OESTROGENS AND ANTI-ANDROGENS IN THE AQUATIC ENVIRONMENT AND THEIR EFFECTS ON FISH

A Thesis Submitted for the Degree of Doctor of Philosophy

By

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CONTENTS

ABSTRACT

Endocrine disrupting chemicals (EDCs) can enter the aquatic environment via wastewater treatment work (WwTW) effluents. Oestrogenic and (anti-)androgenic chemicals present in effluents affect the reproductive health of fish living downstream of these effluents.

The effects of WwTW effluent before and after the addition of an advanced granular activated carbon (GAC) treatment; installed to reduce EDCs, are compared. In laboratory studies, no oestrogenic effects of standard or GAC effluent were observed in fathead minnows (FHM; *Pimephales promelas*). However, the standard effluent elicited possible anti-androgenic effects not observed in the GAC-treated effluent. In the river receiving this effluent, wild roach (*Rutilus rutilus*) were sampled before and after the GAC addition. Here oestrogenic effects were observed in the roach, and these effects were reduced following the GAC addition to the WwTW, but not completely removed.

Over 100 chemicals previously detected in WwTW effluents were examined for (anti-)androgenic activity using an *in vitro* yeast-based assay. Toxicity can cause false positive results in this assay. Investigations, including employing a modified version of the *in vitro* assay, were conducted to improve the assay's reliability. Then, assessments of the most potent anti-androgenic chemicals were made to determine if they were likely to cause a risk to the environment.

Currently, the best *in vivo* fish screen for (anti-)androgenic activity utilises the induction of spiggin, an androgen dependent glue-like protein, normally produced by male 3-spined stickleback (*Gasterosteus aculeatus*) during nest building. Male FHMs also glue their mates' eggs to substrate. Therefore, FHM may have homologous androgen dependant genes/proteins which could serve as biomarkers of (anti-)androgenic activity. Spiggin-like primers were designed and used to look for spiggin-like genes in tissues collected from FHM. However, none of the spiggin-like primers were male specific and further work is needed to determine if similar androgen dependant spiggin-like proteins are present in FHM.

ACKNOWLEDGEMENTS

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Additionally, I would like to thank fish facility staff and Environmental Sciences staff and students at Brunel University London for their support with fish work, along with Hull International Fisheries Institute, University of Hull for electrofishing roach for me in 2013 and Scymaris, Brixham for providing me with fathead minnows in 2018. Additional thanks must go to Dr Anne Lockyer and Ms Marjo Broeder for their support with my molecular chapter.

My long suffering family have also stood by me, particularly my daughter Amelia Dittmann. She was 10 years old when I started my PhD and for most of her secondary school life, I have worked long hours. I can only hope that some of this hard working mindset has rubbed off onto her to make up for my lack of parenting. For so many years family and friends have encouraged me and regularly asked 'how is it going?' and I'm so pleased that I can finally say that I have finished!

PUBLICATIONS

Research that originated from this thesis and has been presented:

- Beresford, N., Baynes, A. and Jobling, S. Testing for (anti-)androgenic activity; pitfalls and possible misinterpretations. Poster Presentation. 16th Environmental Toxicology and Biological Systems Annual Meeting 2018, London, UK.
- Beresford, N., Baynes, A. and Jobling, S. Oral Presentation. 4th Annual College of Health and Life Sciences PG Student Conference 2018, Brunel University London, UK.

Additional research published but not included in this thesis:

- Hettwer, K., Jahne, M., Frost, K., Giersberg, M., Kunze, G., Trimborn, M., Reif, M., Turk, J., Gehrmann, L., Dardenne, F., De Croock, F., Abraham, M., Schoop, A., Waniek, J.J., Bucher, T., Simon, E., Vermeirssen, E., Werner, A., Hellauer, K., Wallentits, U., Drewes, J.E., Dietzmann, D., Routledge, E., Beresford, N., Zietek, T., Siebler, M., Simon, A., Bielak, H., Hollert, H., Muller, Y., Harff, M., Schiwy, S., Simon, K. and Uhlig, S. 2018. Validation of Arxula Yeast Estrogen Screen assay for detection of estrogenic activity in water samples: Results of an international interlaboratory study. Science of the Total Environment 621, 612-625.
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ABBREVIATIONS

AF1	Activation Function region 1
AFSS	Androgenised Female Stickleback Screen
AGD	Anogenital distance
AOP	Adverse outcome pathway
AR	Androgen receptor
AYAS	Yeast anti-androgen screen
A-YES	Yeast oestrogen screen (*Arxula adeninivorans*)
BF3	Binding Function 3
BLAST	Basic Local Alignment Search
cDNA	Complementary deoxyribonucleic acid
CPA	Congenital penile anomaly
CPRG	Chlorophenol red-β-D-galactopyranoside
Cq	Quantification cycle
$CuSO_4$	Copper sulphate
DBD	DNA binding domain
DBP	Dibutyl phthalate
p,p'-DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DEHP	Di(2-ethylhexyl) phthalate
DEP	Diethyl phthalate
DEPC	Diethyl pyrocarbonate
DES	Diethylstilbestrol
DHT	Dihydrotestosterone
DL	Detection limit
Dmrt1	Doublesex and mab-3 related transcription factor 1

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DO-medium	Dropout medium
E1	Oestrone
E2	Oestradiol
E2EQ	Oestradiol equivalent
E2-G	β-Oestradiol 17-(β-D-glucuronide)
E2-S	β-Oestradiol 3-sulphate
EC50	Effective concentration, half maximal
EDA	Effect directed analysis
EDC	Endocrine disrupting chemical
EDDP	Endocrine Disruption Demonstration Programme
EDSP	Endocrine Disrupter Screening Program
EE2	Ethinylestradiol
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
EQS	Environmental Quality Standard
EQSD	Environmental Quality Standards Directive
ER	Oestrogen receptor
EST	Expressed sequence tag
EtOH	Ethanol
EU	European Union
FHM	Fathead minnow
FLUT	Flutamide
FLUTEQ	Flutamide equivalent
Foxl2	Forkhead box l2

FPI	Fatpad Index
FSH	Follicle stimulating hormone
GAC	Granular activated carbon
β-gal	β-galactosidase
GC-MS	Gas chromatography–mass spectrometry
gDNA	Genomic deoxyribonucleic acid
GnRH	Gonadotropin releasing hormone
GOI	Gene of interest
GR	Glucocorticoid receptor
GSI	Gonadosomatic Index
H12	Helix-12
HHCB	Galaxolide
HPG	Hypothalamic-pituitary-gonad axis
HPLC	High-performance liquid chromatography
hprt1	Hypoxanthine phosphoribosyltransferase 1
HSD	Hydroxysteroid dehydrogenase
HTP	High throughput
IC50	Inhibitory concentration, half maximal
ISO	International Organisation for Standardisation
IPC	Inter-plate calibrator
KEH	Kidney epithelial height
11-KT	11-Ketotestosterone
LBD	Ligand binding domain
LBP	Ligand binding pocket
LDH	Lactate dehydrogenase
LH	Luteinising hormone

LogBCF	Log bioconcentration factor
Log/Kow	Octanol/water partition coefficient
MS222	Ethyl 3-aminobenzoate methanesulfonate
MT	Methyltestosterone
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCBI	National Center for Biotechnology
NRT	No reverse transcriptase control
NTC	No template control
NTD	NH_2-terminal domain
OECD	Organisation for Economic Co-operation and Development
oNPG	ortho-Nitrophenyl-β-galactoside
PAH	Polycyclic aromatic hydrocarbon
PNEC	Predicted no effect concentration
PR	Progesterone receptor
PTS	Proline, threonine, serine rich domain
qPCR	Quantitative polymerase chain reaction
QSAR	Quantitative structure–activity relationship
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RNA	Ribonucleic acid
RO	Reverse osmosis
rpl8	Ribosomal protein l8
RQ	Relative quantity
SD-medium	Synthetic defined medium
SHR	Steroid hormone receptor
SSC	Secondary sexual characteristic
T	Testosterone

tbp	Tata box binding protein
TIL	Trypsin inhibitor like cystine rich domain
TSCA	Toxic Substances Control Act
VTG	Vitellogenin
VWD	von Willebrand factor type D domain
VWF	von Willebrand factor
WFD	Water Framework Directive
WwTD	Wastewater Treatment Directive
WwTW	Wastewater Treatment Works
YAS	Yeast androgen screen
YES	Yeast oestrogen screen
ZLG	Zonadhesin-like gene

1. Introduction

Ever since Silent Spring was published in the 1960s (Carson, 1962) there has been a growing interest regarding chemical pollution impacting wildlife reproduction and development. The field of endocrine disruption has gathered momentum since the 1990s, as both scientists and policymakers have started to appreciate the importance of the issue. There are now many papers that address the effects of chemicals on wildlife and collectively they support a connection between environmental pollution and effects on wildlife populations (Bernanke and Kohler, 2009).

In 2010, the international community, with the support of the Convention on Biological Diversity, agreed on 20 biodiversity-related 'Aichi Targets' to be achieved within a decade, and at the mid-term review it was noted that additional effort was required to reduce pressures caused by pollution (Tittensor *et al.*, 2014). In a more recent report, one of the five main causes of biodiversity loss was considered to be pollution, and this anthropogenic driver of biodiversity loss continues to increase globally, despite national efforts to meet the Aichi Targets (IPBES, 2019).

The World Health Organisation defines an EDC as 'an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations' (Damstra *et al.*, 2002). More simply put, an EDC is an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action (Zoeller *et al.*, 2012).

EDCs can interfere with the endocrine system in a number of ways; mimic the effect of endogenous hormones (e.g. oestrogens, androgens, thyroid), antagonise the effect of endogenous hormones, disrupt the synthesis and metabolism of endogenous hormones, and disrupt the synthesis and metabolism of hormone receptors (Sonnenschein and Soto, 1998). This can then lead to a range of developmental, reproductive, immune, neurological or metabolic diseases in humans and animals.

Here I provide some background relating to the endocrine system of vertebrates, specifically concentrating on fish, as endocrine disruption in fish is the main focus of this PhD thesis.

1.1. The endocrine system

1.1.1. The hypothalamic–pituitary–gonadal axis

In common with all vertebrates, reproduction in teleost fish is regulated by coordinated interaction among hormones of the hypothalamic-pituitary-gonad axis (HPG; Figure 1). In

seasonally spawning fish, external factors such as light and water temperature control the timing of gonadal recrudescence and maturation via the brain (Scholz and Mayer, 2008). Kisspeptin stimulates the release of gonadotropin releasing hormones (GnRHs) from the hypothalamus, that in turn stimulate the release of gonadotropins (luteinising hormone (LH) and follicle stimulating hormone (FSH)) from the pituitary gland. The gonadotropins in turn regulate the production and secretion of sex steroid hormones (steroidogenesis) from the gonad (Ji *et al.*, 2013). Through interactions with membrane-bound gonadotropin receptors (e.g. LH receptor and FSH receptor) on the surface of gonad cells, pituitary gonadotropins including LH and FSH play a key role in regulating steroidogenic gene expression via negative or positive feedback mechanisms (Villeneuve *et al.*, 2007). This constant feedback from the target glands to the hypothalamus and pituitary gland ensures homeostasis of the hormone system, and when the correct blood levels of those hormones are reached, the hypothalamus and/or the pituitary cease hormone release, thereby turning off the cascade (Hiller-Sturmhöfel and Bartke, 1998).

In addition, the major carrier of endogenous sex steroids, sex hormone-binding globulin, regulates the plasma levels and biological actions of sex steroids by modulating their bioavailability and accessibility and protecting the sex steroids from rapid degradation and excretion (Liu *et al.*, 2017).

In mammals, LH enables Leydig cells (in the testis) to generate androgens whereas FSH stimulates granulosa cells (in the ovary) to produce oestrogens. Although the precise function of these hormones in teleost is still not clear, much evidence indicates that their roles in steroidogenesis are similar to those in mammals (Gong *et al.*, 2017).

1.1.2. Gonadal steroidogenesis

Steroids are synthesised from cholesterol through a series of reactions catalysed primarily by several cytochrome P450s (Figure 2). Aromatase cyp19 is the key enzyme responsible for the conversion of 19-carbon androgens (mainly testosterone (T) and androstenedione) to 18-carbon oestrogens, regulating local and systemic levels of oestrogens in the body (Cheshenko *et al.*, 2008). The enzyme 5 α-reductase is the enzyme that converts T to dihydrotestosterone (DHT).

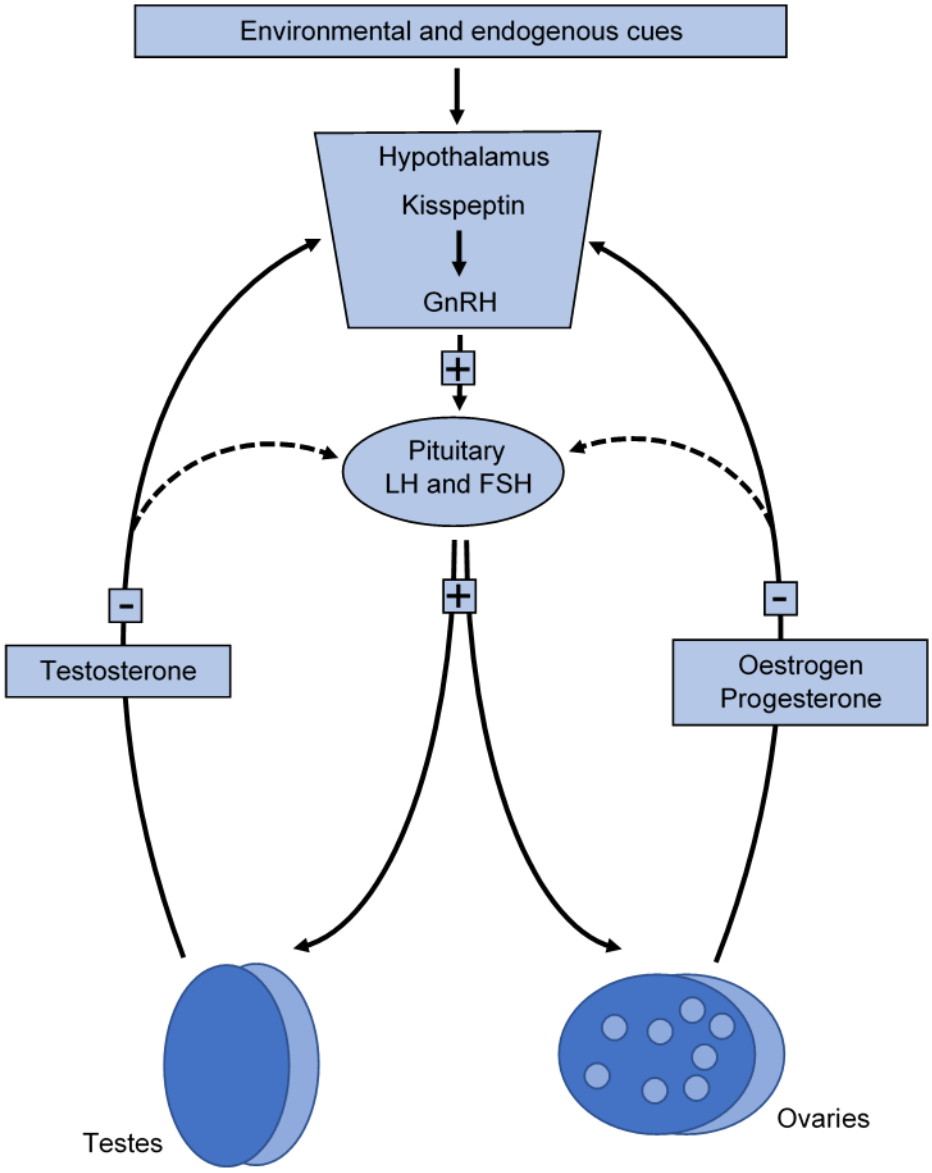
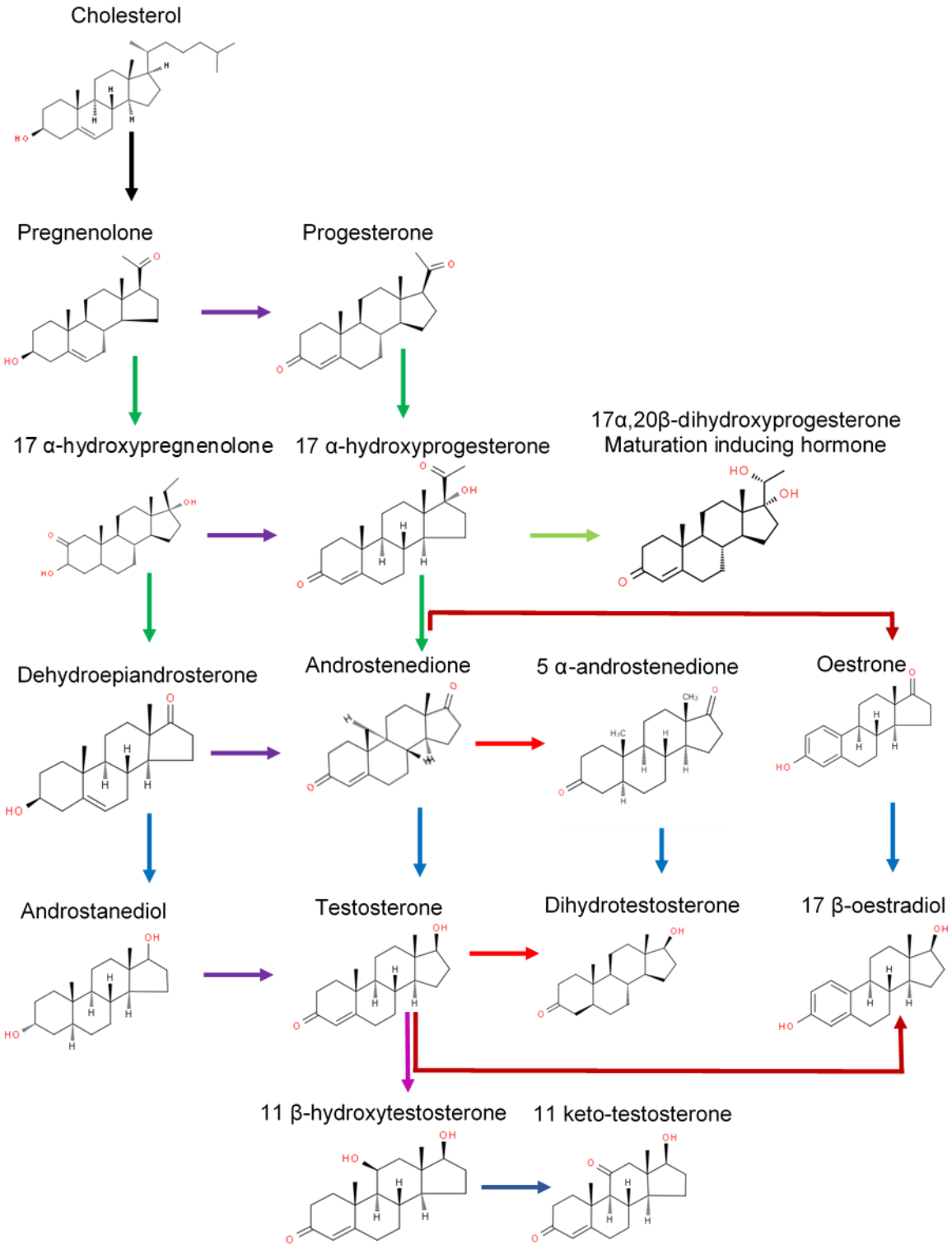


Figure 1. The hypothalamic-pituitary-gonad axis and its role in sex development. Gonadotropin releasing hormones (GnRHs) produced by the hypothalamus stimulate the release of gonadotropins (luteinising hormone (LH) and follicle stimulating hormone (FSH)) from the pituitary gland. The gonadotropins then control the production and secretion of sex steroid hormones by the gonads. The LH and FSH regulate steroidogenic gene expression via negative (-) or positive (+) feedback mechanisms, and this constant feedback from the target glands to the hypothalamus and pituitary gland ensures homeostasis of the hormone system.



Chemical structures – ChemSpider, Royal Society of Chemistry

Figure 2. Schematic representation of the main steroidogenic pathways in teleost fish. The coloured arrows indicate the different conversion enzymes required for the generation of sex steroids from cholesterol;
Black, cytochrome P450 family 11 subfamily A member 1 (P450scc); Cyp11a1
Dark green, cytochrome P450 17α-hydroxylase/C17, 20-lyase; Cyp17
Light blue, 17β-hydroxysteroid dehydrogenase; 17β-HSD
Light red, 5α-reductase; 5α-R
Dark red, aromatase; Cyp19
Purple, 3β-hydroxysteroid dehydrogenase; 3β-HSD
Pink, 11β-hydrolase
Dark blue, 11β-hydroxysteroid dehydrogenase; 11β-HSD
Light green, 20β-hydroxysteroid dehydrogenase; 20β-HSD

Sex steroid hormones such as oestradiol (E2) and T in females, and DHT and T in males, as well as maturation-inducing hormones in both sexes, initiate changes in secondary sexual characteristics (SSCs) and behaviour, as well as development and maturation of gametes (Scholz and Mayer, 2008).

Androgens (based on transcriptomics and proteomics) also have functions in apoptosis, transport and oxidation of lipids, synthesis and transport of hormones, protein metabolism, and cell proliferation (Martyniuk and Denslow, 2012).

Androgens are all steroids with 19 carbons (Marcoccia *et al.*, 2017). The majority of naturally occurring steroids with androgenic activity are, in decreasing order of potency relative to T; DHT (150-200%), androstanediol (65%), androst-4-ene3,17-dione (25%), androsterone (10%) and dehydroepiandrosterone (10%). Over 95% of T is produced and secreted by Leydig cells in the testis, whereas the remaining 5% is produced in the adrenal glands by conversion of precursors. DHT is one of the most important steroids in many male vertebrates with the exception of teleost fish, where T and 11-ketotestosterone (11-KT) were thought to be the major circulating male androgens as well as the most potent ones (Borg, 1994). However, it is now considered that DHT does have a role in the mediation of androgenic responses in teleost fish (Margiotta-Casaluci and Sumpter, 2011).

The traditional view of E2 as the female hormone and T as the male hormone has been challenged in recent years. Now oestrogen is thought to have a regulatory role in the testis because oestrogen biosynthesis occurs in testicular cells and the absence of oestrogen receptors (ERs) causes adverse effects on spermatogenesis and steroidogenesis (Akingbemi, 2005). Also, a direct role for androgens (as the precursor for E2), acting via the

AR in female reproductive function has been confirmed as key to optimal ovarian function (Walters and Handelsman, 2018).

1.1.3. Hormone receptors

Steroid hormone receptors (SHRs) are hormone-activated nuclear transcription factors with distinct specificities for endogenous steroid hormones. In all steroid receptors, the activating hormone binds to an internal cavity within a well-conserved ligand binding domain (LBD), causing the LBD to change conformation, attract co-activator proteins and increase transcription of target genes. Humans have two phylogenetic classes of steroid receptors depending on the endogenous ligand (Eick *et al.*, 2012). In the first class, the ER, the endogenous ligands are 18-carbon steroids with an aromatised A-ring and a hydroxyl attached to the C3 on the steroid skeleton. The other class comprises receptors for androgens (AR), progestogens (PR), glucocorticoids (GR) and mineralocorticoids, and all the ligands for these receptors contain a non-aromatisable A-ring, an additional methyl at C19, and in most cases a ketone at C3.

The AR is consistent with the characteristic nuclear receptor modular structure; consisting of three major functional domains (1) NH_2-terminal domain (NTD) containing the Activation Function region 1 (AF1)), (2) highly conserved deoxyribonucleic acid (DNA)-binding domain (DBD), and (3) a conserved C-terminal domain containing the LBD (Figure 3). The AR-LBD has three different binding or active sites where an agonist or antagonist can bind and alter AR functions (Sakkiah *et al.*, 2018); the ligand binding pocket (LBP), the AF2 site and the Binding Function 3 site (BF3). The hinge region acts as a bridge between the DBD and LBD. The three-dimensional structure of the AR-LBD consists of 12 bundles of helices forming three layers. Among these 12 helices, the Helix-12 (H12) plays a major role in AR activation and when an androgen binds the LBP of the AR, H12 tightly holds co-activator proteins and initiates function.

AR activation can occur through direct or indirect pathways (Lynch *et al.*, 2017). Direct AR activation occurs through a multi-step process (Figure 4). In the absence of a ligand, SHRs are predominantly monomeric and bind to chaperones/ cochaperone complexes to remain stabilised in the cytoplasm (Chen *et al.*, 2018). To dissociate from chaperones/ cochaperones and translocate to the nucleus, the AR must be bound by ligands. Once in the nucleus, the SHRs forms dimers, binding to specific genomic response elements and recruiting coregulators. The SHRs then bind other transcription factors to form transcription regulatory complexes to activate transcription. Hence, the subcellular signalling of SHRs is dynamic and requires the transportation of the SHRs from the cytoplasm to the nucleus.

Figure 3. The linear domain structure of a steroid hormone receptor comprising; an N-terminal domain (NTD), a DNA-binding domain (DBD), a hinge region and a ligand binding domain (LBD). Embedded in the NTD is the Activation Function 1 (AF1), and embedded in the LBD is the AF2, the Binding Function 3 (BF3), and the Helix-12 (H12).

Adapted from Chen *et al.* (2018)

The presence (in some fish species) of multiple AR isoforms with various binding specificities and tissue distributions suggests that different androgens may mediate the androgenic response according to the species and to the differential expression and abundance of AR isoforms in each tissue (Margiotta-Casaluci and Sumpter, 2011). For example, expression analysis of medaka ARα and ARβ in several adult tissues of both sexes revealed both ARs to be highly expressed in male kidney in which the enzymes for androgen production are expressed (Kusakabe *et al.*, 2002). Quantitative polymerase chain reaction (qPCR) suggested that the ARα was more highly expressed in the testis than the ovary whereas ARβ did not show such sexual dimorphic expression in adult gonads.

1.1.4. Gonadogenesis

In almost all vertebrates, sexual reproduction requires two sexes (males and females) to maintain genetic variation. Hence, sex determination and differentiation is one of the most important processes for species survival. The gonad is a unique organ due to its bipotential nature; either testis or ovary can develop from a single primordium (Brennan and Capel, 2004).

However, sex determination mechanisms vary greatly among taxa (Devlin and Nagahama, 2002). In vertebrates, sex is determined either genetically or environmentally, or a combination of both (Chen *et al.*, 2016). In most mammals, sex determination is genetically controlled by the presence or absence of the Y-chromosome, and the initiation of the male pathway depends on the gonadal expression of the Y-linked gene, Sry. The development of SSCs, including external genitalia, depends on whether the gonad differentiates into a testis or ovary and subsequently secretes primarily androgens or oestrogens.

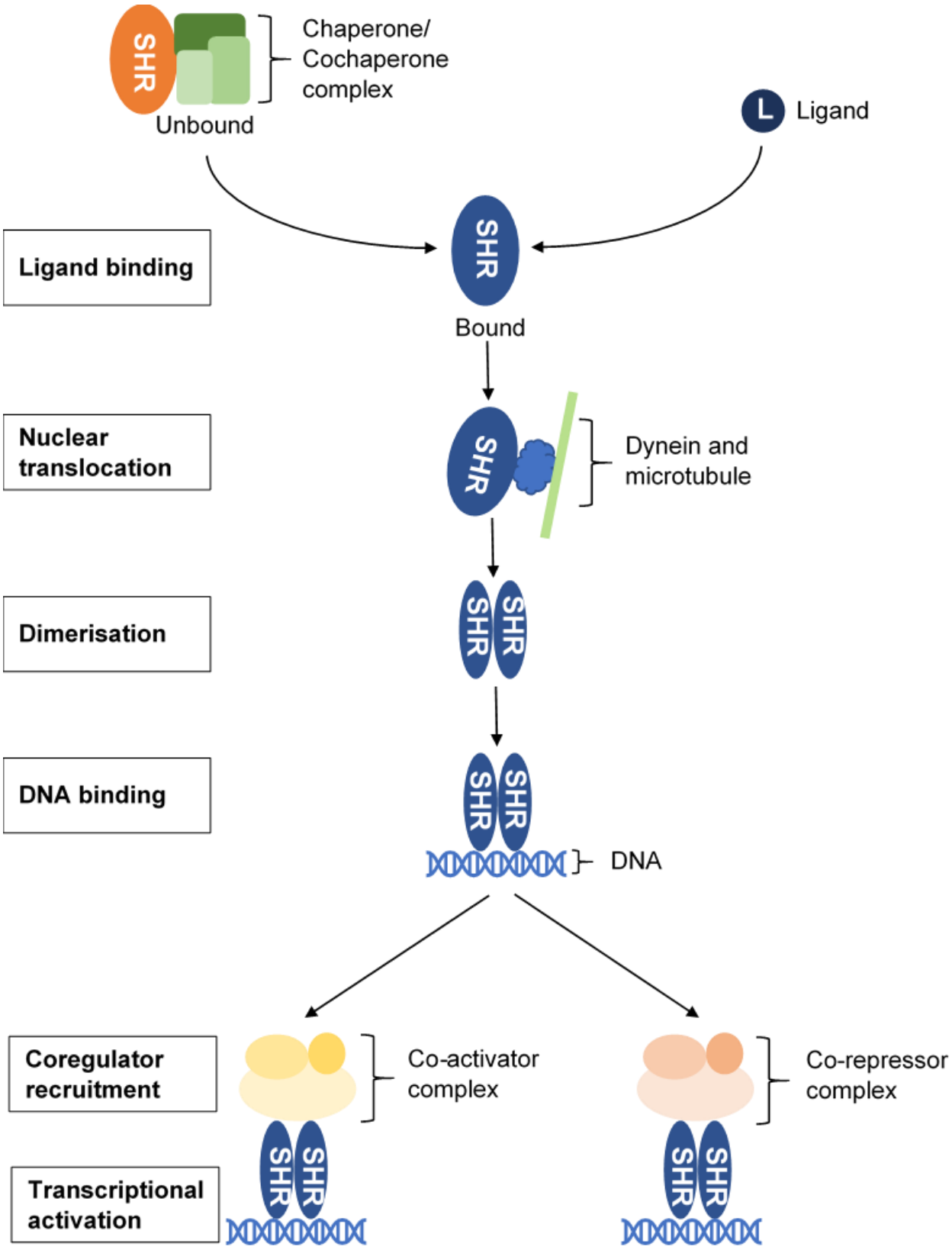

SHR
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Figure 4. An overview of the signalling processes of steroid hormone receptors (SHRs). This multistep process starts with ligand binding and ends with transcriptional activation. Agonists induce an active conformation of the SHR that recruits co-activators and activates transcription. Conversely, an antagonist induces the inactive conformation of the SHR that recruits co-repressors and represses transcription.

Adapted from Chen *et al.* (2018)

Whilst in certain families of fish hermaphroditism or sex reversal represent the normal mode of reproduction, the vast majority of species are gonochoristic, in which distinct males and females are independent and stable (Nolan *et al.*, 2001). In many gonochoristic fish species, sex determination is genetic, but in other cases, sex is determined by environmental factors such as temperature (Baroiller *et al.*, 1999). Sex differentiation is correlated with sex steroid production and receptor signalling pathways involving androgens and oestrogens (Wood *et al.*, 2015). In teleosts, endogenous oestrogens act as the natural inducer of ovarian differentiation, while androgen is not synthesised in the gonad during the critical period of sex determination (Nagahama, 2000). Consequently, sex differentiation and meiosis initiate earlier in female than in male, and this may be because oogenesis takes more time than spermatogenesis (Chen *et al.*, 2016).

The Doublesex and Mab-3 Related Transcription Factor 1 (Dmrt1) gene is proposed as the major player in fish sex differentiation and its expression has been related with testis development and spermatogenesis in several species, for example, Nile tilapia and medaka (Herpin and Schartl, 2011). In addition, the Forkhead Box I2 (foxI2) gene is involved in ovarian development and oocyte maintenance, and is thought to be associated with the regulation of aromatase expression in different fish species (Baron *et al.*, 2004). Alterations of the expression patterns of these genes can lead to modified sex differentiation outcomes in fish.

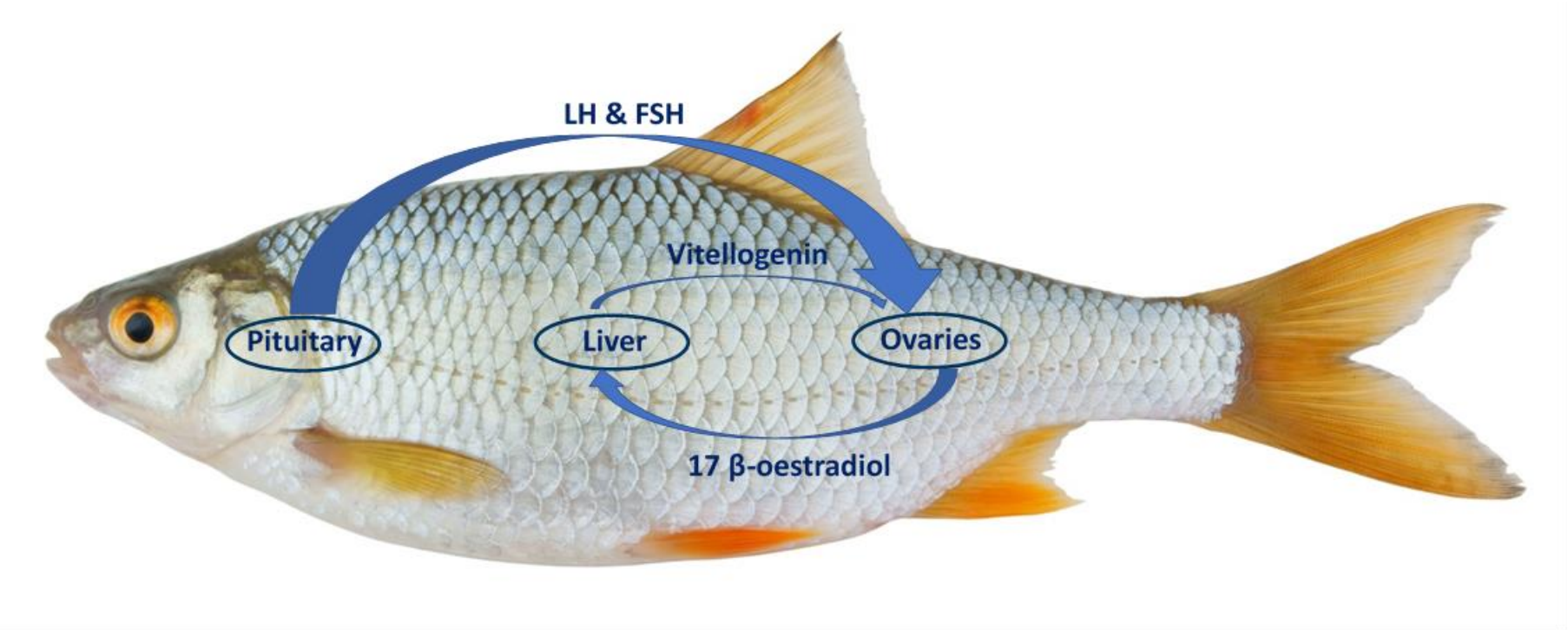


Figure 5. Generalised illustration of the pituitary-gonad axis demonstrating induction of the egg yolk protein, vitellogenin, in female fish.Oestradiol produced by the ovaries stimulates the liver to produce vitellogenin. Vitellogenin is then transported by the blood to the ovary where it is sequestered by developing oocytes. LH, luteinising hormone; FSH, follicle stimulating hormone

1.1.5. Gametogenesis

Gametogenesis is the process by which gametes, or germ cells, are produced in an organism. The formation of egg cells, or ova, is called oogenesis, and the formation of sperm cells, or spermatozoa, is called spermatogenesis.

In the testis, FSH and LH are the most important pituitary hormones regulating fish spermatogenesis; FSH plays a regulatory role in the early stages of spermatogenesis and LH is mainly involved in later stages of maturation, e.g. regulation of spermiation (Gong *et al.*, 2017).

Vitellogenin (VTG) is a female-specific egg-yolk precursor that is synthesised in the liver in response to circulating oestrogens and transported by blood to the ovary, where it is taken up by oocytes and deposited as yolk (Tyler and Sumpter, 1996) (Figure 5). Oocyte growth can be divided into two main stages of development, classified as primary or previtellogenic growth and secondary or vitellogenic growth (Basili *et al.*, 2018). FSH in fish has a dominant role in regulating the vitellogenic growth of follicles, partly through stimulation of E2 biosynthesis by ovarian follicles. LH is involved in final oocyte maturation and ovulation, partly through the stimulation of production of maturation hormone (Nagahama and Yamashita, 2008).

1.2. Mechanisms of disruption

EDCs have been reported to act via several mechanisms which can disrupt the HPG axis, including those that are receptor and non-receptor mediated effects (Wilson *et al.*, 2007). These include the activation/inhibition of hormone receptors, inhibition of hormone synthesis, disruption of hormone transport proteins, and inhibition of hormone metabolism (Liu *et al.*, 2017).

Most studies currently focus on SHRs and this thesis specifically examines chemicals that act as oestrogens and (anti-)androgens. Known androgen antagonists (for example, pharmaceutical anti-androgens flutamide and bicalutamide) have demonstrated that antagonists inhibit AR function by competitive binding with natural androgens (Perera *et al.*, 2017).

One widely proposed mechanism for AR antagonism is the displacement of the H12 upon ligand binding, leading to distortions in the AF2 site, preventing co-activator binding; during binding the H12 functions as a lid, which closes (agonist) or moves away from the LBP (antagonist) (Tan *et al.*, 2015) (Figure 6). AR antagonists are usually bulkier than agonists and thus require a wider binding pocket than agonists. Due to their large size,

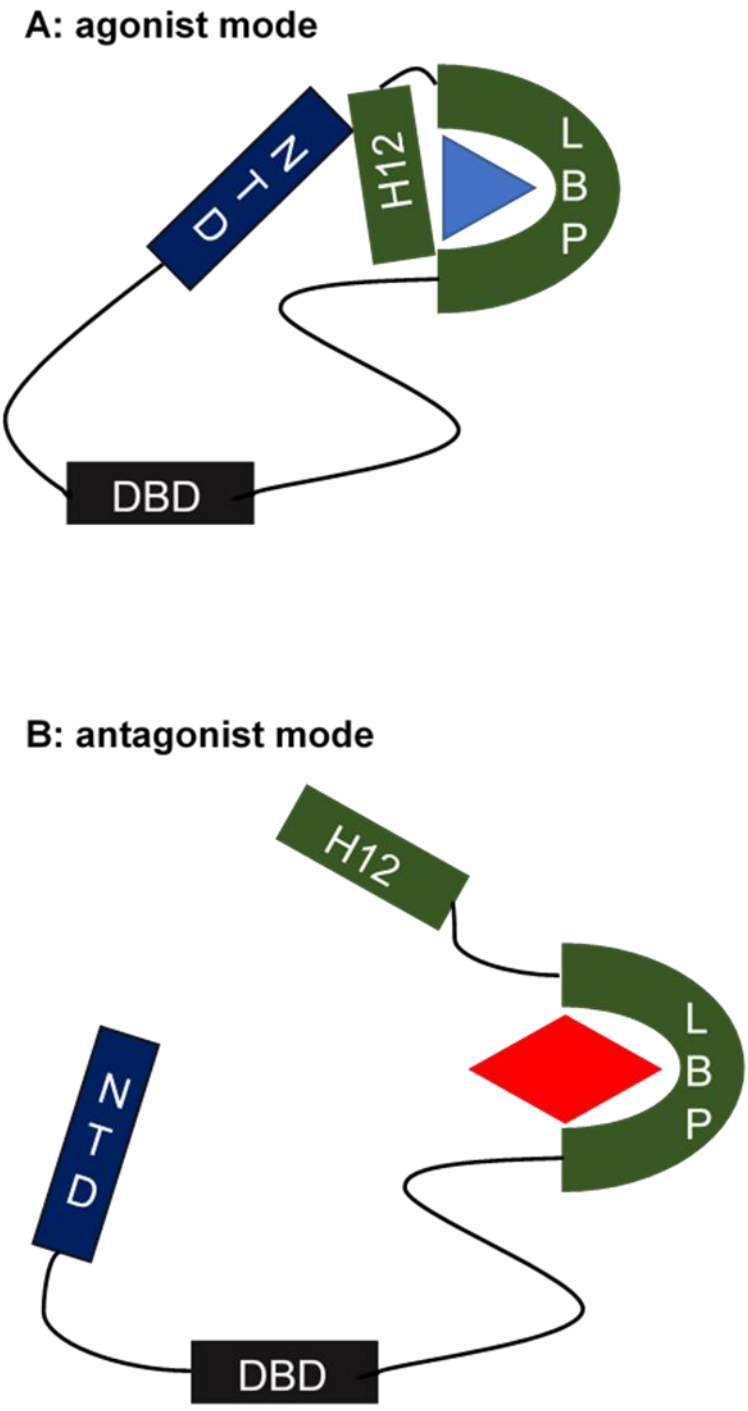


Figure 6. Model for ligand-dependent activation of the androgen receptor. During ligand binding, the H12 functions as a lid and (A) closes when the ligand binding pocket (LBP) is presented with an agonist (illustrated by the blue triangle) or (B) moves away from the LBP in the presence of a bulkier antagonist (illustrated by the red diamond).

Adapted from Tan *et al.* (2015)

antagonists push the residues in H12 outward to expand the active site. These structural changes in the LBP cause the AF2 site to undergo conformational changes, preventing co-activator binding. When a small molecule binds to the AR a conformational change can occur which impacts subsequent binding of the co-regulator proteins and DNA. Thus, an agonist induces an active conformation of the SHR that recruits co-activators and activates transcription (Figure 4). Conversely, an antagonist induces the inactive conformation of the SHR that recruits co-repressors and represses transcription. Co-activators and co-repressors are therefore recruited by SHRs upon binding of agonist and antagonist ligands respectively and play an essential role in the regulation of transcription (Chen *et al.*, 2018).

In addition to receptor mediated effects, steroidogenic enzymes can also be a target for EDCs. For example, cyp19 (aromatase) catalyses the conversion of androgen to oestrogen and therefore changes in aromatase activities (whether up or down regulation) can influence the concentration and balance of sex hormones (Cheshenko *et al.*, 2008). Similarly, 5α-reductase inhibitors will block the conversion of T to DHT and this leads to an increase in T (Azzouni *et al.*, 2012).

1.3. What evidence is there of endocrine disrupting chemicals having harmful effects?

To date, there is evidence of endocrine disruption occurring in many invertebrate and vertebrate species, and here I provide a few key examples of reported reproductive disruption in vertebrates caused by chemical(s) exposure.

1.3.1. Humans and rodent models

Pesticides have been directly linked to endocrine disruption in humans following occupational exposure; for example, reduced sperm counts following handling of the insecticide dichlorodiphenyltrichloroethane (DDT; Singer, 1949) and reduced sperm motility through exposure to the insecticide chlordecone (kepone; Guzelian, 1982).

In addition to these occupational exposure cases, in another well documented case of endocrine disruption, the pharmaceutical oestrogen diethylstilbestrol (DES) was prescribed to pregnant women during the 1940s to 1970s to prevent miscarriage (Ho *et al.*, 2017). In 1971, DES was shown to cause a rare vaginal tumour in girls and women who had been exposed to this medication *in utero* (Swan, 2000). In addition, the gestational exposure to DES increased the infertility rate and poor pregnancy outcomes in daughters and caused an increase in the incidence of genital abnormalities in sons (Schrager and Potter, 2004).

Congenital penile anomalies (CPAs) are amongst the most common birth defects and encompass a range of malformations, including failure of urethral tube closure (hypospadias), penile curvature (chordee), micropenis, and feminisation of male genitalia. Reports of CPAs have risen sharply in recent decades, and the most common of these malformations, hypospadias, now affect 1:125 live male births in the U.S. (Zheng *et al.*, 2015). Matlai and Beral (1985) also examined the rates of malformations of male external genitalia reported at birth and found a significant increase in cases of cryptorchidism (a condition in which one or both of the testes fail to descend from the abdomen into the scrotum), hypospadias and hydrocele (a swelling in the scrotum that occurs when fluid collects in the thin sheath surrounding a testicle) between 1969 and 1983. Existing research indicates that the male reproductive system is the target organ of EDCs, and that exposure to EDCs may result in significantly lower T levels and as a result be responsible for the cryptorchidism, hypospadias, and other urogenital disorders (Liu *et al.*, 2015). Studies of paternal and maternal pesticide exposures reported associations with cryptorchidism and hypospadias but could not pin-point specific chemicals (Pierik *et al.*, 2004).

The anogenital distance (AGD) is naturally shorter in females compared to males and has been used as a marker of a feminised or demasculinised phenotype in rodent models and other mammals. In addition to the CPAs reported in humans, exposures to anti-androgenic pesticides and plasticisers during the critical development window in male reproductive development have also been linked to shortened AGD in humans and rodents (reviewed by Schwartz *et al.*, 2019). In humans, shorter AGD has also been associated with poor semen quality in adult men (Lind *et al.*, 2017).

Testicular dysgenesis syndrome arises from insufficient androgen in foetal life (Arrebola *et al.*, 2015). Due to a disturbance of testicular development, poor semen quality and germ cell cancers can occur (Arrebola *et al.*, 2015). Carlsen *et al.* (1992) carried out a meta-analysis on data from 14,947 men, and this indicated that there had been a decline in semen quality during a period of half a century. Most individual cases of poor semen quality have no known aetiology as people are exposed to low concentrations of thousands of chemicals and so proving specific cause and effect is challenging. Also, individuals are exposed to chemicals as mixtures and these could be acting together to cause effects rather than a single chemical being responsible for the effects (Silva *et al.*, 2002).

The timing of reproductive system differentiation is different in females and males, and female germ cells enter meiosis prenatally and complete initial phases of meiosis before birth, whereas male germ cells only begin to enter meiosis postnatally and continuously do so throughout adulthood. The belief was that androgens induced and completed masculinisation of the reproductive tract during the time when morphological differentiation

could be observed. However, Welsh *et al.* (2008) discovered that androgens could 'preprogram' masculinisation before morphological changes were actually observed in rats, and this was termed the 'masculinisation programming window'. Only within this window did blocking androgen action induce hypospadias and cryptorchidism and alter penile length in male rats, all of which correlated with AGD. Androgen-driven masculinisation of females was also confined to the same window.

1.3.2. Fish

The aquatic environment represents the ultimate sink for a vast number of anthropogenic contaminants, and fish populations are directly exposed to a wide variety of EDCs originating from industrial, agricultural, or municipal effluents. Thus, fish are considered as one of the primary risk organisms for EDCs (Scholz and Mayer, 2008). Sex determination in teleosts is characterised as being very labile and can be disturbed or even functionally reversed by the external application of natural or synthetic hormones and/or aromatase inhibitors (Nolan *et al.*, 2001). Indeed, steroid hormones are widely used to control fish sex in aquaculture (Chen *et al.*, 2016). The labile period when fish are most susceptible to endocrine perturbation is just following hatching or at the juvenile stage (Jobling *et al.*, 1998), and the timing of exposure has been shown to be a crucial factor that determines the potency of EDCs (Gore *et al.*, 2014).

Most teleost fish have external fertilisation, but some species develop copulatory organs formed from an elongation of the anterior region of the anal fin, termed gonopodium, for internal fertilisation (Ogino *et al.*, 2004). The mosquito fish (*Gambusia affinis holbrooki*) has sex dimorphism driven by androgenic hormones mediated by the testis and this becomes apparent in the gonopodium formation at late juvenile stage.

The only androgenic industrial effluent identified to date is pulp mill effluent and reports focus on masculinisation of wild female mosquito fish; both the development of a male-like gonopodium and male-like reproductive behaviours (Howell *et al.*, 1980). Subsequent studies confirmed the presence of androgenic compounds in paper and pulp mill effluent, but these effluents contain a complex mixture of organic and inorganic pollutants and the exact chemical(s) responsible for the androgenic activity have yet to be identified (Kamali *et al.*, 2016; Parks *et al.*, 2001; Singh and Chandra, 2019).

Another widely documented source of environmental androgens are the growth promotor implants used in the cattle industry. Male fathead minnow (FHM; *Pimephales promelas*) collected from rivers downstream of feedlots were found to be demasculinised (decreased plasma T, altered head morphology, smaller testis size) due to androgenic activity either from natural androgens or androgenic pharmaceuticals used in growth implants (trenbolone

acetate is metabolised to the potent 17β-trenbolone; Orlando *et al.*, 2004). Female FHM collected from the same river had a decreased E2:T ratio due to a reduction in E2 synthesis and an increase in T synthesis, indicative of the presence of androgenic compound(s). That is, the presence of endogenous androgen(s) leads to negative feedback via the HPG axis and results in either increased or decreased exogenous T as the FHM attempts to maintain equilibrium (Section 1.1.1).

Additionally, EDCs in the environment have also been shown to feminise fish. For example, exposure to domestic sewage has been shown to feminise male roach (*Rutilus rutilus*), and this effect has been attributed to ethinylestradiol (EE2), the synthetic oestrogen in the oral contraceptive pill found in sewage effluents (Purdom *et al.*, 1994). Jobling *et al.* (1998) also collected roach from a number of UK rivers and found a widespread incidence of intersex, and the presence of oocytes in male testicular tissue, which is not normally seen in this single-sex (gonochoristic) fish species.

In 2004, the Environment Agency sampled roach from 51 UK sites and found 44 of the sites to have feminised male (intersex) fish. The severity of the feminisation correlated with predicted exposure to oestrogens, with the most severely intersex fish being found in the older year classes (Gross-Sorokin *et al.*, 2006). In later studies, the presence of anti-androgenic activity (determined *in vitro*) in UK wastewater treatment works (WwTW) effluent was also found to be statically associated with the feminised responses seen in the wild roach living in those rivers (Jobling *et al.*, 2009).

1.4. What are the sources of chemicals in the aquatic environment?

1.4.1. Steroidal EDCs with understandable biologically activity

Globally, it is likely that over 80% of wastewater is released to the environment without adequate treatment (WWAP, 2017). In Central European countries, connection rates to WwTWs have increased since 1995 and are now at 97%, with about 80% receiving tertiary treatment (European Environment Agency, 2017). In the UK, all wastewater effluent is treated; 43% receiving secondary treatment and 57% receiving tertiary treatment (European Environment Agency, 2017).

One major group of chemicals found in the environment are steroids; either natural (e.g. oestrone (E1), E2 and T) or synthetic (e.g. EE2 and trenbolone). The natural oestrogens, E1 and E2, and the synthetic compound EE2, were found to contribute a large fraction of the total oestrogenicity of wastewater (Arlos *et al.*, 2018b). Thomas *et al.* (2002) also found that 99% of the androgenic activity of WwTW effluent, determined *in vitro*, was due to natural

steroids/metabolites. Of these, androstenedione (direct metabolism of T in the human body followed by excretion) accounted for 33%. Similarly, Houtman *et al.* (2018) analysed 24 steroid hormones in WwTW effluent and androgenic activity was explained by the presence of T and androstenedione.

Wastewater treatment works utilise a number of different processes depending on the amount of effluent that the works receives; ranging from primary settlement, secondary biological treatment, and tertiary treatment with another settlement step (discussed further in Chapter 2). Wastewaters are complex mixtures of chemicals and nutrients that can vary temporally (Fuzzen *et al.*, 2016), and the removal of micropollutants depends on the physical and chemical properties of the compound as well as the characteristics of the WwTW, i.e. the degree and type of treatment process. The incomplete removal of chemicals during wastewater treatment means that some of these compounds are discharged with final effluent, causing aquatic organisms to be continuously exposed (Petrie *et al.*, 2015). Many EDCs are sufficiently stable to survive the wastewater treatment process and can even be reactivated during treatment, for example, conjugated steroids can by hydrolysed to their free form during biological treatment (Zhang and Zhou, 2005). Incomplete mineralisation during wastewater treatment can also result in the formation of transformation products with a potential to produce more toxicity than the parent compound (Maya *et al.*, 2018).

Given that food production consumes more than two-thirds of the world's extracted water and is expected to rise by 70% by 2050 due to population growth (Gilbert, 2012b) there is a need to evaluate the efficiency of current tertiary wastewater treatment processes and to develop advanced treatment processes (Hamilton *et al.*, 2014).

In addition to chemicals entering our aquatic environment via WwTW effluents (point sources), chemicals also enter via non-point sources from land run-off, precipitation, atmospheric deposition, drainage, seepage, or hydrologic modification. Non-point source pollution originates from many diffuse sources and is caused by precipitation moving over and through the ground, picking up and carrying pollutants and finally depositing them into lakes, rivers, wetlands, coastal waters, and ground waters (Gang *et al.*, 2006). Agricultural activities that cause non-point source pollution include poorly located or mismanaged animal feeding; overgrazing; mismanaged ploughing; and improper, excessive, or poorly timed application of fertiliser (e.g. run-off from land-applied animal manure; Abdel-moneim *et al.*, 2017).

Oestrone can be readily measured in run-off, soil, and groundwater, and the major source of this steroid in the environment appears to be cattle and chickens (Shore and Shemesh, 2003). Additionally, wild FHM from a cattle feedlot in the USA were found to be

demasculinised with reduced T synthesis, altered head morphology and smaller testes. As there was no evidence of feminisation (i.e. no oestrogens), the effluent was thought to be androgenic due to the growth promoter administered to the cattle, 17β-trenbolone (Orlando *et al.*, 2004). Whilst use of trenbolone is no longer allowed in the European Union (EU), the use of hormones as production aids is still permitted in North American countries.

1.4.2. Industrial compounds with unforeseen biological activity

Globally, 400 million tons of chemicals were produced in 1995 and Europe was the largest chemical producing region in the world, accounting for 38% of this production (EEA/UNEP, 1998). However, worldwide competition has shifted significantly over the last ten years and now China holds the top ranking in sales (ECIC, 2020). In 2007, the European Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) entered into force and during the first six month period, 143,000 existing substances were pre-registered by 65,000 companies. The REACH deadline for registering existing substances manufactured or imported in quantities from 1 to 100 tonnes per year in the EU was on 31 May 2018 and since 2008, 145,297 substances have now been pre-registered (https://echa.europa.eu/information-on-chemicals/pre-registered-substances, downloaded 22/12/2020) and 23,118 substances have been registered (https://echa.europa.eu/information-on-chemicals/registered-substances, downloaded 22/12/2020). Similarly, the 1976 Toxic Substances Control Act (TSCA) requires the US Environmental Protection Agency (EPA) to compile and keep a current list of every substance that is manufactured or processed in the US, and this list has also continued to grow and now has over 86,000 chemicals on it (https://www.epa.gov/tsca-inventory/about-tsca-chemical-substance-inventory).

The chemical bisphenol A is the monomer used to produce polycarbonate and was, by chance, found to be oestrogenic when the chemical leached from autoclaved tissue culture flasks and caused MCF-7 breast cancer cells to proliferate (Krishnan *et al.*, 1993). Alkylphenol polyexthoxylates, used in the textile industry for wool scouring, were also found to be broken down by wastewater treatment to oestrogenic alkylphenols, e.g. octylphenol and nonylphenol (Desbrow *et al.*, 1998). These chemicals were discharged to rivers and this led to the induction of the female-specific protein, VTG, in caged male trout sited in rivers below WwTWs (Harries *et al.*, 1997). Due to these results, many other chemicals have now been tested for oestrogenic activity, and other classes of chemicals have been found to have activity too, e.g. phthalates (Harris *et al.*, 1997) and parabens (Routledge *et al.*, 1998).

Phthalates or plasticisers enhance the flexibility of plastics and are widely used in consumer and industrial products such as medical devices, food wrappings, toys, paints, finishes, and

upholstery. As a result of their oil solubility and viscous nature, they are similarly constituents of personal care products (Witorsch and Thomas, 2010). Parabens are preservatives widely used in cosmetics and pharmaceuticals and, along with phenols, include some of the highest volume production chemicals in the world (Pollack *et al.*, 2016). Many man-made chemicals find their way into aquatic ecosystems, for example, numerous consumer products are flushed down drains and discharged with wastewater effluent and thence enter the aquatic environment (Jobling *et al.*, 1998), and this wide usage has meant that synthetic chemicals are now ubiquitous in water systems (Wangmo *et al.*, 2018).

The classes of EDCs known to interfere with the androgen signalling pathway include dicarboximide fungicides (e.g. vinclozolin; van Ravenzwaay *et al.*, 2013), organochlorine-based fungicides (e.g. the DDT metabolite, dichlorodiphenyldichloroethylene (p,p'-DDE); Kelce *et al.*, 1995), conazole fungicides (e.g. prochloraz; Vinggaard *et al.*, 2002), plasticisers (e.g. phthalates; Svechnikov *et al.*, 2016), polybrominated diphenyl ether flame retardants (Stoker *et al.*, 2004) and urea-based herbicides (e.g. linuron; Gray *et al.*, 2001).

Man-made chemicals also include pharmaceuticals and there over 4,000 approved drug products for human, veterinary, agriculture and farming use (Lee *et al.*, 2017). Pharmaceuticals are designed to be biologically active and maintain their therapeutic action, resulting in the occurrence of pharmaceuticals in the aquatic environment (Lee *et al.*, 2017). These drugs are currently available under various formulations and comprise a large number of chemical classes and modes of action (Meador *et al.*, 2016).

Whilst for humans, exposure is transient, aquatic organisms will be exposed continually to a cocktail of drugs (Rand-Weaver *et al.*, 2013). Gunnarsson *et al.* (2008) looked at 1318 human drug targets and found 86% to be conserved in zebrafish, 61% in *Daphnia pulex* (water flea) and 35% in *Chlamydomonas reinhardtii* (green algae), indicating the potential for lower vertebrates to respond to the pharmaceuticals present in the aquatic environment. This is of concern as Scott *et al.* (2018) found 40% of mosquitofish sampled from Australian river sites to contain active pharmaceuticals.

Man-made chemicals enter our aquatic environment via non-point sources. The types of chemicals found in the aquatic environment in agricultural landscapes are typically herbicides, fungicides and insecticides, as well as hormones and pharmaceuticals from livestock, and these will differ from those found in urban landscapes that will typically be oil, grease and toxic chemicals (Elliott *et al.*, 2017).

1.5. Methods for assessing oestrogenic and (anti-)androgenic chemicals in the aquatic environment?

1.5.1. Chemical analysis

The highly diversified chemical structures, properties, and mechanisms of action of environmental EDCs present a major challenge to their chemical detection/quantification (Tse *et al.*, 2013). Chemical analysis of environmental samples is required if observed effects need to be linked to specific chemical(s), however, many EDCs often occur at very low concentrations that are often close to or below detection limits (DLs) of analytical chemistry methods (Arlos *et al.*, 2018a).

Water samples can be collected as grab-water samples which enable the determination of water chemistry at a moment in time, but it is difficult to use this method to ascertain the variability in the presence of EDCs and may be more susceptible to extreme events (Perkins *et al.*, 2017). Other approaches such as composite water sampling strategies can compensate for this, although chemical concentrations are then averaged over the time that the composite sample is made, and extreme events may be missed. An alternative is to utilise passive samplers, such as the polar organic chemical integrative sampler, that allow hydrophilic chemicals to permeate the sampler membrane and be taken up by a sorptive sequestering medium over a prolonged exposure period, thus providing a time-integrated record of EDCs present (Jorgenson *et al.*, 2018). Because of accumulation of chemicals over the exposure time, the use of passive samplers allows the detection of EDCs at concentrations much lower than can be achieved with grab-water samples (Lahti *et al.*, 2012) and they have been applied to better understand exposure of biota over time (Hamilton *et al.*, 2016). This information, however, is not readily translated to environmental concentrations of the detected compounds and excludes hydrophobic compounds.

Other substances in water can alter the bioavailability and potency of EDCs, e.g. hydrophobic contaminants in water can associate with dissolved or particulate organic matter such as humic acid (Matthews *et al.*, 1995). Water concentrations may therefore underestimate the concentration present in the environment (Yarahmadi *et al.*, 2018). Indeed, Alvarez-Munoz *et al.* (2015) found that the presence of anti-androgenic activity was significantly correlated with total organic carbon and silt content. That is, the moderately hydrophobic anti-androgenic compounds associated with the organic matter and sorbed onto particles (Rostkowski *et al.*, 2011).

A wide range of EDCs can bioconcentrate in fish bile at concentrations that are many thousands of times greater than in the aquatic environment, facilitating the structural identification of bioavailable contaminants present in that environment (Rostkowski *et al.*,

2011). Some authors have found a significant relationship between oestrogenic activity and levels of oestrogenic substances in surface waters (Larsson *et al.*, 1999; Yang *et al.*, 2014), illustrating that analysis of fish bile could be used to identify EDCs present in water. EDC concentrations in bile and plasma revealed strong accordance in caged trout (Lahti *et al.*, 2012). Good correlation has also been found between bile E2 equivalent concentrations (E2EQs, measured *in vitro*) and plasma VTG (biomarker of oestrogenic exposure) induction in male bream (Legler *et al.*, 2002).

1.5.2. Current tests for assessing chemicals for oestrogenic and (anti-)androgenic activity

In the USA, the original 1976 TSCA did not require chemicals to be tested prior to their use in many consumer products and industrial applications (Trasande and Liu, 2011). Since then, new regulations have come into force to increase the protection of human health and the environment from the risks that can be posed by chemicals.

The development of non-animal test methods for the detection of EDCs has been confirmed as high priority for regulatory authorities in most countries and in 2012 the Organisation for Economic Co-operation and Development (OECD) investigated test methods in the scientific literature that could be standardised and used in chemical regulations to detect and characterise hazards posed by EDCs. This resulted in a Conceptual Framework for Testing and Assessment of Endocrine Disruptors and the issuing of the OECD Standardised Test Guidelines (revised guidelines; OECD, 2018d). These guidelines give the background of each standardised assay, its applicability domain and conceptual framework for testing (Dvorakova *et al.*, 2016). The framework consisted of five levels, each corresponding to a different level of biological complexity; Level 1 using existing data and non-test information, Level 2 utilising *in vitro* assays to provide mechanistic information and Levels 3-5 using *in vivo* test systems for a more definitive understanding of the effects (revised guidelines; OECD, 2018d). The OECD Standardised test guidelines have been updated more recently (OECD, 2018d) and high throughput (HTP) screening techniques are now included, in conjunction with computational methods and information technology, to aid screening of the large number of chemicals still untested.

Similarly, the US EPA Endocrine Disrupter Screening Program (EDSP) was established to identify potential endocrine bioactivity of pesticides and chemicals found in sources of drinking water. The EDSP focussed on chemicals perturbing the oestrogen, androgen and thyroid hormone pathways following a 2-tiered approach consisting of a battery of eleven *in vitro* Tier 1 screening assays and four Tier 2 *in vivo* tests to characterise adverse outcomes (Pinto *et al.*, 2018). While there are about 10,000 chemicals covered by the EDSP chemical universe, only 52 of these chemicals have undergone Tier 1 screening. To evaluate potential

EDCs more quickly and cost-effectively, the EDSP has been transitioning from traditional Tier 1 screening methods to HTP *in vitro* assays to prioritise chemical screening and to provide alternative data for Tier 1 endpoints (Coady *et al.*, 2017).

With the move to HTP assays, more chemicals are being tested, e.g. the US EPAs Tox Cast program has screened thousands of chemicals for biological activity, primarily using HTP *in vitro* bioassays (Fay *et al.*, 2018). However, with over 145,000 pre-registered substances in the EU and more than 86,000 synthetic chemicals registered in the US, only a small fraction of these have been tested for endocrine disrupting activity, and of those tested so far, only around 320 chemicals are considered to be EDCs or probable EDCs (Trasande *et al.*, 2016) (Figure 7). Additionally, these assays are *in vitro* assays, any EDCs identified as endocrine active in *in vitro* tests will then require *in vivo* testing.

There is a lack of data on the hazards (toxicity and ecotoxicity) of chemicals registered with REACH and in the US TSCA Chemical Substance Inventory. This presents a challenge for regulatory decision making, especially as the number and variety of potentially hazardous chemicals continues to increase. Due to the untenable nature of identifying all contaminants at increasingly lower concentrations and then assessing each of these individually, alternate approaches are needed (Davis *et al.*, 2016).

Regulatory authorities have approached the task of assessing all these chemicals by applying rapid and inexpensive quantitative structure-activity relationship (QSAR) Tier 1 methods as decision support tools to address the data gaps and provide provisional data in the toxicity database of a chemical (Morger *et al.*, 2020; Ruiz *et al.*, 2017; Thomas *et al.*, 2019). Also, the call to reduce animal testing in toxicity evaluation has led to an expansion of these tools. QSAR is thus used to prioritise and reduce the number of chemicals requiring further testing, so that efforts can be focussed on those most likely to be endocrine active.

1.5.2.1. *In vitro testing of environmental samples for ED activity*

Tier 1 *in vitro* screening bioassays are more precise at predicting *in vivo* effects than chemical analysis alone (Volker *et al.*, 2016). Environmental samples can contain both agonists and antagonists and *in vitro* bioassays provide information about the net effect of all active chemicals. This means that the presence of antagonists may reduce the observed effect (Grover *et al.*, 2011a). Prior to *in vitro* screening, fractionation of samples can help to separate these effects (Leusch *et al.*, 2017). *In vitro* bioassays are therefore often used as a pre-screening to give an overview of a sample's hormonal activity before analytical detection to determine the compounds that are present (Chamas *et al.*, 2017).

Discrepancies are sometimes found between analytical chemistry measurements and the results of bioassays, and Sun *et al.* (2017) thought that this was because partially decomposed samples were not picked up by gas chromatography–mass spectrometry (GC-MS) but were still active in the yeast oestrogen screen (YES). Similarly, Kanda and Churchley (2008) found considerable discrepancy between E2EQs determined with the YES and steroid oestrogen concentrations determined analytically, and this was thought to be

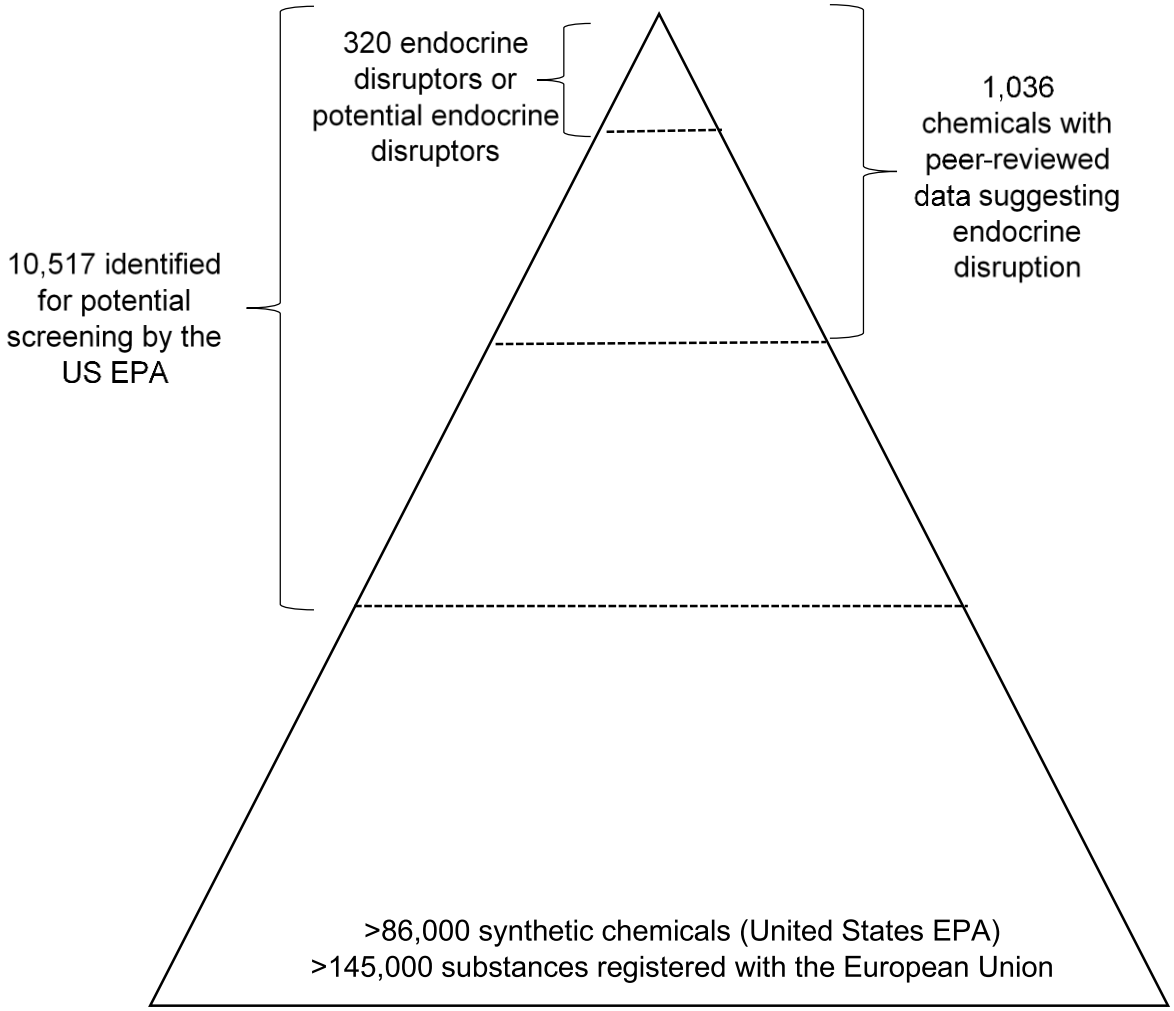


Figure 7. This figure illustrates the >145,000 substances pre-registered with the European Union and >86,000 registered in the United States. Only the 'tip of the iceberg' has been tested for endocrine disrupting activity and many more await testing. EPA, Environmental Protection Agency

Adapted from Trasande *et al.* (2016)

due to matrix effects of the wastewater samples, as these can either suppress or enhance the analytical signals (Taylor, 2005).

In addition to receptor mediated mechanisms, chemicals may also affect processes involved in the synthesis, release, metabolism, transport, and elimination of endogenous hormones, which can lead to alterations in the levels of circulating sex hormones (Pinto *et al.*, 2018). The EPA's EDSP has incorporated the OECD validated H295R-based *in vitro* steroidogenesis assay as part of the Tier 1 screening battery (US Environmental Protection Agency, 2009). As H295R cells synthesise most major steroid hormones, chemically induced toxicity can easily be evaluated using this assay.

In vitro assays provide a rapid, low-cost and sensitive means to assess a wide range of biological effects, and are typically mechanism-based (Cavanagh *et al.*, 2018). However, translating *in vitro* results to *in vivo* is quite complicated and Wangmo *et al.* (2018) only observed good correlations for compounds acting via direct binding to sex steroid receptor. Konig *et al.* (2017) also did not find *in vitro* tests to be directly predictive of effects at the whole organism level and population level. It is often hard to compare results because of variability in assay responses with different cell lines, and a greater focus on standardising bioassay protocols is needed if they are to be incorporated into robust environmental assessment frameworks.

In vitro methods are beneficial as they reduce the need for animal testing and are cheaper to run. However, *in vitro* assays must be used in conjunction with *in vivo* testing, as *in vitro* systems cannot fully take into account bioaccumulation, metabolism, and availability to the target cell, or alternative pathways for endocrine disruption.

Issues and uses of *in vitro* testing are discussed further in Chapter 3.

1.5.2.2. *In vivo testing*

Unlike QSAR models and *in vitro* screening, *in vivo* studies use intact organisms with metabolic function, normal architecture, normal cell-cell and tissue-tissue interactions (Cote *et al.*, 2016). *In vivo* assays therefore take into account the pharmacokinetics of the compounds (uptake, transport, metabolism, differences in binding efficiencies) and thus are measuring final health effects. *In vivo* assays though are less suitable for HTP screening. Hence for comprehensive characterisation of complex environmental pollution, a combination of analytical chemistry, *in vitro* and *in vivo* assessment should be applied.

The gold standard *in vivo* assays for identifying oestrogenic or androgenic compounds were initially developed in mammal models. The uterotrophic assay (Ankley and Gray, 2013) is an *in vivo* assay that utilises rats to detect oestrogenic chemicals. For this assay, immature or

ovariectomised adult female rats are treated with test chemicals for three consecutive days. After exposure, the uterus is weighed, and increases are indicative of chemicals acting as ER agonists (OECD, 2007).

The OECD Hershberger *in vivo* androgenic screen relies on castration removing endogenous androgen in prepubertal male rats, so that exogenous androgens are needed for the target tissues to grow and gain weight. This assay has several advantages; the tissues are the natural targets for androgens, the tissue growth response is relatively rapid, and the tissue weights are quantitative (Owens *et al.*, 2006). This assay can also be used to detect AR antagonists. Another rat assay, the male pubertal system, will more effectively detect other AR antagonists in the intact developing rat (e.g. those affecting steroidogenesis), and is important for confirming observations made in the Hershberger assay (Ankley and Gray, 2013).

Questions remain as to whether the mechanistic information from mammalian studies can be used to predict toxicant effects in fish and wildlife (Wells and Van Der Kraak, 2000).

