplification process.

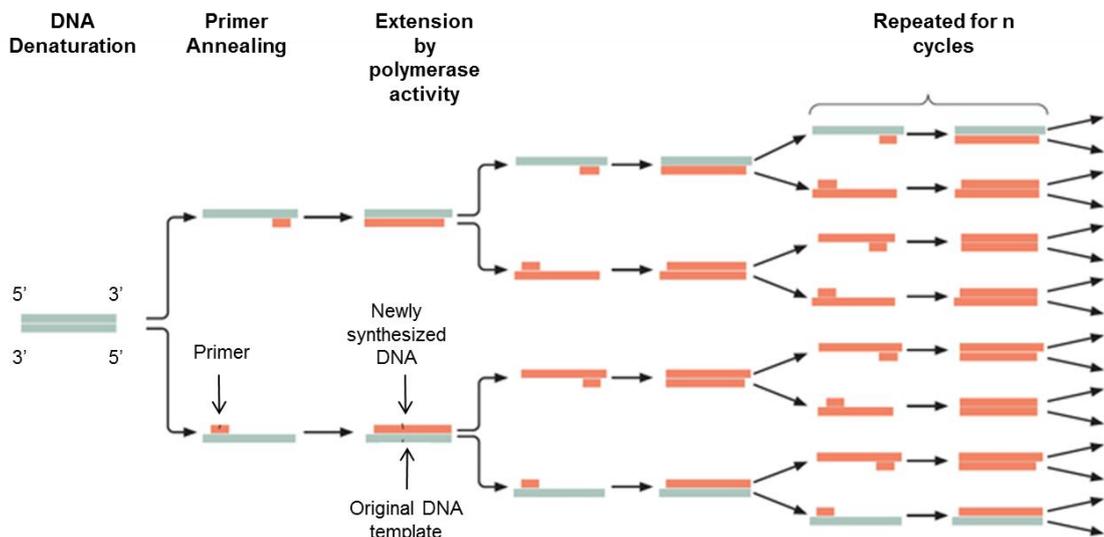


Figure. 47. The process of amplifying small segments of DNA by polymerase chain reaction (PCR). Modified from 2014 *Nature Education* Adapted from Pierce, Benjamin. *Genetics: A Conceptual Approach*, 5th ed., obtained: <http://www.nature.com/scitable/topicpage/the-biotechnology-revolution-pcr-and-the-use-553>

In quantitative real-time PCR (qPCR), a fluorescent dye is used in the reaction for quantification of amplified material. SYBR Green is a green fluorescent dye that binds to the minor groove of dsDNA and produces an amplification signal that can be read by inbuilt fluorescent detection in qPCR thermocyclers. The signal is read at the end of every cycle in real-time, and an amplification plot is produced. The plot is typically sigmoidal in shape. During the initial stage, the baseline phase, the concentration of template DNA is still too low to be detected and only background signal is observed. During the second, exponential phase, the detection threshold is reached and a period of exponential increase is achieved. The third and final stage represents a plateau due to the depletion of the reaction components (information obtained from Sigma-Aldrich product sheet). The cycle threshold (Ct), the cycle when the exponential phase begins, is used for relative comparison between samples to allow for quantification. For example, where the Ct value is lower, less cycles were required to reach the threshold, and therefore the more target cDNA (and therefore mRNA) there was in the starting material.

5.1.4. Reference/Housekeeping genes

In qPCR, analysis of changes in gene transcripts are referenced to 'housekeeping' genes, which are used for normalisation of the gene of interest, to account for variations in the efficiency of the quantification method (i.e. the qPCR assay) (Guénin et al, 2009). Housekeeping genes are stably expressed genes, meaning they are typically expressed at a consistent level in all tissues at all times. Normalisation of the gene of interest to a stably expressed reference gene is crucial, and is typically undertaken using mathematical algorithms. Normalisation therefore accounts for variability and affords reliable comparisons across samples.

5.1.5. FSH and LH expression changes in response to steroid hormones – Evidence from the literature

As previously discussed, pituitary gonadotropins are regulated by feedback from sex steroids, and therefore their levels are likely to change due to exposure to synthetic steroids, such as the pharmaceuticals tested in the presented study. Previous studies have documented evidence supporting this. For example, Harding et al (2013) conducted a study to determine the effects of environmentally relevant concentrations of EE2 using a transcriptome wide gene expression analysis of the pituitary gland (focusing on FSH and LH, and a suite of other genes involved in reproduction) in a teleost fish, the Coho salmon. LH β and FSH β were found to be the most highly up and down regulated transcripts as a result of exposure to EE2. At both the 1 and 6 week exposure time points analysed, LH β expression was significantly upregulated due to exposure of EE2. At the 6 week time point, FSH β was significantly downregulated in the fish. LH β was found to be the most significantly altered transcript, suggesting its high sensitivity to estrogen exposure. These findings are consistent with reports from other teleost species. Although there is some dependence on maturity, species and gender, typically high levels of natural and synthetic steroid hormones (estrogens and androgens) result in high levels of expression of LH β , and depressed levels of FSH β (Borg et al, 1998; Yaron et al, 2003).

Little is known about the effects of synthetic progestins on gonadotropin secretion and regulation. Studies have demonstrated adverse effects on reproduction in fish exposed to synthetic progestins (Zeilinger et al, 2009; Runnalls et al 2013), which produce an androgenic effect in both sexes, suggesting a masculinisation of females leading to reproductive impairment. Many synthetic progestins are known to possess androgenic activity, both *in vitro* and *in vivo*, therefore it can be assumed that such compounds will exert an effect similar to that of androgenic compounds on regulation of gonadotropin subunits. Nothing is known about the effects of synthetic glucocorticoids on the regulation of gonadotropins at the level of the pituitary gland.

Given that the reproductive strategies of fish vary dramatically, the regulation and control of pituitary gonadotropins vary across species. Many studies investigating the control and feedback of gonadotropins have focused on economically important species, such as the salmonids, since manipulation of reproduction can be of huge benefit for aquaculture industries (Borg et al, 1999; Dicky and Swanson, 2000; Ando et al, 2003) However, as previously discussed, regulation of gonadotropins can be very different in other fish. In the fathead minnow, the regulation of pituitary gonadotropins is not thoroughly understood, and the molecular endocrinology of this species is not as well understood as many other commonly used fish models (Villeneuve et al, 2007). However recent studies have become to focus attention on this issue.

Research has shown that changes to transcriptional expression of gonadotropins (particularly FSH) in some teleost species, such as the fathead minnow, can vary with reproductive phase, and feedback from steroids can be either positive or negative, depending on reproductive timing. Given that the fathead minnow is an asynchronous spawner, without a specific timed seasonal spawning event, variation in expression of gonadotropins due to reproductive phase can be largely controlled for. With the pair-breeding assay, fish pairs spawn continuously under laboratory conditions every 3-5 days, and therefore remain in a reproductively active phase. With exposure to a mixture of different synthetic steroids, the effects on the expression of gonadotropins LH and FSH may reflect the pattern of expression of any of the single compounds. However, the expression changes are most likely to be more complex and difficult to predict, being regulated by feedback on the brain-pituitary-gonadal axis after exposed to five dissimilarly (and similarly) acting steroid compounds.

5.1.6. Aims and Objectives

The experiment presented in this chapter served as a continuation of that conducted and described in previous chapters (3 and 4), with the overall aim of assessing expression changes in key pituitary genes involved in regulating reproduction to further understand the effects of a mixture of steroidal pharmaceuticals on reproductive and endocrine function in a small fish model. During a second experiment, breeding pairs of *P. promelas* were exposed to two concentrations of the mixture as described in Experiment 1, to analyse effects on the expression of gonadotropins at the level of the pituitary gland, in order to facilitate further understanding of the mechanistic effects of the mixture on reproductive impairment. Pituitary expression of follicle stimulating hormone (FSH) and luteinizing hormone (LH) were investigated, along with three housekeeping genes; 18S ribosomal RNA (18S), 60S ribosomal protein L8 (rpl8), and tata box binding protein (tbp), genes considered to be unaffected by exposure to the compounds in the mixture. Expression of pituitary FSH and LH was subsequently linked to fecundity of individual breeding pairs to determine the mechanisms of decreased reproductive output, and the variability between individual pairs. The mixture experiment designed and discussed in chapter's 3 and 4 was repeated using the low and medium mixture concentrations. The highest mixture treatment was omitted from this study due to its high potency on egg production. Since egg production in the highest concentration group completely ceased in all breeding pairs after 7 days, expression of key reproductive genes were assumed to provide limited information with regards to mechanisms of decreased fecundity. Furthermore, an additional aim of the study presented in this chapter was to serve as an assessment of the repeatability of the assay based on reproductive performance of breeding pairs, to further validate and strengthen the results presented in earlier chapters.

5.1.7.1. Hypotheses

1. There will be a significant effect on pituitary gland expression of gonadotropin subunits LH β and FSH β genes following a 21-day exposure to a mixture of five dissimilar synthetic steroids.
 - a). There will be an overall increase in the expression of LH β at the level of the pituitary following exposure to a mixture of five steroid pharmaceuticals.
 - b). There will be an overall decrease in the expression of FSH β at the level of the pituitary following exposure to a mixture of five steroid pharmaceuticals.

2. Expressional changes will follow a dose-dependent relationship.

5.1.7.2. Null hypothesis

1. There will be no changes in pituitary expression on gonadotropin subunits LH β and FSH β following a 21-day exposure to a mixture of dissimilar synthetic steroids.
2. Observed changes in pituitary LH β and FSH β will not be dose-dependant.

5.2. Materials and Methods

A reproductive assay was conducted according to the methods described in chapter 3. To allow for assessment of experimental repeatability, and in order to align transcriptomic changes with changes in reproductive performance, mixture ratios and concentrations were identical to those described in chapter 3 (experiment 1). Two mixture concentrations (out of three) were selected for inclusion in the study investigating changes in pituitary gonadotropin expression upon exposure to the five compound steroid mixture (table 23). The low and medium concentrations were selected due to the fact that egg production was reduced to a large enough extent to be significant. However, there was a degree of variability in the response between breeding pairs within in each group that could afford less predictable transcriptomic responses (i.e. some breeding pairs were still producing eggs, albeit at much lower frequencies and in lower numbers). The highest mixture concentration was excluded from the second experiment since all pairs had ceased breeding by day 7 of exposure to the mixture, suggesting a more predicable response in the expression profiles of pituitary FSH β and LH β .

Table 23. Composition of the three mixture concentrations. Mixture ratios remain consistent at an equipotent concentration at each compound's EC10.

Pharmaceutical Compound	Mixture Composition ng/L	
	Low Mix	Medium Mix
EE2	0.16	0.52
Levonorgestrel	0.28	0.93
Beclomethasone dipropionate	19.24	63.49
Trenbolone	31.62	104.35
Desogestrel	299.98	989.93
Total	251.28	1159.22

5.2.1. Experimental Conditions

Experimental conditions have been described in detail in chapter's 3 and 4, and therefore will not be discussed in detail here. Experiment 2 was conducted under the same conditions as Experiment 1 with some modifications, as discussed below.

Aqueous stock solutions of EE2 (Sigma-Aldrich, UK. CAS: 57-63-6, purity, $\geq 98\%$), Levonorgestrel (Sigma-Aldrich, UK. CAS: 797-63-7, purity, $\geq 99\%$), Desogestrel (Sigma-Aldrich, UK. CAS: 54024-22-5, purity, analytical standard), Trenbolone (Sigma-Aldrich, UK. CAS: 10161-33-8, purity, $\geq 93\%$), Beclomethasone dipropionate (Sigma-Aldrich, UK. CAS: 5534—09-8, purity, $\geq 99\%$) were prepared weekly using 2.5L Winchester amber glass bottles and double distilled water. Dosing stock solutions were made at 5000 times concentrate to achieve desired tank concentrations (pump rate 0.2ml/min; flow rate 60L/hour). Master stocks were made up in ethanol and stored at 4°C throughout the experimental period. Master stock concentrations were made up for the medium mixture concentration, and diluted to make the lowest concentration (appendix 1). Master stock, dosing stocks and flow rates were as described in chapter 3. The same master stocks were used throughout the experiment to make up all corresponding dosing stocks, with the aim of reducing variability. Stocks were made up so that ethanol concentrations in the experimental tanks were $<0.00001\%$.

Upon study termination, fish were culled by overdose of anaesthetic (neutral buffered MS222) according to Home Office protocols. Gonad, brain and pituitary tissue were dissected, collected, weighed, and immediately transferred to tubes containing 1.5ml of RNALater. Samples were stored at 4°C for 72 hours to allow complete tissue penetration of RNALater, and then transferred to and stored at -80°C until processed. The remaining body carcass of each fish was stored at -80°C.

Presence or absence of nuptial tubercles, fatpads and fin spots were assessed in females only. Tubercle and fatpad grades, height and weights in males and females were not

assessed due to the need for the dissection of the head in order to obtain the pituitary gland. Hepatosomatic Index (HSI) and Gonadosomatic Index (GSI) were derived from the weights of the liver and gonads, respectively.

5.2.2. Quantification of exposure concentrations

A small sub-set of samples was collected for the quantification of chemical concentrations in exposure tanks. Since the experiment was a repeat of the first mixture experiment, using the same mixture ratios, concentrations and experimental parameters, water samples were collected solely for comparison to experiment 1, to ensure measured concentrations were within similar ranges and that maintenance of mixture ratios was achieved. Therefore, a limited sample set was collected in order to align the observed results with the results from the first experiment. 1L water samples were collected from one tank out of eight (one per treatment group) prior to introduction of chemicals (week 0), and once at the end of the study on day 45 (the final day of the experiment) from four tanks from each treatment group (even tank numbers). On the morning of a water sampling day, fish were not to be fed until after sample collection. 17 α ethinyl estradiol was quantified in the samples according to the method described in chapter 3, and compared with the measured concentrations obtained in experiment 1. Spiked samples were included and run on every assay plate to quantify recoveries. Spiked samples were spiked with the corresponding concentration of the mixture dosing stock, suitably diluted to obtain tank concentrations.

5.2.3. Pilot Studies – RNA extraction efficiency

Pilot studies were conducted with pituitary tissue samples from a small subset of fish not included in either mixture experiment, to ensure that a good quantity of RNA of high quality could be extracted from single tissue samples for successful molecular analysis. Five adult male and three adult female *P. promelas* (ca. 12 months old) were selected

from the general stock held at Brunel University. Fish of varying body sizes were selected for inclusion in the pilot study in order to ensure that RNA yields from fish of varying sizes were quantifiable and sufficient for quantification of gene expression. Fish were euthanized in buffered MS-222 according to Home Office regulations. Pituitary glands were dissected and transferred to 2ml microcentrifuge tubes pre-filled with 1.5ml of RNALater. Samples were stored at 4°C for 72 hours to allow for efficient tissue penetration before being transferred to -80°C until processing. RNA was extracted using the RNeasy Micro Kit (Qiagen, UK) following the manufacturers protocol for tissue extraction.

5.2.4. Tissue Homogenisation, RNA Extraction and Purification

Tissues were homogenised using a bead-beating TissueLyser II (Qiagen, UK) and RNA was extracted using the RNeasy Micro Kit (Qiagen, UK). RNALater was removed from each sample microcentrifuge tube using a small graduation pipette, taking care not to disturb or remove the tissue. A 5mm diameter stainless steel bead was added to each tube, followed by 350µl of lysis buffer (provided in the kit). Since tissues were relatively small, 5µl of poly-A RNA was added to the sample tube to act as carrier RNA in order to improve recovery of total RNA from pituitary tissue. Tissue was subsequently homogenised using a TissueLyser II at 30Hz for 4 minutes, with a short break after 2 minutes to rearrange the tubes in order to ensure balanced and complete homogenisation. RNA was extracted and purified following the manufacturer's protocol for purification of total RNA from animal and human tissues. RNA was eluted in 14µl of RNase-free water and stored at -80°C. RNA yield and quality was quantified using a Nanodrop ND-1000 spectrophotometer. Nucleic acid purity was measured using the 260/280 ratios. Since ratios of ~2.0 are considered to be of optimum purity for RNA, only samples within a given ratio range were utilised. Samples with low 260/280 ratios of below 1.90 were eliminated from the study since low ratios indicate the presence of contaminant

carry-over, such as proteins or phenols. Typically ratios of between 1.90 and 2.10 are considered of adequate purity for downstream molecular applications, however since poly-A RNA (carrier RNA) was added to the samples during the extraction and purification process, higher 260/280 ratios are likely due to the increased absorbance of nucleotides at 260nm. For this reason, samples with ratios of more than 2.10 were also included in the analysis.

5.2.5. cDNA synthesis

First-strand complimentary DNA (cDNA) was synthesised from the RNA by reverse-transcription, using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Life Technologies). For all synthesised samples, 100ng of total RNA was used as starting material (diluted/concentrated accordingly depending on Nanodrop spectrophotometers readings) for the following reaction highlighted in table 24:

Table. 24 SuperScript VILO cDNA Synthesis Kit reaction volumes (Invitrogen, Life Technologies).

Component	Quantity	
5x VILO Reaction Mix	4 µl	
10x SuperScript™ Enzyme Mix	2 µl	
RNA	x µl	} 14µl Total
Molecular Grade Water	x µl	
TOTAL REACTION VOLUME	20 µl	

A master mix containing 5x VILO Reaction Mix and 10x SuperScript Enzyme mix was made up for each batch synthesised (per 10 samples), inverted to mix and spun in a cooled centrifuge for 10 seconds. Calculated volumes of molecular grade water were pipetted to 0.2µl PCR tubes prior to other reaction components to avoid cross

contamination, followed by calculated volumes of RNA. 6µl of master mix solution containing the VILO reaction and Superscript enzyme mix was subsequently added to each PCR tube. Tubes were inverted to mix and spun in a cooled centrifuge for 10 seconds. Synthesis of first-strand cDNA was completed in a conventional thermocycler using the following protocol:

1. Incubation at 25°C for 10 minutes
2. Incubation at 42°C for 60 minutes
3. Termination of the reaction at 85°C at 5 minutes
4. Hold at 4°C

Synthesised first-strand cDNA was subsequently stored at -20°C until used. Prior to use in qPCR assays, cDNA was diluted 1 in 10 with molecular grade water.

5.2.6. Primer selection

All primer sequences were obtained from a review of the literature, and were found to be well designed and had been extensively used. Primer sequences were checked for sequence identity to a region of the target gene, and potential for primer dimerization, whereby primer pairs can hybridise to itself (hairpin) or each other (cross-dimerization) due to complementary base pairs in the sequences. Primers were obtained from Sigma-Aldrich (table 25).

Table 25. Primer sequences utilised in the presented study.

Name	Sequence 5' to 3'	Length (bp)	Reference
FSHβ F	TCGGCTTTCCAATATCTCCATT	22	Villeneuve et al, 2007a
FSHβ R	ATGCAGTTGTGTCGAGATGTGAT	23	Villeneuve et al, 2007a
LHβ F	CCTGGTGTTCAGACCAGCAT	21	Villeneuve et al, 2007a; Villeneuve et al, 2007b
LHβ R	TTGGAGAACGGGCTCTTGTATAC	23	Villeneuve et al, 2007a; Villeneuve et al, 2007b
18S F	AATGTCTGCCCTATCAACTTTC	22	Filby and Tyler, 2005
18S R	TGGATGTGGTAGCCGTTTC	19	Filby and Tyler, 2005
rpl8 F	CTCCGTCTTCAAAGCCCATGT	21	Filby and Tyler, 2005
rpl8 R	TCCTTCACGATCCCCTTGATG	21	Filby and Tyler, 2005
tbp F	CCTATGACCCCATCACTCC	20	Filby and Tyler, 2007
tbp R	GCTGCCAATCGGGACTGT	18	Filby and Tyler, 2007

*The initial concentration of all primers was 100nm. Primers were diluted to 10nm with molecular grade water. Desalt purification (salt-free solution), 0.025 μ m scale. F-forward primer, R-reverse primer. bp-base pair.

5.2.7. Primer Optimisation and Assay Specificity

Prior to primer use in the analysis, primer optimisation was performed on each primer pair to obtain the optimum annealing temperature for each primer pair and to ensure assay specificity. A temperature gradient qPCR was run with a range of temperatures between 55 and 65°C using the assay template shown in table 26, whereby each row was

subjected to a different annealing temperature. cDNA from one individual was used for the assay to maintain consistency in the amplification of gene regions. The sample used in the primer efficiency assay was obtained at random from the pilot study. A No Template Control (NTC) was included for each primer set as a negative control. The qPCR conditions are described in section 5.2.9.

Table 26. qPCR template for primer optimisation assay, using a single cDNA isolate. NTC- Non-template control; X- blank well; S-sample.

Temp (°C)		NTC	FSH β	LH β	18S	rpl8	TBP	NTC
65°C	X	X	S	S	S	S	S	X
64.5°C	X	X	S	S	S	S	S	X
63.3°C	X	X	S	S	S	S	S	X
61.4°C	X	X	S	S	S	S	S	X
59.0°C	X	FSH β	S	S	S	S	S	rpl8
57.0°C	X	LH β	S	S	S	S	S	TBP
55.7°C	X	18S	S	S	S	S	S	X
55.0°C	X	X	S	S	S	S	S	X

*S- sample of cDNA, obtained from RNA yield pilot study.

Optimum primer annealing temperature was selected based on analysis of the melt curve and the melt peak for each primer set. The melting temperature (T_m) was calculated using the standard formula:

$$T_m = ((\text{Number of G+C}) \times 4^\circ\text{C}) + ((\text{Number A+T}) \times 2^\circ\text{C}) \quad \text{McPherson and Møller, 2006}$$

Equation 5. Calculation of melting temperature (T_m) in qPCR

The primer annealing temperature selected was typically 5°C lower than the calculated T_m , in line with standard recommendations. Melt curve and peak analysis were also

undertaken post-qPCR, and were included as an additional step conducted by the qPCR thermocycler after completion of the assay. During melt curve analysis, relative fluorescence (RFU) is monitored as a function of temperature. As the temperature is gradually increased, the denaturing dsDNA releases the fluorophore dye (since the dye only binds to dsDNA) which subsequently decreases the fluorescent signal. When 50% of the dsDNA is denatured to ssDNA, there is a significant drop in the signal, and the temperature at which this occurs (melting temperature, or T_m) is recorded. Melt peak analysis was also performed by the thermocycler post-qPCR, and was subsequently plotted as the negative first regression of RFU versus the temperature ($-d(\text{RFU})/dT$). The peak represents the T_m (melt temperature) of the dsDNA complexes (BioRad; Qiagen). In general, the temperature producing the highest relative fluorescence (RFU, or relative fluorescent unit) melt curve corresponding with the highest peak ($-d(\text{RFU})/dT$) was considered as the optimum annealing temperature, and considered in line with the calculated $T_m - 5^\circ\text{C}$ method (fig. 48).

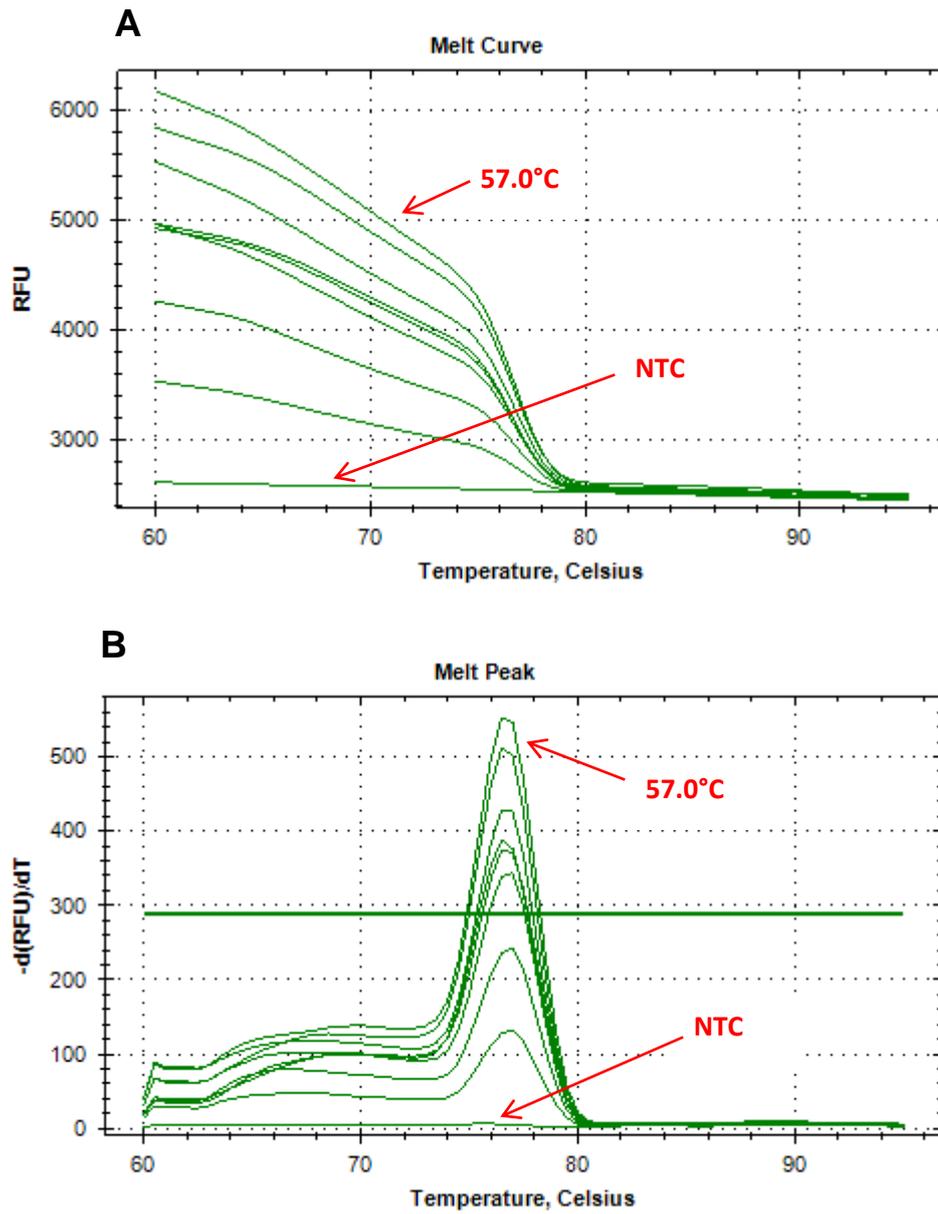


Figure 48. Melt curve (A) and melt peak (B) showing the primer optimisation experiment for the primer pair used to amplify FSH β cDNA. Optimum primer annealing temperature was both calculated (using the formula described), and visually observed, at 57°C.

5.2.8. Amplification efficiency

Primer optimisation and melt curve analysis confirmed the effective annealing temperature conditions for each primer pair. Analysis of the melt curves and peaks revealed a small additional peak in the reactions using the tata-box binding protein (tbp) primers. Since one of these peaks also corresponded to amplification in the NTC, dimerization of this primer pair could be occurring. Primer dimers typically melt at lower temperatures because they are small in size, and thus can be easily identifiable and easily disregarded from the results (BioRad). Given that three housekeeping genes were tested for the purpose of deriving the best reference gene for normalisation of all samples, the tbp primers were excluded from further investigation. All other primers amplified well and no dimerization was observed with any of the remaining primers.

Optimum annealing temperature for three of the primer pairs, FSH β , 18S and rpl8, was 57°C, whilst LH β was optimum at the higher temperature of 61°C, and therefore was run in the qPCR assay separate to the others. A standard curve was prepared for each primer pair to determine that the optimal initial starting concentration of cDNA template in samples was optimal and to assess the efficiency of the amplification process. A dilution series was prepared by diluting the cDNA (initial concentration 100ng/L) to; 100%, 50%, 10% and 1% (table 27).

Table 27. Dilution steps taken for standard curve production in order to assess amplification efficiency of qPCR. (MG-molecular grade).

Step	cDNA Concentration	Dilution Step	Total cDNA	Total Water (MG)	Total Vol
A	100%	1/10 (starting concentration)	30µl	-	30µl
B	50%	1/2 of dilution A	10µl	10µl	20µl
C	10%	1/10 of dilution A	3µl	27µl	30µl
D	1%	1/10 of dilution C	2µl	18µl	20µl

Reaction volumes for production of the standard curve were the same as described for the main assays (table 28, section 5.2.9). Standard curves for three of the four tested primer pairs were conducted in a single assay at 57°C. LHβ primers were run in a separate assay since the optimal annealing temperature of these primers was 4°C higher, at 61°C. The outer wells of the plate were intentionally unused to reduce impacting the results with the microplate edge effect. All samples were assayed in triplicate. A No Template Control (NTC), whereby molecular grade water was substituted for template cDNA, was run on every plate to ensure there was no contamination or amplification of non-target DNA.

Analysis of the melt curve was subsequently undertaken to ensure that only one peak was generated, representing the gene of interest. If multiple peaks were observed, the assumption that non-target DNA had been amplified, or primer dimerization had occurred was adopted, and a review of the primers was undertaken. A small melt peak in the No Template Control (NTC) was deemed acceptable only if the peak was of a different size to the peak of the target gene.

5.2.9. qPCR Assay Conditions

Expression of follicle-stimulating hormone beta subunit (FSHβ) and luteinising hormone beta sub-unit (LHβ) were analysed using real-time qPCR and SYBR Green fluorescence detection. Three housekeeping genes were selected for inclusion in the study. The most

appropriate of the three genes was then used for normalisation of mRNA levels of FSH β and LH β .

All qPCR assays were conducted using Power SYBR Green PCR master mix (Applied Biosystems, Life Technologies), a propriety mix containing SYBR Green I florescent dye, dNTPs, AmpliTaq Gold[®] DNA polymerase Ultra Pure (UP), and optimised buffers. A general stock mix was made comprising of the forward and reverse primers, molecular grade water, and the Power SYBR Green PCR master mix for each assay. 2 μ l of template cDNA diluted 1 in 10 with molecular grade water was added to each well in triplicate, followed by 18 μ l of the master mix described above. For the No Template Control (NTC), 2 μ l of molecular grade water was added in place of cDNA (table 28).

Table 28. Protocol for SYBR Green qPCR fluorescent quantification assay, following manufacturers guidelines. Applied Biosystems, Life Technologies.

Component	Volume per reaction
SYBR Green PCR Mix	10 μ l
Forward Primer	0.5 μ l
Reverse Primer	0.5 μ l
Water (Molecular Grade)	7 μ l
Template cDNA	2 μ l
Total Reaction Volume	20μl

Amplification of target and reference genes were conducted using a BioRad C1000 thermocycler (BioRad Laboratories, Ltd.) according the protocol described in table 29. Assays were conducted in a 96-well PCR plate standard format. The outer wells of the microtiter plates were intentionally left blank to reduce the microplate edge effect, whereby increased evaporation and uneven heating takes place at the circumferential wells

compared with centrally located wells. Reaction volumes were made up to 20µl, consisting of the volumes described in table 28

Table 29. Standard qPCR protocol utilised for the amplification of target genes.

Protocol	Temperature	Duration
Pre-warming	50°C	2 mins
Activate enzyme	95°C	10 mins
Denaturation of DNA	95°C	15secs
Primer annealing/extension	57°C FSH β , 18S and rpl8 61°C LH β	1 mins
Repeat n cycles	Repeat for 40 cycles	
Melt curve generation	65°C-95°C in 0.5°C increments	5 secs per increment

Cycle threshold values (Ct) for each assay were plotted on a scatter graph to assess reproducibility between replicates and across PCR plates. Where erroneous results were observed or where variation between replicates was considerable, the samples were re-run in subsequent assays.