Sex determination in teleosts can be disturbed or even functionally reversed by the external application of hormones, and this forms the basis of a number of fish assays. For assessing aquatic contaminants, fish are ideal, either collected in the field or exposed in laboratories. Small fish model species (zebrafish, FHM, medaka and 3-spined stickleback) are regularly used for biomonitoring, due to their short life-cycles allowing for assessment of reproductive performance, which is one of the most important endpoints for long-term effects, with implications for population development (Scholz and Mayer, 2008).

Small-bodied freshwater fish are commonly used in regulatory testing for EDCs but most lack a sensitive and quantifiable androgen specific biomarker (Muldoon and Hogan, 2016). The FHM is extensively used both for regulatory testing and research for the assessment of chemicals for oestrogenic and androgenic activity. Several endpoints are responsive to oestrogens (VTG, sex-steroid production, decreased nuptial tubercle counts in males, delayed maturation and decreased fecundity and fertility) and others are responsive to androgens (SSCs – nuptial tubercle growth, alterations in size of the dorsal fat pad, alterations in shape, colouration and breeding behaviours). These SSCs are often not sensitive to low concentrations of androgenic compounds and are scored using a subjective scale and thus may be subject to bias. SSCs can also be affected by the hierarchical status of the fish, making it difficult to attribute a change in appearance to exposure to an EDC. Measurement of FHM SSCs in response to androgens and anti-androgens can therefore be ambiguous (Collette *et al.*, 2016).

One of the best-documented physiological responses under tight androgen control is 'spiggin' production in sticklebacks (Sebillot *et al.*, 2014). The kidney of the male stickleback hypertrophies during the breeding season, when the secondary proximal epithelial cells synthesise a glue glycoprotein, spiggin (the Swedish name for the three-spined stickleback being spigg). The spiggin is secreted into the urinary bladder and is then used to stick plant material together to form a nest (Borg, 1994). Spiggin levels are almost undetectable in female stickleback fish under natural conditions, but androgen-dependent spiggin can also be induced in the kidneys of female stickleback in response to exogenous androgens, where it can be determined by measuring the kidney epithelial height (KEH) or by using an enzyme-linked immunosorbent assay (ELISA; Katsiadaki *et al.*, 2002). Since the detection, characterisation and cloning of spiggin, this appropriate and quantifiable biomarker can now be used for the screening of androgens in sticklebacks (Scholz and Mayer, 2008). In the stickleback a clear and quantifiable androgenic response can be measured at 100ng/L methyltestosterone (MT; Muldoon and Hogan, 2016). The stickleback is more sensitive to androgens than the FHM (tubercle formation in females occurs at a concentration of 1 µg/L or greater; Pawlowski *et al.*, 2004), and less sensitive than the Japanese medaka (formation of papillary processes in females occurred at 22.5 ng/L of MT; Kang *et al.*, 2008). The advantage of using stickleback is that it is possible to simultaneously assess androgen and oestrogen exposure in a single fish using fully quantitative endpoints (Muldoon and Hogan, 2016). However, due to the large variability and high levels of spiggin in males, for the anti-androgen screen female co-exposure with androgens has been preferred. Spiggin as a biomarker is only applicable to sticklebacks, but sticklebacks are not as widely used in toxicology as, for example, FHM or medaka. Therefore, fish assays that are sensitive to androgenic/anti-androgenic chemicals and not prone to subjectivity (such as current SSCs) need to be developed. In terms of oestrogen responsiveness, the FHM, medaka and zebrafish may be more suitable species as test methods are better established. These species could therefore hold promise if robust androgenic/anti-androgenic biomarkers or endpoints could be developed. This topic will be developed further in Chapter 5 of this thesis.

1.6. My aims

The aim of my thesis was three-fold:

To determine the extent of endocrine disruption in fish exposed to a WwTW effluent before and after the addition of an advanced water treatment process, Granulated Activated Carbon (GAC), was implemented to investigate the technology's ability to reduce EDCs being

released into the environment. Both laboratory and field-based assessments of the same effluent were conducted to investigate the impacts of the traditional and advanced treatment processes on well characterised markers of endocrine disruption in fish namely, occurrence of intersex phenotypes (both eggs and sperm in the gonad), VTG induction in male fish, and disruption to reproductive output and SSCs.

To examine over 100 chemicals found in WwTW effluents for (anti-)androgenic activity in an *in vitro* yeast test system and determine the potencies of these chemicals.

To search for male-specific molecular markers in a sexually dimorphic fish species, in order to identify possible biomarkers which could provide promising additional endpoints for detecting (anti-)androgenic activity in fish *in vivo* regulatory assays.

1.7. My research questions:

Question 1: Does the addition of an advanced treatment process to a full-scale wastewater treatment plant lead to a reduction in endocrine disruption in fish?

Whilst investigating **Question 1 (described in Chapter 2)**, further questions arose around whether the endocrine effects seen in the effluent exposed fish were driven purely by well characterised oestrogenic chemicals or if they were also caused by less well identified anti-androgenic compounds (**raised in Question 2, described in Chapters 3 and 4**) and if it would be possible to identify a novel specific biomarker of anti-androgenic exposure in the FHM, a well characterised and commonly used fish species in OECD regulatory test guidelines (**raised in Question 4, investigated in Chapter 5**).

Prior to my thesis, an effect directed analysis (EDA) approach was used to tentatively identify chemicals present in environmental samples as having (anti-)androgenic activity using an *in vitro* yeast-based assay. Following on from this work, I then asked the question -

Question 2: Which individual chemicals from wastewater treatment work effluents have (anti-)androgenic activity and how potent are these chemicals?

Whilst testing these chemicals for (anti-)androgenic activity, several issues were encountered that affected data interpretation which could lead to the mislabelling of certain chemicals as endocrine disruptors. Therefore, to refine these results, two *in vitro* assays for the determination of (anti-)androgenic activity of environmental chemicals were then compared, and the question asked was –

Question 3: Do both *in vitro* assays for (anti-)androgenic activity produce the same results and if not, which is the most reliable, i.e. least likely to produce false positive results?

In stickleback, a sexually dimorphic fish species, males secrete a glue-protein, known as spiggin, which is used to build a nest in preparation for female egg laying. Once the eggs are laid the male then cares for the eggs until shortly after they hatch. Spiggin is known to be produced under the control of androgens in males. The presence of spiggin in female sticklebacks indicates exposure to environmental androgens, and a reduction in spiggin in male fish indicates exposure to environmental anti-androgens. Here I ask the question –

Question 4: Do FHMs, another sexually dimorphic fish species exhibiting paternal parental care, possess a similar spiggin-like protein that could be used as a sensitive biomarker of (anti-)androgen exposure, to provide an additional endocrine endpoint to commonly used OECD regulatory test guidelines?

2. Investigating the efficacy of an advanced wastewater treatment process, granular activated carbon (GAC), at removing endocrine-disrupting activity; laboratory and field-based studies of standard and GAC treated wastewater effluents

2.1. Introduction

2.1.1. Brief history of oestrogenic endocrine disruption in wild fish

Whilst in certain families of fish hermaphroditism or sex reversal are the normal mode of reproduction, the vast majority of species are gonochoristic, in which distinct males and females are independent and stable (Nolan *et al.*, 2001). The gonad is a unique organ due to its bipotential nature; either testis or ovary can develop from a single primordium (Brennan and Capel, 2004). In teleosts, endogenous oestrogens act as the natural inducer of ovarian differentiation, while androgen is not synthesised in the gonad during the critical period of sex determination (Nakamura *et al.*, 1998). Consequently, sex differentiation and meiosis initiate earlier in female teleosts than in male teleosts. Under experimental or aquaculture conditions, the phenotypic sex of gonochoristic fish species can be affected by exposure to sex steroids, and, in general, exogenous exposure to oestrogens will produce a female phenotype whereas exposure to androgens will produce a male phenotype (Ankley *et al.*, 1998).

A wide variety of chemicals discharged into the aquatic environment are thought to disrupt endocrine function in fish, leading to reproductive disorders and abnormalities (reviewed in Sumpter, 2005). Roach (*Rutilus rutilus*), of the family Cyprinidae, is a gonochoristic fish species native to the UK. The occurrence of oocytes in testicular tissue in gonochoristic fish (intersex) is thought to be rare (Nolan *et al.*, 2001). However, when Jobling *et al.* (1998) carried out a survey of roach collected from eight UK lowland rivers, the incidence of intersexuality was much higher in those rivers that received sewage effluents (proportion of intersex males at downstream sites ranged from 16-100%) than at the control sites (proportion of intersex males ranged from 4-18.1%). An association was also confirmed between the widespread incidence of intersexuality (gonads containing male and female tissue and/or female-like reproductive ducts) in roach and the degree of exposure to WwTW effluents (Jobling *et al.*, 1998). The severity of intersex in roach was observed to range from single primary oocytes scattered throughout the testicular tissue, to continuous areas of ovarian tissue set apart from the testicular tissue (Jobling *et al.*, 1998).

Jobling *et al.* (1998) found that the number of male roach with normal testes was inversely proportional to the number of intersex fish, indicating that this trend was due to the feminisation of genetically male fish rather than the masculinisation of genetically female

fish. Exposure to WwTW effluents has also been shown to induce the synthesis of VTG (Harries *et al.*, 1997; Purdom *et al.*, 1994) in male fish and to also inhibit testicular growth (Harries *et al.*, 1997). In addition, exposure of juvenile roach to WwTWs effluent has been shown to induce a dose-dependent formation of female-like reproductive ducts (oviducts) in otherwise 'male' looking fish (Rodgers-Gray *et al.*, 2001). More recent work, looking to see if male intersex impacts reproduction, found a significant negative relationship between the severity of intersex and reproductive success; the intersex condition reduced the average contribution to the offspring from 19% for non-intersex fish to 4.5% for severely feminised fish (>100 oocytes within the testicular tissue; Harris *et al.*, 2011).

Intersexuality in fish associated with WwTW effluents is not restricted to roach in the UK, and has also been reported in other fish species and a number of other countries (for example, intersex gudgeon in the UK (van Aerle *et al.*, 2001); intersex roach in France (Geraudie *et al.*, 2017); intersex roach in Denmark (Bjerregaard *et al.*, 2006); intersex rainbow darter in Canada (Bahamonde *et al.*, 2015); intersex smallmouth bass in the USA (Blazer *et al.*, 2014)).

To identify the chemical(s) in the effluent responsible for the feminising effects seen in male roach, Desbrow *et al.* (1998) adopted an EDA approach, using a combination of biotesting, fractionation procedures and chemical analysis. Following the EDA approach, the oestrogenic activity of WwTW effluent was attributed to natural steroid oestrogens; E2, E1, the synthetic birth-control pharmaceutical, EE2 (predominantly excreted by women), and alkylphenols from domestic and industrial sewage effluent, and these chemicals were considered to be the primary agents responsible for the feminisation of fish downstream of the WwTW (Desbrow *et al.*, 1998; Harries *et al.*, 1997).

2.1.2. (Anti-)androgens present in the environment

In addition to work looking at oestrogens in the environment, now chemicals possessing other mechanisms of endocrine modulation are being examined. There are a growing number of anthropogenic chemicals being identified in influents and rivers, and there is increasing evidence showing that discharges are having adverse environmental effects (Prasse *et al.*, 2015). In addition to chemicals in WwTW effluent acting as agonists of the ER, the feminisation of male fish is also thought to be due to anti-androgens, and statistical modelling shows a strong correlation between fish intersex and the predicted exposure to the combination of oestrogens and anti-androgens in effluent (Jobling *et al.*, 2009). However, Johnson *et al.* (2007b) saw no clear relationship between the presence of oestrogenic activity and anti-androgenic activity in individual effluent samples and suggested that different chemical(s) may be causing the oestrogenic and anti-androgenic effects.

In fish, androgens are important as they regulate male sexual differentiation and maturation (Borg, 1994). Most environmental (anti-)androgenic contaminants can bind with the AR to either mimic or block the response, thereby acting as either androgens or anti-androgens (Wong *et al.*, 1995). WwTWs effluents are often found with *in vitro* anti-androgenic activity at concentrations known to cause effects on fish (for example, Fang *et al.*, 2012; Johnson *et al.*, 2007b). Laboratory studies have shown that anti-androgenic chemicals can cause demasculinisation of male fish, for example, the fungicide vinclozolin and the clinical anti-androgen cyproterone acetate induced intersex and inhibited spermatogenesis in medaka (Kiparissis *et al.*, 2003) and reduced SSCs in FHM (Filby *et al.*, 2007). In addition, in laboratory studies using stickleback kidney cell primary culture, spiggin production (a biomarker of androgen exposure) could be inhibited with anti-androgens (Jolly *et al.*, 2009).

2.1.3. Measurement of endocrine disrupting activity in fish

It is not possible to identify every chemical present in an effluent as to do so analytically would be extremely time consuming and costly. Especially as thousands of chemicals are discharged into our rivers (Tyler *et al.*, 2007) and these are transformed in WwTWs and rivers into additional compounds (Escher *et al.*, 2014). A wide range of EDCs have been shown to bioconcentrate in fish bile (to concentrations many thousand times more than concentrations in the environment; Rostkowski *et al.*, 2011), prior to excretion into the intestines as the parent compound or as conjugates (Hill *et al.*, 2010). As with effluent, it would be too time-consuming and costly to measure every chemical present by analytical chemistry. However, the total activity present in fish bile when measured using a bioassay is a much more practical approach and this activity gives a good indication of the types of contaminants that would have been present in the river and that have bioconcentrated in the fish bile over a period.

2.1.4. Wastewater effluents are a major source of aquatic pollution

2.1.4.1. Regulations relating to wastewater and aquatic pollution

The continued occurrence of contaminants in rivers downstream of WwTWs has led to discussion regarding the need for improved wastewater treatment processes. In 1991, the EU Urban Wastewater Treatment Directive (WwTD) 91/271/EEC (European Commission, 1991) was adopted to protect the environment from adverse effects of wastewater discharges from cities and certain industrial sectors. This directive prescribed secondary treatment (e.g. biological treatment with a secondary settlement) for urban areas with a population equivalent of greater than 2,000 and more advanced treatment in places with population equivalents of greater than 10,000 in sensitive areas (e.g. shellfish and freshwater fisheries or where water is used for the abstraction of drinking water) or greater

than 15,000 in areas where the effluent is not discharged into sensitive areas. This EU Urban WwTD established parameters that discharges from urban WwTWs must satisfy; organic content (biological oxygen demand and chemical oxygen demand) and total suspended solids, and for sensitive areas, parameters for total phosphorus and nitrogen must also be satisfied. This directive, however, does not provide acceptable parameters for EDCs.

More recently, the EU Water Framework Directive (WFD) (European Commission, 2000) set out 'Strategies against pollution of water', that assesses the ecological and chemical status of surface waters for biological, hydromorphological, physio-chemical and chemical quality. The WFD outlines a list of priority substances and these substances were selected from amongst those presenting a significant risk to or via the aquatic environment. The original list, agreed in 2001, contained 33 priority substances (Decision 2455/2001) and in 2008, the Environmental Quality Standards (EQSs) for these substances were established (Environmental Quality Standards Directive (EQSD) 2008/105/EC, also known as the Priority Substances Directive). This list was further reviewed in 2012, and the amendment (Directive 2013/39/EU) now lists 45 priority substances and 21 of these are thought to pose the greatest threat and are labelled 'priority hazardous substances'. The Priority Substances Directive requires the progressive reduction and phasing out of these 'priority substances'. These include di(2-ethylhexyl) phthalate (DEHP), nonylphenols, polycyclic aromatic hydrocarbons (PAHs), and tributyltins. In 2015, the most ubiquitous and potent steroid oestrogens, namely the natural steroid oestrogens E1 and E2, and the synthetic steroid oestrogen EE2 were added to the EU WFD chemical '1st watch list' (Carvalho *et al.*, 2015). Standard biological WwTW processes (e.g. trickling filters, activated sludge) do not completely remove steroid oestrogens entering the WwTW, although they can frequently reduce the concentrations down to low ng/L range in the effluent (Bain *et al.*, 2014; Hicks *et al.*, 2017; Kanda and Churchley, 2008; Volker *et al.*, 2016). However, fish are extremely sensitive to these steroids (Caldwell *et al.*, 2012), resulting in predicted no effect concentrations (PNECs) as low as 0.035 ng/L for EE2, 0.4 ng/L for E2 and 3.6 ng/L for E1 (Loos *et al.*, 2018). Therefore, in receiving water with little dilution, surface water concentrations may be close to, or exceed, these PNECs (Johnson *et al.*, 2013). Better analytical techniques will also be required to determine if regulations regarding the concentrations permitted in final effluents entering rivers are being met.

Natural hormones (T, E1, E2) are EDCs excreted by humans and other animals, and are therefore abundant in effluents. Whilst it would be possible to try to find safer alternatives to replace some man-made chemicals (e.g. alternatives that are more readily degraded), it would harder if not impossible to reduce the amount of natural steroid and pharmaceuticals

entering WwTWs. Therefore, other measures are required to reduce their release into the environment, and one option would be to improve effluent treatment technology at the WwTW. Another option would to be to reduce the number of new chemicals produced, unless these are specifically produced as safer alternatives.

2.1.4.2. Traditional wastewater treatment methods

Conventional WwTWs rely on physical, chemical, and biological methods that remove solids, pathogens, organic matter, and nutrients. For removal of EDCs this process would be by biodegradation, sorption to sludge, chemical transformation and volatisation (Kanda and Churchley, 2008). Depending on the chemical structure, biodegradation and/or sorption can lead to differing removal rates during the wastewater treatment process (Prasse *et al.*, 2015).

Figure 8 illustrates different types of WwTWs processes, and the type of chemicals that are likely to be removed at the different treatment stages.

The first step in the wastewater treatment process, the preliminary treatment, involves the removal of grit and gravel by slowing flows down to enable settlement. After this de-gritting step, the effluent is screened to remove large solids. Primary treatment then involves the passive and/or chemically-enhanced process of settlement of suspended solids not removed by preliminary treatment. Removal of chemicals from effluent during primary treatment relies on the hydrophobicity of the chemicals, the suspended solids content and their subsequent settling, and hydrophobic compounds are likely to bind to particles and settle out (Koh *et al.*, 2008).

Secondary treatment is a biological process whereby dissolved organic matter is removed typically by either trickling filters or activated sludge plants. The major process difference is that in trickling filters the biomass is steady (sewage trickled over coarse aggregate coated with bacteria), and in activated sludge plants the biomass is in suspension (aerated agitated liquor). In the England and Wales, most WwTWs use trickling filters for secondary treatment (Johnson *et al.*, 2007a), whereas the activated sludge process is the most widely used wastewater treatment system in the world (Liu *et al.*, 2009b). Typically, the retention time of most European activated sludge systems is between 4 and 14 hours (Johnson and Sumpter, 2001) compared with a trickling filter which might have a retention time of less than one hour. These longer retention times in the activated sludge system will give longer for biodegradation to take place and hence removal rates for EDCs will be better (Zeng *et al.*, 2013). For example, Korner *et al.* (2000) found 90% reduction in oestrogenic activity of effluent following activated sludge plant treatment and concluded that this was the result of

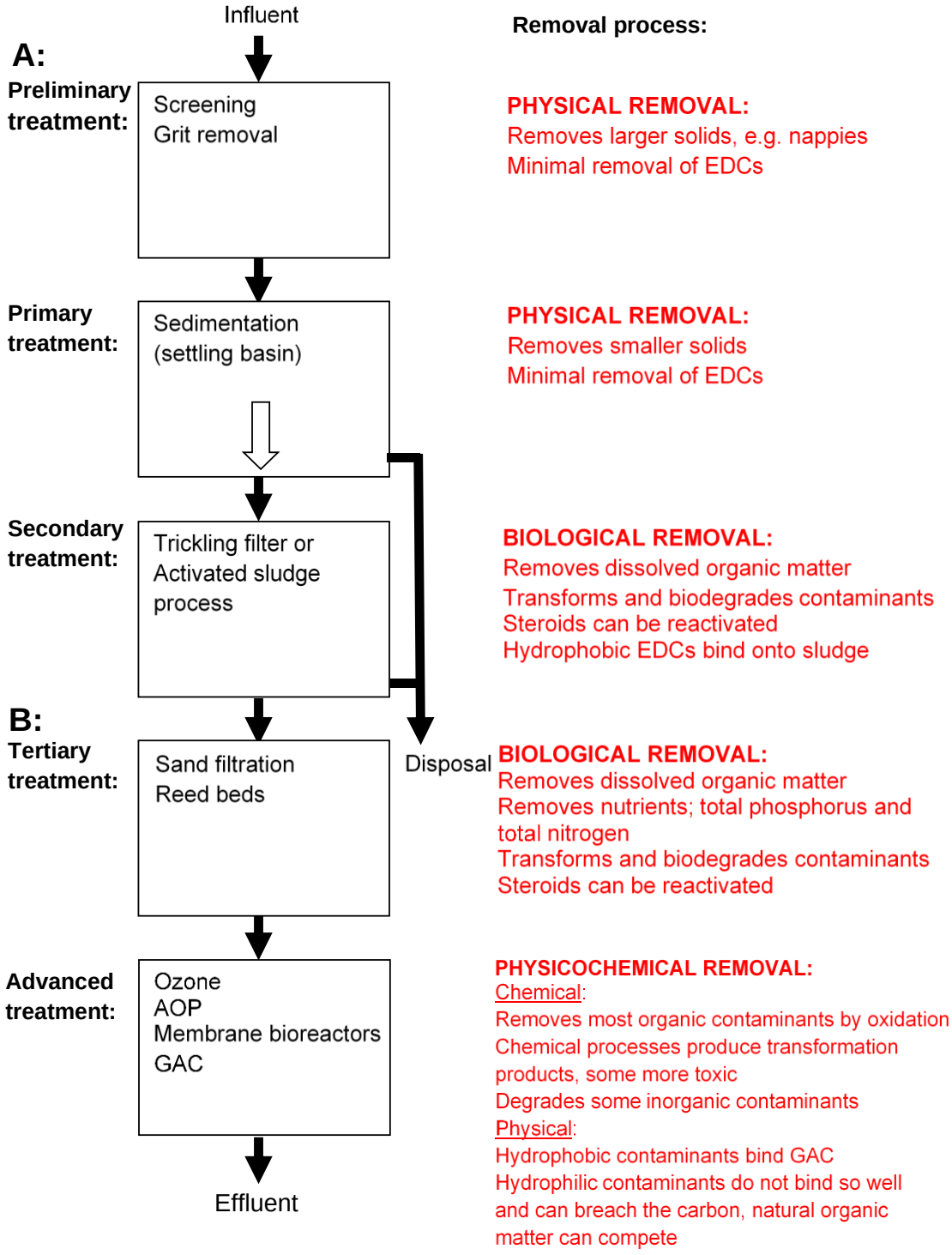


Figure 8. Schematic of (A) a conventional wastewater treatment works, and (B) end-of-pipeline additional (tertiary) and advanced treatments necessary to improve effluent to meet the Environmental Quality Standards of the EU Urban Wastewater Treatment Directive and the Water Framework Directive. The removal process describes the types of compounds that will be removed at the different treatment stages.

biodegradation because only 2.8% of the activity had adsorbed to the sludge.

Tertiary treatments such as sand filtration and reed beds increase the surface area and the retention time of the effluent and allow bacteria to work for longer, thus 'polishing' the effluent further. Baynes *et al.* (2012) found roach exposed to activated sludge process treated effluent to have evidence of significant feminisation compared with the roach exposed to river water, but the effect was much reduced with the addition of sand filtration following the activated sludge process.

There are two approaches for removing EDCs from tertiary treated wastewater; optimising existing treatment technologies or upgrading existing WwTW with new end-of-pipe technologies (Koh *et al.*, 2008), and here we consider end-of-pipe technologies for upgrading existing works with an advanced treatment process. Unlike the tertiary treatments, advanced treatments tend to rely on physical or chemical treatments.

2.1.4.3. Advanced wastewater treatment methods

Advanced treatments include membranes, activated carbon filtration, the application of ozone, and advanced oxidative processes. Removal of EDCs by physical means (e.g. membranes and activated carbon filtration) does not lead to metabolites or by-products, unlike biological or oxidative processes, and removal efficiencies are therefore easier to evaluate (Liu *et al.*, 2009b).

Membrane bioreactors combine conventional activated sludge treatment with micro/ultra-membrane separation and can produce higher quality effluent compared to conventional activated sludge treatment (Li *et al.*, 2015). Trinh *et al.* (2012) examined the efficiency of a membrane bioreactor at removing trace organic chemical contaminants and found 97-100% removal rates for steroid hormones. However, pharmaceuticals such as carbamazepine, diazepam, diclofenac, fluoxetine, and trimethoprim were less effectively removed (24-47%), and this was believed to be because they were not readily biodegraded or adsorbed to particles. Similarly, Snyder *et al.* (2007) tested a membrane bioreactor for its efficiency to remove 18 endocrine disruptors and pharmaceuticals and found that some compounds were well removed whilst others were not. In fact, some compounds appeared to increase in concentration in the membrane bioreactor. Snyder *et al.* (2007) further tested the membrane bioreactor followed by reverse osmosis (RO) and found the RO removed nearly all the compounds tested to below reporting levels. These membrane bioreactors have a lower foot-print than conventional activated sludge but have larger capital costs (Karim and Mark, 2017).

Activated carbon is used as either GAC, as a packed bed filter, or as a powder added directly to the wastewater and subsequently filtered out. The powder form may not be retained by downstream sand filtration, and may therefore enter the environment (Prasse *et al.*, 2015). Additionally, the powder becomes highly contaminated with time and disposal of this waste then also becomes an issue. Granular activated carbon is therefore more widely used and can remove a large variety of organic contaminants (Grover *et al.*, 2011b; Snyder *et al.*, 2007; Ternes *et al.*, 2002). This GAC removal process is based solely on sorption with no formation of potentially reactive transformation products. However, Snyder *et al.* (2007) did find that water soluble compounds can break through the GAC much faster than tightly bound hydrophobic compounds. Similarly, Baynes *et al.* (2012) found good removal of steroid oestrogens following a 6-month exposure period of roach to GAC effluent as indicated by total removal of intersex induction in fish, but did find elevated EE2 concentrations in some of the final samples, suggesting GAC saturation and subsequent breakthrough of the chemical into the effluent.

Ozone has been shown to be an effective means of oxidising trace organic contaminants while also providing effective disinfection (Snyder *et al.*, 2006). Giebner *et al.* (2018) used the YES to check for removal efficiency of oestrogenic activity and found 18.9% receptor activation after secondary treatment and this reduced to 3.2% with ozone treatment and 2.9% with GAC treatment. Giebner *et al.* also measured androgenic activity using the yeast androgen screen (YAS) and found that there was almost total removal of activity following secondary treatment; receptor activation was 2.2%. Removal rates for anti-androgenic activity were not described as no activity was seen in primary treated samples.

Ozonation can remove >90% of oestrogenic activity and 78% of anti-androgenic activity (Stalter *et al.*, 2011), however, the resultant oxidation products can be more toxic to biota. For example, Rosal *et al.* (2009) found that the ozonation of clofibric acid produced oxidation products that were more toxic to *Daphnia magna* due to the formation of open-ring by-products. Furthermore, Prasse *et al.* (2012) found the oxidation product of the antiviral drug acyclovir to be toxic to the bacteria *Vibrio fischeri*, and following 14 days of activated sludge treatment only 40% of this toxic product was removed. Magdeburg *et al.* (2012) tested ozonated effluent on five different invertebrate species and the results also indicated the formation of adverse oxidation products with a tendency towards increased toxicity in three of the species. The toxic effects of ozonation products have, however, been shown to be removed or detoxified by downstream sand filtration; based on studies using invertebrate species (Magdeburg *et al.*, 2012) and rainbow trout (Magdeburg *et al.*, 2014) as bioindicators. Similarly, Giebner *et al.* (2018) found that ozone-induced mutagenicity seen in the Ames test could be removed by a post-ozonation filtration step.

Similar to ozonation, advanced oxidation processes are based on the *in situ* generation of a powerful oxidising agent such as hydroxy-radicals. The generation of hydroxy-radicals by means of various chemical, photochemical, sonochemical, or electrochemical reactions leads to effective removal of chemicals of environmental concern, but also leads to the formation of oxidation products that may be more toxic (reviewed by Oturan and Aaron, 2014).

2.1.5. UK Endocrine Disrupting Chemicals National Demonstration Programme: assessment of the performance of WwTW in removing oestrogenic substances

The Environment Agency, in collaboration with the government and the UK water industry, developed an Endocrine Disruption Demonstration Programme (EDDP) to evaluate different wastewater treatment plant processes for the removal of endocrine disruption. The programme considered the current wastewater treatment technologies for removing steroids from effluent and conducted both pilot-scale (GAC, Cl_2 and ozone) and full-scale demonstration trials of promising advanced treatment technologies (Butwell *et al.*, 2010; Gross-Sorokin *et al.*, 2006; UKWIR, 2009). One of the test sites chosen for demonstrating a full-scale advanced wastewater treatment was Swindon WwTW (Rodbourne, Wiltshire, UK) that serves a population of approximately 180,000. The River Ray is in the non-tidal area of the upper River Thames catchment and the main discharge into the river comes from Swindon WwTW. Due to the small size of the river, there is limited dilution of the effluent on entering the River Ray and in mid-summer approximately 80% of the flow is effluent at the point of discharge (Balaam *et al.*, 2010). Furthermore, even 11 km downstream of the point of discharge just above the confluence of the River Ray with the River Thames, approximately 65% of the flow is effluent.

Prior to the upgrade as part of the Demonstration Program, the Swindon WwTW consisted of primary treatment; screening followed by settlement, and secondary treatment; activated sludge processing and final settlement. As part of this demonstration program, the Swindon WwTW was chosen for investigation into the efficacy of an advanced treatment step; the installation of a full-scale GAC plant, to extend the existing conventional treatment line, was implemented to investigate the technology's ability to reduce concentrations of EDCs being released into the environment. In total, the plant used 1,900 m^3 of GAC (Norit, Glasgow, UK), and had the following properties: 0.50 g/ml apparent density, 1.0 mm effective size, 920 mg/g iodine number (Grover *et al.*, 2011b).

2.1.6. Endocrine sensitive endpoints and biomarkers of endocrine disruption in fish

Vitellogenin is present in high concentrations in the plasma of sexually mature female fish (Sumpter and Jobling, 1995). Vitellogenin is produced in the liver in response to oestrogens from the ovaries. Under normal circumstances, VTG concentrations in male fish are either undetectable or low (about 10,000-100,000 times lower than typical female concentrations; Ankley and Johnson, 2004). However, when male fish are exposed to oestrogenic substances, they are capable of producing a large amount of VTG, and blood concentrations can reach values similar to those in females. Thus, VTG induction in male fish is commonly used as a specific biomarker for the detection of oestrogenic endocrine disruptors in the environment, and also in OECD tests (OECD, 2018a; b) of suspected oestrogenic chemicals (Jobling *et al.*, 1996).

The simultaneous occurrence of male and female reproductive stages in the same gonad at the same time in teleost fish is atypical (Bahamonde *et al.*, 2013) and is referred to as 'intersex', and the presence of oocytes in testicular tissue is termed 'ovotestes'. Sexual differentiation involves gonadogenesis (development of gonads and ducts) and gametogenesis (development of germ cells). Fish (and other animals) are most susceptible to perturbation during early life (Gimeno *et al.*, 1996). Both gametogenesis and gonadogenesis can be altered by exposure to sex steroids and sex steroid mimics during development (Jobling *et al.*, 1998), and the presence of ovotestes and/or an ovarian duct (cavity) in male fish are used as indicators of exposure to EDCs. It is not possible to alter gonadogenesis in adulthood, and the presence of an ovarian cavity in a male fish is an indication of exposure during development rather than adulthood (Jobling *et al.*, 1998).

Severely intersex fish (those with many oocytes in the testes) have been reported to have reduced fertility and fecundity (Fuzzen *et al.*, 2015; Harris *et al.*, 2011; Jobling *et al.*, 2002b; Thorpe *et al.*, 2009). Egg counts give an indication of fecundity, important as changes in fecundity could also lead to population level effects. This is especially significant for short lived species, as was observed by Kidd *et al.* (2007) when a whole lake exposure of FHM to EE2 impacted on gonadal development (intersex in males and altered oogenesis in females) and resulted in near extinction of this species from the lake.

Secondary sexual characteristics are features that appear at sexual maturity and distinguish the two sexes of a species. The development of SSCs, including external genitalia, depends on whether the gonad differentiates into a testis or ovary, and the expression of the male SSCs in fish is under the control of the AR (Borg, 1994). Several SSCs are responsive to androgens, and monitoring for changes in SSCs is the basis of some fish tests for assessing chemicals for (anti-)androgenic activity; examples are gonapodial development in mosquito

fish (Howell and Denton, 1989), papillary process development in medaka (Seki *et al.*, 2006), and tubercle number and size of fatpad in FHM (Ankley *et al.*, 2001).

For my research outlined in this chapter, I have investigated endocrine disruption in two test species, namely the UK native roach (*Rutilus rutilus*), which is frequently found across Europe in effluent impacted rivers, and the FHM (*Pimephales promelas*), a North American species routinely used in regulatory testing for investigating possible EDCs. The following sections provide more details about these two species, their life histories, and specific endocrine sensitive endpoints and biomarkers.

2.1.7. Test species

Roach

The roach is a slow growing and long-lived freshwater species and takes a relatively long time to sexual maturity (2–3 years for males and 3-4 years for females; Epler *et al.*, 2005). For both males and females, the sexual maturation rate is also highly dependent on growth (Hamilton *et al.*, 2015). In addition to fish size, environmental conditions can influence the time roach take to reach sexual maturity (Paull *et al.*, 2008). In most cyprinid species, including roach, the predominant cue for the annual rhythm of reproduction is a combination of annual photoperiod and/or temperature, and also social interactions (Jalabert, 2005). Roach typically spawn April-June, stimulated by an increase in day length and an increase in water temperature to above 12 ^{o}C (Paull *et al*., 2008).

As highlighted above (Section 2.1.1), in the UK the majority of investigations of endocrine disruption in wild fish have focused on roach, involving assessments for intersexuality and VTG induction (for example, Baynes *et al.*, 2012; Hamilton *et al.*, 2015; Jobling *et al.*, 1998; Tyler *et al.*, 2007). The roach has proved a useful model for examining the effects of WwTW effluent on wild fish living in impacted rivers. However, roach are a relatively large (15-25 cm; Geraudie *et al.*, 2010) and slow maturing species that breeds only once a year, and the species lacks sexually dimorphic traits. It therefore has limited utility as a laboratory test species.

Fathead minnow

The FHM is a member of the Cyprinidae family with a broad distribution across North America. FHM males are larger than the female (3-5 g compared with 2-3 g, respectively) and when reproductively active, the male exhibits several SSCs (e.g. nuptial tubercles and dorsal fatpad; Smith and Murphy, 1974). Under optimal conditions the FHM reach maturity within 4-5 months post hatch, and depending on the water temperature and photoperiod, can spawn continuously for several months.

The FHM is extensively used both for regulatory testing and research, especially in North America (Ankley and Villeneuve, 2006), and along with the zebrafish is among the most widely used fish species for testing endocrine disrupting effects (Holbech *et al.*, 2012). For example, the FHM is the recommended fish species in the OECD Fish Short Term Reproduction Assay (OECD, 2018b) and one of three fish species that can be used in the OECD 21-Day Fish Test; A Short-Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition (OECD, 2018a).

Several endpoints are responsive to oestrogens (VTG, sex-steroid production, decreased nuptial tubercles counts in males, delayed maturation and decreased fecundity and fertility) and responsive to (anti-)androgens (SSCs – nuptial tubercle growth, alterations in size of the dorsal fatpad, alterations in body shape, colouration and breeding behaviours). Some of these are short-term transient effects that the FHM is able to recover from once the oestrogen is no longer present (e.g. VTG induction).

2.1.8. The aim of Chapter 2

The aim of this chapter was to determine the extent of endocrine disruption in fish exposed to a WwTWs effluent before and after the addition of an advanced water treatment process; GAC. Two different approaches were used to investigate the impacts of the conventional and advanced treatment processes on well characterised markers of endocrine disruption in fish, namely the occurrence of intersexuality (oocytes in testicular tissue and/or feminised sperm ducts) in male fish, VTG induction in male fish, and disruption to reproductive output and SSCs. The first approach used short-term laboratory studies using the OECD test species, FHM, where the fish were directly exposed to effluent under controlled laboratory conditions. The second approach was chosen due to a wealth of historical data being available for the roach species, including roach from the River Ray, where the wild fish are continuously exposed to effluent in the field under environmental conditions. By using both types of studies, lab-based for short-term effects and field-based for realistic long-term effects, I was able to investigate whether the GAC was actually improving the quality of effluent enough to improve the reproductive health of the fish living in the river.

For the laboratory studies using FHM, flow-through exposure studies were carried out with final effluent from the WwTW, both pre- and post-GAC, to determine the efficacy of GAC at removing endocrine-disrupting activity. At Brunel University London, the effluent was tested using two well documented and sensitive *in vivo* tests of endocrine disruption in fish using the FHM; (1) the VTG Test to measure the induction of VTG in male fish (Thorpe *et al.*, 2008), and (2) the Pair Breeding Test to measure the development of SSCs (tubercle

number and Fatpad Index (FPI)) and the cumulative egg production (an assessment of impacts on reproductive success) (Thorpe *et al.*, 2009).

For the assessment of the GAC addition on wild fish living in the river, wild roach were collected from the River Ray to see if field-based wild fish analysis supported the results found in our lab-based studies. In 2013 roach were collected downstream of the Swindon WwTW 5 years after the upgrade of the works with the GAC, and the results were compared with historical data from roach sampled from the River Ray in 2005 (prior to GAC addition). The effects of the advanced GAC treatment on the fish plasma VTG concentrations and the fish gonad histopathology were examined and compared with the 2005 dataset. In both 2005 and 2013, bile samples were also collected from the fish for the measurement of oestrogenic activity and anti-androgenic activity using yeast-based assays; the YES and yeast anti-androgen screen (AYAS), to look for changes in the amount of activity present.

2.2. Materials and methods

2.2.1. Ethics statement

All fish studies that contributed data to this thesis were carried out in strict accordance with the recommendations of the United Kingdom Animals (Scientific Procedures) Act 1986. Fish were deeply anesthetised (complete loss of response to manual stimulation) prior to blood collection and were sacrificed either by destruction of the brain or trans-spinal severance. All efforts were made to minimise distress to the animals.

For the fish studies carried out at Brunel University London, these were done under both Project and Personnel Licences granted by the UK Home Office, and in accordance with Brunel University London's ethical policies.

2.2.2. Laboratory studies - Fathead minnow flow-through exposure studies to WwTW effluents

2.2.2.1. Experimental design

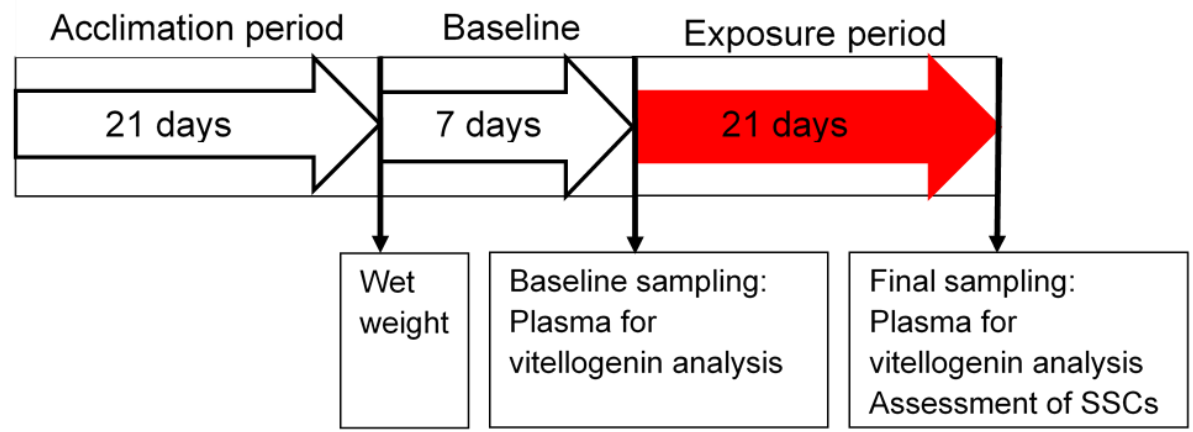
The Swindon WwTW effluent was tested in two different fish tests both before and after the addition of the advanced GAC treatment at the Swindon WwTW (Four experiments in total; Experiments 1 and 2, 21-day VTG Tests, and Experiments 3 and 4, Pair-Breeding Tests). Tests on the same type of effluent were run simultaneously in two adjacent rooms due to space requirements (the standard effluent was tested from 28 May to 18 June 2009 whilst the GAC effluent was tested between 16 March to 6 April 2009). The experimental designs for the two tests are illustrated in Figure 9 and Appendix Figures A 1 and A 2.

For both the VTG Test and the Pair Breeding Test, fish were held in flow-through aquaria which contained various dilutions of the effluent under test (standard effluent at 100%, 50% and 25% dilution, and GAC at 100% only) together with a positive control (10 ng/L EE2 for the VTG Test and 20 ng/L for the Pair Breeding Test) and a 'clean' dilution water control (control) (see Appendix Figures A 3 and A 4).

For the VTG Tests (Experiments 1 (standard effluent) and 2 (GAC effluent)) a total of 160 FHMs were used (Experiment 1; 96 fish and Experiment 2; 64 fish). Two tanks of 8 male fish per treatment (n=8 males in each of 2 replicate tanks) were set up and an additional tank of 16 male fish (in 2 tanks) were set up to determine baseline reproductive physiology. All fish were acclimated to the flow-through for 7 days. After 7 days acclimation, the 16 baseline fish were sampled (see Section 2.2.2.7). The fish in the treatment tanks were then exposed to dilution water (control), 10 ng/L EE2 (+ve control) or effluent (standard effluent at 100%, 50% and 25%, and GAC effluent at 100%). All experimental fish (80 for standard effluent exposure and 48 for GAC effluent exposure) were sampled at the end of the exposure period.

To initiate each Pair Breeding Test (Experiments 3 (standard effluent) and 4 (GAC effluent)), pairs of male and female FHMs were placed into eight replicate glass aquaria per treatment; total of 16 fish per treatment (Experiment 3; 80 fish and Experiment 4; 48 fish). The fish were acclimated to the test conditions for 14 days and the spawning substrates were checked daily at 11 am ± 1 hr to confirm spawning activity. At the end of this acclimation, all fish had fully acclimated to the test conditions and had spawned at least once. The egg number was then determined daily for each pair of fish, over a pre-exposure period of 3 weeks, to provide pair-specific data for egg production. Dosing of control, 20 ng/L EE2 or effluent (standard effluent at 100%, 50% and 25% and GAC at 100%) was then initiated and the number of eggs spawned by each pair of fish determined daily for the 3-week exposure period. All experimental fish were sampled at the end of the exposure period.

A) Vitellogenin Test (Experiments 1 and 2)



B) Pair Breeding Test (Experiments 3 and 4)

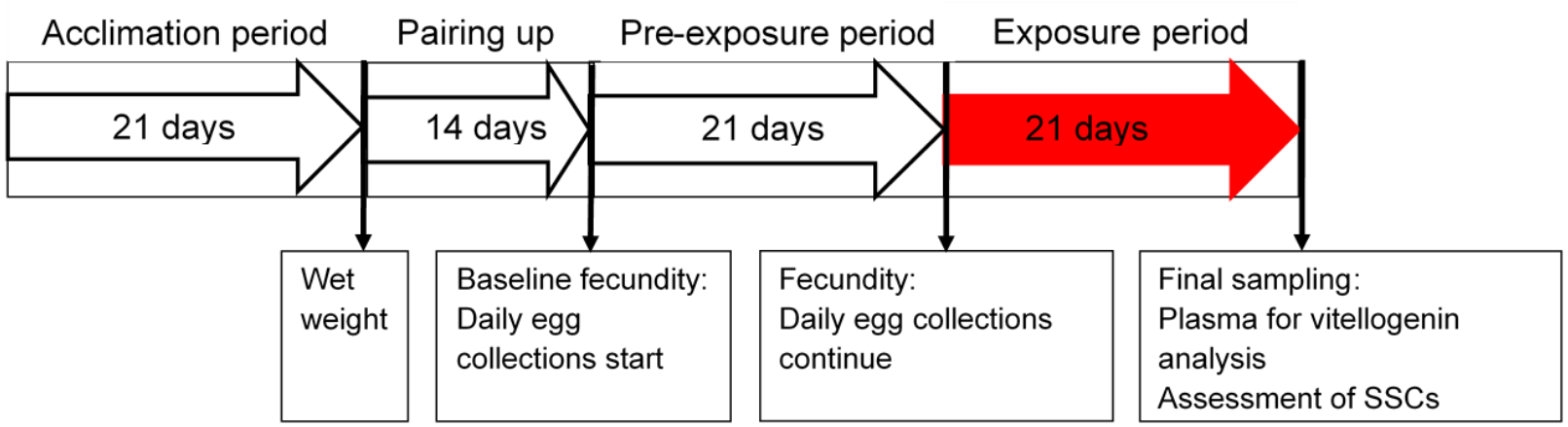


Figure 9. Schematic showing the timescale and endpoints for the fathead minnow exposure studies. (A) the Vitellogenin Test (Experiments 1 and 2), and (B) the Pair Breeding Test (Experiments 3 and 4). SSCs; Secondary sexual characteristics

2.2.2.2. Experimental animals

Fathead minnow used in each experiment were bred from stocks maintained at the aquatic facility at Brunel University London, and were kept according to the United Kingdom Animals (Scientific Procedures) Act 1986 guidelines. The ages and average weights of the fish at the start of the baseline period (VTG Test) and pairing up period (Pair Breeding Test) are shown in Table 1.

Table 1. Age and average weight of fathead minnows at the start of the Vitellogenin Test and Pair Breeding Test exposures to standard and GAC treated effluent.

Effluent	Vitellogenin Test	Pair Breeding Testing
Standard	Experiment 1: 7 months post hatch (average male weight 2.6 g)	Experiment 3: 7 months post hatch (average male weight 3.1 g and female weight 2.5 g)
GAC	Experiment 2: 11 months post hatch (average male weight 3.7 g)	Experiment 4: 11 months post hatch (average male weight 5.3 g and female weight 3.0 g).

For the VTG Test, only male fish were selected for the exposure study instead of a mixture of males and females as previously reported (Thorpe *et al.*, 2008), to minimise the possible influence of female fish-derived oestrogenic steroids as a confounding factor in the assessment of oestrogenic activity of the effluent. This also reduced the number of fish utilised, as female fish were not used for the VTG endpoint assessment.

Three weeks prior to the onset of each experiment, sexually maturing males (onset of the development of the SSCs; nuptial tubercles and a dorsal fatpad) and females (presence of an ovipositor) were selected and separated into single sex tanks to prevent spawning activity. During this 3-week acclimation period, the fish were held in a recirculation system and were acclimated to clean dechlorinated water at 25 ± 1 ^{o}C, with a 16 h light: 8 h dark photoperiod. The fish were fed adult Gamma irradiated frozen brine shrimp twice daily supplemented with Tetramin Tropical Flake food once daily, *ad libitum* during this period.

During pre- and exposure periods, the fish were fed with adult Gamma irradiated frozen brine shrimp (VTG Test fish were fed 50mg/g fish and Pair Breeding Test fish were fed 0.25 g/tank of 2 fish) once daily and were supplemented with Tetramin Tropical Flake food once daily.

2.2.2.3. Water supply and test apparatus

The supply of water to the flow-through laboratory dosing system was dechlorinated tap water (5 and 10 µm carbon-filtered). In all experiments, the tanks were gently aerated at the surface, using a glass pipette, to maintain dissolved oxygen concentrations at >70% of the air saturation value.

Water temperatures were monitored daily while pH levels were checked twice weekly. Ammonia, nitrite, and nitrate were monitored on a daily basis using test strips (Precision Laboratories, Moulton, UK). Dilution water and test chemical flow rates were checked twice weekly. During the tests, flow rates (40 ml/min for VTG Tests and 20 ml/min for Pair Breeding Tests) to the individual aquaria provided >75% replacement in a 12-hour period. (N.B. this flow rate was higher and the feed rate lower than that suggested in the Demonstration Programme protocol because in our own pilot studies, lower flow rates and higher feed rates as specified in the protocol caused accumulation of uneaten food in the aquaria and this led to elevated nitrite and ammonia levels and death of the fish).

For the VTG Test, 20 L tanks each held 8 male fish. For the Pair Breeding Test, one male and one female were housed in 8 L tanks together with a spawning chamber that consisted of a PVC half gutter (80 mm wide 110 mm diameter) on top of a glass tray (130 x 110 x 30 mm (length x width x depth)) containing a 0.5 cm^2 stainless steel mesh (Figure 10). This system allows collection of eggs, including those which have not adhered to the spawning surface.

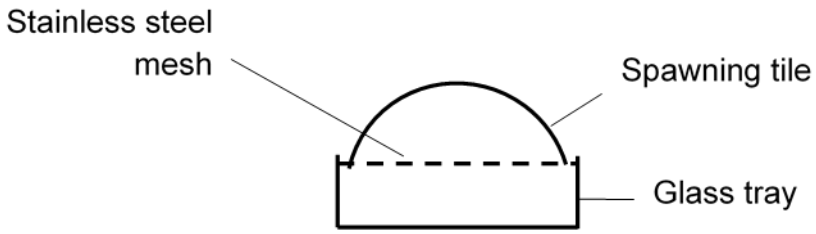


Figure 10. The spawning chamber; PVC half guttering inserted on top of a glass tray containing a stainless steel mesh.

2.2.2.4. Wastewater treatment works effluents

Batches of the standard effluent were delivered twice weekly (6 batches of effluent in total) whilst batches of GAC effluent were delivered once weekly (3 batches in total). All effluent batches were delivered using an industrial stainless steel tanker, collected at the WwTW and immediately transported to the testing facility. On arrival at Brunel University London (between 10.30-11.30 am), the effluent was transferred into a fully enclosed stainless steel holding tank and chilled to between 8 and 10 ^{o}C. The effluent was pumped via a peristaltic pump from the storage tank to the test aquaria via a glass holding tank, where it was first heated to 18 ^{o}C. Tank heaters were then used to raise the temperature in the test aquaria to 25 ^{o}C. The pH of each batch of effluent was checked on arrival. The pH values ranged from 7.8 to 8.0 and dissolved oxygen concentrations were above 80%. The effluent storage system was fully drained and flushed with water immediately prior to the delivery of each batch.

2.2.2.5. Oestrogen positive controls

For the oestrogen positive controls, EE2 (98% purity) was purchased from Sigma-Aldrich Company Ltd., Gillingham, UK. Solvent-free stock solutions were prepared twice a week by adding 1 mL of a concentrated stock solution of EE2 (prepared at 20 mg/L in high-performance liquid chromatography (HPLC) grade acetone; Fisher Scientific UK Ltd., Loughborough, UK) to a 10 L glass vessel. After evaporation of the acetone, 10 L of dilution water was added and the solution was stirred overnight on a magnetic stirrer. The solvent-free stock was then dosed to the glass mixing vessels, where it was mixed with the dilution water to provide a nominal test concentration of 10 ng/L (VTG tanks) and 20 ng/L (pair breeding tanks).

2.2.2.6. Measurement of oestrogenic activity and concentrations of steroid oestrogens

On days 7, 14 and 21, water samples were collected using silanised glassware and transferred to amber bottles containing preservative (Standing Committee of Analysts, 2008). From each VTG Test (Experiments 1 and 2) tank a total of 4.5 L water sample was collected, and a composite 4.5 L water sample was collected from the pair breeding tanks (Experiments 3 and 4). Each batch of effluent delivered to the laboratory was also sampled at the collection point in Swindon, on arrival at the lab and from the effluent storage tank at the end of each batch. All samples were immediately couriered to the analytical lab (Thames Water, Reading, UK) to be solid phase extracted on the day of collection followed by analytical determination of steroid oestrogens (E1, E2 and EE2 analysed by liquid chromatography with tandem mass spectrometry (LC-MS/MS); Standing Committee of Analysts, 2008). For the total oestrogenic activity (analysed by Centre for Environment,

Fisheries and Aquaculture Science, Weymouth, UK), the E2 equivalent concentration (E2EQ) was calculated using the YES (Thomas *et al.*, 2001).

2.2.2.7. Fish sampling

At the end of the study, all fish in all tanks were sacrificed according to UK Home Office regulations. Fish were anaesthetised with ethyl 3-aminobenzoate methanesulfonate salt (MS222; Sigma-Aldrich Company Ltd.) at 500 mg/L and buffered with 1M NaOH to pH 7.4. Whilst under anaesthetic, blood was collected from the caudal peduncle using heparinised capillary tubes and transferred to ice-cold 1.5 mL microfuge tubes (Alpha Laboratories, Eastleigh, UK), each containing one drop (~5 µL) of aprotinin (protease inhibitor; MP Biomedicals Germany GmbH, Eschwege, Germany). Following blood collection, the fish were killed by the destruction of the brain. Microfuge tubes were centrifuged (7000 × g; 5 min, 4 ^{o}C) and the plasma was removed and stored at −80 ^{o}C until required for analysis of VTG. Fork lengths and wet body weights of the fish were recorded to the nearest 1 mm and 0.01 g, respectively, and the condition factor derived by expressing the cube of the fork fish length as a function of the wet body weight. The gonads were removed, wet weighed to the nearest 0.001 g and the Gonadosomatic Index (GSI) derived by expressing the gonad weight as a percentage of the total body weight. The numbers of tubercles on the snout of each fish were recorded and the dorsal fatpad removed and wet weighed to the nearest 0.001 g.

2.2.2.8. Vitellogenin analysis

Plasma VTG concentrations were measured by using a commercially available FHM VTG ELISA kit (Biosense Laboratories AS, Bergen, Norway). The ELISA utilises specific binding between antibodies and VTG to quantify VTG in plasma samples from FHM. The manufacturer's protocol was followed and VTG in standards (0.05-50 ng/ml) and samples (diluted to 1:50, 1:5,000 and 1:500,000) were incubated for 1.5 hr at room temperature (20-25 ^{o}C) in microplate wells that come pre-coated with a specific capture antibody. Between this and all subsequent incubations, plates were washed with a phosphate buffered saline (PBS; pH 7.3) / 0.05% Tween-20 wash buffer using a WellWash (Thermo Scientific, ThermoFisher Scientific, Hemel Hempstead, UK). The plates were then incubated for 30 mins at room temperature, with a VTG-specific detecting antibody labelled with the enzyme horseradish peroxidase, that creates a sandwich of VTG and antibody. The enzyme activity was then determined by addition of a chromogenic substrate (3,3',5,5'-Tetramethylbenzidine) that gave a coloured product measured at 450 nm using a plate reader (Molecular Devices, San Jose, USA). As the colour intensity was directly proportional to the amount of VTG present, regression analysis using a log-log plot of the VTG standard data was used to

calculate the concentrations of VTG, after multiplying by the dilution factor to determine the concentration in the original samples.

In total 7 assays were carried out (5 assays standard effluent and 2 assays GAC effluent), and the DL was 5 ng/ml and the inter-assay variability was 17%.

2.2.2.9. Statistical analysis

Where concentrations were reported at below the DL, for statistical purposes half the DL was used in calculations. All biological results are expressed as a mean ± standard deviation. To investigate effects of the effluent exposure and of the positive control on body weight, length, GSI, SSCs and VTG concentrations, data were compared to the control using GraphPad Prism 8 version 8.0.1 (GraphPad Software, La Jolla California USA; www.graphpad.com). Data were tested using the D'Agostino and Pearson test, and where the assumptions of normality and homogeneity of variance were met, these were analysed using one-way ANOVA followed by Dunnett's multiple comparisons test (F value with degrees of freedom in subscript). Where n was too small or data failed to meet these assumptions, analysis was carried out using the Kruskal-Wallis test followed by Dunn's multiple comparisons test (H value with sample sizes (n)). To investigate the effects on reproductive activity, the mean cumulative distribution of the egg production data over the 21-day pre-exposure and exposure periods were compared for each treatment group using the Kolmogorov-Smirnov test (D value with sample sizes (n)). Differences were considered significant if the p values were ≤0.05 (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

2.2.3. Field studies – wild roach collected from the River Ray downstream of the Swindon WwTW

2.2.3.1. Site selection and fish sampling

In 2005, prior to the GAC installation, 145 mature roach were collected downstream of the Swindon WwTW. Sampling sites were chosen along a 3 km section of the River Ray downstream from the point of discharge, from National Grid Reference: SU124866 at Sparcells to SU122873 at Moredon Bridge (Figure 11). The Environment Agency's fisheries teams collected a total of 145 mature roach by electrofishing. Fish were collected mid-April to avoid the spawning season from late April to early June 2005. Fish were then taken to the Environment Agency's National Fisheries Laboratory, Brampton, UK, and fish scales, plasma, gonads and bile were collected for processing.

For the 2013 sampling, five years after the addition of the GAC treatment to the WwTW, two sites were chosen along the River Ray downstream from the point of discharge; Site 1 at National Grid Reference: SU124866 at Sparcells and Site 2 at SU122873 at Moredon Bridge

(Figure 11 and Figure 12). Power analysis indicated that a minimum sample size of 25 male fish (80% power) was required to determine if there was significant reduction in the incidence of intersex to baseline levels (0.5% of male fish; Geraudie *et al.*, 2010) following the installation of the GAC.

Mature roach were collected by electrofishing in collaboration with Hull International Fisheries Institute, University of Hull, UK (see Appendix A 5 and A 6). Twelve fish were collected from Site 1 and 44 from Site 2; a total of 56 fish. The fish were collected late November 2013 and were transported back to Brunel University London, Uxbridge, UK for processing.

For Home Offices purposes, the fish were anaesthetised with neutrally buffered MS222 (500 mg/L; Sigma-Aldrich Company Ltd.) and blood samples were collected from the caudal artery with a heparinised syringe. Following collection of the blood sample, the fish were killed by destruction of the brain. The blood was then transferred to Eppendorf tubes (Alpha Laboratories, Eastleigh, UK) containing one drop (~5 µL) of aprotinin (Sigma-Aldrich Company Ltd.), and samples were kept on ice until centrifugation at 12,000 g for 5 minutes. The plasma was pipetted to a fresh tube and immediately frozen on dry ice. The plasma samples were then transferred to -80 ^{o}C storage until ready for VTG analysis.

Fork length and wet weight were measured, and the condition factor was calculated by expressing the cube of the length as a percentage of the weight. Scale samples were taken from each fish for age determination.

For histopathological analysis and intersex determination, the gonads were dissected, and the macroscopic sex was determined by eye. The gonads were weighed, and the GSI was calculated by expressing the gonad weight as a percentage of the total weight less the gonad weight. The gonads were then fixed in Bouins (Sigma-Aldrich Company Ltd.) for 24 hours and then stored in 70% industrial methylated spirits (IMS) until ready for tissue processing (histopathology).

For the assessment of possible endocrine activity in the bile of wild roach, the gall bladder was also dissected, and these were placed in cryovials that was snap frozen in liquid nitrogen and stored at -80 ^{o}C until ready for bile analysis.

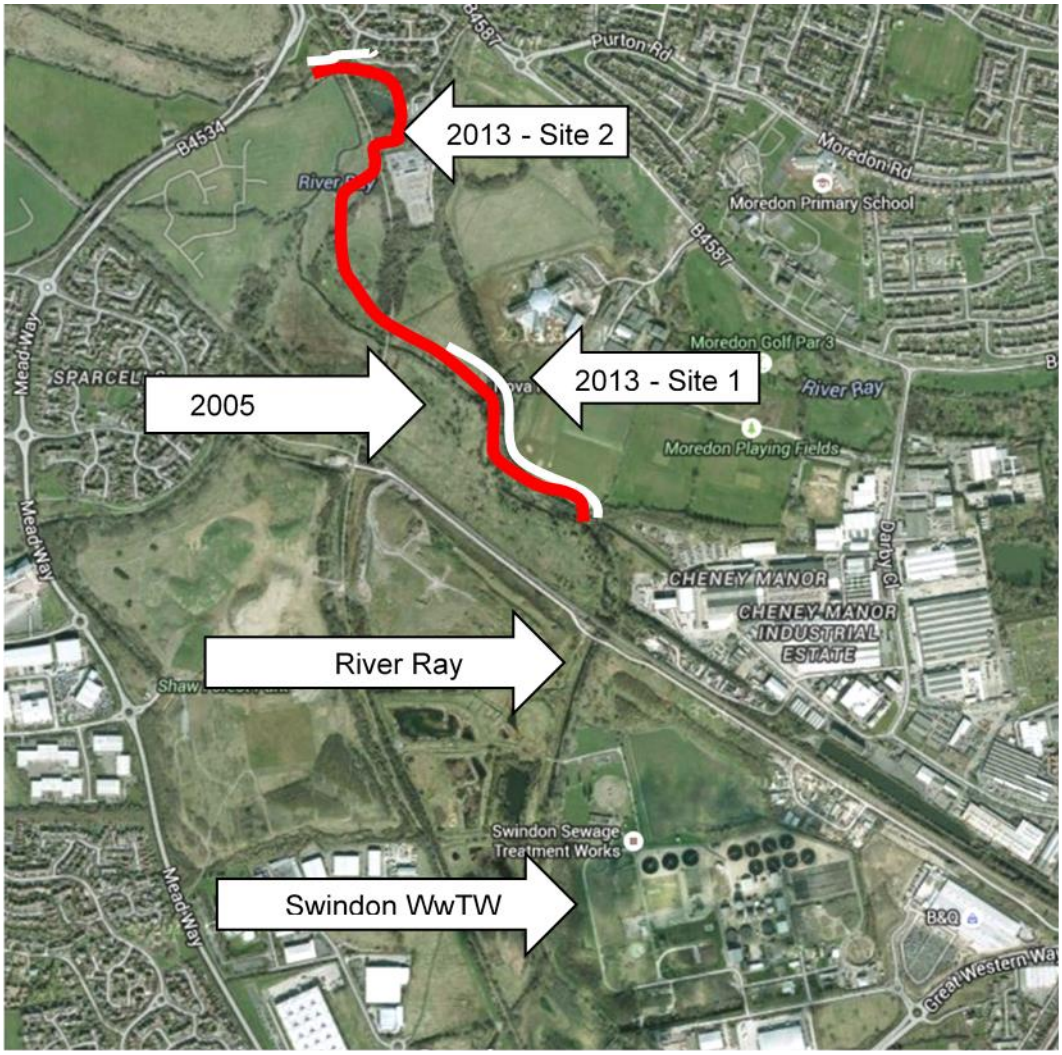


Figure 11. Locations of the 2005 and 2013 fish sampling sites along the River Ray together with the location of the Swindon Wastewater Treatment Works (WwTW). The 2005 sampling site is indicated by the red line and the two 2013 sites by the white lines. (Sourced from Google Maps)

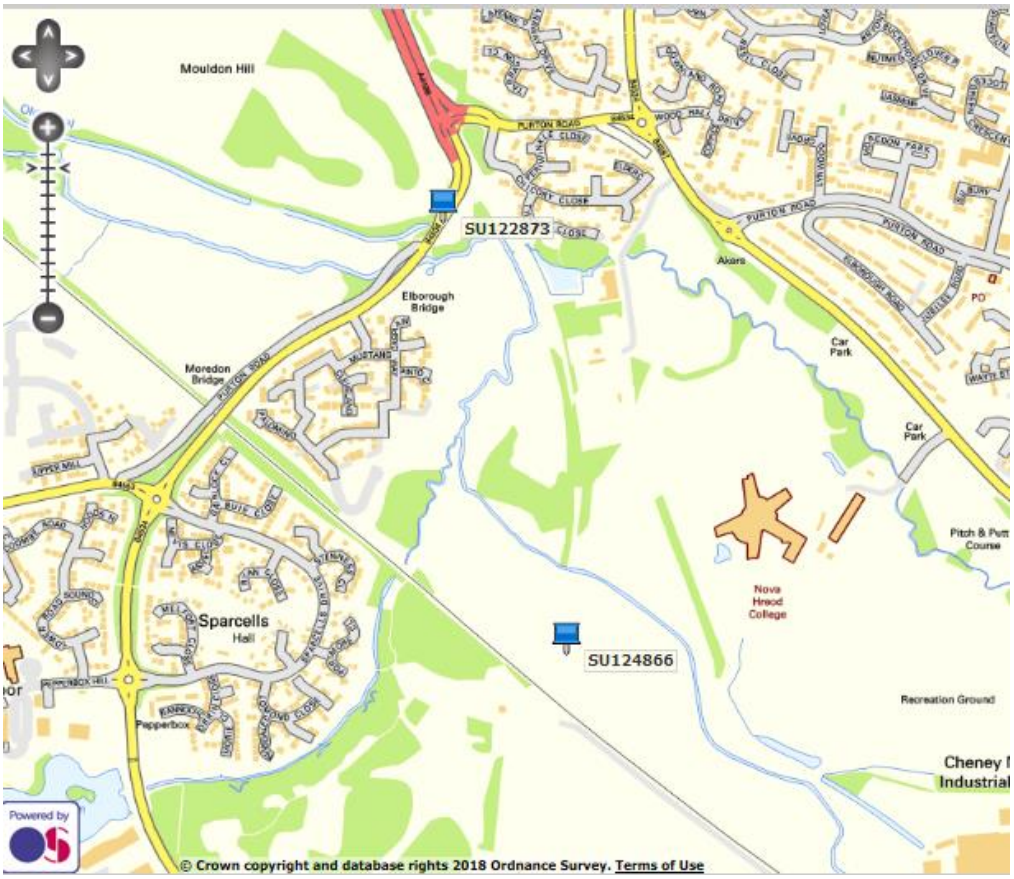


Figure 12. Locations of the two 2013 sampling sites; Site 1, National Grid Reference: SU124866, and Site 2, National Grid Reference: SU122873. (Sourced from Grid Reference Finder)

2.2.3.2. Analysis of fish material

2.2.3.2.1. Fish aging

The fish were aged by counting the number of annuli (winter checks where the circuli are closely spaced) on each fish scale using a microfiche reader (Mann, 1973).

2.2.3.2.2. Gonadal analyses

After histological fixation, each pair of ovaries or testes was cut into three parts (anterior, median and posterior) using a microtome blade (MB35, Thermo Scientific, ThermoFisher Scientific), and from each part a 3-5 mm portion of gonad was taken and placed into a biopsy cassette (Histosette II, Simport™, Fisher Scientific). The cassettes were then processed using a Leica tissue processor (model TP1020, Leica Biosystems (UK) Ltd., Milton Keynes, UK), to dehydrate the tissues and impregnate them with paraffin wax (W1, Thermo Scientific, ThermoFisher Scientific). The processor program was set with the following timings:

Step no.	Treatment	Purpose	Time (hours)
1	70% IMS	Dehydration	3
2	90% IMS	Dehydration	2.5
3	95% IMS	Dehydration	1.5
4	100% IMS	Dehydration	1.5
5	100% IMS	Dehydration	1.5
6	100% IMS	Dehydration	1.5
7	100% IMS	Dehydration	1.5
8	Histoclear	Clearing	1.5
9	Histoclear	Clearing	1.5
10	Histoclear	Clearing	1.5
11	Wax	Wax infiltration	1.25
12	Wax	Wax infiltration	1.25

Following completion of the processing, the tissue samples were embedded in wax blocks (total of 6 pieces of gonad tissue per fish). Wax embedded blocks were sectioned at 3 µm on a rotary microtome (model RM2235, Leica Biosystems (UK) Ltd.), floated out on a water

bath (Electrothermal, Cole Parmer, Stone, UK) and mounted on Polysine™ slides (Menzel Gläser, Thermo Scientific, ThermoFisher Scientific). The slides were dried overnight on a slide drying bench (Electrothermal), and were then stained with Gurr haematoxylin (VWR, East Grinstead, UK) and 1% eosin aqueous (Pioneer Research Chemicals Ltd., Colchester, UK) using an automated stainer (Stainmate, Thermo Scientific, ThermoFisher Scientific) with the following timings:

Step no.	Stain	Purpose	Time (mins)
1	Histoclear	Dissolves wax	15
2	100% IMS	Hydration	2
3	90% IMS	Hydration	2
4	70% IMS	Hydration	2
5	Tap water (running)	Rinse	2
6	Gurr haematoxylin	Stains cell nuclei blue	10
7	Tap water (running)	Remove excess	10
8	Acid / IMS	Dechlorination	20 secs
9	Tap water (running)	Rinse	20 secs
10	$LiCO_3$	Salt	20 secs
11	Tap water (running)	Rinse	20 secs
12	1% eosin (aqueous)	Stains cytoplasm pink	20 secs
13	Tap water (running)	Remove excess	5
14	70% IMS	Dehydration	2
15	90% IMS	Dehydration	2
16	100% IMS	Dehydration	5
17	Histoclear	Remove IMS, binding agent	5

Following staining, slides were sealed with Histomount (National Diagnostics, Hessle, UK) and a glass cover slip (22 x 50 mm, Menzel Gläser, Thermo Scientific, ThermoFisher Scientific).

For each fish sampled, all of the 6 sections were examined by light microscopy and the sex of each fish was determined (Nolan *et al.*, 2001). As several stages of gametogenesis coexist at a given time, the preponderant stage occurring inside the gonad was used to characterise the stage of development (Table 2).

For fish that looked predominantly male when the gametes were examined under light microscopy, the reproductive duct was also examined to see how many points of attachment there were; one point indicated a sperm duct (normal for males) whereas two points signifying an ovarian cavity terminating in an oviduct (normally only observed in females but also an indication of feminisation of male fish). The number of oocytes in the testes of intersex fish was counted microscopically, and each section was scored using an Intersex Index ranging from 0 (normal male testis) to 7 (100% ovarian tissue) (Jobling *et al.*, 2006) (Table 3). The Intersex Index scores for each of the 6 sections were then averaged for each fish.

Because the roach were sampled at different times of the year, the 2013 roach gonads were compared with roach caught from spring-fed Calverton fish farm in November, February, March and April to determine whether the gonadal maturity was as would be expected for the time of year of sampling.

Table 2. Scoring systems used to derive stage of maturity based on Billard (1986) for male fish and Tyler and Sumpter (1996) for female fish.

Sex	Stage	Cell type
Male	I	Spermatagonia only
	II	Mainly spermatagonia with <50% spermatocytes
	III	Some spermatids but >50% spermatocytes
	IV	Some spermatozoa but >50% spermatids
	V	>50% spermatozoa
	VI	Spent
Female	I	Oogonia only
	II	>50% primary oocytes (perinuclear and Balbiani body stage)
	III	Some primary oocytes but >50% cortical alveoli stage
	IV	>50% cortical alveoli and <50% vitellogenic
	V	>50% vitellogenic
	VI	Spent

Table 3. Scoring system used to determine the Intersex Index score for each section (from Jobling *et al.*, 2006).

Intersex Index Score	Description
0	Normal male testis
1	Multifocal ovotestis with 1–5 oocytes (usually singly) scattered among the testicular tissue
2	Multifocal ovotestis, 6–20 oocytes often in small clusters scattered among the testicular tissue
3	Multifocal ovotestis, 21–50 oocytes in clusters
4	>50 and <100 oocytes. Section is usually multifocal and has the appearance of a mosaic of testicular and ovarian tissue
5	>100 oocytes, usually multifocal but could also be focal with clearly identifiable zones of ovarian and testicular tissue separated from the testicular tissue
6	>50 per cent of the gonadal tissue on the section is ovarian and is clearly separated from the testicular tissue by epithelial cells and phagocytic tissues
7	100 per cent of gonadal tissue on the section is ovarian

2.2.3.2.3. Vitellogenin analysis

For the quantification of VTG in plasma samples collected in 2005, a carp VTG ELISA previously validated for use with roach (Tyler *et al.*, 1996) was utilised with a DL of 35 ng/ml VTG (Tyler *et al.*, 1999).

For the quantification of plasma samples collected in 2013, a commercial carp VTG ELISA kit based on the assay developed by Tyler *et al.* (1999) was utilised (Biosense Laboratories AS, Bergen, Norway). The manufacturer's protocol was followed and VTG in standards (0.24-250 ng/ml) and samples (diluted to 1:50, 1:5,000 and 1:500,000) were incubated with a series of antibodies and the enzyme activity was then determined by addition of a peroxidase substrate (o-phenylenediamine; OPD) that gave a coloured product measured at 492 nm using a plate reader. Plasma VTG concentrations were calculated as described in Section 2.2.2.8.

Two assays (male and female plasma in separate ELISAs) were run, both with a DL of 24.5 ng/ml VTG.

To determine comparability of VTG data between the two sampling occasions and methods, six plasma samples from the 2005 roach survey were reanalysed alongside the 2013 female plasma samples using the Biosense carp VTG ELISA kit. The Biosense commercial kit used to analyse the samples collected in 2013 was based on the pre-commercialisation method by Tyler *et al.* (1999) that was also used to analyse the samples collected in 2005. These six 2005 samples were chosen to reflect the range of concentrations recorded in 2005.

The 2013 male and female plasma samples were analysed at different times and therefore a subsample of nine male plasma samples from the 2013 sampling were also reanalysed along with the female plasma samples from the 2013 sampling in the same Biosense carp VTG ELISA assay.

2.2.3.2.4. Bile analysis

All chemicals were purchase from Sigma-Aldrich Company Ltd. unless stated otherwise.