5.2.10. Assessment of Housekeeping genes

Housekeeping genes were carefully selected based on information provided in past and current literature, by selecting genes that are considered to be unaffected by exposure to steroid hormones or similar compounds. 18S ribosomal RNA (18S), ribosomal protein L8 (rpl8) and tata box binding protein (tbp) were selected based on findings in the literature that suggest they are not effected by exposure to any of the chemicals or environmental factors (i.e. reproduction) utilised in the study. The widely-used housekeeping genes beta-actin and elongation factor 1-alpha were excluded from this study since they have been shown to be sensitive to estrogen exposure (Filby and Tyler, 2007).

Housekeeping/reference genes that were found to be suitable by analysis of the optimisation and amplification efficiency experiments were run in each assay. All experimental samples were tested in qPCR with both target genes (FSH β and LH β) and all appropriate housekeeping genes. Selection of the most appropriate housekeeping gene for normalising target gene expression was completed after conducting the qPCR assays. All housekeeping genes run in the assays were assessed for differences across treatments and variability between individuals. All samples were normalised to the same (most appropriate) housekeeping gene.

5.2.11. qPCR Data Analysis

Relative quantification was used to determine the qPCR results. Mean cycle threshold (Ct) was calculated for each sample based on inter and intra plate replicates. Mean Normalised Expression (MNE) of the target genes FSH β and LH β was normalised to the most appropriate housekeeping gene, based on the criteria described previously. Normalisation was performed by QGene96, a Microsoft excel software package for the comparative analysis of quantitative real-time PCR data. Normalised gene expression is proportional to the amount of RNA of the target gene relative to the amount of RNA of the

housekeeping gene (Muller et al, 2002). To calculate MNE for triplicate samples, three independent normalised expression values were calculated and then averaged, based on the following equation developed by Muller et al (2002) (Eq. 6):

$$MNE = \frac{\frac{(E_{target})^{CT_{target, well1}}}{(E_{ref})^{CT_{ref, well1}}} + \frac{(E_{target})^{CT_{target, well2}}}{(E_{ref})^{CT_{ref, well2}}} + \frac{(E_{target})^{CT_{target, well3}}}{(E_{ref})^{CT_{ref, well3}}}}{3}$$

Equation 6. Mean Normalised Expression (MNE) by arithmetic mean of normalised expression values. The formula was used for calculating MNE by averaging three normalised expression values, developed by Muller et al, 2002 for the QGene application, where: NE-normalised expression; E_{target} – amplification efficiency of target gene; E_{Ref} – amplification efficiency of the reference (housekeeping) gene; CT_{target} – threshold cycle of target gene; CT_{ref} – threshold cycle of the reference (housekeeping) gene.

MNE for each treatment group was plotted using a bar graph for ease of visualisation. Data were assessed for normality using the Shapiro-Wilks test, and for homogeneity of variance using either the Levene's test or non-parametric Levene's test (depending on data distribution). Mean MNE levels across treatment groups were compared using either a one-way ANOVA or Kruskal Wallis test (whichever was deemed most appropriate for the data distribution). Where differences in MNE were significant, data were represented on a log scale to ensure that all data points could be represented visually.

5.2.12. Fecundity of breeding pairs

As in the mixture experiment discussed in chapters 3 and 4, fecundity of breeding pairs was assessed in the form of the quantification of egg number and spawning frequency. Assessment of fecundity was undertaken in order to assess the repeatability of the original experiment, to generate further biomarker data, and to investigate any correlation

between the expression of pituitary gonadotropins and reproductive output in individual fish. As in experiment 1, fecundity (egg production) was assessed daily by manual counts and recorded each day on or after 11am. Data were subsequently compared to the data collected from experiment 1, to assess repeatability of results. Fecundity was assessed for normality using the Shapiro-Wilks test, and for homogeneity of variance using either a Levene's test or a non-parametric Levene's test (which uses rank values to compare means, and hence is more suitable for non-parametric data). Comparison of means across treatments was undertaken using one-way analysis of variance (ANOVA), Kruskal Wallis, or the Mood's Median test, whichever was appropriate. Comparison of fecundity data between experiments was investigated using univariate analysis (two-way ANOVA) and pairwise comparison.

5.2.13. Relationship between gene expression changes and reproductive output

A comparison between the reproductive output and pituitary gonadotropin gene expression changes in individual fish was undertaken to determine if any relationship existed between the level of pituitary gonadotropin expression and changes to reproductive output. Mean Normalised Expression (MNE) of FSH β and LH β were directly compared to fecundity using Pearson's correlation coefficient.

5.2.14. Statistical treatment of relationship data

MNE of FSH β and LH β in male fish was plotted on a log scale for better visualisation of the data, and to allow comparisons between the treatments, due to the fact that large variations in MNE was observed in male fish. Change in MNE of FSH β and LH β in females was observed over a smaller range, and was suitably represented on a linear scale. The relationship between fecundity (egg production) and the expression of pituitary gonadotropins was investigated using Pearson's correlation coefficient. Data were

investigated separately for male and female fish, since baseline expression levels of pituitary gonadotropins varies by sex.

5.3. Results

5.3.1. Experimental Conditions

Mortality was below 2% during the duration of the experiment, in line with expectations under normal environmental conditions. Where mortality was observed, the cause was attributed to natural conditions and not due to the chemical exposure concentrations.

5.3.2. Quantification of Exposure Concentrations

Average measured concentrations of 17 α -ethinyl estradiol (EE2) quantified with a commercial ELISA demonstrated that the chemical was present in the mixture. Recoveries of spiked samples and exposure samples were relatively high (151%-226% of nominal concentrations) (table 30). Measured concentrations in both the spiked samples and experimental samples were consistently higher than those recorded from experiment 1 (table 31).

Table. 30. Measured water concentrations of 17 α -ethinyl estradiol (EE2) quantified from experiment 2. Measurements were quantified using a commercial ELISA as in previous experiments (discussed in chapter 3). <DL denotes concentrations below the detection limit of the assay.

Nominal	Week 0	Week 4	Mean	% Nominal (at week 4)
Control	<DL	<DL	<DL	-
	<DL	<DL		
	<DL	<DL		
	<DL	<DL		
0.16ng/L	<DL	0.28	0.27	170%
	<DL	0.32		
	<DL	0.24		
	<DL	0.24		
0.52ng/L	<DL	0.95	1.17	226%
	<DL	1.26		
	<DL	1.20		
	<DL	1.29		
Spiked Samples				
ddH2O	<DL	<DL		
0.16ng/L	0.16	0.28	0.22	137%
0.52ng/L	0.77	0.81	0.79	151%

Table. 31. Measured water concentrations and percentage recoveries of 17 α -ethinyl estradiol (EE2) quantified from both experiment 1 and experiment 2. Measurements were quantified using a commercial ELISA as in previous experiments (discussed in chapter 3). <DL denotes concentrations below the detection limit of the assay.

Nominal	Measured		% Recovery	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Experimental Samples				
Control	<DL	<DL	-	-
0.16ng/L	0.14	0.27	87.9%	170%
0.52ng/L	0.41	1.17	79.9%	226%
Spiked Samples				
0.16ng/L	0.13	0.22	82.5%	137%
0.52ng/L	0.42	0.79	82.5%	151%

5.3.3. RNA extraction yield - Pilot study

Initial pilot studies on individual pituitary glands of unexposed adult *P. promelas* confirmed that enough high quality RNA could be successfully extracted from individual pituitary glands for analysis using qPCR without the need for pooling samples. Quantification of RNA using Nanodrop spectrophotometry quantified all pilot samples at between 7.4 and 36.4ng/L, with 260/280 values of on average 1.83, considered sufficient for downstream analysis. RNA extractions were performed with the aid of carrier RNA (Poly-A RNA) and therefore 260/280 ratios of more than 2.10 were also considered.

5.3.4. Selection of housekeeping genes

18S and rpl8 was expressed in all individuals in the optimisation experiments. Expression of rpl8 was lower than 18S across all individuals, reflected in the higher Ct values. Mean cycle threshold (Ct) of the reference genes 18S and rpl8 was compared across groups to investigate variability between treatment groups that would suggest unsuitable use as a reference gene. 18S Ct data were not normally distributed (Shapiro-Wilks test, 0.747, $df=87$, $p<0.001$), and had equal variances (non-parametric Levene's test, $f=0.879$, $df=2$, $p=0.419$). MNE of 18S was found to be statistically similar across all treatment groups (fig 49), (Kruskal Wallis test, Chi-Square = 3.188, $df=2$, $p=0.203$).

Ct data for rpl8 were not normally distributed (Shapiro-Wilks test, 0.747, $df=96$, $p<0.001$), and found to have equal variances (non-parametric Levene's test, $f=0.522$, $df=2$, $p=0.595$). Mean rpl8 Ct was not statistically different between any of the treatment groups (fig. 50), (Kruskal Wallis, Chi-Square = 2.222, $df=2$, $p=0.329$).

Since no significant different in expression was observed between any of the treatment groups for both 18S and rpl8, it was anticipated that either reference gene could be used for normalisation of target genes. Since rpl8 expression was lower than 18S (reflected in the observation that for rpl8, more cycles are required to reach the cycle threshold), 18S

was selected as the most appropriate housekeeping gene, since there is potentially less variability between individuals when lower number of cycles are required to reach the threshold.

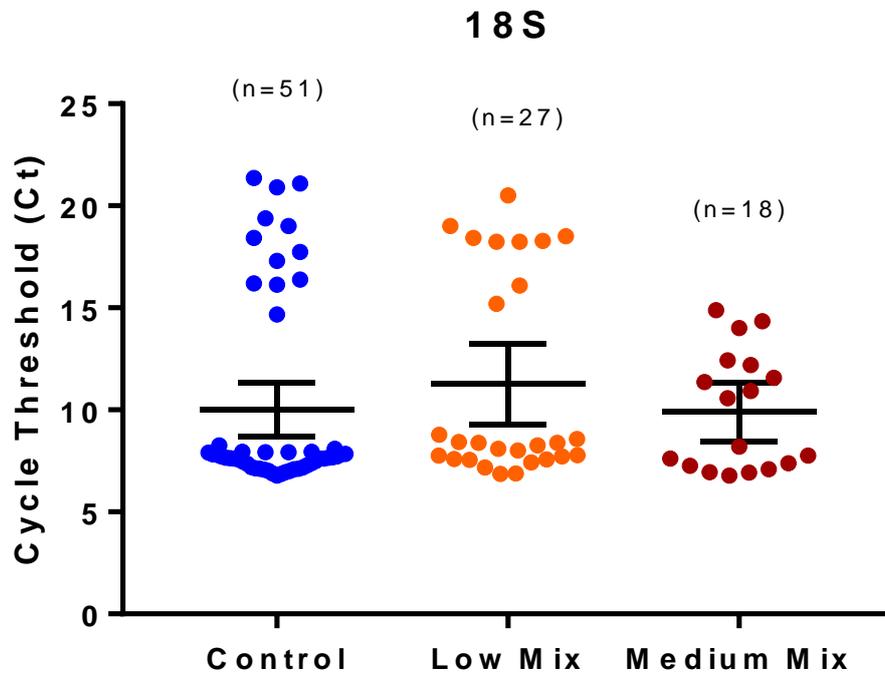


Figure. 49. Cycle threshold values for 18S expression in male and female *P. promelas* across treatment groups. Each dot represents an individual sample (run in triplicate). Horizontal line represents the mean, error bars represent 95% confidence intervals. Cycle threshold is the number of qPCR cycles required for the target gene to reach levels sufficient to be detected above background fluorescence. No statistically significant difference between treatment groups (Kruskal Wallis) was observed.

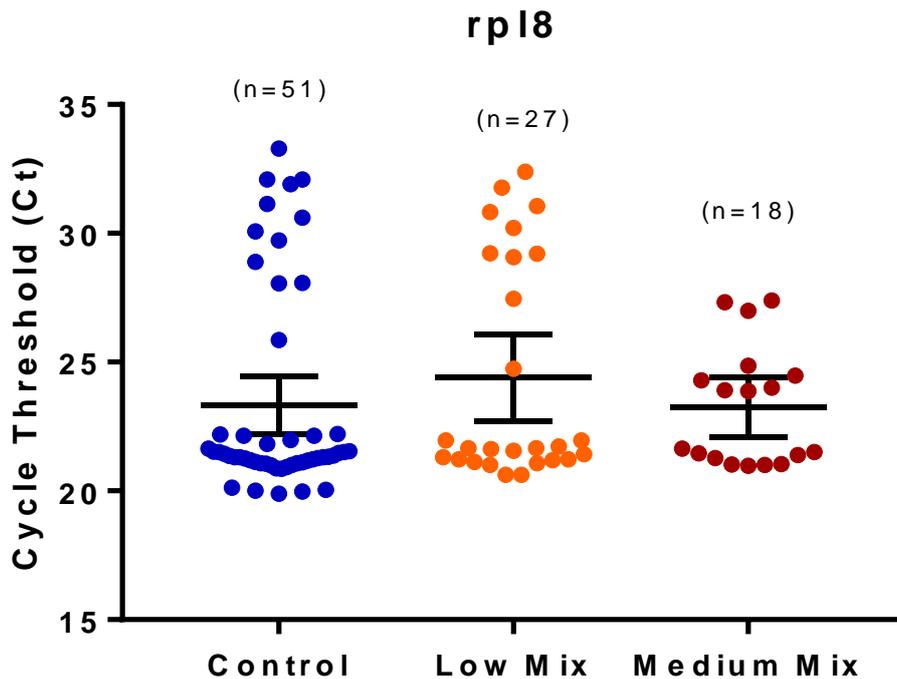


Figure. 50. Cycle threshold values for *rpl8* expression in male and female *P. promelas* across treatment groups. Each dot represents an individual sample (run in triplicate). Horizontal line represents the mean, error bars represent 95% confidence intervals. Cycle threshold is the number of qPCR cycles required for the target gene to reach levels sufficient to be detected above background fluorescence. No statistically significant difference between treatment groups (Kruskal Wallis) was observed.

Furthermore, expression levels of 18S and *rpl8* appear to demonstrate two quite distinct groups (fig's 49 and 50). The most likely explanation for these groupings is that the differences are due to a technical artefact during the RNA extraction, purification and reverse transcription, or during the qPCR assay itself.

5.3.5. Expression of FSH β mRNA (normalised to 18S)

The sample sizes utilised in the assessment of mRNA levels of LH β and FSH β were significantly reduced as a result of technical difficulties during the extraction of high quality RNA. Initially, the pituitary glands from 61 fish (32 males and 29 females) were dissected

and preserved for analysis. However, technical difficulties during the RNA extraction process led to the production of low quality RNA for some samples, and therefore they were excluded from the study. The reasons for these difficulties are discussed further in section 5.4.

5.3.5.1. Males

Expression of FSH β mRNA in male *P. promelas* was downregulated with increasing concentration of the mixture (fig. 51). The down-regulation of FSH β mRNA levels in males were so pronounced that it required the data to be represented on a logarithmic scale. FSH β mRNA expression data were not normally distributed (Shapiro-Wilks test, 0.215, df=23, p<0.001). A non-parametric Levene's test revealed that variances were equal (f=0.154, df=2, p=0.859), and thus a Kruskal-Wallis test was used to compare group means. Downregulation of FSH β mRNA expression with increasing concentration of the mixture in male fish was not statistically significant (Chi-Square 0.048, df=2, p=0.975), however this could be expected due to small and uneven sample sizes.

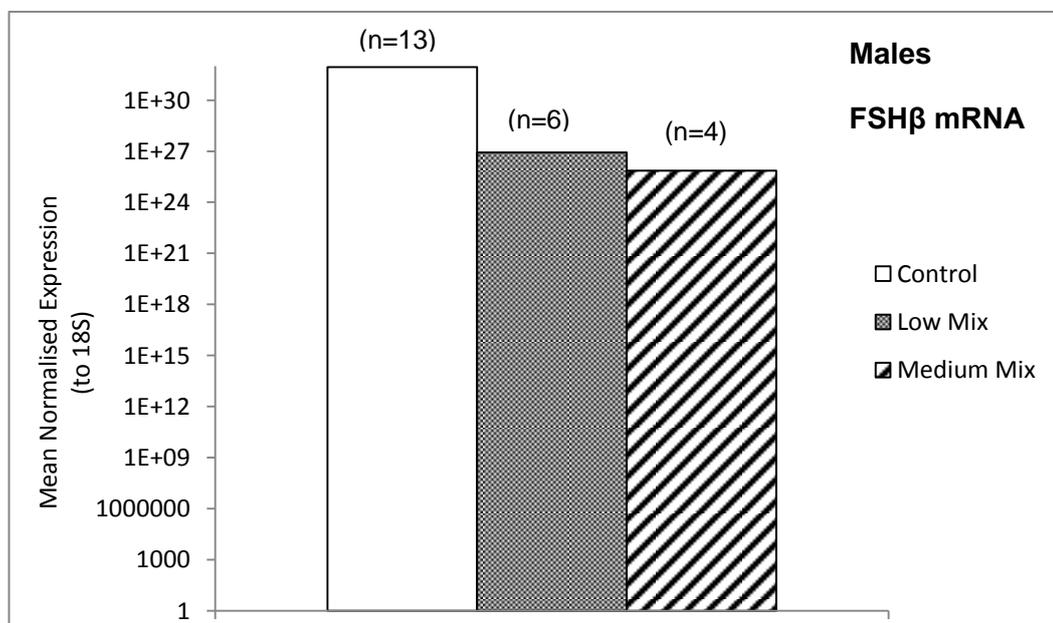


Figure 51. Mean Normalised Expression (MNE) of FSH β mRNA in male *P. promelas*, normalised to 18S. Error bars are not included due to the data representation on a logarithmic scale. n=sample size. The trend in downregulation of FSH β mRNA was not significant (Kruskal Wallis Test).

5.3.5.2. Females

Expression of FSH β mRNA showed a non-dose dependant relationship with concentration of the mixture (fig. 52). The lowest concentration showed a slight upregulation of FSH β mRNA expression compared with the control group. The highest concentration tested (medium mixture) showed a downregulation of FSH β mRNA expression levels compared with both the control and the lowest concentration tested. Data were not normally distributed (Shapiro-Wilks test, 0.390, df=9, p<0.001), and thus homogeneity of variance was assessed using a non-parametric Levene's test. Variances were found to be equal (f=0.718, df=2, p=0.525). No significant difference was observed between any of the treatment groups (Kruskal Wallis test, Chi-Square 2.778, df=2, 0.249).

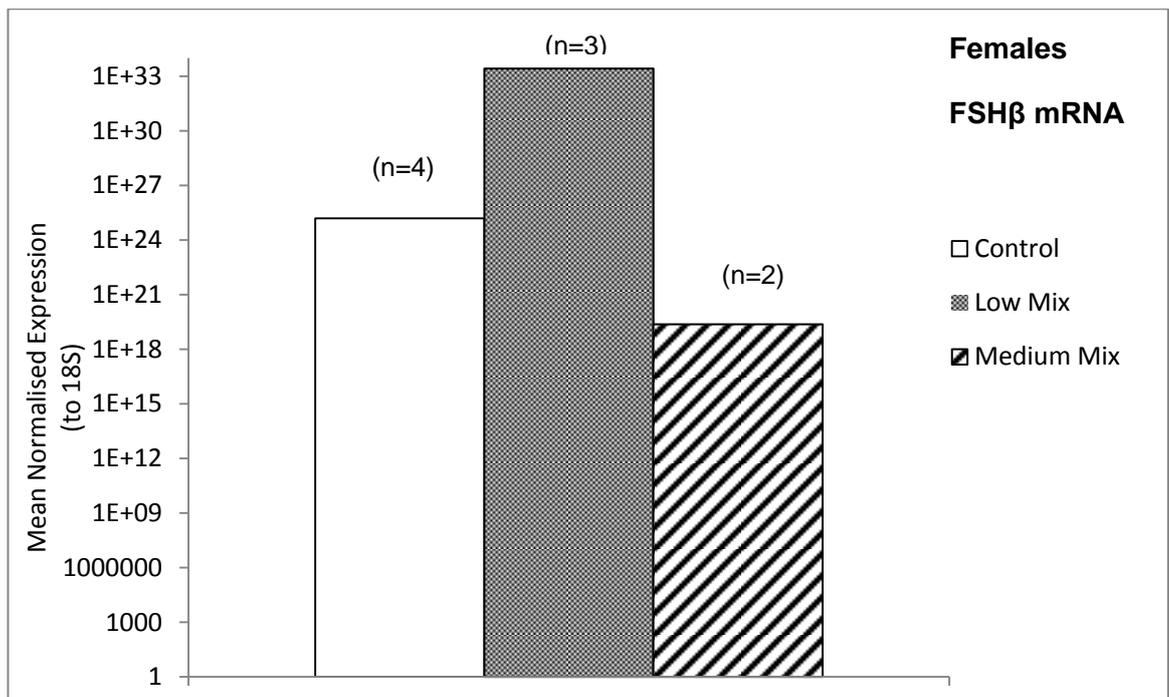


Figure 52. Mean Normalised Expression (MNE) of FSH β mRNA levels in female *P. promelas*, normalised to 18S. Error bars are not included due to the data representation on a logarithmic scale. n=sample size. The trend in downregulation of FSH β mRNA was not significant (Kruskal Wallis Test).

5.3.6. Expression of LHβ mRNA (normalised to 18S)

5.3.6.1. Males

LHβ mRNA expression levels in male *P. promelas* did not follow a dose-dependent trend (fig. 53). LHβ mRNA was upregulated in the lowest concentration relative to the control, and downregulated in the highest concentration group (relative to the control and the lowest treatment dose). LHβ mRNA expression data were not normally distributed (Shapiro-Wilks test, 0.522, df=23, $p < 0.001$). Data were found to have equal variances (non-parametric Levene's test $f = 0.539$, $df = 2$, $p = 0.591$) and was therefore analysed using the Kruskal Wallis test. LHβ mRNA expression levels in male fish was not statistically different between treatment groups (Chi-Square 0.323, $df = 2$, $p = 0.851$).

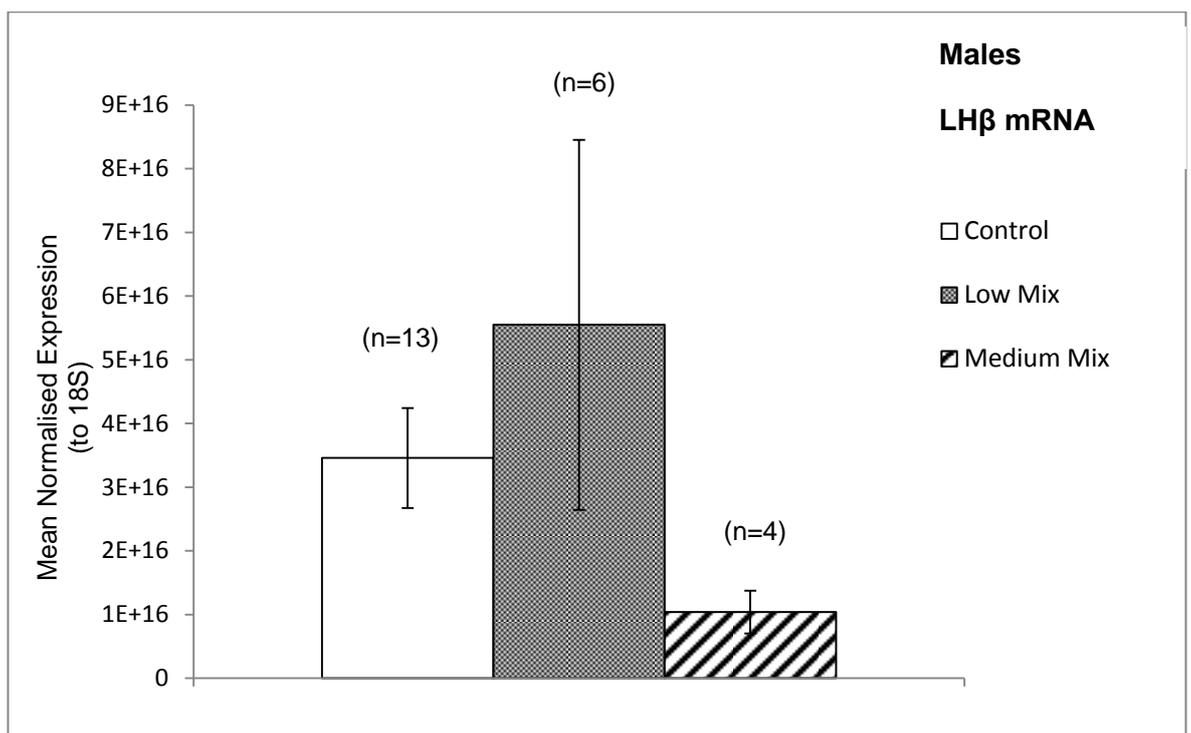


Figure 53. Mean Normalised Expression (MNE) of LHβ mRNA in male *P. promelas*, normalised to 18S. Error bars represent SEM. n= sample size. There was no significant effect of exposure to the mixture (assessed by Kruskal Wallis).

5.3.6.2. Females

Expression of pituitary LH β mRNA was upregulated by the highest mixture concentration relative to the control and the lowest mixture concentration. However, a downregulation was also observed in the lowest concentration group, relative to the control (fig. 54). Data were not normally distributed (Shapiro-Wilks test, 0.664, df=9, $p \leq 0.001$), and had equal variances (non-parametric Levene's test, $f=0.424$, df=2, $p=0.672$). Expression changes in pituitary LH β mRNA was not significantly difference in female *P. promelas* across treatment groups (Kruskal Wallis, Chi-Square 0.400, df=2, $p=0.819$).

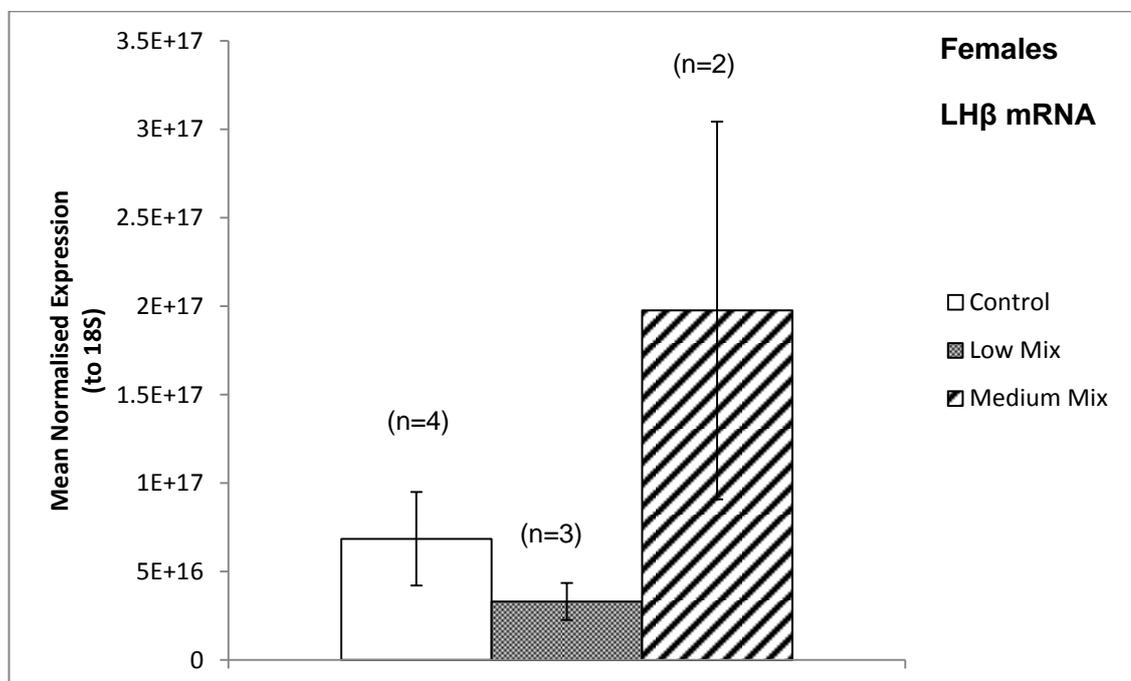


Figure 54. Mean Normalised Expression (MNE) of LH β mRNA in female *P. promelas*, normalised to 18S. Error bars represent SEM. n= sample size. There was no significant effect of exposure to the mixture (assessed by Kruskal Wallis).

However, it should be noted that the low sample sizes in this study inevitably prevents the possibility of obtaining a statistically significant effect, since the statistical methods are not robust enough to account for such low samples sizes. Therefore drawing any conclusions on the significant effects of the mixture or determining any reliable trends is not possible.

5.3.7. Fecundity

Similar to the results observed in the first mixture experiment described in chapters 3 and 4, a dose-dependent reduction in fecundity was observed with exposure to the five compound mixture (fig.55). Data were not normally distributed (Shapiro-Wilks test, 0.865, $df=32$, $p \leq 0.001$) and had equal variances (non-parametric Levene's test, $f=0.663$, $df=2$, $p=0.525$). Mean fecundity across treatment groups was significantly different (Kruskal Wallis, Chi-Square=23.490, $df=2$, $p < 0.001$). Fecundity in the two treatment groups were significantly lower than in the control (Chi-Square=13.95, $df=1$, $p < 0.001$). Fecundity in the highest treatment group (medium mix) was also statistically lower than in the low mixture group (Chi-Square=4.257, $df=1$, $p < 0.05$).

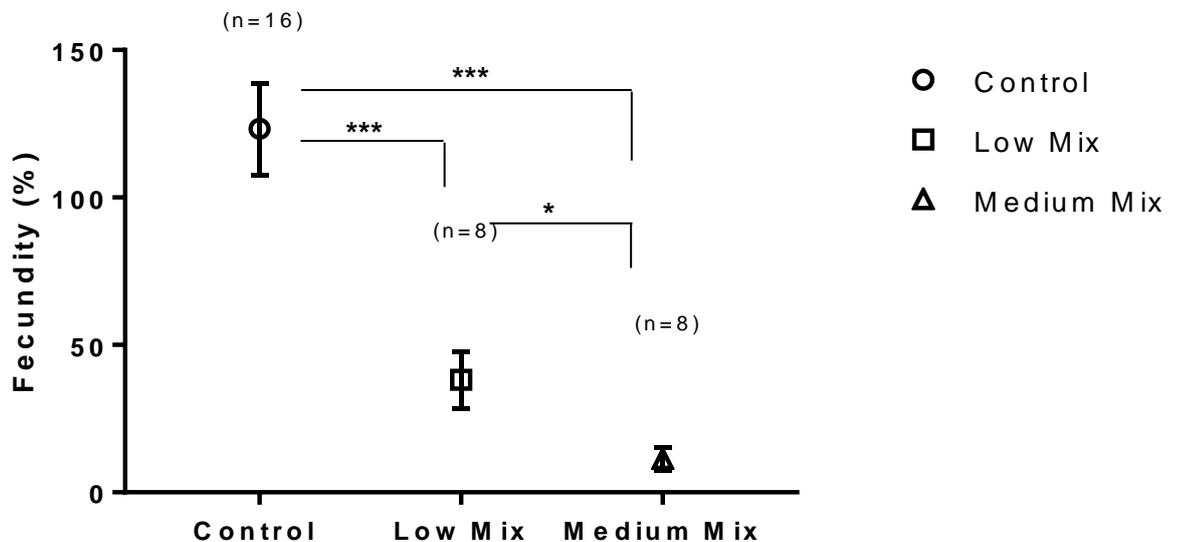


Figure 55. Relative change in fecundity observed in the second mixture experiment, expressed as a percentage of fecundity in the pre-exposure period. Percentage performance refers to cumulative egg production at the end of the exposure period compared to cumulative egg production at the end of the pre-exposure period. 100% equals no change in relative egg performance between the two treatment periods. Error bars represent SEM. Significant differences are indicated by *. n=sample size.

The control group in the second experiment was found to have a relative egg performance of 123% in the exposure period compared with the pre-exposure period (100% egg production), denoting that pairs in the control group spawned more frequently and/or produced more eggs during the second experimental stage (fig. 55).

A univariate analysis was performed on the data from both experiments to assess whether there was a statistically significant difference between the results obtained by the two experiments. A two-way ANOVA using the Type III univariate model (the unweighted means approach) was utilised as the best model when accounting for unequal sample sizes. Data failed to meet the assumption of homogeneity of variances (Levene's test, $F=2.901$, $df=5$, $df=51$, $p=0.22$). Since the assumption of equal variances could not be satisfied, and the sample sizes were unequal, an alpha level of $p<0.001$ was adopted to protect from Type I errors (false positives). There was no statistically significant difference in the reproductive output between the two experiments (two-way ANOVA, $F=0.513$, $df=1$, $p=0.477$), (fig. 56), signifying that the experiment was repeatable and robust, and that the effects on fecundity of the mixture are reliable. Furthermore, there was no significant difference at the interaction level between experiments and treatment groups (two-way ANOVA, $F=1.794$, $df=2$, $p=0.177$), suggesting that differences observed in fecundity across treatment group were not affected by whether they were in experiment 1 or experiment 2.

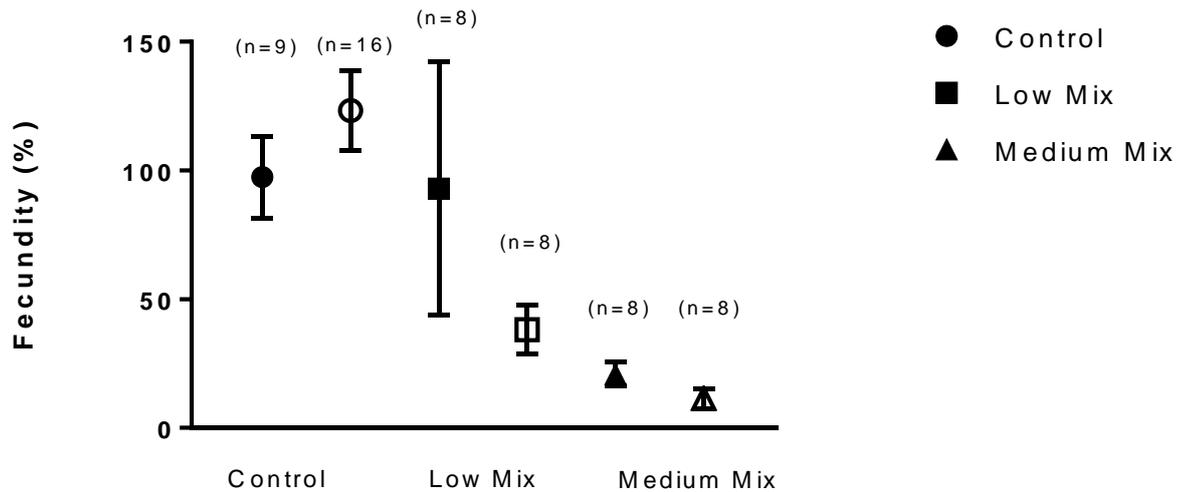


Figure 56. Relative change in fecundity observed in both mixture experiments, expressed as a percentage of fecundity in the pre-exposure period. Closed symbols = experiment 1, open symbols = experiment 2. Percentage fecundity refers to cumulative egg production at the end of the exposure period compared to cumulative egg production at the end of the pre-exposure period. 100% equals no change in relative egg production between the two treatment periods. Error bars represent SEM. n=sample size.

5.3.8. Relationship between expression of pituitary gonadotropin genes and fecundity

The relationship between fecundity and pituitary gonadotropin gene expression was assessed in individual fish. Correlation was assessed collectively and for each concentration individually to reduce biases of additional variables, however data was represented on a single graph. Where data were not normally distributed, the relationship was analysed using Spearman correlation (two-tailed), the non-parametric equivalent of Pearson's correlation coefficient.

In male fish, fecundity and FSH β mRNA expression data did not follow a Gaussian distribution (Shapiro-Wilks, 0.840, df=23, $p < 0.005$; and 0.215, df=23, $p < 0.001$). FSH β

RNA expression was not well correlated to fecundity (Spearman's coefficient, $r=-0.02$, $p=0.92$) (fig. 57). In the control group, fecundity and MNE of FSH β did not follow a Gaussian distribution (Shapiro-Wilks, 0.711, $df=13$, $p\leq 0.001$; 0.311, $df=13$, $p<0.001$ respectively). Fecundity was slightly positively correlated with FSH β mRNA levels (Spearman's coefficient, $r=0.41$). Furthermore, the correlation was not statistically significant ($p=0.1695$) suggesting the probability of obtaining a stronger correlation in these datasets is low. In the group exposed to the lowest concentration of the mixture, fecundity was normally distributed (Shapiro-Wilks 0.774, $df=6$, $p=0.774$), however MNE of FSH β mRNA did not follow a Gaussian distribution (Shapiro-Wilks, $df=0.696$, $df=6$, $p<0.01$), and therefore the Spearman correlation was used since it does not assume a normal distribution. Fecundity in the lowest concentration group was somewhat negatively correlated to FSH β mRNA levels ($r=-0.49$), whereby fecundity decreased with increasingly FSH β mRNA levels, and this was not statistically significant ($p=0.36$).

In the group exposed to the medium concentration of the mixture (the highest tested in this experiment), fecundity was normally distributed (Shapiro-Wilks 0.944, $df=4$, $p=0.677$), however MNE of FSH β mRNA did not follow a Gaussian distribution (Shapiro-Wilks, $df=0.634$, $df=4$, $p\leq 0.001$), and therefore the Spearman correlation was used. As in the low mixture concentration group, fecundity in the medium mixture group was negatively correlated with FSH β mRNA levels ($r=-0.4$), whereby fecundity decreased with increasing FSH β mRNA levels. Furthermore, the correlation was not significant ($p=0.75$), signifying that a strong correlation in this dataset is unlikely.

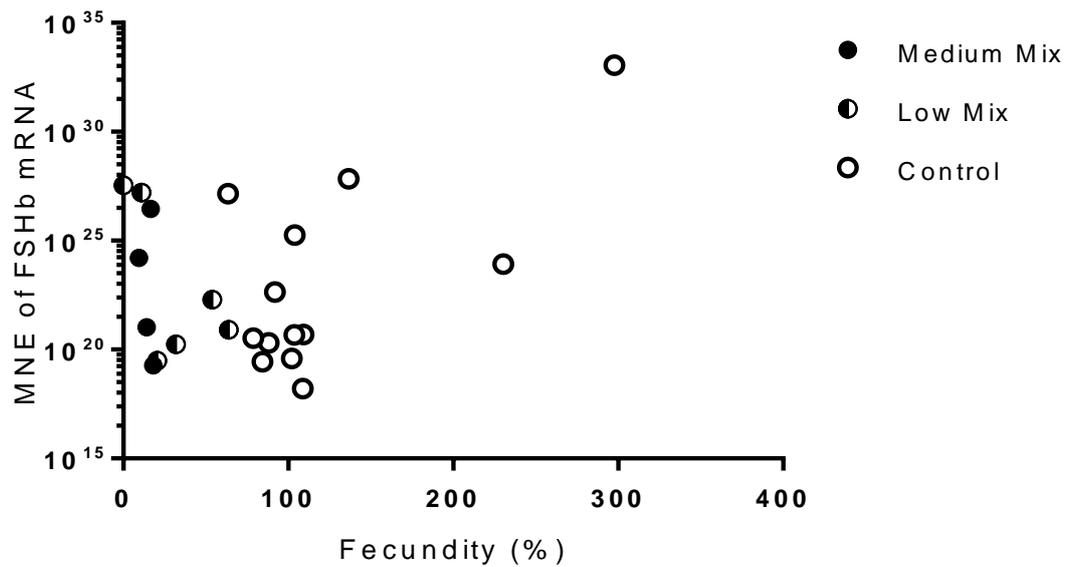
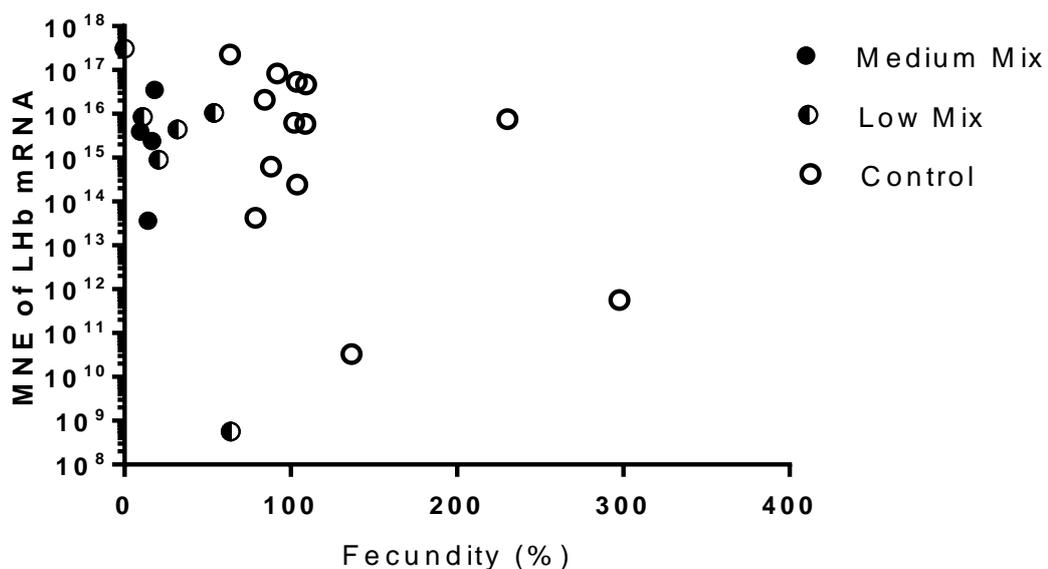


Figure 57. The relationship between Mean Normalised Expression (MNE) of FSH β mRNA and fecundity (egg production) in male *P. promelas* exposed to a mixture of steroidal pharmaceuticals, normalised to 18S expression. Individual dots represent single individual fish. MNE data on the y axis are represented on a logarithmic scale. Fecundity is represented as egg production as a relative ratio, expressed as a percentage of egg production during the exposure period compared with the pre-exposure period (i.e. 100% equals no change in performance). There was a statistically significant positive correlation (Pearson's Correlation Coefficient). Sample size, n=23

LH β mRNA levels in male fish were not correlated with fecundity (fig. 58) (Spearman's coefficient, $r=-0.16$). Although fecundity appeared to decrease with increasing LH β mRNA levels, this correlation was not significant ($p=0.47$), suggesting that random chance could be accounting for the weak trend. Furthermore, the confidence belt of the correlation ranged from -0.54 to 0.28, suggesting that no correlation between fecundity and LH β mRNA was observed. Each treatment group was also analysed individually for correlations. In the control fish, LH β mRNA and fecundity data were not normally distributed (Shapiro-Wilks test, 0.711, $df=13$, $p\leq 0.001$; 0.617, $df=13$, $p<0.001$ respectively). LH β mRNA levels were somewhat negatively correlated with fecundity (Spearman's coefficient $r=-0.40$) however the correlation was not statistically significant ($p=0.17$), suggesting that this correlation could be due to random chance. In the fish exposed to the lowest mixture concentration, fecundity data followed a Gaussian

distribution, however LH β mRNA data did not, and therefore non-parametric analysis was used. LH β mRNA was negatively correlated to fecundity ($r=0.60$), whereby higher expression of the LH β gene was correlated with lower fecundity, however this was not statistically significant ($p=0.24$) suggesting that this apparent correlation could be due to random chance (a likely occurrence since sample sizes were low, $n=6$). In fish exposed to the medium concentration, fecundity was normally distributed, however MNE of LH β mRNA did not follow a Gaussian distribution (Shapiro-Wilks, 0.944, $df=4$, $p=0.677$; 0.720, $df=4$, $p<0.05$ respectively), and therefore non-parametric analysis was used. Fecundity was positively correlated to LH β mRNA levels, however this was not statistically significant ($p=0.75$), indicating that this trend could be (and most like is) due to random chance, due in large to the small sample sizes ($n=4$).



In female fish, pituitary FSH β gene expression was not correlated to fecundity (Spearman's coefficient, $r=-0.02$; non-parametric data (Shapiro-Wilks, 0.932, $df=9$, $p=0.504$; 0.390, $df=9$, $p<0.001$)) (fig.59).

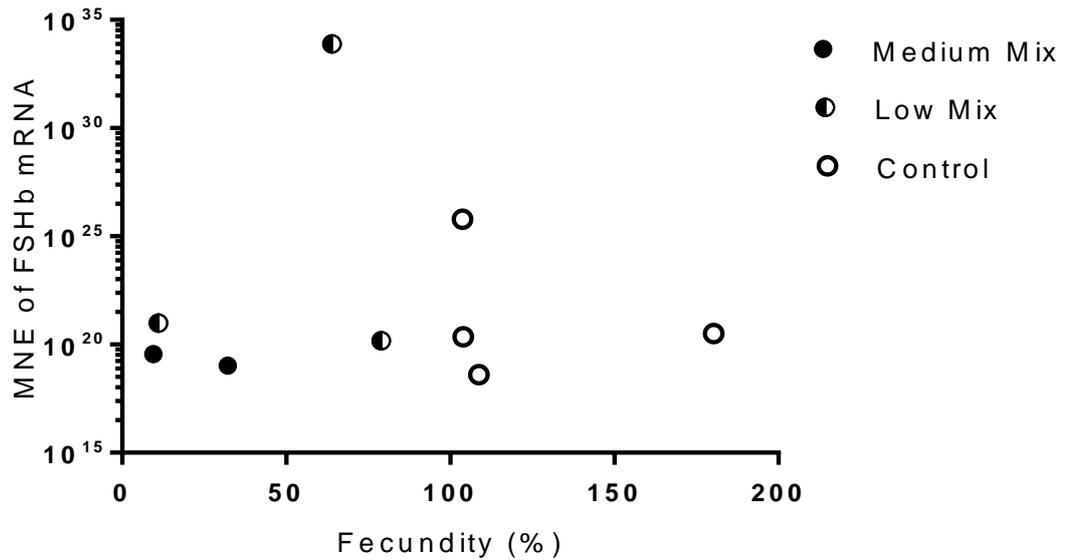


Figure 59. The relationship between Mean Normalised Expression (MNE) of FSH β and fecundity (egg production) in female *P. promelas* exposed to a mixture of steroidal pharmaceuticals, normalised to 18S expression. Individual dots represent a single individual fish. MNE data on the y axis are represented on a logarithmic scale. Fecundity is represented as egg production as a relative ratio, expressed as a percentage of egg production during the exposure period compared with the pre-exposure period (i.e. 100% equals no change in performance). No statistically significant correlation (Spearman's Coefficient). Sample size, $n=9$.

In female fish, an apparent negative correlation was observed in fecundity and LH β gene expression (fig. 60) (assessed by non-parametric analysis) (Spearman's coefficient, $r=-0.47$), however this was not statistically significant (expected due to low sample size) ($p=0.21$) and therefore is likely due to random chance.

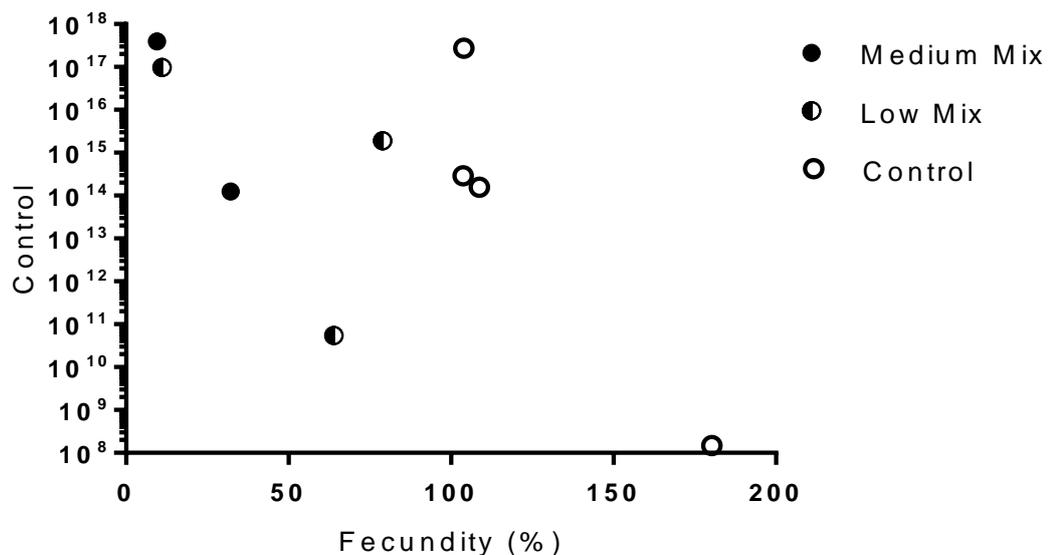


Figure 60. The relationship between Mean Normalised Expression (MNE) of LH β expression and fecundity (egg production) in female *P. promelas* exposed to a mixture of steroidal pharmaceuticals, normalised to 18S expression. Individual dots represent a single individual fish. MNE data on the y axis are represented on a logarithmic scale. Fecundity is represented as egg production as a relative ratio, expressed as a percentage of egg production during the exposure period compared with the pre-exposure period (i.e. 100% equals no change in performance). Sample size, $n=9$.

It is likely that the statistical tests are not robust enough to account for the low sample sizes (n) in this dataset, therefore detecting any statistical significance in the relationship between FSH β and LH β gene expression is unlikely.

5.3.9. Secondary Sexual Characteristics

Hepatosomatic Index (HSI) values were comparable to those recorded in experiment 1 (data presented in chapter 4). There was a small observable difference in HSI in males between treatment groups (fig. 61a), whereby the highest mixture concentration tested (medium mix) appeared to reduce the HSI of male fish. Data were normally distributed (Shapiro-Wilks test, 0.947, $df=32$, $p=0.119$), and variances were found to be equal (Levene's test, 0.318, $df=2$, $df=29$, $p=0.730$). There was no statistically significant difference in HSI across treatment groups, however the difference was close to the 5% significance level (one-way ANOVA, $f=3.231$, $df=2$, $p=0.054$). Post-hoc analysis revealed a statistically significant difference between the low and medium concentrations (Tukey's test, $p<0.05$), however this was not dose-dependent, and no significant difference was observed between the control and either of the two mixture concentrations.

There was little observable difference in HSI of females between treatment groups (fig.61b). Data were normally distributed (Shapiro-Wilks test, 0.977, $df=31$, $p=0.712$) and found to have equal variances (Levene's test, 0.077, $df=2$, $df=28$, $p=0.926$). No significant difference in female HSI was observed across any of the treatment groups (one-way ANOVA, $f=2.814$, $df=2$, $p=0.077$). Post-hoc analysis revealed no significant differences between any of the treatment groups.

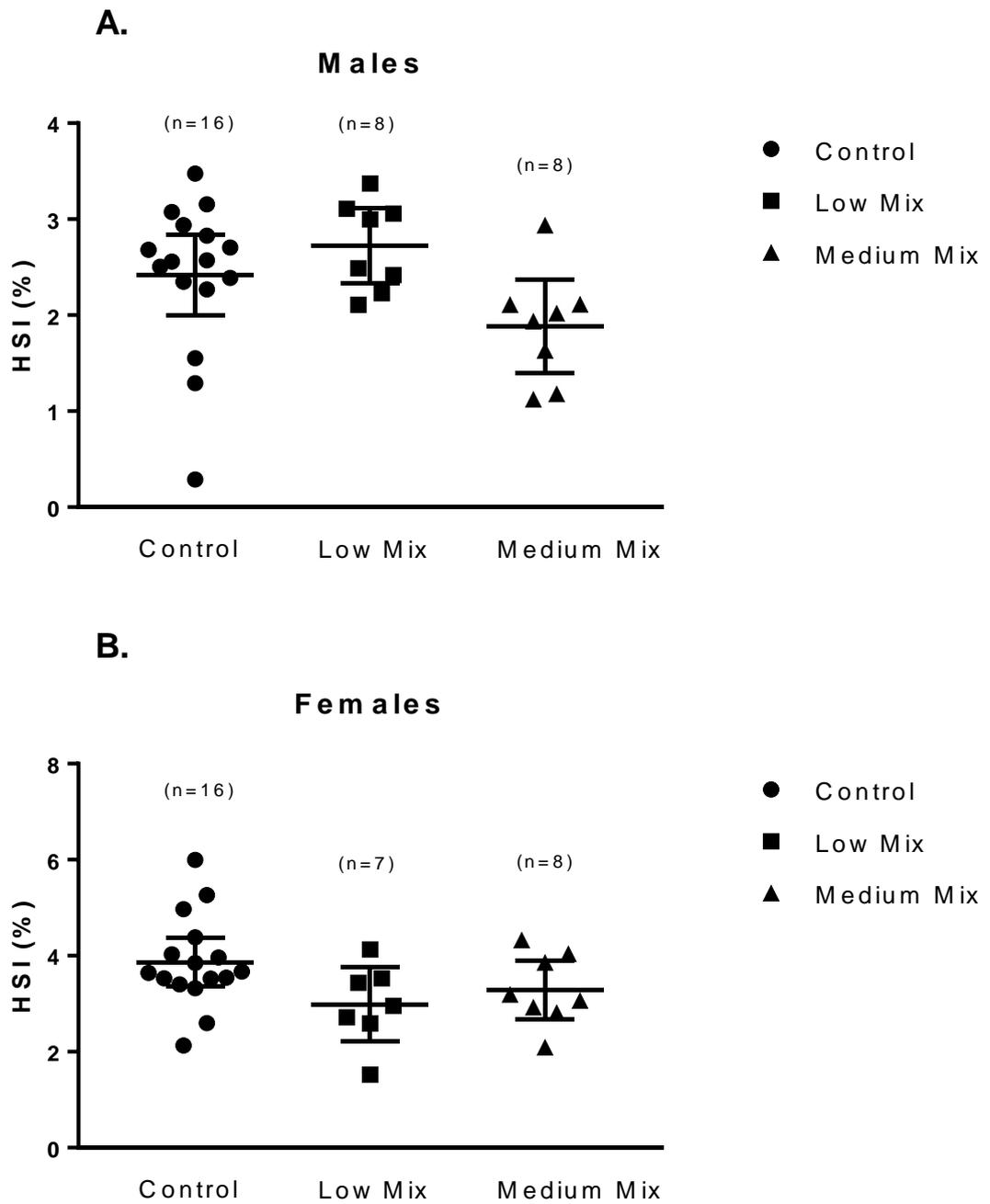


Fig 61. Hepatic somatic index (HSI) for a). male and b). female *P. promelas* exposed to two concentrations of the mixture. Each individual symbol represents an individual fish. Horizontal line indicates the mean. Error bars represent 95% confidence intervals. n=sample size.

Gonadosomatic Indices (GSI) were comparable to those recorded in experiment 1 (data presented in chapter 4). Male GSI appeared to decrease slightly with increasing concentration of the mixture (fig.62a). Data were normally distributed (Shapiro Wilks test, 0.972, $df=32$, $p=0.567$) and had equal variances (Levene's test, 1.691, $df=2$, $df=29$, $p=0.202$). No statistically significant difference was observed between any of the treatment groups (one-way ANOVA, $f=1.548$, $df=2$, $p=0.230$).

Female GSI increased with exposure to the mixture in a dose-dependent manner (fig.62b). At the highest concentration, mean GSI of female fish was almost double that of the controls. Data were normally distributed (Shapiro-Wilks test, 0.938, $df=31$, $p=0.071$), and had equal variances (Levene's test, 3.023, $df=2$, $df=28$, $p=0.065$). A statistically significant difference was observed in the GSI of females between treatments (one-way ANOVA, $f=25.932$, $p<0.001$). Post-hoc analysis revealed that there was a statistically significant difference between the control and the two mixture concentrations (Tukeys test, $p<0.001$), however the two mixture groups were not statistically difference from each other (Tukeys test, $p=0.935$).

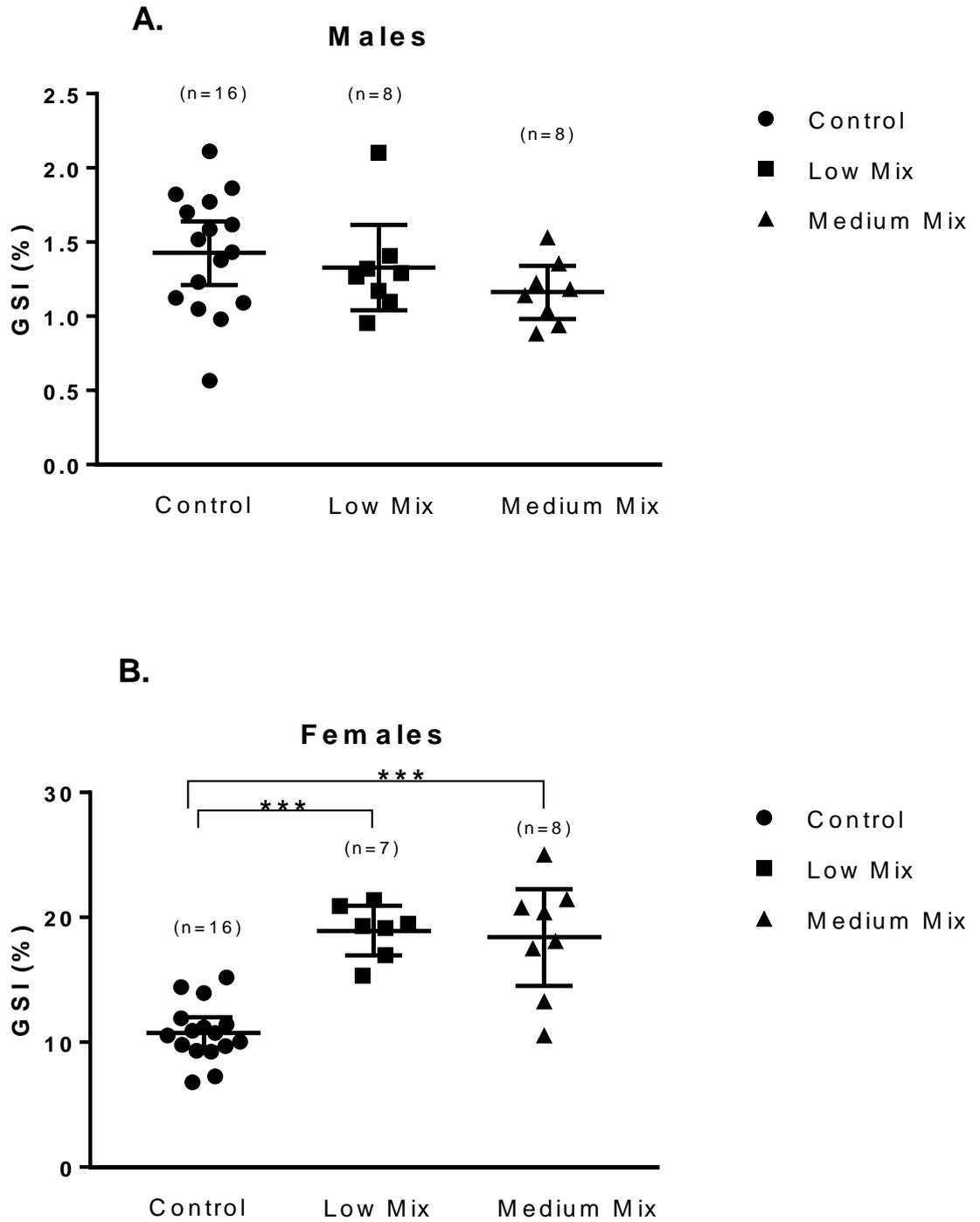


Figure 62. Gonadosomatic Index (GSI) for a). male and b). female *P. promelas* exposed to two concentrations of the mixture. Each individual symbol represents an individual fish. Horizontal line indicates the mean. Error bars represent 95% confidence intervals. * denotes a statistically significance difference. n=sample size.

Fin spot appearance in female fish was induced by exposure to the mixture (fig.63). Data were not normally distributed (Shapiro-Wilks, 0.696, df=31, $p < 0.001$), and had equal variances (non-parametric Levene's test, $f=3.037$, df=2, $p=0.064$). There was a statistically significant difference in the number of fin spots that developed on female fish between treatment groups (Kruskal Wallis, Chi-Square, 27.207, df=2, $p < 0.001$). Both mixture groups were statistically different from the controls ($p < 0.001$), however the low and medium mixture groups were not statistically different from each other (Chi-Square, 1.143, df=1, $p=0.285$).

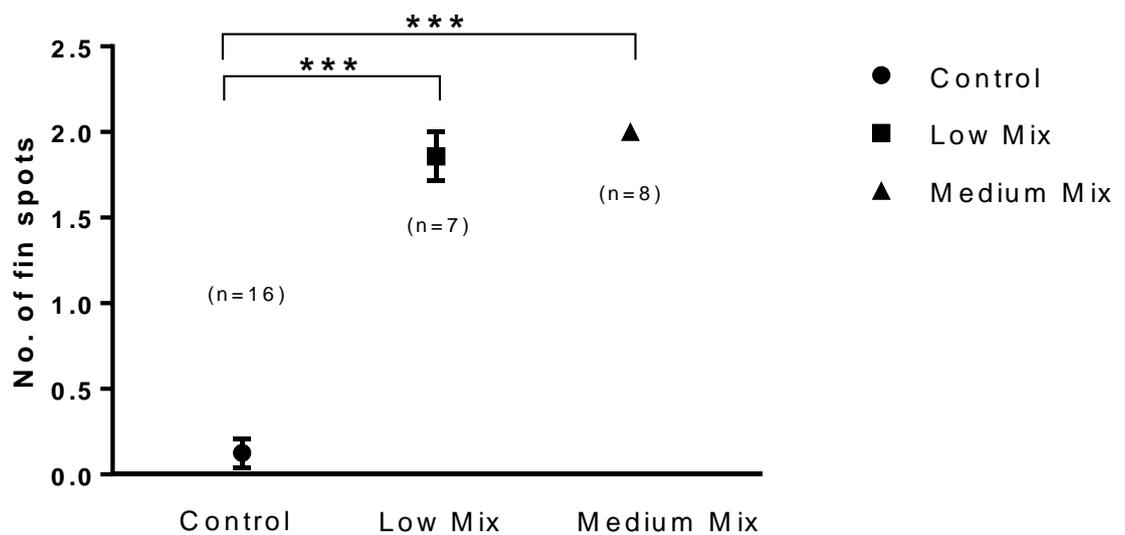


Figure 63. The appearance of fin spots in female *P. promelas* exposed to two concentrations of the mixture, plus a control group.* indicates statistical significance. Data are presented as means \pm SEM.

Nuptial tubercles were observed in all females in the medium mixture group, and in six out of seven females in the lowest mixture concentration. No nuptial tubercles were observed in any females in the control group (n=16) (fig 64). Appearance of a dorsal fatpad was only recorded in one female in the medium mixture group.