2.2.3.2.4.1. Enzymatic hydrolysis of bile samples

For the analysis of the roach bile collected in 2005, the deconjugation, solid phase extraction and analysis for oestrogenic activity and anti-androgenic activity were carried out at the University of Sussex, Brighton, UK, according to Gibson *et al.* (2005). In brief, enzymatic hydrolysis of the samples used glucuronidase type VII-A from *E. coli* (1000 U/ml) and sulphatase type VI from *A. aerogenes* (2 U/ml). The activity of individual enzymes was tested with standard substrates; nitrophenol glucuronide and nitrophenol sulphate (10 µg substrate in 100 µl water). The hydrolysis took place in 0.1 M phosphate buffer (0.2 M sodium dihydrogen orthophosphate: 0.2 M disodium hydrogen orthophosphate: water (43.85:6.15:50.00, v/v/v) at pH 6.0) for 16 hours at 37 ^{o}C and the reactions were stopped with 90 µl glacial acetic acid.

Prior to analysing the bile samples in 2013, the efficiency of the glucuronidase type VII-A from *E. coli* and sulphatase type VI from *A. aerogenes* was rechecked using conjugated oestrogens; β-oestradiol 17-(β-D-glucuronide) sodium salt (E2-G; Santa Cruz Biotechnology, Inc, Dallas, USA) and β-oestradiol 3-sulphate sodium salt (E2-S). The deconjugation was specific for the glucuronidase but there was no deconjugation with the sulphatase (see Appendix Figure A 7). To rectify this, the method was modified by carrying out the enzymatic hydrolysis of the bile with β-glucuronidase type HP-2 from *H. pomatia* (Sigma-Aldrich Company Ltd.; ≥100,000 units/ml with ≤7,500 units/ml sulphatase). For the 2013 samples, the hydrolysis took place using 10 µl *H. pomatia* 'snail juice' in 990 µl 0.1M phosphate buffer for 16 hours at 37 ^{o}C, and the reaction was stopped with 90 µl glacial acetic acid. The deconjugation rate with the 'snail juice' was 103.2% (ranging from 101 to 107%) with the E2-G and 23.7% (ranging from 20.5 to 29.4%) with the E2-S.

2.2.3.2.4.2. Solid phase extraction

In 2005, OASIS HLB cartridges (6 cc, 150 mg; Waters, Milford, MA, USA) were used to extract hydrolysed bile extract. The hydrolysed bile was diluted with the addition of 2 ml HPLC grade water (3 ml total). Cartridges were primed with 5ml methanol (meOH) followed by 5 ml HPLC water acidified with 1% acetic acid and the samples were then loaded onto cartridges and washed through with 2 ml HPLC water. After drying the cartridges under vacuum for 30 mins, samples were eluted sequentially into glass conical tubes (Corning Life Sciences, Tewksbury, USA), using 5 ml meOH and 3 ml acetonitrile (HPLC grade solvents from Fisher Scientific). Samples were then evaporated under a flow of nitrogen, reconstituted in 500 µl ethanol (etOH), and stored at 4 ^{o}C until analysis using recombinant yeast screens.

For the 2013 samples, the same solid phase cartridges and method were followed although the cartridges were eluted with 5 ml meOH, 3 ml dichloromethane and 3 ml hexane (HPLC grade solvents from Fisher Scientific) to improve extraction efficiency of anti-androgens (Rostkowski *et al.*, 2011). The SPE recovery rate for the bile samples hydrolysed in 2013 was 76.3% (ranging from 70.7 to 79.9%) for the E2 spike and 67.3% (ranging from 64 to 71%) for the flutamide spike.

2.2.3.2.4.3. Recombinant yeast oestrogen and anti-androgen screens

The recombinant hER and hAR yeast strains were developed by Glaxo Wellcome. The standard YES and AYAS were used to determine the amount of oestrogenic and anti-androgenic activity in the bile samples collected in 2005, and have been described previously (YES, Routledge and Sumpter, 1996; AYAS, Sohoni and Sumpter, 1998).

These yeast assays were modified for analysis of the 2013 bile extracts, as any toxic activity in the sample can lead to false positive activity in the AYAS using the standard method (discussed further in Chapters 3 and 4).

2.2.3.2.4.4. Modified recombinant yeast oestrogen and anti-androgen screen procedure

2.2.3.2.4.4.1. Preparation of medium and buffers

10x Synthetic defined medium (10x SD-medium): The 10x SD-medium was prepared by dissolving 67 g yeast nitrogen base without amino acids (BD Difco, Wokingham, UK) and 200 g glucose in HPLC grade water and adjusting the final volume to 1 L. The solution was then sterilised by filtration (0.2 µm, cellulose acetate; Nalgene, Thermo Scientific, ThermoFisher Scientific) under sterile conditions and dispensed to polypropylene centrifuge tubes (Alpha Laboratories, Eastleigh, UK). The aliquots were stored at ≤ -18 ^{o}C for up to 12 months.

10x Dropout medium (10x DO-medium): The 10x DO-medium was prepared by adding 2000 mg L-Serine, 1000 mg L-Threonine, 750 mg L-Valine, 500 mg L-Leucine, 250 mg L-Phenylalanine, 150 mg L-Isoleucine, 150 mg L-Tyrosine, 100 mg Adenine, 100 mg L-Arginine, 500 mg L-Aspartic acid, 500 mg L-Glutamic acid, 100 mg L-Histidine-HCl, 150 mg L-Lysine-HCl, 100 mg L-Methionine to HPLC grade water and adjusting the final volume to 0.5L. The solution was then sterilised by filtration (0.2 µm, cellulose acetate) under sterile conditions and dispensed to polypropylene centrifuge tubes. The aliquots were stored at ≤ -18 ^{o}C for up to 12 months.

20 mM Copper (II) sulphate ($CuSO_4$): The $CuSO_4$ solution was prepared by adding 0.1596 g $CuSO_4$ to HPLC grade water and adjusting the final volume to 50 ml. The solution was then sterilised by passing through a syringe filter (0.2 µm, polyethersulfone; Whatman, GE Healthcare Life Sciences, Little Chalfont, UK) into a sterile glass bottle, under sterile conditions.

Growth medium: Under sterile conditions, 5 ml 10 x SD-medium and 5 ml 10 x DO-medium were added to a glass bottle containing 40 ml sterile HPLC grade water.

Exposure medium: The exposure medium was prepared on the day required by adding 125 µl CuSO4 solution to 50 ml of growth medium.

LacZ-buffer (Z-buffer): To 400 ml HPLC grade water, 8.05 g sodium phosphate dibasic, 2.75 g monosodium phosphate, 0.375 g potassium chloride and 0.123 g magnesium sulphate were added, and the pH was adjusted to 7.0. The buffer was made up to 0.5 L and autoclaved at 121 ^{o}C for 15 min.

10% SDS: 10 g sodium dodecyl sulphate was added to sterile HPLC grade water and stirred. This was made up to 100 ml and then transferred to a sterile glass bottle.

Assay buffer (quantities for 1 plate): Just prior to use, 24 mg ortho-Nitrophenyl-β-galactoside (oNPG) was dissolved in 11.9 ml Z-buffer. Once in solution, 32 µl 2-mercaptoethanol and 120 µl 10% SDS were added.

2.2.3.2.4.4.2. Assay procedure

Chemicals were serially diluted in etOH and 10 µl volumes were transferred to 96-well flat-bottom plates (Sarstedt AG & Co, Nümbrecht, Germany) where the etOH was allowed to evaporate to dryness. Then, 200 µl exposure medium containing yeast (final cell number of 5 x 10^5 cells/ml) was added to each well. Included in every assay was the negative control, etOH.

For the oestrogen screens, the positive control E2 was included in each assay (stock concentration at $2x10^{-7}$ M and serially diluted in etOH to achieve final concentrations from 10^{-8} M to $4.88x10^{-12}$ M).

The positive control, flutamide, was included in every AYAS (stock solution at 10^{-3} M and serially diluted in etOH to achieve final concentrations of 5×10^{-5} M to 2.44×10^{-8} M in the wells). For antagonistic screens, DHT at $2x10^{-9}$ M was also added to the medium prior to adding it to all wells.

The plates were taped closed and shaken for 2 mins on a plate shaker. The plates were then incubated at 32 ^{o}C for 68 ± 1 hours. After the incubation period, the yeast cells were resuspended by shaking for 2 mins and then 50 µl of suspension was transferred to a new plate containing 50 µl growth medium (i.e. diluted by half). The plates were then shaken again for 2 mins before taking pre-lysis plate readings at 620 nm to determine yeast turbidity.

The yeast cells were then lysed with 4 freeze/thaw cycles (4 mins on dry ice followed by 4 mins at 42 ^{o}C) and finally shaken for 2 mins. Freshly made assay buffer was then added to each plate (100 µl per well) by reverse pipetting and the plates were transferred to a 37 ^{o}C incubator. The β-galactosidase (β-gal) in the cell lysate then caused the clear medium to turn yellow and plate readings were taken at 420 nm and 620 nm after 30, 60 and 90 mins incubation. The 90 min readings were used for all analyses.

The half maximal effective concentration (EC50) was calculated using 4-parameter plot equations using SoftMax Pro version 5.0.1 (Molecular Devices Limited, Wokingham, UK); the concentration at which 50% of activity of the positive control, E2, was obtained. Similarly, the half maximal inhibitory concentration (IC50) was calculated as the concentration of flutamide required to produce a 50% inhibition of the DHT-induced activity.

Oestradiol equivalent (E2EQ) and flutamide equivalent (FLUTEQ) concentrations were calculated using 4-parameter plot equations after correcting for any dilution or concentration factors.

2.2.3.3. Statistical analysis

To determine the roach sample size, power analysis (CHI-squared test; GraphPad StatMate, San Diago, USA) using the 2005 intersex incidence data was employed.

Where concentrations were reported at below the DL, for statistical purposes half the DL was used in calculations. All biological results are expressed as a mean ± standard deviation. GraphPad Prism 8 was used for statistical analyses. Data were tested using the D'Agostino and Pearson test, and where assumptions of normality and homogeneity of

variance were met, the 2013 data was compared with the 2005 data using a t-test (t value with degrees of freedom in subscript). Where n was too small or data failed to meet these assumptions, data were compared with the 2005 data using the non-parametric Mann-Whitney Test (U value with sample sizes (n)). The proportions of intersex fish and females with uncharacteristically immature ovaries were compared using Fisher's exact test. Where VTG plasma samples were reanalysed at a later date, comparisons were made using the Wilcoxon matched-pairs signed rank test (Spearman correlation coefficient r_s with sample size (n)). Differences were considered significant if p values were ≤0.05 (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

2.3. Results

2.3.1. Laboratory studies - Fathead minnow flow-through exposure studies to WwTW effluents

Water quality parameters (ammonia, nitrite, and nitrate) were within acceptable ranges (see Appendix Tables A 1 - A 12). Tank water temperature, pH, and flow rates (dilution water and test chemical) were also within acceptable ranges (see Appendix Tables A 13 – A 16).

2.3.1.1. Oestrogenic content of the standard and GAC treated effluents within the test system over the course of the in vivo experiments

Both analytical chemistry (LC-MS/MS) measurements for E1, E2 and EE2 (the principal oestrogens prevalent in WwTW effluents), and *in vitro* analysis (E2EQ determined using the YES) for measurement of total oestrogenic activity were used to characterise the oestrogenic content of the effluent delivered to Brunel University London and in the tank water within the exposure system. The mean values derived from these measurements (± standard deviation) are summarised in Figure 13. The variation in oestrogenic content between the individual batches of effluent delivered to the laboratory and the persistence of the measured oestrogenic activity for each batch of effluent, both in transit and during the holding period, can also be seen in Table 4 (standard effluent) and Table 5 (GAC effluent). Whilst there was a variation in the concentrations of steroid oestrogens and in the oestrogenic activity both within (at time of collection, on arrival at Brunel University London, and at the end of the batch) and between batches of effluent, none were significant, indicating their stability within the test system. Unexpectedly, the GAC effluent exposure tanks had higher E2 and EE2 concentrations than the standard treated effluent exposure tanks.

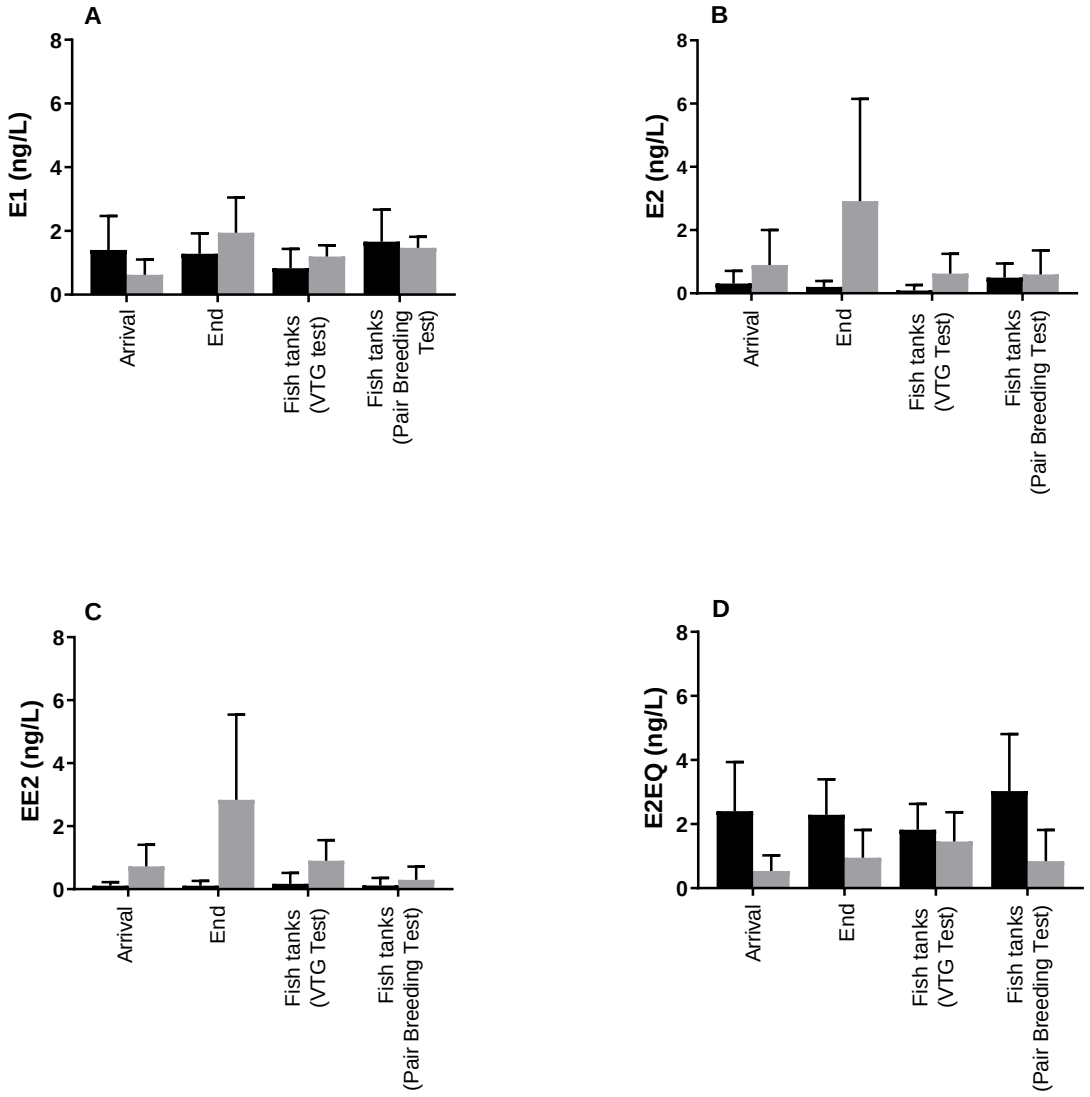


Figure 13. Measurements of steroid oestrogens taken to investigate how the concentrations in either standard treated effluent (black bars) or GAC treated effluent (grey bars) changed through the course of each short-term experiment. Mean concentrations of individual steroid oestrogens (E1, E2 and EE2, measured by LC-MS/MS) and total oestrogenic activity (E2EQ measured in the YES) measured on arrival at Brunel University London, at the end of the batch (taken from the stainless steel holding tank) and in the fish tanks (VTG Test and Pair Breeding Test) receiving 100% effluent. (A) E1, (B) E2, (C) EE2, and (D) E2EQ. For the standard effluent, n=6 on arrival and at end of batch and n=12 in fish tanks, and for the GAC effluent, n=3 on arrival and at end of batch and n=6 in fish tanks. Error bars represent mean ± standard deviation; none of the concentrations changed significantly through the course of the experiments.

Table 4. Stainless steel holding tank concentrations of E1, E2, EE2 and E2 equivalent concentrations (E2EQ) measured in batches of standard effluent on collection, arrival at the laboratory, and at the end of the batch (taken from the stainless steel holding tank).

Effluent Batch	Time Taken	E1 ng/L	E2 ng/L	EE2 ng/L	E2EQ ng/L
1	at collection	0.58	2.2	2.48	1.12
	on arrival	0.18	0.85	<0.04	3.07
	at end	2.37	0.31	<0.04	1.6
2	at collection	0.58	<0.03	1.08	0.58
	on arrival	2.52	0.05	<0.04	1.44
	at end	0.47	0.37	<0.04	3.89
3	at collection	<0.04	<0.03	<0.04	1.6
	on arrival	1.27	0.14	<0.04	5.06
	at end	1.44	0.08	0.37	1.25
4	at collection	1.97	1.19	0.93	0.51
	on arrival	1.29	<0.03	0.25	0.9
	at end	1.31	<0.03	0.23	2.31
5	at collection	6.31	<0.03	0.04	0.58
	on arrival	0.4	<0.03	0.11	1.38
	at end	0.83	0.43	<0.04	1.34
6	at collection	1.12	0.22	<0.04	2.12
	on arrival	2.77	0.8	0.24	2.57
	at end	1.26	<0.03	<0.04	3.34

Table 5. Stainless steel tank concentrations of E1, E2, EE2 and E2 equivalent concentrations (E2EQ) measured in batches of GAC treated effluent on collection, arrival at the laboratory, and at the end of the batch (in the stainless steel holding tank).

Effluent Batch	Time Taken	E1 ng/L	E2 ng/L	EE2 ng/L	E2EQ ng/L
1	at collection	<0.04	<0.03	<0.04	<0.5
	on arrival	0.61	2.14	0.61	<0.5
	at end	2.26	2.00	3.00	<0.5
2	at collection	<0.04	<0.03	<0.04	<0.5
	on arrival	1.11	0.54	1.46	<0.5
	at end	0.72	0.23	0.05	0.68
3	at collection	0.04	<0.03	0.19	<0.5
	on arrival	0.14	<0.03	0.11	0.52
	at end	2.86	6.51	5.46	1.92

The concentrations of steroid oestrogens and oestrogenic activity measured in the laboratory test systems are summarised in Figure 14 and below:

Oestrone and oestradiol:

The concentrations of the natural steroid oestrogens present in the control tanks were generally low but were often higher than their quantitation limits (0.03 ng/L for E2 and 0.04 ng/L for E1), probably due to excretion of these steroids into the water by the fish. Accordingly, the concentrations of these steroids were higher in the control tanks of the pair breeding systems (Experiments 3 and 4, containing males and females) than in the VTG Test system (Experiments 1 and 2, containing only males). In the standard effluent test system, the concentration of E1 in the VTG Test (Experiment 1) fish tanks with the 50% and 100% effluent were significantly greater than the control concentrations ($H=35.86$, $n_1=n_2=n_3=n_4=n_5=12$, $p=0.0086$ and <0.0001, respectively). Likewise, in the GAC effluent VTG Test system (Experiment 2), the concentration of E1 was significantly higher in the 100% effluent fish tanks than the control tanks ($H=12.06$, $n_1=n_2=n_3=6$, $p=0.0375$). In the standard effluent test system, the concentration of E1 in the Pair Breeding Test (Experiment 3) fish tanks with 100% effluent was significantly greater than the control concentrations ($H=20.55$, $n_1=n_2=n_3=n_4=n_5=6$, $p=0.0077$). There were no statistical differences in the E2 concentrations measured in any of the fish treatment tanks.

Ethinylestradiol:

The concentrations of EE2 measured in the control tanks were, as expected, close to or at the limit of quantitation (0.04 ng/L) in each experiment. For the positive control tanks, the amounts of EE2 measured were approximately half the nominal concentrations (nominal 20 ng/L EE2 for the Pair Breeding Test and 10 ng/L for the VTG Test). In the standard effluent and in the GAC effluent, EE2 concentrations were extremely low (the standard effluent mean was 0.17 ± 0.35 ng/L EE2 in the VTG Test (Experiment 1) and 0.12 ± 0.24 ng/L in the Pair Breeding Test (Experiment 3); the GAC mean was 0.9 ± 0.66 ng/L EE2 in the VTG Test (Experiment 2) and 0.30 ± 0.42 ng/L in the Pair Breeding Test (Experiment 4)) and although the effluent EE2 concentrations were higher than in the control tanks (ranging from 0.03 to 0.06 ng/L EE2), this was not statistically significant due to the variability in the concentrations of EE2 between different batches of effluent.

2.3.1.2. Biological effects of effluent exposure under laboratory test conditions

2.3.1.2.1. Experiments 1 and 2 Fathead minnow Vitellogenin Test

There was no evidence that exposure for up to 21 days to the WwTW effluents or the oestrogen positive control affected survival (see Appendix Tables A 17 and A 18).

Biological measurements

The size (length and weight) of the male FHM were not significantly different following exposure to EE2 and effluent in both of the VTG Tests (standard or GAC; Figure 15). Compared with the control fish, there was no significant difference between the condition factor of the male FHM following exposure to EE2 and effluent in both VTG Tests (Experiment 1 standard and Experiment 2 GAC effluent; Figure 16).

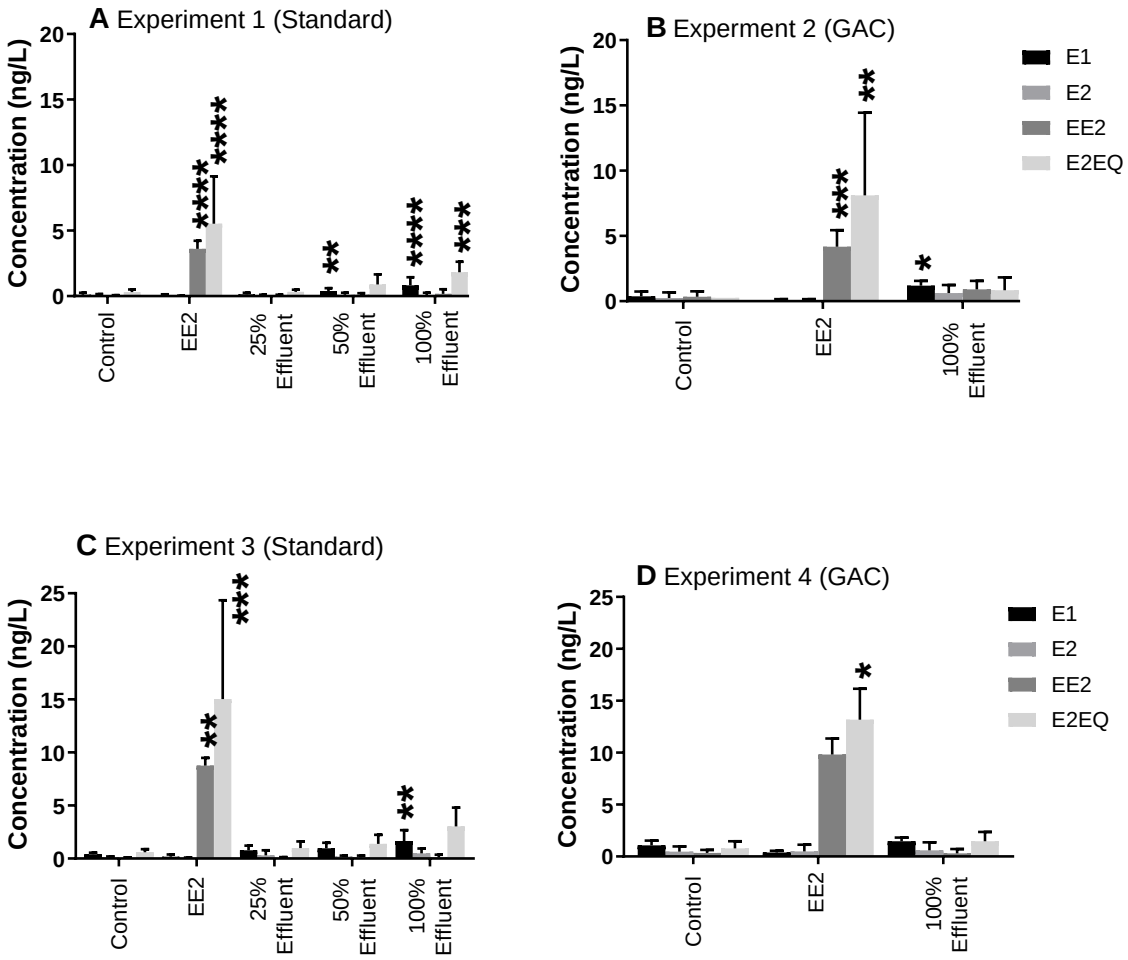


Figure 14. Mean concentrations of individual steroid oestrogens (E1, E2 and EE2, measured by LC-MS/MS) and oestrogenic activity (E2EQ measured in the YES) in the fish tanks (± standard deviation). (A) VTG Test with standard effluent (n=12), (B) VTG Test with GAC treated effluent (n=6), (C) Pair Breeding Test with standard effluent (n=6), and (D) Pair Breeding Test with GAC treated effluent (n=3). The bars represent mean values ± standard deviation. Statistically different from control values; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

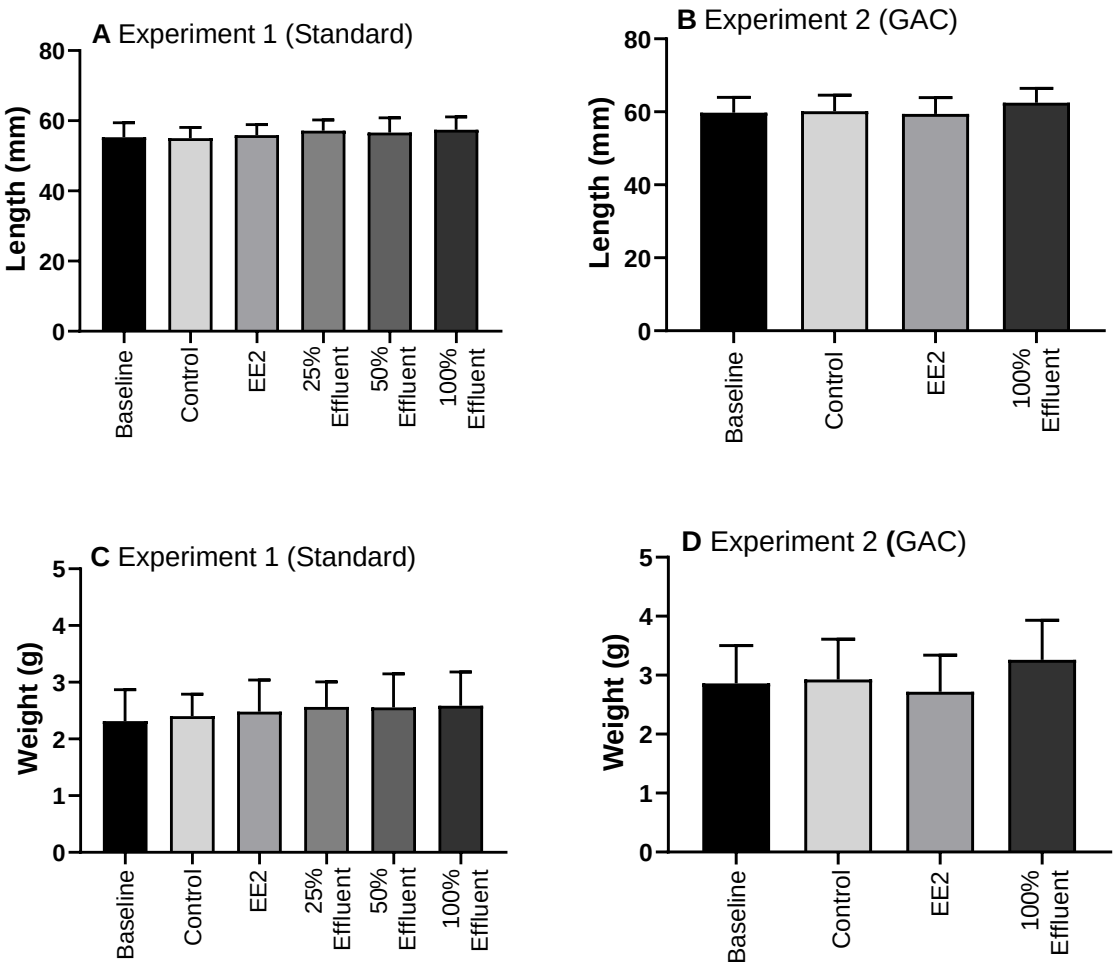


Figure 15. Lengths and weights of male fathead minnows measured at the end of the baseline period and after 21 days exposed in the VTG Test to the standard effluent (A, lengths; C, weights) and GAC treated effluent (B, lengths; D, weights). During the exposure period male fathead minnows (n=8 males in each of 2 replicate tanks) were exposed to effluent, or to the negative (control) or positive (10 ng EE2/L) control. The bars represent mean values ± standard deviation. None of the treatments produced a significant effect compared with the control values.

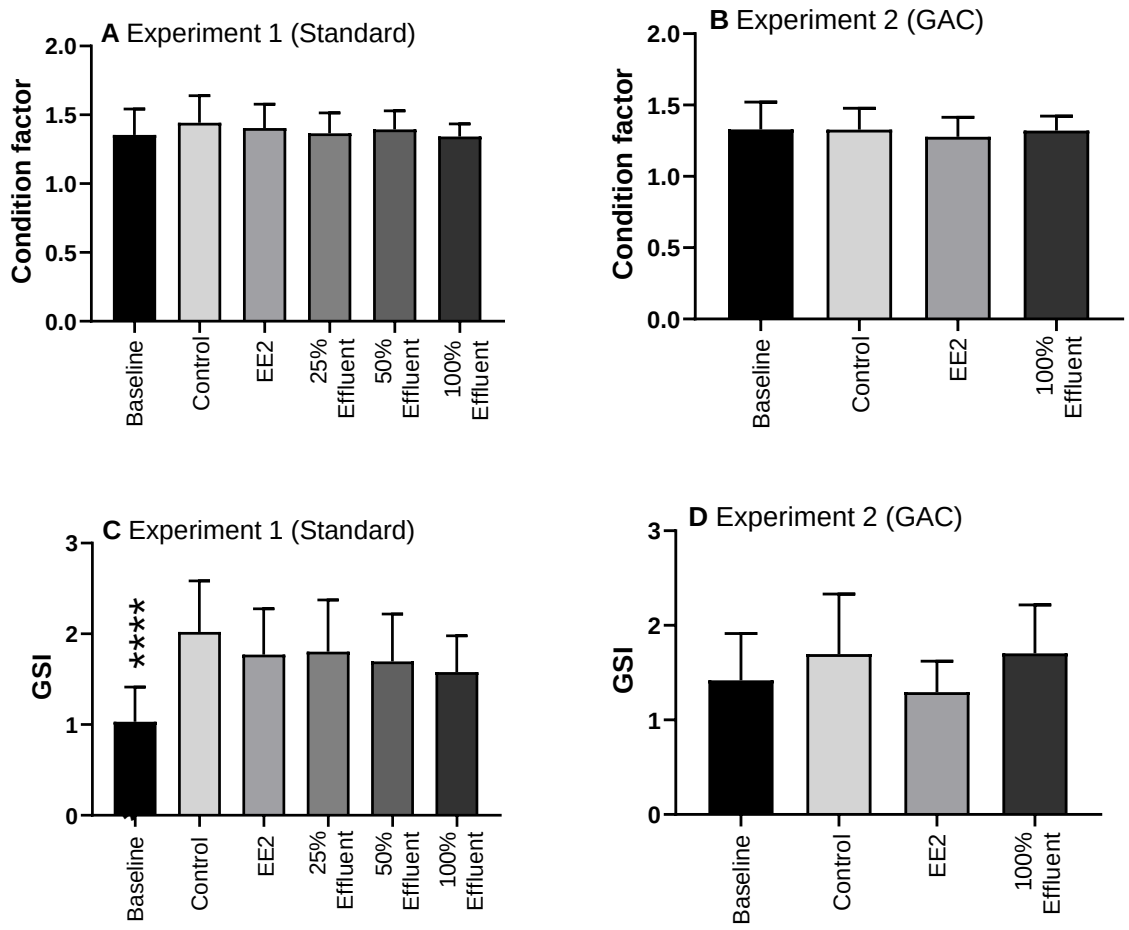


Figure 16. Condition factor and Gonadosomatic Index (GSI) of male fathead minnows measured at the end of the baseline period and after 21 days exposed in the VTG Test to the standard effluent (A, condition factor; C, GSI) and GAC treated effluent (B, condition factor; D, GSI). During the exposure period male fathead minnows (n=8 males in each of 2 replicate tanks) were exposed to effluent, or to the negative (control) or positive (10 ng EE2/L) control. The bars represent mean values ± standard deviation. Statistically different from control values; ****p<0.0001.

In the VTG Tests, the control GSIs were higher than the baseline GSI values and this was particularly apparent for Experiment 1 (standard effluent, $F_{5,90}$=7.338, p<0.0001; Figure 16C) where the baseline values were lower than for Experiment 2.

Male plasma VTG

There was clear evidence of increases in plasma VTG, of five orders of magnitude relative to the controls, in the fish exposed to the positive control EE2 (Figure 17A; H=44.51, $n_1=n_2=n_3=n_4=n_5=n_6$=16, p<0.0001, and Figure 17B; H=35.93, $n_1=n_2=n_3=n_4$=16, p<0.0001). However, there were no significant differences in the VTG concentrations in any of the standard or GAC effluent exposed fish, compared to the controls.

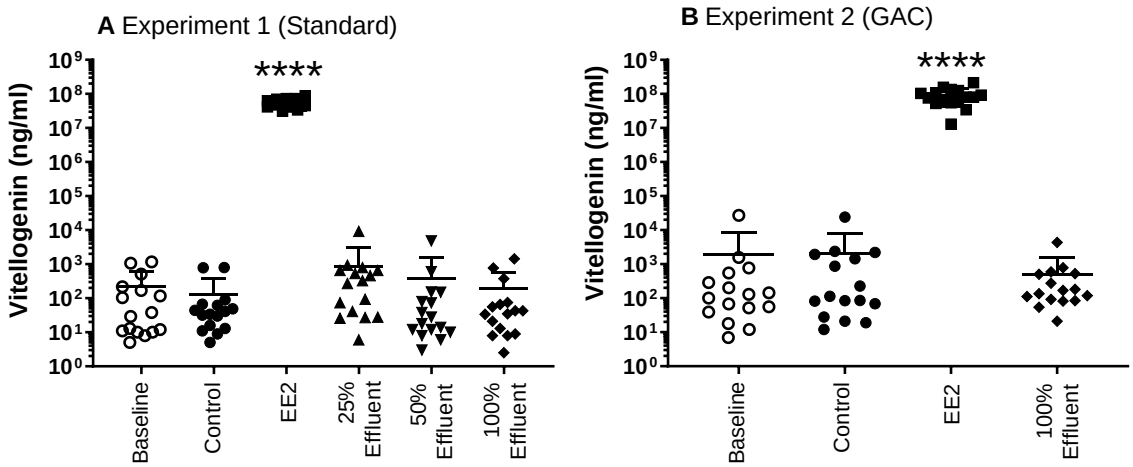


Figure 17. Vitellogenin concentrations measured in the plasma of male fathead minnows at the end of the baseline period and after 21 days exposed in the VTG Test to the standard effluent (A) or GAC treated effluent (B). During the exposure period male fathead minnows (n=8 males in each of 2 replicate tanks) were exposed to effluent, or to the negative (control) or positive (10 ng EE2/L) control. The scatter dot plots show individual values and the whiskers represent the mean VTG concentration ± standard deviation. Statistically different from control values; ****p<0.0001.

Male Fatpad Index

The baseline male FHM sampled just before the start of the exposure period had a significantly lower FPI (mean FPI 0.28) than the control fish (mean FPI 2.37) sampled at the end of the 21-day exposure in the VTG Test with standard effluent (H=37.66,

$n_1=n_2=n_3=n_4=n_5=n_6=16$, $p<0.0001$; Experiment 1; Figure 18A). There was no significant difference in the FPI between the baseline FHM and the control fish in the GAC VTG Test (Figure 18B), but in this experiment the baseline FPI was higher at the start of the exposure period (FPI 0.87).

The FPI was also affected by exposure to the standard treated effluent; a significant suppression of the growth of the fatpad was seen in the both the 50% and 100% effluent treatments relative to the controls ($H=37.66$, $n_1=n_2=n_3=n_4=n_5=n_6=16$, $p=0.0238$ and 0.0009, respectively). No effects on the FPI were seen in the fish exposed to EE2 (Experiment 1 and 2) or the GAC effluent (Experiment 2).

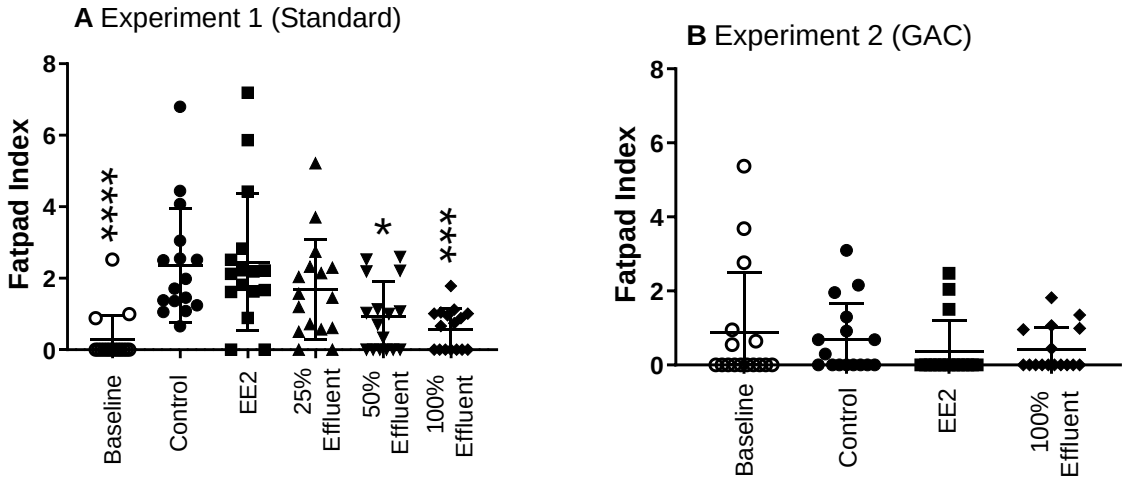


Figure 18. Mean Fatpad Indices (FPI) measured in male fathead minnows at the end of the baseline period and after 21 days exposed in the VTG Test to the standard effluent (A) and GAC treated effluent (B). During the exposure period male fathead minnows (n=8 males in each of 2 replicate tanks) were exposed to effluent, or to the negative (control) or positive (10 ng EE2/L) control. The scatter dot plots show individual values and the whiskers represent the mean FPI ± standard deviation. Statistically different from control values; *p<0.05, ***p<0.001, ****p<0.0001.

Male tubercle number

No statistical differences were seen with male tubercle numbers in any of the fish in the VTG Test with standard effluent (Experiment 1; Figure 19A). In the VTG Test with GAC effluent (Experiment 2; Figure 19B), the control fish (sampled at the end of the 21-day exposure) exposed to dilution water had significantly more tubercles (tubercle number 11.25, $F_{3,60}$=9.469, p=0.0027) compared with the baseline fish sampled at the start of the exposure period (tubercle number 3.88). In addition, there was a significant suppression in male tubercle development with EE2 exposure in Experiment 2 compared with the control fish ($F_{3,60}$=9.469, p=0.0039). No significant differences were seen with male tubercle number with the fish exposed to the GAC effluent.

For raw data for both VTG Tests (standard and GAC effluent) see Appendix Tables A 17 and A 18.

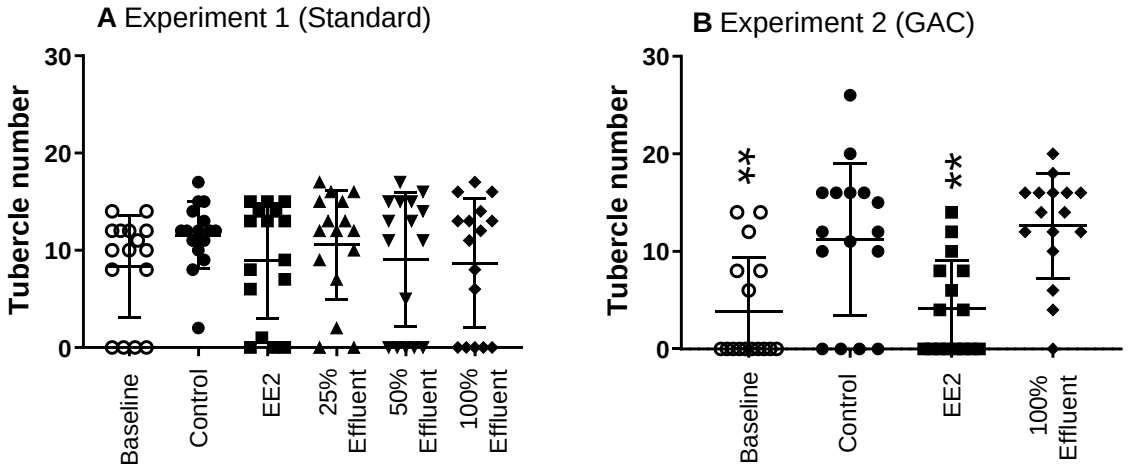


Figure 19. Mean tubercle number measured in male fathead minnows at the end of the baseline period and after 21 days exposed in the VTG Test to the standard effluent (A) and GAC treated effluent (B). During the exposure period male fathead minnows (n=8 males in each of 2 replicate tanks) were exposed to effluent, or to the negative (control) or positive (10 ng EE2/L) control. The scatter dot plots show individual values and the whiskers represent mean tubercle number ± standard deviation. Statistically different from control values; **p<0.01.

2.3.1.2.2. Experiments 3 and 4 Fathead minnow Pair Breeding Test

In Experiment 3 (standard effluent exposure), three male fish died two days prior to the end of the trial and so the remaining EE2 treatment fish were sacrificed two days early to ensure no further deaths. Postmortem examination revealed kidney failure due to over production of VTG. High VTG concentrations have previously been linked with renal failure and elevated mortality (Herman and Kincaid, 1988). During the course of Experiment 3, an additional 4 fish died (control tanks, 1 male and 1 female FHM; 50% effluent tanks, 1 male and 1 female FHM; see Appendix Tables A 19 and A 20). No fish died during the course of Experiment 4 (see Appendix Tables A 21 and A 22).

Biological measurements

The fish length (Figure 20) of both male and female FHM were not significantly different from the control fish following exposure to EE2 and effluent, in both of the Pair Breeding Tests (standard and GAC effluent).

The fish weights (Figure 21) were not significantly different from the control fish for both male and female fish exposed in the Pair Breeding Test with standard effluent (Experiment 3) and also for the female fish exposed to GAC effluent (Experiment 4). However, the male fish exposed to EE2 in the GAC Pair Breeding Test (Experiment 4) did weigh significantly less than the control male fish ($F_{2,21}$=3.454, p=0.0302).

Compared with the control fish, there was no significant difference between the condition factor of the both male and female FHM following exposure to EE2 and effluent in both of the Pair Breeding Tests (Experiment 3 standard effluent and Experiment 4 GAC effluent; Figure 22). Similarly, the GSI values for male and female FHM exposed in both of the Pair Breeding Tests (standard and GAC effluent) were not significantly different from control fish values (Figure 23).

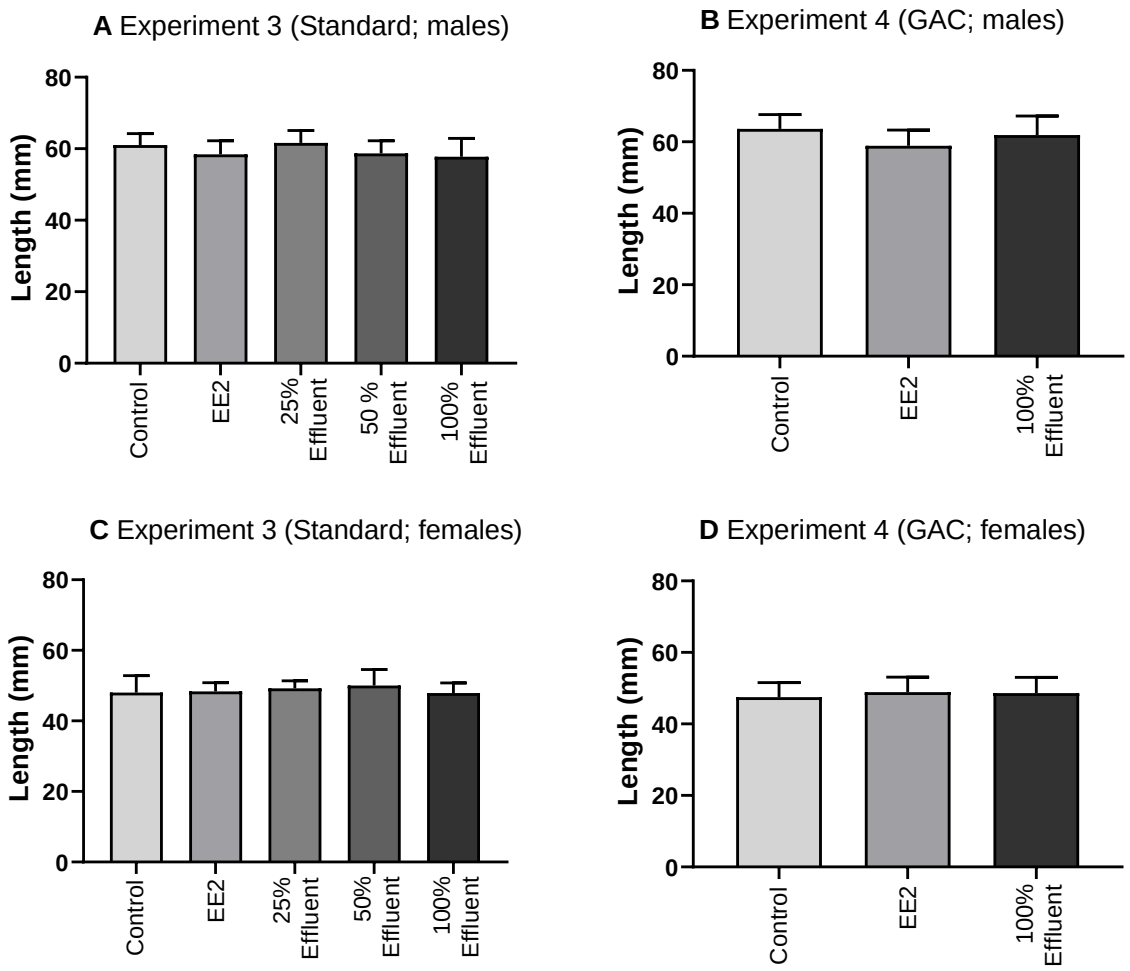


Figure 20. Lengths of male and female fathead minnows measured after being exposed in the Pair Breeding Test for 21 days to the standard effluent (A, males; C, females) and GAC treated effluent (B, males; D, females). During the exposure period 8 replicate pairs of fathead minnows were exposed to effluent, or to the negative (control) or positive control (+ve control; 20 ng EE2/L). The bars represent mean values ± standard deviation. None of the treatments produced a significant effect compared with the control values.

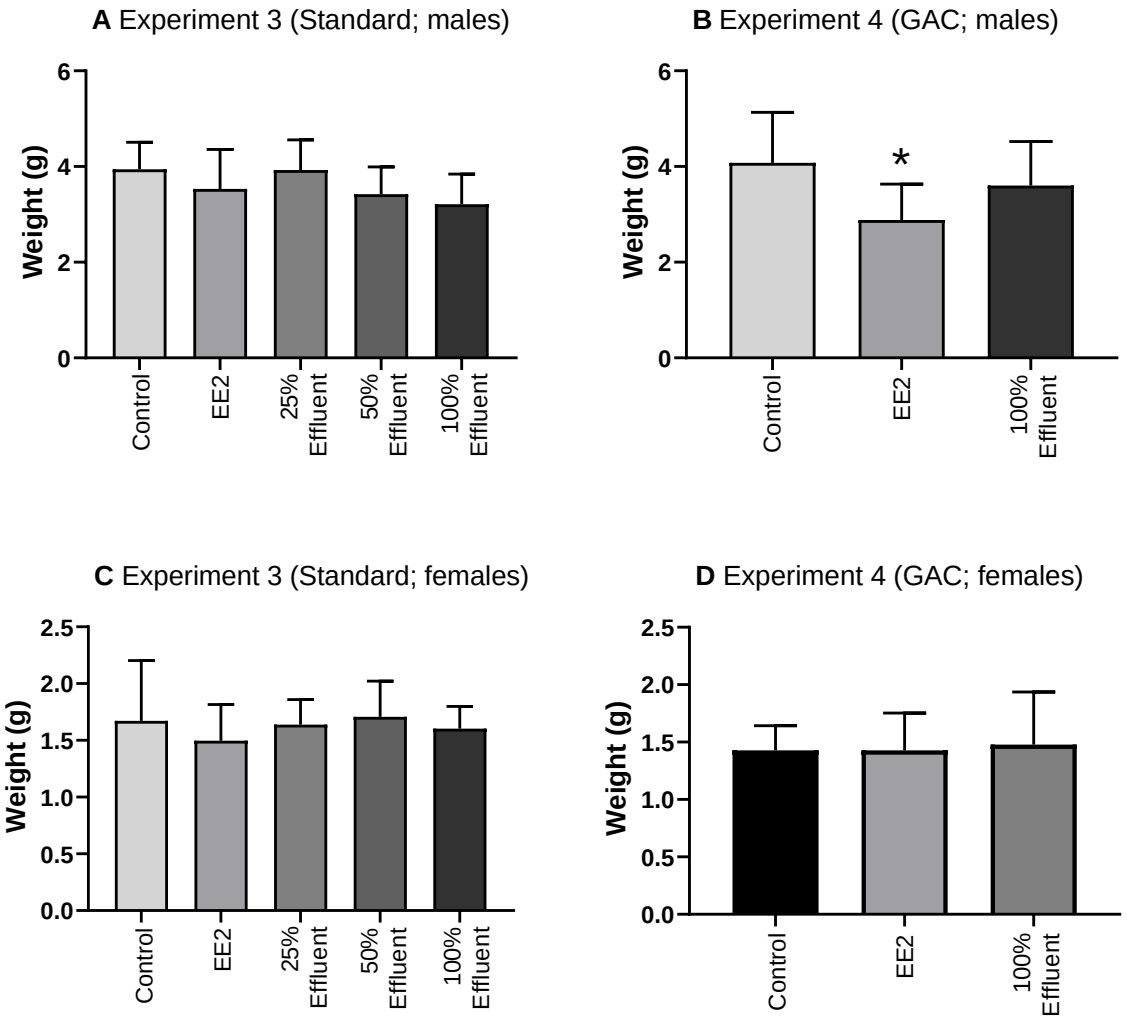


Figure 21. Weights of male and female fathead minnows measured after being exposed in the Pair Breeding Test for 21 days to the standard effluent (A, males; C, females) and GAC treated effluent (B, males; D, females). During the exposure period 8 replicate pairs of fathead minnows were exposed to effluent, or to the negative (control) or positive control (+ve control; 20 ng EE2/L). The bars represent mean values ± standard deviation. Statistically different from control values; *p<0.05.

Plasma vitellogenin

As expected, there was a significant induction of VTG in the EE2-treated male fish compared with the control male fish for both the Pair Breeding Tests (Experiment 3 standard effluent; $H=18.98$, $n_1=7, n_2=7, n_3=8, n_4=7, n_5=8$, $p=0.0002$; Figure 24A, and Experiment 4 GAC; $H=16.21$, $n_1=n_2=n_3=8$, $p=0.0002$; Figure 24B). There was no significant difference between

the male FHM control VTG concentrations and any of the effluent-exposed male fish for both the standard and GAC effluent Pair Breeding Tests.

Similarly, for the female fish, there were also no significant differences between the control VTG concentrations and the values for the fish exposed to standard (Figure 24C) and GAC effluent (Figure 24D). Also, there was no significant difference between the EE2 exposed female FHM (9,641,076 ± 8,834,021 ng/ml VTG) compared with the control female FHM (5,989,596 ± 2,051,597 ng/ml VTG) in the standard effluent Pair Breeding Test (Experiment 3). However, the EE2 exposed female FHM in the GAC Pair Breeding Study (Experiment 4) had significantly elevated VTG concentrations (approximately 10-fold higher) compared with the control female FHMs (61,276,798 ± 57,547,003 ng/ml VTG compared with 5,675,081 ± 1,707,044 ng/ml VTG; $H=7.980$, $n_1=n_2=n_3=8$, $p=0.0218$).

Fatpad Index

The FPIs were not significantly different from the control values for male fish exposed in the Pair Breeding Test with standard effluent (Experiment 3; Figure 25A) and also for the male fish exposed to GAC effluent (Experiment 4; Figure 25B).

Tubercle number

The tubercle numbers for the male fish exposed in both the standard (Experiment 3) and GAC effluent Pair Breeding Tests (Experiment 4) were not significantly different from the values for the control FHM (Figure 26).

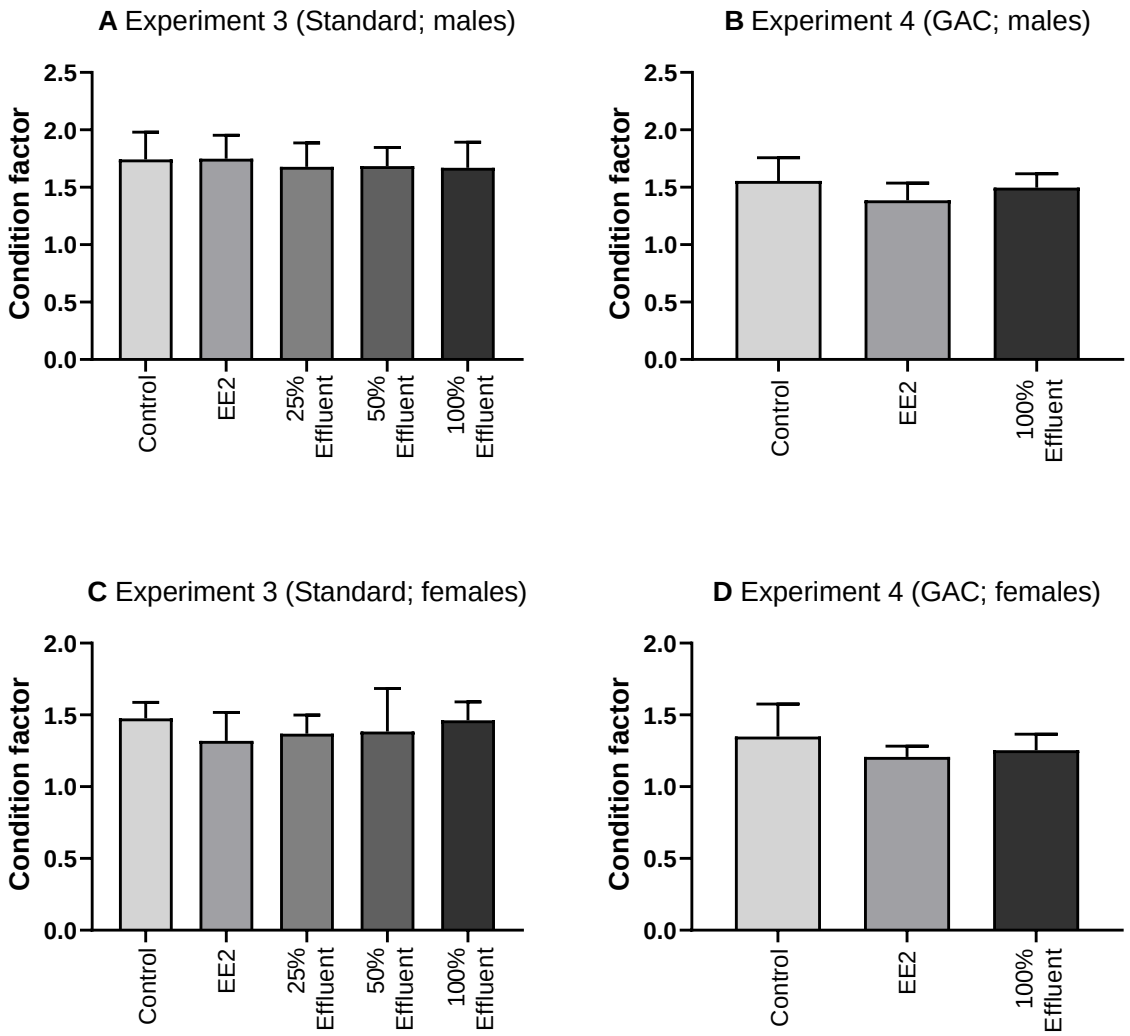


Figure 22. Condition factor of male and female fathead minnows measured after being exposed in the Pair Breeding Test for 21 days to the standard effluent (A, males; C, females) and GAC treated effluent (B, males; D, females). During the exposure period 8 replicate pairs of fathead minnows were exposed to effluent, or to the negative (control) or positive control (+ve control; 20 ng EE2/L). The bars represent mean values ± standard deviation. None of the treatments produced a significant effect compared with the control values.

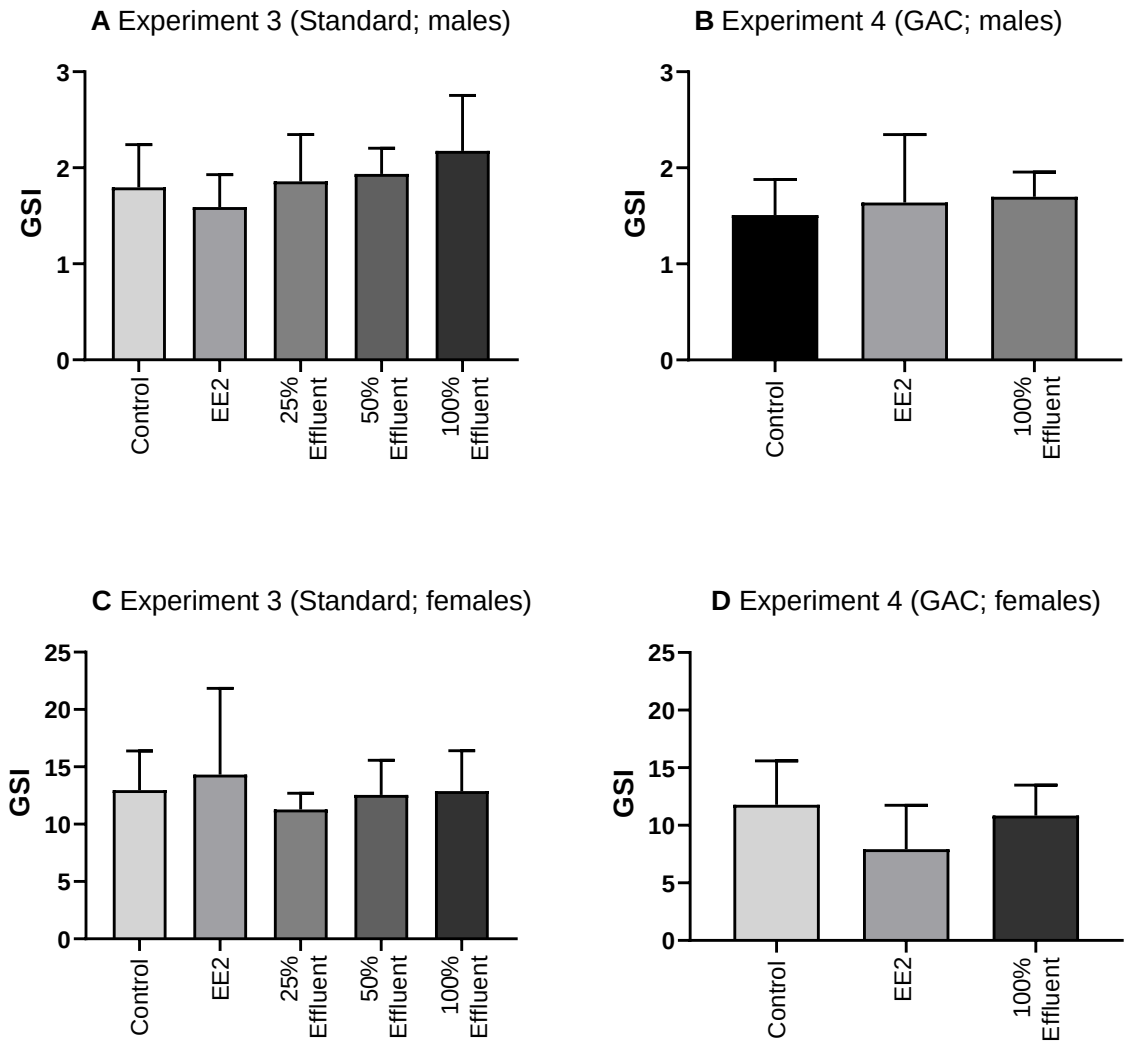


Figure 23. Gonadosomatic Index (GSI) of male and female fathead minnows measured after being exposed in the Pair Breeding Test for 21 days to the standard effluent (A, males; C, females) and GAC treated effluent (B, males; D, females). During the exposure period 8 replicate pairs of fathead minnows were exposed to effluent, or to the negative (control) or positive control (+ve control; 20 ng EE2/L). The bars represent mean values ± standard deviation. None of the treatments produced a significant effect compared with the control values.

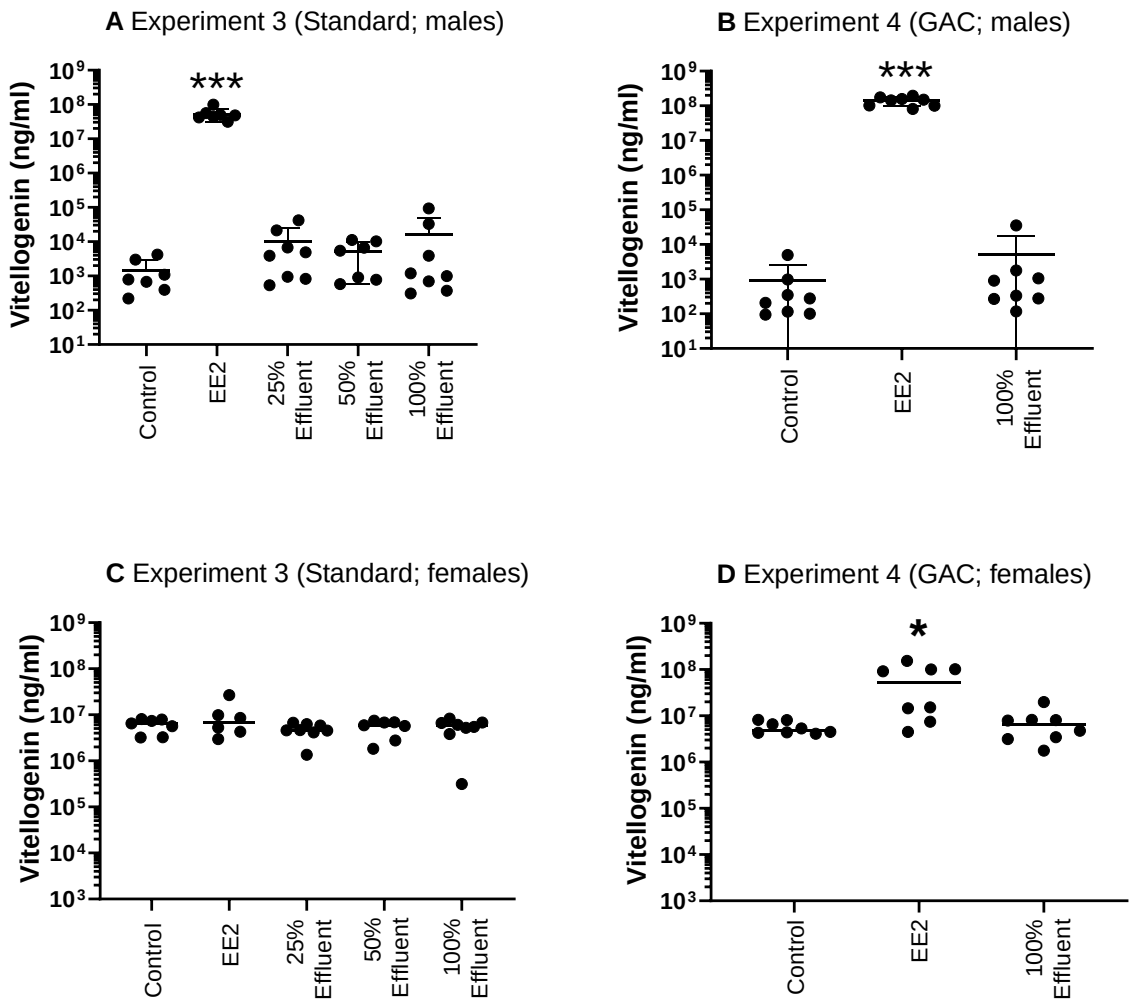


Figure 24. Vitellogenin concentrations measured in the plasma of fathead minnows after 21 days in the Pair Breeding Test exposed to the standard effluent (A; males, C; females) and GAC treated effluent (B; males, D; females). During the exposure period 8 replicate pairs of fathead minnows were exposed to effluent, or to the negative (control) or positive control (+ve control; 20 ng EE2/L). The scatter dot plots show individual values and the whiskers represent mean VTG concentration ± standard deviation. Statistically different from control values; *p<0.05, ***p<0.001.

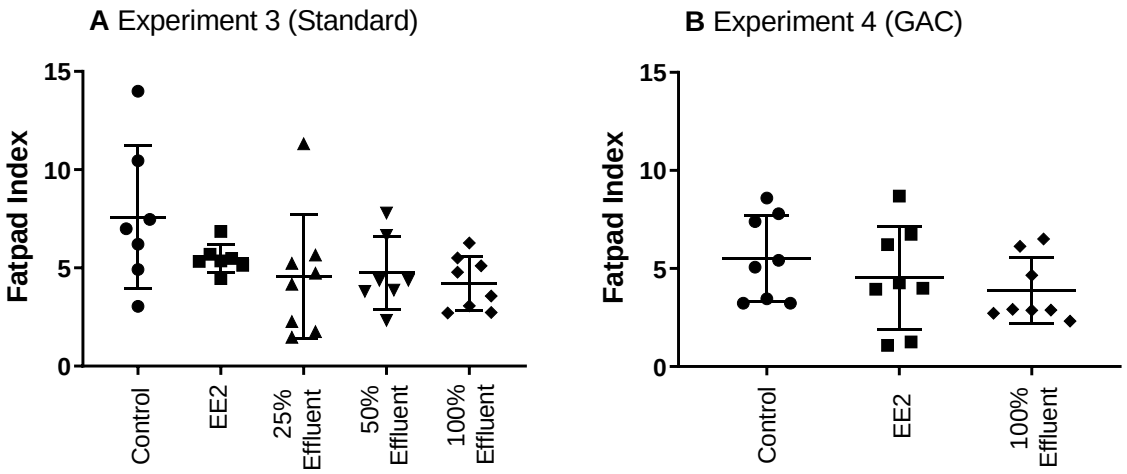


Figure 25. Mean Fatpad Indices (FPI) measured in male fathead minnows after 21 days in the Pair Breeding Test in which they were exposed to the standard effluent (A) and GAC treated effluent (B). During the exposure period 8 replicate pairs of fathead minnows were exposed to effluent or to the negative (control) or positive control (+ve control; 20 ng EE2/L). The scatter dot plots show individual values and the whiskers represent mean FPI ± standard deviation. None of the treatments produced a significant effect compared with the control values.

Egg counts

In the Pair Breeding Test with the standard effluent (Experiment 3), there were reductions in mean cumulative egg production in the exposure period relative to the pre-exposure period for pairs of fish in all treatments except for the 25% standard effluent treatment (Control, 45.4% reduction, D=0.3810, n=21, p=0.0949; 25% effluent, 2.7% increase, D=0.1905, n=21, p=0.8407; 50% effluent, 32% reduction, D=0.1905, n=21, p=0.8407; 100% effluent, 33.6% reduction, D=0.3333, n=21, p=0.1938; Figure 27). Only in the positive control treatment (20 ng/L EE2), however, was the reduction in egg production statistically different from that seen before the start of the exposure (63.2% reduction, D=0.4662, n=19, p=0.0262).

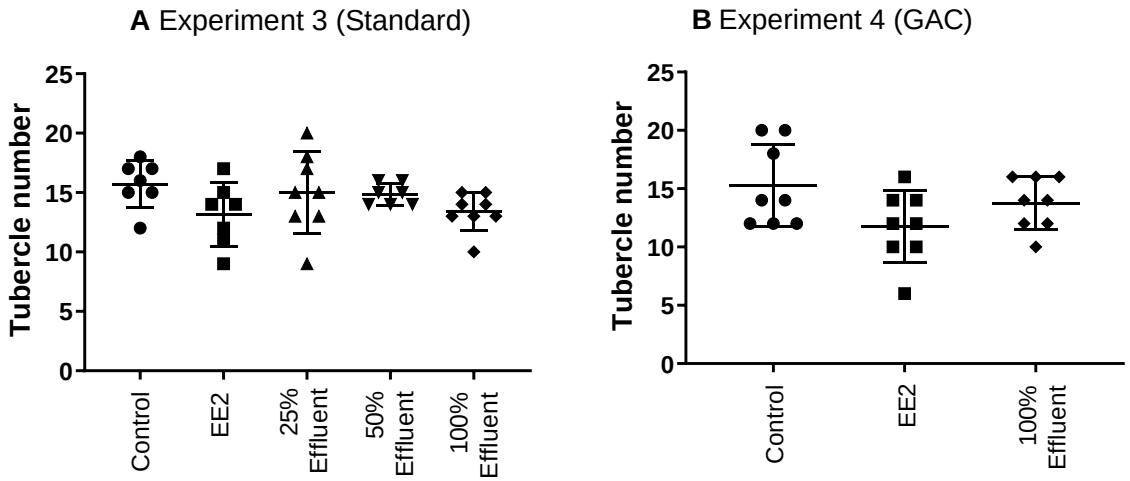


Figure 26. Mean tubercle number measured in male fathead minnows after 21 days in the Pair Breeding Test in which they were exposed to the standard effluent (A) and GAC treated effluent (B). During the exposure period 8 replicate pairs of fathead minnows were exposed to effluent, or to the negative (control) or positive control (+ve control; 20 ng EE2/L). The scatter dot plots show individual values and the whiskers represent mean tubercle number ± standard deviation. None of the treatments produced a significant effect compared with the control values.

In the GAC effluent in Experiment 4, there was a reduction (33.7%) in the mean cumulative egg production relative to the pre-exposure period for pairs of fish exposed to the control treatment, although this difference was not statistically significant (D=0.3810, n=21, p=0.0949; Figure 28). Similarly, both the undiluted GAC effluent and the positive control treatments caused reductions in mean cumulative egg production (100% effluent, 35.9% reduction, D=0.3333, n=21, p=0.1938; EE2, 46.4% reduction, D=0.3810, n=21, p=0.0949) relative to the pre-exposure period.

For raw data for both Pair Breeding Tests (standard and GAC effluent) see Appendix Tables A 19 – A 22 (length, weight, condition factor, GSI, VTG, FPI and tubercle number) and Tables A 23 and A 24 (egg counts).

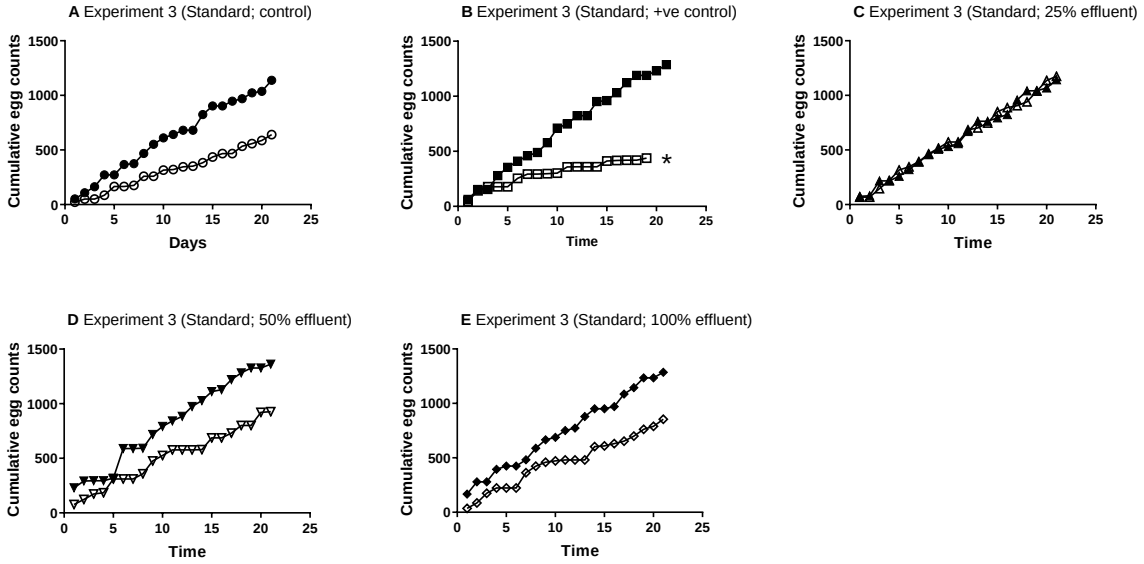


Figure 27. Experiment 3; cumulative egg production in Pair Breeding Test fathead minnows over a 21-day pre-exposure period (all FHMs exposed to control water; filled symbols) and then over a 21-day exposure period (unfilled symbols). During the exposure period 8 replicate pairs of FHMs were exposed to 100%, 50% and 25% effluent, or to the negative (control) or positive control (+ve control; 20 ng EE2/L). Statistically different from pre-exposure period; *p<0.05.