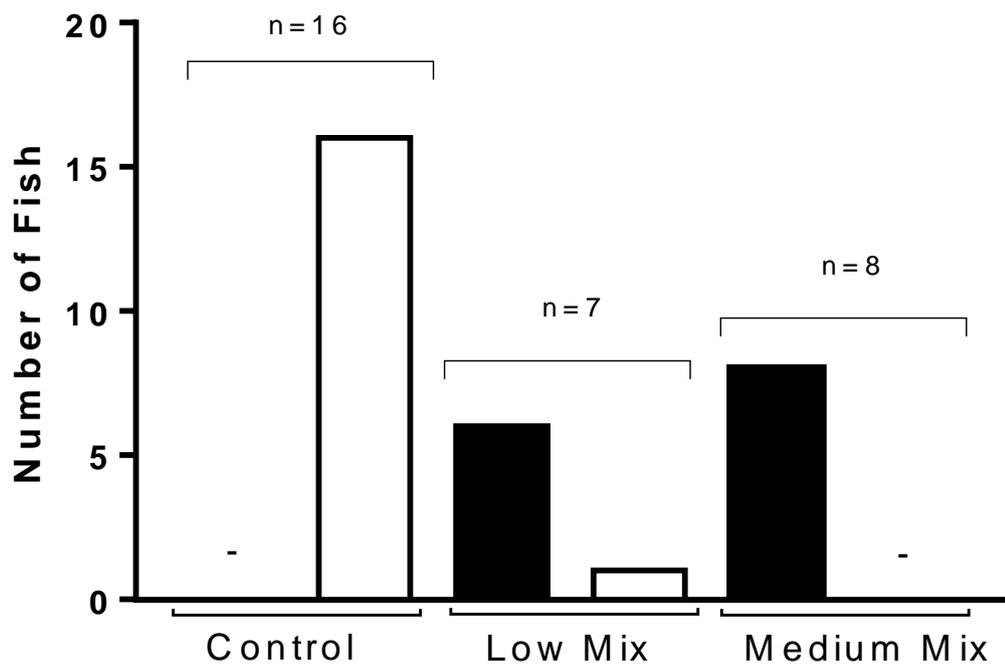


Figure 64. The number of female fish with tubercles present (black shaded bar) or absent (white unfilled bar) after a 21-day exposure to a mixture of five synthetic steroidal pharmaceuticals. n = sample size.

5.4. Discussion

Transcription of the pituitary FSH β and LH β genes was investigated, with the aim of facilitating further understanding of the mechanisms behind the impairment of reproduction and endocrine function by a mixture of steroidal pharmaceuticals observed in experiment 1, as described in chapters 3 and 4, (and confirmed in the experiment described in this chapter).

The results of the mixture experiment presented in this chapter demonstrated that results obtained from the first mixture experiment are repeatable. Fecundity was significantly reduced in a dose-dependent manner in both mixture experiments. Furthermore, univariate analysis revealed no statistically significant differences in the fecundity data between the two experiments. The repeatability of the multicomponent mixture experiments demonstrates that the evidence supporting mixture effects as a result of exposure to relatively low (ng/L) concentrations of synthetic steroids is robust and reliable. In addition, the secondary sexual characteristic endpoints quantified in both mixture experiments demonstrate that the effects are likely to be due to the androgenic properties of the mixture. Since four of the five compounds in the mixture are either androgens or have androgenic properties (i.e. progestagens and glucocorticoids), the results support the hypothesis that the main mechanism causing a reduction in fecundity is androgenicity.

Masculinisation of female fish due to exposure to environmental androgens and androgen mimics has been reported widely in the literature (Ankley et al, 2003), although to a much lesser extent than environmental estrogens. In large this has been due to the lack of androgen biomarkers available for fish (Björkblom et al, 2013). Nevertheless, recent studies have led to the development of sensitive biomarkers of androgen (and antiandrogen) exposure for fish (Katsiadaki et al, 2002; Katsiadaki et al, 2006), and androgenicity of environmental samples can now be investigated using a reliable biomarker. Furthermore, transcriptomic profiling of androgen-responsive genes has been investigated in some fish species, and has revealed candidate genes indicative of

exposure to environmental androgens, including hydroxysteroid (17 β) dehydrogenase 12a (hsd17b12a) in the gonads (Dorts et al, 2009).

In the experiment presented here, female GSI increased significantly in a dose-dependent manner. At the highest concentration, the GSI was almost twice that of control fish. If exposure to the mixture of synthetic steroids is preventing ovulation in females, as suggested by the significant reduction in the production of eggs, the ovary of the fish could be expected to contain more oocytes, and hence would be enlarged, leading to a higher than average GSI. A less pronounced effect on female GSI was observed in the first mixture experiment (presented in chapter 4), however since the effects on fecundity were also less pronounced, the effects on GSI are in line with overall observations in the differences between the two exposures.

Quantification of the exposure concentrations in experimental tanks from experiment 2 (presented in this chapter) demonstrated that they were higher than those observed in experiment 1. For example, measured concentrations for EE2 in experiment 1 were 87.9% and 79.9% of the nominal for the lowest and medium mixture concentrations, respectively. However, in the second experiment, the same nominal mixture concentrations produced much higher measured concentrations, of 170% and 226% respectively, almost 2 and 3-fold higher than in experiment 1. Given that the spiked samples (spiked with the corresponding concentration of the mixture) also produced similar recoveries to the experimental samples, there could be a number of explanations for the higher than nominal recoveries. Standards used in the assays were spiked using the same master stocks of concentrated chemicals used to make the experimental dosing stocks, which could suggest that technical issues or errors led to higher than nominal concentrations in the original master stock solutions. However, following the results of this experiment, stock calculations and dosing rates were rigorously checked for any potential errors, and none were found. Furthermore, stock calculations, dilution rates and flow rates were identical for this experiment as they were in experiment 1 (given that exactly the

same mixture ratio and concentrations were used), and therefore errors in calculations are unlikely. An alternative explanation could be that the discrepancies are due to the quantification process. Although the same commercial ELISA kit was used to quantify EE2 concentrations in both experiments, over-estimation of concentrations of EE2 could have arisen during the ELISA assay process due to a number of factors including pipetting errors, assay dilution step errors and/or data analysis errors. However the later reason is unlikely, since the results were subsequently reviewed and verified by an independent research technician. A further explanation could be due to matrix effects, interference by other unknown substances, or by other compounds in the mixture. Given that four other steroidal compounds were present in the samples, interference could be a factor, and most likely also occurred in experiment 1, although to a different degree. Although nominal concentrations of EE2 were over-estimated, the presence of the chemical in experimental tanks was confirmed, and thus effects on fecundity are likely to be due to the presence of the chemical(s) rather than a random effect. In summary, it is likely that fish were exposed to concentrations similar to nominal (for reasons discussed above), and hence one or more technical problems caused the over-estimation of the actual exposure concentration.

However, in line with the higher measured concentrations of EE2, more pronounced effects on the reproductive capacity of breeding pairs was also observed in the second experiment, in addition to more pronounced effects on some secondary sexual characteristics, suggesting that actual concentrations in the tanks may have been slightly higher, and that the more pronounced effects on fecundity were due to the higher concentrations in the fish tanks of experiment 2.

5.4.1. Extraction efficiency

As a result of RNA extraction pilot studies, it was evident that enough high quality RNA could be extracted from individual pituitary tissues for downstream processing, and therefore there was no requirement to pool samples. The ability to extract and quantify

mRNA from individual pituitary glands proved important for this study, since natural baseline expression between individuals can be highly variable. Furthermore, expression of the LH and FSH beta-subunit genes also depends on sex, age, stress, and reproductive cycle stage at the time of sampling. The ability to quantify without pooling was crucial to enable the robust comparison between individuals exposed to different treatments, as well as enabling the investigation of any correlations between pituitary gonadotropin expression and egg production within individual fish.

Although RNA was efficiently extracted and purified using the RNeasy Micro Kit (Qiagen), some samples had to be disregarded due to low RNA yield or highly degraded RNA. Since the pituitary glands of fathead minnows are extremely small, they can be difficult to dissect and remove, and to extract enough high quality RNA from individual samples to yield efficient amplification. Furthermore, since females are smaller in size than males, these difficulties are usually augmented in female fish. As a result, female sample sizes were low, with only 9 individual female fish yielding enough high quality RNA for downstream analysis (compared with 23 males). Since sample sizes were low (particularly for female fish), conclusions based on the expression analysis of target (and reference) genes were difficult to reach.

RNA degradation can arise from sample storage, or during the extraction and purification process itself. Since samples were immediately transferred to RNALater after dissection, and stored at -20°C, sample storage is unlikely to have caused the degradation, since RNALater-treated samples are highly stable for >12 months at -20°C. In the case of the study presented here, RNA degradation likely arose during the extraction and purification process. Several improvements could be made to improve the RNA extraction and purification process in order to recover high quality RNA from more individuals. For example, additional optimisation of homogenisation methods could be undertaken, since improper homogenisation can result in loss and/or degradation of RNA. Pituitary tissue was homogenised using mechanical lysis in a Tissue Lyser (Qiagen) according to the

protocol supplied with the extraction kit (for extraction from animal tissue). However, since the pituitary glands of fathead minnows are small, extended lysis using the bead-beating technique as employed here could have caused some degradation of the RNA. Additional tests trialling alternate homogenisation methods such as chemical or enzymatic lysis, or extending/reducing bead beating time during mechanical lysis, could ascertain the most appropriate lysis method for this particular tissue. In addition, RNases that are released during the lysis step can cause the degradation of RNA. However, beta-mecaptoethanol (β -ME), a reducing agent that denatures RNases, was added to the lysis buffer prior to homogenisation to ensure the removal of RNases in all samples tested in this experiment, so this is unlikely to have been an issue. Furthermore, genomic DNA or protein carry-over can occur when isolating RNA, which can cause problems with RNA quality and affect downstream processes. Although all samples extracted in the presented study were treated with DNase I to remove genomic DNA, and proteinase K to remove proteins, there may have been some residual carry over, indicated by the 260/280 and 260/230 ratios by Nanodrop spectrophotometry.

Inhibitors can also affect the quality of RNA post extraction. Inhibitors can include proteins, polysaccharides or residual detergents. Column purification such as that used in the RNeasy Micro Kit utilised in this study is typically efficient at removing such inhibitors, however columns can clog and therefore re-purification is sometimes necessary using fresh elute columns. Inappropriate tissue size can also lead to RNA degradation, particularly where the sample volume is too large. However, this is unlikely to have been an issue in this case since tissue volumes were small and within a suitable range for use in the RNeasy Micro extraction kit.

5.4.2. Housekeeping Genes

The results of this study demonstrate that both 18S ribosomal RNA (18S) and ribosomal protein L8 (rpl8) are suitable reference genes for target gene normalisation for this experiment. The absence of variability in product amplification (quantified by comparing

cycle threshold values) between treatment groups and the similar Ct values across samples suggest that either of these reference genes were suitable for normalisation of target genes. Previous studies have obtained similar conclusions. For example, Filby and Tyler (2007) designed an experiment to test several commonly used housekeeping genes in expression profiling studies in fish exposed to environmental estrogens. Similar to the results described here, the Filby and Tyler study reported that expression levels of the 18S and rpl8 genes in adult fathead minnows were unaffected by a 21-day exposure to 10ng/L of EE2, and are therefore considered to be suitable reference genes. Since the 18S gene was (as expected) expressed at higher levels than the rpl8 gene in this study, and therefore may introduce less variability between individuals, 18S was selected as the reference gene. Furthermore, since 18S is more commonly used as a reference gene (Thellin et al, 1999; Schmittgen and Zakrajsek, 2000; Radonić et al, 2004), owing to its invariant expression across different tissue types and with experimental treatments, selecting 18S allows for more consistency and ease of comparison between similar studies in the literature. The appearance of two apparently distinct groups in the expression data of both 18S and rpl8 in all treatment groups was observed, are likely to be technical artefacts of the extraction and analysis process, and considered unimportant in the normalisation process.

5.4.3. LH β mRNA levels in male *P. promelas*

The results obtained in the presented study revealed that pituitary gland LH mRNA expression decreased in male *P. promelas* exposed to the highest concentration (1.16 μ g/L) of the five compound mixture. Levels of LH β mRNA in male fish in the lowest mixture concentration (251ng/L) increased relative to the respective control groups, and therefore the apparent effects did not follow a linear dose-dependent relationship. However, the apparent differences in LH β mRNA levels were not statistically different from the controls, and therefore changes due to random chance could not be ruled out. In line

with the findings observed in this study, many additional studies investigating the effect of steroids on the expression of FSH β and LH β also demonstrated concentration-independent changes (Huggard-Nelson et al, 2002; Lin and Ge, 2009; Harding et al, 2016). In males, exposure to estrogens such as E2 causes increased estradiol binding in the leydig cells in the testis, thereby inhibiting the synthesis of androgens. If the overall effect of the mixture was estrogenic, this could account for the reduction in breeding capacity of males. Furthermore, decreased pituitary LH β transcript levels in male fish could indicate that plasma LH levels were high and that a positive feedback to the pituitary was occurring. However, in contrast to the results presented here and those of other recent studies (discussed), Yadetie and Male (2002) observed no effect on pituitary expression of LH β in juvenile male Atlantic salmon (*Salmo salar*) exposed to either 4-nonylphenol (NP) (125mg/kg body weight) or E2 (5mg/kg body weight).

Concentration-independent changes in expression levels of pituitary gonadotropin genes could be explained by the complex nature of positive and negative feedback mechanisms controlling the release (and therefore mRNA levels) of pituitary gonadotropins. Sex steroids (either endogenous or exogenous) can have a positive or negative feedback effect on the HPG axis, depending on the concentration of the hormone that is required at that time to meet the physiological/reproductive demands of the fish (Arcand-Hoy and Benson, 1998). For example, higher than required testosterone levels in male testis typically lead to negative feedback at the level of the hypothalamus, thereby decreasing the release of GnRH, and subsequently decreasing the release of LH (and FSH) from the pituitary to suppress the production of more testosterone and DHT. Since the mixture produced demonstrable androgenic properties, and caused a suite of androgenic effects in the fish, elevated levels of androgens in circulation (and therefore in the gonads) in male fish could be causing negative feedback to the hypothalamus to suppress the release of gonadotropins. Since LH stimulates leydig cells to synthesise testosterone, the suppression of LH therefore suppresses the synthesis of testosterone. Usually, a

decrease in LH causes a decrease in testosterone and DHT, which then leads to negative feedback stimulating the hypothalamus to release gonadotropins, but since androgenic effects may be maintaining the levels of androgens in circulation, LH expression in the pituitary remains low. Since testosterone is essential for the production of spermatozoa, this could suggest that androgen levels (testosterone and DHT) in the gonads of male fish remain elevated and spermatogenesis is still occurring. If this hypothesis is correct, it could be suggested that the reduction in fecundity is largely due the effects of the mixture on reproductive processes in female fish. Furthermore, the increase in LH β mRNA levels in males at the lowest concentration of the mixture may be due to the levels of gonadal androgens in male fish not reaching a sufficient threshold to initiate the feedback to the pituitary to cease synthesising LH. In this manner, LH β mRNA levels remain elevated and the synthesis of endogenous androgens still occurs. Although not within the remit of this study, this hypothesis could be further investigated by measuring the levels of testosterone and DHT (and other sex steroids) in plasma and in gonadal tissues, for example using a radioimmunoassay, and correlating it with the levels of LH β mRNA in the pituitary. However, there was no correlation between LH β gene expression and fecundity in males, and as previously discussed, relatively low sample sizes in this study prevent any substantial conclusions on the effect of the mixture on LH β mRNA levels in male fish from being drawn.

5.4.4. LH β mRNA levels in female *P. promelas*

The results from this study observed an increase in LH β mRNA levels in the highest mixture treatment group compared with the control and the lowest treatment group in female fish. These results are in line with expectations based on reports from the literature. For example, the endogenous estrogen E2 is known to stimulate LH β mRNA levels and LH synthesis in fish (Yaron et al, 2003). Previous studies have reported similar findings in pituitary LH β mRNA levels from studies with salmonids. For example, Yadetie and Male (2002) reported a 6-fold induction in the level of LH β mRNA in juvenile female

Atlantic salmon (*Salmo salar*) exposed to either 4-nonylphenol (NP) or E2 via injection (125mg/kg and 5mg/kg body weight respectively). Earlier research has found that exposing immature trout to estrogens or androgens causes an increase in pituitary LH β mRNA levels (Breton et al, 1997; Dicky and Swanson, 1998). For example, Dickey and Swanson (1998) reported that a seven-day exposure to both estradiol (E2) and testosterone (individually) resulted in a significant increase in pituitary LH β mRNA levels in maturing female coho salmon (*Oncorhynchus kisutch*) relative to a control group in two separate exposure experiments. However unlike the presented study, increased LH β mRNA was also observed in male coho salmon in one experiment.. Although the findings are similar for LH β mRNA levels in females, the fishes employ very different reproductive approaches, some being seasonal spawners with an annual reproductive cycle, and therefore the gonadotropin and sex steroid profiles are typically different. Fathead minnows, unlike many fish, are not periodic annual spawners, and therefore it is reasonable to expect differential regulation of pituitary gonadotropins in this species.

Similar results have, however, also been documented *in vitro*, from cultured zebrafish pituitary cells. Lin and Ge (2009) exposed primary zebrafish pituitary cell cultures to 1, 10 and 100nM of estradiol and testosterone and found a significant upregulation in LH β mRNA levels. Furthermore, upregulation following exposure to EE2 did not follow a dose-response relationship. Rather, expression levels were elevated at all concentrations relative to the control, but were statistically similar to each other, suggesting a potential maximum expression of LH β .

Similar to the findings presented here for female pituitary mRNA levels of LH β , Harding et al (2016) reported a significant increase in the levels of LH β mRNA at 2 and 10ng/L of EE2 after exposing juvenile coho salmon (*Oncorhynchus kisutch*) for 72 hours. However, the androgenic compound trenbolone did not initiate any changes in LH β mRNA levels at concentrations of 20 and 200ng/L. The results from the Harding et al study suggest that the changes in LH β mRNA levels are largely due to estrogenic, not androgenic, activity, at

the concentrations tested, in contrast to the results observed here. However, the Harding et al study was a chronic exposure, in which fish were exposed for 72 hours only, whilst fish in the presented study were exposed to the mixture of steroids for 21(\pm 3) days. Furthermore, profiles of gonadotropins and sex steroids in juvenile fish are significantly different to the profiles in fully sexually mature adults, and therefore comparing the results of the two studies can only offer limited conclusions.

The results obtained in this experiment demonstrated no correlation between LH β mRNA levels and fecundity in female fish. A surge of plasma LH in females typically induces egg maturation and ovulation, therefore higher LH β mRNA levels could be expected to correlate well with increased fecundity. However, the results of the correlation described here suggest that the females with the highest LH β mRNA levels are not strictly the same females with the highest fecundity. Surges or dips in LH mRNA levels observed in this experiment could be due to the stage of the reproductive cycle of individual females (i.e. time since last spawning) than exposure to the mixture, since the reproductive cycle of fish pairs were not in sync. This theory is supported by other recent studies. For example, studies in salmonids have revealed that FSH and LH are differentially synthesised during different stages of the reproductive cycle (Dicky and Swanson, 2000). However, sample sizes obtained for the assessment of LH β and FSH β mRNA levels in female fish were low and unequal across treatment groups, therefore reducing the power of the correlation analysis. Due to the small sample sizes, the variation in LH β mRNA expression observed in this study could be due to random chance. Further investigation of the effects of the mixture on pituitary gonadotropin expression incorporating larger sample sizes would be required before any substantial conclusions could be drawn.

5.4.5. FHH β b mRNA levels in male and female *P. promelas*

In the presented study, FSH β mRNA levels in male fish were apparently downregulated with increasing concentration of the mixture. Although the effect was pronounced (and thus represented on a logarithmic scale in the results section), the effect of the mixture

was not statistically significant. As previously discussed, since sample sizes were relatively low, the statistical power of the tests used (and that of any other statistical test) to analyse the data were not robust enough to determine whether the results were related to the exposure or whether they were due to random chance. Thus it is more appropriate to conclude that, as a result of exposure to the mixture of synthetic steroids presented in this thesis, no determinable effects were observed on FSH β mRNA levels were observed, though it is likely that effects do exist, and effects would likely be demonstrated if sample sizes were expanded.

Other studies have demonstrated effects on FSH β expression in fish exposed to similar compounds. For example, Dickey and Swanson (1998) observed a decrease in pituitary mRNA levels of FSH β in male coho salmon exposed to estradiol (E2). FSH synthesis in male fish stimulates primary spermatocytes to undergo meiosis to form secondary spermatocytes, and thus leads to the promotion of spermatogenesis. Therefore, if FSH levels are decreased, spermatogenesis may not be happening as efficiently, potentially leading to reduced fecundity in males. Furthermore, since FSH is involved in steroidogenesis and gametogenesis, decreased levels of LH β mRNA could lead to depleted FSH plasma levels and therefore lower steroidogenesis and gametogenesis in males. Although the statistical significance of the results presented here could not be determined, it could be that the decrease in FSH β mRNA levels in male fish is related to exposure to the mixture (and not random chance), and that increasing the sample sizes in the experiment would reveal a statistically significant dose-dependent effect.

In female fish, FSH β mRNA levels were downregulated at the highest concentration (1.6 μ g/L total mixture concentration) relative to the control, however at the lowest concentration (252ng/L total mixture concentration) there was an upregulation of FSH β (relative to the control). However, since these results were not statistically significant (due to the low sample sizes, as previously discussed), it should be concluded that this study showed no demonstrable effects on FSH β mRNA levels in female fish as a result of

exposure to the mixture. Previous studies investigating the effects of sex steroids on expression of pituitary gonadotropin transcripts have reported variable findings. For example, in contrast to the results observed in the presented study, Dickey and Swanson (1998) reported no effect of exposure to E2 in females. In contrast to the results presented here and those of Dickey and Swanson (1998), Lin and Ge (2009) observed an increase in FSH β mRNA levels in primary pituitary cell cultures from zebrafish, after being exposed to 1, 10 and 100nM of E2 and testosterone (individually). The conflicting results from this study, and those of Dickey and Swanson (1998) and Lin and Ge (2009), suggest that the nature of FSH regulation via feedback mechanisms is complex, not easily predicted, and rarely reflects a linear dose-dependent relationship.

Furthermore, temporal variations between sampling time and the stage of the reproductive cycle of experimental fish could account for the large variation and non-dose-dependent relationship in pituitary gonadotropin expression levels. A key potential limitation of analysing gene expression profiles is the temporal scale restrictions inherent with the process. For example, the fish in this study were exposed to the mixture for 21(\pm 3) days, and the tissues were collected and preserved at this time point. The profile of pituitary gonadotropin expression will inevitably vary depending on the stage of the reproductive cycle of the individual fish. Thus, if a breeding pair had recently spawned just prior to sampling, or were just about to spawn, the FSH β and LH β mRNA levels could be expected to be quite different to a fish that had not spawned for a number of days, irrespective of exposure conditions.

Previous reports have attempted to generalise the relationship between steroids and gonadotropin regulation. In general, it is supposed that E2 and testosterone have a negative feedback effect on FSH, and a positive feedback on LH (Dickey and Swanson 1998; Hellqvist et al, 2008). However the results observed in the current study and in those in the literature are often conflicting, and therefore it is apparent that the nature of

change in gonadotropins in relation to exposure to steroids is not typically linear or predictable.

In the study presented, concentration-independent changes were observed, although none of the changes in mRNA levels were statistically different. Concentration-independent changes in pituitary gonadotropins have also been observed elsewhere. In a similar study by Harding et al (2016), exposure of juvenile coho salmon to EE2 caused changes in gonadotropin expression that did not follow a dose-dependent relationship. For example, EE2 exposure caused a decrease in LH β mRNA levels at 2ng/L relative to the control, and an increase at 10ng/L, although, as with the results presented here, none of these results were statistically significant. In the same study by Harding and colleagues, exposure to trenbolone caused no change in LH β mRNA levels.

In this study, the absence of significant effects of the mixture on FSH β and LH β expression is likely due to the low sample sizes, particularly for the females, and the high levels of variability between individuals. Although the statistical methods applied to the data should account for different sample sizes across groups, the small sample sizes due to the selection of samples with good quality RNA yield only, could weaken the conclusions reported from the data.

Nevertheless, the results from the presented study and that of Harding et al (2016) suggest that the positive/negative regulation of gonadotropins in fish exposed to these compounds is complex and does not always follow a dose-dependent relationship. The low sample sizes used in this study likely account for the variable results, since the effects could be due to random chance, however larger sample sizes were used in the Harding et al study (n=20), suggesting that even higher sample sizes still are required in order to obtain reliable and robust information on changes in LH β and FSH β mRNA levels due to exposure from these compounds.

The difficulties in regulating exposure to chemical mixtures present an inherent challenge for risk assessment. One concept that has been proposed as a useful tool in addressing these issues is that of the Adverse Outcome Pathway (AOP) (Ankley et al, 2010; Villeneuve et al, 2014). An AOP is a conceptual framework that uses existing knowledge of the link between a molecular change and an adverse outcome at a biologically-relevant level of organisation to the risk assessment in question. Therefore in using an AOP, mechanistic toxicological data can be applied to risk assessment, for example at the species or population level (Ankley et al, 2010; US EPA, 2013). AOPs have many applications, including enabling the use of biomarkers of exposure, and extrapolating effects to other species, however within the context of the work presented here, AOPs can offer support and mechanistic understanding for the prediction of mixture effects. Several organisations including the US Environmental Protection Agency (EPA), the European Commission (EC) and the Organisation for Economic Cooperation and Development (OECD) have developed the Collaborative AOPWiki, an open resource for accessing existing AOP's and for constructing new ones based on shared information (US EPA, 2013). The experimental data generated as part of the research presented here would serve to test the strength and validity of AOPs with regards to exposure to multicomponent mixtures.

5.4.6. Limitations

Although analysing changes in gene transcripts in genes associated with the regulation of reproduction is extremely useful in determining mechanisms of disruption, there are several inherent limitations. Phenotypic anchoring, the process by which observed expression changes (i.e. in LH β transcripts) are linked to changes in phenotype (i.e. fecundity) is important in order to determine actual effects of a toxicant on an organism, and is sometimes omitted from toxicogenomics studies. Predicting phenotypic effects from changes in expression of key genes can be notoriously difficult, and changes initiated at

gene transcript level are thought to be much more sensitive than changes observed on the phenotype. For example, a low concentration of EE2 might cause upregulation of the estrogen receptor and a host of estrogen-responsive genes, however the concentrations required to produce an effect significant enough to cause a phenotypic effect are typically much higher. This can lead to the over-sensitivity of some genomics assays in predicting toxicity of certain compounds. Nevertheless, the use of transcriptional profiling is instrumental in toxicant mode of action studies, in addition to predictions of phenotypic effects caused by chronic or high concentration exposure. The study presented here attempted phenotypic anchoring of changes in pituitary gonadotropin expression to reduced egg production in breeding pairs of fathead minnows exposed to a five compound steroid mixture. Although the nature of changes in mRNA levels of LH β and FSH β were not concentration-dependant, nor statistically significant, trends in mRNA levels of the two target genes were observed. Furthermore, sensitivities to changes were significantly more pronounced in the treatment groups exposed to the highest concentrations.

A further limitation of this study is inherent in the nature of tissue sample collection. Since tissue can only be collected once, and the commencement of the exposure study, the changes in gonadotropin expression observed in this study represent only a snapshot in time in a particular tissue. However, toxic effects are governed by spatial and temporal factors that interact across biological levels (Neumann and Galvez, 2002). For example, transcriptional changes observed 24 hours after continual exposure to a steroid or steroid mixture are likely to be quite different from those observed 21 days after exposure. Furthermore, changes in expression of these genes are unlikely to be uniform and consistent, rather, would represent a series of peaks and dips in the cycle to maintain homeostasis. Therefore, the choice of sampling time and day has a strong effect on the transcript profiles.

Ultimately, the major limitation and principle challenge encountered in this study was the low sample sizes. The inability to obtain enough high quality RNA from the pituitary gland

of several of the test fish (particularly the smaller females) led to high variability in the data, and therefore no conclusive effects could be established from the data analysis.

Improvements to the RNA extraction and purification process, such as investigating alternative lysis and RNA precipitation methods could help improve the sample size by increasing the quality of the RNA. Furthermore, for this study, RNA quality was determined based on 260/280 and 260/230 ratios obtained from a Nanodrop spectrophotometer. A more robust assessment of RNA quality that could have been adopted is gel electrophoresis. Amplified products could have been visualised on agarose gel to check RNA quality, with only clear bands at the correct size being used for downstream processes. However, if the technical problems experienced in this study did in fact originate as a result of the RNA extraction and purification processes (as previously discussed), then using gel electrophoresis would not serve to increase the sample size. Furthermore, since it is a more robust method of assessing RNA quality, the likelihood is that adoption of this step could have reduced sample sizes further.

5.4.7. Further work

Although the results from this study and those of others presented in the literature demonstrate the effects of synthetic steroids and their mixtures on pituitary LH β and FSH β mRNA transcripts, regulation of reproduction and sexual characteristics is in fact controlled by vast numbers of genes, gene networks and feedback mechanisms. For example, a quick search in the Comparative Toxicogenomics Database (ctdbase.org) reveals over 250 key genes involved in male sexual characteristic development in humans, a situation that is likely to be found across the vertebrate taxa. Dorts et al, (2009) investigated transcriptional responses in the gonadal tissue of female fathead minnows exposed to 1 μ g/L of the synthetic androgen 17 β -trenbolone for 4 days and observed the up- and downregulation of 99 and 741 genes, respectively. For example, kisspeptin, a G-protein coupled receptor ligand encoded by the *kiss1* gene, has been identified as a central signalling pathway in the control of reproduction in vertebrates including teleost fish

via the regulation of GnRH (Gopurappilly et al, 2013), the hormone that stimulates the synthesis of the gonadotropins LH and FSH. The transport protein 'steroidogenic acute regulatory protein (StAR), encoded by the StARD1 gene in the gonads, is also a key component regulating reproduction, and is the rate-limiting factor in steroidogenesis. Further investigation of other key genes involved in regulating reproduction, such as kisspeptin in brain tissue and steroidogenic acute regulatory protein (StAR) in gonadal tissue, could facilitate more insight into the mechanisms of action of the mixture on reproduction in pair-breeding fathead minnows. Moreover, since a validated 2000-gene microarray has been developed for the fathead minnow (Larkin et al, 2007), undertaking an array-based study on brain and gonad tissue could provide further information on the mechanisms of disrupted reproduction in breeding fathead minnows exposed to a mixture of synthetic steroids.

Many additional studies have demonstrated the effects on steroids on LH and FSH beta sub-unit mRNA levels. However, results from the literature are somewhat conflicting, and do not represent a simple linear concentration-response relationship. Furthermore, while evidence for the effect of synthetic steroids of LH β expression is convincing, research appears to suggest that FSH levels are not significantly affected by exposure to some steroids, such as estrogens and/or androgens (Dickey and Swanson, 1998; Mateos et al, 2002; Harding et al, 2016).

Since the mixture was largely androgenic (as demonstrated by the masculinisation of females through the development of male secondary sexual characteristics), it could be assumed that androgenicity of the mixture is exerting a potent effect on the HPG axis of the fish. However, results from the literature report less pronounced effects of androgens on expression of pituitary gonadotropins compared with the effects of estrogens. This could suggest that whilst the androgenic properties of the mixture are exerting a strong influence over some endpoints (i.e. the development of male secondary sexual characteristics in females), it could be likely that it is the estrogenic properties of the

mixture that are exerting a stronger effect on gonadotropin expression. Furthermore, there are limited studies on the effects of glucocorticoids and progestagens on gonadotropin expression, and therefore the likely mechanisms of these compounds on mRNA levels of FSH β and LH β in the mixture are somewhat unknown.

Next steps in this research should be to repeat the experiment with a higher number of fish in each treatment group, and to develop better molecular techniques for the extraction and purification of RNA, and the running of qPCR assays to assess changes in mRNA levels of the LH β and FSH β genes.

5.5. Conclusion

The results presented in this chapter demonstrate the repeatability and robustness of the pair breeding fathead minnow assay in assessing the effects of multicomponent steroidal mixtures on an ecologically-important endpoint, egg production. The experiment produced comparable results with the mixture experiment presented in chapters 3 and 4, and therefore facilitates confidence in the results. Since large *in vivo* studies such as the pair breeding assay are inherently variable in nature, the repeatability of the experiment is important.

The results also demonstrated a pronounced effect on the mixture of gonadosomatic index (GSI) of female fish, with GSI indices in fish at the highest concentration almost twice that of the control fish. Furthermore, the induction of male secondary sexual characteristics (fin spots, nuptial tubercles, dorsal fatpad) support the theory that the adverse effects on reproduction are likely due to the androgenic activity of the mixture and the resultant masculinisation of females.