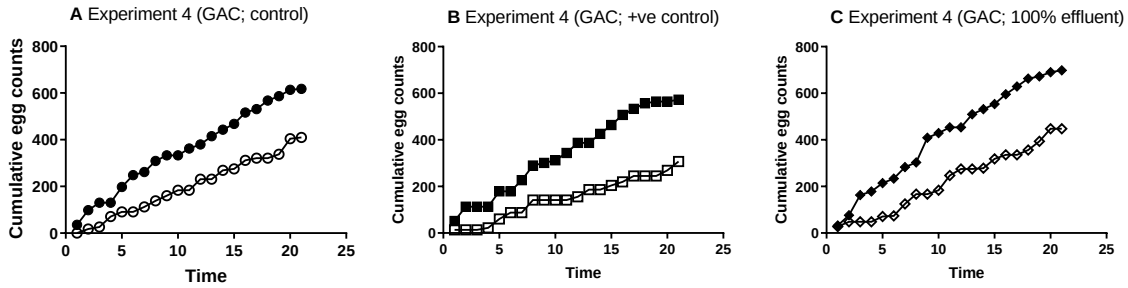


Figure 28. Experiment 4; cumulative egg production in Pair Breeding Test fathead minnows over a 21-day pre-exposure period (all FHMs exposed to control water; filled symbols) and then over a 21-day exposure period (unfilled symbols). During the exposure period 8 replicate pairs of FHMs were exposed to 100% GAC treated effluent, or to the negative (control) or positive (20 ng EE2/L) control. None of the treatments produced a significant effect.

2.3.2. Field based studies – wild roach collected from the River Ray downstream of the Swindon WwTW

2.3.2.1. Biological measurements

The male and female fork lengths of roach collected in 2013 were significantly longer (t_{66}=2.305, p=0.0243 and U= 994.5, n_1=92, n_2=36, p=0.0004, respectively) than those collected in 2005 (Figure 29). The fork length for the roach sampled in 2013 were between 113-237 mm, and whilst these were significantly longer, they fitted within the broader range of the fish lengths from the 2005 sampling; namely 63-286 mm. The mean weights of the male and female roach sampled in 2005 were 71.2 g and 112.7 g, respectively. Both male and female roach sampled in 2013 weighed significantly more than those sampled in 2005 (95.6 g, U=293.5, n_1=48, n_2=20, p=0.0113 and 145.5 g, U=1074, n_1=91, n_2=36, p=0.0023, respectively; Figure 30).

The condition factor for fish collected in 2013 were compared with those collected in 2005 and the values were not significantly different (U=3943, n_1=144, n_2=56, p=0.8087; Figure 31).

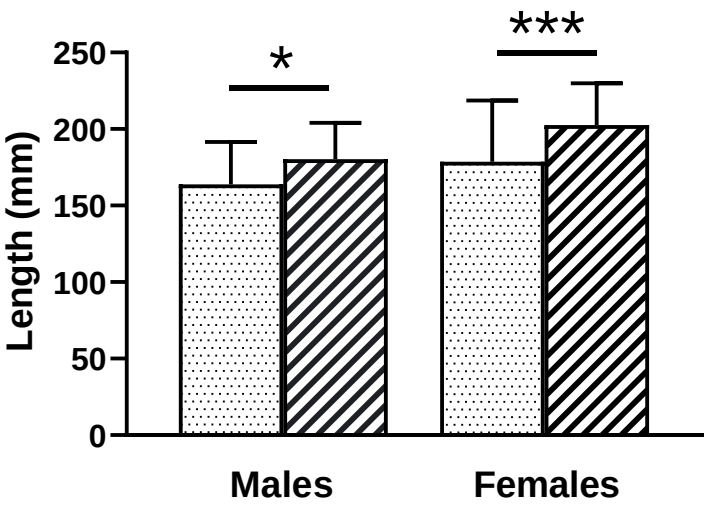


Figure 29. Fork lengths of male and female roach sampled from the River Ray in 2005 (dotted plots) and 2013 (striped plots). The bars represent mean values ± standard deviation. Statistically different from the 2005 sampling values; *p<0.05, ***p<0.001.

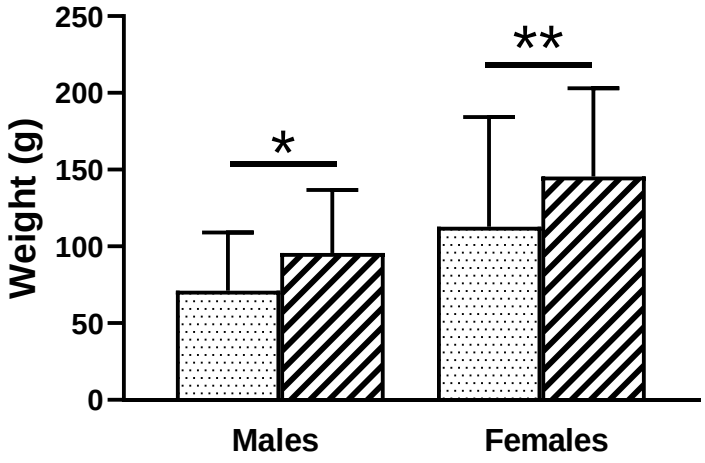


Figure 30. Weights of male and female roach sampled from the River Ray in 2005 (dotted plots) and 2013 (striped plots). The bars represent mean values ± standard deviation. Statistically different from the 2005 sampling values; *p<0.05, **p<0.01.

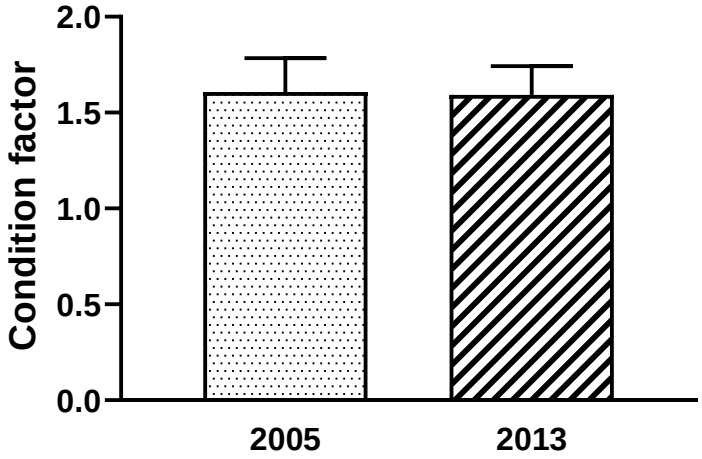


Figure 31. Condition factor of roach sampled from the River Ray in 2005 (dotted plot) and 2013 (striped plot). The bars represent mean values ± standard deviation. Not statistically different from the 2005 sampling values.

2.3.2.2. Age structure of River Ray roach

The age structure of the roach caught at the 2005 sampling sites appeared to have a normal distribution and ranged from 1+ to 8+ years old. (Table 6). The age structure of the roach caught in 2013 ranged from 3+ to 6+ years (Table 7).

The fish length and age structures of the fish sampled in 2013 were compared with those sampled in 2005 in Figure 32.

Table 6. Mean length and age of wild roach sampled from the River Ray in 2005. One fish could not be aged.

Age	1+ 2004	2+ 2003	3+ 2002	4+ 2001	5+ 2000	6+ 1999	7+ 1998	8+ 1997
Mean length (mm)	63	127	154	186	206	233	258	286
Standard deviation		9	18	15	14	18		
Number	1	28	37	38	33	5	1	1

Table 7. Mean length and age of wild roach sampled from the River Ray in 2013. One fish could not be aged.

	1+ 2012	2+ 2011	3+ 2010	4+ 2009	5+ 2008	6+ 2007	7+ 2006	8+ 2005
Mean length (mm)			163	193	212	218		
Standard deviation			28	49	17	13		
Number			12	22	17	4		

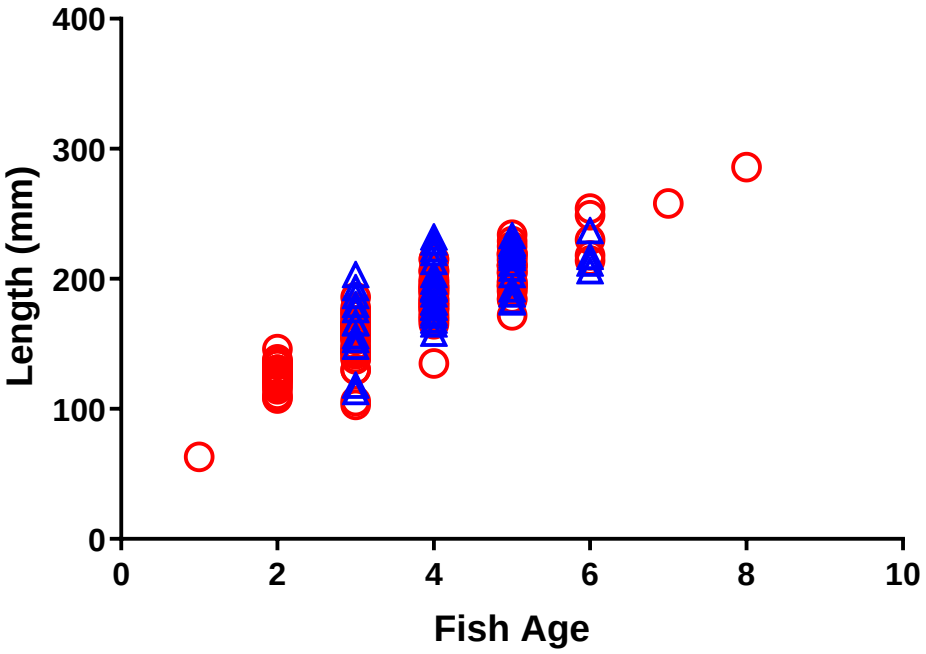


Figure 32. Age structure and length of roach sampled from the River Ray in 2005 (red circles) and 2013 (blue triangles).

2.3.2.3. Sex ratio, gonadal histology and gonad growth of River Ray roach

In the 2005 survey, a total of 145 fish were sampled and it was possible to sex 140 individuals; 92 females and 48 males. Of the male fish, 27.1% were intersex (n=13) and the ages of these intersex fish ranged from 2 to 6 years. In 2013, 56 fish were sampled; 36 females and 20 males. Of these male fish, 15% were intersex (n=3; all collected from Site 2) and were either 3 or 4 years of age. Although the overall percentage of males with intersex was lower in 2013, this reduced proportion of intersex fish was not significantly different from proportion of intersex fish observed in 2005 (p=0.3594; Fisher's exact test). The fish sampled in 2005 had a sex ratio of 66% female: 9% intersex: 25% male compared with a sex ratio of 64% female: 5% intersex: 30% male for those sampled in 2013 (Figure 33).

For the intersex roach sampled in 2005, the Intersex Index ranged from 0.167 (a few primary oocytes on one gonadal section of one gonad only) to 2 (all sections with oocyte numbers ranging from 1-21 oocytes per section) and averaged 0.513 ± 0.56. The distribution of these oocytes was multifocal rather than focal (Nolan *et al.*, 2001). In 2013, there were only 3 intersex fish collected these had Intersex Index scores of 0.167, 0.167 and 0.5, and

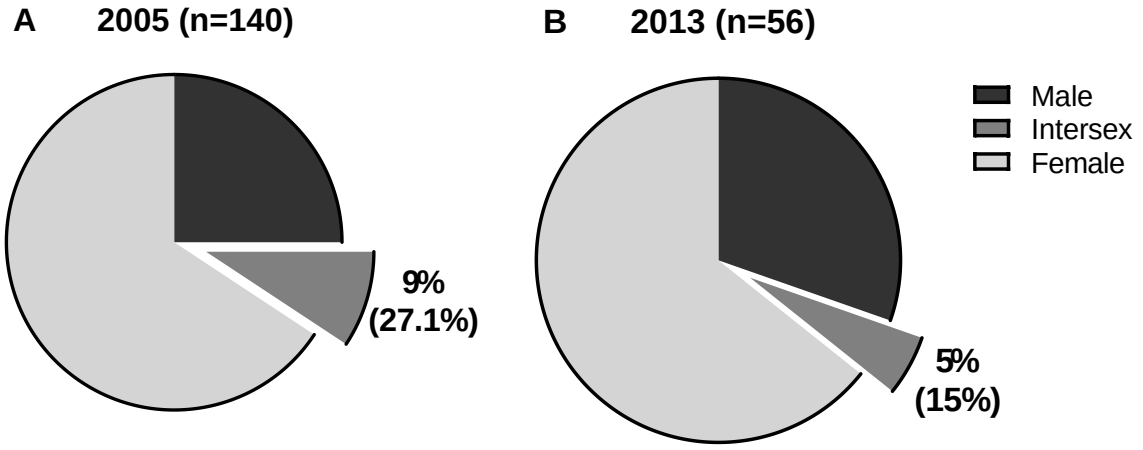


Figure 33. Sex ratio of the fish collected from the River Ray downstream of the Swindon WwTW; (A) in 2005, and (B) in 2013. The percentage of fish that were intersex was 9% (2005 sampling; n=13) and 5% (2013 sampling; n=3). The percentage of male fish that were intersex are indicated in brackets; 27.1% in 2005 and 15% in 2013.

averaged 0.278 ± 0.19. These Intersex Index scores were not significantly different from the 2005 scores (U= 9.500, n_1=11, n_2=3, p=0.3159). The severity of intersex from the two samplings was compared in Figure 34. Whilst some of the male fish collected in 2005 had a feminised sperm duct with 2 points of attachment (i.e. an ovarian cavity), all male fish collected in 2013 had normal sperm ducts with only one point of attachment.

The GSI for the male testes were significantly higher for the fish collected in November 2013 than for the fish collected in spring 2005 (t_{50}=2.878, p=0.0059; Table 8). Unlike the male fish, the GSI for the female ovaries were significantly lower for the fish collected November 2013 than for the fish collected spring 2005 (U=1259, n_1=91, n_2=36, p=0.0421; Table 8). The GSIs of the intersex fish collected in 2013 were not significantly different from the intersex fish sampled in 2005 (U=11, n_1=13, n_2=3, p=0.2964).

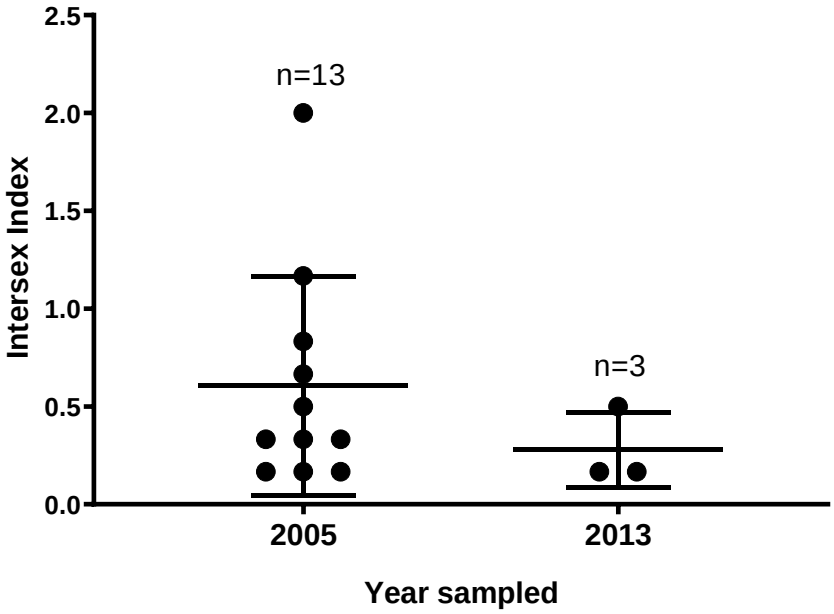


Figure 34. Severity of intersex of the male roach with intersex gonads (testicular oocytes and/or an ovarian cavity). In 2005, 13 male fish were found to be intersex and in 2013, 3 of the male fish were intersex. The scatter dot plot shows individual values and the whiskers represent the mean ± standard deviation.

Table 8. Gonadosomatic indices (GSIs) of wild roach sampled from the River Ray in 2005 and 2013 (mean ± standard deviation). The 2013 GSI values which are significantly different from respective 2005 values are denoted by stars; *p<0.05, **p<0.01.

GSI	Roach sampled in 2005 (n=139)	Roach sampled in 2013 (n=56)
Male	4.298 ± 1.333	5.299 ± 0.74 **
Intersex	4.089 ± 1.519	5.086 ± 0.658
Female	16.128 ± 11.004	12.580 ± 3.264 *

Most studies investigating endocrine endpoints in fish gonads focus primarily on intersex occurrence, intersex severity and feminised reproductive ducts (Bahamonde *et al.*, 2013; Sumpter, 2005). However, additional assessments on gonad development in both males and females can also shed light on reproductive health.

In 2005, 79% of males had spermiating gonads (Stage V), whereas in 2013 100% of males had less mature Stage II gonads (Figure 35). These findings are typical of both the month/season they were sampled (Geraudie *et al.*, 2010).

In 2005, just over 80% of the females had 'typical' gonads for their age and time of sampling (Figure 36). Of the 92 females sampled in 2005, 59 female fish were in the final stages of maturation with a high GSI (Figure 37) and greater than 50% vitellogenic oocytes (Stage V) and one female was spent (Stage VI), as might be expected for the mid-April sampling time (Geraudie *et al.*, 2010) . A further sixteen females were 1 or 2 years old and ranged from Stage II (only primary oocytes) to Stage IV (mainly cortical alveolus stage oocytes), typical of younger fish (Geraudie *et al.*, 2010). However, another sixteen females of mature age (3-7 years of age) had gonads that were uncharacteristically immature in appearance (Stage I-IV; 17.4%; Figure 38) (Jobling *et al.*, 2002a; Tyler and Sumpter, 1996). Whilst two of these uncharacteristically immature female fish were 3 years old and the developmental stage may be linked to the young age, the remaining 14 fish were 4+ years old. In Figure 38, the dotted line indicates the 3+ age that one would expect female roach to have reached maturity (stage V-VI) (Epler *et al.*, 2005), and for fish sampled in 2005 many 3+ fish had uncharacteristically immature (stage I-IV) gonads.

In 2013, one female had immature Stage II ovaries, and this developmental stage may be linked to the fish's relatively young age of 3 years (Figure 38). None of the other females in the 2013 sampling had under-developed for age gonads as seen in the 2005 samples. The remainder of the females (97.2%) had ovaries with greater than 50% vitellogenic oocytes (Stage V). On comparing the proportion of uncharacteristically immature female roach, there was a significant difference between the number of female fish in 2005 (n=16, 17.4%) and the single female fish (n=1, 2.8%) in 2013 ($p=0.0394$, Fisher's exact test).

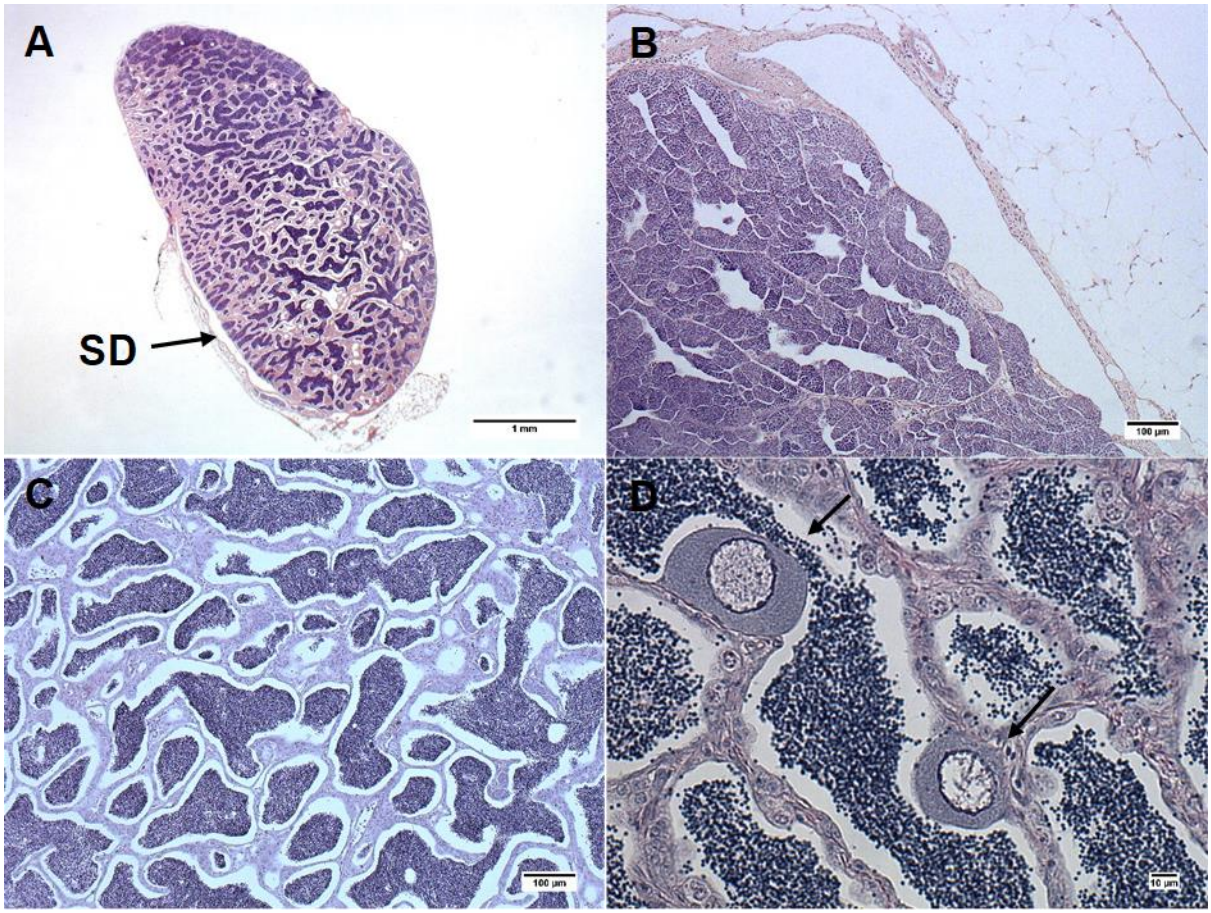


Figure 35. Male histology. Image A shows a normal testis sampled April 2005, at x 20 magnification. It is close to spermiation with the arrow indicating the sperm duct (SD). Image B at x 100 magnification is an example of a testis collected from a fish in November 2013, and at stage II the predominant cells are spermatogonia. Image C at x 100 magnification is an example of a testis collected from a fish in 2005, and at stage V the predominant cells are spermatozoa. Image D at 400 x magnification shows an intersex gonad section (Intersex Score 1) from a roach testis collected in 2005. A few scattered oocytes (shown with arrows) were visible in amongst the testicular tissue.

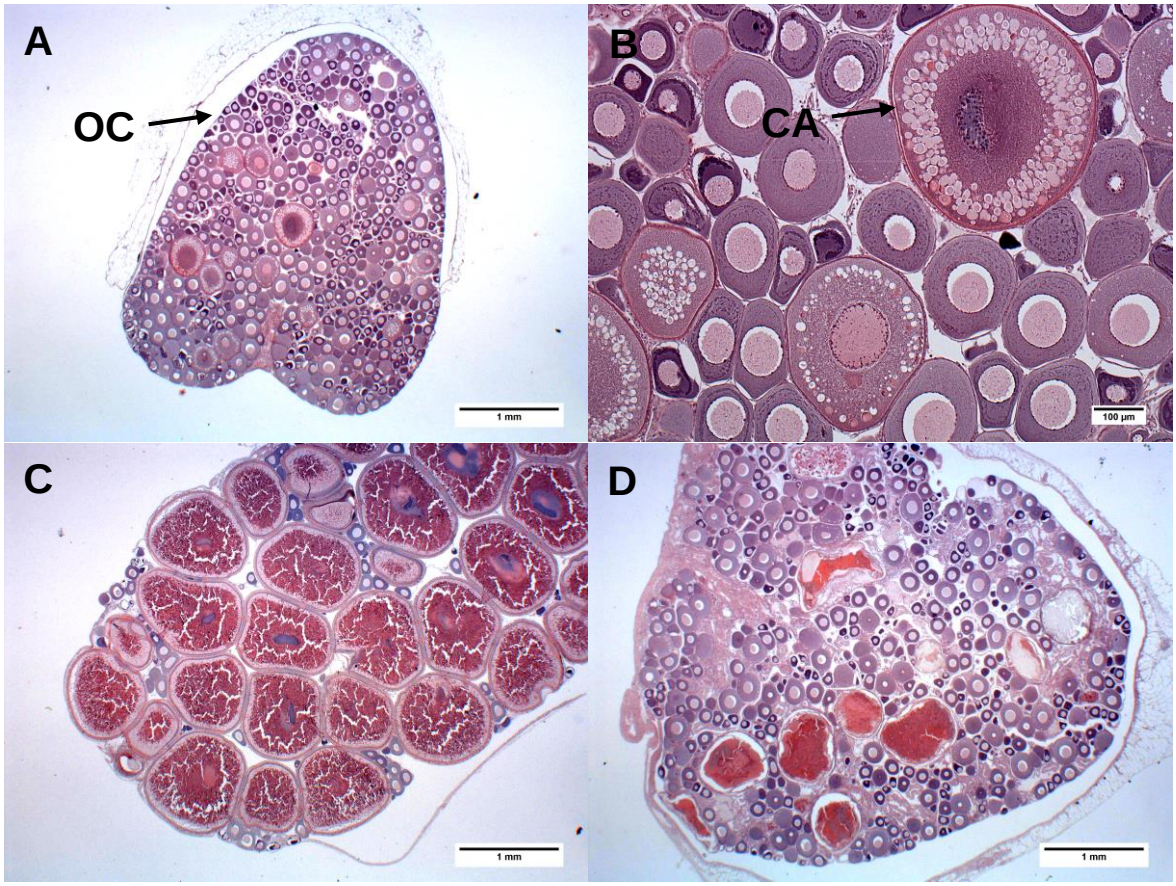


Figure 36. Female histology. Images showing normal immature ovary at x 20 (A) and x 100 (B) magnification. The ovarian cavity can be observed in image A (OC) and a developing cortical alveolus oocyte can be observed in image B (CA). Image C shows a mature ovary at x 20 with many vitellogenic oocytes (filled with yolk and surrounded by a thick cell wall or zona radiata) compared with an ovary from a fish of a similar age but with immature and degenerating oocytes (x 20; D). All images are of ovaries from the 2005 sampling.

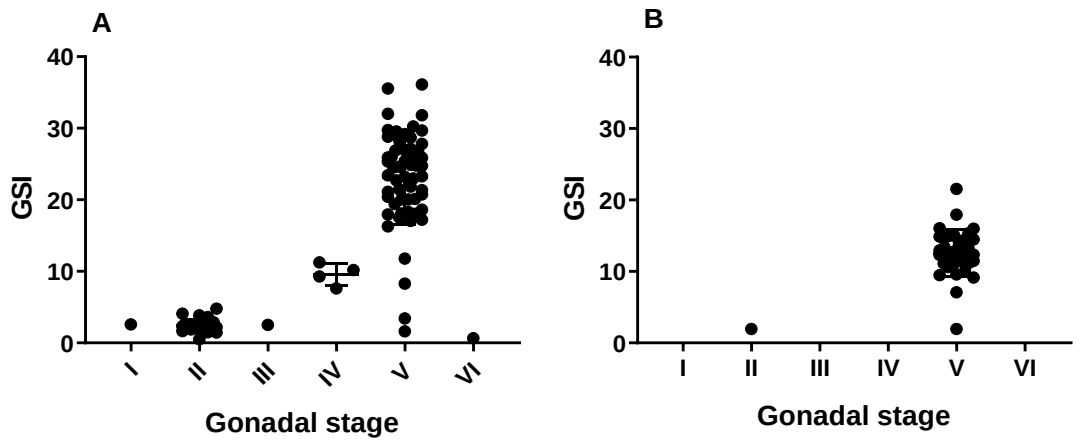


Figure 37. Gonadal stage of maturity of female roach sampled from the River Ray in 2005 (A) and 2013 (B) plotted against GSI. The scatter dot plots show individual values and the whiskers represent the mean ± standard deviation.

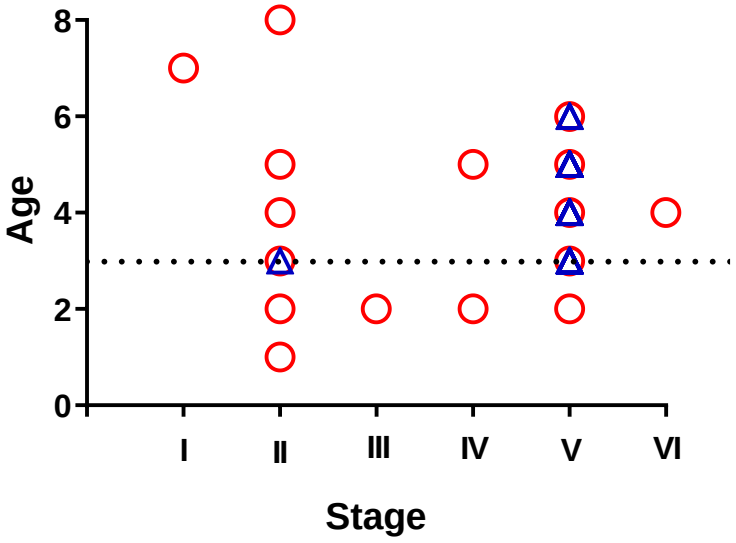


Figure 38. Gonadal stage of maturity of female roach sampled from the River Ray in 2005 (red circles) and 2013 (blue triangles) plotted against fish age. The dotted line indicates the age that one would expect female roach to have reached maturity (Epler *et al.*, 2005). Note that for fish sampled in 2005, many 3+ fish had immature stage I-IV oocytes.

Many of the female roach sampled in both 2005 and 2013 contained atretic oocytes, and in 2005 the average number of atretic oocytes per section ranged from 0 to 48.3, whereas in 2013 the number ranged from 0 to 15 (Table 9). Whilst there were lower numbers in the ovaries of the roach sampled in 2013 than for those sampled in 2005, this difference was not significant ($U=939$, $n_1=67$, $n_2=35$, $p=0.1002$).

Table 9. Number of atretic oocytes (average of 6 sections) found in the ovaries of wild roach sampled from the River Ray downstream of Swindon WwTW in 2005 and 2013 (mean ± standard deviation). The 2005 sampling was prior to the installation of GAC to the WwTW, and the 2013 sampling was 5 years after GAC was installed to the WwTW.

Atretic oocytes (average per 6 sections)	Roach sampled in 2005	Roach sampled in 2013
Range	0 - 48.3	0 - 15
Mean ± standard deviation	5.564 ± 8.813	5.443 ± 4.613

2.3.2.4. Plasma vitellogenin of River Ray roach

For the quantification of plasma samples collected in 2013, a commercial carp VTG ELISA kit based on the assay developed by Tyler *et al.* (1999) was utilised (Biosense Laboratories AS, Bergen, Norway). For an example standard curve see Appendix Figure A 8.

The quantification of VTG in plasma samples used two different ELISA methods (the pre-commercialisation method by Tyler *et al.* (1999) and the Biosense commercial kit) and also took place over a number of years. To determine comparability of VTG data, subsamples of plasma from 2005 and male plasma from 2013 were also included (reanalysed) in the ELISA used to determine the 2013 female plasma VTG concentrations; the VTG values were adjusted accordingly. The VTG concentrations of the six 2005 samples reanalysed at a later date were significantly different from the values obtained when analysed at the time of collection ($r_s= 0.8286$, $n=6$, $p=0.0313$; Table 10 and Appendix Figure A 9). These 2013 concentrations were on average around 5-fold lower, and the 2005 plasma sample values were adjusted to enable comparisons to be made.

Additionally, a significant difference in VTG concentrations was found when a subsample of 9 male samples from the 2013 sampling were reanalysed with the 2013 female plasma

samples at a later date (the male samples analysed in the first instance were on average 0.65 lower than when reanalysed with the female samples; r_s= 0.9833, n=9, p=0.0195; Table 11 and Appendix Figure A 9).

Unadjusted VTG concentrations can be seen in Table 12 and Appendix Figure A 10, and adjusted VTG concentrations can be seen in Table 13 and Figure 39.

Table 10. Comparison of 2005 plasma sample vitellogenin concentrations analysed by the two different carp ELISA methods (a carp VTG ELISA, previously validated for use with roach (Tyler *et al.*, 1996) and the commercial Biosence ELISA). The regression coefficient R^2 = 0.979 (see Appendix Figure A 9). The concentrations were significantly different (p=0.0313) and the 2005 sample concentrations were adjusted by a factor of 5.1 to take into account this difference.

Fish no.	Mean vitellogenin concentration (ng/ml)	
	Roach sampled 2005, analysis based on Tyler *et al.* (1999) ELISA	Roach sampled 2005, reanalysed in 2017 using Biosense ELISA kit
10	4,300,000	992,834
17	81	12
24	2,500,000	1,111,732
49	2,755,000	664,000
111	16,794	1,463
117	84	61

Table 11. Comparison of 2013 male plasma sample vitellogenin concentrations analysed using the same method (Biosense carp VTG ELISA kit) but in different years (2015 and 2017). The regression coefficient R^2 = 0.9916 (see Appendix Figure A 9). The concentrations were significantly different (p=0.0195) and the 2013 male sample concentrations were adjusted by 0.65 to take into account this difference.

Fish no.	Mean vitellogenin concentration (ng/ml)	
	Male roach sampled 2013, analysed in 2015 using Biosense ELISA	Male roach sampled 2013, reanalysed in 2017 using Biosense ELISA
1	23	25
3	208	545
8	385	933
11	12	12
16	45,133	118,156
25	198	519
26	12,801	33,750
27	12	12
29	48	47

Table 12. Comparison of unadjusted VTG concentrations measured in wild roach samples collected from the River Ray in 2005 and 2013. Unadjusted mean values are expressed ± standard deviation. VTG values significantly different from respective 2005 values are denoted by stars; **p<0.01, ***p<0.001, ****p<0.0001.

Histological Sex	Mean vitellogenin concentration (ng/ml)	
	Roach sampled 2005 (n=118)	Roach sampled 2013 (n=56)
Male	722,009 ± 1,518,246	3,560 ± 11,142 **
Intersex	1,759,236 ± 2,048,997	1,056 ± 1,763
Male and intersex	993,663 ± 1,709,877	3,185 ± 10,281 ***
Female	2,128,028 ± 2,186,094	7,785,099 ± 3,842,592 ****

Table 13. Comparison of adjusted VTG concentrations measured in wild roach samples collected from the River Ray in 2005 and 2013. Adjusted mean values are expressed ± standard deviation. VTG values significantly different from respective 2005 values are denoted by stars; ****p<0.0001.

Histological Sex	Mean adjusted VTG concentration (ng/ml)	
	Roach sampled 2005 (n=118)	Roach sampled 2013 (n=56)
Male	142,689 ± 300,049	5,477 ± 17,141
Intersex	347,675 ± 404,940	1,625 ± 2,713
Male and intersex	196,376 ± 337,920	4,900 ± 15,817
Female	420,559 ± 432,034	7,785,099 ± 3,842,592 ****

In 2005, prior to the GAC upgrade, the average plasma VTG concentration for male (including intersex) roach was 196,376 ± 337,920 ng/ml, whereas in the 2013 survey, after the GAC upgrade, the mean male (including intersex) roach plasma VTG concentration was 4,900 ± 15,817 ng/ml; although this reduction was not significant ($U=394$, $n_1=42$, $n_2=20$, $p=0.7008$; Table 13; Figure 39).

In contrast to the males, there was a significant increase in female VTG concentrations in 2013 (7,785,099 ± 3,842,592 ng/ml) when compared with those females sampled in 2005 (420,559 ± 432,034 ng/ml, $U=73$, $n_1=76$, $n_2=36$, $p<0.0001$; Table 13; Figure 39).

When GSIs were plotted against VTG concentrations for the fish collected in 2005, there appear to be two different populations of VTG concentrations (‘low responders’ and ‘high responders’), but these did not correlate with GSI (Figure 40). When comparing the 2013 fish GSI and plasma VTG concentrations, two distinct populations were not observed (Figure 41).

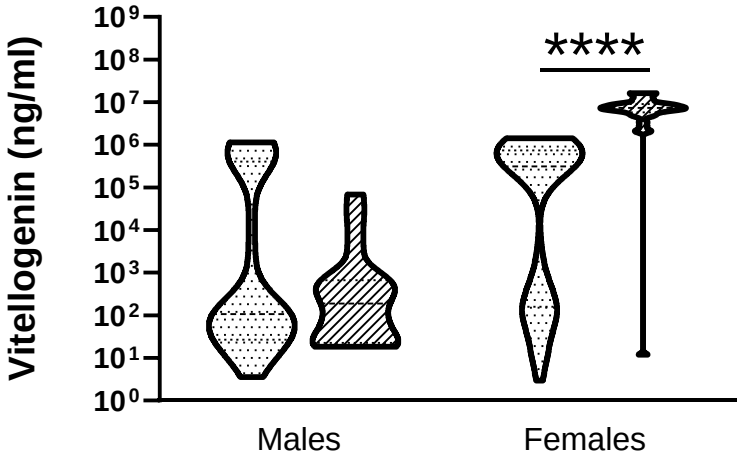


Figure 39. Adjusted plasma VTG concentrations in male (including intersex) and female roach sampled from the River Ray in 2005 (dotted plots; prior to the GAC installation) and in 2013 (striped plots; after the GAC WwTW upgrade). The violin plots include lines at the median and quartiles. VTG values significantly different from respective 2005 values are denoted by stars; ****p<0.0001.

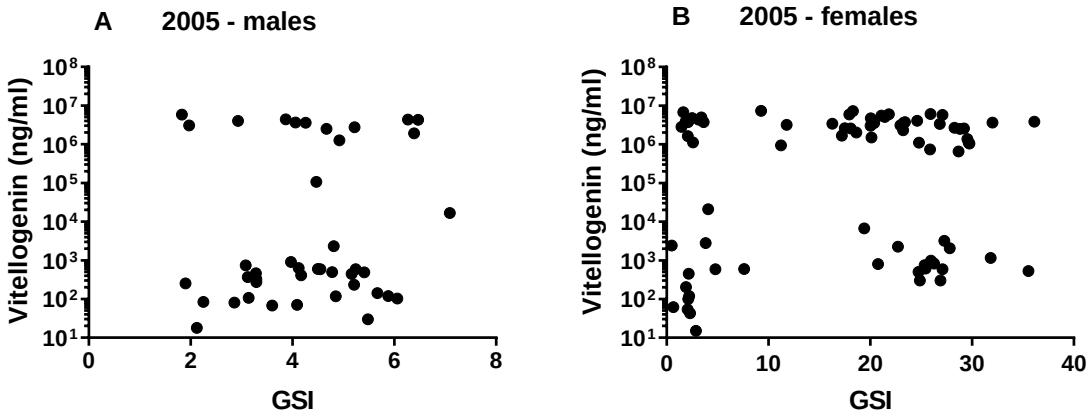


Figure 40. Comparing plasma VTG concentrations in (A) male and (B) female roach sampled from the River Ray in 2005 (prior to the GAC WwTW upgrade) plotted against GSI. Scattered dot plots represent individual values.

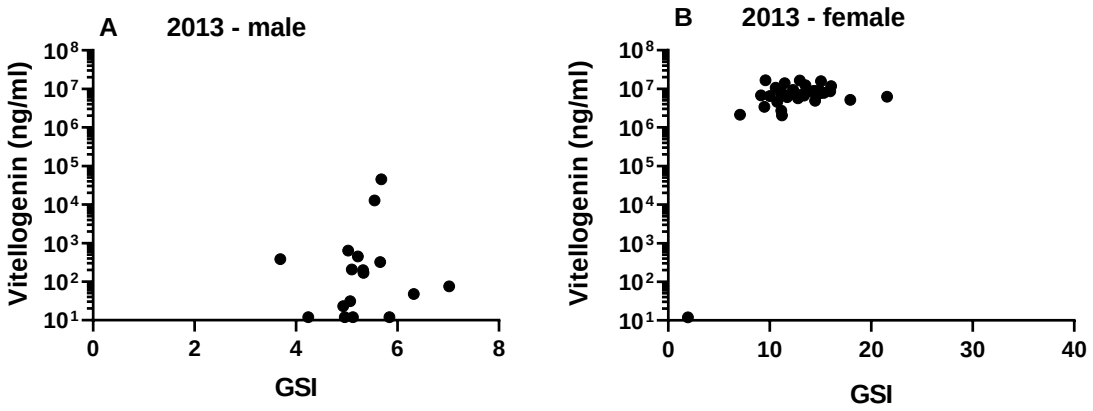


Figure 41. Comparing plasma VTG concentrations in (A) male and (B) female roach sampled from the River Ray in 2013 (after the GAC WwTW upgrade) plotted against the GSI. Scattered dot plots represent individual values.

2.3.2.5. Oestrogenic and anti-androgenic activity of River Ray roach bile

In 2005, bile concentrations were not determined for the female roach, so comparisons can only be made between male and intersex fish.

When male and intersex fish bile E2EQ concentrations were compared, there were no significant differences between the 2005 male bile concentrations and the 2005 intersex bile concentrations. Similarly, the E2EQ concentrations did not differ significantly between the 2013 male bile and 2013 intersex bile concentrations. However, in the 2013 samples the bile E2EQ concentrations were significantly higher for both male ($U=35$, $n_1=25$, $n_2=15$, $p<0.0001$) and intersex fish ($U=3$, $n_1=11$, $n_2=3$, $p=0.0385$) than those collected in 2005 (Figure 42). For bile anti-androgenic activity quantification, the FLUTEQ concentrations in around 90% of the samples were below the DL of the assays carried out in 2005 and 2013 (2005; DL=0.30 ng/ml and 2013; DL=0.34 ng/ml) and therefore statistical comparisons cannot be made.

For raw data for the roach sampled from the River Ray in 2005 and 2013, see Appendix Tables A 25 and A 26.

The results from the *in vivo* lab-based exposure studies with FHM and the roach collected from the River Ray in 2005 and 2013 are summarised in Table 14.

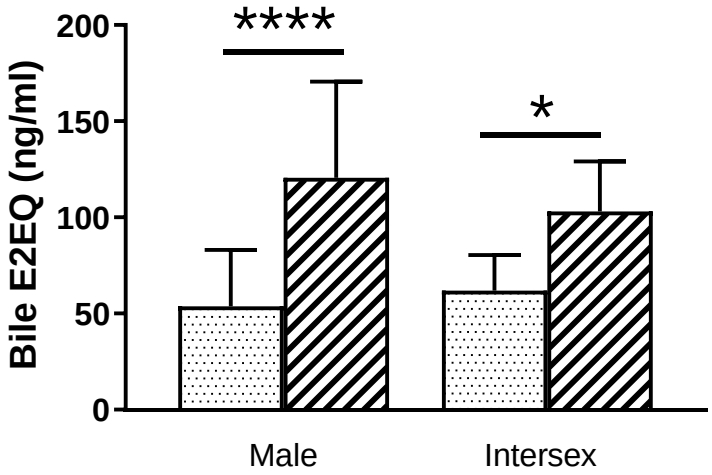


Figure 42. Bile oestradiol equivalent (E2EQ) concentrations collected from male and intersex fish in 2005 (dotted plots; prior to the GAC WwTW upgrade) and 2013 (striped plots; after the GAC WwTW upgrade). The bars represent mean values ± standard deviation. Statistically different from the 2005 sampling values; *p<0.05, ****p<0.0001.

2.4. Discussion

2.4.1. Lab-studies - Fathead minnow flow-through exposure studies to WwTW effluents

2.4.1.1. Oestrogenic content of the standard and GAC effluents

It has been previously demonstrated that the FHM tests can be used to assess the effects of oestrogenic chemicals and oestrogenic effluents on reproduction (Ankley *et al.*, 2001; Brian *et al.*, 2007; Harries *et al.*, 2000; Kramer *et al.*, 1998; Thorpe *et al.*, 2007).

Measurement of the natural steroidal oestrogens, E1 and E2, the synthetic oestrogen EE2, and the *in vitro* assessment of total oestrogenic activity (E2EQ YES measurements) showed that there were low levels of steroid oestrogens in both the standard and the GAC effluents. As observed by others, E1 was often the most abundant oestrogen of those measured (Ekman *et al.*, 2018). Indeed, Liu *et al.* (2020) detected E1, E2 and EE2 in 96%, 81 and 68% of Chinese surface water samples, respectively. Due to the lower potency of E1 (Lange *et al.*, 2012) this steroid has been less of an environmental concern, but recent work suggests that fish are able to metabolise E1 back into the more potent E2 (Ankley *et al.*, 2017; Tapper *et al.*, 2020) and overlooking it could underestimate risk to the environment.

Table 14. Summary table of changes (one arrow) and statistically significant changes (two arrows) of different parameters. Endpoints following exposure of fathead minnow (FHM) to standard and GAC-treated effluent were compared with dilution water (control) in two *in vivo* studies (Vitellogenin Assay and Pair Breeding Assay). Endpoints were also compared for roach collected from the River Ray in 2013 after the addition of GAC to the Swindon Wastewater Treatment Works with fish collected before the GAC plant was installed in 2005. NC; no change.

Endpoints	FHM - Vitellogenin Assay		FHM - Pair Breeding Assay		Roach from the River Ray
	Experiment 1 Standard	Experiment 2 GAC	Experiment 3 Standard	Experiment 4 GAC	
Length	NC	NC	NC	NC	♂↑↑ ♀↑↑
Weight	NC	NC	NC	NC	♂↑↑ ♀↑↑
Condition Factor	NC	NC	NC	NC	NC
GSI	NC	NC	NC	NC	♂↑↑ ♀↓↓
Tubercle number	NC	NC	NC	NC	
Fatpad Index	♂↓↓	NC	♂↓	NC	
Intersex incidence					♂↓
Uncharacteristically immature					♀↓↓
Atretic eggs					♀↓
Vitellogenin	NC	NC	NC	NC	♂↓ ♀↑↑
Egg counts			NC	NC	
Bile E2EQ					♂↑↑ ♀↑↑

The higher E2 and EE2 concentrations in the GAC effluent than the standard effluent, was not seen when the total oestrogenic activity (E2EQ) was measured via the *in vitro* YES. However, the concentrations of steroids in the standard treated effluent are likely to be underestimated due to matrix effects (Itzel *et al.*, 2017; Taylor, 2005) or because the steroids were present as conjugates/metabolites (Kumar *et al.*, 2011). The use of the YES bioassay gives an estimate of total oestrogenicity whereas the analytical methods look to identify the individual steroids and do not necessarily detect metabolites. The E2EQ YES values are therefore more likely to give a better prediction of *in vivo* effects than the analytical chemistry results (Volker *et al.*, 2016). Direct comparisons of the absolute levels of oestrogens in the standard and GAC effluents as measures of removal efficiency are not possible here though because the standard and GAC effluent exposures were conducted at different times of the year.

Panter *et al.* (2002) exposed FHMs to EE2 and their tank concentrations were between 40 and 50% of nominal values, and it was established that the low measured EE2 concentrations were at least in part due to losses because of adsorption to the test system. Our positive control EE2 tank concentrations were similarly about half the nominal values and may also be due to adsorption to the test system. Despite the sometimes biologically active concentrations of steroid oestrogens present in the effluents leaving the storage tank, particularly at the end of the batch, the concentrations measured in the aquaria were often below biologically active concentrations and sometimes even below DLs. This could be due to adsorption but might also be due to uptake by the fish (Panter *et al.*, 2002).

2.4.1.2. The sensitive biomarker, vitellogenin, was not induced by the standard or GAC effluent

Male FHMs were exposed to either control, effluent (both standard and GAC), or positive control (EE2) in the VTG Test (Experiments 1 and 2) and the Pair Breeding Test (Experiment 3 and 4) for a period of 21 days. Whilst the male fish exposed to EE2 had a significant induction of plasma VTG in all four experiments, none of the male fish exposed to effluent (either standard or GAC) showed induction of plasma VTG, indicating insufficient levels of oestrogens in the Swindon effluent to elicit this well-known biomarker of oestrogenic activity. This result was unexpected, given that the analytical chemistry reported concentrations of EE2 ≥1 ng/L in the effluent storage tank feeding the test aquaria that are known to induce VTG in male FHM (Flick *et al.*, 2014; Lange *et al.*, 2001; Pawlowski *et al.*, 2004). Because the effluent failed to induce a VTG response in male FHM, it seems that either the analytical measurements in the effluent of >1 ng/L EE2 were overestimated or the vitellogenic response was underestimated because the EE2 was in a form that was not

bioavailable to the fish (e.g. conjugated or bound to particles). This shows the importance of using a combination of chemical analysis and biological endpoints for assessing the effects of sewage effluents.

In the Pair Breeding Test (Experiment 3 and 4), exposure of the female FHMs to the EE2 positive control did not increase the plasma VTG in Experiment 3 (standard effluent) but caused a 10-fold increase ($p=0.0218$) in the females exposed to EE2 in Experiment 4 (GAC effluent). The females used in Experiment 3 (7 months) were younger than those used in Experiment 4 (11 months), but if this was the reason for the different sensitivities, the younger female FHM might have been expected to be more sensitive. However, some fish did die in these EE2 tanks in Experiment 3 and the remainder were sampled 2 days early, and this is likely to have affected the mean VTG concentration.

2.4.1.3. Observed reductions in fecundity may have been due to lower feeding rates rather than the oestrogenic effects of effluent

Decreases in egg production following exposure to steroidal oestrogen are often seen at concentrations of the oestrogen that induce VTG concentrations in males above a threshold of 1 mg/ml (Kramer *et al.*, 1998; Thorpe *et al.*, 2007). In this investigation, concentrations of plasma VTG were often 10 times higher than this 1 mg/ml and so the suppression of egg production seen in the Experiment 3 positive control (EE2) was as expected.

Surprisingly, there were also reductions in the egg production in the control tanks with both standard (Experiment 3) and GAC effluent (Experiment 4) Pair Breeding Tests. This may have been due to alterations to the prescribed feeding regime, which were introduced to prevent the build-up of nitrite and ammonia (both toxic to fish) with the low flows to the fish tanks. There was also a reduction in egg production seen with the 50% and 100% standard effluent and as all replicates and treatments were fed in the same way, this reduction could have been due to the reduced feed too. Interestingly, the 25% effluent exposed fish was the only treatment where the cumulative egg production was not lower than the pre-exposure period. Others have reported an increase in fecundity following exposure of FHM to 20% WwTW effluent (Cavallin *et al.*, 2016), and whilst it is possible that the extra nutrients in the 25% standard effluent were sufficient for egg production to be sustained, this was not reflected in the biological parameters, such as, fish weight and condition factor. Filby *et al.* (2010) carried out pair-breeding exposures with FHMs using standard and GAC effluent and found that with both effluents there was a similar inhibition of egg production but no inhibition with the EE2 positive control. They therefore considered this fall in egg production to be due to the chemical/physical properties of the effluent and not due to the oestrogenicity of the effluent.

For the pair-breeding test with the GAC effluent (Experiment 4), there was a similar reduction in egg production in all treatments. This reduction was most pronounced (although not significant) with the EE2 treatment.

2.4.1.4. The development/suppression of SSCs were variable

For the VTG Test (Experiment 1 and 2), the male FHM FPI (Experiment 1, standard effluent; $p<0.0001$) and tubercle number (Experiment 2, GAC; $p=0.0027$) increased significantly during the exposure period. This was as expected as the FHM in the male-only tanks would have had a further 3 weeks to set up social hierarchies of dominance leading to increased SSCs (Ivanova *et al.*, 2017). The increase in FPI (only significant in standard effluent VTG Test, Experiment 1) and tubercle number (only significant in GAC effluent VTG Test, Experiment 2) during the exposure period may also be due to fish of two different ages being used for the exposures; 7 months old male FHM for Experiment 1 and 11 months old for Experiment 2. However, Coady *et al.* (2017) found SSCs to be correlated with body weight and one would therefore have expected to see the significant increases in the 11 months old FHM. Increases in FPI and tubercle number might also have been more likely in the younger FHM than the older fish, but these endpoints did not develop consistently.

Whilst standard effluent did not induce VTG production in male fish (Experiment 1), the male fish exposed to the standard effluent had a significant suppression of fatpad development (Experiment 1; with 50% and 100% effluent) compared with the control fish during the exposure period. There was no suppression of the fatpad growth in the EE2 exposed fish in this experiment, indicating that the disruption to normal fatpad development could be due to the presence of other EDCs in effluent, such as anti-androgens (Bahamonde *et al.*, 2013; Filby *et al.*, 2010; Gross-Sorokin *et al.*, 2004).

In the Pair Breeding Tests, both the FPI and the tubercle number values were higher than for the VTG Test fish, and this was as expected due to the presence of female fish. In both the VTG Tests (Experiments 1 and 2) and the Pair Breeding Tests (Experiments 3 and 4), there were no reductions in number of tubercles with either the standard or GAC effluent. Therefore, the FPI was a more sensitive endpoint than the tubercle number, as has been observed by others (Filby *et al.*, 2010; Harries *et al.*, 2000; Miles-Richardson *et al.*, 1999). We did see a significant repression in the growth of tubercles in Experiment 2 (VTG Test) with the EE2 compared with the control males, such that they did not develop in line with the tubercle development of the control fish between the start and the end of the 21-day exposure period. This was not observed with the Pair Breeding Test EE2 treatment, perhaps because the males were actively breeding at the start of this test, and therefore likely to have higher tubercle numbers before the exposure period started.

Although fish from all experiments were within the age range specified in the EDDP protocol (between 4 and 12 months at the start of the study), the OECD 21-day Fish Assay (OECD, 2018a) does specify a FHM age of 20 ± 2 weeks, and for all experiments the FHM were older than OECD guidelines. Age and timing of exposure could be critical when assessing SSC and as for others (Armstrong *et al.*, 2016; Harries *et al.*, 2000; Panter *et al.*, 2012), the age and timing of maturity may have affected our FPI and tubercle number results. Additionally, here all-male tanks were used whereas the OECD guidelines specify mixed sex tanks (2 male and 4 female FHM) and this ratio was chosen to optimise reproduction (Armstrong *et al.*, 2016) and may also lead to more consistent SSC scores. It is also worth noting that the OECD fish test guidelines (OECD, 2018a; b) give details for rating tubercles, but whilst the guidelines cite the size of the fatpad as a potentially important SSC, no guidance is given and standard practise is to limit assessments to visual observations (Wheeler *et al.*, 2020).

2.4.2. Field studies – wild roach collected from the River Ray downstream of the Swindon WwTW

2.4.2.1. Roach sampled following addition of GAC at the WwTW were longer and heavier

When comparing the biological parameters of the wild fish collected in 2013 from the River Ray with those collected in 2005, there was no statistical difference in condition factor. However, for the fish collected in 2013, both males and females were significantly longer and heavier than those collected in 2005 were. Fish collected downstream of sewage discharges have been found by others to have greater growth (longer and heavier; McMaster *et al.*, 2005; Tetreault *et al.*, 2011) and this was thought to be due to nutrient enrichment in nutrient-limited rivers. However, we observed greater growth following the addition of the GAC where the nutrients were likely to have been lower. It is possible that following the GAC, cleaner river water meant a healthier river with more macroinvertebrates for the roach to feed on, although Johnson *et al.* (2019) did not observe any noticeable impact on River Ray macroinvertebrates during this period of GAC treatment.

Although there are differences between the two samples, prevalence and severity of intersex increase with age and size (Gross-Sorokin *et al.*, 2006; Jobling *et al.*, 2006), therefore it is unlikely we have underestimated possible endocrine disrupting effects in 2013. Pottinger *et al.* (2011), investigating non-reproductive endpoints in sticklebacks collected from the River Ray both before and after the GAC addition to the Swindon WwTW, also found a higher growth rate post-remediation, and proposed that factors present in the effluent before the GAC addition influenced the health of the stickleback populations downstream.

2.4.2.2. Improved male roach reproductive health following the installation of GAC at the WwTW

Spermatogonia were the most abundant cell type in the testes of fish sampled in late November 2013 and spermatozoa were the most abundant cell type in April to June 2005, as observed by Billard (1986).

The percentage of female fish was very similar when comparing the sex ratios of the fish collected in 2013 (64%) with those collected in 2005 (66%).

When Geraudie *et al.* (2010) sampled roach from a low contaminated site, 54.5% were found to be female fish and this value was not considered significantly different from the expected 1:1 ratio. Pottinger *et al.* (2011) observed a female-skewed sex ratio of sticklebacks sampled from the River Ray before the GAC addition but following the addition of the GAC to the Swindon WwTW this ratio was not significantly different to unity. However, using a genetic sex probe (Lange *et al.*, 2020), Baynes *et al.* (2020) found female-skewed sex ratios in roach at many sites in the UK with various WwTW effluent inputs, and the sex ratio observed in our study of the River Ray is therefore not atypical.
For the fish sampled in 2013, 15% of male fish were intersex compared with 27.1% of the male fish collected in 2005. Unfortunately, because only 20 of the fish caught in 2013 were males, even though there was a reduction in the number of intersex males, this was not significant. In a study carried out by Jobling *et al.* (1998), between 4% and 100% of male fish in different UK rivers were found to be intersex, and later Jobling *et al.* (2002a) found approximately 10 percent of fish from reference sites to be intersex. In addition, Bjerregaard *et al.* (2006) examined male roach collected from Danish reference sites and found a background presence of 4.8% intersex, and roach collected from the site with the highest proportion of sewage effluent were found to be 26.5% intersex. Lukšienė *et al.* (2000) found only one intersex fish out of 949 roach sampled in Sweden and in another study by Geraudie *et al.* (2010), where 474 roach were collected, no intersex fish were recorded. Geraudie *et al.* concluded that the incidence of intersex is extremely low (below 0.5%) under natural conditions. The intersex incidence seen in 2013 could therefore represent continued exposure to low concentrations of oestrogens and/or anti-androgens, even following the addition of GAC to the WwTW. As with the fish collected in 2013, the fish collected from reference sites by Jobling *et al.* (2002a) had a very low Intersex Index scores (<1). At this low incidence observed in 2013, it is unlikely that this intersex condition alone would lead to population level effects (Harris *et al.*, 2011), especially as Hamilton *et al.* (2014) found roach populations living in effluent-contaminated rivers with widespread feminisation to be self-sustaining.

The roach collected in 2013 were from 2008 to 2011-year classes. As the GAC at the WwTW was installed in 2008, some of these roach from the River Ray may have been exposed to standard effluent, especially the 6+ fish. However, our 6+ year old fish collected in 2013 did not have feminised ducts (thought to be permanent, indicating early exposure; Jobling *et al.*, 1998). Also, whilst some have observed the severity of intersex to be influenced by age (Gross-Sorokin *et al.*, 2006; Jobling *et al.*, 2009; Lange *et al.*, 2011), for both sets of roach sampled in 2005 and 2013 there was no indication that the severity was linked to age (i.e. length of exposure to the effluent in the river).

2.4.2.3. Improved female reproductive health following the installation of GAC at the WwTW

Ovarian atresia is a common degenerative process and can be observed at all stages of the reproductive cycle, but normally occurs most frequently post spawning (Lange *et al.*, 2011), and these residual oocytes (atretic) are then digested and absorbed by macrophages invading the ovaries (Geraudie *et al.*, 2010). Not surprisingly, atresia is thought to be largely a result of environmental stress, but reduced feeding also causes a progressive increase in atresia (Tyler and Sumpter, 1996). Investigators have reported ovarian disruption with oestrogenic compounds (Kiparissis *et al.*, 2003) and anti-androgens (Makynen *et al.*, 2000), as well as exposure to high concentrations of WwTW effluent (100% effluent, ovaries contained degenerative primary and secondary oocytes; Lange *et al.* (2011) and effluent contaminated rivers had a greater level of ovarian atresia; Jobling *et al.* (2002a)). Atretic oocytes were seen in several of the 2005 females and for some gonads the atretic oocytes predominated. This atresia may therefore be the result of oestrogenic and/or anti-androgenic activity in the River Ray prior to the addition of the GAC at the WwTW. There was a significant reduction in the number of female roach with uncharacteristically immature ovaries in the 2013 roach collected following the addition of the GAC at the WwTW. This result could be due to a reduced EDC concentrations in the GAC effluent. In the 2013 females there were small numbers of atretic oocytes on the edges of the gonad sections. Geraudie *et al.* (2010) observed biphasic development of ovaries and considered that this indicated that gonadal maturation was interrupted, and oocytes were probably partially absorbed in December. The presence of degenerating oocytes in the 2013 ovaries collected at the end of November may therefore be part of a normal process, and not be linked to the presence of EDCs in the river.

2.4.2.4. Reduced male plasma vitellogenin concentrations following addition of GAC to the WwTW, but concentrations were still elevated

The VTG concentration measured in the male roach collected in 2013 from Site 1 was 241 ng/ml and in the male (including intersex) roach collected from Site 2 was 6,064 ng/ml, with an average of 4,900 ng/ml (Site 1 plus Site 2). Considering that Site 1 was closer to Swindon WwTW, one might have expected to see intersex fish and the plasma VTG concentrations to have been higher here. However, as only four male fish were collected from this site one cannot draw any conclusions, but it is possible that other waste streams enter the River Ray between the two sites.

The fish collected from the River Ray in 2005 were collected at a different time of year (late April to early June) to those collected in 2013 (late November). Since the season has been shown to affect the oestrogenic potency of effluents (Harries *et al.*, 1999), comparisons were made between the 2013 male plasma VTG concentrations and with the potable water control male fish sampled at the same time of year as part of another study (Baynes *et al.*, 2012). The 2013 male fish were found to have significantly lower VTG concentrations than the potable water control fish, but Baynes *et al.* noted that their VTG concentrations were higher than expected and linked these to exposure to natural oestrogens present in the fish tanks. Jobling *et al.* (2002a) measured VTG concentrations in roach from two reference sites collected at the end of October, and found the concentrations to be 100 and 15 ng/ml. Jobling *et al.* (2002a) considered that the concentration at 100 ng/ml was above basal concentrations, and was possibly a result of exposure to endogenous oestrogens via the diet and/or water, but also considered that the difference may be due to normal interpopulation variability. Geraudie *et al.* (2010) measured roach VTG concentrations over a period of 18 months and found male plasma basal concentrations to be 24 ng/ml and rising to 120 ng/ml during the spawning period (February to April). Our male (including intersex) plasma VTG concentrations of 4,900 ng/ml are over an order of magnitude higher than typical basal concentrations, indicating that the fish may still be being exposed to EDCs even after the addition of the GAC treatment to the WwTW. Therefore, these elevated VTG concentrations may be due to water soluble EDCs not being retained by the GAC (Snyder *et al.*, 2007) or breakthrough due to saturation of the GAC (Baynes *et al.*, 2012).

Kidd *et al.* (2007) reported a positive correlation between increased hepatic VTG expression and plasma VTG and intersex incidence following exposure of FHMs to EE2 in a whole lake experiment. However, we observed no such correlation between plasma VTG and intersex with either the fish sampled in 2005 or those sampled in 2013, and the driver for the intersex that we observed may therefore be non-oestrogenic mediated pathways, as suggested by Bahamonde *et al.* (2013). The 2005 male plasma VTG concentrations were highly variable

and seemed to fall into two different populations irrespective of GSI, with some fish being more sensitive than others to oestrogenic contaminants in the river water. This has been reported previously by Beresford *et al.* (2011), who found FHM VTG to be highly variable in lab-based studies, even within the same treatment group. Biales *et al.* (2007) thought that this variation could be due to genetic variation in the degree of the response of the fish as, of the genes examined, only the VTG gene was variable. Purdom *et al.* (1994) also looked at plasma VTG in wild rainbow trout exposed to effluents and found the concentrations were raised above the baseline by 500 to 50,000 times. Similarly, the 'high male responders' collected from the River Ray in 2005 had plasma VTG values about 10,000 times higher than the 'low male responder' values. More recently, Wheeler *et al.* (2019) examined control data from 49 FHM studies and found high intra- and inter-laboratory variability for VTG and therefore considered this endpoint to be of limited use during study interpretation.

The female roach had significantly higher plasma VTG concentrations when collected in November 2013 than in spring 2005. Whilst the spring 2005 concentrations were similar to concentrations reported by Geraudie *et al.* (2010) and Scott *et al.* (2013) for female roach sampled at this time of year, these same authors reported lower VTG concentrations when they sampled female fish in November. Whilst different methods were used for the detection of the VTG at the two different times of the year, both methods were based on work by Tyler *et al.* (1996) and are not likely to be the reason for this discrepancy. Other factors (e.g. water temperature) may also be playing a part here, but due to the different seasons, it is not possible to make any definite conclusions.

The male GSIs were significantly different between the two samplings and one would have expected the testes collected in 2005 to have a higher GSI than those collected in November, in preparation for spawning. Oestrogenic and/or anti-androgenic effluent entering the river could also have led to lower circulating plasma T concentrations in the fish, resulting in reduced GSIs. However, as many of the sperm ducts of the 2005 male fish were full of sperm, it is just as likely that the lower GSIs were because some were part spent.

2.4.2.5. Bile E2EQ and FLUTEQ values do not reflect the intersex or vitellogenin results

In 2005, bile E2EQ and FLUTEQ values were only determined for the male and intersex fish. The method utilised separate glucuronidase and sulphatase enzymes and the efficiencies of these enzymes had been previously determined using nitrophenyl glucuronide and nitrophenol sulphate (Gibson *et al.*, 2005). However, when analysing the bile samples collected in 2013 using E2-G and E2-S, the sulphatase from *Aerobacter aerogenes* did not deconjugate the E2-S. Steroid sulphates are known to be difficult to deconjugate using

enzymatic hydrolysis and some authors have used acid hydrolysis (Hauser *et al.*, 2008) or digestive juice from *Helix pomatia* instead (Pedersen *et al.*, 2017). Therefore, for the later samples analysed in 2013, *H. pomatia* digestive 'snail juice' was used for deconjugations. Whilst the 'snail juice' sulphatase activity has the broadest specificity and is commonly used in bioanalysis for hydrolysing both glucuronide and sulphatase conjugates (Gomes *et al.*, 2009), our results show that the sulphate hydrolysis was still incomplete; only 23.7% deconjugation occurred. However, as most conjugates are thought to be in the glucuronide moiety (Gomes *et al.*, 2009), we did not try to further improve this sulphate deconjugation efficiency.

Houtman *et al.* (2004) carried out bioassay-directed fractionation of bile and identified many xenobiotic compounds at relatively high concentrations but found that the majority of the oestrogenic activity in the bile was due to the presence of E2. Female E2EQs from bile collected in 2013 were significantly higher than the males E2EQ values, and this higher activity is thought to be due to the natural oestrogens present in the female bile.

The male and intersex E2EQ determined for the 2005 samples averaged 53.43 and 61.91 ng/ml, respectively, and these are lower than values determined by Gibson *et al.* (2005) for fish exposed to WwTW effluents, but higher than values from their reference fish bile. Values from fish collected following the GAC treatment in 2013 were significantly higher both for the male and intersex fish (120.5 and 103.0 ng/ml, respectively), but these values may be higher due to the improved enzymatic hydrolysis method used.

Some authors have found a correlation between plasma VTG induction and bile E2EQ (e.g. Legler *et al.*, 2002) but others have not (e.g. Ros *et al.*, 2015). We found no correlation between plasma VTG induction and bile E2EQ in male and intersex fish collected in both 2005 and 2013. This suggests that the incidence of intersex is not the result of oestrogens but of EDCs with non-oestrogenic activity.

Many of the endpoints that we have examined suggest that anti-androgens are present in the effluent, particularly in the standard effluent. For FLUTEQ, we were unable to compare our results with previously reported values as so many were below the DL (only 12% (2005) and 15% (2013) had detectable concentrations). For our bile samples with anti-androgenic activity, no efforts were made to identify the anti-androgenic culprits as this was beyond the scope of this work, but even when chemicals have been identified these often only account for a proportion of the activity seen (Hill *et al.*, 2010; Rostkowski *et al.*, 2011).

2.4.3. Has the reproductive health of the fish living in the river improved following the addition of the GAC to the WwTW?

Grover *et al.* analysed the oestrogen content of the final effluent entering the River Ray prior to the installation of the GAC plant, and found the E2EQ concentration to be unexpectedly low at <5 ng/L (Grover *et al.*, 2011a; Grover *et al.*, 2011b). Balaam *et al.* (2010) also found the concentration of oestrogens in the River Ray to be lower than concentrations predicted by a model utilised by Johnson and Williams (2004), and Balaam *et al.* thought that this was due to biological nutrient removal leading to better than expected performance at the WwTW. Following the addition of GAC to the Swindon WwTW, Grover *et al.* (2011b) could not detect E1, E2 and EE2 analytically, and there was a significant reduction in many of the pharmaceuticals determined (84-99%) although for some of the pharmaceuticals the removal efficiency by the GAC was poor (e.g. 17% for propranolol).

In addition, Katsiadaki *et al.* (2012) measured spiggin concentrations (a biomarker of androgenic exposure) in wild female stickleback fish collected from the River Ray. When the stickleback fish were collected from the River Ray prior to the addition of the GAC at the WwTW, the spiggin concentration increased with distance from the effluent discharge point, indicative of the effluent containing anti-androgenic compounds that were suppressing the spiggin production closer to the WwTW. In addition, following the addition of the GAC treatment, the spiggin concentrations in female fish returned to the levels seen in the fish collected from the control site. Pottinger *et al.* (2011) measured the activity of monooxygenase cytochrome P4501A (to determine exposure to PAHs) in sticklebacks collected from the River Ray, but did not find a decline in the activity following the addition of GAC to the Swindon WwTW, and considered that the primary route of exposure was not via the water column but attached to particles.

From this work, it seems there may have been a reduction of oestrogenicity in the Swindon WwTW effluent even before the GAC addition, as reflected by the low VTG concentrations measured in the male FHM in 2009 compared with the high plasma VTG concentrations measured in male roach in 2005. Before the installation of the GAC, disc filters were introduced at the Swindon WwTW, and this may have been the reason for the reduced oestrogenic activity before the GAC addition. Post addition of the GAC, roach collected from the River Ray in 2013 had much reduced plasma VTG concentrations and a reduction in the incidence (and severity) of intersex. However, it is clear from some of the FHM results seen (e.g. Fatpad Indices), that there were chemicals in the standard effluent with other modes of action present (i.e. not oestrogens and more likely anti-androgens). When Grover *et al.* (2011a) carried out spot water sampling of the River Ray, the anti-androgenic activity reduced from 148.8 to 22.4µg FLUTEQ/L following addition of the GAC. Grover *et al.* also found that the YES and AYAS values were related to each other, suggesting a coexistence of both types of activities. However, we did not find any VTG induction in any of our FHM lab

tests to be able to relate VTG concentrations with the oestrogenic content of the effluent. Where the VTG concentrations were elevated in the roach, even after the addition of the GAC, these did not mirror the bile E2EQ or FLUTEQ. Recent work by Hamilton *et al.* (2020) looked for evidence of adaptation to oestrogenic pollution but found none for selection in oestrogen-dependent genes. However, Hamilton *et al.* found an allele shift at the AR in the River Lee population (historically contaminated with WwTW effluents) and thought that this resulted from historical contamination with endocrine disrupting pesticides, i.e. due to anti-androgens and not oestrogens.

Volker *et al.* (2016) examined advanced water treatments for the removal of EDCs and found that activated sludge removed >59-91%. However, high anti-androgenic activity persisted in the final effluent following activated sludge treatment, despite the fact that several of the known anti-androgens are hydrophobic and hence should be well removed by sorption to the sludge particles, e.g. the antibacterial and antifungal agent, triclosan. Not only was anti-androgenic activity ineffectively removed by the activated sludge, but at some of the sampling periods anti-androgenic activity was formed; either activity pre-treatment was masked by androgens or degradation resulted in more active compounds. Similarly, Bain *et al.* (2014) also found an increase in anti-androgenic activity after secondary treatment compared with influent and concluded that androgens masked the anti-androgenic activity in the influent. There are also reports of androgenic and oestrogenic activity being almost completely removed by the wastewater treatment process but that anti-androgenic compounds resist removal and are more likely to then enter our rivers, even with the addition of advanced treatments (Gehrmann *et al.*, 2018). As for the bile samples where glucuronide conjugates were easy to deconjugate and sulphates were harder, so glucuronide steroid conjugates in effluent will be completely transformed in wastewater treatment whilst sulphate conjugates will only be partially removed (Kumar *et al.*, 2011).