There were no detectable effects of the mixture on expression of pituitary FSH β and LH β genes, due in large to the low sample sizes. Low sample sizes were a direct result of

technical difficulties during the tissue excision, and the extraction and purification of good quality RNA. However, although no discernible effects were demonstrated on mRNA levels of LH β and FSH β genes, the significant decrease in the production of eggs, and the significant induction of male secondary sexual characteristics and GSI in females demonstrate that the mixture is having a pronounced effect. This further supports the theory that the absence of change in mRNA levels of the gonadotropin genes in exposed fish is likely due to the random variability caused by the low sample size, rather than the fact that the mixture is truly having no effect. The results demonstrate the potential usefulness of pituitary gonadotropin expression changes in assessing the adverse effects of synthetic steroids on reproduction in teleost fish, given that the technical difficulties encountered here could be overcome.

The work presented in this chapter could be further developed by the analysis of FSH β and LH β transcript changes as a result of exposure to each of the compounds individually at the concentrations present in the mixture. This could also further develop the work on mixtures toxicity assessment, and determine whether this endpoint is robust enough for mixture prediction studies, as was undertaken for the egg production data. Although not within the remit of the project presented here, this complementary research could strengthen the findings documented in this study, and add to the current research knowledge on the effects of synthetic steroids on pituitary gonadotropins in relation to the regulation of reproduction.

Chapter 6 General Discussion and Conclusions

6.1. Summary of Results

The purpose of this research was to assess potential combination effects of mixtures of pharmaceuticals on ecologically relevant endpoints in aquatic organisms. The main focus of the research was to assess combination effects from mixtures of synthetic steroids at individually low concentrations on reproductive success in a small laboratory fish model species, the fathead minnow (*Pimephales promelas*). Preliminary research was also undertaken to investigate the effects of antineoplastic pharmaceuticals and their mixtures on growth in a unicellular microalga, *Raphidocelis subcapitata*, to facilitate training in theoretical and practical mixtures toxicology research.

6.1.1. Effects of antineoplastic pharmaceuticals and their mixtures on growth of the microalga *Raphidocelis subcapitata*

The AlgalTox F microalga growth inhibition assay proved a robust and suitable assay for testing the effects on antineoplastic compounds on an ecologically relevant endpoint. The results obtained from this research demonstrated adverse effects of antineoplastic agents on growth in *R. subcapitata*. However, inhibition of algal growth was observed only at concentrations several orders of magnitude higher than those that have been measured (and predicted) in the environment. Furthermore, concentrations required to induce an effect were also much higher than those measured from hospital effluent, the environmental matrix where concentrations would be expected to be at their highest due to point source discharge. From the results presented here and in line with other findings presented throughout the literature, it is reasonable to conclude that antineoplastic pharmaceuticals do not presently pose a great concern to aquatic wildlife, even when present as mixtures. Although combination effects on growth inhibition in *R. subcapitata* were observed in this study as a result of exposure to a mixture of five antineoplastic compounds with varying mechanisms of action, since the mixture design undertaken was relatively crude, substantial conclusions cannot be drawn. However, the study was beneficial in highlighting the issues with a basic arithmetic summation approach to

assessing mixture effects (highlighted in section 2.4.1). This facilitated enhanced knowledge and understanding of predictive mixture experimental designs and supported the development of an improved mixture study in investigating the effects of mixtures on a more biological complex endpoint in a higher organism. Since the algal growth inhibition assay is less time and cost intensive, and less biologically complex compared with the pair-breeding *P. promelas* assay, it was rational to utilise the opportunity presented to gain experience and knowledge in how, and how not, to design a mixture experiment, before undertaking experimental *in vivo* research using fish.

6.1.2. Effects of synthetic steroidal mixtures on reproductive capability in pair-breeding adult fathead minnows (*Pimephales promelas*)

The results from this study demonstrate that combination effects as a result of exposure to steroidal pharmaceutical mixtures containing individual compounds at low concentrations, are possible, and can produce dose-dependent additive effects on an ecologically relevant endpoint. Concentrations close to those measured (in the case of EE2, levonorgestrel and trenbolone) and predicted (in the case of desogestrel and beclomethasone dipropionate) in sewage effluents in the UK were found to cause a significant reduction in the reproductive output of breeding pairs of *P. promelas* that was greater than the effect of any individual compound. A further key finding of this research was that five dissimilar synthetic steroids can act in combination in a mixture to produce a ‘something from nothing’ effect on an ecologically important endpoint. At the lowest tested mixture concentration, each compound was present individually at a concentration producing an effect well below the detection limit of the assay (10% reduction in eggs). However the mixture reduced egg production by breeding pairs by almost 60% in both mixture experiments.

This study also found that these effects could be adequately predicted by the models Concentration Addition (CA) and Independent Action (IA), with the actual observed mixture effects falling between the two predictions. The results suggest that egg

production is a robust and sensitive endpoint and can be used to efficiently predict mixture effects.

The results presented here reveal that compounds with dissimilar mechanisms of action can act additively on certain endpoints (i.e. egg production), whilst displaying no mixture effects on other endpoints (i.e. gonadal histopathology, somatic index), conclusions that are based on observations from comparisons between the work presented in this thesis and that of Runnalls et al, 2013; Kugathas et al, 2013; and Runnalls et al, 2015) (table's 32a and 32b). Furthermore, to the author's knowledge, this study is the first to demonstrate more pronounced predicted mixture effects from the IA model. In general, CA typically predicts more pronounced mixture effects than IA, and is therefore often used as a worst-case approximation for risk-based mixture assessments. This study not only demonstrated that for an ecologically important endpoint (reproduction), IA can predict more pronounced mixture effects, but that observed effects can also be larger than those predicted by CA. A reduction in egg production greater than that predicted by CA occurred in both mixture studies presented in this thesis, at all concentrations tested. Thus the use of the CA model for conservative 'worst-case' approximation in risk assessments should be adopted with caution. The IA model appeared to predict mixture effects of the five compound steroid mixture more accurately than the CA model.

Table 32a. Summary of the effects of a five compound steroidal mixture, and a single compound exposure to Beclomethasone dipropionate, on pair-breeding fathead minnow (*P. promelas*) exposed for 21 days. ↓ signifies an overall decrease, ↑ signifies an overall increase, ↔ indicates no change. Solid arrows denote statistically significant results. Open arrows signify an overall trend but were either not significant or the significance was questionable. Grey shaded region – data not quantified.

Test Compound	Fecundity (egg production)	GSI	HSI	Condition Factor	Ovipositor Length (Females)	Nuptial Tubercles (Males)		Nuptial Tubercles (Females)		Dorsal Fatpad (Males)	
						Number	Grade	Number	Grade	Height/Weight	Grade
Mix Low	↓	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔
Mix Med	↓	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔
Mix High	↓	↔	↔	↔	↔	↔	↔	↑	↑	↔	↔
Bec 25ng/L	↘										
Bec 100ng/L	↔										
Bec 1000ng/L	↔										

Table 32b. Summary of the effects of a five compound steroidal mixture, and a single compound exposure to Beclomethasone dipropionate, on pair-breeding fathead minnow (*P. promelas*) exposed for 21 days. ↓ signifies an overall decrease, ↑ signifies an overall increase, ↔ indicates no change. Solid arrows denote statistically significant results. Open arrows signify an overall trend but were either not significant or the significance was questionable.

Compound	Sperm Maturation	Oocyte Development	Embryo Hatching Success 3dpf		Embryo Hatching Success 5dpf		Abdominal Girth	Fin Spots
			% Dead	% Hatched	% Dead	% Hatched		
Mix Low	Not Quantified	Not Quantified	↔	↔	↔	↔	↔	↔
Mix Medium	Not Quantified	Not Quantified	↔	↔	↔	↔	↔	↑
Mix High	↔	↔	↑	↔	↑	↓	↑	↑

The results observed here also demonstrate that reproductive impairment as a result of exposure to a five compound steroidal pharmaceutical mixture is likely due to disruption of endocrine function and steroid feedback mechanisms on the HPG axis, at and above the level of pituitary gonadotropins. Follicle-stimulating hormone beta subunit (FSH β) mRNA levels were on average downregulated in male and female fish, whilst luteinizing hormone beta subunit (LH β) mRNA was downregulated in males and upregulated in females. However, changes in FSH β and LH β mRNA expression observed in this study were not especially convincing, due to the low sample sizes as a result of technical difficulties in obtaining high quality RNA. These issues lead to the possibility that any observed changes could be due to random change and not as a result of exposure to the mixture. Repeating this experiment with larger sample sizes would serve to establish the effects of the steroid mixture of pituitary gonadotropin gene expression.

Biomarker changes observed in this study suggest that impaired reproduction is likely due to the masculinisation of female fish as a result of the androgenic properties of the mixture (indicated by the significant development of androgen controlled male secondary sexual characteristics in female fish).

6.2. Environmental relevance

The concentrations of antineoplastic pharmaceuticals tested in the algal growth inhibition assay were orders of magnitude higher than those documented to be present in the environment (even in point source matrices such as hospital effluent). However, concentrations of synthetic steroids used in the experiments presented here were within range of those measured and predicted in sewage effluent receiving waters, and were shown to significantly reduce reproductive output in breeding fish when present as a mixture. The results presented here demonstrate that environmental concentrations of synthetic steroidal pharmaceuticals have the potential to cause significant adverse effects to aquatic wildlife when present as complex chemical mixtures. Furthermore, the

demonstration of the 'something from nothing' effect of five synthetic steroids on reproduction in fish could have implications for mixture toxicity risk assessment. However, most laboratory studies could be classed as chronic exposures when compared with real-world temporal scales, where exposure to contaminants would be extended and constant. Whether this has the potential to increase adverse effects in wildlife, or whether prolonged exposure would result in some element of acclimation/adaptation, remains to be determined.

The results presented here demonstrate the importance of conducting risk assessments for aquatic organisms on mixtures of chemicals, as opposed to individual chemicals. However, the quantity and variety of 'real-world' mixtures is likely to be infinite, and thus highlights the unattainability of this requirement. In reality, the question of toxicity as a result of mixtures is currently addressed by including a so-called 'safety factor', a margin of error to act as a safety buffer – typically around a 10-fold change. Thus the Predicted No Effect Concentration (PNEC) is divided by 10 before factoring into mixture assessments to address any uncertainties or potential variations in mixture effects. However, this is an arbitrary number, and currently there is no information of whether this is robust enough to provide an adequate safety margin when considering mixture effects from mixtures containing hundreds of individual chemicals, as recent research is beginning to indicate is the case. In the case of the results presented in this thesis, a safety factor of 10 would sufficiently safeguard against mixture effects, however, the mixture presented here is composed of only 5 chemicals. Real-world chemical mixtures would be expected to contain hundreds, if not thousands of individual chemicals. If each compound (or any given number of compounds) in a mixture is contributing to the overall effect, the effect would be amplified with increasing number of compounds present. Therefore, it is somewhat difficult to determine whether the safety factor is adequate for all mixtures based on the results presented, particularly those that are likely to be present in the environment.

Furthermore, there is also the question of whether multicomponent mixtures could produce unique toxic effects when present simultaneously that individual chemicals do not. The question of synergisms and antagonisms in chemical mixtures has been debated widely in the literature, however there is no overall consensus on whether true synergisms are likely, and under what conditions they may arise, and the situation is likely to be different for most 'real-world' mixtures. Finally there is the question of the ratio of each compound in the mixture. The mixtures tested in the fish experiments presented here were all present at only one mixture ratio (equipotent, EC10s). It could be that the observed effects on fish reproduction are significantly different when this ratio is altered. Furthermore, in a 'real-world' situation, chemicals would not be present at equipotent concentrations, and therefore some chemicals would truly dominate the effect whilst some may have no effect at all.

The data presented here has significant implications for several concepts used in assessing chemical toxicity risk, discussed throughout this thesis. Predictive mixture modelling using the Concentration Addition and Independent Action models are becoming more widely used in chemical risk assessments, particularly the CA model, which is seen as a conservative 'worst-case' approximation of risk due to its additively assumptions. However, the data presented here highlight the potential danger of CA underestimating mixture effects, and therefore may not always serve as an absolute worst-case prediction. This is likely due to the fact that the presently-utilised mathematical models do not account for biological factors. Therefore there is substantial justification for the further development of these models to account for inherent biological parameters, for example the mathematical elements could be weighted to account for the fact that some biological components are more significant to the contribution towards an adverse effect than others. Furthermore, since both CA and IA assume no interaction between the components, which is not a true reflection of the reality of chemical mixtures, it would be

beneficial to further develop the models to incorporate the possibility of mixture interactions (either agonistic or antagonistic), for example using statistical probabilities.

The read-across hypothesis, a concept used to predict adverse effects of a compound in a non-target organism, states that an adverse effect will occur if, firstly, the molecular target is present, and secondly, if internal concentrations reach that of human therapeutic levels. Therefore adverse effects can be predicted. However, the data described in this thesis suggest that when present in a mixture, certain chemicals have the potential to cause adverse effects even when not present at levels equivalent to the human therapeutic dose. The results presented here could be strengthened further by quantifying each pharmaceutical steroid in the plasma of the fish and comparing internal concentrations to human therapeutic doses. Furthermore, the results demonstrated pronounced effects on some endpoints (i.e. fecundity) whilst exhibiting low or negligible effects on others (condition factor, HSI, gonadal histopathology), suggesting that effects can be endpoint specific, and that the read-across hypothesis may work well for certain endpoints but not for others. Nevertheless, the read-across approach is a valuable tool in determining potential effects of chemicals in non-target species, and serves to highlight potentially problematic chemicals.

The principle of risk ranking chemical pollutants, as discussed by Caldwell et al, 2014; Donnachie et al, 2015; and Guo et al, 2016, can be a valuable tool in prioritising chemical contaminants. However there are several limitations when considering mixtures. Hypothetically, chemicals ranked as low risk individually may become high risk when present in combination with another chemical, with the potential to act additively or even synergistically on certain endpoints. One approach in addressing this is to consider the primary toxic mode of action of the mixture in question (Guillén et al, 2012). Grouping chemicals with similar modes of action, for example in line with the Anatomical Therapeutic Chemical Category (ATC) guidelines, (Roos et al, 2012; Caldwell et al, 2014), and assessing cumulative risk should highlight high risk drug groups, and therefore

mixtures could be assessed for risk based on the number of chemicals present from these high risk groups. Though this would require detailed information and assessment of each unique mixture, and therefore the practicalities of wider applications are somewhat limited.

6.3 General conclusion

The research presented here, in line with many studies presented throughout the literature, highlights the potential for adverse effects to wildlife as a result of exposure to low concentrations of synthetic steroidal pharmaceuticals. Furthermore, the demonstration that combination effects on ecologically important processes can occur from exposure to mixtures of these compounds, each present individually at a very low concentration, highlights the need for mixture effects to be taken into consideration in environmental risk assessment, particularly for this group of compounds.

6.4 Further work and improvements

There is substantial scope to develop this research further with additional studies and refinement of the conducted studies. Several improvements, refinements and additions to the research presented, as suggested by the author, include:

- Development of methodologies for simultaneous detection and quantification of compounds in tank water samples for a full suite of measurements from all five compounds (requiring the skills of an analytical chemist). This would provide important information on the actual concentrations that the fish were exposed to, and importantly, whether the equipotent mixture ratios were maintained.
- Quantification and measurement of endogenous steroids (estradiol, testosterone and 11-ketotestosterone) in plasma of experimental fish to enhance knowledge of how steroidogenesis is disrupted by the mixture.

- Quantification and measurement of all five synthetic steroids (EE2, levonorgestrel, desogestrel, beclomethasone dipropionate and 17 β -trenbolone) in plasma of experimental fish in order to improve the knowledge on the mechanism of disrupted reproduction in pair-breeding fish. Determining whether concentrations in fish plasma need to reach human therapeutic concentrations before effects can be observed (in line with the read-across hypothesis).
- Refinement of gonadotropin gene expression experiment by increasing sample numbers. This could be achieved by additional method development during the RNA extraction and purification process. For example, testing alternative lysis techniques, such as adapting mechanical bead beating times, or including enzymatic or chemical lysis steps, and alternative RNA precipitation (i.e. using alcohol and linear polyacrylamide solution in place of elute spin columns) could aid the recovery of higher quality RNA from more samples, increasing the robustness of the data.
- Measurement of one (or more) highly specific biomarkers, for example, quantification of plasma vtg or hepatic VTG mRNA levels, in experimental fish exposed to the mixture could enhance knowledge on the mechanisms of disrupted egg production by revealing whether EE2 was significantly contributing the effect of the mixture. This could also be achieved by quantification of steroid receptor mRNA levels (i.e. er α and er β).
- Analysis of mRNA levels of a number of other key genes involved in the regulation of steroidogenesis and reproduction in a number of endocrine tissues such as steroidogenic acute regulatory protein (StAR) in the gonads, kisspeptin (Kiss1 and Kiss2) in the brain, and the steroid receptors (er, ar, gr, and pr) in the gonads (discussed above). These data could facilitate improved understanding of the mechanisms causing a decline in fecundity in pair-breeding fish.

7.0 References

- Africander, D., Verhoog, N., & Hapgood, J. P. (2011). Molecular mechanisms of steroid receptor-mediated actions by synthetic progestins used in HRT and contraception. *Steroids*, 76(7), 636–52.
- Ågerstrand, M., Berg, C., Björlenius, B., Breitholtz, M., Brunström, B., Fick, J., Rudén, C. (2015). Improving environmental risk assessment of human pharmaceuticals. *Environmental Science and Technology*. <http://doi.org/10.1021/acs.est.5b00302>
- Aherne, G. W., English, J., & Marks, V. (1985). The role of immunoassay in the analysis of microcontaminants in water samples. *Ecotoxicology and Environmental Safety*, 9(1), 79–83.
- Aherne, G. W., Hardcastle, A., & Nield, A. H. (1990). Cytotoxic drugs and the aquatic environment: estimation of bleomycin in river and water samples. *The Journal of Pharmacy and Pharmacology*, 42(10), 741–2.
- Al-Odaini, N. A., Zakaria, M. P., Yaziz, M. I., & Surif, S. (2010). Multi-residue analytical method for human pharmaceuticals and synthetic hormones in river water and sewage effluents by solid-phase extraction and liquid chromatography–tandem mass spectrometry. *Journal of Chromatography A*, 1217(44), 6791–6806.
- Algae tox kit Standard Operating Procedure. MicroBioTests. Accessed 2013. <http://www.microbiotests.be/SOPs>
- Algaebase: Listing the World's Algae. (Accessed 2016.). Retrieved from <http://www.algaebase.org/>
- American Cancer Society. Accessed 2016. Retrieved from <http://www.cancer.org/>
- Altenburger, R., Backhaus, T., Boedeker, W., Faust, M., Scholze, M., & Grimme, L. H. (2000). Predictability of the toxicity of multiple chemical mixtures to *Vibrio fischeri*: Mixtures composed of similarly acting chemicals. *Environmental Toxicology and Chemistry*, 19(9), 2341–2347.
- Altenburger, R., Krüger, J., & Eisenträger, A. (2010). Proposing a pH stabilised nutrient medium for algal growth bioassays. *Chemosphere*, 78(7), 864–870.
- Altenburger, R., Walter, H., & Grote, M. (2004). What Contributes to the Combined Effect of a Complex Mixture? *Environmental Science & Technology*, 38(23), 6353–6362.
- Amores, A., Force, A., Yan, Y. L., Joly, L., Amemiya, C., Fritz, A., ... Postlethwait, J. H. (1998). Zebrafish hox clusters and vertebrate genome evolution. *Science (New York, N.Y.)*, 282(5394), 1711–4.
- Ando, H., Swanson, P., & Urano, A. (2003). Regulation of LH synthesis and release by GnRH and gonadal steroids in masu salmon. *Fish Physiology and Biochemistry*, 28(1–4), 61–63.
- Angerer, J., Bernauer, U., Chambers, C., Chaudhry, Q., Degen, G., Nielsen, E., White, I. R. (2012). Toxicity and Assessment of Chemical Mixtures. European Commission.

- Ankley, G. T., Bencic, D. C., Breen, M. S., Collette, T. W., Conolly, R. B., Denslow, N. D., Watanabe, K. H. (2009). Endocrine disrupting chemicals in fish: developing exposure indicators and predictive models of effects based on mechanism of action. *Aquatic Toxicology* 92(3), 168–78.
- Ankley, G. T., Bennett, R. S., Erickson, R. J., Hoff, D. J., Hornung, M. W., Johnson, R. D., Villeneuve, D. L. (2010). Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environmental Toxicology and Chemistry / SETAC*, 29(3), 730–41.
- Ankley, G. T., Jensen, K. M., Makynen, E. A., Kahl, M. D., Korte, J. J., Hornung, M. W., ... Gray, L. E. (2003). Effects of the androgenic growth promoter 17-beta-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. *Environmental Toxicology and Chemistry / SETAC*, 22(6), 1350–60.
- 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), 469–83.
- Ankley, G. T., & Villeneuve, D. L. (2006). The fathead minnow in aquatic toxicology: past, present and future. *Aquatic Toxicology (Amsterdam, Netherlands)*, 78(1), 91–102.
- Antonopoulou, E., Swanson, P., Mayer, I., & Borg, B. (1999). Feedback control of gonadotropins in Atlantic salmon, *Salmo salar*, male parr.II. Aromatase inhibitor and androgen effects. *General and Comparative Endocrinology*, 114(1), 142–50
- Arcand-Hoy, L. D., & Benson, W. H. (1998). Fish reproduction: An ecologically relevant indicator of endocrine disruption. *Environmental Toxicology and Chemistry*, 17(1), 49–57.
- Arena, F. (2008). Dutasteride in the treatment of hormone refractory prostate cancer. *Minerva Urologica E Nefrologica = The Italian Journal of Urology and Nephrology*, 60(2), 71–6.
- Armstrong, B. M., Lazorchak, J. M., Jensen, K. M., Haring, H. J., Smith, M. E., Flick, R. W., Biales, A. D. (2016). Reproductive effects in fathead minnows (*Pimphales promelas*) following a 21 d exposure to 17 α -ethinylestradiol. *Chemosphere*, 144, 366–373.
- Arnold, S. F., Klotz, D. M., Collins, B. M., Vonier, P. M., Guillette, L. J., & McLachlan, J. A. (1996). Synergistic activation of estrogen receptor with combinations of environmental chemicals. *Science (New York, N.Y.)*, 272(5267), 1489–92.
- Arnold-Reed, D., Hazon, N., & Balment, R. J. (1991). Biological actions of atrial natriuretic factor in flatfish. *Fish Physiology and Biochemistry*, 9(3), 271–7.
- Arthurson, V. (2008). Proper sanitization of sewage sludge: A critical issue for a sustainable society. *Applied and Environmental Microbiology*.
- Aruoja, V. (2011). *Algae pseudokirchneriella subcapitata in environmental hazard evaluation of chemicals and synthetic nanoparticles*. Thesis submitted for award of Doctor of Philosophy. Estonian University of Life Sciences.

- Ashby, J., Lefevre, P. A., Odum, J., Harris, C. A., Routledge, E. J., & Sumpter, J. P. (1997). Synergy between synthetic oestrogens? *Nature*, 385(6616), 494.
- Backhaus, T., Altenburger, R., Boedeker, W., Faust, M., Scholze, M., & Grimme, L. H. (2000). Predictability of the toxicity of a multiple mixture of dissimilarly acting chemicals to *Vibrio fischeri*. *Environmental Toxicology and Chemistry*, 19(9), 2348–2356.
- Baronti, C., Curini, R., D'Ascenzo, G., Di Corcia, A., Gentili, A., & Samperi, R. (2000). Monitoring Natural and Synthetic Estrogens at Activated Sludge Sewage Treatment Plants and in a Receiving River Water. *Environmental Science & Technology*, 34(24), 5059–5066.
- BC Cancer Agency - Fluorouracil. (2012). http://www.bccancer.bc.ca/drug-database-site/Drug%20Index/Fluorouracil_monograph_1Sep2015.pdf
- BCCA Cancer drugs manual. Accessed 2016. <http://www.bccancer.bc.ca/>
- Belfroid, A. ., Van der Horst, A., Vethaak, A. ., Schäfer, A. ., Rijs, G. B. ., Wegener, J., & Cofino, W. (1999). Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. *Science of The Total Environment*, 225(1), 101–108.
- Berenbaum, M. C. (1989). What is synergy? *Pharmacological Reviews*, 41(2), 93–141.
- Bergmann, A., Fohrmann, R., & Weber, F.-A. (2011). Zusammenstellung von Monitoringdaten zu Umweltkonzentrationen von Arzneimitteln.
- Besse, J.-P., Latour, J.-F., & Garric, J. (2012). Anticancer drugs in surface waters: what can we say about the occurrence and environmental significance of cytotoxic, cytostatic and endocrine therapy drugs? *Environment International*, 39(1), 73–86.
- Björkblom, C., Mustamäki, N., Olsson, P.-E., Katsiadaki, I., & Wiklund, T. (2013). Assessment of reproductive biomarkers in three-spined stickleback (*Gasterosteus aculeatus*) from sewage effluent recipients. *Environmental Toxicology*, 28(4), 229–37.
- Blagosklonny, M. V. (2004). Analysis of FDA approved anticancer drugs reveals the future of cancer therapy. *Cell Cycle (Georgetown, Tex.)*, 3(8), 1035–42.
- Blair, R. M., Fang, H., Branham, W. S., Hass, B. S., Dial, S. L., Moland, C. L., Sheehan, D. M. (2000). The Estrogen Receptor Relative Binding Affinities of 188 Natural and Xenochemicals: Structural Diversity of Ligands. *Toxicological Sciences*, 54(1), 138–153.
- Blake, L. S., Martinović, D., Gray, L. E., Wilson, V. S., Regal, R. R., Villeneuve, D. L., & Ankley, G. T. (2010). Characterization of the androgen-sensitive MDA-kb2 cell line for assessing complex environmental mixtures. *Environmental Toxicology and Chemistry / SETAC*, 29(6), 1367–76.
- Bliss, C. (1939). The toxicity of poisons applied jointly. *Annals of Applied Biology*, 26, 585–615.

- Blüthgen, N., Sumpter, J. P., Odermatt, A., & Fent, K. (2013). Effects of low concentrations of the antiprogestin mifepristone (RU486) in adults and embryos of zebrafish (*Danio rerio*): 2. Gene expression analysis and in vitro activity. *Aquatic Toxicology (Amsterdam, Netherlands)*, 144-145, 96–104.
- Boedeker, W., Drescher, K., Altenburger, R., Faust, M., & Grimme, L. H. (1993). Combined effects of toxicants: the need and soundness of assessment approaches in ecotoxicology. *Science of The Total Environment*, 134, 931–939.
- Booker, V., Halsall, C., Llewellyn, N., Johnson, A., & Williams, R. (2014). Prioritising anticancer drugs for environmental monitoring and risk assessment purposes. *The Science of the Total Environment*, 473-474, 159–70.
- Borg, B., Antonopoulou, E., Mayer, I., Andersson, E., Berglund, I., & Swanson, P. (1998). Effects of gonadectomy and androgen treatments on pituitary and plasma levels of gonadotropins in mature male Atlantic salmon, *Salmo salar*, parr--positive feedback control of both gonadotropins. *Biology of Reproduction*, 58(3), 814–20.
- Bound, J. P., & Voulvoulis, N. (2005). Household disposal of pharmaceuticals as a pathway for aquatic contamination in the United Kingdom. *Environmental Health Perspectives*.
- Brausch, J. M., Connors, K. A., Brooks, B. W., & Rand, G. M. (2012). Human pharmaceuticals in the aquatic environment: a review of recent toxicological studies and considerations for toxicity testing. *Reviews of Environmental Contamination and Toxicology*, 218, 1–99.
- Breton, B., Sambroni, E., Govoroun, M., & Weil, C. (1997). Effects of steroids on GTH I and GTH II secretion and pituitary concentration in the immature rainbow trout *Oncorhynchus mykiss*. *Comptes Rendus de l'Académie Des Sciences. Série III, Sciences de La Vie*, 320(10), 783–9.
- Bretz, F., Pinheiro, J. C., & Branson, M. (2005). Combining multiple comparisons and modeling techniques in dose-response studies. *Biometrics*, 61(3), 738–48.
- Brezovšek, P., Eleršek, T., & Filipič, M. (2014). Toxicities of four anti-neoplastic drugs and their binary mixtures tested on the green alga *Pseudokirchneriella subcapitata* and the cyanobacterium *Synechococcus leopoliensis*. *Water Research*, 52, 168–77.
- Brezovšek, P., Eleršek, T., & Filipič, M. (2014). Toxicities of four anti-neoplastic drugs and their binary mixtures tested on the green alga *Pseudokirchneriella subcapitata* and the cyanobacterium *Synechococcus leopoliensis*. *Water Research*, 52, 168–77.
- Brian, J. V, Harris, C. A., Scholze, M., Backhaus, T., Booy, P., Lamoree, M., Sumpter, J. P. (2005). Accurate prediction of the response of freshwater fish to a mixture of estrogenic chemicals. *Environmental Health Perspectives*, 113(6), 721–8.
- Brian, J. V, Harris, C. A., Scholze, M., Kortenkamp, A., Booy, P., Lamoree, M., Sumpter, J. P. (2007). Evidence of estrogenic mixture effects on the reproductive performance of fish. *Environmental Science & Technology*, 41(1), 337–44.
- Brion, F., Tyler, C. R., Palazzi, X., Laillet, B., Porcher, J. M., Garric, J., & Flammarion, P. (2004). Impacts of 17beta-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.
- Brion, F., Le Page, Y., Piccini, B., Cardoso, O., Tong, S.-K., Chung, B., Gustafsson, J. (2012). Screening Estrogenic Activities of Chemicals or Mixtures In Vivo Using Transgenic (cyp19a1b-GFP) Zebrafish Embryos. *PLoS ONE*, 7(5), e36069.
- British National Formulary. (2014). Accessed 2016. <https://www.bnf.org/>
- Broderius, S. J., Kahl, M. D., & Hoglund, M. D. (1995). Use of joint toxic response to define the primary mode of toxic action for diverse industrial organic chemicals. *Environmental Toxicology and Chemistry*, 14(9), 1591–1605.
- Brooks, S., Tyler, C. R., & Sumpter, J. P. (1997). Egg quality in fish: what makes a good egg? *Reviews in Fish Biology and Fisheries*, 7(4), 387–416.
- Buerge, I. J., Buser, H.-R., Poiger, T., & Müller, M. D. (2006). Occurrence and fate of the cytostatic drugs cyclophosphamide and ifosfamide in wastewater and surface waters. *Environmental Science & Technology*, 40(23), 7242–50.
- Bury, N. R., Sturm, A., Le Rouzic, P., Lethimonier, C., Ducouret, B., Guiguen, Y., Prunet, P. (2003). Evidence for two distinct functional glucocorticoid receptors in teleost fish. *Journal of Molecular Endocrinology*, 31(1), 141–56.
- Cancer Research UK. <http://www.cancerresearchuk.org> Accessed 2016.
- Calabrese, E. J., & Baldwin, L. A. (2001). Hormesis: U-shaped dose responses and their centrality in toxicology. *Trends in Pharmacological Sciences*, 22(6), 285–291.
- Caldwell, D. J., Mastrocco, F., Anderson, P. D., Länge, R., & Sumpter, J. P. (2012). Predicted-no-effect concentrations for the steroid estrogens estrone, 17 β -estradiol, estriol, and 17 α -ethinylestradiol. *Environmental Toxicology and Chemistry*, 31(6), 1396–1406.
- Caldwell, D. J., Mastrocco, F., Margiotta-Casaluci, L., & Brooks, B. W. (2014). An integrated approach for prioritizing pharmaceuticals found in the environment for risk assessment, monitoring and advanced research. *Chemosphere*, 115, 4–12.
- 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.
- 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.
- 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.
- Carvan, M. J., Gallagher, E. P., Goksøyr, A., Hahn, M. E., & Larsson, D. G. J. (2007). Fish models in toxicology. *Zebrafish*, 4(1), 9–20.

- Cedergreen, N., Christensen, A. M., Kamper, A., Kudsk, P., Mathiassen, S. K., Streibig, J. C., & Sørensen, H. (2008). A review of independent action compared to concentration addition as reference models for mixtures of compounds with different molecular target sites. *Environmental Toxicology and Chemistry*, 27(7), 1621.
- Cedergreen, N., & Streibig, J. C. (2005). Can the choice of endpoint lead to contradictory results of mixture-toxicity experiments? *Environmental Toxicology and Chemistry / SETAC*, 24(7), 1676–83.
- Celiz, M. D., Tso, J., & Aga, D. S. (2009). Pharmaceutical metabolites in the environment: analytical challenges and ecological risks. *Environmental Toxicology and Chemistry / SETAC*, 28(12), 2473–84. <http://doi.org/10.1897/09-173.1>
- Česen, M., Kosjek, T., Buseti, F., Kompore, B., & Heath, E. (2016). Human metabolites and transformation products of cyclophosphamide and ifosfamide: analysis, occurrence and formation during abiotic treatments. *Environmental Science and Pollution Research International*, 23(11), 11209–23.
- Chang, H., Hu, J., & Shao, B. (2007). Occurrence of natural and synthetic glucocorticoids in sewage treatment plants and receiving river waters. *Environmental Science & Technology*, 41(10), 3462–8.
- Cheshenko, K., Pakdel, F., Segner, H., Kah, O., & Eggen, R. I. L. (2008). Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. *General and Comparative Endocrinology*, 155(1), 31–62. <http://doi.org/10.1016/j.ygcen.2007.03.005>
- Chong, K. L., Koh, M., & Melamed, P. (2005). Molecular Regulation of Gonadotropin Gene Expression in Teleosts. In P. Melamed & N. Sherwood (Eds.), *Hormones and their receptors in fish reproduction* (pp. 76–104). World Scientific.
- Christian, T., Schneider, R. J., Färber, H. A., Skutlarek, D., Meyer, M. T., & Goldbach, H. E. (2003). Determination of Antibiotic Residues in Manure, Soil, and Surface Waters. *Acta Hydrochimica et Hydrobiologica*, 31(1), 36–44.
- Christiansen, J. J., Djurhuus, C. B., Gravholt, C. H., Iversen, P., Christiansen, J. S., Schmitz, O., Møller, N. (2007). Effects of cortisol on carbohydrate, lipid, and protein metabolism: studies of acute cortisol withdrawal in adrenocortical failure. *The Journal of Clinical Endocrinology and Metabolism*, 92(9), 3553–9.
- Chu, E., & Sartorelli, A. C. (2007). Cancer Chemotherapy. In B. Katzung (Ed.), *Basic and Clinical Pharmacology* (10th ed., pp. 878–907). McGraw Hill Company.
- Clark, M. M., & Galef, B. G. (1995). Prenatal influences on reproductive life history strategies. *Trends in Ecology & Evolution*, 10(4), 151–3.
- Clements, P. J., Yu, D. T. Y., Levy, J., Paulus, H. E., & Barnett, E. V. (1974). Effects of cyclophosphamide on B- and T-lymphocytes in rheumatoid arthritis. *Arthritis & Rheumatism*, 17(4), 347–353.
- Cleuvers, M. (2004). Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. *Ecotoxicology and Environmental Safety*, 59(3), 309–315.

- Cleuvers, M. (2003). *Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects*. *Toxicology Letters* (Vol. 142).
- Cole, K. S., & Smith, R. J. F. (1987). Male courting behaviour in the fathead minnow, *Pimephales promelas*. *Environmental Biology of Fishes*, 18(3), 235–239.
- Collette, T. W., Teng, Q., Jensen, K. M., Kahl, M. D., Makynen, E. A., Durhan, E. J., ... Ekman, D. R. (2010). Impacts of an anti-androgen and an androgen/anti-androgen mixture on the metabolite profile of male fathead minnow urine. *Environmental Science & Technology*, 44(17), 6881–6.
- 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), 319–28.
- Colvin, M. (2003). Alkylating Agents. In D. Kufe, R. Pollock, & R. Weichselbaum (Eds.), *Holland-Frei Cancer Medicine* (6th ed.). BC Decker.
- Comparative Toxicogenomics Database. <http://doi.org/10.1289/EHP> Accessed 2013.
- Conley, J. M., Evans, N., Mash, H., Rosenblum, L., Schenck, K., Glassmeyer, S., Wilson, V. S. (2016). Comparison of in vitro estrogenic activity and estrogen concentrations in source and treated waters from 25 U.S. drinking water treatment plants. *The Science of the Total Environment*. In Press.
- 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.
- Coward, K., Bromage, N. R., Hibbitt, O., & Parrington, J. (2002). Gamete physiology, fertilization and egg activation in teleost fish. *Reviews in Fish Biology and Fisheries*, 12(1), 33–58.
- Craig, C. R., & Stitzel, R. E. (2004). *Modern pharmacology with clinical applications* (6th ed.). Lippincott Williams & Wilkins.
- Crowther, J. R. (2009). *The ELISA Guidebook*. Totowa, NJ: Humana Press.
- Cui, C. W., Ji, S. L., & Ren, H. Y. (2006). Determination of steroid estrogens in wastewater treatment plant of a contraceptives producing factory. *Environmental Monitoring and Assessment*.
- 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, 39–45.
- Daniel, M., Sharpe, A., Driver, J., Knight, A. W., Keenan, P. O., Walmsley, R. M., ... Rawson, D. (2004). Results of a technology demonstration project to compare rapid aquatic toxicity screening tests in the analysis of industrial effluents. *Journal of Environmental Monitoring : JEM*, 6(11), 855–65.

- Danylchuk, A. J., & Tonn, W. M. (2006). Natural disturbance and life history: consequences of winterkill on fathead minnow in boreal lakes. *Journal of Fish Biology*, 68(3), 681–694.
- Daston, G. P. (2008). Gene expression, dose-response, and phenotypic anchoring: applications for toxicogenomics in risk assessment. *Toxicological Sciences : An Official Journal of the Society of Toxicology*, 105(2), 233–4.
- Daston, G. P., Cook, J. C., & Kavlock, R. J. (2003). Uncertainties for endocrine disruptors: our view on progress. *Toxicological Sciences : An Official Journal of the Society of Toxicology*, 74(2), 245–52.
- Daughton, C. G., & Ternes, T. A. (1999). Pharmaceuticals and Personal Care Products in the Environment: Agents of Subtle Change? *Health Perspect*, 1(6), 7907–938.
- Davis, S. R., Tran, J., Secreto, G., al., et, Secreto, G., al., et, al., et. (2001). Testosterone influences libido and well being in women. *Trends in Endocrinology & Metabolism*, 12(1), 33–37.
- De Groot, W. T. (1983). Modelling the multiple nutrient limitation of algal growth. *Ecological Modelling*, 18(2), 99–119. [http://doi.org/10.1016/0304-3800\(83\)90049-2](http://doi.org/10.1016/0304-3800(83)90049-2)
- de Jongh, C. M., Kooij, P. J. F., de Voogt, P., & ter Laak, T. L. (2012). Screening and human health risk assessment of pharmaceuticals and their transformation products in Dutch surface waters and drinking water. *Science of the Total Environment*, 427-428, 70–77.
- DeMayo, F. J., Zhao, B., Takamoto, N., & Tsai, S. Y. (2002). Mechanisms of action of estrogen and progesterone. *Annals of the New York Academy of Sciences*, 955, 48–59; discussion 86–8, 396–406.
- de Mes, T., Zeeman, G., & Lettinga, G. (2005). Occurrence and Fate of Estrone, 17 β -estradiol and 17 α -ethynylestradiol in STPs for Domestic Wastewater. *Reviews in Environmental Science and Bio/Technology*, 4(4), 275–311.
- Department for Environment, Food and Rural Affairs (DEFRA). (2014). Water Framework Directive implementation in England and Wales: new and updated standards to protect the water environment. Retrieved from www.gov.uk/defra
- Department of Health 2004. Report. Variations in usage of cancer drugs approved by NICE. Report of the review undertaken by the National Cancer Director. Crown http://webarchive.nationalarchives.gov.uk/+/dh.gov.uk/en/publicationsandstatistics/publications/publicationspolicyandguidance/dh_4083901
- Denslow, N. D., Chow, M. C., Kroll, K. J., & Green, L. (1999). Vitellogenin as a Biomarker of Exposure for Estrogen or Estrogen Mimics. *Ecotoxicology*, 8(5), 385–398.
- Denslow, N. D., Garcia-Reyero, N., & Barber, D. S. (2007). Fish “n” chips: the use of microarrays for aquatic toxicology. *Molecular bioSystems*, 3(3), 172–7.
- DeQuattro, Z. A., Peissig, E. J., Antkiewicz, D. S., Lundgren, E. J., Hedman, C. J., Hemming, J. D. C., & Barry, T. P. (2012). Effects of progesterone on reproduction

and embryonic development in the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry / SETAC*, 31(4), 851–6.

Deroo, B. J., & Korach, K. S. (2006). Estrogen receptors and human disease. *The Journal of Clinical Investigation*, 116(3), 561–70.

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

Dickey, J. T., & Swanson, P. (1998). Effects of sex steroids on gonadotropin (FSH and LH) regulation in coho salmon (*Oncorhynchus kisutch*). *Journal of Molecular Endocrinology*, 21(3), 291–306.

Dickey, J. T., & Swanson, P. (2000). Effects of salmon gonadotropin-releasing hormone on follicle stimulating hormone secretion and subunit gene expression in coho salmon (*Oncorhynchus kisutch*). *General and Comparative Endocrinology*, 118(3), 436–49.

Dietrich, D. R., Webb, S. F., & Petry, T. (2002). Hot spot pollutants: pharmaceuticals in the environment. *Toxicology Letters*, 131, 1–3.

Donnachie, R. L., Johnson, A. C., & Sumpter, J. P. (2016). A rational approach to selecting and ranking some pharmaceuticals of concern for the aquatic environment and their relative importance compared with other chemicals. *Environmental Toxicology and Chemistry / SETAC*, 35(4), 1021–7.

Dorts, J., Richter, C. A., Wright-Osment, M. K., Eilersieck, 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, 17 β -trenbolone. *Aquatic Toxicology*, 91(1), 44–53.

DrugBank. <http://www.drugbank.ca/> Accessed 2013.

Ducouret, B., Tujague, M., Ashraf, J., Mouchel, N., Serval, 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), 3774–83.

Duffull, S. B., & Robinson, B. A. (1997). Clinical pharmacokinetics and dose optimisation of carboplatin. *Clinical Pharmacokinetics*, 33(3), 161–83.

Efron, B., & Tibshirani, R. (1994). *An introduction to the bootstrap*. Chapman & Hall.

Eguchi, K., Nagase, H., Ozawa, M., Endoh, Y. S., Goto, K., Hirata, K., Yoshimura, H. (2004). Evaluation of antimicrobial agents for veterinary use in the ecotoxicity test using microalgae. *Chemosphere*, 57(11), 1733–1738.

Eleršek, T. (2012). The advantages of flow cytometry in comparison to fluorometric measurement in algal toxicity test Prednosti merjenja s pretočno citometrijo v primerjavi s fluorimetričnim merjenjem v strupenostnih testih z algami. *Acta Biologica Slovenica*, 55(552), 3–11.

- Elersek, T., Milavec, S., Korošec, M., Brezovsek, P., Negreira, N., Zonja, B., Filipič, M. (2016). Toxicity of the mixture of selected antineoplastic drugs against aquatic primary producers. *Environmental Science and Pollution Research International*, 23(15), 14780–90.
- Ellis, R. J., van den Heuvel, M. R., Bandelj, E., Smith, M. A., McCarthy, L. H., Stuthridge, T. R., & Dietrich, D. R. (2003). In vivo and in vitro assessment of the androgenic potential of a pulp and paper mill effluent. *Environmental Toxicology and Chemistry*, 22(7), 1448–1456.
- Ema. (2007). Adoption by chmp for release for consultation. Retrieved from <http://www.emea.europa.eu>
- Encyclopedia of Life (EOL) <http://eol.org/> Accessed 2016
- Environmental Protection Agency. US EPA fathead minnow acute toxicity database. Epa.gov. http://www.epa.gov/ncct/dsstox/sdf_epafhm.html Accessed 2014.
- Ermiler, S., Scholze, M., & Kortenkamp, A. (2013). Seven benzimidazole pesticides combined at sub-threshold levels induce micronuclei in vitro. *Mutagenesis*, 28(4), 417–26.
- European Commission. Commission Directive 92/18/European Economic Community. (1992).
- European Commission. Directive on Environmental Quality Standards 2008/105/EC. (2008).
- European Commission. Priority Substances Directive 2013/39/EC. (2013).
- European Commission. Water Framework Directive 2000/60/EC. (2000).
- European Commission (EC). (2001). Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community Code relating to medicinal products for human use.
- European Commission Joint Research Centre. ECB. European Chemicals Bureau. (2003). Technical guidance document on risk assessment. Part II. EUR 20418 EN/2
- European Medicines Agency (EMA). Committee for medicinal products for human use (CHMP) (2006). Guideline on the environmental risk assessment of medicinal products for human use. SWP/4447/00 cor2.
- Fasano, A. (2012). Leaky gut and autoimmune diseases. *Clinical Reviews in Allergy & Immunology*, 42(1), 71–8.
- Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P., ... Grimme, L. H. (2001). Predicting the joint algal toxicity of multi-component s-triazine mixtures at low-effect concentrations of individual toxicants. *Aquatic Toxicology*, 56(1), 13–32.

- Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P., ... Grimme, L. . (2003). Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independent action. *Aquatic Toxicology*, 63(1), 43–63.
- Fenske, M., Maack, G., Schäfers, C., & Segner, H. (2005). An environmentally relevant concentration of estrogen induces arrest of male gonad development in zebrafish, *Danio rerio*. *Environmental Toxicology and Chemistry / SETAC*, 24(5), 1088–98.
- Fent, K., Weston, A. A., & Caminada, D. (2006a). Ecotoxicology of human pharmaceuticals. *Aquatic Toxicology*, 76, 122–159.
- Fick, J., Lindberg, R. H., Tysklind, M., & Larsson, D. G. J. (2010). Predicted critical environmental concentrations for 500 pharmaceuticals. *Regulatory Toxicology and Pharmacology*, 58(3), 516-523
- Fick, J., Söderström, H., Lindberg, R. H., Phan, C., Tysklind, M., & Larsson, D. G. J. (2009). Contamination of surface, ground, and drinking water from pharmaceutical production. *Environmental Toxicology and Chemistry / SETAC*, 28(12), 2522–7.
- Filby, A. L., & Tyler, C. R. (2005). Molecular characterization of estrogen receptors 1, 2a, and 2b and their tissue and ontogenic expression profiles in fathead minnow (*Pimephales promelas*). *Biology of Reproduction*, 73(4), 648–62.
- Filby, A. L., Thorpe, K. L., Maack, G., & Tyler, C. R. (2007). Gene expression profiles revealing the mechanisms of anti-androgen- and estrogen-induced feminization in fish. *Aquatic Toxicology*, 81(2), 219–231.
- Fischer, S. J., Benson, L. M., Fauq, A., Naylor, S., & Windebank, A. J. (2008). Cisplatin and dimethyl sulfoxide react to form an adducted compound with reduced cytotoxicity and neurotoxicity. *Neurotoxicology*, 29(3), 444–52.
- Fishbase.org Accessed 2013.
- Fitzsimmons, P. N., Fernandez, J. D., Hoffman, A. D., Butterworth, B. C., & Nichols, J. W. (2001). Branchial elimination of superhydrophobic organic compounds by rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology (Amsterdam, Netherlands)*, 55(1-2), 23–34.
- Folmar, L. C., Denslow, N. D., Rao, V., Chow, M., Crain, D. A., Enblom, J., Guillette, L. J. (1996). Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. *Environmental Health Perspectives*, 104(10), 1096–101.
- Folmar, L. C., Hemmer, M. J., Denslow, N. D., Kroll, K., Chen, J., Cheek, A., Grau, E. G. (2002). A comparison of the estrogenic potencies of estradiol, ethynylestradiol, diethylstilbestrol, nonylphenol and methoxychlor in vivo and in vitro. *Aquatic Toxicology* 60(1-2), 101–10.
- Frei, W. (1913). Versuche u̇ber Kombinationen von Desinfektionsmitteln. *Zeitschrift Hygiene Infektionskrankheiten*, 75, 433–496.

- Fuhrmann, U., Slater, E. P., & Fritzemeier, K.-H. (1995). Characterization of the novel progestin gestodene by receptor binding studies and transactivation assays. *Contraception*, *51*(1), 45–52.
- Gan, S. D., & Patel, K. R. (2013). Enzyme Immunoassay and Enzyme-Linked Immunosorbent Assay. *Journal of Investigative Dermatology*, *133*(9), 1–3.
- Gilmour, K. M. (2005). Mineralocorticoid receptors and hormones: fishing for answers. *Endocrinology*, *146*(1), 44–6.
- Gopurappilly, R., Ogawa, S., & Parhar, I. S. (2013). Functional significance of GnRH and kisspeptin, and their cognate receptors in teleost reproduction. *Frontiers in Endocrinology*, *4*, 24.
- Gore, A. C., Crews, D., Doan, L. L., La Merrill, M., Patisaul, H., & Zota, A. (2014). Introduction to endocrine disrupting chemicals (edcs) a guide for public interest organizations and policy-makers. Endocrine Society Publication. IPEN.
- Green, R. E., Taggart, M. A., Das, D., Pain, D. J., Sashi Kumar, C., Cunningham, A. A., & Cuthbert, R. (2006). Collapse of Asian vulture populations: Risk of mortality from residues of the veterinary drug diclofenac in carcasses of treated cattle. *Journal of Applied Ecology*, *43*, 949–956.
- Green, C., Brian, J., Kanda, R., Scholze, M., Williams, R., & Jobling, S. (2015). Environmental concentrations of anti-androgenic pharmaceuticals do not impact sexual disruption in fish alone or in combination with steroid oestrogens. *Aquatic Toxicology*, *160*, 117–127.
- 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), 4226–36.
- Grung, M., Källqvist, T., Sakshaug, S., Skurtveit, S., & Thomas, K. V. (2008). Environmental assessment of Norwegian priority pharmaceuticals based on the EMEA guideline. *Ecotoxicology and Environmental Safety*, *71*(2), 328–340.
- Guénin, S., Mauriat, M., Pelloux, J., Van Wuytswinkel, O., Bellini, C., & Gutierrez, L. (2009). Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. *Journal of Experimental Botany*, *60*(2), 487–93.
- Guillén, D., Ginebreda, A., Farré, M., Darbra, R. M., Petrovic, M., Gros, M., & Barceló, D. (2012). Prioritization of chemicals in the aquatic environment based on risk assessment: analytical, modeling and regulatory perspective. *The Science of the Total Environment*, *440*, 236–52.
- 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 & Technology*, *42*(15), 5807–5813.

- Guo, J., Sinclair, C. J., Selby, K., & Boxall, A. B. A. (2016). Toxicological and ecotoxicological risk-based prioritization of pharmaceuticals in the natural environment. *Environmental Toxicology and Chemistry / SETAC*, 35(6), 1550–9.
- Hagenbuch, I. M., & Pinckney, J. L. (2012). Toxic effect of the combined antibiotics ciprofloxacin, lincomycin, and tylosin on two species of marine diatoms. *Water Research*, 46(16), 5028–5036.
- Hahlbeck, E., Katsiadaki, I., Mayer, I., Adolfsson-Erici, M., James, J., & Bengtsson, B.-E. (2004). The juvenile three-spined stickleback (*Gasterosteus aculeatus* L.) as a model organism for endocrine disruption II--kidney hypertrophy, vitellogenin and spiggin induction. *Aquatic Toxicology (Amsterdam, Netherlands)*, 70(4), 311–26.
- Halling-Sorensen, B., Nielsen, S. N., Lanzky, P. F., Ingerslev, F., Liitzhofl, H. C. H., & Jorgensen, S. E. (1998). Occurrence, Fate and Effects of Pharmaceutical Substances in the Environment-A Review. *Pergamon Chemosphere*, 36(2), 357–393.
- Hansen, P.-D., Dizer, H., Hock, B., Marx, A., Sherry, J., McMaster, M., & Blaise, C. (1998). Vitellogenin – a biomarker for endocrine disruptors. *TrAC Trends in Analytical Chemistry*, 17(7), 448–451.
- Harding, L. B., Schultz, I. R., da Silva, D. A. M., Ylitalo, G. M., Ragsdale, D., Harris, S. I., Swanson, P. (2016). Wastewater treatment plant effluent alters pituitary gland gonadotropin mRNA levels in juvenile coho salmon (*Oncorhynchus kisutch*). *Aquatic Toxicology*, 178, 118–31.
- Harding, L. B., Schultz, I. R., Goetz, G. W., Luckenbach, J. A., Young, G., Goetz, F. W., & Swanson, P. (2013). High-throughput sequencing and pathway analysis reveal alteration of the pituitary transcriptome by 17 α -ethynylestradiol (EE2) in female coho salmon, *Oncorhynchus kisutch*. *Aquatic Toxicology* 142-143, 146–63.
- Harries, J. E., Janbakhsh, A., Jobling, S., Matthiessen, P., Sumpter, J. P., & Tyler, C. R. (1999). Estrogenic potency of effluent from two sewage treatment works in the United Kingdom. *Environmental Toxicology and Chemistry*, 18(5), 932–937.
- Harries, J. E., Sheahan, D. A., Jobling, S., Matthiessen, P., Neall, P., Sumpter, J. P., Zaman, N. (1997). Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environmental Toxicology and Chemistry*, 16(3), 534–542.
- Harries, J.E., Runnalls, T., Hill, E., Harris, C.A., Maddix, S., Sumpter, J.P., and, & Tyler, C.R.. (2000). Development of a Reproductive Performance Test for Endocrine Disrupting Chemicals Using Pair-Breeding Fathead Minnows (*Pimephales promelas*). *Environmental Toxicology and Chemistry* 34(14), 3003-3011,.
- Harris, H. A., Bapat, A. R., Gonder, D. S., & Frail, D. E. (2002). The ligand binding profiles of estrogen receptors alpha and beta are species dependent. *Steroids*, 67(5), 379–84.
- Hashimoto, S., Watanabe, E., Ikeda, M., Terao, Y., Strüssmann, C. A., Inoue, M., & Hara, A. (2009). Effects of ethinylestradiol on medaka (*Oryzias latipes*) as measured by sperm motility and fertilization success. *Archives of Environmental Contamination and Toxicology*, 56(2), 253–9.

Health and Social Care Information Centre, 2013 <http://digital.nhs.uk/>

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.

Heberer, T., & Stan, H.-J. (1997). Determination of Clofibric Acid and N-(Phenylsulfonyl)-Sarcosine in Sewage, River and Drinking Water. *International Journal of Environmental Analytical Chemistry*, 67(1-4), 113–124.

Heberer, T., Reddersen, K., & Mechliniski, A. (2002). From municipal sewage to drinking water: Fate and removal of pharmaceutical residues in the aquatic environment in urban areas. In *Water Science and Technology*.

Heggie, G. D., Sommadossi, J. P., Cross, D. S., Huster, W. J., & Diasio, R. B. (1987). Clinical pharmacokinetics of 5-fluorouracil and its metabolites in plasma, urine, and bile. *Cancer Research*, 47(8), 2203–6.

Hellqvist, A., Schmitz, M., & Borg, B. (2008). Effects of castration and androgen-treatment on the expression of FSH- β and LH- β in the three-spine stickleback, *gasterosteus aculeatus*—Feedback differences mediating the photoperiodic maturation response? *General and Comparative Endocrinology*, 158(2), 178–182.

Henschel, K. P., Wenzel, A., Diedrich, M., & Fliedner, A. (1997). Environmental hazard assessment of pharmaceuticals. *Regulatory Toxicology and Pharmacology : RTP*, 25(3), 220–5.

Hindák, F. (1990). Studies on the chlorococcal algae (Chlorophyceae). *Biologické Práce*, 36, 1–227.

Hirsch, R., Ternes, T., Haberer, K., & Kratz, K.-L. (1999). Occurrence of antibiotics in the aquatic environment. *Science of The Total Environment*, 225(1), 109–118.

Hishida, T.-O., & Kawamoto, N. (1970). Androgenic and male-inducing effects of 11-ketotestosterone on a teleost, the medaka (*Oryzias latipes*). *Journal of Experimental Zoology*, 173(3), 279–283.

Huang, Y. S., Schmitz, M., Le Belle, N., Chang, C. F., Quérat, B., & Dufour, S. (1997). Androgens stimulate gonadotropin-II beta-subunit in eel pituitary cells *in vitro*. *Molecular and Cellular Endocrinology*, 131(2), 157–66.

Huang, C. H., & Sedlak, D. L. (2001). Analysis of estrogenic hormones in municipal wastewater effluent and surface water using enzyme-linked immunosorbent assay and gas chromatography/tandem mass spectrometry. *Environmental Toxicology and Chemistry / SETAC*, 20(1), 133–9.

Huggard-Nelson, D. L., Nathwani, P. S., Kermouni, A., & Habibi, H. R. (2002). Molecular characterization of LH-beta and FSH-beta subunits and their regulation by estrogen in the goldfish pituitary. *Molecular and Cellular Endocrinology*, 188(1-2), 171–93.

Huggett, D. B., Cook, J. C., Ericson, J. F., & Williams, R. T. (2003). A Theoretical Model for Utilizing Mammalian Pharmacology and Safety Data to Prioritize Potential Impacts

of Human Pharmaceuticals to Fish. *Human and Ecological Risk Assessment: An International Journal*, 9(7), 1789–1799.

- Hughes, S. R., Kay, P., & Brown, L. E. (2013). Global synthesis and critical evaluation of pharmaceutical data sets collected from river systems. *Environmental Science and Technology*
- Hutchinson, T. H., Ankley, G. T., Segner, H., & Tyler, C. R. (2006). Screening and testing for endocrine disruption in fish-biomarkers as “signposts,” not “traffic lights,” in risk assessment. *Environmental Health Perspectives*. 114(Suppl 1): 106–114.
- Hwang, P. P., Wu, S. M., Lin, J. H., & Wu, L. S. (1992). Cortisol content of eggs and larvae of teleosts. *General and Comparative Endocrinology*, 86(2), 189–96.
- Ikeuchi, T., Todo, T., Kobayashi, T., & Nagahama, Y. (1999). cDNA cloning of a novel androgen receptor subtype. *The Journal of Biological Chemistry*, 274(36), 25205–9.
- Ikeuchi, T., Todo, T., Kobayashi, T., & Nagahama, Y. (2001). Two subtypes of androgen and progesterone receptors in fish testes. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 129(2), 449–455.
- IMS Health : Data collection. (2011.). Retrieved from <http://csdmruk.cegedim.com/our-data/data-content.shtml>
- International Programme on Chemical Safety (IPCS). World Health Organisation (WHO) (2016). <http://www.who.int/ipcs/en/> Accessed 2016
- Jay, A. El. (1996). Toxic Effects of Organic Solvents on the Growth of *Chlorella vulgaris* and *Selenastrum capricornutum*. *Bull. Environ. Contam. Toxicol*, 57, 191–198.
- 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. Toxicology & Pharmacology : CBP*, 128(1), 127–41.
- Jensen, K. M., Makynen, E. A., Kahl, M. D., & Ankley, G. T. (2006). Effects of the Feedlot Contaminant 17 α -Trenbolone on Reproductive Endocrinology of the Fathead Minnow. *Environmental Science & Technology*, 40(9), 3112–3117.
- Joakim Larsson, D. G., & Fick, J. (2009). Transparency throughout the production chain—a way to reduce pollution from the manufacturing of pharmaceuticals? *Regulatory Toxicology and Pharmacology*. <http://doi.org/10.1016/j.yrtph.2009.01.008>
- Jobling, S., Beresford, N., Nolan, M., Rodgers-Gray, T., Brighty, G. C., Sumpter, J. P., & Tyler, C. R. (2002b). Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. *Biology of Reproduction*, 66(2), 272–81.
- Jobling, S., Coey, S., Whitmore, J. G., Kime, D. E., Van Look, K. J. W., McAllister, B. G., Sumpter, J. P. (2002a). Wild intersex roach (*Rutilus rutilus*) have reduced fertility. *Biology of Reproduction*, 67(2), 515–24.

- 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., ... Brighty, G. (2006). Predicted exposures to steroid estrogens in U.K. rivers correlate with widespread sexual disruption in wild fish populations. *Environmental Health Perspectives*, (Suppl 1), 32–9.
- Jobling, S., Williams, R., Johnson, A., Taylor, A., Gross-Sorokin, M., Nolan, M., Brighty, G. (2006). Predicted exposures to steroid estrogens in U.K. Rivers correlate with widespread sexual disruption in wild fish populations. *Environmental Health Perspectives*, 114, 32–39.
- Johannessen, M., Fontanilla, D., Mavlyutov, T., Ruoho, A. E., & Jackson, M. B. (2011). Antagonist action of progesterone at σ -receptors in the modulation of voltage-gated sodium channels. *American Journal of Physiology. Cell Physiology*, 300(2), C328–37.
- 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, 167–175.
- Johnson, A. C., & Williams, R. J. (2004). A model to estimate influent and effluent concentrations of estradiol, estrone, and ethinylestradiol at sewage treatment works. *Environmental Science and Technology*, 38, 3649–3658.
- Johnson, A. C., Dumont, E., Williams, R. J., Oldenkamp, R., Cisowska, I., & Sumpter, J. P. (2013a). Do concentrations of ethinylestradiol, estradiol, and diclofenac in European rivers exceed proposed EU environmental quality standards? *Environmental Science & Technology*, 47(21), 12297–304.
- Johnson, A. C., Oldenkamp, R., Dumont, E., & Sumpter, J. P. (2013b). Predicting concentrations of the cytostatic drugs cyclophosphamide, carboplatin, 5-fluorouracil, and capecitabine throughout the sewage effluents and surface waters of Europe. *Environmental Toxicology and Chemistry / SETAC*, 32(9), 1954–61.
- Joining Research Centre (JRC). 2010. River Basin Specific Pollutants – Identification and Monitoring. ISBN: 978-92-79-18471-0
- Jones, A. W. (2011). Early drug discovery and the rise of pharmaceutical chemistry. *Drug Testing and Analysis*. <http://doi.org/10.1002/dta.301>
- Jones, O. A. H., Voulvoulis, N., & Lester, J. N. (2001). Human Pharmaceuticals in the Aquatic Environment a Review. *Environmental Technology*, 22, 12–1383.
- Jones, O. A., Voulvoulis, N., & Lester, J. N. (2001). Human pharmaceuticals in the aquatic environment a review. *Environmental Technology*, 22(12), 1383–94.
- Jones, O. A., Lester, J. N., & Voulvoulis, N. (2005). Pharmaceuticals: A threat to drinking water? *Trends in Biotechnology*. <http://doi.org/10.1016/j.tibtech.2005.02.001>

- Jukosky, J. A., Watzin, M. C., & Leiter, J. C. (2008). The effects of environmentally relevant mixtures of estrogens on Japanese medaka (*Oryzias latipes*) reproduction. *Aquatic Toxicology (Amsterdam, Netherlands)*, 86(2), 323–31.
- Juneau, P., Sumitomo, H., Matsui, S., Itoh, S., Kim, S.-G., & Popovic, R. (2003). Use of chlorophyll fluorescence of *Closterium ehrenbergii* and *Lemna gibba* for toxic effect evaluation of sewage treatment plant effluent and its hydrophobic components. *Ecotoxicology and Environmental Safety*, 55(1), 1–8.
- Kapoor, D., Malkin, C. J., Channer, K. S., & Jones, T. H. (2005). Androgens, insulin resistance and vascular disease in men. *Clinical Endocrinology*, 63(3), 239–50.
- Kang, I. J., Yokota, H., Oshima, Y., Tsuruda, Y., Yamaguchi, T., Maeda, M., ... Honjo, T. (2002). Effect of 17beta-estradiol on the reproduction of Japanese medaka (*Oryzias latipes*). *Chemosphere*, 47(1), 71–80.
- Karels, A., Markkula, E., & Oikari, A. (2001). Reproductive, biochemical, physiological, and population responses in perch (*Perca fluviatilis* L.) and roach (*Rutilus rutilus* L.) downstream of two elemental chlorine-free pulp and paper mills. *Environmental Toxicology and Chemistry / SETAC*, 20(7), 1517–27.
- Katsiadaki, I., Scott, A. ., & Mayer, I. (2002). The potential of the three-spined stickleback (*Gasterosteus aculeatus* L.) as a combined biomarker for oestrogens and androgens in European waters. *Marine Environmental Research*, 54 (3-5), 725-728
- Katsiadaki, I., Morris, S., Squires, C., Hurst, M. R., James, J. D., & Scott, A. P. (2006). Use of the three-spined stickleback (*Gasterosteus aculeatus*) as a sensitive *in vivo* test for detection of environmental antiandrogens. *Environmental Health Perspectives*, (Suppl 1), 115–21.
- Katsiadaki, I., Sanders, M. B., Henrys, P. A., Scott, A. P., Matthiessen, P., & Pottinger, T. G. (2012). Field surveys reveal the presence of anti-androgens in an effluent-receiving river using stickleback-specific biomarkers. *Aquatic Toxicology*, 122, 75–85.
- Katsiadaki, I., Scott, A. P., Hurst, M. R., Matthiessen, P., & Mayer, I. (2002). Detection of environmental androgens: A novel method based on enzyme-linked immunosorbent assay of spiggin, the stickleback (*Gasterosteus aculeatus*) glue protein. *Environmental Toxicology and Chemistry*, 21(9), 1946–1954.
- Katsiadaki, I., Williams, T. D., Ball, J. S., Bean, T. P., Sanders, M. B., Wu, H., Chipman, J. K. (2010). Hepatic transcriptomic and metabolomic responses in the Stickleback (*Gasterosteus aculeatus*) exposed to ethinyl-estradiol. *Aquatic Toxicology*, 97(3), 174–87.
- Katzung, B. (2006). *Basic and Clinical Pharmacology*. McGraw-Hill Companies.
- Khan, B., Lee, L. S., & Sassman, S. A. (2008). Degradation of Synthetic Androgens 17 α - and 17 β -Trenbolone and Trendione in Agricultural Soils. *Environmental Science & Technology*, 42(10), 3570–3574.
- 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 estrogen. *Proceedings of the National Academy of Sciences*, 104(21), 8897–8901.