Chemicals are present in the Swindon WwTW effluent and are having effects on fish. The most pronounced effects on FHM in the lab-based studies were the demasculinising effects (reduced male FPI) when exposed to the standard effluent in the lab. In the absence of plasma VTG induction in these male fish, the culprits are likely to be anti-androgenic rather than oestrogenic compounds. In the wild roach collected from the River Ray, there was a marked improvement following the GAC treatment; a reduction in the incidence and severity of intersex and a reduction in the plasma VTG concentration. Both are indicative of a marked decrease in the amount of oestrogens entering the River Ray, but a low incidence of intersex is still present and male VTG concentrations are still at least an order of magnitude above baseline concentrations. This work considers that chemicals enter the River Ray solely from the Swindon WwTW, but chemicals may also enter via non-point routes. These include

chemicals associated with agricultural land (e.g. herbicides, veterinary pharmaceuticals; Fairbairn *et al.*, 2016) and chemicals associated with buildings and roads (e.g. PAHs; Zgheib *et al.*, 2012), that may enter the River Ray through runoff. In addition, in February 2005 9800L of diesel spilled into the River Ray upstream of the sampling site (Smith *et al.*, 2010). Thus, both before and after the addition of the GAC to the WwTW, chemicals present in the river may also come from non-point sources.

In 2014, the GAC plant at the Swindon WwTW was switched off; GAC plants are expensive and energy intensive to run and their utility as a cost-effective treatment have been questioned (Baynes *et al.*, 2012; Gilbert, 2012a). It remains to be seen if endocrine disruption in fish living downstream of the effluent outfall will return to the levels seen in 2005. My next chapter sets out to identify anti-androgenic compounds that are present in WwTW effluents and river water and to use the AYAS to determine how potent the compounds present are.

3. *In vitro* testing of chemicals for (anti-)androgenic activity

3.1. Introduction

Endocrine disrupting chemicals in the environment are a cause for concern as they may be inadvertently affecting the reproductive health of wildlife and humans (reviewed by Colborn *et al.*, 1993; Hotchkiss *et al.*, 2008). For example, in humans, exposure to EDCs has been associated with delayed onset of puberty and cancer in women (Colborn *et al.*, 1993) and reduced sperm counts and increased rates of testicular cancer in men (Sharpe and Skakkebaek, 1993; Toppari *et al.*, 1996).

Most of the early research in this field concentrated on natural and man-made chemicals that mimic steroidal oestrogens (called xenoestrogens). However, the field of EDCs has now developed beyond oestrogens, and chemicals with other types of endocrine activity are also being studied (reviewed by Sumpter, 2005). Androgens are a major class of steroid hormones in vertebrates that have key functions in the development and maintenance of the male reproductive system, acting through binding to nuclear ARs or by interfering with the production of steroidogenic enzymes that catalyse the production of steroids (Gray *et al.*, 2006). Environmental chemicals can interfere with androgen action and disrupt these processes. For example, Howell *et al.* (1980) discovered that pulp mill effluent was responsible for the masculinisation of female mosquitofish, *Gambusia affinis holbrooki*, living downstream of where the effluent entered the river. Due to the complex nature of pulp mill effluent, the exact chemical(s) responsible for this masculinisation, an elongation of the anal fin, have yet to be identified (Kamali *et al.*, 2016; Parks *et al.*, 2001; Singh and Chandra, 2019). In another study, Larsson and Forlin (2002) observed male-biased sex ratios of eelpout collected from close to another pulp mill, and suggested this to be due to masculinising agents in the pulp mill effluent. Similarly, Orlando *et al.* (2004) reported that cattle feedlot effluent (containing 17 β-trenbolone, the active metabolite of the androgenic growth promotor trenbolone acetate) entering rivers was responsible for significant alterations in the reproductive biology of wild FHM, with a demasculinisation of male fish and a defeminisation of female fish. The demasculinisation of the male reproductive system has also been observed in the aquatic environment. For example, following a spill of DDT, which was metabolised to the potent anti-androgen p,p'-DDE, male alligators in Lake Apopka (Florida, USA) were found to be have a reduction in penis size and plasma T concentrations (Guillette *et al.*, 1996).

An increasing number of structurally diverse non-steroidal compounds are now being identified as AR agonists and/or antagonists (able to bind to the AR but block its transcriptional activity) in vertebrates. Whilst the modes of action of oestrogens and anti-

androgens differ, they can result in similar phenotypic effects (Filby *et al.*, 2007; Sohoni and Sumpter, 1998). Androgen antagonists that competitively bind to the AR and block the action of the endogenous androgens can disrupt the fine balance of oestrogens and androgens, resulting in an oestrogenic internal environment where androgenic activity is reduced, and oestrogens predominate (Sohoni and Sumpter, 1998). The resulting effects are suggestive of oestrogenic exposure. Many of these compounds are extensively used every day in personal care products, fire retardants, pesticides, food products and pharmaceutical products, and their high volume use has led to detectable concentrations in the environment (Liscio *et al.*, 2014; Urbatzka *et al.*, 2007). This has raised interest in characterising and identifying further contaminants that might interact with the AR, especially as there are growing concerns over the possible impacts on human health, such as reduced sperm production and altered genital development in males (Skakkebaek *et al.*, 2001). Whilst fewer chemicals appear to possess androgenic activity than possess oestrogenic activity, many oestrogen mimics, e.g. bisphenol A, have also been shown to have some structural features that give them anti-androgenic activity as well (Paris *et al.*, 2002; Sohoni and Sumpter, 1998).

Traditional animal-based testing methods are slow and costly, and the low efficiency and high cost have generated a huge backlog of untested existing chemicals, and untested new chemicals awaiting approval prior to entering production (Zhang *et al*., 2018). There is a need to develop new approach methodologies that satisfy the regulatory requirements and are acceptable and affordable to society (Fischer *et al*., 2020).

New approaches that support the humane treatment of experimental animals through the 3R principles (reduction, refinement, and replacement; Russell and Burch, 1959), with a shift away from traditional animal studies to target-specific, mechanism-based, biological observations, are largely obtained using *in vitro* assays (Tice *et al*., 2013). Although *in vivo* assays such as the rat pubertal and Hershberger assay remain “gold standard” for determining androgenic activity, multiple *in vitro* assays for detecting endocrine activity have now been developed for assessing chemical toxicity and health risk (Szafran *et al*., 2020). *In vitro* studies include fewer confounding factors, and are suitable for determining the direct effects of chemicals on a specific tissue or cell type (Celino-Brady *et al*., 2021). Whilst *in vitro* methods can be carried out using HTP screening and are cheaper in comparison with animal models, they must be used in conjunction with *in vivo* testing as existing *in vitro* systems cannot fully take into account bioaccumulation, metabolism, and availability to the target cell, or alternative pathways for endocrine disruption.

Most *in vitro* methods of compound screening use receptor-ligand binding assays, transient reporter gene assays (nucleic acids introduced into transfected cells that are not permanently incorporated into the cellular genome), mammalian cell lines that have been stably transfected with the androgen-responsive reporter gene (the plasmid DNA successfully integrated into the cellular genome and passed on to future generations of the cell), or recombinant yeast assays. All these assays have limitations. Screening for receptor-ligand binding is not able to discern whether a chemical has the ability to act as an agonist or an antagonist (Schapaugh *et al.*, 2015). Transient reporter gene assays do not reflect physiological conditions because target DNA sequences are overexpressed, and the transfections maintain their responsiveness for only a limited time (Terouanne *et al.*, 2000). These assays often produce highly variable results due to different transfection efficiencies from replicate to replicate (Wilson *et al.*, 2002).

Currently there are several reliable *in vitro* tests for (anti-)androgenic activity, and these are generally based on either stably transfected mammalian cell lines (Korner *et al.*, 2004) or yeast cells (Sohoni and Sumpter, 1998). Mammalian cell lines have been stably transfected with androgen-responsive reporter genes and are widely used, for example, the human prostatic PALM cell line (Terouanne *et al.*, 2000), the human AR CALUX cell line (Sonneveld *et al.*, 2005), and the MDA-kb2 cells stably expressing the androgen responsive luciferase reporter gene (Wilson *et al.*, 2002). Stable reporter gene assays have been described for androgens but these sometimes use slow growing prostatic cell lines or are not selective in their response because of expression of other nuclear receptors (Sonneveld *et al.*, 2005; Szafran *et al.*, 2020), for example, the expression of PRs or GRs in addition to the AR (CHO-AR-Luc, Roy *et al.*, 2004; PALM, Terouanne *et al.*, 2000; MDA-kb2, Wilson *et al.*, 2002). Yeast assay systems have some advantages, for example, increased robustness, easier handling and low cost of reagents (Lopreside *et al.*, 2019). They do also have some drawbacks, such as the poor transport of substances across the cell wall (Gaido *et al.*, 1997) and limited metabolic capability, although Beresford *et al.* (2000) found their YES to have the means to metabolise methoxychlor. Whilst some chemicals can be converted to either more or less active metabolites, neglecting to determine metabolism can lead to both false negative and false positive results. The relevance of metabolic activation is widely recognised, especially as most immortalised cell lines have very low metabolic competence (van Vugt-Lussenburg *et al*., 2018). This was addressed by van Vugt-Lussenburg *et al*. (2018) who successfully incorporated metabolic enzymes to improve predictability of reporter gene assay results with the CALUX system, and found the endocrine activity of 23 of the 27 chemicals tested to be affected by metabolism.

Yeast-based assays can have lower sensitivity than similar assays using vertebrate cells (Lopreside *et al*., 2019; Wangmo *et al*., 2018). Therefore, extraction/preconcentration is generally required and this increases the assay time and may cause higher specific toxicity that could cause artefacts (Lopreside *et al*., 2019). However, whilst Leusch *et al*. (2017) found that yeast-based assays were somewhat less sensitive than their mammalian counterparts, the higher tolerance of yeast-based assays to solvents meant that the most sensitive yeast assay (Sohoni and Sumpter, 1998) was comparable to the mammalian reporter gene assays.

When testing some samples for antagonistic activity using the YAS and AR-CALUX assay, significant differences were seen between the YAS and AR-CALUX results, with the YAS showing strong antagonistic activity that was not replicated using the AR-CALUX (Mertl *et al.*, 2014). Mertl *et al.* (2014) considered the YAS results to be false positives resulting from interference with the yeast cell. Mansouri *et al.* (2020) found a significant discrepancy between *in vitro* AR activity and the results of the *in vivo* Hershberger assay, especially in the antagonist mode, and most of the discrepancies were because the *in vivo* activity occurred at internal concentrations well above the upper limit of testing in the *in vitro* assays. Challenging chemicals that give false positives (e.g. caused by cell stress, or only active in metabolically competent cells) are problematic when only a few assays are carried out, and a battery of tests is therefore required (Judson *et al*., 2017).

In 1998, the OECD set about revising and developing a new Test Guidelines Programme for testing and assessing chemicals for endocrine disrupting activity (Huet, 2000). The framework consisted of five levels, each corresponding to a different level of biological complexity; Level 1 using existing data and non-test information, Level 2 utilising *in vitro* assays to provide mechanistic information and Levels 3-5 using *in vivo* test systems for a more definitive understanding of the effects. This OECD guidance document on standardised tests was updated in 2018 to reflect scientific advances in the use of test methods and assessment of the endocrine activity of chemicals (OECD, 2018d). The Level 2 *in vitro* assays provide data about selected endocrine mechanism(s)/pathway(s) using mammalian and non-mammalian methods. Assays to determine oestrogenic and androgenic activity use stably transfected mammalian cell lines; ER (ERα-HeLa-9903 and VM7Luc4E2 human cell lines) and AR (AR-EcoScreen, derived from a Chinese hamster ovary cell line). Recently, three bioassays for the determination of oestrogenic activity in water and wastewater have been standardised and have resulted in international standards (two yeast-based assays using *Saccharomyces cerevisiae* and *Arxula adeninivorans*, and one human cell-based reporter assay (ER-CALUX); Wangmo *et al*, 2018).

To more quickly and cost effectively evaluate potential EDCs, *in vitro* screening is shifting towards HTP screening, potentially as replacements for lower-throughput *in vitro* and *in vivo* tests (reviewed in Fischer *et al.*, 2020). These HTP screening techniques are now routinely used in combination with computational methods and information technology to investigate how chemicals interact with biological systems both *in vitro* and *in vivo* (e.g. Huang *et al.*, 2016; Szafran *et al.*, 2020; Thomas *et al.*, 2019). HTP assays, though, can be used to better capture the diverse array of chemicals currently in use and to allow the development of computational models to better predict *in vivo* EDC activity (Szafran *et al.*, 2020).

Recent work with ToxCast/Tox21 mammalian HTP assays showed a high degree of conservation between human and vertebrate ARs (McArdle *et al*., 2020). The high degree of cross-species conservation of structural and functional aspects of key endpoints (e.g. ER and AR) suggests that these systems should also be useful for nonmammalian vertebrates, at least at the level of screening and prioritising chemicals for endocrine activity (Ankley *et al*., 2016). Translating *in vitro* results to *in vivo* has been found to be complicated and Wangmo *et al.* (2018) found good correlations were only observed for those compounds acting via direct binding to the sex steroid receptor.

However, whilst HTP screening represents an efficient and standardised approach to chemical testing, there is uncertainty as to applicability of the data for hazard identification and risk assessments (Fay *et al.*, 2018). The adverse outcome pathway (AOP) framework has been proposed as a means to help provide this linkage (Kleinstreuer *et al*., 2016).

Knowledge of physiological and toxicological pathways has allowed the development of AOPs, which consist of a series of key events linking a molecular initiating event (e.g. receptor binding) to an adverse outcome (e.g. individual/population response) via a series of causal key event relationships (e.g. organ/tissue responses) (Martyniuk *et al*., 2020). The AOP framework combines relevant information and defines how measurable perturbations in response to environmental stressors lead to an adverse outcome or an event of regulatory concern (Fischer *et al*., 2020). One adverse outcome of concern is the interference of chemicals with sex hormone synthesis, regulation, and function, potentially disturbing reproduction and foetal development (WHO-UNEP, 2013).

The REACH legislation entered into force in 2007 and is a regulation of the EU adopted to increase the protection of human health and the environment from the risks that can be posed by chemicals. The REACH deadline for registering existing substances manufactured or imported in quantities from 1 to 100 tonnes per year in the EU was on 31 May 2018. Pre-registration a total of 145,297 unique substances/entries were submitted to the European Chemicals Agency, and since 2008 a total of 23,118 substances have been registered

(https://echa.europa.eu/information-on-chemicals/, downloaded 22/12/2020). Similarly, the US EPA TSCA Chemical Substance Inventory, first published in 1979, now lists more than 86,000 chemicals (https://www.epa.gov/tsca-inventory/about-tsca-chemical-substance-inventory).

These EDCs enter the aquatic environment via WwTW, and endocrine activity is regularly found when river water and sediments are extracted and tested in *in vitro* assays (reviewed in Wangmo *et al*., 2018). Some have sought to identify the chemicals responsible for the observed endocrine activity, but the chemicals identified often only account for a proportion of the activity (for example, Hill *et al.*, 2010; Rostkowski *et al.*, 2011; Tousova *et al.*, 2017). It would not be feasible to test environmental samples for all the registered substances in an attempt to identify the substances responsible for the endocrine disrupting activity, so a different approach needs to be adopted. As a result of the wide range of potential EDCs and the fact that they will be present in water as a complex mixture of contaminants, chemical analysis alone is insufficient to monitor EDCs. Instead, *in vitro* bioassays indicative of hormonal activity can be applied to assess endocrine activity in environmental waters (Leusch *et al*., 2017). Some environmental contaminants can act as antagonists which, if present in a sample, can reduce the agonist response *in vitro*, emphasising the importance of evaluating both agonism and antagonism in environmental samples. Further sample processing, such as fractionation, may help to separate the effect of agonists and antagonists (Leusch *et al*., 2017).

Prior to my thesis, Green (2014) set out to identify the anti-androgenic chemicals responsible for anti-androgenic activity observed in WwTW effluent and river samples from the UK, using an EDA approach (Desbrow *et al.*, 1998). The samples were extracted by solid phase extraction and then fractionated by HPLC. The fractions were subsequently tested in the YAS and AYAS, and positive fractions then underwent a broad scan by GC-MS to tentatively identify the constituent chemicals. The highest number of chemicals were detected in two of the effluent samples (1,063 and 982 chemicals), but only 16% and 25% of the chemicals could be identified by this analytical method and the mass spectral library. Many of the chemicals were of domestic origin, and included fatty acids, fragrances and flavourings, personal care products, and pharmaceuticals. Other industrial chemicals were also identified such as flame retardants and plasticisers.

In total 109 chemicals found in the river water and effluent samples were identified by Green (2014), and this chapter of my thesis aims to further characterise these chemicals; to find out what individual chemicals from WwTW effluents have (anti-)androgenic activity and how potent these chemicals are.

3.2. Materials and methods

3.2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (Dorset, UK), with the exception of diethyl phthalate (DEP), DEHP and dibutyl phthalate (DBP) that were purchased from Greyhound Chemical Service (Birkenhead, UK).

Positive control chemicals, DHT (≥99% pure) and flutamide (99% pure) were prepared in etOH (>99.7%, Hayman Speciality Products, Essex, UK), at 10^{-6}M and 10^{-3}M, respectively. Details of test chemicals are listed in Table 15. At the time of purchase, two of the chemicals were not available through Sigma-Aldrich, 12-methyl tetradecanoic acid methyl ester and 11-H-benzo(b)fluorene, and these chemicals were therefore not tested.

Unless otherwise stated, all test chemicals were prepared at a starting concentration of 10^{-2}M in etOH. Four chemicals were not soluble in 100% etOH and methyl nonadecanoate, carbamazepine and carbamazepine 10,11-epoxide were dissolved in 50:50 etOH:ethyl acetate (stock at $5x10^{-3}$M), and chrysene was dissolved in 25:75 etOH:ethyl acetate (stock at $2.5x10^{-3}$M). Ethyl acetate is known to dissolve some plastics at high concentrations. To mitigate this issue, the chemicals prepared in etOH:ethyl acetate were diluted in the first well of the plate into etOH to give a further 1:2 dilution of the stock, leading to concentrations of either $2.5x10^{-3}$M or $1.25x10^{-3}$M.

In initial yeast (anti-)androgen screens, some chemicals caused a reduction in yeast turbidity (i.e. they were toxic) and were diluted for repeat testing. Dodecanoic acid, 1H-benzimidazole, 2,4-di-tert-butylphenol, 4-chloro-3,5-dimethyl-phenol, 9H-fluorene, dehydroabietic acid, dihydromethyl jasmonate, diphenylacetic acid methyl ester and N-ethyl-4-methyl benzenesulphonamide were further diluted in etOH to a stock concentration of 10^{-3}M, and chlorophene, N,N-dimethyl-1-dodecanamine and terbutryn were diluted to a stock concentration of 10^{-4}M. For other chemicals, strong anti-androgenic activity in the initial screen was such that the chemical also had to be diluted to 10^{-3} or 10^{-4}M to be able to obtain the full dose-response curve (fluoranthene, triclosan, triphenyl phosphate and methyl triclosan).

All stocks were stored in sealed glass vials at 4 ^{o}C.

3.2.2. Recombinant yeast (anti-)androgen screens

The recombinant hAR yeast strain was developed by Glaxo Wellcome and details of the YAS and AYAS have been described previously (Sohoni and Sumpter, 1998; Tyler *et al.*, 2000).

In brief, yeast cells were transfected with the human AR gene together with expression plasmids; the androgen response element and the *lac-Z* gene encoding the enzyme β-gal. In the screen for agonistic activity, the yeast cells were incubated in medium containing the test chemical and substrate (chlorophenol red-β-D- galactopyranoside; CPRG), and active ligands induced β-gal expression. The β-gal was then secreted into the medium and caused the substrate to change colour from yellow to red, and this was measurable by absorbance. When screening for antagonist activity, DHT was added to the assay medium and the ability of the test chemical to inhibit the colour change was determined.

3.2.3. Recombinant yeast (anti-)androgen screen procedure

The medium components were prepared, and the standard assay procedure was followed (Sohoni and Sumpter, 1998; Tyler *et al.*, 2000). Chemicals were serially diluted in etOH and 10 µl volumes were transferred to 96-well flat-bottom plates (Sarstedt AG & Co, Nümbrecht, Germany) where the etOH was allowed to evaporate to dryness. Then, 200 µl medium containing CPRG and yeast (final cell number of 5×10^5 cells/ml) were added to each well. Included with every assay were two negative controls; plus and minus etOH. For the androgen screen the positive control, DHT, was included in each assay (stock concentration at 10^{-6}M and serially diluted in etOH to achieve final concentrations from 5×10^{-8}M to 2.44×10^{-11}M). Test chemicals, when diluted in the assay medium, resulted in a maximum well concentration of 5×10^{-4}M (those with solubility issues had a maximum well concentration of 2.5×10^{-4}M or 1.25×10^{-4}).

For antagonistic screens, DHT was added to all wells at a non-saturating concentration that raised the background to 65% of the maximal response (2×10^{-9}M) so as to distinguish between agonistic and antagonistic activity. The pharmaceutical anti-androgen, flutamide, was included as the positive control in every AYAS (stock solution at 10^{-3}M and serially diluted in etOH to achieve final concentrations of 5×10^{-5}M to 2.44×10^{-8}M in the wells after the addition of the medium). Three negative controls were included; plus and minus etOH with medium including DHT, and plus etOH and medium without DHT.

Table 15. This table gives details of all the chemicals tests, including alternate names, a description, the Catalogue Number, CAS Number, molecular weight, and purity:

No.	Chemical	Alternate name	Description	Cat. no.	CAS no.	Mol. wt.	Purity
1	Dimethyl adipate	Hexanedionic acid dimethyl ester	Fatty acid	186252	627-93-0	174.19	≥99%
2	Methyl heptadecanoate	Heptadecanoic acid methyl ester	Fatty acid	H4515	1731-92-6	284.48	≥99%
3	Methyl nonadecanoate	Nonadecanoic acid methyl ester	Fatty acid	N5377	1731-94-8	312.53	≥98%
4	Methyl Palmitate	Hexadecanoic acid methyl ester	Fatty acid	P5177	112-39-0	270.45	≥99%
5	Monomethyl phthalate	Methyl phthalate	Plasticiser	36926	4376-18-5	180.16	
6	Methyl palmitoleate	9-Hexadecanoic acid methyl ester	Fatty acid	P9667	1120-25-8	268.43	≥99%
7	Methyl octanoate		Fatty acid	260673	111-11-5	158.24	99%
8	Benzophenone		Photo initiator/UV absorber	442842	119-61-9	182.22	
9	12-Methyl tetradecanoic acid methyl ester		Fatty acid	M3664	5502-94-3	242.4	≥98%
10	N-Butylbenzene sulfonamide	NBSS	Plasticiser	B90653	3622-84-2	213.3	99%
11	Myristic acid	Tetradecanoic acid	Fatty acid	M3128	544-63-8	228.37	≥99%
12	Decanoic acid		Fragrance/flavouring	C1875	334-48-5	173.26	≥98%
13	Tris (2-butoxyethyl) phosphate		Plasticiser/flame retardant	130591	78-51-3	398.47	94%

No.	Chemical	Alternate name	Description	Cat. no.	CAS no.	Mol. wt.	Purity
14	Triphenylphosphine oxide		Flame retardant	T84603	791-28-6	278.28	98%
15	Dodecanoic acid	Lauric acid	Personal care product	L556	143-07-7	220.32	98%
16	Fluoranthene		PAH	423947	206-44-0	202.25	98%
17	Triclosan	5-chloro-2-(2-4-dichlorophenoxy) phenol	Antimicrobial	72779	3380-34-5	289.54	≥97%
18	Triphenyl phosphate		Plasticiser/flame retardant	241288	115-86-6	326.28	≥99%
19	Pyrene		PAH	82648	129-00-0	202.25	≥99%
20	Methyl triclosan		Metabolite/Antimicrobial	34228	4640-01-1	303.57	
21	HHCB	Galaxolide 1 and 2 isomers	Fragrance	W520608	1222-05-5	258.4	50% in diethyl phthalate
22	Pentadecanoic acid		Fatty acid	P6125	1002-84-2	242.4	~99%
23	Methyl myristate	Tetradecanoic acid methyl ester	Flavouring/detergent intermediate/surfactant	M3378	124-10-7	242.4	≥99%
24	Methyl decanoate	Decanoic acid methyl ester	Fatty acid	W505501	110-42-9	186.29	≥99%
25	Methyl pentadecanoate	Pentadecanoic acid methyl ester	Fatty acid	P6250	7132-64-1	256.42	98.5%
26	Methyl stearate	Octadecanoic acid methyl ester	Fatty acid	S5376	112-61-8	298.5	~99%
27	3-Methylphenol		Precursor to pesticides	442391	108-39-4	108.14	
28	2-Ethyl-1-hexanol		Precursor for DEHP/emollient	08607	104-76-7	130.23	

No.	Chemical	Alternate name	Description	Cat. no.	CAS no.	Mol. wt.	Purity
29	Triethyl phosphate		Flame retardant/plasticiser	538728	78-40-0	182.15	≥99.8%
30	Phthalic anhydride		Precursor compound for plasticisers	320064	85-44-9	148.12	≥99%
31	Tributyl phosphate		Flame retardant/plasticiser	00675	126-73-8	266.31	≥99%
32	2-Ethylhexanoic acid		Paint and varnish driers/ plasticisers/stabilisers for PVC	538701	149-57-5	144.21	≥99%
33	Tris(2-chloroethyl) phosphate		Flame retardant	119660	115-96-8	285.49	97%
34	Benzo(a)pyrene		PAH	B1760	50-32-8	252.31	96%
35	Methyl oleate	cis-9-Octadecenoic methyl ester	Fatty acid	O4754	112-62-9	296.49	≥99%
36	Diethyl phthalate	DEP	Plasticiser	PT-20	84-66-2	222.24	97-99%
37	Chrysene		PAH	245186	218-01-9	228.29	98%
38	Isoproturon		Herbicide	36137	34123-59-6	206.28	
39	Di(2-ethylhexyl) phthalate	DEHP	Plasticiser	PT-7	117-81-7	390.56	97-99%
40	Dibutyl phthalate	DBP	Plasticiser	PT-17	84-74-2	278.34	97-99%
41	Chlorophene	2-Benzyl-4-chlorophenol	Preservative	548618	204-385-8	218.68	95%
42	11-H-benzo(b)fluorene		PAH	123595	243-17-4	216.28	98%
43	Phenobarbital		Barbiturate - sedative-hypnotic/ anticonvulsant	P1636	50-06-6	232.24	≥99%

No.	Chemical	Alternate name	Description	Cat. no.	CAS no.	Mol. wt.	Purity
44	Diclofenac sodium salt		Non-steroidal anti-inflammatory	D6899	15307-79-6	318.13	≥98%
45	Ibuprofen		Non-steroidal anti-inflammatory	I4883	15687-27-1	206.28	≥98%
46	Methyl 2-(4-isobutyl phenyl)propanoate	Ibuprofen methyl ester	Photoproduct of ibuprofen	CDS017839	61566-34-5	220.31	
47	Carbamazepine		Anticonvulsant/analgesic	C4024	298-46-4	236.27	≥98%
48	Carbamazepine 10,11-epoxide		Active metabolite of carbamazepine	C4206	36507-30-9	252.27	≥98%
49	1-(4-aminophenyl)-ethanone	1-(4-Aminophenyl) ethanone hydrochloride		CDS000448	41784-08-1	171.62	
50	1,4 Dioxane		Stabilizer for 1,1,1-trichloroethane used in aluminium containers	296309	123-91-1	88.11	99.8%
51	1-[4-(hydroxy-1-methylethyl)phenyl] ethanone			CDS011908	54549-72-3	180.2	
52	10-oxo-octadecanoic acid methyl ester	Methyl 10-undecenoate	Flavouring/odour removal	115126	111-81-9	198.3	96%
53	12-Methyl tetradecanoic acid methyl ester	Methyl 12-methylmyristate	Fatty acid	M3789	5129-66-8	256.42	≥97%
54	1-Butoxy-2-propanol	1-*tert*-Butoxy-2-propanol	Industrial solvent, cleaner, degreaser	433845	57018-52-7	132.2	99%
55	1H-Benzimidazole		Fungicide	194123	51-17-2	118.14	98%

No.	Chemical	Alternate name	Description	Cat. no.	CAS no.	Mol. wt.	Purity
56	2-(methylthio)benzothiazole		Vulcanisation accelerator/ degradation product of a biocide	168653	615-22-5	181.28	97%
57	2-(Methylthio)pyridine			07655	18438-38-5	125.19	≥95%
58	2,4,7,9-Tetramethyl-5-dicyne-4,7-diol	2,4,7,9-Tetramethyl-5-decyne -4,7-diol, mixture of (±) and *meso*	Surfactant	278386	126-86-3	226.36	98%
59	2,4-Di-tert-butylphenol	2,4-Di-*tert*-butylphenol	Intermediate in manufacture of UV stabiliser, pharmaceuticals & fragrances	137731	96-76-4	206.32	99%
60	2,6-Di-tert-butyl-p-benzoquinone		Agent to improve germination, plant health and yield	153931	719-22-2	220.31	98%
61	2-Ethylhexyl diphenyl phosphate		Plasticiser/Flame retardant	34064	1241-94-7	362.4	
62	2-Methyl butanoic acid methyl ester	Methyl 2-methylbutyrate	Flavouring	W271918	868-57-5	116.16	≥98%
63	2-Methyl-5-(1-methylethenyl)-2-cyclohexen-1-one	(*R*)-(−)-Carvone	Fragrance/flavouring	124931	6485-40-1	150.22	98%
64	2-Methyl-5-(1-methyl ethenyl)-2-cyclohexen-1-one	(*S*)-(+)-Carvone	Fragrance/flavouring	435759	2244-16-8	150.22	96%

No.	Chemical	Alternate name	Description	Cat. no.	CAS no.	Mol. wt.	Purity
65	2-Methylbutanoic acid	Isovaleric acid	Fragrance/flavouring	129542	503-74-2	102.13	99%
66	2-Phenyl-2-propanol		Fragrance/catalyst/solvent	P30802	617-94-7	136.19	97%
67	2-Toluene sulphonamide	*o*-Toluenesulfonamide	Intermediate for production of saccharin/plasticiser	257990	88-19-7	171.22	99%
68	3-(4-methoxyphenyl)-2-propenoic acid 2ethylhexyl ester	2-Ethylhexyl *trans*-4-methoxycinnamate	UV absorber	437174	83834-59-7	290.4	98%, contains BHT as stabiliser
69	3,3,5-Trimethyl-2-cyclohexen-1-one	Isophorone	Solvent in printing inks, paints etc./ intermediate	I18709	78-59-1	138.21	97%
70	3,4-Dimethyl-2,5-furandione	2,3-Dimethylmaleic anhydride	Intermediate	D167800	766-39-2	126.11	98%
71	3,7-Dimethyl-1,6-Octadien-3-ol	Linalool	Fragrance	L2602	78-70-6	154.25	97%
72	4-Chloro-3,5-dimethyl-phenol		Antimicrobial	C38303	88-04-0	156.61	99%
73	4-hydroxy-3-methoxy-benzaldehyde	Vanillin	Flavouring/fragrance	V1104	121-33-5	152.15	99%
74	4-Methoxycinnamic acid		Synthesis of pharmaceutical intermediates/cosmetics UV absorption	65420	943-89-5	178.18	≥98%
75	4-Methoxymethylphenol		Flavouring	W267104	93-51-6	138.16	≥98%
76	4-tert-Butylcyclohexanone		Fragrance	B92303	98-53-3	154.25	99%

No.	Chemical	Alternate name	Description	Cat. no.	CAS no.	Mol. wt.	Purity
77	6,10-Dimethyl-5,9-undecadien-2-one		Fragrance/flavouring	W354201	3796-70-1	194.31	≥97%
78	9,12-Octadecadienoic acid methyl ester	*trans*-9,12-Octadecadienoic acid methyl ester	Fatty acid	46951-U	2566-97-4	294.5	10 mg/ml in heptane
79	9H-Fluorene		PAH	128333	86-73-7	166.22	98%
80	Acetyl tri-n-butyl citrate	Tributyl *O*-acetylcitrate	Plasticiser/flavouring	388378	77-90-7	402.48	98%
81	Acetylcedrene	Methyl cedryl ketone	Fragrance	W522805	32388-55-9	246.39	
82	alpha cedrol	Cedrol	Fragrance	W521418	77-53-2	222.37	
83	Benzaldehyde		Flavouring	B1334	100-52-7	106.12	≥99%
84	Benzeneacetaldehyde	Phenylacetaldehyde	Fragrance/flavouring	107395	122-78-1	120.15	≥90%
85	Benzothiazole		Precursor of vulcanisation accelerator, flavouring, antimicrobial	101338	95-16-9	135.19	96%
86	Butanedioic acid dimethyl ester	Dimethyl succinate	Flavouring	W239607	106-65-0	146.14	98%
87	Camphor		Preparation of mothballs/ plasticiser	148075	76-22-2	152.23	96%
88	Dehydroabietic acid		Synthesis of plastics (PP), surfactants	SMB00089	1740-19-8	300.44	≥95%
89	Dihydromethyl jasmonate	Methyl dihydrojasmonate, mixture of *cis* and *trans*	Fragrance	W340804	24851-98-7	226.31	≥96%
90	Dihydromyrcenol	Dihydromyrcenol	Fragrance	W516406	18479-58-8	156.27	≥99%

No.	Chemical	Alternate name	Description	Cat. no.	CAS no.	Mol. wt.	Purity
91	Diphenylacetic acid methyl ester	Methyl diphenylacetate	Chemical, organic intermediate	386359	3469-00-9	226.27	99%
92	Hexamethylbenzene		Pharmaceutical intermediate	322377	87-85-4	162.27	99%
93	Hexamethyldisiloxane		Solvent in organic synthesis	52630	107-46-0	162.38	≥98.5%
94	Hexanal dimethyl acetal	1,1-Dimethoxyhexane	Fragrance/flavouring	547174	1599-47-9	146.23	98%
95	Ketoisophorone	4-Oxoisophorone	Fragrance/flavouring	W342106	1125-21-9	152.19	≥98%
96	Methyl propyl ketone	2-Pentanone	Surface coating/flavouring	471194	107-87-9	86.13	99.5%
97	Methylbenzaldehyde	*p*-Tolualdehyde	Fragrance/flavouring/ pharmaceutical & dyestuff intermediate	W306800	104-87-0	120.15	≥97%
98	N,N-Dimethyl-1-dodecanamine	*N*,*N*-Dimethyldodecylamine	Raw material for surfactants, germicides, and bactericides	284386	112-18-5	213.4	97%
99	N-ethyl-4-methyl benzenesulphonamide	*N*-Ethyl-*p*-toluenesulfonamide	Plasticiser	415367	80-39-7	199.27	98%
100	Nonanal		Fragrance	N30803	124-19-6	142.24	95%
101	Nonanoic acid methyl ester	Methyl nonanoate	Fragrance/flavouring	76368	1731-84-6	172.26	
102	N-tert-Butylacrylamide		Production of polymers/ indirect food additive	411779	107-58-4	127.18	97%
103	Octamethylcyclotetrasiloxane		Used in production of silicone/ textiles/personal care products	235695	556-67-2	296.62	98%

No.	Chemical	Alternate name	Description	Cat. no.	CAS no.	Mol. wt.	Purity
104	Phenoxyacetic acid methyl ester	Methyl phenoxyacetate	Pharmaceutical intermediate	275638	2065-23-8	166.17	99%
105	Propyl benzene	(2,2-Dimethyl-1-propyl) benzene	Fuel/fuel additive	359076	1007-26-7	148.24	≥97%
106	Squalene		Moisturiser in cosmetics/ adjuvant in vaccines	S3626	111-02-4	410.72	≥98%
107	Terbutryn		Herbicide/pesticide	45677	886-50-0	241.36	
108	Tridecanoic acid methyl ester	Methyl tridecanoate	Fatty acid	T0627	1731-88-0	228.37	≥97%
109	Xylene isomer	Xylenes	Fuel/solvent in pharmaceutical, printing, rubber, and leather industries	247642	1330-20-7	106.17	≥98.5%

The plates were taped closed and shaken for 2 mins on a plate shaker. The plates were then incubated at 32 ^{o}C for 36 hours followed by 36 hours at room temperature. After the incubation period, absorbance readings were taken at 540 and 620 nm using a Spectramax 340PC microplate reader (Molecular Devices Limited, Wokingham, UK). The second absorbance (620 nm) was a measure of cell density and hence yeast growth, and this was especially important as high concentrations of some chemicals can inhibit the growth of the yeast cells or cause cell lysis (producing clear yellow wells). The absorbance values were corrected for cell density using to the following equation:

Corrected value = $chemical_{540nm}$-($chemical_{620nm}$-etOH $blank_{620nm}$).

All chemicals were tested in duplicate. For initial screenings, chemicals were not tested in the androgen screen, because in the AYAS, by raising the background to 65% of the maximal response, it is possible to distinguish between agonistic and antagonistic activity. For later experiments, chemicals were tested in both the YAS and AYAS.

Where activity was observed in the first test, the chemicals were retested. Active chemicals were tested between 2 and 7 times in a total of 13 YAS and 16 AYAS.

The EC50 for the positive control, DHT, was calculated using 4-parameter plot equations produced with SoftMax Pro version 5.0.1 (Molecular Devices Limited, Wokingham, UK). Similarly, the IC50 was calculated as the concentration of flutamide producing 50% inhibition of the DHT-induced activity.

Potency values were calculated by dividing the flutamide IC50 by the chemical IC50, and the higher the number the more potent the chemical relative to flutamide.

Potencies for anti-androgens are given the following symbols:

+	very weak; <0.02 times the potency of flutamide
++	weak; 0.02-0.199 times the potency of flutamide
+++	moderate; 0.2-5 times the potency of flutamide
++++	strong; >5 times the potency of flutamide

The anti-androgenic activity of both 9H-fluoranthene and acetylcedrene ‘crept’ across the assay plate (i.e. activity was found in wells not directly exposed to the test chemical) and these 2 chemicals had to be retested on separate plates to make sure this behaviour did not impact on other test chemicals or controls.

Where chemicals were toxic, resulting in clear yellow wells, the stocks were further diluted, and the chemicals were retested at a lower concentration.

3.3. Results

Photos to demonstrate typical multiwell plates with positive control standard curves (YAS; DHT, AYAS; flutamide) and negative control (etOH) can be seen in Figure 43.

The mean EC50 for the 13 androgen experiments was $9.49x10^{-10}$M ± $4.87x10^{-11}$M or 275 ± 14 ng/L, as indicated by the dotted line in Figure 44. The mean IC50 for the 16 AYAS experiments was $3.78x10^{-6}$M ±$3.13x10^{-7}$M or 1066 ± 118µg/L, as indicated by the dotted line in Figure 45. See Appendix Tables A 27 and A 28 for raw data.

Examples of the 4-parameter plots used to calculate DHT positive control EC50s and flutamide positive control IC50s are shown in Figure 46.

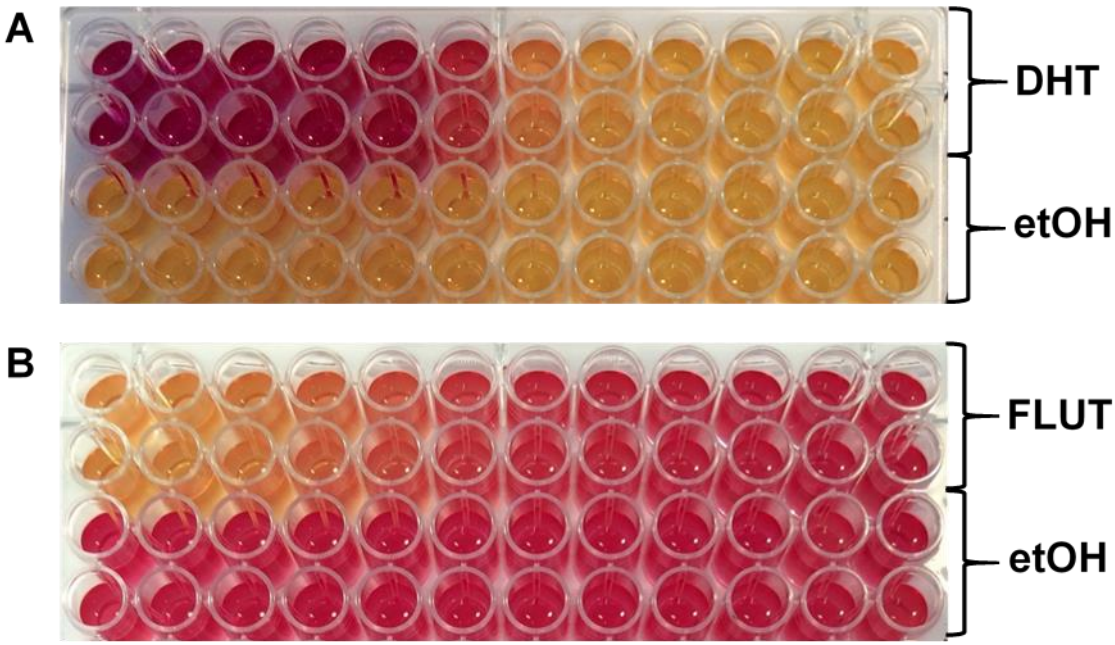


Figure 43. Photos showing typical yeast screen results after 3 days incubation with the chromogenic substrate CPRG. (A) Androgen screen; the positive control, dihydrotestosterone (DHT), standard curve with red wells (left hand side) at highest concentrations caused by β-gal converting the yellow CPRG substrate to chlorophenol red, and the negative control, ethanol (etOH) remaining yellow in the absence of β-gal. (B) Anti-androgen screen (all well containing DHT); the positive control, flutamide (FLUT) standard curve, with red wells at all but the highest flutamide concentrations where the flutamide is blocking the AR receptor so that no β-gal is released, and the wells remain yellow. For the negative control (etOH) all the wells have turned red due to the presence of DHT.

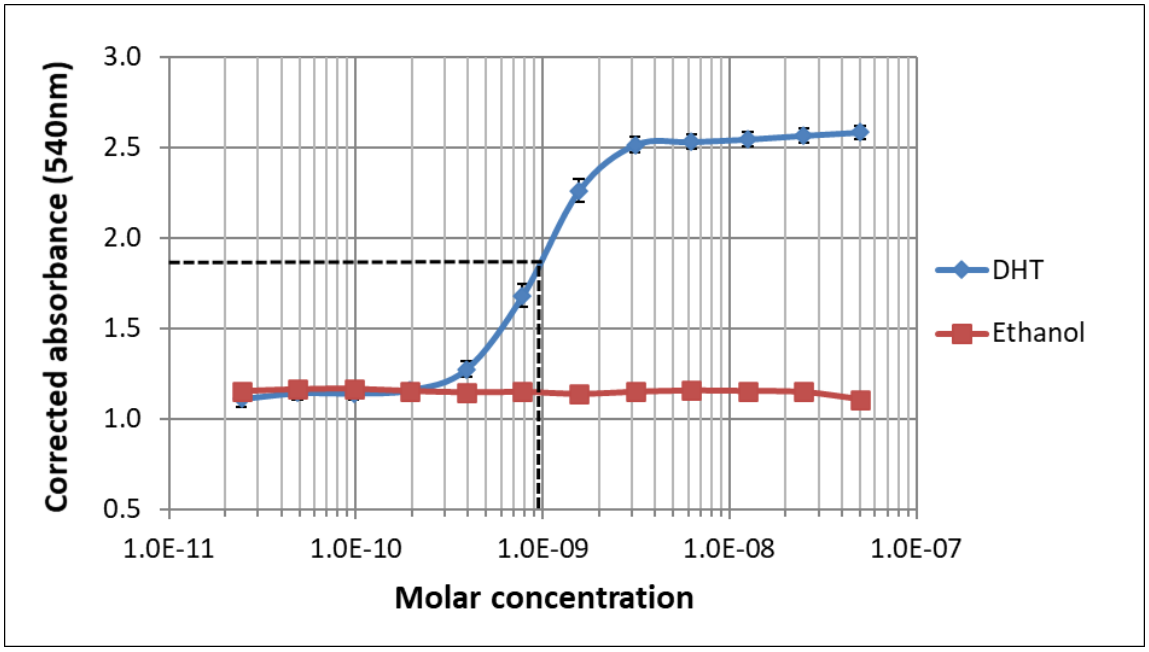


Figure 44. Response of the yeast androgen screen to dihydrotestosterone (DHT) positive control and ethanol negative control (mean ± SE). The mean EC50 for the 13 experiments was 9.49×10^{-10}M ± 4.87×10^{-11}M or 275 ± 14 ng/L, as indicated by the dotted line (see Appendix Table A 28 for raw data).

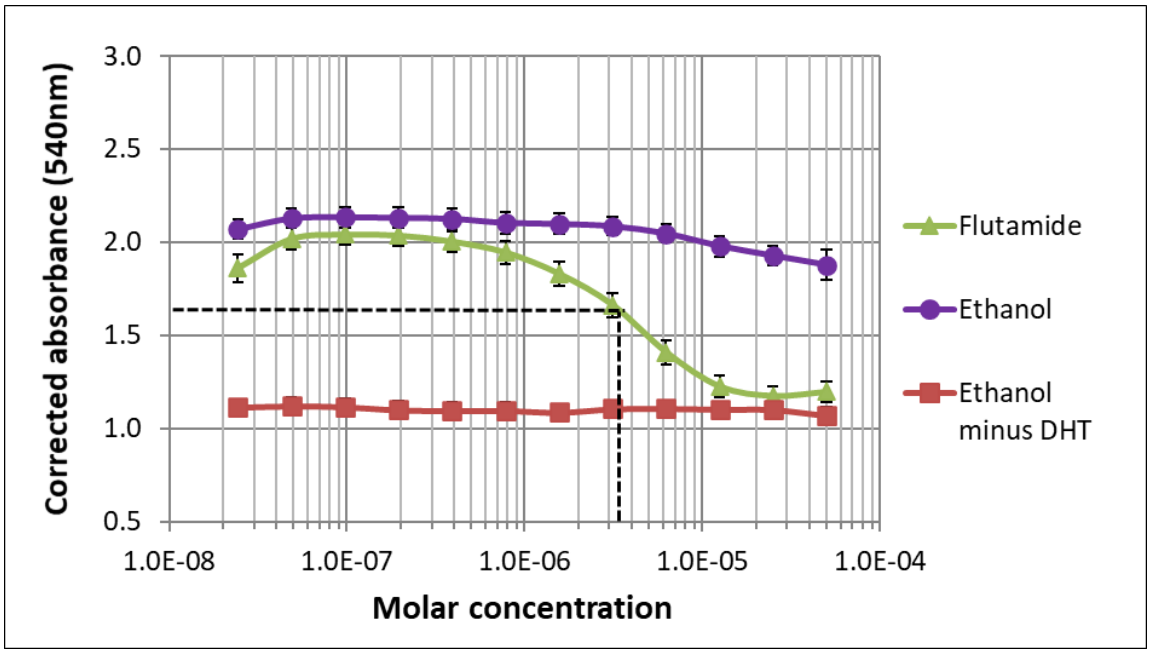


Figure 45. Response of the yeast anti-androgen screen to flutamide positive control and ethanol negative controls (ethanol and ethanol minus DHT in the medium) (mean ± SE; mean ± SE). The IC50 for the 16 experiments was 3.78×10^{-6}M ±3.13×10^{-7}M or 1066 ± 118 µg/L, as indicated by the dotted line (see Appendix Table A28 for raw data).

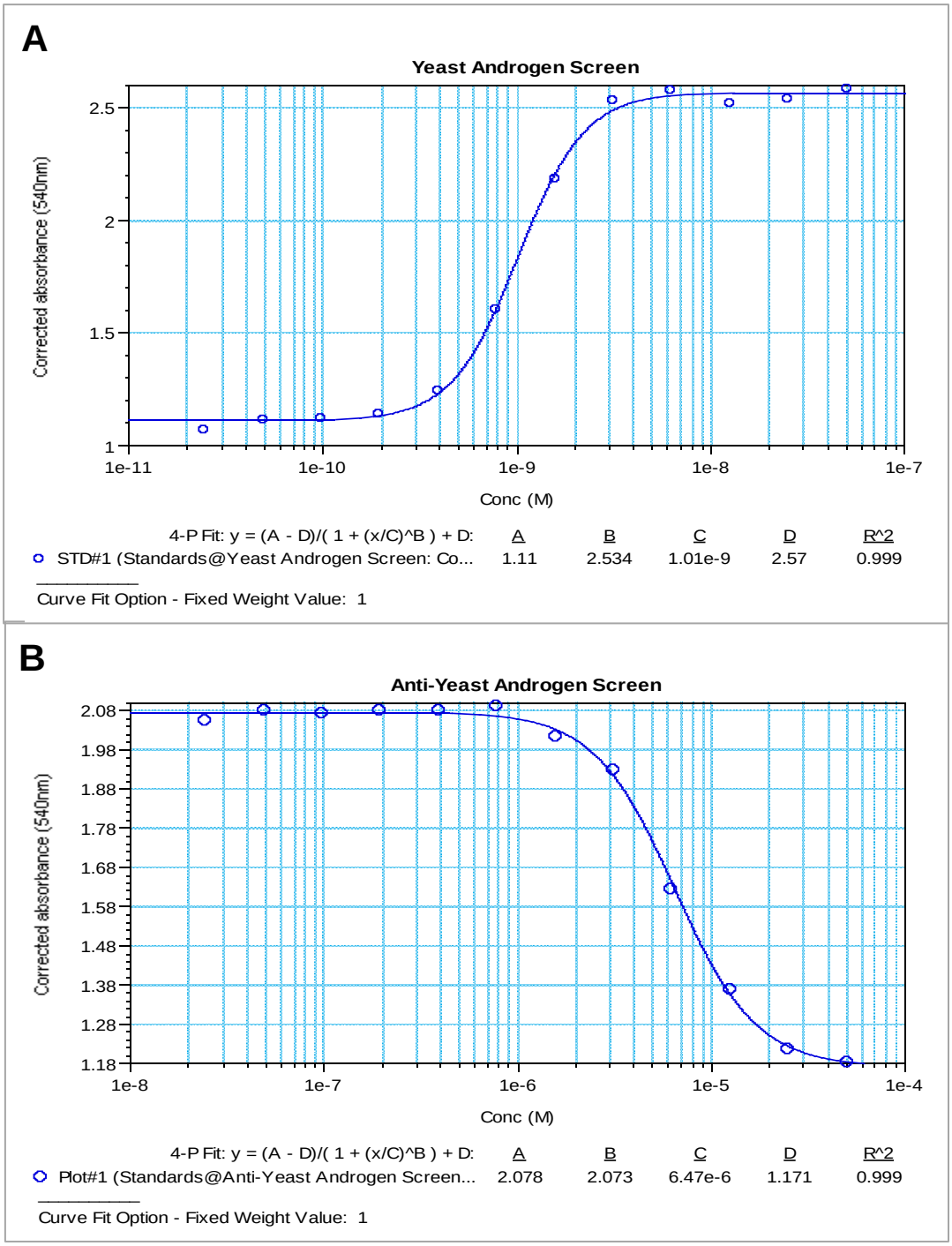


Figure 46. Examples of standard curves produced using SoftMax Pro version 5.0.1 (Molecular Devices Limited, Wokingham, UK). (A) Androgen screen positive control DHT standard curve and (B) Anti-androgen screen positive control flutamide standard curve. A 4-parameter fit has been applied to each curve. The C-value gives the point of inflection; (A) the EC50, and (B) the IC50.

3.3.1. Possible androgens

The results of all yeast experiments are presented in Table 16. Of the 107 chemicals tested, only three showed androgenic activity in the YAS; methyl decanoate, 2-ethylhexanoic acid and ibuprofen (Figure 47). All three chemicals were very weak androgens with a maximal absorbance below the DHT EC50 and therefore potency measurements could not be calculated, although by extrapolation of the curves the potencies of these chemicals would have been at least a million times less than that of DHT. The highest ibuprofen concentrations (above 10^{-4}M) showed a reduction in 540nm absorbance readings and this was accompanied by a reduction in turbidity (620nm) in these wells, indicating toxicity.

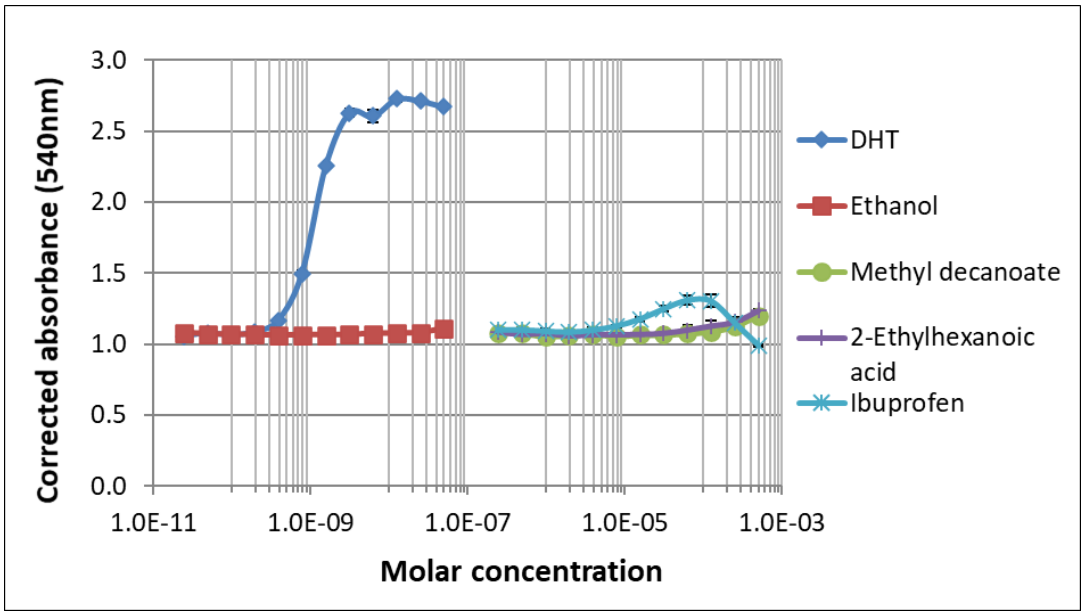


Figure 47. Yeast androgen results showing plots for the DHT positive control, ethanol negative control, and the 3 test chemicals showing very weak androgenic activity; methyl decanoate, 2-ethylhexanoic acid and ibuprofen. Each plot is the mean of duplicate values ± standard error.

3.3.2. Possible anti-androgens

In the AYAS, there was a reduction in the yeast turbidity at the highest concentrations for 18 chemicals and when this was observed it is mentioned in Table 16. Twelve of these 18 chemicals (tris (2-butoxyethyl) phosphate, triphenylphosphine oxide, HHCB, tributyl phosphate, 1H-benzimidazole, 2,4-di-tert-butylphenol, 4-chloro-3,5-dimethyl-phenol, dihydromethyl jasmonate, N,N-Dimethyl-1-dodecanamine, N-ethyl-4-methyl benzenesulphonamide, terbutryn, and tridecanoic acid methyl ester) appeared to be anti-androgenic, although the raised background was accompanied by a noticeable reduction in turbidity, indicating that the chemicals were likely to be toxic. This was confirmed when these chemicals were retested at a lower (non-toxic) concentration and the apparent anti-androgenic activity was no longer present.

Twenty of the chemicals showed anti-androgenic activity (Table 16; benzophenone, N-butylbenzene sulphonamide, myristic acid, dodecanoic acid, fluoranthene, triclosan, triphenyl phosphate, pyrene, methyl triclosan, chlorophene, 1-[4-(hydroxy-1-methylethyl)phenyl] ethanone, 2-(methylthio)benzothiazole, 9,12-octadecadienoic acid methyl ester, 9H-fluorene, acetylcedrene, alpha cedrol, benzeneacetaldehyde, dehydroabietic acid, diphenylacetic acid methyl ester, and hexamethylbenzene), although for two of these chemicals repeat assays gave inconsistent results (N-butylbenzene sulphonamide and triphenyl phosphate).

Of the chemicals showing anti-androgenic activity, fluoranthene, triclosan, methyl triclosan and chlorophene were the most potent, with a potency great than the positive control, flutamide. Triphenyl phosphate, pyrene, 9H-fluorene, and dehydroabietic acid had similar potency values to flutamide. Benzophenone, N-butylbenzene sulphonamide, myristic acid, dodecanoic acid, 2-(methylthio)benzothiazole, acetylcedrene, alpha cedrol, and diphenylacetic acid methyl ester were less potent than flutamide, and 1-[4-(hydroxy-1-methylethyl)phenyl] ethanone, 9,12-octadecadienoic acid methyl ester, benzeneacetaldehyde, and hexamethylbenzene had only very weak activity.

An example of four of these chemicals with anti-androgenic activity in the AYAS can be seen in Figure 48.

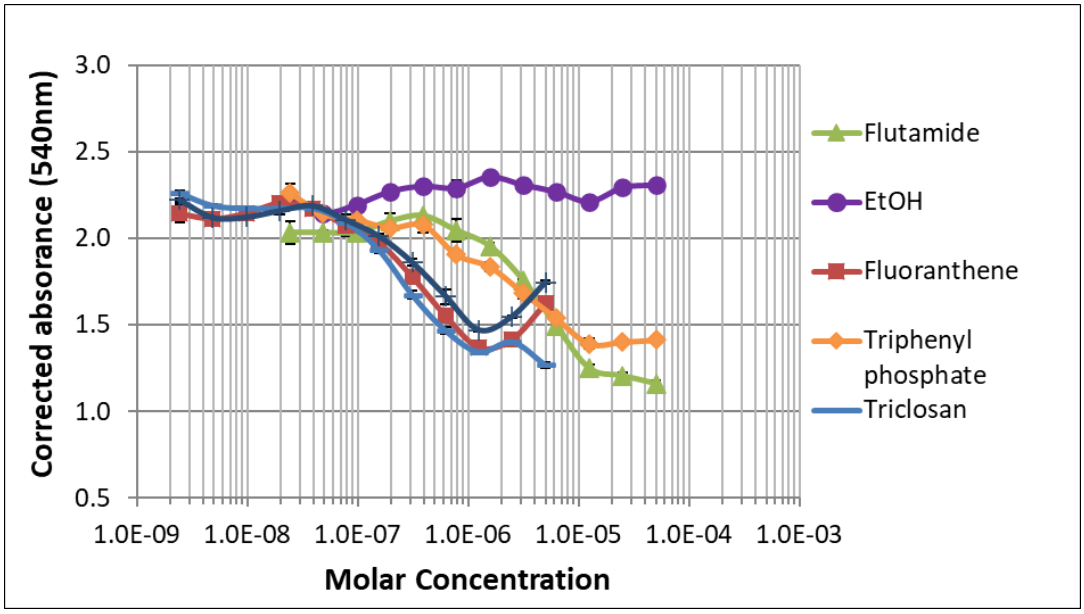


Figure 48. Yeast anti-androgen results showing plots for the flutamide positive control and ethanol (etOH) negative control, together with 4 of the test chemicals showing anti-androgenic activity; fluoranthene, triphenyl phosphate, triclosan and methyl triclosan. Each plot is the mean of duplicate values ± standard error.

3.3.3. Possible superagonists

For twelve of the chemicals, when co-administered with DHT in the AYAS, there was a heightened response above the raised background, suggesting that these 12 chemicals possessed androgenic activity. As the purpose of this testing was to look for (anti-)androgenic activity, the chemicals were then tested in the YAS and not retested in the AYAS. On testing these twelve chemicals in the YAS, no androgenic activity was observed. This heightened response above the DHT raised background is normally indicative of androgenic activity, and hence here we term this response as 'superagonism' (Table 16; dimethyl adipate, methyl heptadecanoate, methyl palmitate, monomethyl phthalate, methyl octanoate, decanoic acid, methyl myristate, methyl pentadecanoate, 2-ethyl-1-hexanol, methyl oleate,4-methoxycinnamic acid, and methylbenzaldehyde).

Table 16. Results of all chemicals tested in the yeast (anti-)androgen. Potencies for anti-androgens are given the following symbols; + (very weak; <0.02 times the potency of flutamide), ++ (weak; 0.02-0.199 times the potency of flutamide), +++ (moderate; 0.2-5 times the potency of flutamide), and ++++ (strong; >5 times the potency of flutamide), and for inconsistent results the symbols are in brackets.

No.	Chemical	Cas no.	Androgen screen	Anti-androgen screen	Comments
1	Dimethyl adipate	627-93-0	No activity	Superagonist	
2	Methyl heptadecanoate	1731-92-6	No activity	Superagonist	
3	Methyl nonadecanoate	1731-94-8	No activity	No activity	
4	Methyl Palmitate	112-39-0	No activity	Superagonist	
5	Monomethyl phthalate	4376-18-5	No activity	Superagonist	
6	Methyl palmitoleate	1120-25-8	Not tested	No activity	
7	Methyl octanoate	111-11-5	No activity	Superagonist	
8	Benzophenone	119-61-9	Not tested	**++**	Reduced turbidity at 10^{-2}M
9	12-Methyl tetradecanoic acid methyl ester	5502-94-3			Not tested
10	N-Butylbenzene sulfonamide	3622-84-2	Not tested	**(++)**	Inconsistent results
11	Myristic acid	544-63-8	Not tested	**++**	Toxic at 10^{-2}M
12	Decanoic acid	334-48-5	No activity	Superagonist	
13	Tris (2-butoxyethyl) phosphate	78-51-3	Not tested	No activity at 10^{-3}M	Top 2 wells at 10^{-2}M - reduction in turbidity
14	Triphenylphosphine oxide	791-28-6	Not tested	No activity at 10^{-3}M	Top 2 wells at 10^{-2}M - reduction in turbidity
15	Dodecanoic acid	143-07-7	Not tested	**++**	Toxic at 10^{-2}M
16	Fluoranthene	206-44-0	Not tested	**++++**	
17	Triclosan	3380-34-5	Not tested	**++++**	
18	Triphenyl phosphate	115-86-6	Not tested	**(+++)**	Inconsistent results
19	Pyrene	129-00-0	Not tested	**+++**	

No.	Chemical	Cas no.	Androgen screen	Anti-androgen screen	Comments
20	Methyl triclosan	4640-01-1	Not tested	**++++**	
21	HHCB	1222-05-5	Not tested	No activity at 10^{-3}M	Top 2 wells at 10^{-2}M - reduction in turbidity
22	Pentadecanoic acid	1002-84-2	No activity	No activity	
23	Methyl myristate	124-10-7	No activity	Superagonist	
24	Methyl decanoate	110-42-9	**+**	No antagonistic activity	
25	Methyl pentadecanoate	7132-64-1	No activity	Superagonist	
26	Methyl stearate	112-61-8	Not tested	No activity	
27	3-Methylphenol	108-39-4	No activity	No activity	
28	2-Ethyl-1-hexanol	104-76-7	No activity	Superagonist	
29	Triethyl phosphate	78-40-0	No activity	No activity	
30	Phthalic anhydride	85-44-9	No activity	No activity	
31	Tributyl phosphate	126-73-8	Not tested	No activity at 10^{-3}M	Top 2 wells at 10^{-2}M - reduction in turbidity
32	2-Ethylhexanoic acid	149-57-5	**+**	No antagonistic activity	
33	Tris(2-chloroethyl) phosphate	115-96-8	Not tested	No activity	
34	Benzo(a)pyrene	50-32-8	Not tested	No activity	
35	Methyl oleate	112-62-9	No activity	Superagonist	
36	Diethyl phthalate	84-66-2	Not tested	No activity	
37	Chrysene	218-01-9	Not tested	No activity	
38	Isoproturon	34123-59-6	Not tested	No activity	
39	Di(2-ethylhexyl) phthalate	117-81-7	Not tested	No activity	
40	Dibutyl phthalate	84-74-2	Not tested	No activity	
41	Chlorophene	204-385-8	Not tested	**++++**	Toxic at 10^{-3}M

No.	Chemical	Cas no.	Androgen screen	Anti-androgen screen	Comments
42	11-H-benzo(b)fluorene	243-17-4			Not tested
43	Phenobarbital	50-06-6	Not tested	No activity	
44	Diclofenac sodium salt	15307-79-6	Not tested	No activity	
45	Ibuprofen	15687-27-1	+	No antagonistic activity	
46	Methyl 2-(4-isobutyl phenyl)propanoate	61566-34-5	Not tested	No activity	
47	Carbamazepine	298-46-4	Not tested	No activity	
48	Carbamazepine 10,11-epoxide	36507-30-9	Not tested	No activity	
49	1-(4-aminophenyl)-ethanone	41784-08-1	No activity	No activity	
50	1,4 Dioxane	123-91-1	No activity	No activity	
51	1-[4-(hydroxy-1-methylethyl) phenyl] ethanone	54549-72-3	No activity	+	
52	10-oxo-octadecanoic acid methyl ester	111-81-9	No activity	No activity	
53	12-Methyl tetradecanoic acid methyl ester	5129-66-8	No activity	No activity	
54	1-Butoxy-2-propanol	57018-52-7	Not tested	No activity	
55	1H-Benzimidazole	51-17-2	No activity	No activity at 10^{-3}M	Reduced turbidity at 10^{-2}M.
56	2-(methylthio)benzothiazole	615-22-5	No activity	++	
57	2-(Methylthio)pyridine	18438-38-5	No activity	No activity	
58	2,4,7,9-Tetramethyl-5-dicyne-4,7-diol	126-86-3	No activity	No activity	

No.	Chemical	Cas no.	Androgen screen	Anti-androgen screen	Comments
59	2,4-Di-tert-butylphenol	96-76-4	Toxic at 10^{-2}M. Retest at 10^{-3}M.	No activity when diluted to 10^{-3}M	Creeps/toxic at 10^{-2}M
60	2,6-Di-tert-butyl-p-benzoquinone	719-22-2	No activity	No activity	
61	2-Ethylhexyl diphenyl phosphate	1241-94-7	No activity	No activity	
62	2-Methyl butanoic acid methyl ester	868-57-5	No activity	No activity	
63	2-Methyl-5-(1-methylethenyl)-2-cyclohexen-1-one	6485-40-1	No activity	No activity	
64	2-Methyl-5-(1-methylethenyl)-2-cyclohexen-1-one	2244-16-8	No activity	No activity	
65	2-Methylbutanoic acid	503-74-2	No activity	No activity	
66	2-Phenyl-2-propanol	617-94-7	No activity	No activity	
67	2-Toluene sulphonamide	88-19-7	No activity	No activity	
68	3-(4-methoxyphenyl)-2-propenoic acid 2ethylhexyl ester	83834-59-7	No activity	No activity	
69	3,3,5-Trimethyl-2-cyclohexen-1-one	78-59-1	No activity	No activity	
70	3,4-Dimethyl-2,5-furandione	766-39-2	No activity	No activity	
71	3,7-Dimethyl-1,6-Octadien-3-ol	78-70-6	No activity	No activity	
72	4-Chloro-3,5-dimethyl-phenol	88-04-0	Toxic at 10^{-2}M. Retest at 10^{-3}M.	No activity when diluted to 10^{-3}M	Toxic at 10^{-2}M
73	4-hydroxy-3-methoxy-benzaldehyde	121-33-5	No activity	No activity	
74	4-Methoxycinnamic acid	943-89-5	No activity	Superagonist	

No.	Chemical	Cas no.	Androgen screen	Anti-androgen screen	Comments
75	4-Methoxymethylphenol	93-51-6	No activity	No activity	
76	4-tert-Butylcyclohexanone	98-53-3	No activity	No activity	
77	6,10-Dimethyl-5,9-undecadien-2-one	3796-70-1	No activity	No activity	
78	9,12-Octadecadienoic acid methyl ester	2566-97-4	No activity	+	
79	9H-Fluorene	86-73-7	No activity	+++	Creeps
80	Acetyl tri-n-butyl citrate	77-90-7	No activity	No activity	
81	Acetylcedrene	32388-55-9	No activity	++	Creeps
82	alpha cedrol	77-53-2	No activity	++	
83	Benzaldehyde	100-52-7	No activity	No activity	
84	Benzeneacetaldehyde	122-78-1	No activity	+	
85	Benzothiazole	95-16-9	No activity	No activity	
86	Butanedioic acid dimethyl ester	106-65-0	No activity	No activity	
87	Camphor	76-22-2	No activity	No activity	
88	Dehydroabietic acid	1740-19-8	Toxic at 10^{-2}M. Retest at 10^{-3}M.	+++	Toxic at 10^{-2}M
89	Dihydromethyl jasmonate	24851-98-7	No activity	No activity when diluted to 10^{-3}M	Reduced turbidity at 10^{-2}M.
90	Dihydromyrcenol	18479-58-8	No activity	No activity	
91	Diphenylacetic acid methyl ester	3469-00-9	No activity	++	Reduced turbidity at 10^{-2}M
92	Hexamethylbenzene	87-85-4	No activity	+	
93	Hexamethyldisiloxane	107-46-0	No activity	No activity	
94	Hexanal dimethyl acetal	1599-47-9	No activity	No activity	

No.	Chemical	Cas no.	Androgen screen	Anti-androgen screen	Comments
95	Ketoisophorone	1125-21-9	No activity	No activity	
96	Methyl propyl ketone	107-87-9	No activity	No activity	
97	Methylbenzaldehyde	104-87-0	No activity	Superagonist	
98	N,N-Dimethyl-1-dodecanamine	112-18-5	No activity	No activity when diluted to 10^{-4}M	Creeps/toxic at 10^{-2}M
99	N-ethyl-4-methyl benzenesulphonamide	80-39-7	Retest at 10^{-3}M	No activity at 10^{-3}M	Reduced turbidity at 10^{-2}M
100	Nonanal	124-19-6	No activity	No activity	
101	Nonanoic acid methyl ester	1731-84-6	No activity	No activity	
102	N-tert-Butylacrylamide	107-58-4	No activity	No activity	
103	Octamethylcyclotetra-siloxane	556-67-2	Need to retest due to creeping on plate	No activity	
104	Phenoxyacetic acid methyl ester	2065-23-8	No activity	No activity	
105	Propyl benzene	1007-26-7	No activity	No activity	
106	Squalene	111-02-4	No activity	No activity	
107	Terbutryn	886-50-0	Toxic at 10^{-2}M. Retest at 10^{-4}M.	No activity at 10^{-4}M	Toxic at 10^{-2}M
108	Tridecanoic acid methyl ester	1731-88-0	Need to retest due to creeping on plate	No activity at 10^{-3}M	Reduced turbidity at 10^{-2}M
109	Xylene isomer	1330-20-7	Need to retest due to creeping on plate	No activity	

3.4 Discussion

The European Chemicals Agency currently contains information on around 145,000 chemicals, and this list is continuously growing. It is not possible to test all of these as individual chemicals and what has been monitored for so far represents only a small proportion of the chemicals potentially present in the environment (Heiger-Bernays *et al.*, 2018; Scott *et al.*, 2018). When Alygizakis *et al.* (2019) examined WwTW effluent samples across 9 countries, of the 280 chemicals detected, 25–67% were pharmaceuticals, 5-30% were industrial chemicals, 3-21% were pesticides, 3-23% were psychoactive drugs and 2-17% antibiotics. Due to the structurally diverse nature of these chemicals many end up in the aquatic environment, and undetectable concentrations often means that chemical analysis is not sufficiently sensitive rather than that the chemicals are absent (Itzel *et al.*, 2019).

Our yeast YAS was only able to detect androgenic activity in three of the 107 chemicals tested (<3%) and found anti-androgenic activity in 20 of the chemicals tested (19%). In a study by Kojima *et al.* (2004), 200 pesticides were tested for (anti-)androgenic activity by a reporter gene assay using Chinese hamster ovary cells. Of the chemicals tested, none were found to have androgenic activity, but 66 were found to have anti-androgenic activity (33%) with 34 of these having both oestrogenic and anti-androgenic activity (17%). Araki *et al.* (2005), in another study, screened 253 industrial chemicals using AR-EcoScreen cells but only found two AR agonists (<1%) and nine AR antagonists (3.6%). Our results are very much in agreement with these findings; that is, more chemicals possessed anti-androgenic activity than possessed androgenic activity.

3.4.1. Possible androgens

In my chemical set, the three androgenic chemicals had only very weak (unquantifiable) activity, and of these, ibuprofen produced the highest response. Ibuprofen is a widely used non-steroidal anti-inflammatory drug, and a study by Kristensen *et al.* (2011) found an association between intrauterine exposures and risk factors for developing male reproductive disorders. In an *in vitro* yeast luciferase assay, Ezechias *et al.* (2016) found ibuprofen to exhibit a dose-dependent antagonism towards the AR, rather than the agonism seen in this study. Ji *et al.* (2013) also tested ibuprofen in adult zebrafish and, following a 14-day exposure, found that females had increased concentrations of E2 and T and males had decreased T concentrations, although this effect was not thought to be due to androgenic activity but rather by alteration of aromatase activity, a steroidogenic enzyme involved in converting androgens to oestrogens.

Whilst no reports of methyl decanoate having androgenic activity could be found in the literature, 2-ethyl hexanoic acid has been linked with developmental toxicity, although being positive to the peroxisome proliferator-activated receptor-alpha rather than the AR in the CALUX assay (Kroese *et al.*, 2015).