- Kime, D. ., Nash, J. ., & Scott, A. (1999). Vitellogenesis as a biomarker of reproductive disruption by xenobiotics. *Aquaculture*, 177(1), 345–352.
- Kime, D. E. (1993). Classical and non-classical reproductive steroids in fish. *Reviews in Fish Biology and Fisheries*, 3(2), 160–180.
- Kiparissis, Y., Metcalfe, T. L., Balch, G. C., & Metcalfe, C. D. (2003). Effects of the antiandrogens, vinclozolin and cyproterone acetate on gonadal development in the Japanese medaka (*Oryzias latipes*). *Aquatic Toxicology (Amsterdam, Netherlands)*, 63(4), 391–403.
- 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 & Technology*, 36(6), 1202–1211.
- Kortenkamp, A., & Altenburger, R. (1998). Synergisms with mixtures of xenoestrogens: a reevaluation using the method of isoboles. *The Science of the Total Environment*, 221(1), 59–73.
- Kortenkamp, A. (2008). Low dose mixture effects of endocrine disruptors: implications for risk assessment and epidemiology. *International Journal of Andrology*, 31(2), 233–40.
- Kortenkamp, A. (2007). Ten years of mixing cocktails: a review of combination effects of endocrine-disrupting chemicals. *Environmental Health Perspectives*, 98–105.
- Kortenkamp, A., & Altenburger, R. (2011). Toxicity from Combined Exposure to Chemicals. In C. A. M. van Gestel (Ed.), *Mixture toxicity: linking approaches from ecological and human toxicology* (p. 280). CRC Press.
- Kosjek, T., & Heath, E. (2011). Occurrence, fate and determination of cytostatic pharmaceuticals in the environment. *TrAC Trends in Analytical Chemistry*, 30(7), 1065–1087.
- Kostich, M. S., Batt, A. L., & Lazorchak, J. M. (2014). Concentrations of prioritized pharmaceuticals in effluents from 50 large wastewater treatment plants in the US and implications for risk estimation. *Environmental Pollution*, 184, 354–359.
- Krisfalusi, M., & Nagler, J. J. (2000). Induction of gonadal intersex in genotypic male rainbow trout (*Oncorhynchus mykiss*) embryos following immersion in estradiol-17 beta. *Molecular Reproduction and Development*, 56(4), 495–501.
- Kroupova, H. K., Trubiroha, A., Lorenz, C., Contardo-Jara, V., Lutz, I., Grabic, R., ... Kloas, W. (2014). The progestin levonorgestrel disrupts gonadotropin expression and sex steroid levels in pubertal roach (*Rutilus rutilus*). *Aquatic Toxicology*, 154, 154–162.
- Kuch, H. M., & Ballschmiter, K. (2001). Determination of Endocrine-Disrupting Phenolic Compounds and Estrogens in Surface and Drinking Water by HRGC-(NCI)-MS in the Picogram per Liter Range. *Environmental Science & Technology*, 35(15), 3201–3206.

- Kugathas, S., Runnalls, T. J., & Sumpter, J. P. (2013). Metabolic and Reproductive Effects of Relatively Low Concentrations of Beclomethasone Dipropionate, a Synthetic Glucocorticoid, on Fathead Minnows. *Environmental Science & Technology*, 47(16), 9487–9495.
- Kugathas, S., & Sumpter, J. P. (2011). Synthetic glucocorticoids in the environment: first results on their potential impacts on fish. *Environmental Science & Technology*, 45(6), 2377–83.
- Kuhl, A. J., Manning, S., & Brouwer, M. (2005). Brain aromatase in Japanese medaka (*Oryzias latipes*): Molecular characterization and role in xenoestrogen-induced sex reversal. *The Journal of Steroid Biochemistry and Molecular Biology*, 96(1), 67–77.
- Kümmerer, K., & Al-Ahmad, A. (1997). Biodegradability of the Anti-tumour Agents 5-Fluorouracil, Cytarabine, and Gemcitabine: Impact of the Chemical Structure and Synergistic Toxicity with Hospital Effluent. *Acta Hydrochimica et Hydrobiologica*, 25(4), 166–172.
- Kümmerer, K., & Henninger, A. (2003). Promoting resistance by the emission of antibiotics from hospitals and households into effluent. *Clinical Microbiology and Infection*, 9(12), 1203–1214.
- Kümmerer, K. (2008). *Pharmaceuticals in the Environment - Sources, Fate, Effects*. Springer.
- Kümmerer, 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), 957–969.
- Kümmerer, K. (2009a). Antibiotics in the aquatic environment - A review - Part I. *Chemosphere*, 75, 417–434. <http://doi.org/10.1016/j.chemosphere.2008.11.086>
- Küster, A., & Adler, N. (2014). Pharmaceuticals in the environment: scientific evidence of risks and its regulation. *Philosophical Transactions of the Royal Society B*. 369: 20130587.
- Lai, K. M., Johnson, K. L., Scrimshaw, M. D., & Lester, J. N. (2000). Binding of Waterborne Steroid Estrogens to Solid Phases in River and Estuarine Systems. *Environmental Science & Technology*, 34(18), 3890–3894.
- LaLone, C. A., Villeneuve, D. L., Olmstead, A. W., Medlock, E. K., Kahl, M. D., Jensen, K. M., Ankley, G. T. (2012). Effects of a glucocorticoid receptor agonist, dexamethasone, on fathead minnow reproduction, growth, and development. *Environmental Toxicology and Chemistry*, 31(3), 611–622.
- Länge, R., Hutchinson, T. H., Croudace, C. P., Siegmund, F., Schweinfurth, H., Hampe, P., Sumpter, J. P. (2001). Effects of the synthetic estrogen 17 α -ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry*, 20(6), 1216–1227.
- Larkin, P., Villeneuve, D. L., Knoebel, I., Miracle, A. L., Carter, B. J., Liu, L., Ankley, G. T. (2007). Development and validation of a 2,000-gene microarray for the fathead

- minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry / SETAC*, 26(7), 1497–506.
- Larsson, D. G. J., Adolfsson-Erici, M., Parkkonen, J., Pettersson, M., Berg, A. H., Olsson, P.-E., & Förlin, L. (1999). Ethinyloestradiol — an undesired fish contraceptive? *Aquatic Toxicology*, 45, 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*.
- Larsson, J. D. G., & Fick, J. (2009). Transparency throughout the production chain--a way to reduce pollution from the manufacturing of pharmaceuticals? *Regulatory Toxicology and Pharmacology : RTP*, 53(3), 161–3.
- Lee, H.-R., Kim, T.-H., & Choi, K.-C. (2012). Functions and physiological roles of two types of estrogen receptors, ER α and ER β , identified by estrogen receptor knockout mouse. *Laboratory Animal Research*, 28(2), 71–6.
- Levin, E. R. (2009). Plasma membrane estrogen receptors. *Trends in Endocrinology and Metabolism: TEM*, 20(10), 477–82.
- Li, X., Huang, J., Yi, P., Bambara, R. A., Hilf, R., & Muyan, M. (2004). Single-chain estrogen receptors (ERs) reveal that the ER α /beta heterodimer emulates functions of the ER α dimer in genomic estrogen signaling pathways. *Molecular and Cellular Biology*, 24(17), 7681–94.
- Li, D., Yang, M., Hu, J., Ren, L., Zhang, Y., & Li, K. (2008a). Environmental Research in China determination and fate of oxytetracycline and related compounds in oxytetracycline production wastewater and the receiving river. *Environmental Toxicology and Chemistry*, 27(1), 80–86.
- Li, D., Yang, M., Hu, J., Zhang, Y., Chang, H., & Jin, F. (2008b). Determination of penicillin G and its degradation products in a penicillin production wastewater treatment plant and the receiving river. *Water Research*.
- Li, Z.H., and Randak, T., (2009). Residual pharmaceutically active compounds (PhACs) in aquatic environment - status, toxicity and kinetics: a review. *Veterinarni Medicina - UZEI (Czech Republic)*, 54(7), 295–314.
- Liao, S., Liang, T., Fang, S., Castañeda, E., & Shao, T. C. (1973). Steroid structure and androgenic activity. Specificities involved in the receptor binding and nuclear retention of various androgens. *The Journal of Biological Chemistry*, 248(17), 6154–62.
- Lin, S.-W., & Ge, W. (2009). Differential regulation of gonadotropins (FSH and LH) and growth hormone (GH) by neuroendocrine, endocrine, and paracrine factors in the zebrafish--an in vitro approach. *General and Comparative Endocrinology*, 160(2), 183–93.
- Lin, A. Y. C., & Tsai, Y. T. (2009). Occurrence of pharmaceuticals in Taiwan's surface waters: Impact of waste streams from hospitals and pharmaceutical production facilities. *Science of the Total Environment*.

- Liu, S., Ying, G.-G., Zhao, J.-L., Chen, F., Yang, B., Zhou, L.-J., & Lai, H. (2011). Trace analysis of 28 steroids in surface water, wastewater and sludge samples by rapid resolution liquid chromatography–electrospray ionization tandem mass spectrometry. *Journal of Chromatography A*, 1218(10), 1367–1378.
- Loos, R., Carvalho, R., António, D. C., Comero, S., Locoro, G., Tavazzi, S., ... Gawlik, B. M. (2013). EU-wide monitoring survey on emerging polar organic contaminants in wastewater treatment plant effluents. *Water Research*.
- Lowe, S., & Muischnek, H. (1926). Combined effects I Announcement - Implements to the problem. *Naunyn-Schmiedebergs Archiv Fur Experimentelle Pathologie Und Pharmakologie*, 114, 313–326.
- Lutterbeck, C. A., Machado, Ê. L., & Kümmerer, K. (2015). Photodegradation of the antineoplastic cyclophosphamide: a comparative study of the efficiencies of UV/H₂O₂, UV/Fe²⁺/H₂O₂ and UV/TiO₂ processes. *Chemosphere*, 120, 538–46.
- Ma, J., Lin, F., Wang, S., & Xu, L. (2004a). Acute Toxicity Assessment of 20 Herbicides to the Green Alga *Scenedesmus quadricauda* (Turp.) Breb. *Bulletin of Environmental Contamination and Toxicology*, 72(6), 1164–1171.
- Ma, J. (2005). Differential sensitivity of three cyanobacterial and five green algal species to organotin and pyrethroids pesticides. *The Science of the Total Environment*, 341(1-3), 109–17.
- Ma, J., Lin, F., Zhang, R., Yu, W., & Lu, N. (2004v). Differential sensitivity of two green algae, *Scenedesmus quadricauda* and *Chlorella vulgaris*, to 14 pesticide adjuvants. *Ecotoxicology and Environmental Safety*, 58(1), 61–67.
- Ma, J., Xu, L., Wang, S., Zheng, R., Jin, S., Huang, S., & Huang, Y. (2002a). Toxicity of 40 Herbicides to the Green Alga *Chlorella vulgaris*. *Ecotoxicology and Environmental Safety*, 51(2), 128–132.
- Ma, J., Zheng, R., Xu, L., & Wang, S. (2002b). Differential Sensitivity of Two Green Algae, *Scenedesmus obliquus* and *Chlorella pyrenoidosa*, to 12 Pesticides. *Ecotoxicology and Environmental Safety*, 52(1), 57–61.
- Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Evans, R. M. (1995). The nuclear receptor superfamily: the second decade. *Cell*, 83(6), 835–9.
- Marcucci, G., Perrotti, D., & Caligiuri, M. A. (2004). Understanding the Molecular Basis of Imatinib Mesylate Therapy in Chronic Myelogenous Leukemia and the Related Mechanisms of Resistance. *Clinical Cancer Research*, 9(4), 1248–1252.
- Margiotta-Casaluci, L., Owen, S. F., Cumming, R. I., De Polo, A., Winter, M. J., Panter, G. H., Sumpter, J. P. (2014). Quantitative cross-species extrapolation between humans and fish: The case of the anti-depressant fluoxetine. *PLoS ONE*, 9(10), 1–18.
- Margiotta-Casaluci, L., Owen, S. F., Huerta, B., Rodríguez-Mozaz, S., Kugathas, S., Barceló, D., ... Sumpter, J. P. (2016). Internal exposure dynamics drive the Adverse Outcome Pathways of synthetic glucocorticoids in fish. *Scientific Reports*, 6, 21978.

- Margiotta-Casaluci, L., & Sumpter, J. P. (2011). 5 α -Dihydrotestosterone is a potent androgen in the fathead minnow (*Pimephales promelas*). *General and Comparative Endocrinology*, 171(3), 309–18.
- Mateos, J., Mañanos, E., Carrillo, M., & Zanuy, S. (2002). Regulation of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) gene expression by gonadotropin-releasing hormone (GnRH) and sexual steroids in the Mediterranean Sea bass. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, 132(1), 75–86.
- Matthews, J., Celius, T., Halgren, R., & Zacharewski, T. (2000). Differential estrogen receptor binding of estrogenic substances: a species comparison. *The Journal of Steroid Biochemistry and Molecular Biology*, 74(4), 223–34.
- Mattson, M. P. (2008). Hormesis defined. *Ageing Research Reviews*, 7(1), 1–7.
- McCormick, M. I. (1999). Experimental test of the effect of maternal hormones on larval quality of a coral reef fish. *Oecologia*, 118(4), 412–422.
- McCormick, S. D. (2001). Endocrine Control of Osmoregulation in Teleost Fish¹. *American Zoologist*, 41(4), 781–794.
- McCormick, S. D., Regish, A., O’Dea, M. F., & Shrimpton, J. M. (2008). Are we missing a mineralocorticoid in teleost fish? Effects of cortisol, deoxycorticosterone and aldosterone on osmoregulation, gill Na⁺,K⁺ -ATPase activity and isoform mRNA levels in Atlantic salmon. *General and Comparative Endocrinology*, 157(1), 35–40.
- Mcneff, G., Schmidt, W., & Quinn, B. (2014). Pharmaceuticals in the Aquatic Environment: A Short Summary of Current Knowledge and the Potential Impacts on Aquatic Biota and Humans. *EPA Research Programme*. Retrieved from www.epa.ie
- McDonald, K. ., Gross, T. ., Denslow, N. ., & Blazer, E. . (2000). Reproductive Indicators. In C. J. Schmitt, M. & G. Dethloff (Eds.), *Biomonitoring of Environmental Status and Trends (BEST) Program: selected methods for monitoring chemical contaminants and their effects in aquatic ecosystems* (pp. 30–42). U.S Geological Survey.
- McEwan, I. J., & Kumar, R. (2015). *Nuclear Receptors: From Structure to the Clinic*. Cham: Springer International Publishing.
- McPherson, M. J., & Møller, S. G. (2006). *PCR: The Basics* (2nd ed.). Taylor and Francis.
- Medicines & Healthcare Products Regulatory Agency. (MHRA). *Public Assessment Report. Decentralised Procedures. Desogestrel 75 microgram Film-coated Tablet*.
- Medicines and Healthcare Products Regulatory Agency (MHRA) (2016). A guide to what is a medicinal product. MHRA Guidance Note 8.
- Melamed, P., Rosenfeld, H., Elizur, A., & Yaron, Z. (1998). Endocrine regulation of gonadotropin and growth hormone gene transcription in fish. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, 119(3), 325–338.

- Metcalfe, C. D., Metcalfe, T. L., Kiparissis, Y., Koenig, B. G., Khan, C., Hughes, R. J., ... Potter, T. (2001). Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by in vivo assays with Japanese medaka (*Oryzias latipes*). *Environmental Toxicology and Chemistry*, 20(2), 297–308.
- MicroBioTests Inc. | Welcome. <http://www.microbiotests.be/> Accessed 2013.
- Miller, D. H., Jensen, K. M., Villeneuve, D. L., Kahl, M. D., Makynen, E. A., Durhan, E. J., & Ankley, G. T. (2007). Linkage of biochemical responses to population-level effects: a case study with vitellogenin in the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry / SETAC*, 26(3), 521–7.
- Millington, L. A., Goulding, K. H., & Adams, N. (1988). The influence of growth medium composition on the toxicity of chemicals to algae. *Water Research*, 22(12), 1593–1597.
- Monteiro, S. C., & Boxall, A. B. A. (2010). Occurrence and fate of human pharmaceuticals in the environment. *Reviews of Environmental Contamination and Toxicology*, 202, 53–154.
- Morgan, W. . (1939). A test for the significance of the difference between the two variances in a sample from a normal bivariate population. *Biometrika*, 31, 13–19.
- Moynihan, H., & Crean, A. (2009). *Physicochemical Basis of Pharmaceuticals*. OUP Oxford.
- Muller, P. Y., Janovjak, H., Miserez, A. R., & Dobbie, Z. (2002). Processing of Gene Expression Data Generated by Quantitative Real-Time RT-PCR. *Biotechniques*, 32(6).
- Nagler, J. J., Cavileer, T., Sullivan, J., Cyr, D. G., & Rexroad, C. (2007). The complete nuclear estrogen receptor family in the rainbow trout: discovery of the novel ERalpha2 and both ERbeta isoforms. *Gene*, 392(1-2), 164–73.
- Nakayama, K., Sato, K., Shibano, T., Isobe, T., Suzuki, G., & Kitamura, S.-I. (2016). Occurrence of glucocorticoids discharged from a sewage treatment plant in Japan and the effects of clobetasol propionate exposure on the immune responses of common carp (*Cyprinus carpio*) to bacterial infection. *Environmental Toxicology and Chemistry*, 35(4), 946–952.
- Nash, J. P., Kime, D. E., Van der Ven, L. T. M., Wester, P. W., Brion, F., Maack, G., Tyler, C. R. (2004). Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. *Environmental Health Perspectives*, 112(7), 1725–1733.
- Nash, R., Valencia, A. ., & Geffen, A. . (2006). The origin of Fulton's condition factor - Setting the record straight. *Fisheries*, 31(5), 236–238.
- Nelson, E. R., & Habibi, H. R. (2013). Estrogen receptor function and regulation in fish and other vertebrates. *General and Comparative Endocrinology*, 192, 15–24.

- Nett, T. M., Turzillo, A. M., Baratta, M., & Rispoli, L. A. (2002). Pituitary effects of steroid hormones on secretion of follicle-stimulating hormone and luteinizing hormone. *Domestic Animal Endocrinology*, 23(1-2), 33–42.
- Neubig, R. R., Spedding, M., Kenakin, T., & Christopoulos, A. (2003). International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on Terms and Symbols in Quantitative Pharmacology.
- Neumann, N. F., & Galvez, F. (2002). DNA microarrays and toxicogenomics: applications for ecotoxicology? *Biotechnology Advances*, 20(5-6), 391–419.
- NHS Digital, 1 Trevelyan Square, Boar Lane, Leeds, LS1 6AE, United Kingdom. (n.d.). More than 1 billion prescription items dispensed in a year - or 1,900 a minute. NHS Digital, 1 Trevelyan Square, Boar Lane, Leeds, LS1 6AE, United Kingdom.
- Norrgren, L. (2012). Fish models for ecotoxicology. *Acta Veterinaria Scandinavica*, 54(Suppl 1), S14.
- Nussey, S., & Whitehead, S. (2001). *Endocrinology. Endocrinology: An Integrated Approach*. BIOS Scientific Publishers.
- Nygaard, G., Komárek, J., Kristiansen, J., & Skulberg, O. . (1986). Taxonomic designations of the bioassay alga NIVA-CHL 1 (*Selenastrum capricornum*) and some related strains. *Opera Botanica*, 90, 1–46.
- Oaks, J. L., Gilbert, M., Virani, M. Z., Watson, R. T., Meteyer, C. U., Rideout, B. A., ... Khan, A. A. (2004). Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature*, 427(6975), 630–3.
- OECD GUIDELINE FOR TESTING OF CHEMICALS. (1992). 301a and b <http://www.oecd.org/chemicalsafety/risk-assessment/1948209.pdf> Accessed 2013
- OECD Guidelines for the Testing of Chemicals, Section 2 /Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test. (2011), 1–25.
- OECD Guidelines for the Testing of Chemicals, Section 2: Effects on Biotic Systems. <http://www.oecd.org/env/ehs/testing/section-2-effects-biotic-systems.htm> Accessed 2013
- Ogino, Y., Katoh, H., Kuraku, S., & Yamada, G. (2009). Evolutionary history and functional characterization of androgen receptor genes in jawed vertebrates. *Endocrinology*, 150(12), 5415–27.
- Okada, Y. K., & Yamashita, H. (1944). Experimental investigation of the manifestation of secondary sexual characters in fish, using the medaka, *Oryzias latipes* (Temminck and Schlegel) as material. *Journal of the Faculty of Science, Imperial University of Tokyo. IV Zoology*, 6, 383–437.
- Orlando, E. F., Bass, D. E., Caltabiano, L. M., Davis, W. P., Gray, L. E., & Guillette, L. J. (2007). Altered development and reproduction in mosquitofish exposed to pulp and paper mill effluent in the Fenholloway River, Florida USA. *Aquatic Toxicology*, 84(4), 399–405.

- Orlando, E. F., Kolok, A. S., Binzcik, G. A., Gates, J. L., Horton, M. K., Lambright, C. S., Guillette, L. J. (2004). Endocrine-disrupting effects of cattle feedlot effluent on an aquatic sentinel species, the fathead minnow. *Environmental Health Perspectives*, 112(3), 353–8.
- Overington, J. P., Al-Lazikani, B., & Hopkins, A. L. (2006). How many drug targets are there? *Nature Reviews. Drug Discovery*, 5(12), 993–6.
- Overturf, M. D., Overturf, C. L., Carty, D. R., Hala, D., & Huggett, D. B. (2014). Levonorgestrel exposure to fathead minnows (*Pimephales promelas*) alters survival, growth, steroidogenic gene expression and hormone production. *Aquatic Toxicology*, 148, 152–161.
- Page, L. M., & Burr, B. M. (1991). *A Field Guide to Freshwater Fishes: North America North of Mexico*. Houghton Mifflin.
- 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.
- Panter, G. H., Hutchinson, T. H., Länge, R., Lye, C. M., Sumpter, J. P., Zerulla, M., & Tyler, C. R. (2002). Utility of a juvenile fathead minnow screening assay for detecting (anti-)estrogenic substances. *Environmental Toxicology and Chemistry / SETAC*, 21(2), 319–26.
- 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.
- Parker, J. A., Webster, J. P., Kover, S. C., & Kolodziej, E. P. (2012). Analysis of trenbolone acetate metabolites and melengestrol in environmental matrices using gas chromatography-tandem mass spectrometry. *Talanta*, 99, 238–46.
- Parks, L. G., Lambright, C. S., Orlando, E. F., Guillette, L. J., Ankley, G. T., & Gray, L. E. (2001). Masculinization of female mosquitofish in Kraft mill effluent-contaminated Fenholloway River water is associated with androgen receptor agonist activity. *Toxicological Sciences: An Official Journal of the Society of Toxicology*, 62(2), 257–67.
- 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), 131–41.
- 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(2), 256–262.
- Payne, J., Rajapakse, N., Wilkins, M., & Kortenkamp, A. (2000). Prediction and assessment of the effects of mixtures of four xenoestrogens. *Environmental Health Perspectives*, 108(10), 983–7.

- Peakall, D. B. (1994). The role of biomarkers in environmental assessment (1). Introduction. *Ecotoxicology*, 3(3), 157–160.
- Pentikainen, V., Erkkilä, K., Suomalainen, L., Parvinen, M., & Dunkel, L. (2000). Estradiol Acts as a Germ Cell Survival Factor in the Human Testis in Vitro*. *J Clin Endocrinol Metab*, 85, 2057–2067.
- PerkinElmer. Radioimmunoassays | Application Support Knowledgebase | Lab Products and Services | PerkinElmer.. Retrieved from <http://www.perkinelmer.com/lab-products-and-services/application-support-knowledgebase/radiometric/radioimmunoassays.html> Accessed 2015.
- Petrie, B., McAdam, E. J., Scrimshaw, M. D., Lester, J. N., & Cartmell, E. (2013). Fate of drugs during wastewater treatment. *TrAC - Trends in Analytical Chemistry*.
- Petrovic, M., Solé, M., López De Alda, M. J., & Barceló, D. (2002). Endocrine disruptors in sewage treatment plants, receiving river waters, and sediments: Integration of chemical analysis and biological effects on feral carp. *Environmental Toxicology and Chemistry*, 21(10), 2146–2156.
- The Pharmacogenomics Knowledgebase (PharmGKB). <https://www.pharmgkb.org/> Accessed 2013.
- Phillips, P. J., Smith, S. G., Kolpin, D. W., Zaugg, S. D., Buxton, H. T., Furlong, E. T., ... Stinson, B. (2010). Pharmaceutical formulation facilities as sources of opioids and other pharmaceuticals to wastewater treatment plant effluents. *Environmental Science and Technology*.
- 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.
- Pierce, B. A. (2014). *Genetics: A Conceptual Approach* (2nd ed.). W.H. Freeman & Co Ltd.
- Piha, H., Dulio, V., & Hanke, G. (2010). Workshop Report River Basin-Specific Pollutants Identification and Monitoring Identification and Monitoring A collaboration between NORMAN and JRC in support of the Water Framework Directive.
- Pitman, E. J. . (1939). A note on normal correlation. *Biometrika*, 31, 9–12.
- Pitt, J. (2009). Principles and Applications of Liquid Chromatography. *Clinical Biochemist Reviews*, 30, 19–34.
- 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 & Technology*, 40(7), 2442–2447.
- Pottinger, T. G., Katsiadaki, I., Jolly, C., Sanders, M., Mayer, I., Scott, A. P., Scholze, M. (2013). Anti-androgens act jointly in suppressing spiggin concentrations in androgen-primed female three-spined sticklebacks - prediction of combined effects by concentration addition. *Aquatic Toxicology*, 140-141, 145–56.

- 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.
- Pu, C., Wu, Y.-F., Yang, H., & Deng, A.-P. (2008). Trace analysis of contraceptive drug levonorgestrel in wastewater samples by a newly developed indirect competitive enzyme-linked immunosorbent assay (ELISA) coupled with solid phase extraction. *Analytica Chimica Acta*, 628(1), 73–79.
- Purdom, C. E., Hardiman, P. A., Bye, V. V. J., Eno, N. C., Tyler, C. R., & Sumpter, J. P. (1994). Estrogenic Effects of Effluents from Sewage Treatment Works. *Chemistry and Ecology*, 8(4), 275–285.
- Radonić, A., Thulke, S., Mackay, I. M., Landt, O., Siegert, W., & Nitsche, A. (2004). Guideline to reference gene selection for quantitative real-time PCR. *Biochemical and Biophysical Research Communications*, 313(4), 856–62.
- Rafi, I. (2005). *An Introduction to the Use of Anticancer Drugs*. Elsevier Ltd. Butterworth Heinemann. .
- Rajapakse, N., Silva, E., & Kortenkamp, A. (2002). Combining xenoestrogens at levels below individual no-observed-effect concentrations dramatically enhances steroid hormone action. *Environmental Health Perspectives*, 110(9), 917–21.
- Randall, V. A. (1994). Role of 5 alpha-reductase in health and disease. *Baillière's Clinical Endocrinology and Metabolism*, 8(2), 405–31.
- Rand-Weaver, M., Margiotta-Casaluci, L., Patel, A., Panter, G. H., Owen, S. F., & Sumpter, J. P. (2013). The read-across hypothesis and environmental risk assessment of pharmaceuticals. *Environmental Science and Technology*, 47, 11384–11395.
- Rang, H., Ritter, J., Flower, R., & Henderson, G. (2015). *Rang and Dale's Pharmacology* (8th ed.). Elsevier.
- Raney, R. E. (1977). Comparative metabolism of 17 α -ethynyl steroids used in oral contraceptives. *Journal of Toxicology and Environmental Health*, 3(1-2), 139–166.
- Razandi, M., Pedram, A., Greene, G. L., & Levin, E. R. (1999). Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. *Molecular Endocrinology (Baltimore, Md.)*, 13(2), 307–19.
- Reed, m. J., fotherby, k., & steele, s. J. (1972). Metabolism of ethynylloestradiol in man. *Journal of Endocrinology*, 55(2), 351–361.
- Reynolds, A. R. (2009). Potential relevance of bell-shaped and u-shaped dose-responses for the therapeutic targeting of angiogenesis in cancer. *Dose-Response: A Publication of International Hormesis Society*, 8(3), 253–84.
- Richards, S. M., Wilson, C. J., Johnson, D. J., Castle, D. M., Lam, M., Mabury, S. A., Solomon, K. R. (2004). Effects of pharmaceutical mixtures in aquatic microcosms. *Environmental Toxicology and Chemistry / SETAC*, 23(4), 1035–42.

- Rixe, O., & Fojo, T. (2007). Is cell death a critical end point for anticancer therapies or is cytostasis sufficient? *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, 13(24), 7280–7.
- 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*.
- Roos, V., Gunnarsson, L., Fick, J., Larsson, D. G. J., & Rudén, C. (2012). Prioritising pharmaceuticals for environmental risk assessment: Towards adequate and feasible first-tier selection. *The Science of the Total Environment*, 421–422, 102–110.
- Rose, J., Holbech, H., Lindholst, C., Nørum, U., Povlsen, A., Korsgaard, B., & Bjerregaard, P. (2002). Vitellogenin induction by 17beta-estradiol and 17alpha-ethinylestradiol in male zebrafish (*Danio rerio*). *Comparative Biochemistry and Physiology. Toxicology & Pharmacology : CBP*, 131(4), 531–9.
- Routledge, E. J., Sheahan, D., Desbrow, C., Brighty, G. C., Waldock, M., & Sumpter, J. P. (1998). Identification of Estrogenic Chemicals in STW Effluent. 2. In Vivo Responses in Trout and Roach. *Environmental Science & 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 / SETAC*, 28(12), 2733–43.
- Rudrabhatla, P., Reddy, M. M., & Rajasekharan, R. (2006). Genome-wide analysis and experimentation of plant serine/ threonine/tyrosine-specific protein kinases. *Plant Molecular Biology*, 60(2), 293–319.
- Runnalls, T. J., Beresford, N., Kugathas, S., Margiotta-Casaluci, L., Scholze, M., Scott, A. P., & Sumpter, J. P. (2015). From single chemicals to mixtures—Reproductive effects of levonorgestrel and ethinylestradiol on the fathead minnow. *Aquatic Toxicology*, 169, 152–167.
- Runnalls, T. J., Beresford, N., Losty, E., Scott, A. P., & Sumpter, J. P. (2013). Several Synthetic Progestins with Different Potencies Adversely Affect Reproduction of Fish. *Environmental Science & Technology*, 47(4), 2077–2084.
- Säfholm, M., Jansson, E., Fick, J., & Berg, C. (2015). Mixture effects of levonorgestrel and ethinylestradiol: Estrogenic biomarkers and hormone receptor mRNA expression during sexual programming. *Aquatic Toxicology*, 161, 146–153.
- Savory, J. G., Préfontaine, G. G., Lamprecht, C., Liao, M., Walther, R. F., Lefebvre, Y. A., & Haché, R. J. (2001). Glucocorticoid receptor homodimers and glucocorticoid-mineralocorticoid receptor heterodimers form in the cytoplasm through alternative dimerization interfaces. *Molecular and Cellular Biology*, 21(3), 781–93.
- Schaaf, M. J. M., Champagne, D., van Laanen, I. H. C., van Wijk, D. C. W. A., Meijer, A. H., Meijer, O. C., ... Richardson, M. K. (2008). Discovery of a functional glucocorticoid receptor beta-isoform in zebrafish. *Endocrinology*, 149(4), 1591–9.

- Scherholz, M. L., & Curtis, W. R. (2013). Achieving pH control in microalgal cultures through fed-batch addition of stoichiometrically-balanced growth media. *BMC Biotechnology*, *13*(1), 39.
- Schiffer, B., Daxenberger, A., Meyer, K., & Meyer, H. H. (2001). The fate of trenbolone acetate and melengestrol acetate after application as growth promoters in cattle: environmental studies. *Environmental Health Perspectives*, *109*(11), 1145–51.
- Schmittgen, T. D., & Zakrajsek, B. A. (2000). Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. *Journal of Biochemical and Biophysical Methods*, *46*(1-2), 69–81.
- Scholze, M., Boedeker, W., Faust, M., Backhaus, T., Altenburger, R., & Grimme, L. H. (2001). A general best-fit method for concentration-response curves and the estimation of low-effect concentrations. *Environmental Toxicology and Chemistry*, *20*(2), 448–457.
- Scholze, M., Boedeker, W., Faust, M., Backhaus, T., Altenburger, R., & Grimme, L. H. (2001). A general best-fit method for concentration-response curves and the estimation of low-effect concentrations. *Environmental Toxicology and Chemistry*, *20*(2), 448–457.
- Schreck, C. B. (2010). Stress and fish reproduction: The roles of allostasis and hormesis. *General and Comparative Endocrinology*, *165*(3), 549–556.
- Schreck, C. B., Contreras-Sanchez, W., & Fitzpatrick, M. S. (2001). Effects of stress on fish reproduction, gamete quality, and progeny. *Aquaculture*, *197*(1), 3–24.
- 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 & Technology*, *44*(12), 4766–4774.
- Schubert, S., Peter, A., Schönenberger, R., Suter, M. J.-F., Segner, H., & Burkhardt-Holm, P. (2014). Transient exposure to environmental estrogen affects embryonic development of brown trout (*Salmo trutta fario*). *Aquatic Toxicology*, *157*, 141–149.
- Sebire, M., Katsiadaki, I., Taylor, N. G. H., Maack, G., & Tyler, C. R. (2011). Short-term exposure to a treated sewage effluent alters reproductive behaviour in the three-spined stickleback (*Gasterosteus aculeatus*). *Aquatic Toxicology (Amsterdam, Netherlands)*, *105*(1-2), 78–88.
- Selye, H. (1942). Correlations between the chemical structure and the pharmacological actions of the steroids. *Endocrinology*, *30*(3), 437–453. <http://doi.org/10.1210/endo-30-3-437>
- Sever, R., & Glass, C. K. (2013). Signaling by Nuclear Receptors. *Cold Spring Harbor Perspectives in Biology*, *5*(3), a016709–a016709.
- Shultz, S., Baral, H. S., Charman, S., Cunningham, A. A., Das, D., Ghalsasi, G. R., ... Prakash, V. (2004). Diclofenac poisoning is widespread in declining vulture populations across the Indian subcontinent. *Proceedings. Biological Sciences / The Royal Society*, *271* Suppl(Suppl_6), S458–60.

- Schwabl, H. (1993). Yolk is a source of maternal testosterone for developing birds. *Proceedings of the National Academy of Sciences of the United States of America*, 90(24), 11446–50.
- Schwabl, H. (1996). Maternal testosterone in the avian egg enhances postnatal growth. *Comparative Biochemistry and Physiology Part A: Physiology*, 114(3), 271–276.
- Silva, E., Rajapakse, N., & Kortenkamp, A. (2002). Something from nothing;--eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. *Environmental Science & Technology*, 36(8), 1751–6.
- Silverman, M. N., Pearce, B. D., Biron, C. A., & Miller, A. H. (2005). Immune modulation of the hypothalamic-pituitary-adrenal (HPA) axis during viral infection. *Viral Immunology*, 18(1), 41–78.
- Silverman, M. N., & Sternberg, E. M. (2012). Glucocorticoid regulation of inflammation and its functional correlates: from HPA axis to glucocorticoid receptor dysfunction. *Annals of the New York Academy of Sciences*, 1261, 55–63.
- Singh, M., Su, C., & Ng, S. (2013). Non-genomic mechanisms of progesterone action in the brain. *Frontiers in Neuroscience*, 7, 159.
- Small Molecular Pathway Database (SMPDB) <http://smpdb.ca/> Accessed 2015.
- Smeets, J. M., van Holsteijn, I., Giesy, J. P., Seinen, W., & van den Berg, M. (1999). Estrogenic potencies of several environmental pollutants, as determined by vitellogenin induction in a carp hepatocyte assay. *Toxicological Sciences: An Official Journal of the Society of Toxicology*, 50(2), 206–13.
- Smeets, J. M., van Holsteijn, I., Giesy, J. P., Seinen, W., & van den Berg, M. (1999). Estrogenic potencies of several environmental pollutants, as determined by vitellogenin induction in a carp hepatocyte assay. *Toxicological Sciences: An Official Journal of the Society of Toxicology*, 50(2), 206–13.
- Society for Endocrinology. (Accessed April 2016). You and Your Hormones | Hormones | Dihydrotestosterone. <http://www.yourhormones.info/>
- Soltysik, K., & Czekaj, P. (2013). Membrane estrogen receptors - is it an alternative way of estrogen action? *Journal of Physiology and Pharmacology: An Official Journal of the Polish Physiological Society*, 64(2), 129–42.
- Song, M. K., & Choi, S. H. (2001). Growth Promoters and Their Effects on Beef Production - Review -. *Asian-Australasian Journal of Animal Sciences*, 14(1), 123–135.
- Soto, A. M., Calabro, J. M., Prechtel, N. V., Yau, A. Y., Orlando, E. F., Daxenberger, A., Sonnenschein, C. (2004). Androgenic and estrogenic activity in water bodies receiving cattle feedlot effluent in Eastern Nebraska, USA. *Environmental Health Perspectives*, 112(3), 346–52.
- Sowers, M. F., Beebe, J. L., McConnell, D., Randolph, J., & Jannausch, M. (2001). Testosterone concentrations in women aged 25-50 years: associations with lifestyle,

- body composition, and ovarian status. *American Journal of Epidemiology*, 153(3), 256–64.
- Speroff, L., Glass, R. and Kase, N.G. 1999. Clinical gynecology and endocrinology and infertility. Lippincott Williams and Wilkins.
- 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), 1602–11.
- Staudinger, J. L., Ding, X., & Lichti, K. (2006). Pregnane X receptor and natural products: beyond drug-drug interactions. *Expert Opinion on Drug Metabolism & Toxicology*, 2(6), 847–57.
- Steger-Hartmann, T., Kümmerer, K., & Hartmann, A. (1997). Biological Degradation of Cyclophosphamide and Its Occurrence in Sewage Water. *Ecotoxicology and Environmental Safety*, 36(2), 174–179.
- Steger-Hartmann, T., Kümmerer, K., & Schecker, J. (1996). Trace analysis of the antineoplastics ifosfamide and cyclophosphamide in sewage water by twostep solid-phase extraction and gas chromatography-mass spectrometry. *Journal of Chromatography A*, 726(1-2), 179–184.
- Straub, J. O. (2010). Combined environmental risk assessment for 5-fluorouracil and capecitabine in Europe. *Integrated Environmental Assessment and Management*, 6 Suppl, 540–66.
- Streit, B. (1998). Bioaccumulation of contaminants in fish. In *Fish Ecotoxicology* (pp. 353–387). Basel: Birkhäuser Basel.
- Sumpter, J. P. (2009). Protecting aquatic organisms from chemicals: the harsh realities. *Phil. Trans. R. Soc. A*, 367, 3877–3894.
- Sumpter, J. P., & Jobling, S. (1995). Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environmental Health Perspectives*, (Suppl 7), 173–8.
- 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 & Technology*, 40(17), 5478–5489.
- Sun, L., Zha, J., & Wang, Z. (2009). Effects of binary mixtures of estrogen and antiestrogens on Japanese medaka (*Oryzias latipes*). *Aquatic Toxicology*, 93(1), 83–89.
- Supalkova, V., Beklova, M., Baloun, J., Singer, C., Sures, B., Adam, V., Kizek, R. (2008). Affecting of aquatic vascular plant *Lemna minor* by cisplatin revealed by voltammetry. *Bioelectrochemistry*, (72(1)), 59–65.
- Svensson, J., Fick, J., Brandt, I., & Brunström, B. (2013). The Synthetic Progestin Levonorgestrel Is a Potent Androgen in the Three-Spined Stickleback (*Gasterosteus aculeatus*). *Environmental Science & Technology*, 47(4), 2043–2051.

- Swanson, P., & Dickhoff, W. (1988). Effects of exogenous gonadal steroids on gonadotropins I and II in juvenile coho salmon. *American Zoologist*, 28, 55A.
- Tait, A. S., Butts, C. L., & Sternberg, E. M. (2008). The role of glucocorticoids and progestins in inflammatory, autoimmune, and infectious disease. *Journal of Leukocyte Biology*, 84(4), 924–31.
- Takeo, J., & Yamashita, S. (1999). Two distinct isoforms of cDNA encoding rainbow trout androgen receptors. *The Journal of Biological Chemistry*, 274(9), 5674–80.
- Tanaka, M., Tanangonan, J. B., Tagawa, M., de Jesus, E. G., Nishida, H., Isaka, M., ... Hirano, T. (1995). Development of the pituitary, thyroid and interrenal glands and applications of endocrinology to the improved rearing of marine fish larvae. *Aquaculture*, 135(1), 111–126.
- Tanaka, T., Masuda, H., Naito, M., & Tamai, H. (2001). Pretreatment with 5-fluorouracil enhances cytotoxicity and retention of DNA-bound platinum in a cisplatin resistant human ovarian cancer cell line. *Anticancer Research*, 21(4A), 2463–9.
- Tarazona, J. V., Escher, B. I., Giltrow, E., Sumpter, J., & Knacker, T. (n.d.). Targeting the Environmental Risk Assessment of Pharmaceuticals: Facts and Fantasies. *Integrated Environmental Assessment and Management*, 6(1), 606–613.
- Taves, M. D., Gomez-Sanchez, C. E., & Soma, K. K. (2011). Extra-adrenal glucocorticoids and mineralocorticoids: evidence for local synthesis, regulation, and function. *American Journal of Physiology. Endocrinology and Metabolism*, 301(1), E11–24.
- Ternes, T., Stumpf, M., Mueller, J., Haberer, K., Wilken, R.-D., & Servos, M. (1999). Behaviour and occurrence of estrogens in municipal sewage treatment plants — I. Investigations in Germany, Canada and Brazil. *Science of The Total Environment*, 225(1), 81–90.
- Ternes, T. A. (1998). Occurrence of drugs in German sewage treatment plants and rivers. *Water Research*, 32(11), 3245–3260.
- Ternes, T. A., Joss, A., & Siegrist, H. (2004). Peer Reviewed: Scrutinizing Pharmaceuticals and Personal Care Products in Wastewater Treatment. *Environmental Science & Technology*, 38(20), 392A–399A.
- Ternes, T. A., Stüber, J., Herrmann, N., McDowell, D., Ried, A., Kampmann, M., & Teiser, B. (2003). Ozonation: A tool for removal of pharmaceuticals, contrast media and musk fragrances from wastewater? *Water Research*, 37, 1976–1982.
- Ternes, T.A., Joss, A. Siegrist, H. 2004. Scrutinizing pharmaceuticals and personal care products in wastewater treatment. American Chemical Society. Environmental Science and Technology 393A.
- The PubChem Project. <https://pubchem.ncbi.nlm.nih.gov/> Accessed 2015
- Thellin, O., Zorzi, W., Lakaye, B., De Borman, B., Coumans, B., Hennen, G., ... Heinen, E. (1999). Housekeeping genes as internal standards: use and limits. *Journal of Biotechnology*, 75(2-3), 291–5.

- Thomas Backhaus, A.-P., & Faust, M. (2009). State of the Art Report on Mixture Toxicity.
- Thomas, P. (2012). Rapid steroid hormone actions initiated at the cell surface and the receptors that mediate them with an emphasis on recent progress in fish models. *General and Comparative Endocrinology*, 175(3), 367–83.
- Thompson, I. M., Goodman, P. J., Tangen, C. M., Parnes, H. L., Minasian, L. M., Godley, P. A., ... Ford, L. G. (2013). Long-term survival of participants in the prostate cancer prevention trial. *The New England Journal of Medicine*, 369(7), 603–10.
- Thorpe, K. L., Hutchinson, T. H., Hetheridge, M. J., Scholze, M., Sumpter, J. P., & Tyler, C. R. (2001). Assessing the Biological Potency of Binary Mixtures of Environmental Estrogens using Vitellogenin Induction in Juvenile Rainbow Trout (*Oncorhynchus mykiss*). *Environmental Science & Technology*, 35(12), 2476–2481.
- Tilton, S. C., Foran, C. M., & Benson, W. H. (2005). Relationship between ethinylestradiol-mediated changes in endocrine function and reproductive impairment in Japanese medaka (*Oryzias latipes*). *Environmental Toxicology and Chemistry / SETAC*, 24(2), 352–9.
- Tokarz, J., Möller, G., Hrabě de Angelis, M., & Adamski, J. (2013). Zebrafish and steroids: What do we know and what do we need to know? *The Journal of Steroid Biochemistry and Molecular Biology*, 137, 165–173.
- Tomas, C., Newton, J., Watson, S., Tomas, C., Newton, J., & Watson, S. (2013). A Review of Hypothalamic-Pituitary-Adrenal Axis Function in Chronic Fatigue Syndrome. *ISRN Neuroscience*, 2013, 1–8.
- Trudeau, V. L., Metcalfe, C. D., Mimeault, C., & Moon, T. W. (2005). Chapter 17 Pharmaceuticals in the environment: Drugged fish? *Biochemistry and Molecular Biology of Fishes*.
- Tyler, C. R., van der Eerden, B., Jobling, S., Panter, G., & Sumpter, J. P. (1996). Measurement of vitellogenin, a biomarker for exposure to oestrogenic chemicals, in a wide variety of cyprinid fish. *Journal of Comparative Physiology B*, 166(7), 418–426.
- Tyler, C. R., van Aerle, R., Hutchinson, T. H., Maddix, S., & Trip, H. (1999). An in vivo testing system for endocrine disruptors in fish early life stages using induction of vitellogenin. *Environmental Toxicology and Chemistry*, 18(2), 337–347.
- UBA, Umwelt Bundesamt - German Federal Environmental Agency. Pharmaceuticals in the Environment. Occurrence, effects and potential cooperation action under SAICM. 2014.
- Undevia, S. D., Gomez-Abuin, G., & Ratain, M. J. (n.d.). Pharmacokinetic variability of anticancer agents.
- U.S Environmental Protection Agency. Office of Research and Development. (2013). *Adverse Outcome Pathway Wiki: Science in Action*.
- Vajda, A. M., Barber, L. B., Gray, J. L., Lopez, E. M., Woodling, J. D., & Norris, D. O. (2008). Reproductive Disruption in Fish Downstream from an Estrogenic Wastewater Effluent. *Environmental Science & Technology*, 42(9), 3407–3414.

- Valenti, T. W., Gould, G. G., Berninger, J. P., Connors, K. A., Keele, N. B., Prosser, K. N., & Brooks, B. W. (2012). Human therapeutic plasma levels of the selective serotonin reuptake inhibitor (SSRI) sertraline decrease serotonin reuptake transporter binding and shelter-seeking behavior in adult male fathead minnows. *Environmental Science and Technology*.
- van Aerle, R., Pounds, N., Hutchinson, T. H., Maddix, S., & Tyler, C. R. (2002). Window of sensitivity for the estrogenic effects of ethinylestradiol in early life-stages of fathead minnow, *Pimephales promelas*. *Ecotoxicology (London, England)*, *11*(6), 423–34.
- van der Linden, S. C., Heringa, M. B., Man, H.-Y., 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 & Technology*, *42*(15), 5814–5820.
- 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), 459–65.
- Vasquez, M. I., Lambrianides, A., Schneider, M., Kümmerer, K., & Fatta-Kassinos, D. (2014). Environmental side effects of pharmaceutical cocktails: What we know and what we should know. *Journal of Hazardous Materials*, *279*, 169–189.
- Velasco-Santamaría, Y. M., Bjerregaard, P., & Korsgaard, B. (2013). Evidence of small modulation of ethinylestradiol induced effects by concurrent exposure to trenbolone in male eelpout *Zoarces viviparus*. *Environmental Pollution*, *178*, 189–96.
- Venkataramani, E. S., Carlin, J. R., Dolling, U., Christofalo, P., Magliette, R. J., Arison, B. H., & Stearns, R. A. (1994). Biotransformation of finasteride (MK-0906) by *Selenastrum capricornutum* (green algae). *Annals of the New York Academy of Sciences*, *745*, 51–60.
- Verhoeven, C. H. ., Gloudemans, R. H. ., Peeters, P. A. ., van Lier, J. ., Verheggen, F. T. ., Groothuis, G. M., Vos, R. M. (2001). Excretion and metabolism of desogestrel in healthy postmenopausal women. *The Journal of Steroid Biochemistry and Molecular Biology*, *78*(5), 471–480.
- Viglino, L., Aboufadi, K., Prévost, M., & Sauvé, S. (2008). Analysis of natural and synthetic estrogenic endocrine disruptors in environmental waters using online preconcentration coupled with LC-APPI-MS/MS. *Talanta*, *76*(5), 1088–1096.
- Villeneuve, D. L., Crump, D., Garcia-Reyero, N., Hecker, M., Hutchinson, T. H., LaLone, C. A., Whelan, M. (2014). Adverse outcome pathway (AOP) development I: strategies and principles. *Toxicological Sciences: An Official Journal of the Society of Toxicology*, *142*(2), 312–20.
- Villeneuve, D. L., Miracle, A. L., Jensen, K. M., Degitz, S. J., Kahl, M. D., Korte, J. J., ... Ankley, G. T. (2007a). Development of quantitative real-time PCR assays for fathead minnow (*Pimephales promelas*) gonadotropin beta subunit mRNAs to support endocrine disruptor research. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP*, *145*(2), 171–83.

- Villeneuve, D. L., Blake, L. S., Brodin, J. D., Greene, K. J., Knoebel, I., Miracle, A. L., ... Ankley, G. T. (2007b). Transcription of key genes regulating gonadal steroidogenesis in control and ketoconazole- or vinclozolin-exposed fathead minnows. *Toxicological Sciences: An Official Journal of the Society of Toxicology*, 98(2), 395–407.
- Vulliet, E., Baugros, J.-B., Flament-Waton, M.-M., & Grenier-Loustalot, M.-F. (2007). Analytical methods for the determination of selected steroid sex hormones and corticosteroids in wastewater. *Analytical and Bioanalytical Chemistry*, 387(6), 2143–51.
- Vulliet, E., & Cren-Olivé, C. (2011). Screening of pharmaceuticals and hormones at the regional scale, in surface and groundwaters intended to human consumption. *Environmental Pollution (Barking, Essex: 1987)*, 159(10), 2929–34.
- Vulliet, E., Wiest, L., Baudot, R., & Grenier-Loustalot, M.-F. (2008). Multi-residue analysis of steroids at sub-ng/L levels in surface and ground-waters using liquid chromatography coupled to tandem mass spectrometry. *Journal of Chromatography A*, 1210(1), 84–91.
- Wang, H., Wang, N., Wang, B., Zhao, Q., Fang, H., Fu, C., ... Jiang, Q. (2016). Antibiotics in Drinking Water in Shanghai and Their Contribution to Antibiotic Exposure of School Children. *Environmental Science & Technology*, 50(5), 2692–2699.
- Waterhouse, R. N., Chang, R. C., Atuehene, N., & Collier, T. L. (2007). *In vitro* and *in vivo* binding of neuroactive steroids to the sigma-1 receptor as measured with the positron emission tomography radioligand [18F]FPS. *Synapse (New York, N.Y.)*, 61(7), 540–6.
- Watts, C., Maycock, D., Crane, M., & Fawell, J. (2007). Desk based review of current knowledge on pharmaceuticals in drinking water and estimation of potential levels (Defra Project Code: CSA 7184/WT02046/DWI70/2/213) Final Report.
- Webb, S., Ternes, T., Gibert, M., & Olejniczak, K. (2003). Indirect human exposure to pharmaceuticals via drinking water. *Toxicology Letters*, 142, 157–167.
- Weber, L. P., Hill, R. L., & Janz, D. M. (2003). Developmental estrogenic exposure in zebrafish (*Danio rerio*): II. Histological evaluation of gametogenesis and organ toxicity. *Aquatic Toxicology*, 63(4), 431–446.
- Weber, F.-A., Aus Der Beek, T., Carius, A., Grüttner, G., Hickmann, S., Ebert, I., ... Stolzenberg, H.-C. (2014). Pharmaceuticals in the environment – the global perspective Occurrence, effects, and potential cooperative action under SAICM. Retrieved from www.umweltbundesamt.de
- Wessels, J. A. M., van der Kooij, S. M., le Cessie, S., Kievit, W., Barerra, P., Allaart, C. F., ... Guchelaar, H.-J. (2007). A clinical pharmacogenetic model to predict the efficacy of methotrexate monotherapy in recent-onset rheumatoid arthritis. *Arthritis and Rheumatism*, 56(6), 1765–75.
- Wilkinson, G.R. (2001). Pharmacokinetics: The dynamics of drug absorption, distribution and elimination. Pp3-29. In: Hardman, J.J., Limbird, L.E., and Gilman, A.G. Goodman and Gilman's The Pharmacological Basis of Therapeutics. 10th edition. McGraw-Hill, New York.

- Willen, E. (2000). Phytoplankton in water quality assessment. In P. Heinonen, G. Ziglio, & A. Van der Beken (Eds.), *Hydrological and Limnological* (pp. 57–808). John Wiley.
- Williams, R. (2005). *Human Pharmaceuticals: Assessing the Impacts on Aquatic Ecosystems*. Allen Press/ACG Publishing.
- Williams, R. J., Keller, V. D. J., Johnson, A. C., Young, A. R., Holmes, M. G. R., Wells, C., ... Benstead, R. (2009). A national risk assessment for intersex in fish arising from steroid estrogens. *Environmental Toxicology and Chemistry*, 28(1), 220.
- Wilson, V. S., Lambright, C., Ostby, J., & Gray, L. E. (2002). In vitro and in vivo effects of 17beta-trenbolone: a feedlot effluent contaminant. *Toxicological Sciences : An Official Journal of the Society of Toxicology*, 70(2), 202–11.
- Wilson, K. S., Matrone, G., Livingstone, D. E. W., Al-Dujaili, E. A. S., Mullins, J. J., Tucker, C. S., ... Denvir, M. A. (2013). Physiological roles of glucocorticoids during early embryonic development of the zebrafish (*Danio rerio*). *The Journal of Physiology*, 591(24), 6209–20.
- 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), 600–12
- World Health Organisation (WHO) (2012) State of the science of endocrine disrupting chemicals. WHO/UNEP.
- World Health Organisation. (2016). WHO | International Programme on Chemical Safety. Retrieved August 29, 2016, from <http://www.who.int/ipcs/en/>
- Yadete, F., & Male, R. (2002). Effects of 4-nonylphenol on gene expression of pituitary hormones in juvenile Atlantic salmon (*Salmo salar*). *Aquatic Toxicology*, 58(1-2), 113–29.
- Yalow, R. S., & Berson, S. A. (1960). Immunoassay of endogenous plasma insulin in man. *The Journal of Clinical Investigation*, 39(7), 1157–75.
- Yaron, Z., Gur, G., Melamed, P., Rosenfeld, H., Elizur, A., & Levavi-Sivan, B. (2003). Regulation of fish gonadotropins. *International Review of Cytology*, 225, 131–85.
- Yang, L.-H., Ying, G.-G., Su, H.-C., Stauber, J. L., Adams, M. S., & Binet, M. T. (2008). Growth-inhibiting effects of 12 antibacterial agents and their mixtures on the freshwater microalga *Pseudokirchneriella subcapitata*. *Environmental Toxicology and Chemistry / SETAC*, 27(5), 1201–8.
- 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 / SETAC*, 28(12), 2663–70.
- Zhang, Y., Luo, X., Wu, D., & Xu, Y. (2015). ROR nuclear receptors: structures, related diseases, and drug discovery. *Acta Pharmacologica Sinica*, 36(1), 71–87.

- Zhao, L., Au, J. L.-S., & Wientjes, M. G. (2010). Comparison of methods for evaluating drug-drug interaction. *Frontiers in Bioscience (Elite Edition)*, 2, 241–9. Retrieved from
- Zhao, Y., Castiglioni, S., & Fent, K. (2015). Synthetic progestins medroxyprogesterone acetate and dydrogesterone and their binary mixtures adversely affect reproduction and lead to histological and transcriptional alterations in zebrafish (*Danio rerio*). *Environmental Science & Technology*, 49(7), 4636–45.
- Zohar, Y., Muñoz-Cueto, J. A., Elizur, A., & Kah, O. (2010). Neuroendocrinology of reproduction in teleost fish. *General and Comparative Endocrinology*, 165(3), 438–55. <http://doi.org/10.1016/j.ygcen.2009.04.017>
- Zounkova, R., Kovalova, L., Blaha, L., & Dott, W. (2010). Ecotoxicity and genotoxicity assessment of cytotoxic antineoplastic drugs and their metabolites. *Chemosphere*, 81(2), 253–60.
- Zounková, R., Odráska, P., Dolezalová, L., Hilscherová, K., Marsálek, B., & Bláha, L. (2007). Ecotoxicity and genotoxicity assessment of cytostatic pharmaceuticals. *Environmental Toxicology and Chemistry / SETAC*, 26(10), 2208–14.
- Zucchi, S., Castiglioni, S., & Fent, K. (2012). Progestins and antiprogestins affect gene expression in early development in zebrafish (*Danio rerio*) at environmental concentrations. *Environmental Science & Technology*, 46(9), 5183–92.
- Zucchi, S., Mirbahai, L., Castiglioni, S., & Fent, K. (2014). Transcriptional and Physiological Responses Induced by Binary Mixtures of Drospirenone and Progesterone in Zebrafish (*Danio rerio*). *Environmental Science & Technology*, 48(6), 3523–3531.

Appendix 1: Dosing and master stock calculations for a five compound steroid mixture experiment

Peristatic Pump Rate	0.2ml/min	Flow Rate	60L/hour	Dilution Factor
	12ml/hour		1000ml/min (1L/min)	1000/8 tanks = 125ml/min/tank
	288ml/24 hours 2016ml/7 days		1000/0.2	5000 dilution factor

Compound	EC10 ng/L	Dosing Stock ng/L	Master Stock ng/L	x3.3 ng/L	Dosing Stock ng/L	Master Stock ng/L
EE2	0.16	792.31	792309.1664	EE2	0.52	2614.62
Levonorgestrel	0.28	1409.71	1409705.068	Levonorgestrel	0.93	4652.03
Trenbolone	31.62	158104.80	158104797	Trenbolone	104.35	521745.83
Beclomethasone	19.24	96192.48	96192481.84	Beclomethasone	63.49	317435.19
Desogestrel	299.98	1499897.80	1499897799	Desogestrel	989.93	4949662.74
Total ng/L	351.28	1756397.09	1756397092	Total ng/L	1159.22	5796110.40
µg/L		1756.40	1756397.1	µg/L	1.16	5796.11
mg/L		1.76	1756.4	mg/L	5.77	5796.1

Compound	x6.6 ng/L	Dosing Stock ng/L	Master Stock ng/L
EE2	1.046	5229.2405	5222940.498
Levonorgestrel	1.816	9304.0535	9304053.452
Trenbolone	208.698	1043491.66	1043491660
Beclomethasone	126.974	634870.38	634870380.1
Desogestrel	1979.865	9899325.48	9899325475
Total ng/L	2318.444	11592220.81	11592220809
µg/L	2.32	11592.22	11592220.8
mg/L		11.59	11592.2

Appendix 2. Fathead minnow pair-breeding classification sheet

FAB Regular & Lots	REALLY GOOD Less Regular but Lots	GOOD Low/Med Numbers But regular	OK ½ spawning's In pairing period or new pair - unsure	POOR Not spawned or really low number
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Appendix 3. Protocol for the assessment of second sexual characteristics in adult

Fathead minnow (*Pimephales promelas*)

Fatpad

The dorsal fatpad is divided into two distinct regions, the triangular head pad (that starts as a point just above the nuptial tubercles and spreads between the eyes for the full length of the head) and the dorsal pad that runs from the head pad and narrows to a point that terminates at the dorsal fin.

The prominence of the dorsal fatpad on each male and female fish must be graded using the following criteria:

- Grade 0 no fatpad visible
- Grade 1 formation of a small fatpad evident but this is not raised by more than 1 mm above the body surface
- Grade 2 fatpad is clearly visible and is protruding above the body surface by 2 to 5 mm
- Grade 3 fatpad is very prominent and is raised by more than 5 mm above the body surface, but has not formed folds
- Grade 4 fatpad is very prominent (see grade 3) above the body surface and has formed folds

Nuptial tubercles

The tubercles are visible as white disks projecting above the body surface on the snout of the fish. The tubercles are often arrayed in a bilaterally symmetrical pattern. The prominence of the nuptial tubercles on each fish must be graded using the criteria below (originally described by Smith, 1977).

- | | |
|---|--|
| 0 | no visible sign of tubercles |
| 1 | tubercles visible as white disks, not protruding above body surface |
| 2 | tubercles project above body surface |
| 3 | tubercles prominent but not sharp |
| 4 | tubercles prominent and sharp |
| 5 | tubercles have started to run together and not all individual tubercles can be distinguished |