3.4.2. Possible anti-androgens

Twelve of our test chemicals caused a reduction in the raised background alongside a reduction in the turbidity (i.e. the number of yeast cells). We concluded that this reduction was due to toxicity, because when these chemicals were diluted to remove the toxicity, there was no activity. Of these twelve chemicals, dihydromethyl jasmonate has also been reported by others to have no anti-androgenic activity (Araki *et al.*, 2005). Similar to our results, Simon *et al.* (2016) found 2,4-di-tert-butylphenol to be inactive, whereas Mertl *et al.* (2014) reported anti-androgenic activity. However, tris (2-butoxyethyl) phosphate, HHCB, 4-chloro-3,5-dimethyl-phenol and terbutryn have all been reported in the literature as having anti-androgenic activity. Some of these results may be actually a misinterpretation of toxicity, as no tests for toxicity were carried out during the *in vitro* testing for anti-androgenic activity of tris (2-butoxyethyl) phosphate (Liscio *et al.*, 2014), 2,4-di-tert-butylphenol (Mertl *et al.*, 2014), and 4-chloro-3,5-dimethyl-phenol (Liscio *et al.*, 2014). Likewise, when Huang *et al.* (2011b) examined data from the screening of approximately 3000 chemicals against a panel of 10 human nuclear receptors, more compounds were active in the antagonist mode than the agonist mode, and the authors considered this due to cytotoxicity interfering with results in the antagonist mode. However, others did carry out parallel tests for toxicity (terbutryn, Araki *et al.*, 2005; HHCB, Ermler *et al.*, 2011) and the different results observed may therefore be due to differences in the test systems used, i.e. mammalian versus yeast cells. For two of our toxic chemicals (2,4-di-tert-butylphenol and N,N-Dimethyl-1-dodecanamine), this toxicity did ‘creep’ across the plate to affect other wells. Previously, oestrogenic activity of alkylphenols, in the YES, has also been reported to creep across plates to wells not dosed with the compound, but the toxicity at the higher concentrations did not creep in these oestrogenic compounds (Beresford *et al.*, 2000).

In agreement with others, we found no anti-androgenic activity for 3-methyl phenol (Satoh *et al.*, 2005), 2-ethyl-1-hexanol (Araki *et al.*, 2005), 2,6-Di-tert-butyl-p-benzoquinone (Simon *et al.*, 2016), 2-phenyl-2-propanol (Simon *et al.*, 2016), 3-(4-methoxyphenyl)-2-propenoic acid 2ethylhexyl ester (Vinggaard *et al.*, 2008), 3,3,5-trimethyl-2-cyclohexen-1-one (Araki *et al.*, 2005; Simon *et al.*, 2016), benzaldehyde (Araki *et al.*, 2005), and camphor (Araki *et al.*, 2005; Simon *et al.*, 2016). Likewise, we

found no activity with isoproturon and this is in agreement with Vinggaard *et al.* (2008) and Ait-Aissa *et al.* (2010), but not with Orton *et al.* (2009) where the 'Brunel' yeast AYAS was also used. However, this chemical when tested by Orton *et al.* showed a sharp decline in response at the higher concentrations (often indicative of toxicity), and this apparent activity may therefore not in be real. We also saw no anti-androgenic activity when testing carbamazepine, whereas Liscio *et al.* (2014) found weak anti-androgenic activity.

For one of the groups of chemicals tested, phthalates, there are mixed reports in the literature regarding anti-androgenic activity, depending on the paper or test method employed. In our yeast-based assay, DEP, DEHP and DBP did not show any anti-androgenic activity, which is in agreement with Roy *et al.* (2004), Vinggaard *et al.* (2008), Simon *et al.* (2016), and Freyberger *et al.* (2010). However, Tamura *et al.* (2006) and Christen *et al.* (2010) did find DEP to show anti-androgenic activity using MDA-kb2 cells, and Lorenzetti *et al.* (2010) did observe a weak effect that was considered indicative of a mixed agonist/antagonist effect with LNCaP cells. As no anti-androgenic activity was observed by other laboratories using different mammalian cell lines (PALM, Freyberger *et al.*, 2010; CHOK1, Roy *et al.*, 2004; TARM-Luc, Simon *et al.*, 2016; Vinggaard *et al.*, 2008), the lack of activity is not restricted to yeast-based assays.

There have also been discrepancies in the *in vivo* literature (reviewed in Svechnikov *et al.*, 2016), and whilst some phthalates alter reproductive development in an anti-androgenic fashion, the mechanism of action does not appear to involve either the ER or AR (Gray *et al.*, 2001; Marcoccia *et al.*, 2017). The presence of anti-androgenic activity in other *in vitro* assays could therefore be false positive results or involve other mechanisms of action that are not AR receptor mediated.

Twenty of our test chemicals showed anti-androgenic activity, although for two of these chemicals this activity was inconsistent. Triphenol phosphate produced inconsistent anti-androgenic results in our repeat assays, although others have also observed anti-androgenic activity *in vitro* (Liscio *et al.*, 2014) and *in vivo* it has also been shown to significantly decrease fecundity following a 21-day adult zebrafish exposure study (Liu *et al.*, 2013). Our anti-androgenic activity was observed with triphenol phosphate only in our first testing, and it is possible that this chemical became inactive during storage due to degradation. Additionally, N-butylbenzene sulphonamide gave inconsistent results in our AYAS assay and this compound has been reported by others (Simon *et al.*, 2016) to be inactive. However, the assay utilised by Simon *et al.*, derived from the human

mammary gland adenoma T47-D cell line, appears to be less sensitive than our yeast assay because, whilst they saw oestrogenic activity with benzophenone, they saw no anti-androgenic activity with benzophenone. However, in support of our benzophenone results, others have similarly reported anti-androgenic activity (Suzuki *et al.*, 2005; Tamura *et al.*, 2006).

For a number of the active chemicals tested by us, others have also reported anti-androgenic activity in the literature. We found fluoranthene to be a potent anti-androgen (more potent than flutamide) and this has also been reported by both Vinggaard *et al.* (2008) and Araki *et al.* (2005). Triclosan is also a potent androgen antagonist in our study and many other publications have previously reported similar activity. For example, Vinggaard *et al.* (2008) reported an IC25 of 3-10 µM and Tamura *et al.* (2006) reported an IC50 of 7.5µM. However, Christen *et al.* (2010) observed an enhancement of DHT induced activity (superagonism) using MDA-kb2 cells and this activity could be blocked by flutamide. In this study we found chlorophene to be a potent antagonist (more potent than flutamide) and similarly Rostkowski *et al.* (2011) found chlorophene to be more potent than flutamide. However, Lange *et al.* (2015) carried out an *in vitro* study of 11-ketotesterone (an androgen found in fish) induced activation of stickleback ARα and ARβ but saw no inhibition with chlorophene. Lange *et al.* considered that the lack of an inhibition was due to the relatively low sequence identity between the human AR and stickleback AR. Pyrene was of a similar anti-androgenic potency to flutamide and has also been found to be anti-androgenic by others (Tamura *et al.*, 2006; Vinggaard *et al.*, 2008). We also found 9H-fluorene to have a similar potency to flutamide, although no activity was seen when it was tested by Araki *et al.* using AR-EcoScreen^{TM} cells derived from a Chinese hamster ovary cell line (Araki *et al.*, 2005).

Many of our results are similar to those already reported by others in the literature. However, whilst both Vinggaard *et al.* (2008) and Tamura *et al.* (2006) found chrysene to be very weakly anti-androgenic, no activity was seen by ourselves. Also, when benzo[a]pyrene was tested for anti-androgenic activity, we found no activity. This is different to results reported by both Ermler *et al.* (2011) and Tamura *et al.* (2006), who found benzo[a]pyrene to be androgenic at higher concentrations and anti-androgenic at lower concentrations using MDA-kb2 cells, although Ermler *et al.* did encounter solubility issues. Others report benzo[a]pyrene to have only anti-androgenic activity (i.e. no androgenic activity at the higher concentrations; Charles *et al.*, 2005; Vinggaard *et al.*, 2008). However, whilst many cite the anti-androgenic effect of benzo[a]pyrene *in vitro*, Charles *et al.* (2005) found no activity in the Hershberger assay. Additionally, an *in vivo*

zebrafish exposure study linked the developmental outcomes to aromatase inhibition (i.e. less androgens were converted to oestrogens; Alharthy *et al.* (2017), so the *in vitro* activity reported in the literature may in fact be artefactual.

Where others have reported seeing anti-androgenic activity in *in vitro* based assays, often these reports have used mammalian cell-based assays rather than yeast cell-based assays. In addition to the cells potentially behaving differently (including having different susceptibilities to chemical toxicity), many mammalian cell-based assays deliver the chemical in dimethyl sulphoxide (DMSO), and solubilities (and therefore availability) of chemicals may thus be different to yeast-based assays typically using etOH as solvent. Additionally, some report using higher chemical stock concentrations than tested by myself (1M; Araki *et al.*, 2005) and this may also account for discrepancies when lower concentrations were tested.

3.4.3. Possible superagonists

In androgen responsive mammalian tissues, T is converted to the more potent DHT by 5 α-reductase enzymes, and inhibiting these enzymes leads to a reduction in DHT synthesis (Aggarwal *et al.*, 2010). Liang and Liao (1992) discovered that certain naturally occurring unsaturated fatty acids were able to inhibit 5 α-reductase in cultured cells. Liu *et al.* (2009a) further found fatty acids were able to inhibit the proliferation of LNCaP prostate cancer cells. The most potent was dodecanoic acid, followed by myristic acid and then pentadecanoic acid. In our yeast screen for anti-androgenic activity we saw anti-androgenic activity with dodecanic acid and myristic acid, but not with pentadecanoic acid. However, as T was not present in our yeast assay, it is not clear how blocking the conversion of T to DHT would create an anti-androgenic effect.

As it is unlikely that the conversion of T to DHT is the reason for the superagonism, another possibility might be due to an alteration in the yeast cell membrane fluidity. When Alexandre *et al.* (1996) exposed *Saccharomyces cerevisiae* to decanoic acid, a medium-chain fatty acid, the plasma membrane H+-ATPase activation induced by the decanoic acid correlated with an alteration in membrane lipid constituents, with a resultant increase in membrane fluidity. It is therefore conceivable that in our studies fatty acids behaving as superagonists might alter the yeast cell membrane and allow the DHT to more readily enter the cell. *S. cerevisiae* do possess 3-oxo-5-alpha-steroid 4-dehydrogenase that contributes to dolichol-linked oligosaccharide biosynthesis and polyprenol degradation and is homologous to human SRD5A3 steroid 5 alpha-reductase 3 (www.yeastgenome.org). These compounds might therefore be interfering with this pathway and as dolichol is an important cellular membrane component may be altering

the ability of the DHT to enter though the yeast cell wall. Additionally, there is evidence showing that the fatty acids methyl palmitate and methyl oleate have an androgenic effect in the Hershberger rat assay (Seres *et al.*, 2014), but when we tested these two chemicals in the absence of DHT, no androgenic activity was seen. However, it could be that these fatty acids both affect the permeability of the cell wall and act as very weak androgens, but it is only in the presence of the DHT that this 'superagonism' is apparent.

Many of the chemicals that behaved as superagonists were fatty acids, although monomethyl phthalate, 4-methoxycinnamic acid and methylbenzadlehyde were not. Others have similarly seen superagonism, for example, Ermler *et al.* (2010) found that genistein had little to no activity when tested on its own but when tested with DHT induced an androgenic response greater than that of DHT. In the MDA-kb2 cell line, Christen *et al.* (2010) found the flame retardant BDE-100 and antimicrobials triclosan and triclocarbon to have no or weak agonistic activity, but they enhanced the DHT-dependent activation of AR-responsive gene expression by 150%, 180% and 130%, respectively. This superagonism is not specific to the AR, and superagonism when testing genistein has also been observed by Legler *et al.* (1999), Sonneveld *et al.* (2005) and Berckmans *et al.* (2007), in different oestrogen responsive reporter gene assays. Additionally, the potentiation of the feminising effects of EE2 have been observed *in vivo* in stickleback fish and also rats exposed to EDCs (triclosan, chlorophene and dichlorophene; Lange *et al.* (2015), and triclosan; Stoker *et al.* (2010).

These examples of previously reported superagonism are not with fatty acids, and whilst there are reports of superagonism with triclosan (Christen *et al.*, 2010; Lange *et al.*, 2015; Stoker *et al.*, 2010), we saw strong anti-androgenic activity with our yeast-based assay instead. Araki *et al.* (2005) suggested that superagonism could be due to synergism via AR and GR transcriptional mechanisms, although our yeast cells do not have a GR receptor, and therefore this mechanism cannot explain our results. Stoker *et al.* (2010) also proposed that triclosan either enhanced the interaction of the EE2 with the ER or it increased endogenous oestrogen concentrations by inhibiting their clearance or catabolism. As our test system did not have an ER or EE2 to interact with it, triclosan might be capable of directly inhibiting the AR and having feminising effects.

3.4.4. Further work

In addition to checking for toxicity, the potential for false positives can be reduced by checking that a strong antagonist is able to block the ability of an agonist to bind to a receptor, i.e. whether the response is specific or non-specific (Dent *et al.*, 2019; Juberg *et al.*, 2014). For chemicals with anti-androgenic activity, the addition of DHT would

enable recovery of the signal if the response was mediated through the AR (Fic *et al.*, 2014). Parallel inhibition of activity in YES and YAS would also indicate that there was non-specific inhibition of induction, i.e. not mediated through the receptor (Fic *et al.*, 2014).

These tests were not investigated as part of this study, although they might have helped to clarify where toxicity was confounding results. Especially as inconsistencies were evident, both when retesting chemicals and also when comparing our results in the literature. Instead, for chemicals that tested positive, these were then retested in a modification of the yeast (anti-)androgen screen, for confirmation of activities. This work is presented in Chapter 4.

4. Comparison of two *in vitro* assays for the determination of (anti-) androgenic activity of environmental chemicals

4.1. Introduction

As outlined earlier (Chapter 3, Section 3.1), the OECD conceptual framework for testing EDCs consists of five levels of testing, investigating different levels of biological complexity and sources of data, including *in vitro* assays (Level 2) which provide vital foundation data prior to higher level tests (Levels 3-5) using animal models (OECD, 2018d).

In Chapter 3, I tested a large number of chemicals (>100) identified from WwTW effluent, for androgenic and anti-androgenic activity using a yeast-based *in vitro* assay system. Whilst testing these chemicals for (anti-)androgenic activity, I came across several unexpected results (inconsistencies when retesting chemicals with the AYAS, and also some inconsistencies with results published by others) that highlighted some of the difficulties that can arise when interpreting results from yeast-based *in vitro* screens. These issues could affect data interpretation and lead to the mislabelling of some of the chemicals as EDCs, and a false positive result might lead to *in vivo* investigations leading to unnecessary costs and wasted resources. In contrast, false negatives might mean that chemicals do not get investigated further, leading to exposure to the environment and people.

The primary issues identified in Chapter 3 were 1) possible false positives for anti-androgenic activity which may be caused by chemical toxicity reducing cell viability, or 'edge effect' caused by evaporation, both leading to an altered colourmetric response in the assay (presented in Sections 3.3.2 and 3.4.2), and 2) the possibility that some chemicals may be 'superagonists' (presented in Sections 3.3.3 and 3.4.3).

Therefore, to refine these results, the *in vitro* assay was modified, and the results from the two assays were then compared to find out which was the most reliable. In this Chapter, I used the modified yeast-based assay system to retest all of the androgenic, anti-androgenic and superagonist chemicals to confirm the activity observed in Chapter 3. Additionally, the issues of cytotoxicity and the 'edge effect' were investigated further to see if they could lead to false positive anti-androgenic activity.

As found in Chapter 3, Sections 3.3.3, some chemicals had no androgenic activity but were able to enhance the activity of DHT, thus acting as 'superagonists'. We do not yet know how superagonists might function, but it is possible that these chemicals are able to alter the yeast cell wall permeability and enhance the transport of the chemical into

the cell. For example, many of the 'superagonists' were fatty acids and this group of compounds have been described as 'penetration enhancers' (Williams and Barry, 2004). Methoxyacetic acid, the active metabolite of ethylene glycol, is an established testicular toxicant (Bagchi *et al.*, 2011). In co-transfection studies by Jansen *et al.* (2004) using human hepatic carcinoma (HepG2) cells, two short-chain fatty acids (methoxyacetic acid and valproaic acid) were found to markedly increase cellular sensitivity to nuclear hormone receptor ligands by enhancing the transcriptional efficiency of ligand activated nuclear hormone receptors. The short-chain fatty acids did not affect the EC50 of the receptor ligands in the presence of methoxyacetic acid, so Jansen *et al.* (2004) concluded that the effect was not exerted at the ligand binding step. Other short-chain fatty acids were also tested, and the carboxylic acid component was found to be important for the enhanced hormone activity. Bagchi *et al.* (2011) also investigated the effects of methoxyacetic acid using an androgen-responsive mouse testicular Leydig cell line, either on its own or in combination with T. Methoxyacetic acid was able to both enhance and antagonise AR activity, and affected many cellular processes including ion transport, apoptosis, cell adhesion, phosphorylation and transcription.

Assays for cytotoxicity measure whether a compound or sample is toxic to cells and in most assays either the cell number, cell proliferation or cell viability are measured. The determination of cell number in yeast-based assays is usually carried out by monitoring absorbance at 600 or 620nm and looking for a reduction in turbidity indicative of toxicity (Archer and van Wyk, 2015; Liscio *et al.*, 2014; Ma *et al.*, 2014). Yeast cells have also been observed microscopically for damage as an indication of toxicity (Tamura *et al.*, 2006).

There are a number of tests available for toxicity that measure different end points of cell death. The tryphan blue exclusion assay relies on the fact that viable cells are selectively permeable to certain dyes, and membranes of dead cells lack this selectivity. The ratio of live cells (clear cytoplasm) to dead cells (blue cytoplasm) is determined visually by use of a haemocytometer (Strober, 1997). Another parameter for cell death is the integrity of the cell membrane, which can be measured by the leakage of lactate dehydrogenase (LDH) by damaged cells. In addition, the metabolic activity of viable cells is the basis of a number of colourimetric assays, for example, the 'MTT assay' relies on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by the mitochondrial dehydrogenase of viable cells to a blue formazan product that can be measured spectrophotometrically (Mosmann, 1983).

It is more common to use an assay to directly determine cytotoxicity, but often this involves setting up plates in parallel (one for antagonist and one for cytotoxic activity), although Berckmans *et al.* (2007) developed a method using transgenic MELN for testing for oestrogenicity and LDH-leakage using a single plate. Weyermann *et al.* (2005) used the LDH, MTT, neutral red and adenosine triphosphate cytotoxicity assays to determine the toxicity of triton X-100, chloroquine, sodium azide and ketamine on fibroblast cells. The results seen with triton X-100 were comparable with all four assays. However, for chloroquine and sodium azide major differences were found with the four assays. Assays like LDH and MTT, which rely on enzymatic reactions, could be influenced by enzyme inhibitors, leading to misleading results. Hamid *et al.* (2004) compared alamar blue and MTT assays for the HTP screening of 177 drugs using the hepatoma cell line, HepG2. Both assays performed consistently, although the alamarBlue® assay was slightly more sensitive for most of the drugs tested.

Cellular screening experiments are often carried out in 96-well plates and multiple day incubations can lead to loss of growth medium due to evaporation, particularly in the outer wells (Berg *et al.*, 2001; Zimmermann *et al.*, 2003). This can lead to an 'edge effect' or 'hook effect' to the elevated background around the edge of the plate and, depending on the size of the 'edge effect', this could be misled for anti-androgenic activity (anti-androgenic activity also leads to a reduction in colour).

Prior to testing anticancer drugs, Faessel *et al.* (1999) characterised the statistical properties of an *in vitro* cell growth inhibition assay. Multiwell plate row and column parabolic growth patterns were seen with the most pronounced being the column effect. Faessel *et al.* examined several different parameters, e.g. different multiwell plates, multichannel pipette, plate reader, position in incubator, and differential evaporation. However, the underlying cause was not discovered so a randomisation step was developed for the growth assay. Others have also reported methods, for example, removing controls from the periphery plates, to eliminate edge effects (Juberg *et al.*, 2014).

Similarly, Patel *et al.* (2005), using a 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2 H-tetrazolium assay, found that with evaporation in the outer wells there was an increase in absorbance compared to the inner wells. When the cells were counted, though, there was no difference in the cell numbers between the rows, and the decreased volume was consistent with the increased absorbance. However, whilst there may not be a quantifiable difference in cell number in these outer wells, Walzl *et al.* (2012) found significantly different cell

metabolism when using the alamarBlue® assay; both an increase in dye concentration and a reduction in cellular metabolic activity. Walzl *et al.* (2012) managed to restore these differential effects with the use of a humidity chamber to reduce evaporation.

It is often hard to compare results of different *in vitro* assays, as their responses can vary due to use of different cell lines and protocols. Therefore, International Organisation for Standardisation (ISO) standardised bioassay protocols have been developed, including two for yeast-based assays for oestrogenic activity:

- ISO 19040-1. Water quality – Determination of the estrogenic potential of water and wastewater – Part 1: Yeast estrogen screen (YES, *Saccharomyces cerevisiae*)
- ISO 19040-2. Water quality – Determination of the estrogenic potential of water and wastewater – Part 2: Yeast estrogen screen (A-YES, *Arxula adeninivorans*; Hettwer *et al.*, 2018)

The former of these yeast-based assays uses a similar recombinant yeast strain as used for my research outlined in Chapter 3. However, both of these assays are for testing for oestrogens and none have yet been developed for (anti-)androgenic assessment.

In this chapter, the chemicals identified in Chapter 3 with androgenic (3 chemicals, Section 3.3.1), anti-androgenic (20 chemicals; Section 3.3.2) or superagonistic activity (able to enhance activity of DHT in the AYAS; 12 chemicals; Section 3.3.3), were retested using a modified method based on the International Organization for Standardization protocol; ISO 19040-1:2018.

In addition, the issues highlighted in Chapter 3 have been further scrutinised to try to identify the reason for the observed results:

- toxic chemicals can lead to reduction in the colourimetric response that can be misinterpreted as anti-androgenic activity. Nystatin (a fungicide) was used to kill the yeast cells to assess its effect on the DHT-elevated background in both the AYAS and the MTT assay. Comparisons were then made between colourimetric absorbance readings and the absorbance readings for turbidity.
- the ‘edge effect’ (a reduction in the colourimetric absorbance around the periphery of the multiwell plate) can be misdiagnosed as anti-androgenic activity. Different sealing tapes, to prevent evaporation during the 3 day incubation period, were used to see if the ‘edge effect’ could be minimised.

- the position that a chemical is pipetted into a multiwell plate may affect the response due to the ‘edge effect’. The well position in multiwell plate and its effect on anti-androgenic potency relative to flutamide was investigated.

This investigation illustrates potential issues encountered which may affect data interpretation and lead to the mislabelling of certain chemicals as EDCs.

4.2. Materials and methods

4.2.1. Chemicals

All chemicals used to prepare medium and components for the assays were purchased from Sigma-Aldrich (Dorset, UK) unless stated otherwise.

Positive control chemicals, namely DHT (≥99% pure) and flutamide (99% pure), were purchased from Sigma-Aldrich. DHT and flutamide were prepared at stock concentrations of 10^{-6}M and 10^{-3}M in etOH (>99.7%; Hayman Speciality Products, Essex, UK), respectively. All test chemicals were prepared at a starting concentration of 10^{-2}M unless otherwise stated (see Chapter 3 for further details). All stocks were stored in sealed glass vials at 4 ^{o}C.

4.2.2. Recombinant yeast (anti-)androgen procedures

4.2.2.1. Yeast (anti-)androgen assay without yeast cell lysis

Using the standard YAS (Sohoni and Sumpter, 1998), in the presence of androgens β-gal was secreted into the medium, causing the substrate, CPRG, to change colour from yellow to red and this was measurable by reading the absorbance at 540 nm. For the standard AYAS, DHT was added to all the wells and where anti-androgens were present this blocked the colour development and the substrate remained yellow. Both standard methods are described in Chapter 3.

4.2.2.2. Yeast (anti-)androgen assay with yeast cell lysis (adapted from ISO 19040-1:2018 Water quality - Determination of the estrogenic potential of water and wastewater - Part 1: Yeast estrogen screen (Saccharomyces cerevisiae))

The preparation of medium and buffers is described in Chapter 2, Section 2.2.3.2.4.3.

For the modified YAS the positive control, DHT, was included in each assay (stock concentration at 10^{-6}M and serially diluted in etOH to achieve final concentrations from $5x10^{-8}$M to $2.44x10^{-11}$M).

The positive control, flutamide, was included in every modified AYAS (stock solution at 10^{-3}M and serially diluted in etOH to achieve final concentrations of 5 x 10^{-5}M to 2.44 x 10^{-8}M in the wells). For antagonistic screens, DHT at 2x10^{-9}M was also added to the medium prior to addition to all wells.

The assay procedure is described in Chapter 2, Section 2.2.3.2.4.3. In brief, chemicals were serially diluted in etOH and 10 µl volumes were transferred to 96-well flat-bottom plates where the etOH was allowed to evaporate to dryness. Then, 200 µl exposure medium containing yeast (final cell number of 5 x 10^5 cells/ml) was added to each well, and the plates were sealed and incubated at 32 ^oC. After incubation the yeast cells are lysed with freeze/thaw cycles and assay buffer containing the substrate oNPG was added to all wells. Any β-gal in the cell lysate caused the clear medium to turn yellow and plate readings were taken at 420 nm (colour) and 620 nm (turbidity) after 30, 60, and 90 mins incubation. The 90 min readings were used for all analyses.

4.2.2.3. Investigation of issues with the potential to cause false positive results when screening for anti-androgenic activity

4.2.2.3.1. MTT assay for investigating the effects of toxicity on anti-androgenic activity

Nystatin was used to observe the effects of toxicity on the DHT response in the AYAS. Nystatin was prepared at 120 mg/L in meOH and stored in a sealed vial at 4 ^oC. MTT (used to measure metabolic activity), was prepared at 5 mg/ml in PBS, filter sterilised and stored in aliquots at -20 ^oC.

For the MTT assay, the nystatin was serially diluted in etOH at 1/1.3 dilutions to elongate the transition from toxic to non-toxic concentrations, and this gave final well concentrations of 6 to 0.3 mg/L. As described in Chapter 3, 10 µl volumes were then transferred to the assay plate and allowed to evaporate to dryness. Then, 180 µl medium containing yeast (final cell number of 5 x 10^5 cells/ml) and DHT at 2x10^{-9}M was added to each well. The plates were taped closed and shaken for 2 mins on a plate shaker. The plates were then incubated at 32 ^oC for 68 ± 1 hours.

After the 3 day incubation period, 20 µl of MTT was added to each well containing 180 µl medium (total volume 200 µl). Plates were covered with foil and incubated at 32 ^oC for 3 hours (shaking after 0, 1 and 2 hours) and during this time yellow tetrazolium MTT was reduced by metabolically active cells into intracellular purple formazan crystals. The medium was carefully aspirated from each well (150 µl) so as not to disturb the cells, and this was replaced with 150 µl DMSO. The plates were then shaken for 30 mins to

solubilise the crystals prior to reading at 570 nm (colour) and 620 nm (turbidity) on a plate reader.

Alongside this MTT assay plate, a further two plates were set up with the same serial dilutions of nystatin for running in the standard AYAS, with absorbance reading at 540 nm (colour) and 620 nm (turbidity), and the modified AYAS with cell lysis with absorbance readings at 420 nm (colour) and 620 nm (turbidity).

4.2.2.3.2. Different sealing tapes to prevent evaporation causing the 'edge effect' that can be misdiagnosed as anti-androgenic activity

To investigate if possible 'edge effect' can be influenced by different sealing tapes, both the standard AYAS and the modified method with lysis were performed with just etOH (normally negative control), i.e. no additional chemicals were included in these assays. Ethanol was added to all wells in a 96-well plate and was allowed to evaporate to dryness. Media containing DHT was then added to all wells and plates were sealed with the different tapes, namely tape (i), Browne autoclave indicator tape, and tape (ii) Bel-Art® Write-on™ Label Tape, prior to incubation at 32 ^{o}C.

4.2.2.3.3. Well position in multiwell plate and its effect on anti-androgenic potency relative to flutamide

Benzeneacetaldehyde was chosen to investigate the issue of well position, as it had a clear dose-response curve in the AYAS with no indication of toxicity. Benzeneacetaldehyde along with the positive control (flutamide) and negative control (etOH) were tested in the AYAS using the method without lysis. The two chemicals (flutamide and benzeneacetaldehyde) were pipetted to different rows in multiwell plates; into the outer rows or inner rows (see Figure 60 insets illustrating plate layouts). Sealing tape (i) was used to seal the plates prior to incubation at 32 ^{o}C for 3 days.

4.2.3. Data Analysis

All test chemicals were tested in duplicate in every experiment, and each chemical was tested in at least two separate experiments. Plotted values are means ± standard error.

The absorbance values were corrected for cell density using the following equation: Corrected value = $chemical_{420\ or\ 540nm}$-($chemical_{620nm}$-etOH $blank_{620nm}$).

The EC50s and IC50s were calculated using 4-parameter plot equations with SoftMax Pro version 5.0.1. Potency values were calculated by dividing the flutamide IC50 by the chemical IC50, and the higher the number the more potent the chemical relative to flutamide.

4.3. Results

Photos illustrating typical standard curves can be seen in Figure 49. The EC50 for the YAS using the method with lysis was $8.89x10^{-10} \pm 4.51x10^{-11}$M or 258 ± 13 ng/L (n=6, Figure 50). The IC50 for the AYAS using the method with lysis was $1.03x10^{-5} \pm 8.59x10^{-7}$M or 3834 ± 237 µg/L (n=11, Figure 51). See Appendix Tables A 29 and A 30 for raw data.

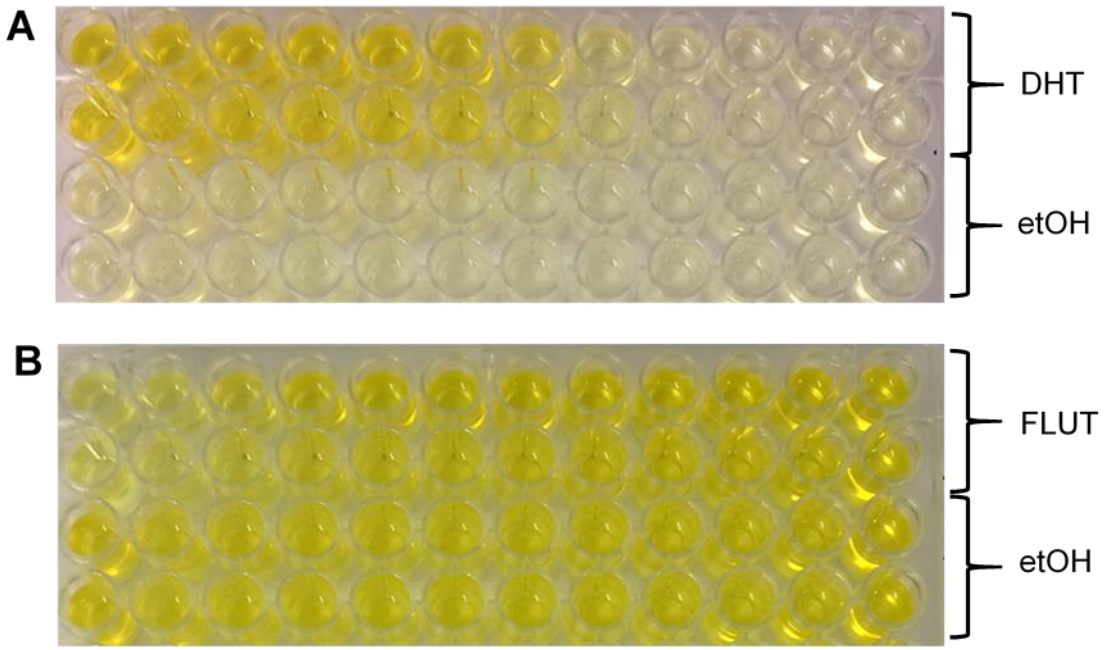


Figure 49. Photos showing typical yeast screen results after 3 days incubation followed by cell lysis and incubation with the chromogenic substrate oNPG. Chemicals were diluted from left (high concentration) to right (low concentration). (A) androgen screen; the positive control, dihydrotestosterone (DHT), standard curve with yellow wells at highest concentrations caused by β-gal converting the clear oNPG substrate to the yellow o-nitrophenol, and the negative control, ethanol (etOH) remaining clear in the absence of β-gal. (B) anti-androgen screen (all wells containing DHT); the positive control, flutamide (FLUT) standard curve, with yellow wells at all but the highest flutamide concentrations where the flutamide is blocking the AR receptor so that no β-gal is released, and the wells remain clear. For the negative control (etOH) all the wells have turned yellow due to the presence of DHT.

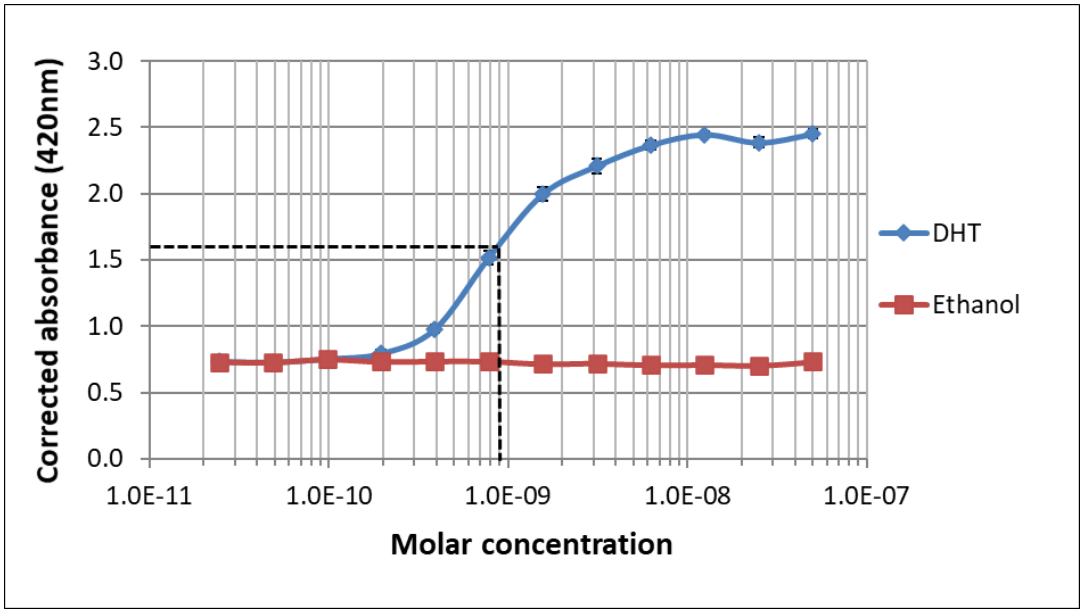


Figure 50. Mean ± SEM of the positive control, DHT, and the negative control, ethanol, from 6 androgen screens using the cell lysis method. The DHT EC50 for the 6 experiments was 8.89×10^{-10}M ± 4.51×10^{-11}M, as indicated by the dotted line (see Appendix Table A 30 for raw data).

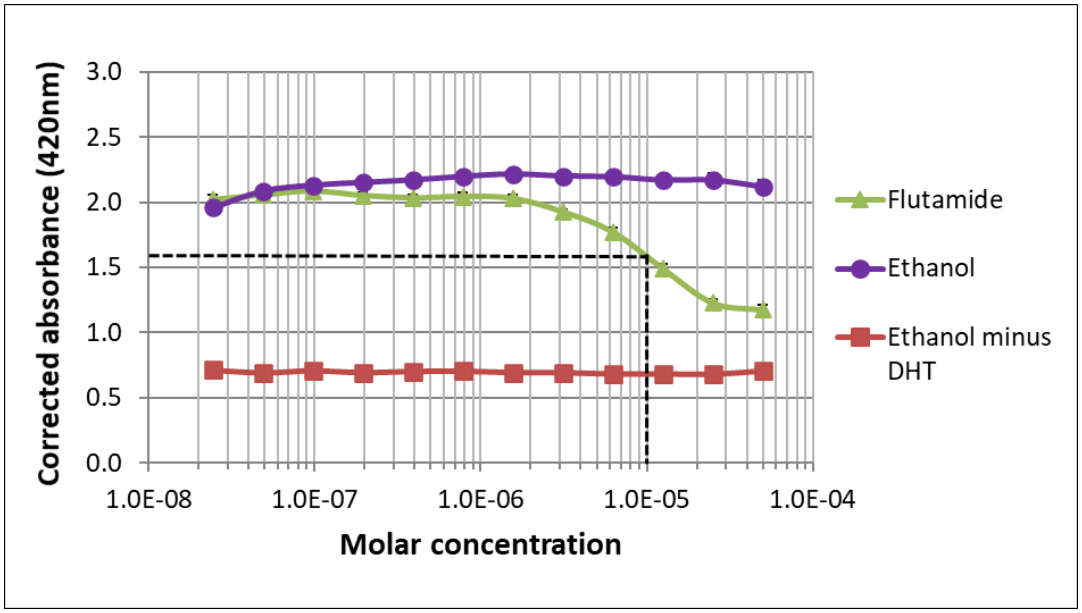


Figure 51. Mean ± SEM of the positive control, flutamide, and the ethanol negative controls (ethanol and ethanol minus DHT in the medium) from 11 anti-androgen screens using the cell lysis method. The IC50 for the 11 experiments was 1.03×10^{-5}M ± 8.59×10^{-7}M, as indicated by the dotted line (see Appendix Table A 30 for raw data). The ethanol minus DHT in the medium is included and note that flutamide does not fully block the DHT.

4.3.1. Comparison of the standard YAS with the lysis method for detecting androgenic, anti-androgenic and superagonism activity

Over 100 chemicals were tested in the standard AYAS (presented in Chapter 3, Section 3.2.1). Where chemicals tested positive for (anti-)androgenic activity or behaved as superagonists, these were repeated in the modified AYAS with yeast cell lysis.

4.3.1.1. Androgenic activity

Using the standard YAS with no lysis, three chemicals were found to have very weak androgenic activity (methyl decanoate, 2-ethyl hexanoic acid and ibuprofen; Figure 52A). The activity of these 3 androgens was too weak to quantify. At the higher ibuprofen concentrations (>10^{-4}M) there was a reduction in the androgenic activity due to a reduction in the yeast turbidity (i.e. at these test concentrations this chemical was toxic). When these 3 chemicals were tested for androgenic activity using the modified method with cell lysis, no androgenic activity was seen (Figure 52B), although there was a reduction in absorbance with ibuprofen due to toxicity at concentrations above 10^{-5}M (the top 6 wells).

4.3.1.2. Anti-androgenic activity

Thirty two chemicals showed anti-androgenic activity in the standard AYAS with no lysis. For twelve of these chemicals there was a distinct reduction in turbidity and on diluting these chemicals to non-toxic concentrations, anti-androgenic activity was no longer observed. Therefore, twenty chemicals were anti-androgenic using the method without lysis, with no apparent reduction in the turbidity readings. Of these twenty chemicals, 18 also showed anti-androgenic activity in the assay with cell lysis and the potencies using the two methods correlated well (R^2 = 0.8789; Table 17 and Figure 53). Two chemicals were anti-androgenic using the standard method with no lysis but had no activity using the modified method with cell lysis; myristic acid and triphenyl phosphate. The structures of the eighteen chemicals showing (anti-)androgenic activity with both methods are shown in Figure 54. The chemical properties and environmental concentrations of the most potent of these anti-androgens (moderate and strong potency) are shown in Table 18.

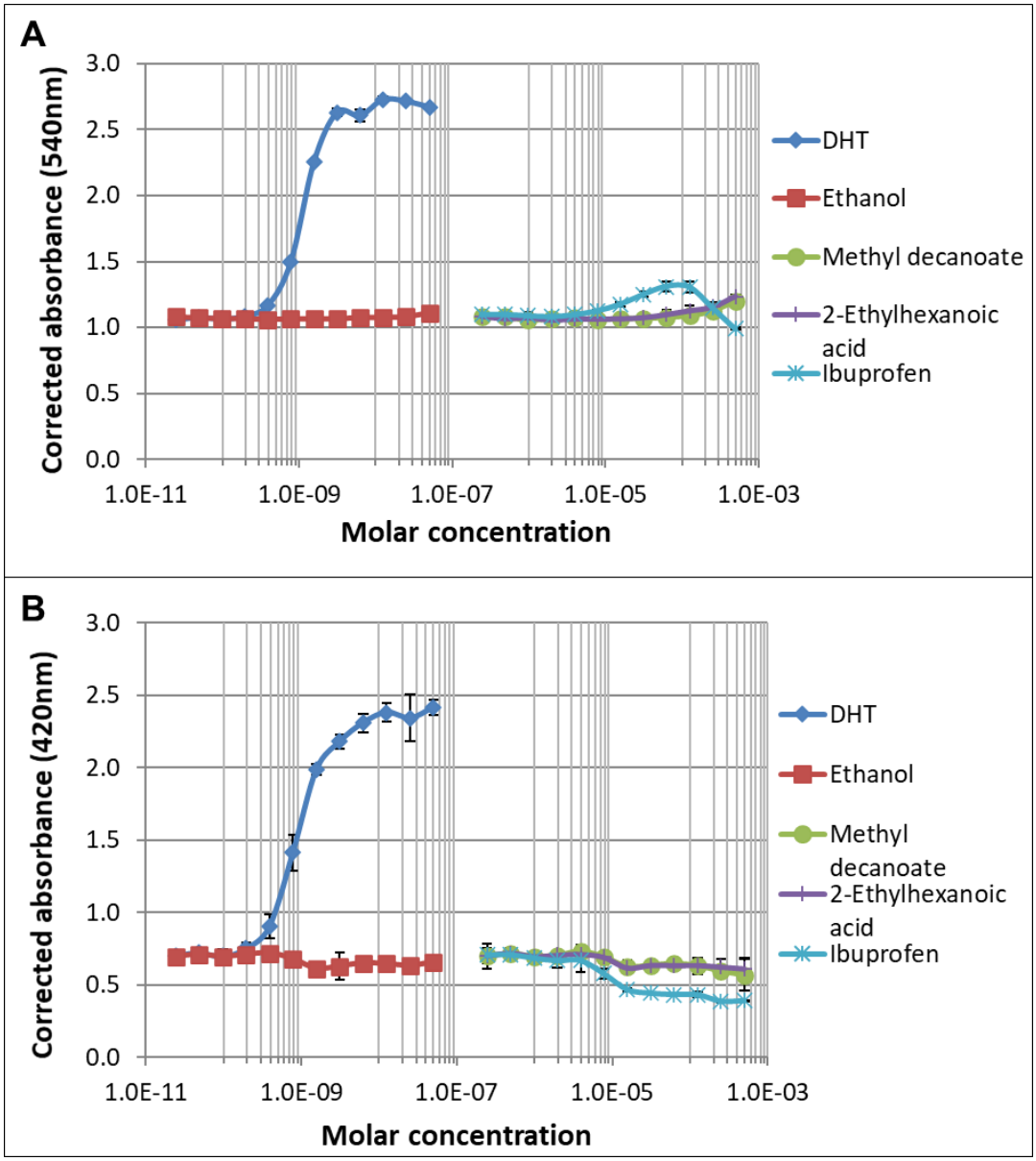


Figure 52. Androgen screen results seen in (A) the standard androgen screen with no cell lysis and (B) the modified anti-androgen screen with cell lysis. The plots are for mean values ± SEM for dihydrotestosterone (DHT) positive control, negative control (ethanol) and the three test chemicals; methyl decanoate, 2-ethyl hexanoic acid and ibuprofen.

Table 17. Comparison of potency values for 20 chemicals possessing anti-androgenic activity using the standard yeast anti-androgen screen without cell lysis and the modified method with cell lysis. Potency values are relative to the positive control, flutamide, and values above 1 are more potent than flutamide. The results are colour coded; strong in red (>5 times the potency flutamide), moderate in orange (0.2-5 times the potency of flutamide), weak in yellow (0.02-0.199 times the potency of flutamide) and very weak in white <0.02 times the potency of flutamide). See Appendix Tables A 31 and A 32 for raw data.

Chemical no.	Chemical	Cas no.	Anti-androgenic potency relative to flutamide	
			No lysis with CPRG	Lysis with oNPG
17	Triclosan	3380-34-5	9.865	15.736
41	Chlorophene	120-32-1	12.662	10.166
20	Methyl triclosan	4640-01-1	8.488	8.859
16	Fluoranthene	206-44-0	5.113	6.768
88	Dehydroabietic acid	1740-19-8	1.158	2.194
19	Pyrene	129-00-0	3.268	1.520
79	9H-Fluorene	86-73-7	0.485	0.475
81	Acetylcedrene	32388-55-9	0.151	0.294
91	Diphenylacetic acid methyl ester	3469-00-9	0.161	0.237
82	alpha cedrol	77-53-2	0.030	0.144
84	Benzeneacetaldehyde	122-78-1	0.034	0.134
56	2-(methylthio)benzothiazole	615-22-5	0.063	0.129
10	N-Butylbenzene sulfonamide	3622-84-2	0.172	0.117
8	Benzophenone	119-61-9	0.020	0.104
15	Dodecanoic acid	143-07-7	0.081	0.102
78	9,12-Octadecadienoic acid methyl ester	2566-97-4	0.032	0.072
51	1-[4-(hydroxy-1-methylethyl) phenyl] ethanone	54549-72-3	0.016	0.071
92	Hexamethylbenzene	87-85-4	0.018	0.014
18	Triphenyl phosphate	115-86-6	0.517	0.000
11	Myristic acid	544-63-8	0.086	0.000

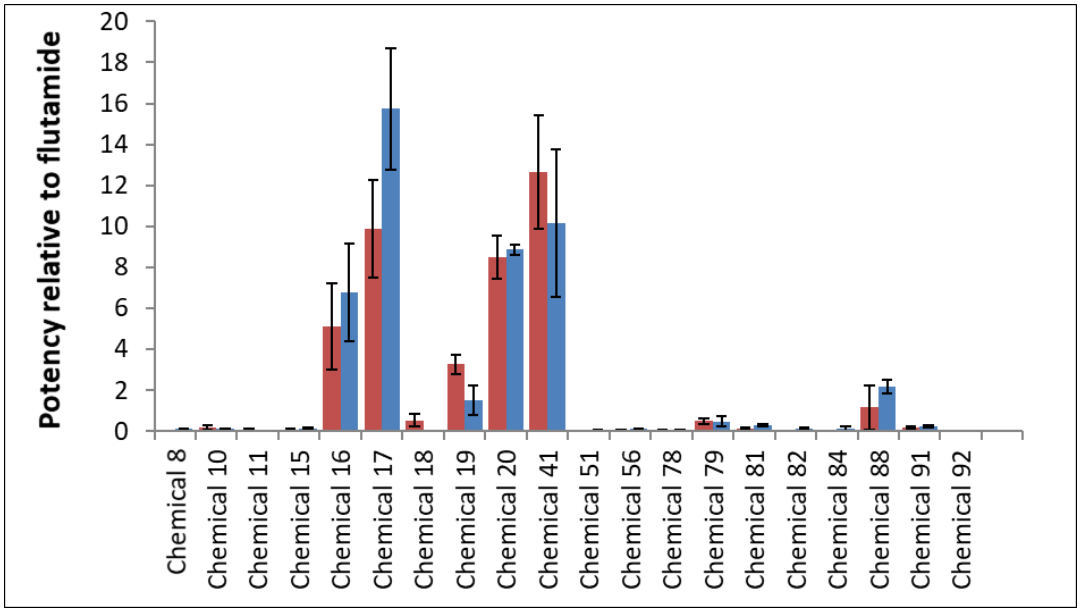
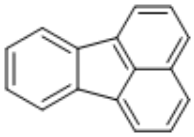


Figure 53. Anti-androgen screen results; mean potency values (± SEM; n≥2) for 20 chemicals (see Table 17 for chemical details) using the standard anti-androgen screen with no cell lysis (red bars) and the modified method with cell lysis (blue bars).

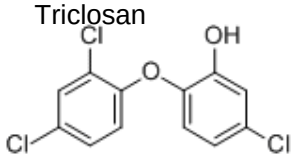
Potency values are relative to the positive control, flutamide, and values above 1 are more potent than flutamide. R^2 = 0.8789. See Appendix Tables A 31 and A 32 for raw data.

Anti-androgens – potent:

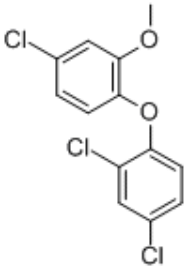
Fluoranthene



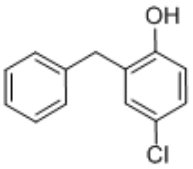
Triclosan



Methyl triclosan

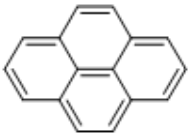


Chlorophene

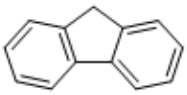


Anti-androgens – moderate:

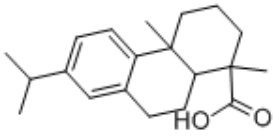
Pyrene



9H-Fluorene

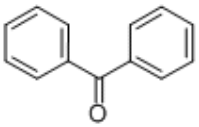


Dehydroacietic acid

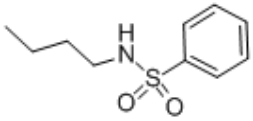


Anti-androgens – weak:

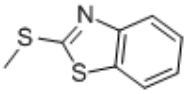
Benzophenone



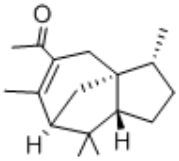
N-Butylbenzene sulphonamide



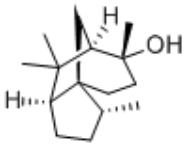
2-(methylthio)benzothiazole



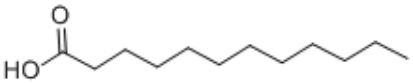
Acetylcedrene



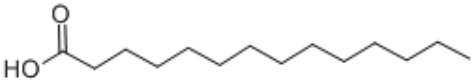
Alpha cedrol



Dodecanoic acid

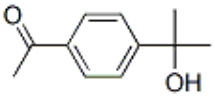


Myristic acid

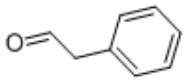


Anti-androgens – very weak:

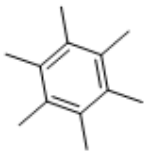
1-[4-(hydroxyl-1-methylethyl)phenyl] ethanone



Benzeneacetaldehyde



Hexamethylbenzene



9,12-Octadecadienoic acid methyl ester

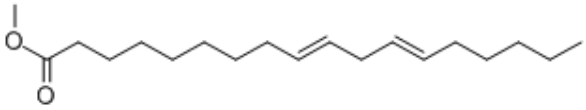


Figure 54. Structures of the eighteen anti-androgenic chemicals with activity observed using both yeast screen methods, grouped in order of activity; strong to very weak activity. Most have one or more benzene ring within the structure and below are examples of different side chain structures that can be attached to the ring.

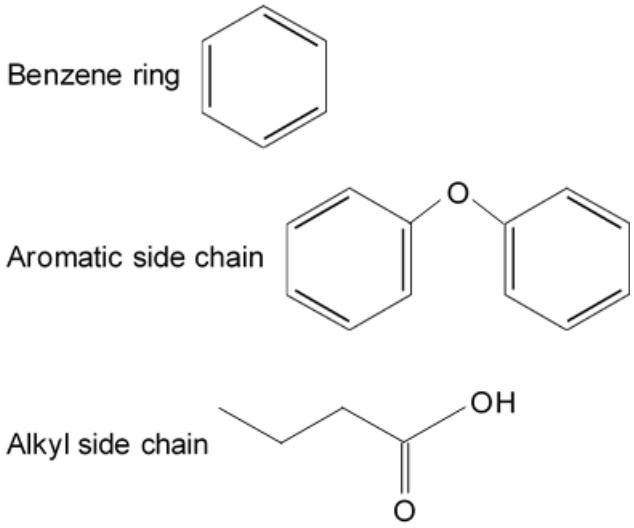


Table 18. Chemical characteristics of the most potent anti-androgens (strong and moderate activity) together with their reported environmental concentrations (no information was available for methyl triclosan as this is a metabolite and not a parent compound).

Chemical no.	Chemical	Cas no.	Anti-androgenic potency relative to flutamide	Solubility (mg/L)	Log Kow	Log BCF [a]	Half-life (days) [b]	Environmental concentrations (surface water) (ng/L)
17	Triclosan	3380-34-5	15.736	4.621	4.76	2.81	3.29	<3-135[c]
41	Chlorophene	120-32-1	10.166	112	3.6	2.04	0.268	86-191[c]
20	Methyl triclosan	4640-01-1	8.859					
16	Fluoranthene	206-44-0	6.768	0.1297	5.16	3.07	2.57	<1-29.6[d]
88	Dehydroabietic acid	1740-19-8	2.194	2.412	4.8	0.5	5.69	130-170[e]
19	Pyrene	129-00-0	1.520	0.2249	4.88	2.89	0.556	-840[f] and 750-4890[g]
79	9H-Fluorene	86-73-7	0.475	1.339	4.18	2.42	1.37	
81	Acetylcedrene	32388-55-9	0.294	1.278	5.02	2.98	7.82	<10[h]
91	Diphenylacetic acid methyl ester	3469-00-9	0.237	21.41	3.72	2.12	0.067	

a – Log bioconcentration factor (LogBCF) = 0.6598 LogKow - 0.333 + correction

b - normalised for 10g fish

c – Arlos *et al.* (2015); d – Tousova *et al.* (2017); e – McMartin *et al.* (2002); f – Kolpin *et al.* (2002); g – Kotti *et al.* (2018); h – Klaschka *et al.* (2013)

Values for LogKow, LogBCF and Half-life were taken from the Environmental Protection Agency Estimation Program Interface™ Suite v4.11 https://www.epa.gov/tsca-screening-tools/download-epi-suitetm-estimation-program-interface-v411

4.3.1.3. Chemicals able to enhance activity of DHT in anti-androgen screen (superagonists)

In 2013 and 2014 when the chemicals were initially tested in the standard AYAS with no lysis, twelve chemicals (many being fatty acids) had no obvious androgenic activity in the YAS but in the AYAS they were able to heighten the response above the raised background in the presence of DHT, normally suggestive of androgenic activity (see Chapter 3, Section 3.3.3). In the absence of androgenic activity, these 12 chemicals were enhancing the DHT response, i.e. behaving as superagonists.

At a later date in 2017, four of the more active fatty acids (dimethyl adipate, methyl heptadecanoate, methyl palmitate and methyl octanoate) were used as examples in the AYAS to compare the results observed using the method without lysis (Figure 55A) and the method with lysis (Figure 55B).

In 2016 and later in 2018, assays were run to try to get a better understanding for the reason for the enhanced activity in the AYAS but in these years no superagonism was apparent. That is, the earlier results did not repeat.

4.3.1.4. Investigation of issues with the potential to cause false positive results when screening for anti-androgenic activity

4.3.1.4.1. MTT assay for investigating the effects of toxicity on anti-androgenic activity

Nystatin was used to observe if toxicity could induce false positive results in the standard AYAS, i.e. cause a reducing effect on the DHT-elevated absorbance readings. Results are shown for the standard AYAS (540 nm and 620 nm absorbance readings are compared; Figure 56A), and the modified AYAS with lysis (420 nm and 620 nm absorbance readings are compared; Figure 56C). The standard AYAS 540 nm absorbance readings are also compared with the MTT assay 570 nm readings (Figure 56B). For the MTT assay, at the top four nystatin concentrations (wells 1-4; 6-2.7 mg/L; Figure 56B) the 570 nm absorbance readings are low, due to toxicity. When the 540 nm absorbance results from the standard AYAS were overlaid with the MTT 570 nm results (Figure 56B), where there was a lack of metabolic activity due to the nystatin's toxicity at 570 nm, there was also no colour development at 540nm that would normally be seen with DHT in the AYAS. At the nystatin concentration that was partially toxic (well 5; 2.1 mg/L) there was an increase in both the 540nm (standard AYAS) and 570 nm (MTT assay) absorbance readings, and at the other nystatin concentrations (wells 6-12; 1.6-0.3 mg/L) none of the absorbance readings were affected by the nystatin and developed

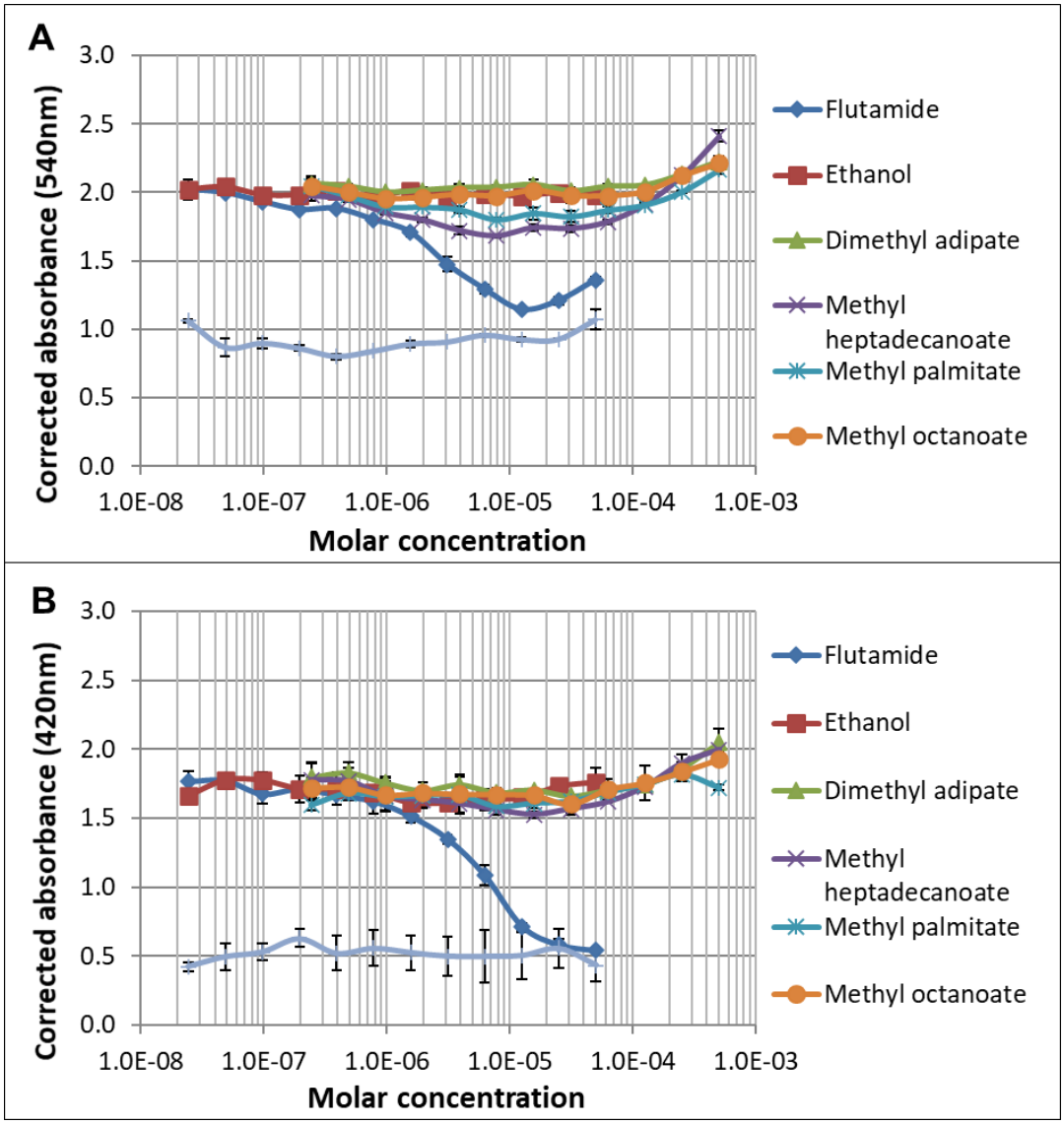


Figure 55. Superagonist results seen in (A) the standard anti-androgen screen with no cell lysis and (B) the modified anti-androgen screen with cell lysis. The plots are for mean values ± SEM for the flutamide positive control, the negative control (ethanol), and four fatty acids; dimethyl adipate, methyl heptadecanoate, methyl palmitate and methyl octanoate. Also included is an ethanol standard curve from the androgen screen run alongside the anti-androgen screen (ethanol minus DHT).

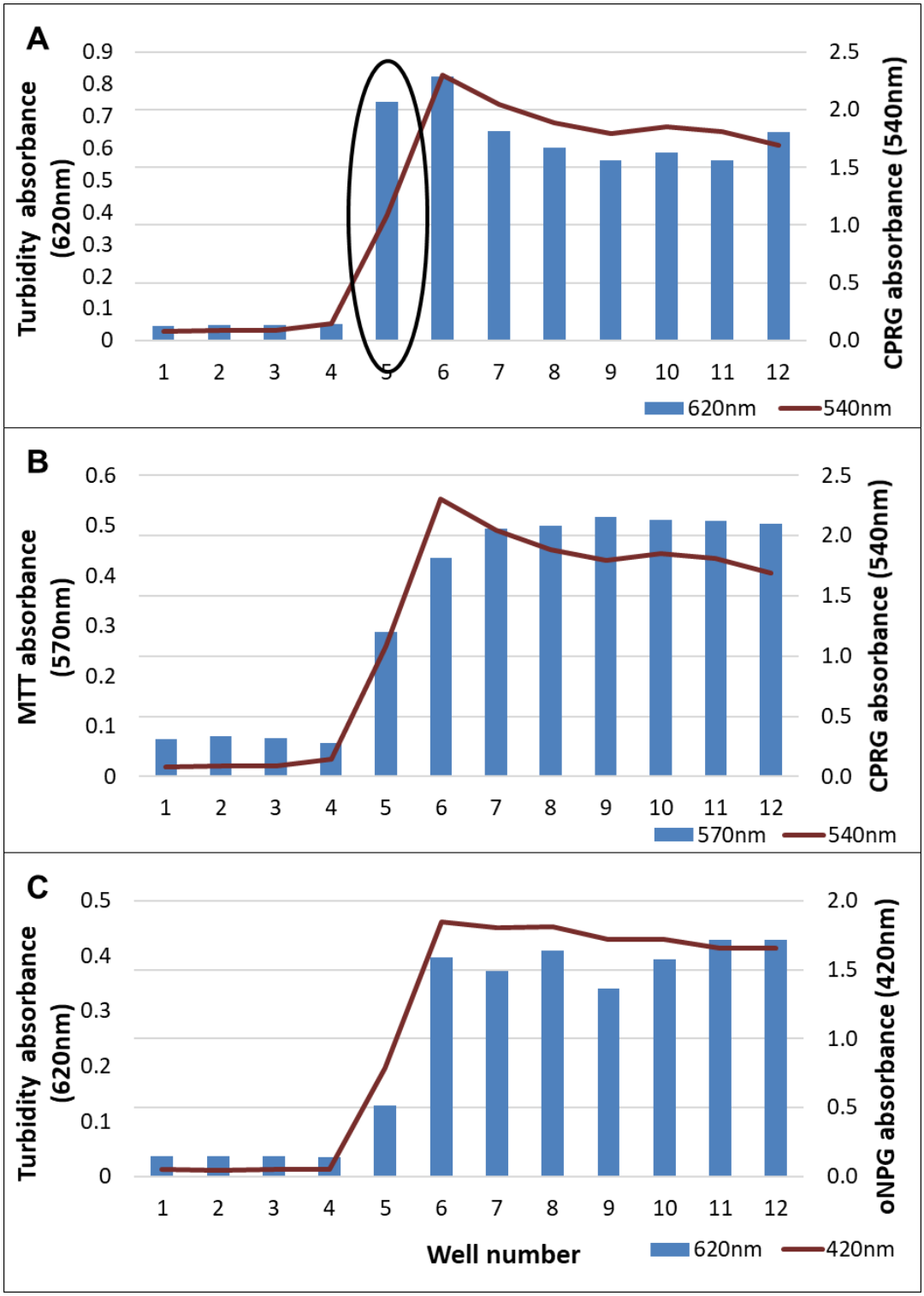

A
Turbidity absorbance (620nm)
CPRG absorbance (540nm)
620nm
540nm
B
MTT absorbance (570nm)
CPRG absorbance (540nm)
570nm
540nm
C
Turbidity absorbance (620nm)
oNPG absorbance (420nm)
Well number
620nm
420nm

Figure 56. Results from screens monitoring yeast turbidity using nystatin as a positive control to kill the yeast (highest concentration in Well 1 (6 mg/L nystatin) diluting to lowest concentration in Well 12 (0.3 mg/L nystatin)). The background absorbance was raised by adding DHT to the medium in all wells and at the highest nystatin concentrations, there was a drop in both colour and turbidity; (A) anti-androgen screen using the standard method without lysis, (B) standard anti-androgen screen compared with the MTT assay, and (C) the modified anti-androgen screen with lysis. The bars represent the colourimetric absorbance at 3 different wavelengths (CPRG; 540 nm, MTT; 570 nm, oNPG; 420 nm) and the lines shown the yeast turbidity read at 620 nm (A and C) or 540 nm colour (B). In (A) the oval marks the discrepancy between the CPRG (540 nm) and turbidity (620 nm) readings when using the standard method without lysis. In (A) this drop in the 540 nm reading without a corresponding drop in turbidity could be interpreted as anti-androgenic activity, whereas in the modified yeast method with lysis (C) the corresponding drop in turbidity indicates the toxicity observed in the MTT assay (B).

as would be expected due to the DHT present in the wells. Similarly, for the modified AYAS with lysis, the nystatin was toxic in the first 4 wells (620 nm and 420 nm; Figure 56C) and at the more dilute nystatin concentrations there was a decrease in the toxicity matched by an increase colourimetric response due to the DHT in the wells.

However, for the standard AYAS without lysis, in well 5 (Figure 56A) the nystatin was not visibly toxic (no reduction in the 620 nm absorbance), but there was less colour development at 540 nm. That is, in the standard screen the nystatin did not appear to be toxic but there was less colour development that might indicate anti-androgenic activity, if only turbidity was used as an indicator of toxicity (which it frequently is).

4.3.1.4.2. Different sealing tapes to prevent evaporation causing the 'edge effect' that can be misdiagnosed as anti-androgenic activity

In the standard screen the 'edge effect' (measured by absorbance at 540 nm) was a pronounced reduction in the 540 nm absorbance reading with sealing tape (i) (Figure 57A), as well as a visible reduction in the well volume around the edge of the plate. This 'edge effect' around the edge of the plate was not accompanied by a reduction in yeast turbidity (measured by absorbance at 620 nm; Figure 57B).

For the standard AYAS and the method with lysis, two different sealing tapes were compared to determine if they influenced 'edge effect' (a drop in the raised absorbance around the edge of the plate). The sealing tapes were used for the duration of the 3 day incubation. For the sealing tape (i), the 'edge effect' was obvious (540 nm absorbance; Figure 58A) and similarly, there was no reduction of the turbidity readings around the edge of the plate (620 nm absorbance; Figure 58B). For the sealing tape (ii), the 'edge effect' was not obvious (540 nm absorbance; Figure 58C) and similarly, there was no reduction of the turbidity readings around the edge of the plate (620 nm absorbance; Figure 58D).

The two types of sealing tape were also tested in the modified AYAS with cell lysis. The 'edge effect' with tape (i) was much reduced (420 nm absorbance; Figure 59A), and similarly there was no effect on the turbidity readings (620 nm absorbance; Figure 59B). When carrying out the AYAS with lysis, for the plate sealed with tape (ii) there was no 'edge effect' (420 nm absorbance; Figure 59C), or effect on the turbidity readings (620nm absorbance; Figure 59D).

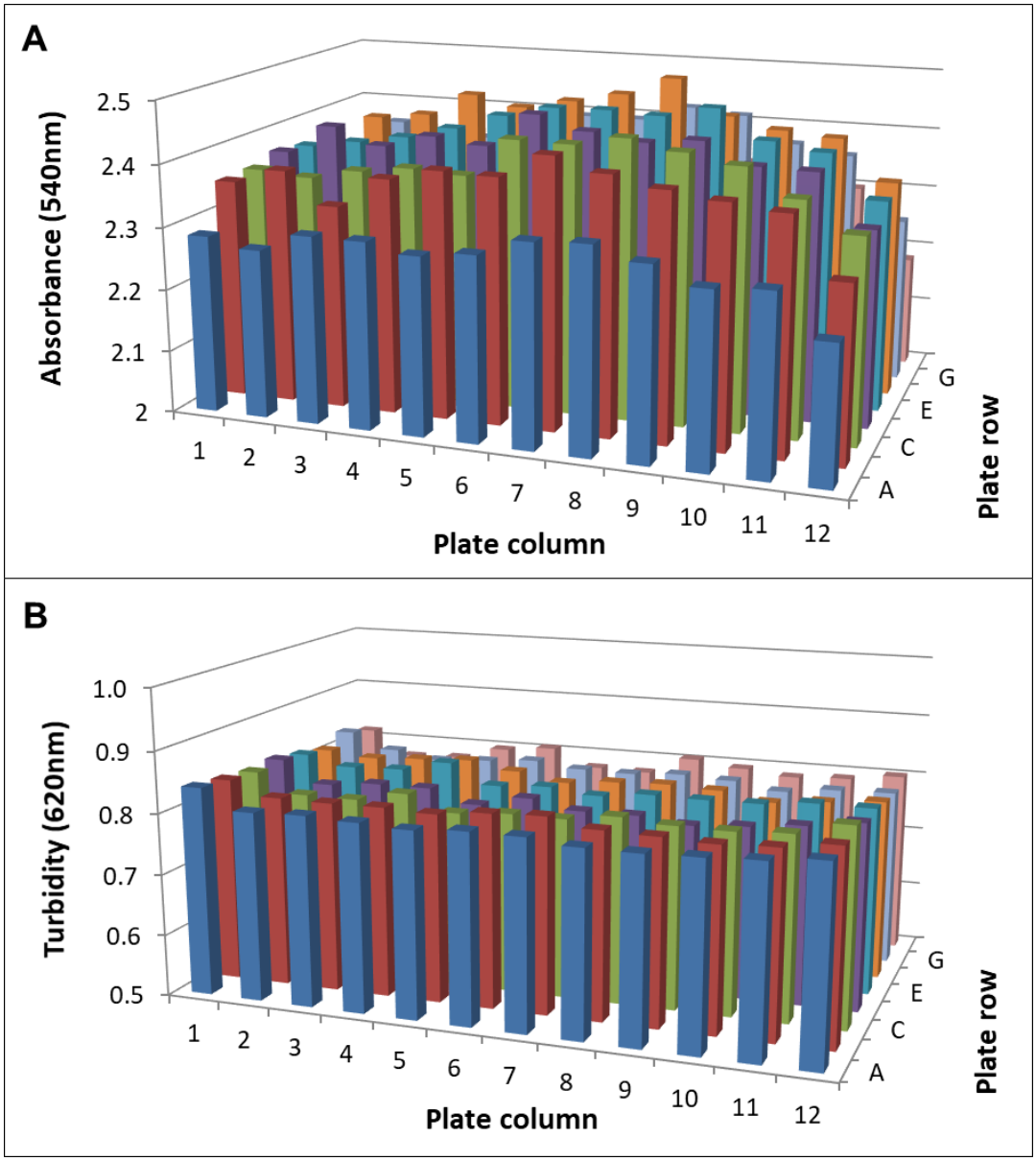


Figure 57. The influence of the plate column and plate row on the 'edge effect' at 540 nm (colourimetric) and 620 nm (turbidity) absorbance values using the standard anti-androgen screen method without lysis. All wells contained ethanol (evaporated to dryness) and media containing DHT, and plates were sealed with tape (i), to prevent evaporation during the 3 day incubation. (A) shows a pronounced 'edge effect' with a reduction in the 540 nm absorbance reading, most visible in the outside rows; Row A and H, and also the outside columns; Column 1 and 12. Each well has the same concentration of DHT, however the lower 540 nm absorbance in the outer rows/columns, could be misinterpreted as anti-androgenic activity. (B) shows the turbidity, 620 nm absorbance readings, in the same plate. The turbidity (620 nm) does not have the same reduction as the colourmetric (540 nm) readings. Both plots represent individual values from one 96-well plate.

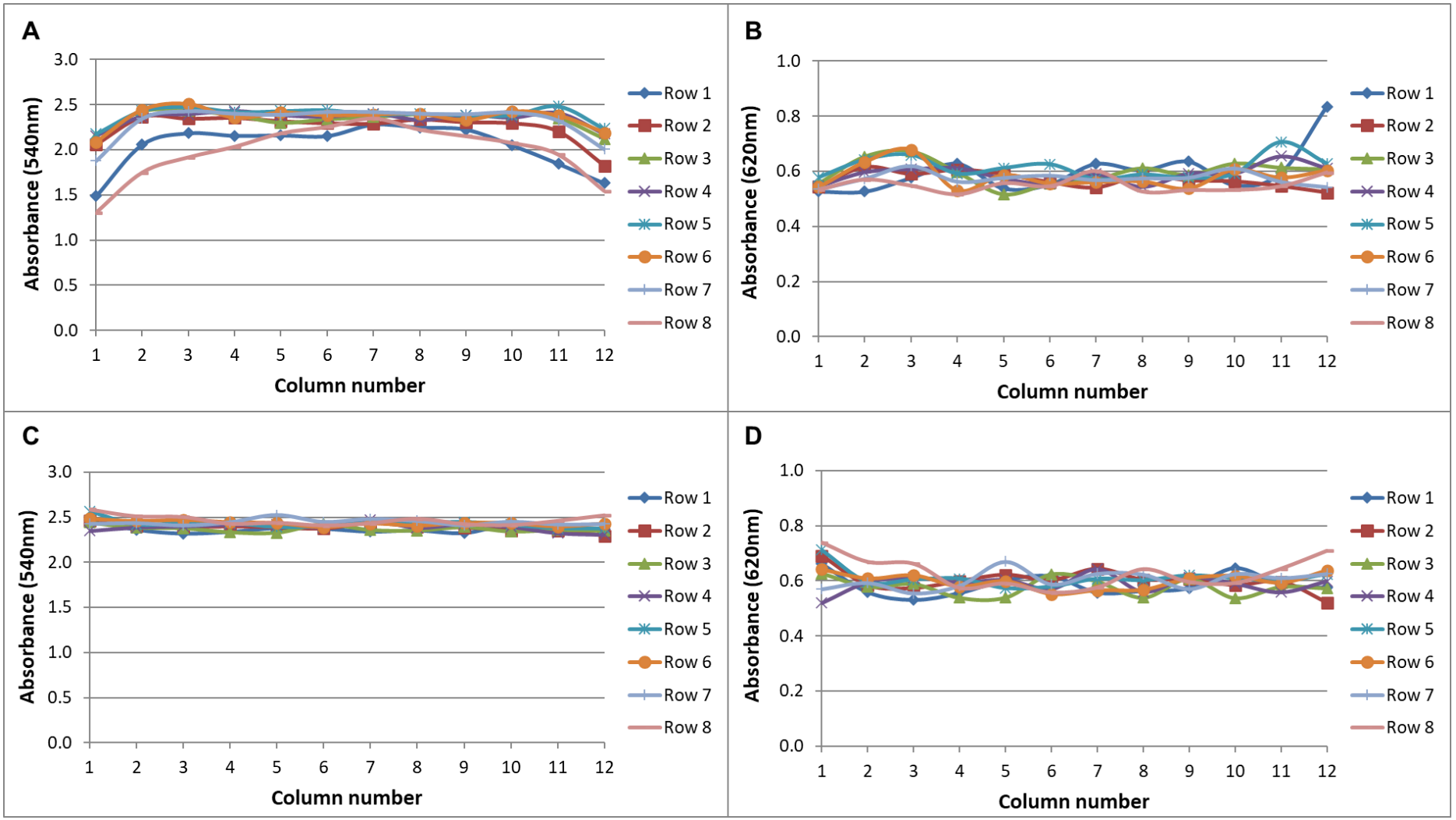

A
Absorbance (540nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1 2 3 4 5 6 7 8 9 10 11 12
Column number
Row 1
Row 2
Row 3
Row 4
Row 5
Row 6
Row 7
Row 8
B
Absorbance (620nm)
1.0
0.8
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12
Column number
Row 1
Row 2
Row 3
Row 4
Row 5
Row 6
Row 7
Row 8
C
Absorbance (540nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1 2 3 4 5 6 7 8 9 10 11 12
Column number
Row 1
Row 2
Row 3
Row 4
Row 5
Row 6
Row 7
Row 8
D
Absorbance (620nm)
1.0
0.8
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12
Column number
Row 1
Row 2
Row 3
Row 4
Row 5
Row 6
Row 7
Row 8

Figure 58. Different sealing tapes and their influence on the ‘edge effect’ values in the standard AYAS without cell lysis. All wells contain ethanol (evaporated to dryness) and media containing DHT, and plates were sealed with tape to prevent evaporation during the 3 day incubation. (A) shows the 540 nm absorbance readings for the plate sealed with sealing tape (i) and (B) the 620 nm absorbance values (turbidity) for the same plate. (C) shows the 540 nm absorbance readings for the plate sealed with sealing tape (ii) and (D) the 620 nm absorbance values (turbidity) for the same plate. Plots represent individual well values.

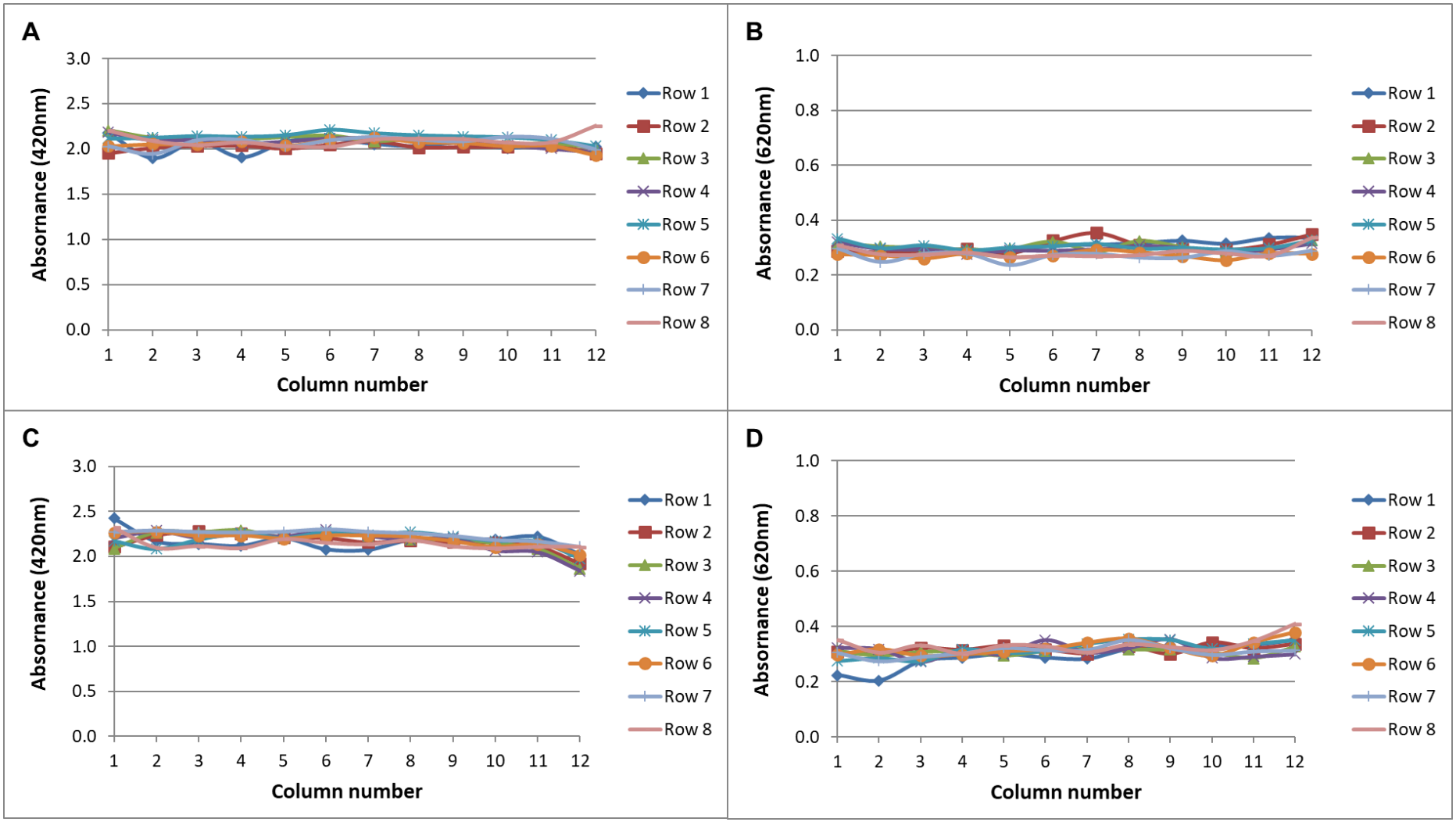

A
Absornance (420nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1 2 3 4 5 6 7 8 9 10 11 12
Column number
Row 1
Row 2
Row 3
Row 4
Row 5
Row 6
Row 7
Row 8
B
Absornance (620nm)
1.0
0.8
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12
Column number
Row 1
Row 2
Row 3
Row 4
Row 5
Row 6
Row 7
Row 8
C
Absornance (420nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1 2 3 4 5 6 7 8 9 10 11 12
Column number
Row 1
Row 2
Row 3
Row 4
Row 5
Row 6
Row 7
Row 8
D
Absornance (620nm)
1.0
0.8
0.6
0.4
0.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12
Column number
Row 1
Row 2
Row 3
Row 4
Row 5
Row 6
Row 7
Row 8

Figure 59. Different sealing tapes and their influence on the ‘edge effect’ values in the modified AYAS with cell lysis. All wells contain ethanol (evaporated to dryness) and media containing DHT, and plates were sealed with tape to prevent evaporation during the 3 day incubation. (A) shows the 420 nm absorbance readings for the plate sealed with sealing tape (i) and (B) the 620 nm absorbance values (turbidity) for the same plate. (C) shows the 420 nm absorbance readings for the plate sealed with sealing tape (ii) and (D) the 620 nm absorbance values (turbidity) for the same plate. Plots represent individual well values.

4.3.1.4.3. Well position in multiwell plate and its effect anti-androgenic potency relative to flutamide

When flutamide was pipetted into outer rows A and H, the maximum absorbance value was 2.373 and the IC50 was $2.76x10^{-6}$M (Figure 60A), whereas when flutamide was pipetted in to middle rows D and E the maximum absorbance value was 2.529 and the IC50 was $2.58x10^{-6}$M (Figure 60B). The reverse was true when the positions of the positive control and the test chemical were swapped, and when benzeneacetaldehyde was pipetted into middle rows D and E, the maximum absorbance was 2.474 and the IC50 was $7.37x10^{-5}$M (Figure 60A), whereas when benzeneacetaldehyde was pipetted in to outer rows A and H the highest absorbance was 2.421 and the IC50 was $4.87x10^{-5}$M (Figure 60B). That is, the outer rows have lower maximal absorbance values as they are affected by the 'edge effect' and chemicals are less affected when they are pipetted into the middle rows. The position that the chemical was pipetted on the plate did also alter the position of the slope of the curves and the potency of benzeneacetaldehyde relative to flutamide in plate layout A (Figure 60) was 0.037 and for plate layout B the potency of benzeneacetaldehyde relative to flutamide was 0.054.

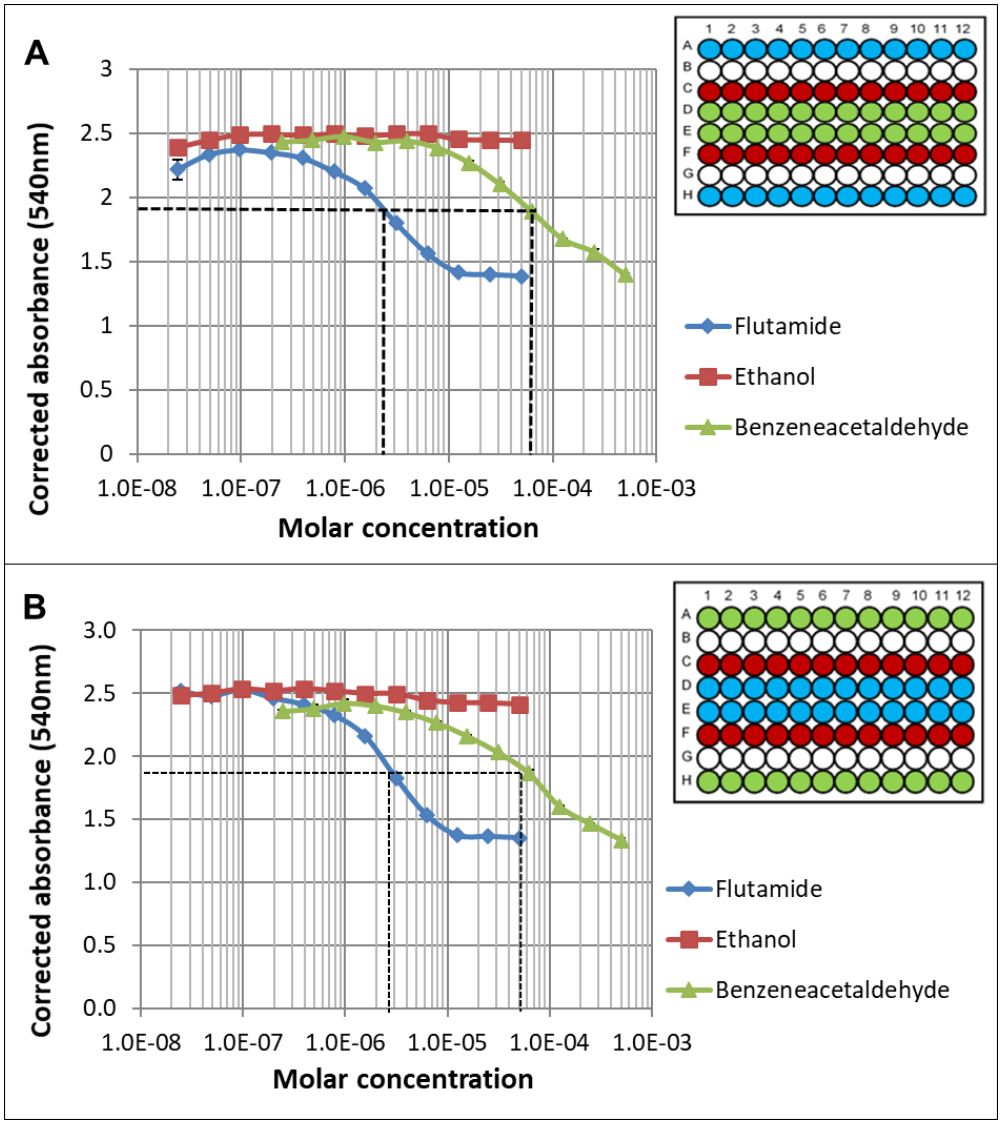


Figure 60. Pipetting to different positions in the multiwell plate and effect on chemical potency. Plots describing how the position of the chemical on a 96-well plate can influence perceived activity in the standard anti-androgen screen method without cell lysis.The positive control (flutamide; blue diamonds), the negative control (ethanol; red squares), and the example test chemical (benzeneacetaldehyde; green triangles) were pipetted into different positions in the multiwell plate (see insets). In (A) flutamide is pipetted at the edge of the plate in rows A and H and benzeneacetaldehyde is pipetted to rows D and E. In (B) the positions are reversed and flutamide is pipette to rows D and E and benzeneacetaldehyde is pipetted to rows A and H. Sealing tape (i) was used to seal the plates prior to incubation. For both plate layouts, ethanol is pipetted to rows C and F. The plots are for mean values ± SEM.

4.4. Discussion

4.4.1. Comparison of the standard YAS with the lysis method for detecting androgenic, anti-androgenic and superagonism activity

There are a number of reliable *in vitro* tests for (anti-)androgenic activity and these are generally based on either mammalian cell lines (Korner *et al.*, 2004) or yeast cells (Sohoni and Sumpter, 1998). When testing chemicals for (anti-)androgenic activity in this thesis using the standard YAS and AYAS (Sohoni and Sumpter, 1998), a number of issues were encountered that had the potential to influence the results and could lead to a chemical being wrongly labelled.

4.4.1.1. Androgenic activity

Using the standard YAS to test 107 chemicals for activity, only three had androgenic activity (methyl decanoate, 2-ethyl hexanoic acid and ibuprofen) and all gave sub-maximal responses that were too weak to quantify. There are no reports in the literature of methyl decanoate or 2-ethylhexanoic acid possessing either *in vitro* or *in vivo* androgenic activity (latest Web of Science search 22/12/2020) and a search of PubChem (https://pubchem.ncbi.nlm.nih.gov/, downloaded 22/12/2020) of HTP screens utilising AR bioassays with MDA cells showed either no activity or inconclusive activity for both methyl decanoate or 2-ethylhexanoic acid. Differences in activity when using different test systems have been explained by others as being due to a difference in toxicity, different permeability of the cell wall/membrane, or the fact that yeast cells lack mammalian-specific coactivators (Christiaens *et al.*, 2005). However, yeast cells would be more likely to underestimate activity rather than overestimate it, due to the less permeable cell wall (Soto *et al.*, 2006). Given that no androgenic activity was seen when using the modified YAS with cell lysis, the results seen here may be due to artefacts.

For ibuprofen, at concentrations above 10^{-4} M there was a reduction in turbidity, and this meant that the curve did not attain its maximal absorbance. However, even by extrapolating the curve, the potency would have been at least a million times less than the potency of DHT. No androgenic activity was seen with ibuprofen when using the modified yeast screen with cell lysis, but the ibuprofen was toxic to the yeast cells at the highest concentrations, so androgenic activity may not have been seen for this reason. Why the ibuprofen would be more toxic using the method with cell lysis is unclear, as whilst the preparation of the medium was different, the concentrations of the individual components were almost identical. I could find no reports in the literature of ibuprofen possessing androgenic activity (latest Web of Science search 22/12/20), but ibuprofen has been demonstrated to have anti-androgenic activity in a bioluminescent yeast assay (Ezechias *et al.*, 2016) and has been

linked with anti-androgenic effects in humans (depression of T production; Kristensen *et al.*, 2018). As ibuprofen is able to interact with the AR, the yeast may have behaved differently to the chemical (as an agonist and not an antagonist) due to the near toxic concentrations, or produced false positive androgenic activity triggered by cell stress at concentrations approaching toxicity (Escher *et al.*, 2019; Fay *et al.*, 2018; Judson *et al.*, 2017).

4.4.1.2. Anti-androgenic activity

For some of the chemicals tested in this thesis, other laboratories found them to have anti-androgenic activity, but we did not. For example, tris (2-butoxyethyl) phosphate and triphenyl phosphate (Liscio *et al.*, 2014), HHCB (Orton *et al.*, 2014), benzo[a]pyrene (Tamura *et al.*, 2006; Vinggaard *et al.*, 2008), DEP (Christen *et al.*, 2010; Tamura *et al.*, 2006), chrysene (Vinggaard *et al.*, 2008) and (Tamura *et al.*, 2006), and carbamazepine (Liscio *et al.*, 2014). For a number of these chemicals we noticed toxicity that might have been misinterpreted as antagonistic activity by others, e.g. tris (2-butoxyethyl) phosphate. At concentrations close to toxicity cells activate numerous defence mechanisms (described as cytotoxic burst), and these can potentially lead to non-specific activation of reporter genes (Escher *et al.*, 2019). However, for other chemicals, e.g. benzo[a]pyrene, we did not notice any sign of toxicity, but in this case it is worth noting that the benzo[a]pyrene was poorly soluble and may not have been taken up by the yeast cells.

On searching PubChem (https://pubchem.ncbi.nlm.nih.gov/, downloaded 22/12/2020) for confirmation of AR antagonistic activity, the results were a mix of inactive, inconclusive and active for a number of these chemicals. The HTP bioassays mainly use MDA-kb2 cells with the test chemicals dissolved in DMSO, and therefore even with similar bioassay protocols, consistent results are limited.

4.4.1.3. Chemicals able to enhance activity of DHT in anti-androgen screen (superagonists)

Using the standard AYAS method, twelve chemicals produced an enhanced response above that produced by baseline DHT. However, in the YAS, in the absence of DHT, these chemicals had no androgenic activity and hence we term them ‘superagonists’. Some of the ‘superagonist’ compounds were fatty acids. These chemicals could be acting as penetration enhancers rather than actually enhancing the activity at the AR, i.e. the fatty acid could be aiding DHT entry into the cells or the secretion of β-gal into the medium.

This superagonism was seen with both our standard and modified AYAS methods (without cell lysis and with cell lysis) but the results were not reliable in that the superagonism was noticed in 2013, 2014, and 2017, but not in 2016 and 2018. Fresh chemicals were

purchased at the start of this work in 2013 and 2014, and again in 2017 when the superagonists were repeated in the modified AYAS with cell lysis. It is therefore possible that the superagonism was observed when the chemicals were fresh but not after they had been stored. However, due to the long periods between assays, there would have been different batches of yeast and media used for many of the experiments, making it difficult to be consistent despite following a Standard Operating Procedure. These differences may have impacted on the sensitivity of the cells to the various chemicals, and perhaps the cell wall was more/less penetrable at different times, depending on this sensitivity. Unfortunately, due to time constraints I was not able to investigate this phenomenon further.

This potentiation of the response has also been observed with genistein using both gene reporter assays for anti-oestrogens (Legler *et al.*, 1999; Sonneveld *et al.*, 2005) and for anti-androgens (Ermler *et al.*, 2010). Similarly, the potentiation of the response has also been observed with triclosan in gene reporter assays for both anti-oestrogens (Ahn *et al.*, 2008) and for anti-androgens (Ahn *et al.*, 2008; Christen *et al.*, 2010).

Superagonism observed *in vitro* has also been observed *in vivo*. For example, when Knag *et al.* (2013) exposed stickleback fish to naphthenic acids, no androgenic effect was observed when tested alone, but when tested with DHT there was a potentiation of the spiggin induction (androgenic) response in a dose-dependent manner. But when this experiment was repeated with seawater rather than freshwater, there was no longer a potentiation of the spiggin signal, and Knag *et al.* (2013) suggested that possibly the composition of the naphthenic acids was different when in the saltwater. The potentiation of the feminising effects of EE2 have also been observed *in vivo* in roach (Lange *et al.*, 2015) and rats exposed to EDCs (Stoker *et al.*, 2010), and given that this phenomenon has been observed in a number of different instances (with different chemicals, different cell lines, different receptors, *in vitro* and *in vivo*) it seems likely that the response is not receptor mediated.

4.4.1.4. MTT assay for investigating the effects of toxicity on anti-androgenic activity

Toxic effects of chemicals *in vitro* can cause misleading results in the AYAS, as both anti-androgenic activity and toxicity cause a drop in the DHT-elevated absorbance, either because the action of DHT is blocked (anti-androgenic) or cell death means that the cells no longer respond to the DHT (toxic), and both lead to a reduction in β-gal. *In vitro* testing is therefore often carried out alongside tests for toxicity. Using the standard AYAS (see Chapter 3, Section 3.3.2), a number of the chemicals that we tested were either toxic (giving a clear yellow well with no red colour developing) or caused a reduction in the yeast turbidity at the higher concentrations (together with an inhibition in the development of the red colour). Hence where anti-androgenic activity was observed for chemicals that were toxic at

the higher concentrations, the results may in fact be due to cytotoxicity. Similarly, when Huang *et al.* (2011a) examined data for approximately 3000 environmental chemicals from the Tox21 pilot-phase collection against a panel of 10 nuclear receptors including the AR, a total of three hundred and twenty three compounds were identified as potentially cytotoxic. False positive activities are often caused by cell stress at concentrations approaching toxicity (Judson *et al.*, 2016).

Due to the potential for chemicals at/close to cytotoxic concentrations to cause false positive results, the antifungal nystatin was used to see how 'killing' the yeast cells affected the DHT enhanced colourimetric absorbance readings. The reduction in the DHT colourimetric response in the AYAS due to nystatin (Figure 56B) and modified method with lysis (Figure 56C), was compared with the respective turbidity readings at 620 nm and the absorbance at 570 nm in the MTT assay.

In the MTT assay (a measure of metabolic activity), the four highest concentrations of nystatin (6-2.7 mg/L) caused toxicity and these same results were mirrored in the standard AYAS (Figure 56B). Similarly, for the modified AYAS with cell lysis, the toxicity was mirrored by low colourimetric values (Figure 56C). Therefore, a drop in the MTT values in the MTT assay and the turbidity values in the modified AYAS with lysis both gave a good indication of toxicity. However, for the standard AYAS, whilst there was a lack of colour change at 540 nm due to the toxicity of nystatin, this was not reflected at almost toxic concentrations (Figure 56A), where there was little alteration in the yeast turbidity absorbance value. Because the standard AYAS relies on the secretion of the β-gal into the medium, under mildly toxic conditions or prior to cell death, β-gal secretion could continue even as the cells are being killed by the nystatin, and hence false positive anti-androgenic activity might be observed.

Chemicals may also be non-toxic but exert their effects in other ways. For example, Liscio *et al.* (2014) used a yeast-based assay to identify two antifungal agents with high relative potency in anti-androgenic fractions following fractionation of river water samples; these were propiconazole and miconazole. However, when using the AR-CALUX assay, which uses mammalian cells, these two compounds were found to have very weak anti-androgenic activity. Conazole fungicides inhibit ergosterol synthesis, which is necessary for creating yeast cell wall membranes, and although Liscio *et al.* observed no effects on yeast growth, it is possible that the antifungal agents affected the yeast cell function in other ways. Kjaerstad *et al.* (2010) examined the effects of conazole antifungals using a panel of *in vitro* assays and found the critical mechanism for endocrine disruption to be the disturbance of steroid biosynthesis, rather than receptor binding.

4.4.1.5. Different sealing tapes to prevent evaporation causing the 'edge effect' that can be misdiagnosed as anti-androgenic activity

Using the standard AYAS procedure using autoclave tape (i) to seal the 96-well assay plates, we observed a distinct 'edge effect' or 'hook effect' to the 540nm absorbance readings. Similar to Patel *et al.* (2005), we saw no reduction in turbidity across the plate but there was a visible reduction in the volume in the outer wells of the plate. In the standard AYAS, this 'edge effect' causes a reduction in colour in the outer wells that could be misinterpreted as anti-androgenic activity. On using a different, less porous tape to seal the plate, this 'edge effect' was much reduced. For the modified screen where the cells are lysed, only a proportion (25%) of the well contents was removed from the assay plate and transferred to a fresh plate for cell lysis, and using this method the 'edge effect' was also much reduced with both tapes. Zimmermann *et al.* (2003) also examined different tapes and materials to compare their ability to minimise water evaporation from a 96-well plate while maintaining oxygen supply as high as possible. Evaporation was quantified by weighing the plate filled with water but an optimal product with high oxygen permeability and low evaporation was not found.

Whilst a tighter seal did reduce evaporation in our AYAS and resulted in a reduced 'edge effect', by using a less porous tape it is possible that less oxygen was available for aerobic growth. Snoek and Steensma (2007) compared the composition of yeast cell walls when growing under aerobic and anaerobic conditions, and under anaerobic conditions the yeast cells could no longer synthesise sterols but instead required them to be added to the media. As sterol is necessary for producing the cell wall, there is a possibility that the cell wall could have been altered when using the less porous tape, but nevertheless there did not appear to be any difference in the turbidity measurements with the second less porous tape.

4.4.1.6. Well position in multiwell plate and its effect anti-androgenic potency relative to flutamide

The position that a chemical is pipetted into a plate did alter the potency of that chemical relative to flutamide. This was due to the 'edge effect' with the standard AYAS and with sealing tape (i). Reducing the 'edge effect' by using the less porous tape to seal the plate and/or the modified method with cell lysis rather than the standard AYAS, is likely to minimise this difference in potency depending on the pipetting position (although due to time constraints this was not confirmed). Alternatively, the outer wells could be left unused (although this does not make good use of the wells) or chemicals pipetted into different positions when retesting chemicals.

4.4.2. Advantages and disadvantages of the two different yeast screens for (anti-) androgenic activity

The standard yeast screen methods without cell lysis has the advantage that the colour development of the screen can easily be monitored over a period of time, because the yeast secretes the β-gal into the medium, whereas with the modified cell lysis method no colour development is seen until after lysis and incubation with the substrate. The standard method without lysis is a quicker screen to perform, as the method with cell lysis requires transferring a proportion of the medium in each well to another plate for lysing. This transfer process can also increase variability if the cells are not well resuspended. The cell lysis method, though, could be sped up by lysing the cells with lyticase rather than freeze/thawing, and this might additionally produce less variable readings.

The standard screen method could lead to misinterpretations due to toxicity, as it did for two of the chemicals presented in Chapter 3. Also, when comparing the two methods (with or without cell lysis) in the YAS, three chemicals were shown to be androgens in the standard YAS method (methyl decanoate, 2-ethylhexanoic acid and ibuprofen) but not in the modified method with cell lysis, and hence these activities were possibly false positives. I found the modified method with cell lysis to be less prone to false positives for both androgenic and anti-androgenic activity, and therefore believe it to be a more robust method.

4.4.3. Are the most potent anti-androgens likely to cause harm to the environment?

The structures of chemicals containing AR antagonist properties are extremely diverse (Vinggaard *et al.*, 2008). During AR binding, the H12 functions as a lid, which closes (agonist) or moves away from the LBP (antagonist), and AR antagonists are therefore usually bulkier than agonists and thus require a wider binding pocket than agonists (presented in Chapter 1, Section 1.2).

The most potent of the anti-androgens that I tested were four carbon ring structures (as for DHT) (Table 17 and Figure 54). In general, the smaller the structure the less the activity (most of the compounds with moderate activity had a 3-ring structures, those with weak activity had a 2-ring structures and those with very weak activity had a single-ring. An aromatic side chain has been shown to have a significant role in the anti-androgenic potency of parabens (Ding *et al.*, 2017), and for many of the more potent anti-androgen that I tested this was also the case. Three of the potent chemicals had one or more chlorines and this has also been shown to increase AR binding (Singh *et al.*, 2000). Ding *et al.* (2017), reported that larger side chains were unable to dock into the AR LBD, and perhaps for the three fatty acids that only had weak activity this was due to their longer chain length.

The most potent anti-androgens were assessed for their ability to cause harm to the environment. For these chemicals, the potency, the water solubility, octanol/water partition coefficient (LogKow), log bioconcentration factor (LogBCF), and half-life are presented in Table 18.

Chemicals with a high water solubility (>1 g/L) will partition to water and not to particles such as sludge, sediments, and soil, and *vice versa* for chemicals with low solubility (<0.1 g/L). Highly soluble chemicals are more likely to present a potential exposure concern for aquatic organisms in surface waters, and for humans via drinking water, but the more potent compounds identified in this chapter were only slightly soluble (e.g. triclosan, has a solubility of 4.621 mg/L). In addition, chemicals with a LogKow greater than 4 are hydrophobic and are again more likely to partition with particles, as was the case for the LogKow for most of our compounds. Of more concern are compounds with a LogKow between 2 and 4 (e.g. chlorophene and diphenylacetic acid), as these can be readily absorbed and are more likely to accumulate in aquatic organisms; Meador *et al.* (2016) found most of the compounds detected in fish to have high LogKow values. Stackelberg *et al.* (2007) also found the primary route of removal for hydrophobic analytes (LogKow > 4) was adsorption onto GAC during the wastewater treatment process and in surface waters it was the most hydrophilic classes that were detected, namely pharmaceuticals with LogKow value less than 1. According to the Sustainable Futures/P2 Framework Manual, 2012 (EPA-748-B12-001), any compound with a LogBCF ≥ 3.7 has a high bioconcentration potential, a value of 3 has a moderate bioconcentration potential and below 3 has a low bioconcentration potential. Many of our active compounds have a LogBCF close to 3 (triclosan, pyrene and acetylcedrene; Table 18) but only one exceeded 3 (fluoranthene; Table 18).

Another chemical property is the half-life; anything with a value greater than 2 days meets the Persistence Criteria set by the EPA for new chemicals (http://www.epa.gov/oppt/newchems/pubs/pbtpolcy/htm). Four of the anti-androgenic compounds identified in this chapter fall into this category; triclosan, fluoranthene, dehydroabietic acid and acetylcedrene. Longer half-life means that triclosan, fluoranthene, dehydroabietic acid and acetylcedrene are more likely to bioaccumulate in in animals and bioconcentrate through the food chain (Wangmo *et al.*, 2018), although the LogBCF for dehydroabietic acid is very low (Table 18). The most soluble of our active compounds, chlorophene and diphenylacetic acid methyl ester, did have very short half-lives but, despite this, chlorophene has been detected in surface waters at concentrations from 86-191ng/L (Arlos *et al.*, 2015). In addition, whilst acetylcedrene has a long half-life, it is only at low concentrations in the aquatic environment and therefore not such a concern.

Fluoranthene is one of the 48 compounds on the List of Priority Substances in the Field of Water Policy, with an EQS of 6.3 ng/L (WFD (Classification, Priority Substances and Shellfish Waters) Regulations (Northern Ireland) 2015). However, fluoranthene is only on this list as an indicator of other, more dangerous, PAHs. About 817,800 tonnes of high temperature coal tar pitch, which contains fluoranthene and pyrene, was produced in one year and production leads to PAH emissions (Fluoranthene EQS dossier, 2011). Given the production volume, potency, chemical properties, and the environmental concentrations of fluoranthene and pyrene, these compounds should be considered as hazardous. Especially as, whilst they are likely to be associated with particles rather than partitioned to water, zebrafish exposed to freely dissolved fluoranthene and pyrene in the presence of suspended particles had an elevated uptake rate due to exposure via ingestion (Zhai *et al.*, 2018).

Triclosan and its metabolite, methyl triclosan, were both potent anti-androgens and methyl triclosan is more persistent and toxic and found more commonly in fish than triclosan (Rudel *et al.*, 2013). Due to its potency, the US Food and Drug Administration has banned triclosan from some household products and it is being considered for banning and/or replacement in products around the world (Cavanagh *et al.*, 2018). Since this ban the production of triclosan has dropped and in 2013, 4,760 tonnes were produced worldwide compared with 6,581 tonnes in 2011 (Triclosan Market Report, 2016). Triclosan has been widely detected in surface water (e.g. Arlos *et al.*, 2015; Fuzzen *et al.*, 2016; Kolpin *et al.*, 2002; Meador *et al.*, 2016; Scott *et al.*, 2018) and in human urine (mean of spot samples 23.3 µg/L; Pollack *et al.*, 2016). Whilst I found triclosan to be a potent anti-androgen *in vitro*, Pernoncini *et al.* (2018) found no evidence of (anti-)androgenic activity in the *in vivo* Hershberger assay at a dose as high as 8 mg/kg. Furthermore, Mihaich *et al.* (2017) weighted up the evidence in over 35 peer-reviewed *in vitro* and *in vivo* studies for an assessment of the endocrine activity of triclosan, and concluded that their assessment indicated that triclosan was not acting as an agonist or antagonist within the oestrogen, androgen, thyroid or steroidogenic pathway. Considering that triclosan is able to bioconcentrate (900 - 2100 times in algae; Coogan *et al.*, 2007) and has a long half-life with a potent metabolite, this chemical is still a potential concern despite the questionable *in vivo* activity. Indeed, due to the frequency of detection in surface waters and exceedance of the PNEC (30 ng/L; Liu *et al.*, 2020), triclosan appears on several priority lists as a high to moderate risk to the environment (Liu *et al.*, 2020; Tousova *et al.*, 2017; Zhou *et al.*, 2019)

Whilst several of the chemicals tested in this chapter possessed anti-androgenic activity that was weaker than the potent anti-androgen, flutamide, it is important to consider that the chemicals present in the environment will occur as a mixture. For compounds that exhibit the

same mode of action one would expect additive effects (Ermler *et al.*, 2011), and many weak anti-androgenic chemicals might together be sufficient to cause disruption.

In this chapter I have looked extensively at two *in vitro* tests for (anti-)androgenic activity. However, no single assay can be expected to be 'the best' and any activity seen *in vitro* needs to be confirmed *in vivo* to ensure that the bioaccumulation, metabolism, and availability to the target cell, or alternative pathways for endocrine disruption of the compounds, and thus the final health effects, are assessed. Only by using a suite of assays, as described in the OECD conceptual framework for evaluating chemicals for endocrine disruption (OECD, 2018d), is it possible to lower the likelihood of mislabelling chemicals as EDCs. Currently there are no validated fish tests for anti-androgens, and although the Androgenised Female Stickleback Screen (AFSS; a variant of the 21-Day Fish Assay; OECD, 2018c) has more power to identify anti-androgens than the OECD TG 229 or TG 230 (OECD, 2018a; b), this test has a more limited range of endpoints. The AFSS is an assay for identifying endocrine active chemicals with (anti-)androgenic activity in fish using sexually mature female sticklebacks, that are specifically dosed with DHT to induce spiggin production (androgenised females). Chemicals blocking the AR receptor reduce this spiggin production, indicating anti-androgenic effect (Katsiadaki *et al.*, 2006). An alternative *in vivo* assay with the ability for identifying anti-androgens is the Juvenile Medaka Anti-Androgen Screening Assay (OECD, 2018c), but this assay awaits validation.

In Chapter 5, next I investigate potential biomarkers for (anti-)androgenic activity in the FHM, a species regularly used in OECD test guidelines to identify oestrogenic, androgenic, and steroidogenic activity of chemicals *in vivo.*

5. Do fathead minnows possess a spiggin-like protein that could be used as a sensitive bio-marker of (anti-)androgen exposures?

5.1. Introduction

Small-bodied freshwater fish are commonly used in regulatory testing for EDCs. Currently there are only a few fish tests that assess chemicals for (anti-)androgenic activity, and these do so by monitoring for changes in SSCs; gonopodial development in mosquito fish (Howell and Denton, 1989), papillary process development in medaka (Seki *et al.*, 2006), and tubercle number and size of fatpad in FHM (Ankley *et al.*, 2001). These endpoints often lack sensitivity (i.e. are not responsive to low concentrations of androgenic compounds; Muldoon and Hogan, 2016) and some are scored on a subjective scale so may be subject to bias (Ankley *et al.*, 1998). SSCs can also be affected by the hierarchical status of the fish, making it difficult to attribute a change in appearance to exposure to an EDC (Muldoon and Hogan, 2016).

To date there is only one adequate androgen dependant biomarker that has been developed in fish for the screening of (anti-)androgens (Katsiadaki *et al.*, 2006; Katsiadaki *et al.*, 2002). This screen uses the three-spined stickleback (*Gasterosteus aculeatus*), a fish native to most inland coastal waters north of 30°N. The kidney of the male stickleback hypertrophies during the breeding season, when the secondary proximal epithelial cells synthesise a glue glycoprotein, spiggin (the Swedish name for the three-spined stickleback being spigg). The spiggin is secreted into the urinary bladder and is then used to stick plant material together to form a nest (Borg, 1994). Following fertilisation of the eggs, the male subsequently cares for the eggs until shortly after they hatch.

Androgen-dependent spiggin can also be induced in the kidneys of female stickleback fish in response to endogenous androgens, where it can be determined by measuring the KEH or by using an ELISA for spiggin (Katsiadaki *et al.*, 2002). European bullhead exposed to trenbolone acetate also had dose-dependent kidney hypertrophy (linked to the production of glycoprotein containing mucus), which was quantified using the KEH measurement (Villeret *et al.*, 2013). However, the bullhead KEH measurement was a less sensitive endpoint than stickleback KEH. Androgenised female stickleback fish can also be used to screen for endogenous anti-androgens (Katsiadaki *et al.*, 2006) and is partially validated as an OECD test (OECD, 2018c). More recently, spiggin transcription was significantly down regulated when stickleback fish were exposed to the anti-androgen flutamide although surprisingly the flutamide did not affect the transcription of AR receptors (Fitzgerald *et al.*, 2020).

Jones *et al.* (2001) characterised three different spiggin subunits (α, β and γ), and these spiggin subunits were found to have highest similarity to Xenopus mucin B.1 (28%), rat

MUC2 (27%), human MUC5AC (27%), human von Willebrand factor (VWF; 26%) and murine otogelin (25%). Later, a phylogenetic study by Kawahara and Nishida (2006) showed that spiggin was related to vertebrate mucins, avian ovomucins, zebrafish otogelins, and mammalian zonadhesin, although more closely so to mucins than otogelins (Kawahara and Nishida, 2006).

Mucins are major glycoprotein components of mucus and are either membrane bound (in humans and mice; MUC1, MUC3, MUC4, MUC12, MUC13, MUC15, MUC16 and MUC17) or secreted (in human and mice; MUC2, MUC5B, MUC5AC, MUC6 and MUC19) (Lang *et al.*, 2007). The secreted mucins are large glycoproteins that cover the epithelial cell surfaces of the respiratory, digestive and urogenital tracts, forming gel-like structures, thereby protecting against harmful molecules and microorganisms (Lang *et al.*, 2007). Most vertebrates have 5 to 6 secreted gel-forming mucin genes and these share a similar structure with considerable sequence homology in the conserved regions (Perez-Vilar and Hill, 1999). A characteristic protein structural domain of the gel-forming mucins is the von Willebrand factor type D domain (VWD) named after its occurrence in the VWF (Figure 61).

The MUC2, MUC5AC, MUC5B have a domain architecture which is –

(VWD-C8-TIL)-(VWD-C8-TIL)-(VWD-C8-TIL)-PTS-(VWD-C8-TIL)

The MUC6 and MUC19 have the same domain structure but lack the C-terminal VWD-C8-TIL unit. There is a highly variable region in the 5’ end of the MUC19 that is lacking in other gel-forming mucins.

Although MUC19 is present in fish, amphibians, and mammals, it does not appear to be present in birds. In contrast, MUC6 is present in birds but is missing in most teleost species (Lang *et al.*, 2016). Whilst spiggin is found in the kidney of male stickleback fish (Kawahara and Nishida, 2006), human and mouse MUC19, the most closely related mucin to spiggin, are found in the sub-maxillary gland (Chen *et al.*, 2004). Phylogenetic analysis revealed porcine sub-maxillary gland mucin to be the same as pMUC19 (Zhu *et al.*, 2011). Thus, the expression pattern of the spiggin gene may have changed after the divergence of tetrapods and fish (Kawahara and Nishida, 2006).

More recent work by Kawahara and Nishida (2007) using the genome sequence of the three-spined stickleback showed that there are at least five spiggin genes and analyses of these sequences suggested that an ancestral spiggin gene originated from a member of the mucin gene family. The occurrence of a single spiggin homologue was also demonstrated in zebrafish, medaka, torafugu and spotted green puffer fish (Kawahara and Nishida, 2006; Kawahara and Nishida, 2007). Expression analysis was carried out on zebrafish and torafugu, and whilst spiggin was not expressed in zebrafish, kidney-specific expression was

Adapted from Lang *et al.*, 2007

Figure 61. Gel-forming mucins are evolutionarily related to the von Willebrand factor and possess domain structures that are highly conserved (D1-D4). Organisms represented are human (hMUC2, hMUC5AC, hMUC6, hMUC19, hOtogelin, hVWF and hZonadhesin) and the stickleback *G. aculeatus* (Spiggin α).

Abbreviations: PTS; proline, threonine, serine rich domains, VWD; von Willebrand factor type D domain, Cys-knot; C-terminal cystine knot-like domain, C8; Cysteine rich C8 domains, TIL; Trypsin Inhibitor like cystine rich domain, VWC; von Willebrand factor type C domain, VWA; von Willebrand factor type A domain, MAM; meprin, A-5 protein, and receptor protein-tyrosine phosphatase mu, AbfB; Apha-L-arabinofuranosidase B, PhaB; Large exoprotein involved in heme utilisation or adhesion

found in both male and female torafugu. No glue-like protein produced in the torafugu kidney has been reported, which suggests that the function of this spiggin differs from that in the stickleback. All the spiggins and the medaka spiggin homologue shared the VWD domain structure, but no other domains characteristic of the translated products of secreted mucins (MUC2, 5AC, 5B and 19) were identified (Kawahara and Nishida, 2006).

In addition to male stickleback fish producing spiggin, several other fish species have sexually dimorphic mucus production as part of their reproductive processes, generally involving parental care. For example, the male grass goby has a pair of sperm-duct glands that produce mucus bands in which sperm are embedded (Giacomello *et al.*, 2006), the male Bristlenose catfish has snout mucin-producing goblet cells whilst females have none (Giacomello *et al.*, 2008), and the pharyngeal organ of the Siamese fighting fish produces mucus to aid nest building by male fish (Kang and Lee, 2010). Specialised renal mucus production has also been identified in the Australian catfish, but the presence of the mucus was not seasonal, so the function was thought not to be related to reproduction (Kelly and Gibson-Kueh, 2017).

Since spiggin is androgen dependent, one would expect to find higher AR expression in male kidney compared with other tissues, and no difference in kidney AR expression compared to other tissues in species without spiggin. Indeed, for most fish species, no sexual dimorphism in AR expression in the kidney is known, however, in the stickleback, zebrafish, half-smooth tongue sole and rice field eel, AR expression is higher in the kidney than in other tissues (Hoffmann *et al.*, 2012).

In North America, the FHM is the most common small model fish used to assess oestrogenic and androgenic chemicals. Sexually mature male fish develop large nuptial tubercles on the snout and a dorsal fatpad that is rich in mucus (Smith and Murphy, 1974). Breeding males are territorial and actively defend against other males intruding on their nest site. Following spawning, the male guards the eggs from predation as well as rubbing its fatpad against the spawning substrate to clean the eggs. In the FHM, exposure of female fish to androgenic chemicals results in an induction of nuptial tubercles (Smith and Murphy, 1974). The adult males and females are easily distinguished from one another when in breeding condition; the males are usually larger and darker than the females. The FHM has a rapid lifecycle, reaching reproductive maturity in four to five months post hatch, and the reproductive cycle can be controlled with temperature and photoperiod manipulation (Harries *et al.*, 2000). In the FHM, several endpoints are responsive to oestrogens (vtg, sex-steroid production, decreased nuptial counts in males, delayed maturation and decreased fecundity and fertility) and to androgens (SSCs – nuptial tubercle growth, alterations in size of the dorsal fatpad, alterations in shape, colouration and breeding behaviours); for example, Miles-Richardson *et*

al. (1999) and Ankley *et al.* (2003). In terms of oestrogen responsiveness, the FHM, medaka and zebrafish may be more suitable species with more established test methods. However, the stickleback can be used to simultaneously assess androgen and oestrogen exposure in a single fish using fully quantitative endpoints (Muldoon and Hogan, 2016). In addition, Katsiadaki *et al.* (2006) found the spiggin endpoint measured by the ELISA to be 400-4000x more sensitive than FHM nuptial tubercle formation following MT exposure, and is the preferred method for determining spiggin concentration because KEH measurements are more time consuming.

Expression of the male SSCs in fish is under the control of the AR (Borg, 1994). In teleost fish, 11-KT is generally considered to be the major circulating androgen as well as the most potent one (Borg, 1994) although it has recently been demonstrated that DHT plays a role in early development and reproduction (Martyniuk *et al.*, 2013). Female fish seem to be more strongly affected by androgen exposure than males (Ekman *et al.*, 2011; Martinovic *et al.*, 2008; Pawlowski *et al.*, 2004). Martinovic *et al.* (2008) hypothesised that this was due to the lower concentrations of endogenous T in females, making it easier for androgen mimics to compete. Similarly, juveniles with lower circulating androgen concentrations should be more sensitive than adults, although Sone *et al.* (2005) found adults to be more sensitive than fry/juveniles. Whilst females appear to be more sensitive to androgens than male fish, this may not necessarily be true for anti-androgens. Indeed, (Martinovic-Weigelt *et al.*, 2011) found zebrafish male reproductive systems to be more extensively impacted following exposure to the known anti-androgens vinclozolin and flutamide.

Spiggin induction in female stickleback fish is currently the best test for (anti-)androgens, but spiggin is specific to stickleback fish and cannot be used as an endpoint in the any other laboratory test species. For studies where several endpoints have been measured, molecular endpoints appear to be among the most sensitive (Scholz and Mayer, 2008). However, when examining molecular end-points, the changes in gene expression are transient (Ankley and Johnson, 2004; Sone *et al.*, 2005) and proteins are therefore expected to be more stable biomarkers of EDCs (Denslow *et al.*, 2012). FHM also have sexually dimorphic mucus production as part of their reproductive process, and FHM may possess a spiggin-like protein that could be used as a sensitive bio-marker of (anti-)androgen exposure, to provide an additional endocrine endpoint to commonly used OECD regulatory fish tests. This spiggin-like protein, if found, could be collected non-invasively, and fish would therefore not need to be sacrificed (Bahamonde *et al.*, 2019; Barkowski and Haukenes, 2014; Tarnawska *et al.*, 2019).

Whilst my long-term aim was to find a new protein biomarker, the preliminary work described in this chapter looked for the sexually dimorphic expression of spiggin-like targets in different

tissues collected from male and female FHM. The tissues chosen were external tissues where sexually dimorphic features have been previously described (dorsal fin, snout, fatpad), other tissues where publications have reported sexually dimorphic mucus production (kidney, mouth), and positive control tissues (brain, gonad, liver). Gene expression was quantified using qPCR. Of interest, were spiggin-like genes with low expression in females and higher expression in male FHM. Tubercle number and FPI were also determined, to enable a comparison of these SSCs with the molecular expression data.

5.2. Materials and methods

5.2.1. Bioinformatics; Basic Local Alignment Search Tool searching and phylogenetic analysis

The National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov) was checked for spiggin and spiggin-like genes using the search term 'spiggin'. Cluster analysis of the 'spiggin' sequences was then carried out using the Welcome Sanger Institute SeqTools (https://www.sanger.ac.uk/science/tools/seqtools). One spiggin sequence from each cluster was then used to search for FHM 'spiggin-like' sequences using the translated Basic Local Alignment Search Tool (BLAST; www.blast.ncbi.nlm.nih.gov/). Potential FHM nucleotide sequences were then translated using ExPASy (www.web.expasy.org/translate/), and reverse BLAST to confirm similarity to spiggin.

The NCBI website (www.ncbi.nlm.nih.gov/) was then searched for mucin and mucin-like 2, 5AC, 5B, 6 and 19 protein sequences in human, mouse, and different fish species. These sequences together with the translated FHM 'spiggin' sequences were aligned using the multiple sequence alignment program Clustal Omega, available from the European Bioinformatics Institute (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Geneious Prime 2019 (version 2019.0.4; https://www.geneious.com/geneious-prime-faq/) was used to build a distance-based Neighbour Joining tree, and this tree was rooted to Human MUC2.

The bioinformatics work carried out in 2013 was repeated in 2018, just prior to starting the practical aspect of this chapter.

5.2.2. Fish sampling

5.2.2.1. First sampling of different tissue from male and female fathead minnows

An actively reproducing pair of FHMs (approx. 14 months old) kept under flow-through conditions were humanely sacrificed according to UK Home Office procedures using a lethal dose of MS222 (500 mg/L adjusted to pH 7.4; Sigma-Aldrich, Gillingham, UK) followed by trans-spinal severance. The following tissues were collected; snout, mouth area, fatpad, dorsal fin, brain, gonad, liver. Between obtaining samples, dissection instruments were cleaned with RNase AWAY® (Sigma-Aldrich). Following collection, the tissues were immediately snap frozen in cryovials in liquid nitrogen and then transferred to -80 ^{o}C for long-term storage.

5.2.2.2. Second sampling of snout tissues from male and female fathead minnows

Fathead minnows (10 male and 10 female fish, approx. 16 months old) were obtained from Scymaris (Brixham, UK). The male fish were kept in separate flow-through tanks due to aggressive behaviour towards the females, and whilst not reproductively active, the males did have obvious SSCs (fatpads; Figure 62, and tubercles; Figure 63). Fish were humanely sacrificed according to UK Home Office procedures using a lethal dose of benzocaine (500 mg/L; Alfa Aesar by Thermo Fisher Scientific, Heysham, UK), followed by trans-spinal severance. Measurements were taken of fork length, wet weight, and the male fish nuptial tubercles and fatpads were photographed. Dissection instruments were cleaned between fish tissues using sequential washes of 10% household bleach, MilliQ water and 70% etOH. The snout, mouth area, and dorsal fin were dissected. The fatpad was also dissected from male fish and weighed, and the FPI calculated (FPI = [fatpad weight/body weight] × 100). Tissues were then transferred to cryovials containing 5-10 volumes of RNA*later*® Solution (Life Technologies, Carlsbad, USA). Further tissues were collected; namely brain, gonad, liver and kidney. The gonad was weighted and the GSI calculated (GSI = [gonad weight/body weight] × 100). Similarly, these were transferred to cryovials containing RNA*later*® Solution, but due to the size of the ovaries, after weighing only one was transferred to the cryovial for storage.

All cryovials were stored at 4 ^{o}C overnight and were then transferred to -20 ^{o}C for longer-term storage.

The male fish nuptial tubercles photographs were examined using ImageJ (https://imagej.nih.gov/ij/) and this software was used to count the number of tubercles.

Figure 62. Photograph showing tank of male FHMs with dorsal fatpads of varying sizes. (taken at Scymaris, Brixham, UK)

Figure 63. Photograph of male FHM snout showing raised nuptial tubercles. (Panasonic Lumix DC Vario camera, macro setting)

5.2.3. Ribonucleic acid extraction and complementary deoxyribonucleic acid synthesis

All surfaces were routinely decontaminated using 1% Distel (Tristel Solutions Ltd., Snailwell, UK) to prevent DNA contamination.

Ribonucleic acid (RNA) extractions were carried out using the NucleoSpin® RNA Plus kit (Macherey Nagel Bioanalysis™, Düren, Germany). Tissue homogenisation and lysis utilised 5 mm stainless steel beads (Qiagen, Manchester, UK) that were prepared by washing with 100% etOH and autoclaving. Eppendorf 2 ml Safe-Lock centrifuge tubes (Stevenage, UK) were used and into each tube, one sterile bead and 350 µl kit Lysis Buffer were added. The FHM tissues were then added to the tubes (tissues stored in RNA*later*® were blotted on tissue to remove excess liquid) and immediately homogenised using a TissueLyser II (Qiagen) for 1 min at 30 Hz followed by a further 1 min after inverting the tube inserts in the holders.

Homogenised lysates were added to the NucleoSpin® gDNA Removal Column and after spinning, the column (together with the bound genomic DNA; gDNA) was discarded. Binding solution (100 µl) was added to each of the flow-through samples and was mixed well by pipetting up and down. This mix was then transferred to the NucleoSpin® RNA Plus Column and after spinning the column, the bound RNA was then washed twice with Wash Buffer. The bound RNA was then eluted with two 20 µl volumes of RNase-free water. RNA was then quantified spectrophotometrically using a Nanodrop One (Thermo Fisher Scientific, Paisley, UK), and purity was assed using a ratio of absorbance at A_{260nm} and $A_{280nm.}$ Ratios above 1.8 indicated good quality RNA that was devoid of contamination.

To further check the structural integrity of the extracted RNA (i.e. to check the RNA was not degraded), an aliquot was run on a 0.8% agarose gel. To 100 ml 1 x Tris-borate EDTA buffer (TBE; Sambrook and Russell, 2001), 0.8 g of agarose (Sigma-Aldrich) was added and was melted using a microwave. Once cooled to hand-hot, 2 µl of GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA) was added and the gel was cast in a gel mould with a comb in position.

Equal volumes of RNA (2 µl) and 2 x RNA Loading Buffer (2 µl; Thermo Fisher Scientific) were pipetted to microfuge tubes and heat denatured at 72 ^{o}C for 2 mins on a Dri-Block® (Techne, Cole-Parmer, Stone, UK), then immediately transferred to ice. The denatured samples were run on an agarose gel along with lanes containing 2 µl of 1kb ladder (Bioline Reagents Ltd., London, UK), for 30 mins at 110 Volts. Structural integrity was confirmed by visual inspection of the 28S and 18S rRNA bands using a BioDoc-It™ imaging system (Analytik Jena AG, Jena, Germany). For most of the samples extracted, the RNA quality was good and two distinct ribosomal RNA bands were seen (28S and 18S; Figure 64).

Unfortunately, the RNA quality for the testis and ovary were poor and the two ribosomal bands were not clear, possibly due to overloading of the NucleoSpin® column. Additionally, the quantity of RNA extracted from the female fatpad was too low (<50 ng/µl). No qPCR results are therefore available for these three tissues.

To remove any residual gDNA, the RNA samples were further treated with DNase I recombinant (Roche Diagnostics, Mannheim, Germany), a DNA-specific endonuclease that hydrolyses DNA. The following volumes were used:

40 µl RNA in RNase free H_2O

4 µl 10x Incubation Buffer (included with DNase I recombinant)

1 µl DNase I recombinant (10 units)

The tubes were then incubated on a dry block at 37 ^oC for 10 mins.

Following DNase I treatment, the RNA was precipitated overnight as follows, using the co-precipitant, GlycoBlue™ (Invitrogen, Thermo Fisher Scientific) to aid RNA precipitation and increase the pellet visibility:

1 µl GlycoBlue

4 µl 5M sodium acetate (0.1 x volume)

125 µl 100% etOH (2.5 x volume; stored at -20 ^oC)

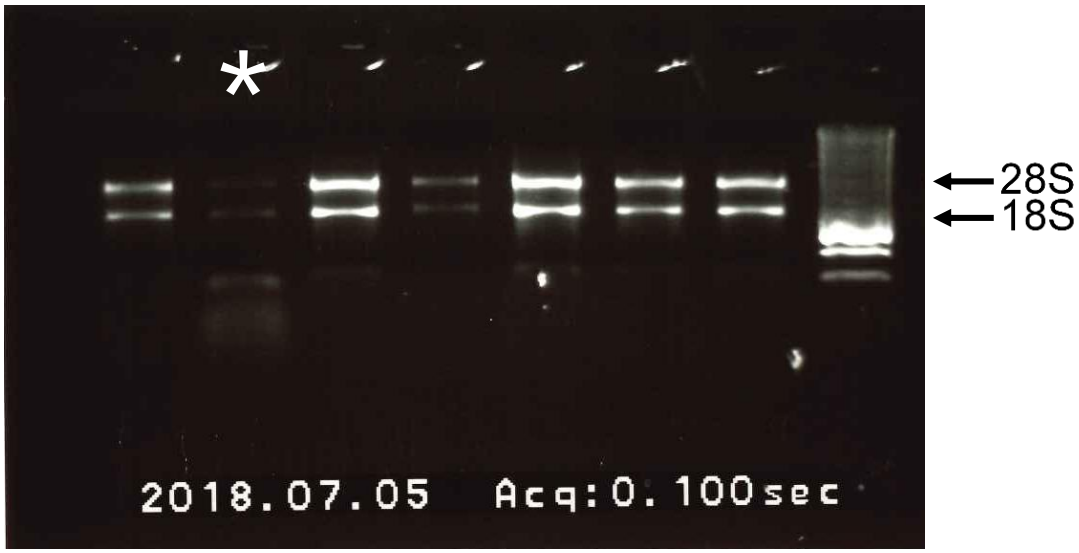


Figure 64. Image of RNA samples run on a 0.8% agarose gel alongside a 1 kb HyperLadder™. The 28S and 18S bands indicate good quality RNA. The lane indicated by the white asterisk shows a sample with poor quality RNA.

The tubes were then stored at -20 ^{o}C overnight. The following day, the tubes were spun for 30 mins at 13,000 rpm in a microfuge pre-cooled to 4 ^{o}C. The supernatants were then carefully removed and 150 µl 70% etOH (stored at -20 ^{o}C) was added to wash the RNA pellet. Following a further spin for 15 mins at 13,000 rpm and at 4 ^{o}C, the supernatants were carefully removed, and the pellet air-dried for 10 mins. Depending on the size of the pellet, between 20 and 60 µl RNase-free water was added and the pellet gently resuspended. The RNA was again quantified using the Nanodrop One, and a total of 2 µg RNA was required for complementary DNA (cDNA) synthesis.

For first-strand cDNA synthesis, the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific) was used. RNA samples were diluted to 1 µg in 10 µl diethyl pyrocarbonate (DEPC) H_2O (Life technologies, Paisley, UK) in duplicate, for minus (no reverse transcriptase control; NRT) and plus reverse transcriptase.

Into a PCR tube strip (BRAND GmbH, Wertheim, Germany) aliquots of the 2x RT master mix were added, each containing:

2 µl 10x RT Buffer

0.8 µl 25x dNTP Mix (100mM)

2 µl 10x RT Random Primers

1 µl MultiScribe™ Reverse transcriptase (or 1µl DEPC H_2O for NRT control)

4.2 µl DEPC H_2O

To this PCR tube strip, the 10 µl diluted RNA samples were added, followed by pipetting up and down two times to mix the contents (total volume 20 µl). The tube strips were then sealed and briefly centrifuged to spin down contents and eliminate air bubbles.

The tube strips were subsequently loaded onto the thermal cycler (Biometra GmbH, Göttingen, Germany) and the program was run using the following conditions:

Settings	Step 1	Step 2	Step 3	Step 4
Temperature (^{o}C)	25	37	85	4
Time (minutes)	10	120	5	∞

At the end of the program, 130 µl DEPC H_2O was added to all tubes (150 µl total volume) and they were then stored at -20 ^{o}C until ready for qPCR.

5.2.4. Primer design

Previously reported reference gene primers were used for ribosomal protein l8 (*rpl8*), hypoxanthine phosphoribosyltransferase 1 (*hprt1*) and tata box binding protein (*tbp*) (Filby and Tyler (2007); Table 19), for normalising for differences in the amount of starting template between samples.

Two sex specific primers were also chosen; VTG primers specific to female fish (Cavallin *et al.*, 2015), and Hushi tarazu factor-1 (FTZ-F1) homologue primers specific to male fish (ff1d, previously reported as male specific in zebrafish; von Hofsten and Olsson, 2005). The ff1d primers together with the spiggin-like primers were designed using NCBI Primer-BLAST to have a melting temperature (T_m) of 60 ^oC and an amplicon size between 70 and 120bp (Table 20).

5.2.5. Quantitative PCR

For qPCR, the following controls were included on each plate:

- no template control (NTC; cDNA was substituted with DEPC H_2O) to check buffers and solutions for DNA contamination and to assess for primer-dimers
- NRT prepared in Section 5.2.3 to check that there was no amplification due to the presence of gDNA in the sample
- inter-plate calibrator (IPC; a common sample used in every plate with the hpbt primer set)

Primer pairs were prepared by diluting each 1 in 100:

1 µl Forward primer + 1 µl Reverse primer + 98 µl DEPC H_2O (total volume 100 µl)

qPCR reactions were performed using iTaq™ Universal SYBR Green® Supermix (Bio-Rad, Mercules, CA, USA), and a master mix was prepared as follows:

5 µl SYBR® Green

2.5 µl diluted primer pair

1.5 µl DEPC H_2O

Table 19. qPCR primers and product size for FHM reference genes (Filby and Tyler, 2007)

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product (bp)
rpl8	CTCCGTCTTCAAAGCCCATGT	TCCTTCACGATCCCCTTGATG	102
hprt1	GATGAAGAGCAAGGTTATGAC	ACACAGAGCAACGATATGG	165
tbp	CTCAAGGGCTGGCTTCTC	ACTGGCTGTGGTGTAAGAC	97

Table 20. qPCR primers and product size for FHM ff1d, vtg and spiggin-like ESTs designed using NCBI Primer-BLAST

Target gene	Accession number	Forward primer (5'-3')	Reverse primer (5'-3')	Product (bp)
ff1d	DT343801.1	GGCCCCATGTACAAACGAGA	TGGTGATGACAGCAAAGGGG	105
vtg	AF13034	TCACCACATACGCCAAAAAGC	CAAGTCTAAAGCCCGTCTGGTT	69
Spiggin-like?	DT131813.1	AGAAGACCCCCTGAAACCCT	GGGGTGGAGGTGGAGGTATT	80
Spiggin-like?	DT267220.1	AAGGCACTTGTACTGAGCCC	CTCCTTGAAGGGCACACACT	89
Spiggin-like?	DT347638.1	ACCACTACCAGGGAGCGTAT	GACACTGAAAGGGGTGAGGG	77

The Master Mix was pipetted to each well (9 µl per well) of the qPCR plate (Bio-Rad) using a multichannel pipette. The cDNA (1 µl) was then added, pipetting samples in triplicate, to give a final volume of 10 µl. Plates were sealed with optically transparent film (Bio-Rad) and spun briefly to 4000 rpm to remove any air bubbles and to spin contents down. All reactions were carried out using a CFX96™ Real-Time System (Bio-Rad).

The first qPCR runs did not include a melt curve so that the products could be run on a 2% agarose gel (as previously described) to check that the amplicon was the expected size. Following qPCR, when products were run on an agarose gel, these all produced a single band of the correct size (see Figure 65 for an example of an amplification plot and Figure 66 for an example of qPCR products run on a gel).

Later, melt curve analyses were performed to validate the specificity of the PCR amplicons and the following cycling protocol was used:

	Amplification (40 cycles)		
Polymerase activation and DNA denaturation at 95 ^{o}C	Denaturation at 95 ^{o}C	Annealing/ extension and plate read at 60 ^{o}C	Melt curve analysis (0.5 ^{o}C increments)
3 mins	10 secs	30 secs	65-95 ^{o}C

For all tested genes, the dissociation (melt curve) analysis demonstrated that only one peak existed at the corresponding melting temperature, indicating specific amplification (

Figure 67).

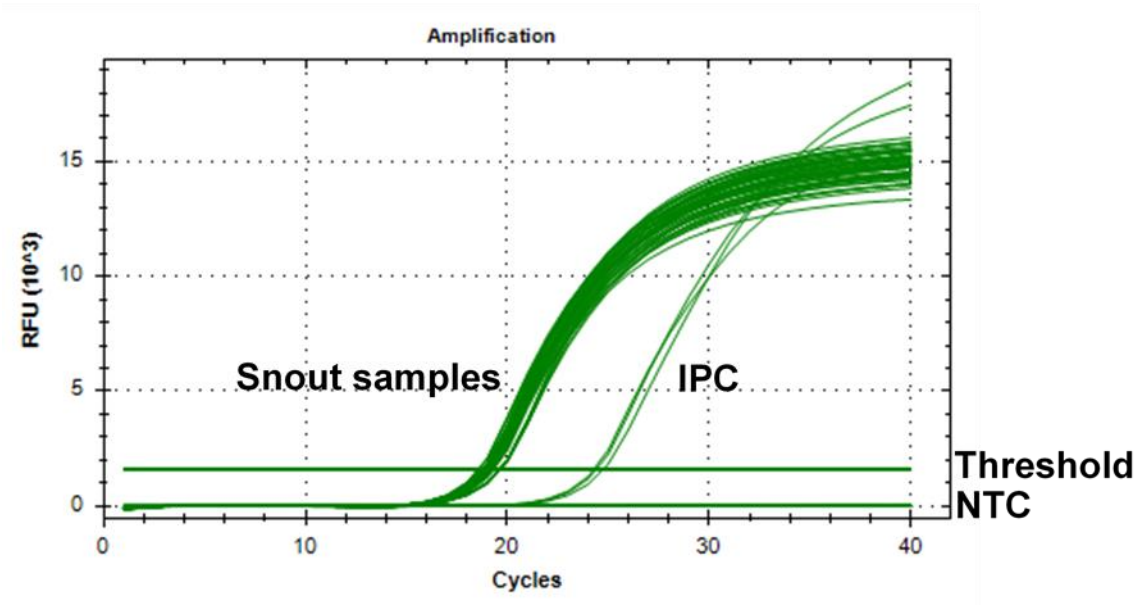


Figure 65. An example of an amplification plot; NTC (no amplification), inter-plate calibrator (IPC; FHM liver; Cq ~25) and FHM snout samples with one primer set (Cq ~20). The horizontal line indicates the quantification cycle (Cq).

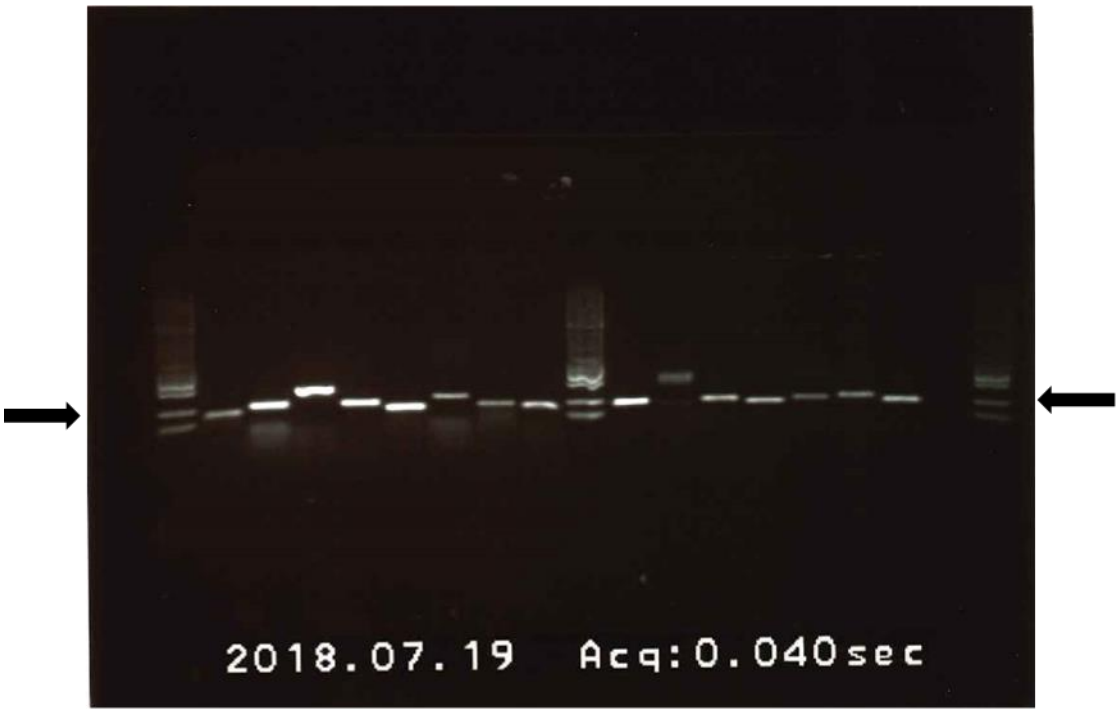


Figure 66. Image of qPCR products run on a 2% agarose gel alongside a 50 bp HyperLadder™ (loaded into the outer and middle wells). The arrows indicate the ladder's 100bp band.

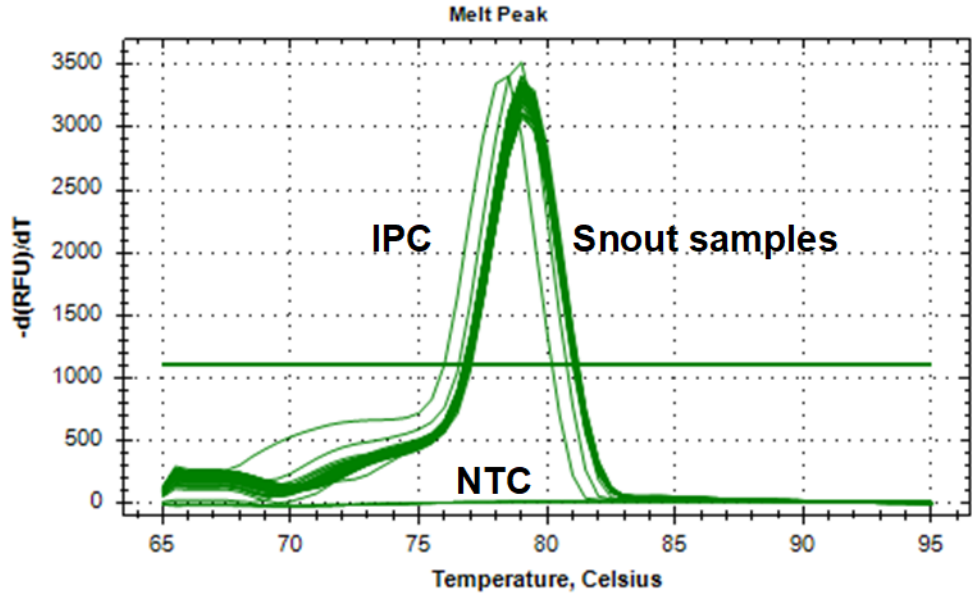


Figure 67. Typical denaturation curve (melt peak) performed after qPCR cycling; NTC (no melt peak), inter-plate calibrator (IPC; FHM liver) and FHM snout samples with one primer set with melt peak at approximately the same temperature (~79 ^{o}C).

Guidelines for Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) were considered (Bustin *et al.*, 2009; Taylor *et al.*, 2010).

The qPCR data were analysed using the gene study option in the CFX Manager™ software (Bio-Rad). The quantification cycle (Cq), the cycle at which the amplification plot crosses the threshold, was set automatically by the software. Amplification curves were checked to confirm that the Cq for each sample occurred in the log-linear phase of amplification.

The NRTs and NTCs were evaluated on each plate to rule out contamination, and plots were checked for amplification occurring before a Cq of 35, thus indicating contamination. Target stability was calculated as coefficient of variance and the expression stability, M value. Relative quantification was carried out with normalisation against three reference targets genes (*rpl8*, *hprt1* and *tbp*) to account for loading differences or other variations represented in each sample. The IPC was used to calculate inter-plate variability (coefficient of variance).

The normalised expressions of the Genes of Interest (GOIs) were analysed to determine differences in expression between different tissues. Normalised gene expression was calculated using CFX Manager™ software equations as follows:

The Relative Quantity (RQ; ΔCq) for each GOI was calculated with the formula:

$$RQ_{\text{sample (GOI)}} = E_{GOI}^{(Cq_{(MIN)} - Cq_{(sample)})}$$

Where:

- E = Efficiency of primer and probe set. This efficiency was calculated with the formula (% Efficiency * 0.01) + 1, where 100% efficiency = 2
- Cq (MIN) = Average Cq for the Sample with the lowest average Cq for GOI
- Cq (sample) = Average Cq for the Sample
- GOI = Gene of interest (one target)

The Normalised Expression (ΔΔCq) was the RQ of target normalised to the quantities of the reference targets in the biological system. This calculation for normalised expression used the calculated RQ:

$$\text{Normalised Expression}_{\text{sample (GOI)}} = \frac{RQ_{\text{sample (GOI)}}}{(RQ_{\text{sample (Ref 1)}} \times RQ_{\text{sample (Ref 2)}} \times \ldots\ldots \times RQ_{\text{sample (Ref n)}})^{1/n}}$$

Where:

- RQ = Relative Quantity of a sample
- Ref = Reference target in a run that includes one or more reference targets in each sample
- GOI = Gene of interest (one target)

5.2.6. Statistics

For the first FHM sampling, only one male and one female were examined, and no statistical comparisons were therefore made.

For the second FHM sampling, t-tests with GraphPad Prism 8 were used to compare male and female data. Where data was not normally distributed and could not be transformed, the Mann-Whitney test was employed. Correlation analysis was used to investigate the relationship between the SSCs and the normalised expressions of the GOIs. Differences were statistically significant when $p<0.05$.

5.3. Results

5.3.1. Bioinformatics; BLAST searching and phylogenetic analysis

On checking the NCBI website in 2013, there were found to be 27 spiggin nucleotide sequences (21 for the three-spined stickleback *Gasterosteus aculeatus* and 6 for nine-spined stickleback *Pungitius pungitius*). When cluster analysis was carried out using the Welcome Sanger Institute SeqTools there were eight unique sequences amongst the 27 sequences retrieved, which could be organised into three clusters.

In 2013, Taxonomy Browser on the NCBI website identified 391 nucleotide Entrez records and 258,504 nucleotide expressed sequence tag (EST) records for FHM. When the TBLASTN was used to look for FHM nucleotide sequences with similarities to the spiggin sequences (one from each cluster; spiggin α, spiggin β and spiggin 4), there were no relevant nucleotide hits. However, there was one potential FHM spiggin-like EST hit (Accession Number DT131813). This FHM EST was 722 base pairs long and following translation using ExPASy*,* a BLAST search to check for similarity with spiggin mainly came up with mucin-19-like hits, the most closely related mucin to spiggin. Whilst the DT131813 appears to be aligning with the mouse and human MUC-19 in a non-conserved domain in the multisequence alignments, when doing a BLAST search for FHM ESTs, there was poor alignment/no hits. With the multiple sequence alignment, and therefore also the phylogenetic tree, the DT131813 may not be in the correct position. However, when doing a BLAST search looking for FHM ESTS against spiggin α and β, the DT131813 aligned with spiggin α in a VWD domain (36% identity; see Appendix Figure A 11) and with spiggin β there was alignment with two different VWD domains (43% identity and 25% identity, see Appendix Figure A 12). The DT131813 also aligned well with mucin-19-like from the cyprinid *Sinocyclocheilus graham* (81% identity; Appendix Figure A 13).

In 2018, further searches of the NCBI website came up with two more potential FHM spiggin-like EST hits (Accession Numbers DT267220 (826 base pairs) and DT347638 (797 base pairs)). Following translation using ExPASy, BLAST searches to check for similarity to spiggin came up with zonadhesin as well as mucins hits.

The NCBI website was then searched for mucin and mucin-like 2, 5AC, 5B, 6 and 19 protein sequences in human, mouse and different fish species (see Table 21 for Accession Numbers). For two of the spiggin homologues identified by Kawahara and Nishida (2007), the torafugu and medaka ‘spiggin’, were almost identical to torafugu and medaka mucin-19-like hits (98 and 94% identity, respectively) found on the NCBI website, and they were therefore assumed to be mucin-19 rather than spiggin. However, the zebrafish ‘spiggin’ was less similar (65% identity) to muc-19-like and was included in the phylogenetic analysis. The

Table 21. Accession numbers for mucin and spiggin protein sequences found on the NCBI website.

Species	Protein	Abbreviation	Accession no.
Homo sapiens	MUC2	HS__MUC2	NP_002448.4
	MUC5B	HS_MUC5B	NP_002449.2
	MUC5AC	HS_MUC5AC	NP_001291288.1
	MUC6	HS_MUC6	NP_005952.2
	MUC19	HS_MUC19	NP_775871.2
Mus musculus	MUC2	MM_MUC2	NP_076055.3
	MUC5B	MM_MUC5B	NP_083077.2
	MUC5AC	MM_MUC5AC	NP_034974.1
	MUC6	MM_MUC6	NP_001316930.1
	MUC19	MM_MUC19	NP_997126.2
Maylandia zebra	mucin-2-like	MZ_MUC2	XP_024659054.1
	mucin-5AC-like	MZ_MUC5AC	XP_024655160.1
	mucin-19-like	MZ_MUC19	XP_014262801.2
Danio rerio	mucin-2-like	DR__MUC2	XP_021326453.1
	mucin-5B-like	DR_MUC5B	XP_021333301.1
	mucin-19-like	DR_MUC19	XP_002667130.6
Takifugu rubripes	mucin-2-like	TR__MUC2	XP_011605972.1
	mucin-5B-like	TR_MUC5B	XP_011605978.1
	mucin-5AC-like	TR_MUC5AC	XP_011618317.1
	mucin-19-like	TR_MUC19	XP_011617030.1
Oryzias latipes	mucin-19-like	OL_MUC19	XP_023808397.1
Oreochromis niloticus	mucin-2-like	ON_MUC2	XP_025758309.1
	mucin-5B-like	ON_MUC5B	XP_019202525.1
	mucin-5AC-like	ON_MUC5AC	XP_019210361.2
Gasterosteus aculeatus	Spiggin α	GA_SPIG_ALPHA	AAK15297.1
	Spiggin β	GA_SPIG_B	BAS02336.1
	Spiggin 4	GA_SPIG_4	BAE92625.1
Pungitius pungitius	Spiggin α	PP_SPIG_ALPHA	AAY52022.1

mucin-2s, mucin-5ACs, mucin-5Bs, mucin-6s, mucin-19s and zebrafish ‘spiggin’ sequence, together with the four spiggin sequences (three *Gasterosteus aculeatus* spiggin sequences (one from each cluster) and one *Pungitius pungitius* spiggin sequence), were aligned with the translated FHM ESTs using the multiple sequence alignment program, Clustal Omega, available from the European Bioinformatics Institute.

Trimming, so that poorly aligned regions are eliminated, can increase the accuracy of resulting sequence alignments (Talavera and Castresana, 2007). However, the FHM ESTs aligned with different conserved regions, and it was therefore decided to carry out phylogenetic analysis on untrimmed sequences.

The gel-forming mucin genes had strong consensus in the region of the three conserved (VWD-C8-TIL)-(VWD-C8-TIL)-(VWD-C8-TIL) domain structures from approximately 500-2000 amino acids (Illustrated by red box; Figure 68). Most of the fish mucin proteins map to this specific VWD region and their amino acid sequences do not extend beyond this part, including the zebrafish ‘spiggin’. Also, two of the three FHM ESTs (DT267220 and DT347638) aligned within this conserved region although in different positions, and the third EST (DT131813) aligned with human and mouse MUC19, at a less conserved region.

Spiggin proteins aligned best with the mucin-19 proteins including the zebrafish ‘spiggin-like protein’.

The distance-based Neighbour Joining tree rooted to Human MUC2 contained a side-branch with the mammalian MUC19 proteins, *M. zebra* mucin 19-like, *O. niloticus* mucin-5B like, and the FHM EST DT131813. The DT131813 ‘branch’ of the phytogenetic tree was very long, indicating that there was much genetic difference between the DT131813 and the *M. zebra* mucin 19-like. The mammalian MUC6 was only weakly linked to the mucin-19 proteins.

The EST clone DT267220 clustered with the mucin-2 proteins, and DT347638 with the mucin 5B and 5AC proteins.

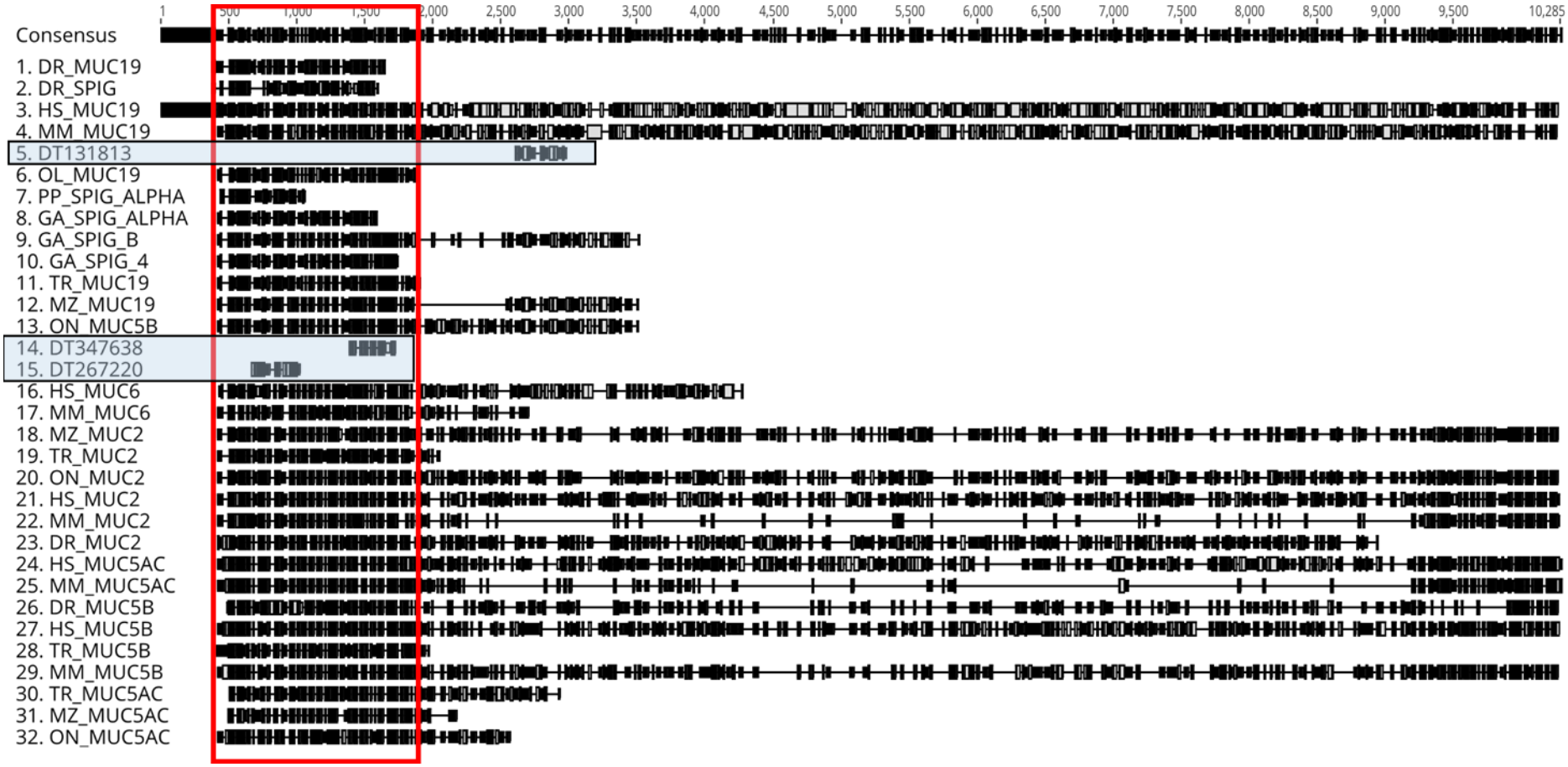


Figure 68. Multiple alignments of gel-forming mucin sequences together with the three FHM spiggin-like ESTs (DT131813, DT347638 and DT267220, highlighted in blue), created using Clustal Omega with default settings. The red box indicates the first three conserved VWDs and the ruler indicates amino acid length. For abbreviations see Table 21.

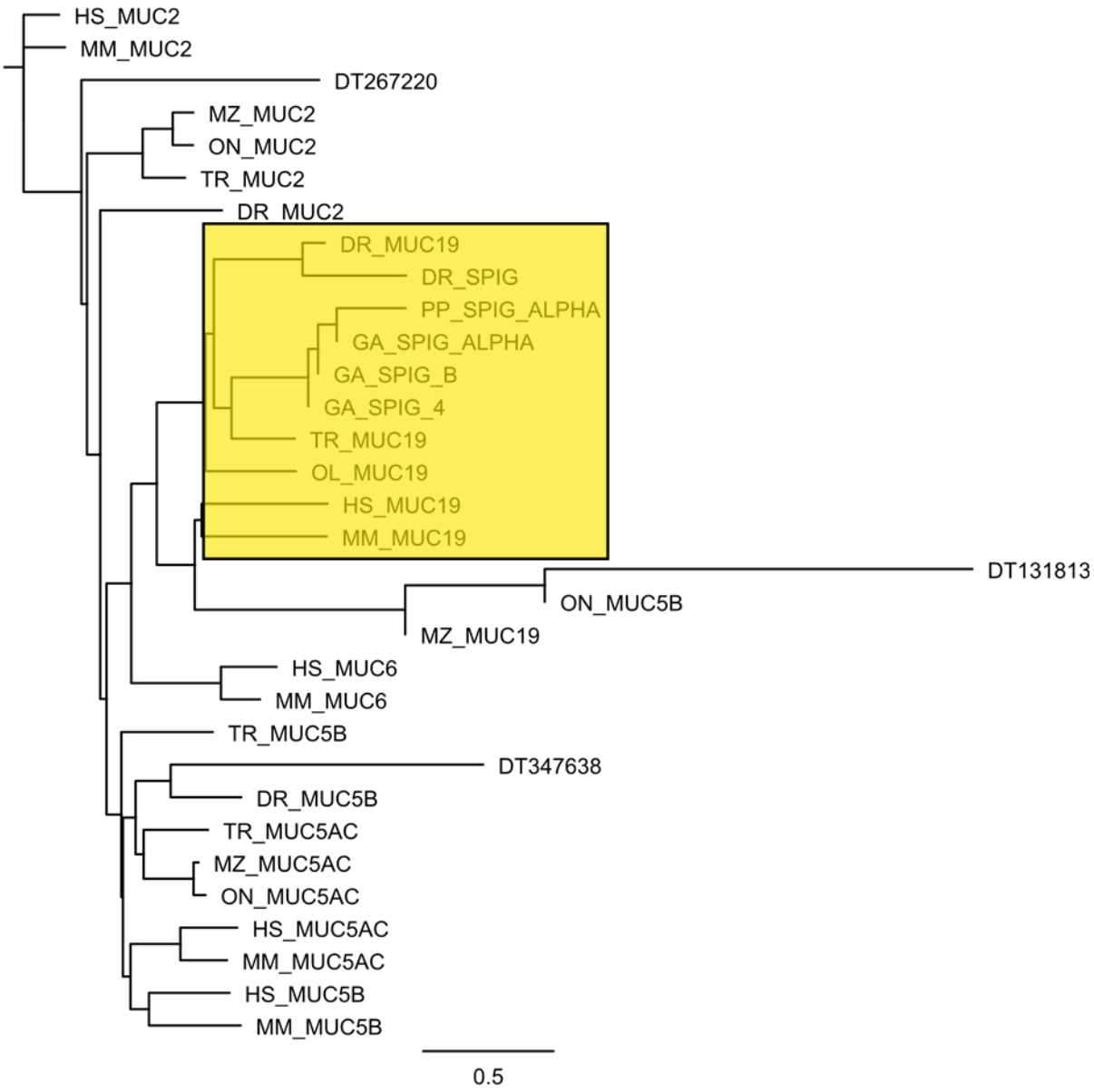


Figure 69. Phylogenetic tree analysis of the gel-forming mucins together with the three FHM spiggin-like ESTs, created with Geneious Tree Builder using the Neighbour-Joining method. The tree is rooted to Homo sapiens MUC2. The highlighted box in yellow indicates the spiggin/MUC19 cluser. The scale bar indicates genetic change; the number of substitutions per site**.** For abbreviations, see Table 21.

5.3.2. Gene expression results for fathead minnow tissue samples

5.3.2.1. First sampling; comparing expression levels in different tissues from a male and female fathead minnow

5.3.2.1.1. Quality control and positive controls for sex specificity

For the different male and female FHM tissues with the positive control and spiggin-like genes of interest, a total of 7 plates were run, and the inter-run calibrator had a Cq of 26.31 ± 0.014. The mean coefficient of variance for the three housekeeping genes (*hprt1*, *tbp* and *rpl8*) was 0.4763, and the mean expression stability (M value) was 1.2126. The mean coefficient of variance values for these heterogeneous samples were within the acceptable range of <0.5, but the M value was slightly higher than the acceptable upper limit of 1.0 (Hellemans *et al.*, 2007).

The highest expression of the ff1d was with the male brain tissue, with some expression also in the female brain and male mouth tissues (Figure 70A and Appendix Table A 33). Vitellogenin was strongly expressed in female liver tissue and not in any other tissue (Figure 70B and Appendix Table A 33).

5.3.2.1.2. Spiggin-like primers

Of interest was expression that was sexually dimorphic where expression was higher in male tissues than female tissues. For primer set DT131813 (Figure 71A and Appendix Table A 34), highest expression was in the female kidney (only low expression in the male kidney). In addition, DT131813 showed higher expression in the male snout compared with the female snout, although this difference was not as apparent. For both the spiggin-like FHM primer sets, developed for the DT267220 and DT347638 ESTs (Figure 71B and C and Appendix Table A 34), expression was highest in the male snout tissues. As only one male and one female fish were sampled for this preliminary work, no statistical analysis was carried out. Future work looked solely at FHM snout tissue from male and female fish and 20 snout tissues (10 from male FHM and 10 from female FHM) were examined.

5.3.2.2. Comparing expression levels in snout tissues from male and female FHM

5.3.2.2.1. Biological results

The biological endpoints (weight, fork length, condition factor and GSI) of the 10 male FHM and 10 female FHM from the second sampling can be seen in Table 22 and Table 23, respectively. The male FHM weighed significantly more than the female FHM (6.46 ± 2.26 g compared with 3.39 ± 0.44 g; p<0.0001). Similarly, the male FHM fork lengths were significantly longer than the female fork lengths (69.10 ± 5.38 mm compared with 59.90 ± 3.03 mm; <0.0001). For the condition factor, the male values were also significantly higher

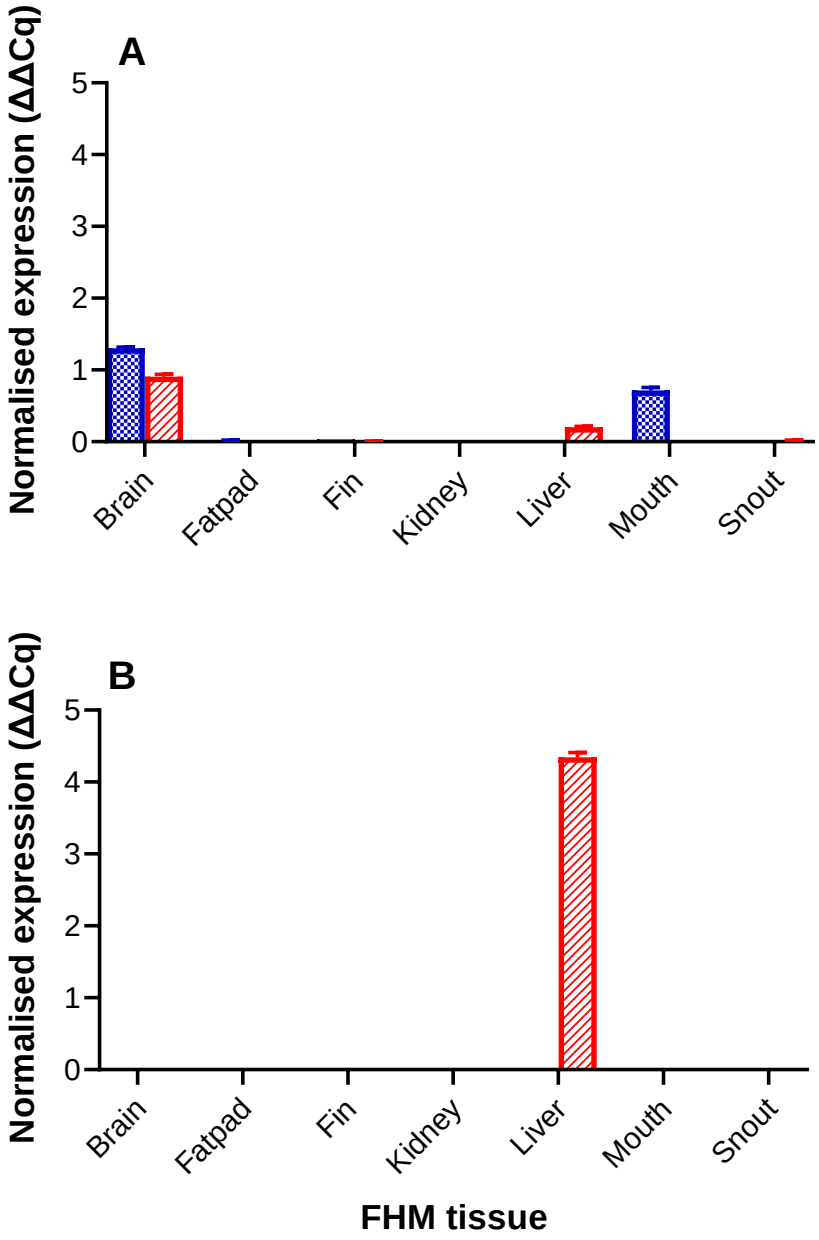


Figure 70. Bar chart showing normalised expression (ΔΔCq) of two positive control genes of interest (A; ff1d and B; vtg) in different tissues from one male (blue bars) and one female (red bars) FHM. Bars represent the mean of triplicate values ± SEM. See Appendix Table A 33 for raw data.

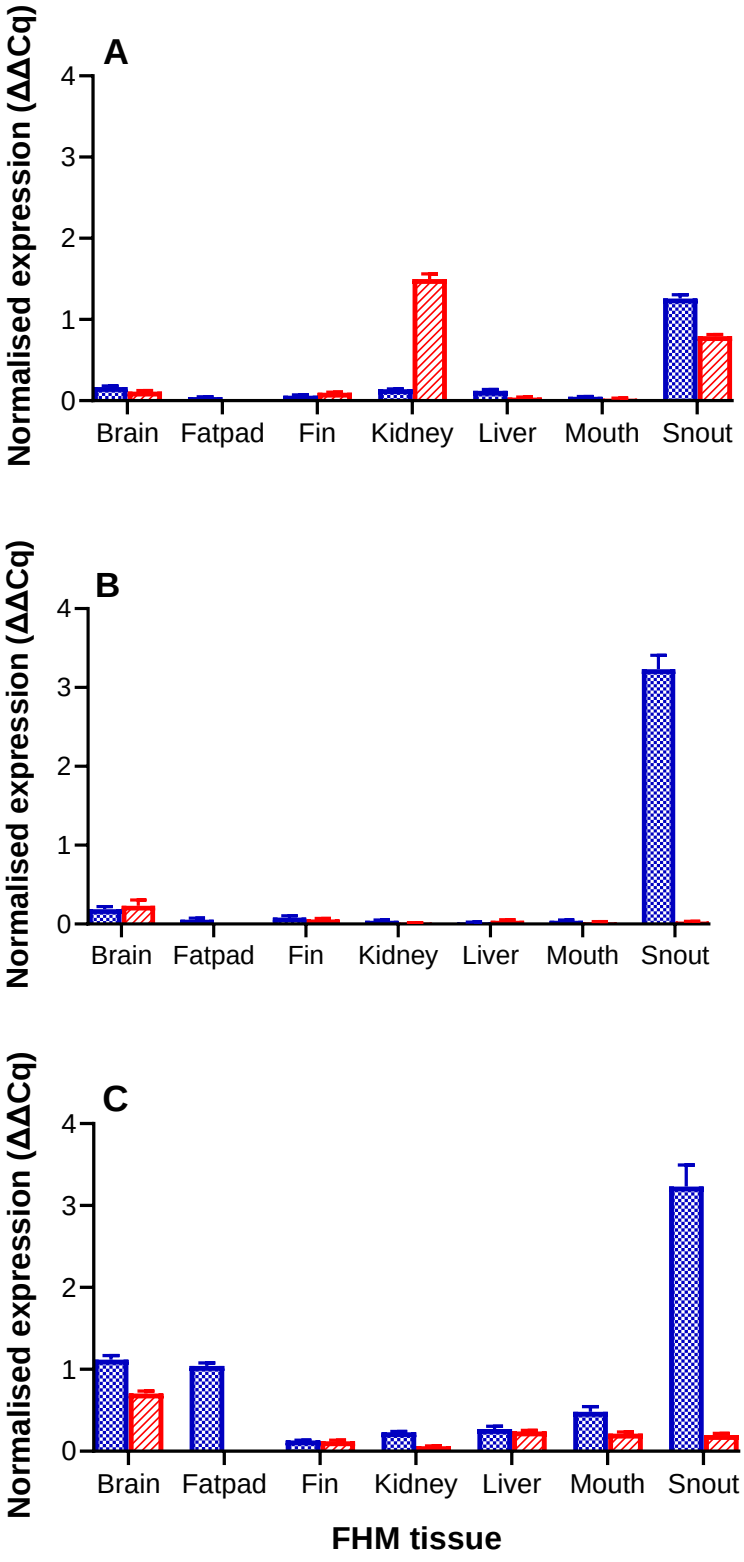


Figure 71. Bar chart showing normalised expression (ΔΔCq) of three spiggin-like genes (A; DT131813, B; DT267220 and C; DT347638) in different tissues from one male FHM (blue bars) and one female FHM (red bars). Bars represent the mean of triplicate values ± SEM. See Appendix Table A 34 for raw data.

Table 22. Biological endpoints for the ten male FHM fish including the secondary sexual characteristics; tubercle number and Fatpad Index (FPI).

Fish no.	Sex	Wet wt. (g)	Fork Length (mm)	Gonad weight (mg)	Condition factor	GSI*	Dorsal fatpad weight (mg)	Tubercle number	FPI
1	M	3.92	62	47	1.427	1.199	342	17	8.724
2	M	6.88	74	133	1.507	1.933	329	17	4.782
3	M	5.33	65	79	1.695	1.482	388	15	7.280
4	M	4.10	65	80	1.304	1.951	229	15	5.585
5	M	7.00	71	186	1.659	2.657	885	14	12.643
6	M	5.08	65	69	1.616	1.358	280	12	5.512
7	M	4.63	64	75	1.539	1.620	379	18	8.186
8	M	10.76	75	189	2.267	1.757	1581	17	14.693
9	M	8.90	76	124	1.805	1.393	788	18	8.854
10	M	7.99	74	102	1.750	1.277	701	20	8.773
Mean		6.46	69.10	108.40	1.657	1.663	590.20	16.30	8.503
Standard Deviation		2.26	5.38	48.89	0.262	0.437	416.60	2.31	3.138

* Gonadosomatic Index

Table 23. Biological endpoints for the ten female FHM fish.

Fish no.	Sex	Wet wt. (g)	Fork Length (mm)	Gonad weight (mg)	Condition factor	GSI*
1	F	3.48	60	457	1.328	13.132
2	F	3.84	60	534	1.398	13.906
3	F	3.34	62	463	1.216	13.862
4	F	3.52	60	444	1.343	12.614
5	F	3.84	59	669	1.611	17.422
6	F	2.80	56	494	1.363	17.643
7	F	2.48	54	271	1.208	10.927
8	F	3.62	64	426	1.204	11.768
9	F	3.68	63	446	1.280	12.120
10	F	3.28	61	283	1.194	8.628
Mean		3.39	59.90	448.70	1.314	13.202
Standard Deviation		0.44	3.03	114.36	0.128	2.751

* Gonadosomatic Index

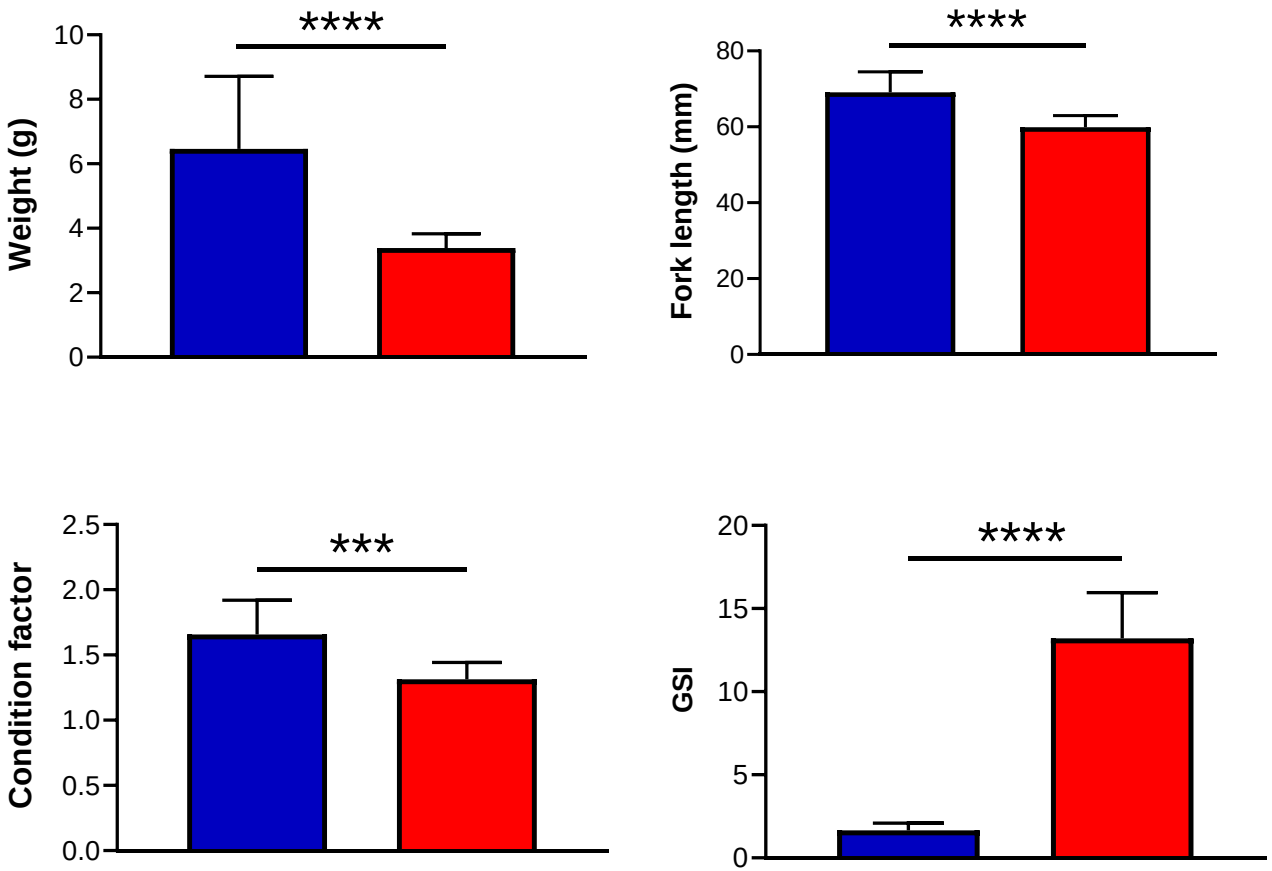


Figure 72. Graphs for the biological endpoints (weight, fork length, condition factor and Gonadosomatic Index (GSI)) of the ten male (blue bars) and ten female (red bars) FHM fish. Bars represent mean and standard deviation. Statistical comparisons between male and female measurements; ***p<0.001, ****p<0.0001.

than the female values; 1.657 ± 0.262 and 1.314 ± 0.128, respectively (p=0.007). The female FHM ovaries were large and filled with ripe eggs and, as a result, the GSI values were significantly higher than the male FHM GSI values (13.202 ± 2.751 compared with 1.663 ± 0.437; p<0.0001).

The plots for the biological endpoints (weight, fork length, condition factor and GSI) are presented in Figure 72.

Correlation analysis was carried out on the male tubercle number and FPI, but there was no significant relationship between these two endpoints (p=0.6184) (Figure 73).

5.3.2.2.2. Molecular results

5.3.2.2.2.1. Quality control

For three of the female snout tissues there was either not enough RNA or the RNA quality was too poor to proceed further. The n-values were therefore ten for male snout tissues but only seven for female snout tissues.

For the snout tissues, a total of 7 plates were run, and the inter-run calibrator (a different sample to that used for the first sampling) had a Cq of 24.72 ± 0.04616. The mean target stability value of the three housekeeping genes (*rpl8*, *hprt1*, and *tbp*) was 0.1721 and the mean M value was 0.4418. The mean coefficient of variance and M value for these homogeneous samples were within the acceptable range of <0.25 and <0.5, respectively (Hellemans *et al.*, 2007).

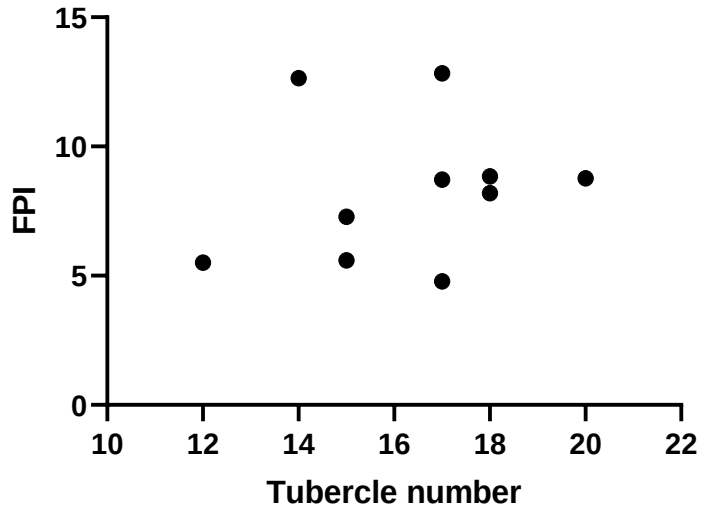


Figure 73. Plot of tubercle number against Fatpad Index (FPI) for the ten male FHM fish. Correlation analysis p=0.6184, Pearson r=0.1802, R^2=0.0325.

5.3.2.2.2.2. Spiggin-like primers

DT131813 gene expression was significantly greater in female FHM snout than male FHM snout (p=0.0001; Figure 74). With the DT267220 primer set, there appeared to be higher expression of this target in the male snout tissue than the female snout tissue, but due to biological variation this was not significant (p=0.2295; Figure 74). There was also no significant difference between the expression of the DT347638 target in male and female snout tissues (p=0.9623; Figure 74).

Correlation analysis was carried out between the tubercle numbers and the normalised expression with the three ESTs (DT131813, p=0.3279; DT267220, p=0.4680; DT347638, p=0.7257; Appendix Figure A 14) and also between the FPIs and the normalised expression with the three ESTs (DT131813, p=0.5367; DT267220, p=0.6321; DT347638, p=0.5135; Appendix Figure A 15), but no significant relationships were found.

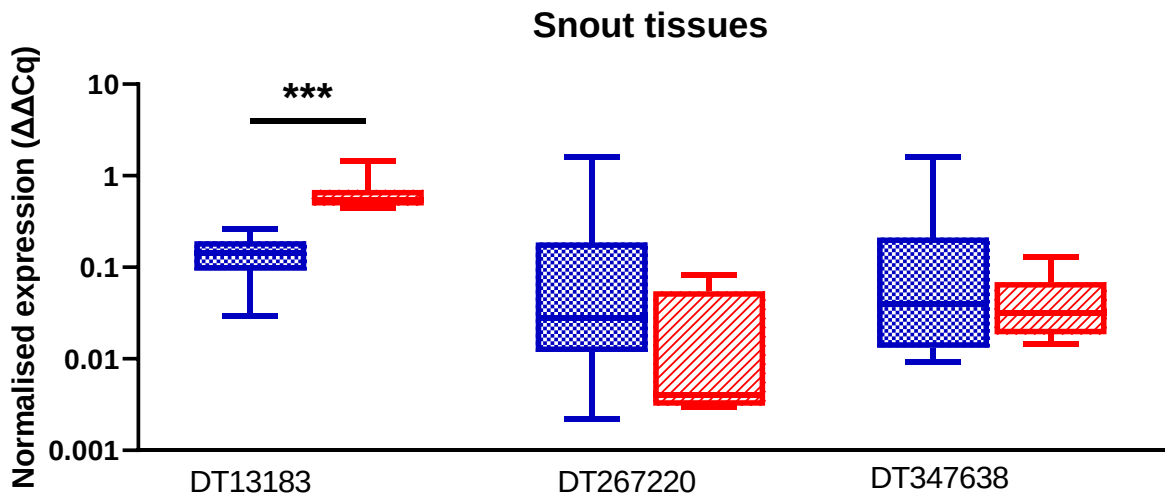


Figure 74. Bar chart showing normalised expression (ΔΔCq) of the three spiggin-like genes (DT131813, DT267220 and DT347638 primer sets) in snout tissues from ten male FHM (blue bars) and seven female FHM (red bars). The bars represent mean ± standard deviation. For statistical comparisons between male and female values for each primer set; ***p<0.001. See Appendix Table A 35 for raw data.

5.4 Discussion

5.4.1 Bioinformatics; BLAST searching and phylogenetic analysis

The release of 250,000 FHM ESTs deposited in the NCBI GenBank in 2005 stimulated the development of high-density microarray tools for FHM, and the sequencing and assembly of the FHM genome reported by Burns *et al.* (2016) was an important step in advancing molecular characterisation of the FHM. However, compared with zebrafish and medaka, there is still a lack of molecular information for the FHM (Burns *et al.*, 2016; Lavelle *et al.*, 2018).

Because of this poorly characterised FHM genome, the best approach was to look to see if any of the available FHM ESTs showed similarity to spiggin. However, due to the highly conserved nature of mucin and related proteins, the use of other teleost species to identify spiggin-like proteins in FHM were likely to fit within conserved regions of mucins (Martyniuk *et al.*, 2012), and therefore likely to amplify related proteins as well. As expected, there was high sequence consensus with the fish and mammalian mucins, and whilst two of the FHM ESTs (DT267220 and DT347638) aligned within the conserved (VWD-C8-TIL)-(VWD-C8-TIL)-(VWD-C8-TIL) domain structure, the DT131813 did not. The phylogenetic tree analysis showed that the DT267220 and DT347638 were located with the MUC2 and MUC5 clusters, respectively.

The DT131813 was in a separate cluster from spiggin and was located with *M. zebra* mucin 19-like and *O. niloticus* mucin 5B-like. From the multiple alignments comparison as well in the phylogenetic tree, the *O. niloticus* mucin 5B-like clustered with *M. zebra* MUC-19 and could be wrongly annotated as MUC-5B. Genbank does contain a significant proportion of erroneous protein sequences (Lang *et al.*, 2016) and a protein may be missed/ wrongly annotated because it was overlooked in the process of genome annotation. When collecting additional information by analysing the genomic sequence more carefully against other mucin 5s and mucin 19s, the *O. niloticus* mucin 5B-like had been misannotated and was in fact mucin 19-like.

As reported by (Kawahara and Nishida, 2006), in the phylogenetic tree analysis the mucin-6 genes clustered with mucin-19 genes, and the mucin-19 genes clustered closely with spiggin. Therefore, the FHM EST DT131813 was the most similar to spiggin, based on its relationship with mucin 19 in both the sequence alignments and the phylogenetic analysis.

5.4.2 Gene expression

5.4.2.1 First sampling; comparing expression levels in different tissues from male and female FHM

The mean stability value (M) for the qPCR reference genes with the different tissues from a male and female FHM was slightly above the highest value expected for heterogeneous samples. Due to the wide range of tissues from different sexes, the samples were highly heterogenous and this slightly higher value might therefore have been expected. In any case, the mean coefficient of variation for the reference genes was within range.

The expression of *ff1d* in adult zebrafish is restricted to brains, gonads and liver with higher expression in testis than ovary (von Hofsten and Olsson, 2005). *Ff1d* is highly expressed in testicular tissue, suggesting an involvement in gonadal steroid synthesis. Unfortunately, the quality of the RNA from FHM testis and ovary was insufficient to be able to carry out qPCR and therefore looking for testis-specific expression was not possible. Despite the lack of gonad tissue due to RNA degradation, there was still higher expression of *ff1d* in the male brain than female brain, as seen in zebrafish (von Hofsten and Olsson, 2005).

For the female-specific positive control, the VTG expression was restricted to the liver. This agrees with other publications where expression was almost exclusively in the liver (zebrafish; Islinger *et al.*, 2003).

For the different tissue samples, there was female specific expression with the DT131813 target in the kidney, but as this thesis focuses on male specific biomarkers this was not considered to be of interest for this study. In contrast, for both the DT267220 and DT347638 targets, with the snout tissues there was male-specific expression, although as only one fish of each sex was sampled, more fish samples need to be collected before any conclusions can be drawn. This was of interest because it is possible to sample mucus from fish snouts non-invasively (Mosley *et al.*, 2018). In addition, whilst biofluids (e.g. blood, urine, mucus) are generally thought to have higher variability than tissues, Ekman *et al.* (2015) did find that the range of variability for the FHM mucus metabolome was similar to other biofluids and therefore snout mucus could be a potential biofluid for monitoring influences of (anti-)androgens.

5.4.2.2 Second sampling; comparing biological endpoints and expression levels in snout tissues from male and female FHM

As expected, the male FHM fish were both heavier and longer than the female FHM. The female GSI was significantly higher than the male GSI, and this is probably also why the female condition factor was lower for the female fish, as energy was being put into egg

production. Compared with the FHM used in the Pair Breeding Test in Chapter 2, these FHM were older and subsequently larger (both heavier and longer).

There was no significant correlation between tubercle number and FPI. The FHM were photographed at the time of the sampling as the traditional method for counting tubercles (freezing snouts in liquid nitrogen just before counting by eye) was not used so as not to destroy the tissues required for molecular work. Due to the three-dimensional nature of the snout, counting the tubercles using photographs was not easy, as not all were in focus. This might be the reason why there was no correlation between our tubercle numbers and FPIs. However, the two endpoints do not always respond in parallel; Miles-Richardson *et al.* (1999) found the FHM tubercle endpoint in male fish to be more sensitive to E2 exposure compared with the fatpad. In contrast, (Filby *et al.*, 2010) found the male fatpad to be more responsive than the tubercles following exposure to WwTW effluent, so the mechanism for response may be different.

The DT131813 target had significantly higher expression in the female snout tissue than the male snout tissue. This was not reflective of the first sampling, although in the first instance only one male and one female fish were sampled, and conclusions should therefore not be drawn from the preliminary work. Seear *et al.* (2014) speculated that coding for the different spiggin genes might be selected to suit local conditions and found that spiggin α was more highly expressed than other spiggins in flow-through conditions compared with static conditions. More recent analysis of spiggin transcripts found spiggin α to have diverged the least from the ancestral MUC19 gene, and Seear *et al.* (2015) predicted that spiggin α and β are secreted as long mucin-like polymers but that further spiggins (C1 and C2) are secreted as short monomers, with putative antimicrobial properties. Thus, spiggin α and β are more likely to be the 'glue' proteins and further spiggins may have different functional properties. This DT131813 EST did align better with spiggin β and not so well with α (see Appendix Figures A 11 and A 12), and perhaps this is why the expression pattern was not as expected, i.e. as the fish were from flow-through tanks, the conditions perhaps selected for spiggin α and not β. As this thesis focussed on male specific biomarkers this was not considered to be relevant for monitoring for (anti-)androgenic activity but might be suitable for (anti-)oestrogenic activity. This difference in expression with the target most closely related to spiggin (DT131813) is unlikely to be spiggin-like and more likely to be another mucin, especially considering the conserved nature of mucins. Unfortunately, due to the highly conserved nature of mucin and related proteins, without additional sequence information it would not be possible to further identify this FHM EST DT131813.

The expression pattern for the other two targets (DT267220 and DT347638) looked promising in the preliminary sampling, as snout expression looked to be sexually dimorphic,

i.e. had higher expression levels in male than female tissue. However, when more FHM were sampled there was no significant difference in the expression levels between snout tissues collected from male and female FHM. Particularly with the male expression there was high variability, and whilst there was higher expression in the male snout tissues with the DT267220 target, this was not significant.

For none of the ESTs was there correlation with either the tubercle number or the FPI as might have been expected, although due to the males being separate from the females and not actively breeding, the SSCs could have been reflective of territoriality and not reproduction.

Transcript variability can be high and Cowie *et al.* (2015) estimated that a sample size of greater than 20 FHM would be required to detect a 2-fold change (0.8 power). Cowie *et al.* considered that variability in the gene expression of reproductive transcripts might be more pronounced for individuals actively undergoing sex maturation and be less variable in older individuals. Our n-values of ten (male FHM) and seven (female FHM) may have been too small, although all were sexually mature. For the first sampling, the male and female FHM fish pair were set up in a single tank with a spawning tile and were breeding prior to the sampling. Unfortunately, for the second sampling the male FHM were kept in separate tanks from the female fish due to the aggressive behaviour of the male FHM. Whilst SSCs were evident, the expression levels for these fish may have been different, as they were not actively breeding.

Both DT267220 and DT347638 targets were similar to zonadhesin as well as mucin when carrying out BLAST searches. Zonadhesin is homologous to both mucins and VWF and is a sperm membrane protein that mediates sperm adhesion to the extracellular matrix (zona pellucida) of the egg (Hardy and Garbers, 1995). Hardy and Garbers (1995) detected porcine zonadhesin mRNA only within the testis, where it was expressed primarily in haploid spermatids. Hunt *et al.* (2005) has also identified a zonadhesin-like gene (ZLG) in fish. Unlike mammalian zonadhesin, zebrafish and salmon ZLG were expressed in the gut and not the testes and although the function of fish ZLG is not known, expression in the gut and absence in the testes, combined with their similarity to gut-expressed genes of the mucosal immune system (i.e. FCG Binding Protein and mucin), suggests a non-reproductive function for the ZLG. However, when Skillman *et al.* (2006) exposed rainbow trout to EE2 and measured changes in expression of *vtg* and *zlg* in liver tissues, a 10.42 fold induction in *vtg* and a 5.05 fold reduction of *zlg* following the exposure were observed. From the results seen here, it is unlikely that the expression seen for DT267220 and DT347638 targets are spiggin-like. Instead, it is more likely that they are zonadhesin-like.

Unfortunately, ESTs only contain partial cDNA sequences and not full-length sequences. As a result, it is not possible to design primers that span exons, and without this the primers can also bind to gDNA. Designing primers to span exons would make the qPCR step easier, as otherwise gDNA can amplify alongside the cDNA. ESTs are also highly likely to be in conserved regions of proteins and this makes it difficult to identify specific proteins. For future work it would be better to PCR the FHM GOI first from gDNA and to have performed a 5'RACE to get a full-length sequence. Primers could then be designed to span exons and also to avoid conserved domains. Amplification would then be specific to the GOI and not pick up similar spiggin-like genes.

Could there be a zonadhesin-like protein produced in the male snout tissues used to help glue the eggs to substrate? For future research with larger numbers of actively breeding FHM, it would be interesting to determine if DT267220 and DT347638 increase in female FHMs following exposure to androgens.

6. Conclusions

6.1. Overall aims and approach:

Firstly, to determine if the addition of a full scale GAC, advanced wastewater treatment plant, could mitigated endocrine disrupting effects frequently observed in fish exposed to standard treated WwTW effluents.

- Both laboratory and field-based assessments of the same effluent were conducted to investigate the impacts of the traditional and advanced treatment processes on well characterised markers of endocrine disruption in fish, namely, occurrence of intersex phenotypes (both eggs and sperm in the gonad) (field assessment), VTG induction in male fish (laboratory and field assessments), and disruption to reproductive output and SSCs (laboratory assessment).

To determine if chemicals found in WwTW effluents possessed (anti-)androgenic activity.

- Over 100 chemicals were investigated via an *in vitro* yeast test system.

Thirdly, to identify possible biomarkers which could provide promising additional endpoints for detecting (anti-)androgenic activity in a sexually dimorphic fish species i.e. for subsequent addition to ecotoxicology regulatory assays.

- Bioinformatic searches and expression analysis were conducted for male-specific molecular markers in a sexually dimorphic fish species.

6.2. Research questions and findings:

These aims were addressed through 4 research questions, the first being **'Does the addition of an advanced treatment process to a full-scale wastewater treatment plant lead to a reduction in endocrine disruption in fish?'** (investigated in Chapter 2). This question was addressed using short-term *in vivo* laboratory exposure studies and a field collection of wild roach exposed under natural conditions, to see if the wild fish analysis supported the lab-based studies. Whilst a few papers have been published following the addition of the GAC to the Swindon WwTW, none looked at long-term effects (5 years after the addition of GAC) on fish living downstream of the WwTW.

Prior to the addition of the GAC treatment, the WwTW effluent had significant demasculinising effects on FHM in the lab-based studies. These were not observed post GAC. The chemical monitoring of the standard/GAC effluents for E1, E2 and EE2 in the FHM tests gave inconsistent results, but the yeast bioassay used to determine the E2EQ did not give any better predictions of *in vivo* effects than the analytical chemistry results, as the

total oestrogenic content was at concentrations likely to induce VTG, but no VTG induction was observed. This was unexpected as, when roach were sampled from the River Ray in 2005 to assess the suitability of the site for the end-of-pipe GAC plant at the Swindon WwTW, the VTG concentrations in males were elevated to concentrations similar to those found in female roach, indicating the presence of oestrogens. However, the FHM exposure studies did take place in 2009, 4 years after the 2005 sampling of roach from the River Ray, and during this time other modifications that resulted in better treatment of the effluent prior to the installation of the GAC plant at the Swindon WwTW, may have occurred. Additional monitoring for chemicals with other activities (e.g. anti-androgens) would have made it easier to identify what type of activity was responsible for the observed demasculinising effects. In the absence of VTG induction, the culprits were thought to be anti-androgens.

For the lab-based Pair Breeding Test with FHM, only the positive control (EE2) significantly reduced egg production; no effects on egg production were observed with the effluent treatments. This was unexpected, as it was hypothesised that EDCs thought to be present in the effluent would have reduced breeding (in a similar way to the positive control). There are a couple of possible explanations as to why egg laying was not reduced by the effluent exposures. Firstly, that the effluent did not contain enough oestrogens to inhibit egg laying in the FHM (which is supported by the lack of VTG induction in the male FHMs), or secondly, that the reduced feeding (to minimise ammonia issues) lowered the reproductive output in the control fish (effluent tanks had additional organic matter in comparison), thus reducing the ability to detect any subtle effects of the effluent. Increasing the feeding regime for the FHM, so that egg production remained constant for control fish during both the pre-exposure and exposure period, might have improved the sensitivity of this test.

Ideally, short-term fish studies assessing effluents should take place at the WwTW, so that the effluent does not have to be transported/stored, although the Pair Breeding Test is labour intensive, and it would not be practical to carry out such tests at the WwTW. Carrying out testing at the WwTW would have allowed for the parallel testing of the standard and GAC effluent (not possible at Brunel University London due to the availability of only one cooled storage tank) and enabled easier comparisons, especially if enough of the same age FHMs were used for both effluent studies. Unfortunately, because FHM are not native to the UK, they require a water temperature of 25 ^{o}C, and this would add to the expense of experiments if sited at the WwTW.

On comparing the endpoints for the roach collected from the River Ray in 2013 with results from the historic sampling in 2005, whilst there were reductions in the biomarkers of oestrogen exposure in male roach (VTG, intersex), they were not significant. This lack of statistical significance was probably because neither the VTG concentrations nor the

proportion of intersex in male fish had reduced to baseline “natural” levels following the GAC addition to the WwTW. However, it could also be due to the smaller number of fish collected in 2013, as well as the variability of some of the endpoints (e.g. VTG). Comparisons between the 2005 and 2013 roach samplings were also complicated because the fish were sampled at different times of the year. In contrast, for the female endpoints investigated, there were significantly less uncharacteristically immature female roach after the GAC addition, indicating an improvement in female reproductive health.

Whilst there were improvements in the female roach reproductive health, it was not clear if this was due to reduced oestrogen concentrations in the river and/or reductions in chemicals possessing other type of activities (e.g. anti-androgens). This is because the sampling of the roach from the River Ray was not accompanied by any measurements of EDCs (analytical chemistry or total activity using bioassays), and this lack of water analytical chemistry/bioassay data made it harder to interpret the results. Also, whilst it was important to monitor the reproductive health of roach over a longer period of time, this did mean that some of the methods used at the first sampling were not exactly the same as the second sampling (e.g. the VTG ELISA technique), making comparisons between the different results even more challenging.

On comparing the VTG endpoint in the short-term (lab) and long-term (field) tests, whilst the male FHM in 2009 did not have elevated concentrations when exposed to either standard or GAC effluent, in 2013 after the GAC addition the male roach still had VTG concentrations about an order higher than baseline concentrations. This may have been due modifications to the Swindon WwTW between 2005 and 2009 having an effect on the quality of the effluent, but may also be because the FHM exposures were to effluent alone compared with the field sampling where the river water would have also contain chemicals from non-point sources, such as agricultural run-off. The lab-based studies therefore simplify the exposure and make the identification of the presence of non-oestrogenic compounds possible and helped to pin-point the likely chemicals to anti-androgens. Comparing the short-term and long-term tests was not so easy as many of the endpoints were different, however, this was also advantageous as it helped to build a bigger picture. By combining the short-term and long-term tests, it was possible to consider that the effects on the female roach reproductive health could just as likely be due to the presence of other chemicals.

In fish, EDCs are linked to reproductive disorders, and whilst some studies show that roach with mild intersex are able to successfully reproduce (Hamilton *et al.*, 2015), others have linked severe intersex in rainbow darter with poor reproductive success (Fuzzen *et al.*, 2015). Post addition of the GAC, the reproductive effects appear to be minor, but early life adverse effects may not manifest until later life, or even later generations (Hamilton *et al.*,

2016). The use of advanced treatments such as GAC are expensive and whilst the GAC did succeed in removing the possible anti-androgenic effect of the effluent seen in the FHM, perhaps other less intensive methods, such as increasing retention times or tertiary treatments such as sand filtration, might have also been sufficient to reduce risks to acceptable levels. Especially as it seems, from the FHM study, that even before the addition of the GAC in 2009, the oestrogenic effect of the effluent was already much reduced compared with when the historic sampling of the roach took place in 2005.

My second research question, **'Which individual chemicals from wastewater treatment work effluents have (anti-)androgenic activity and how potent are these chemicals?'**, was addressed by testing 107 chemicals found in WwTW effluent and river water using a yeast-based (anti-)androgen screen (investigated in Chapter 3). When testing the chemicals in the (A)YAS, some of the results were inconsistent (on retesting chemicals and compared with results published by others) and toxicity was considered a possible reason for false positives. Therefore, the active chemicals were then retested using a modification of the standard method that utilised a final cell lysis step (based on the ISO 19040-1 protocol). The results related to the third question **'Do both *in vitro* assays for (anti-)androgenic activity produce the same results and, if not, which is the most reliable, i.e. least likely to produce false positive results?'** (investigated in Chapter 4) are summarised in the following table:

Assay / Activity	Standard (A)YAS	Modified (A)YAS
Androgenic	3	0
Anti-androgenic	20	18
Superagonist	Inconsistent	Inconsistent

Whilst three of the 107 chemicals were very weakly androgenic (methyl decanoate, 2-ethylhexanoic acid and ibuprofen) using the standard YAS method (investigated in Chapter 3), this activity was not repeatable when the YAS was modified with an additional cell lysis step (investigated in Chapter 4). Whilst the most potent of the androgenic chemicals, ibuprofen, is widely used for its pain-relieving and anti-inflammatory effects, the potency was over a million times less potent than DHT (positive control) and the discrepancy between the standard YAS method and the modified assay was therefore small. These results seen with the standard YAS were thought to be false positives.

Additionally, in the standard AYAS, twenty of the chemicals tested (19%) were found to be anti-androgenic, some more potent than the pharmaceutical flutamide. However, for two of the chemicals (triphenyl phosphate and myristic acid) identified in the standard AYAS, the anti-androgenic activity could not be repeated when the assay was modified, probably because toxicity was interfering with the results in the standard assay. Some of the anti-androgenic chemicals were more potent than the pharmaceutical flutamide (triclosan and its metabolite methyl triclosan, chlorophene, fluoranthene and pyrene, and the resin acid dehydroabietic acid) used as the anti-androgenic standard. Whilst the most potent of the anti-androgens have already been reported in the literature, for many of the less potent chemicals these results were novel, for example, acetylcedrene, diphenylacetic acid methyl ester, alpha cedrol, benzeneacetaldehyde, 2-(methylthio)benzothiazole.

Chemicals identified as anti-androgenic in the modified (A)YAS		
Strong (more potent than flutamide)	Moderate (similar potency to flutamide)	Weak/very weak (less potent than flutamide)
Triclosan	Dehydroabietic acid	alpha cedrol
Chlorophene	Pyrene	Benzeneacetaldehyde
Methyl triclosan	9H-Fluorene	2-(methylthio)benzothiazole
Fluoranthene	Acetylcedrene	N-Butylbenzene sulfonamide
	Diphenylacetic acid methyl ester	Benzophenone
		Dodecanoic acid
		9,12-Octadecadienoic acid methyl ester
		1-[4-(hydroxy-1-methylethyl) phenyl] ethanone
		Hexamethylbenzene

For the anti-androgenic activity thought to be the result of toxicity, the modified AYAS with cell lysis was much less likely to give false positive results, and this was especially important as many of the chemicals tested were toxic at higher concentrations. This was because with

the modified assay with cell lysis, the reduction in colourimetric absorbance due to toxicity mirrored the reduction in turbidity readings, but this was not the case with the standard assay where toxicity led to reductions in colour before turbidity was reduced. Some laboratories routinely carry out assays for toxicity in parallel with the assays for (anti-)androgenic activity, but this often doubles both the time and the cost of running the assay. This modified assay was more complex and lengthier to run, particularly at the cell lysis step at the end of the incubation period, but was also more robust, i.e. less prone to false positives. This is beneficial as a false positive result might lead to *in vivo* investigations, leading to unnecessary costs, wasted resources and unnecessary use of animals.

Some chemicals were able to enhance the activity of DHT in the anti-androgen screen rather than block the activity, i.e. were superagonists and not anti-androgens. As pollutants in the natural environment are most often present as mixtures rather than discrete chemicals, chemicals present that enhance the activity of androgens (and possibly chemicals with other types of activity), yet have no activity when tested singly, may get overlooked. In fact, most regulatory testing is of single chemicals and not mixtures and this type of activity might go unnoticed yet has the potential to increase the potency of other chemicals. In this case the activity of the steroid DHT was enhanced, which is important as steroids are a major group of chemicals found in WwTW effluents.

In terms of limitations, due to the part-time nature of my PhD, the *in vitro* yeast assays were carried out over a five-year period, and this meant that there was less consistency in the assay components. This might also have been the reason for the variable superagonist activity. For cost saving, I also used freeze/thawing to lyse the yeast cells rather than lyticase, as detailed in the ISO 19040-1:2018 protocol, and this meant that the final stages of the assay took longer and smaller assays had to be run, again possibly reducing the consistency.

In stickleback, a sexually dimorphic fish species, males secrete spiggin under the control of androgens, which is used to build a nest in preparation for female egg laying. The presence of spiggin in female sticklebacks indicates exposure to environmental androgens, and a reduction in spiggin in male fish can indicate exposure to environmental anti-androgens. The Androgenised Female Stickleback Screen (AFSS) is now partially validated as an OECD test. However, spiggin as a biomarker is only applicable to sticklebacks, and this led to my fourth and final question, **‘Do FHMs, another sexually dimorphic fish species exhibiting paternal parental care, possess a similar spiggin-like protein that could be used as a sensitive biomarker of (anti-)androgen exposure, to provide an additional endocrine endpoint to commonly used OECD regulatory test guidelines?’.** To answer this question, I designed primers to FHM ESTs with similarity to spiggin, and carried out qPCRs

to look for sexually dimorphic expression in tissues where sexually dimorphic features/ mucus production have previously been described. Whilst a spiggin-like protein was not discovered, two zonadhesin-like targets were identified in the FHM snout tissues (providing potential for non-invasive sampling). As zonadhesin is another glue-like protein that mediates sperm adhesion, if sexually dimorphic in FHM, it could be used to provide an additional endocrine endpoint to commonly used OECD regulatory test guidelines. The initial results looked promising, albeit zonadhesin-like and not spiggin-like gene expression, but on repeating the work with larger fish numbers, whilst the expression levels were higher in the male FHM, these levels were not significantly different from the female FHM snout expression levels.

Whilst the FHM used for my first expression analysis work were available at Brunel University London, this was not the case when it came to analysing these tissues or for my second sampling. This meant that, when I was not able to get enough RNA or the RNA was degraded, I was not easily able to collect further tissues, and important tissues (testis and ovary) were missing from the first analysis. As we no longer had FHM at Brunel University London for my second sampling, I was able to obtain the FHM for this expression analysis work from another aquatic ecotoxicology facility; Scymaris, Brixham, but as these were supplied off site, it was not possible to set these fish up as breeding pairs. This may have been why results that initially looked promising for the first sampling were not significant for the second sampling with larger fish numbers.

Whilst there are several fish species used to measure the effects of oestrogenic chemicals in the environment (e.g. zebrafish, medaka, fathead minnow), for anti-androgens the stickleback is still the recommended species (OECD, 2018c). Sebillot *et al.* (2014) has also thought to use the biomarker ‘spiggin’ in another fish species and developed a sensitive specific transgenic medaka model bearing an androgen responsive fluorescent reporter construct (green fluorescent protein gene driven by the spiggin promoter) for whole organism based environmental screening of (anti-)androgens. This assay (RADAR: Rapid Androgen Disruption Adverse Outcome Reporter Assay) is now being considered as an OECD test for AR agonists and antagonists.

The effects of EDCs have only been investigated in a few fish species, mainly because of limited genomic information for non-model species including the FHM. Whilst this OECD RADAR test looks to be a reliable test for (anti-)androgens, this uses freshly hatched medaka that may have limited metabolic capability compared with adult fish. As FHM are one of the three recommended species for two OECD tests (OECD TG 229: Fish Short-Term Reproduction Assay and OECD TG 230: 21-Day Fish Assay), both using adult fish, it

would be beneficial to add endpoints to this species rather than add extra species/tests for different endpoints (3Rs). If sexually dimorphic 'zonadhesin-like' expression was found to be androgen dependent, it could be a useful endpoint for monitoring for (anti)-androgens, especially as there is the possibility it could be sampled non-invasively.

6.3. Recommendations for future work

For future work it would be very interesting to investigate the superagonists further. As a starting point it would be important to purchase the 12 superagonists once more and to carry out the testing with fresh yeast assay components, to determine if the inconsistent activity was because they had reached the end of their shelf-life. It would also be interesting to run the same chemicals through the yeast anti-oestrogen screen to see if these 12 chemicals were similarly able to enhance the activity of the oestradiol present in the medium. If these chemicals were then consistently active as superagonists, it would be important to get a better understanding of the mechanism by which one chemical is able to enhance the activity of another, as this would help to understand if the issue could also be affecting other assays, e.g. HTP assays. As the real concern regarding these superagonists is that they enhance the uptake of chemicals into fish, assuming that the superagonist response was consistent, it would be interesting to determine whether these chemicals were able to affect the uptake of other chemicals into fish.

Because the snout tissue has the potential to be sampled non-invasively, it would be of interest to look further at zonadhesin-like expression in FHM, with larger numbers of sexually active FHMs, as well as looking at differences in expressions levels in tissues of female fish treated with androgens. Further information is now available regarding a FHM zonadhesin-like gene on the NCBI website, and this would enable the design of primers to span introns and avoid amplifying gDNA or other mucins with conserved domains.

6.4. Final conclusions

There is a need to discover and apply new approaches to replace, reduce and refine the use of animals in scientific procedures. The use of *in vitro* assays can reduce the need for *in vivo* testing, but inappropriate *in vitro* assays could affect data interpretation and lead to the mislabelling of some of the chemicals as EDCs. Modifying and improving the robustness of this standard (A)YAS could lead to a reduction in false positive results and subsequent reduction in *in vivo* investigations, thus reducing unnecessary costs/wasted resources along with reducing animal usage.

Currently there are no validated fish tests for anti-androgens, and although the AFSS (a variant of the 21-Day Fish Assay) has more power to identify anti-androgens than the 21-Day Fish Assay (utilising zebrafish, FHM or medaka), this test has a more limited range of endpoints. In the 21-Day Fish Assay, this protocol describes SSC measurements as biomarkers of exposure to (anti-)androgens, yet these endpoints often lack sensitivity and some are scored on a subjective scale, so may be subject to bias (Ankley et al., 1998; Muldoon and Hogan, 2016). An additional biomarker for (anti-)androgens in the FHM would greatly enhance this fish test and could reduce the need for the AFSS. This would reduce the number of fish tests required when testing chemicals. The zonadhesin-like also looked to have the potential to be sampled non-invasively, and this would refine the *in vivo* test by reducing stress/suffering.

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APPENDIX - FIGURES

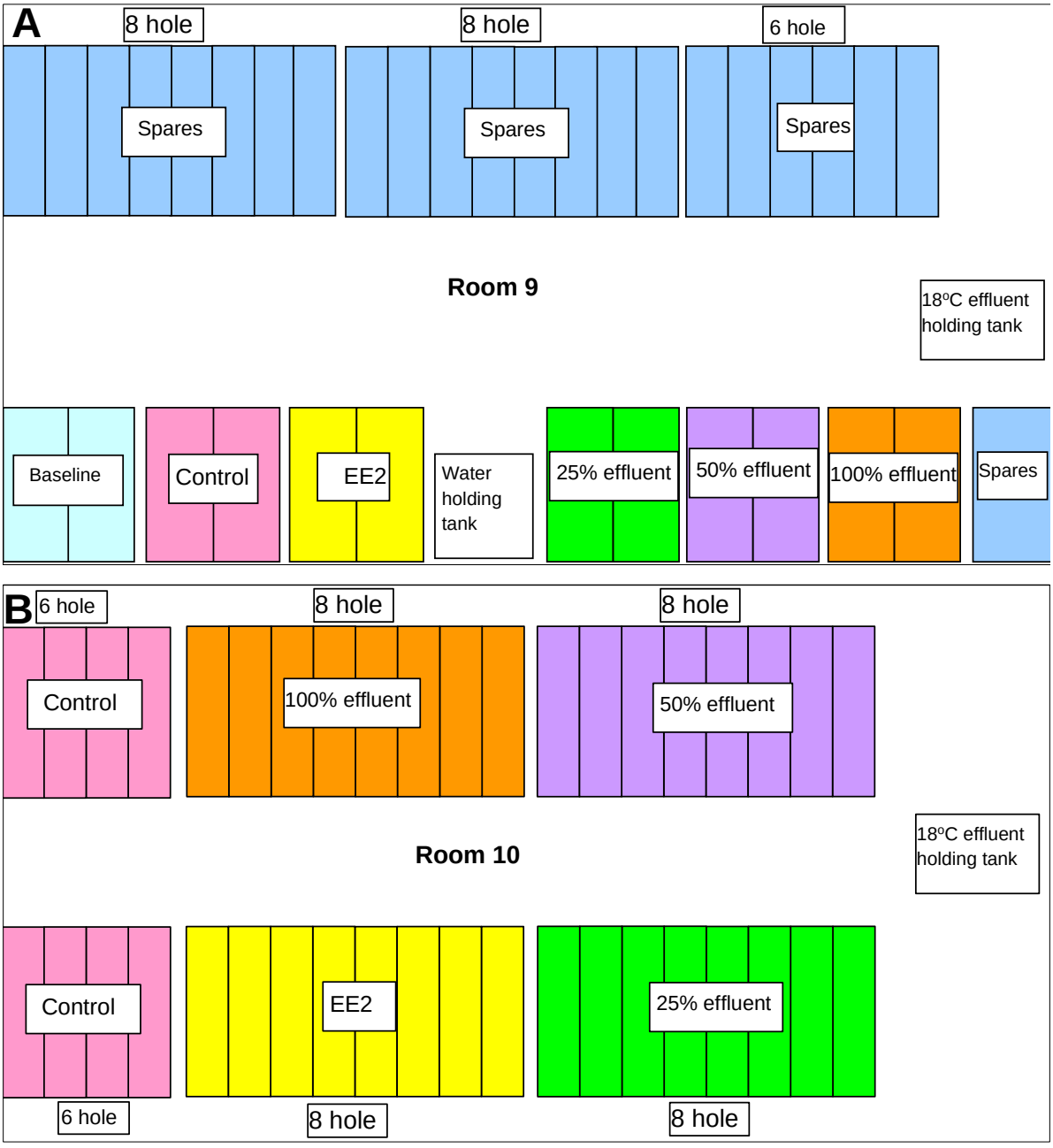

Figure A 1. Fish tank room set ups with standard treated effluent. (A) Experiment 1; VTG Test, dilution water, positive control (10ng/L EE2) and 100%, 50% or 25% effluent, and (B) Experiment 3, Pair Breeding Test, dilution water, positive control (20ng/L EE2) and 100%, 50% or 25% effluent, sets of 6 or 8 tanks fed dilution water/EE2/effluent via 6- or 8-hole mixing chambers.

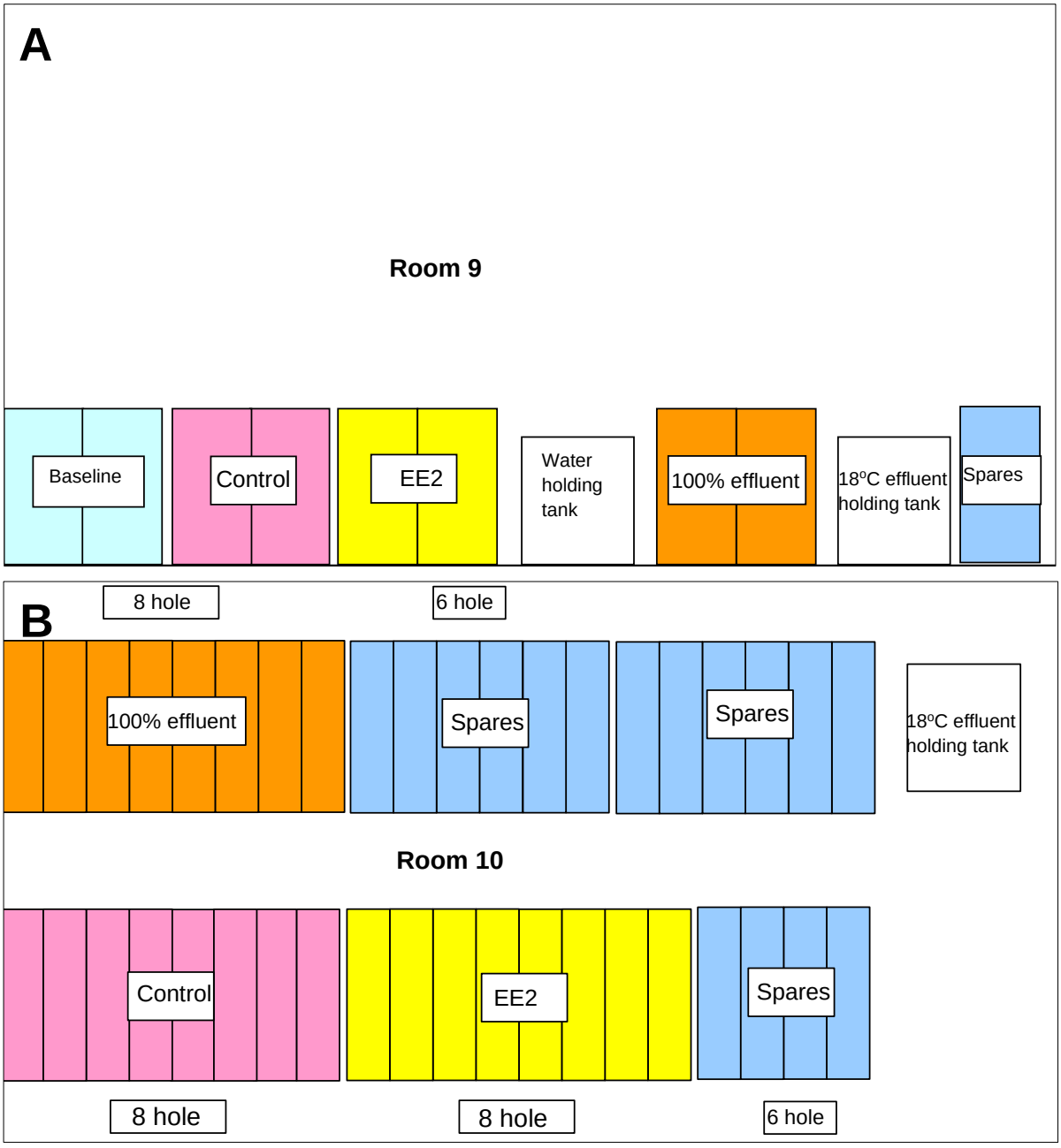

Figure A 2. Fish tank room set ups with GAC treated effluent. (A) Experiment 2; VTG Test, dilution water, positive control (10ng/L EE2) and 100% effluent, and (B) Experiment 4; Pair Breeding Test, dilution water, positive control (20ng/L EE2) and 100% effluent, sets of 6 or 8 tanks fed dilution water/EE2/effluent via 6- or 8-hole mixing chambers.

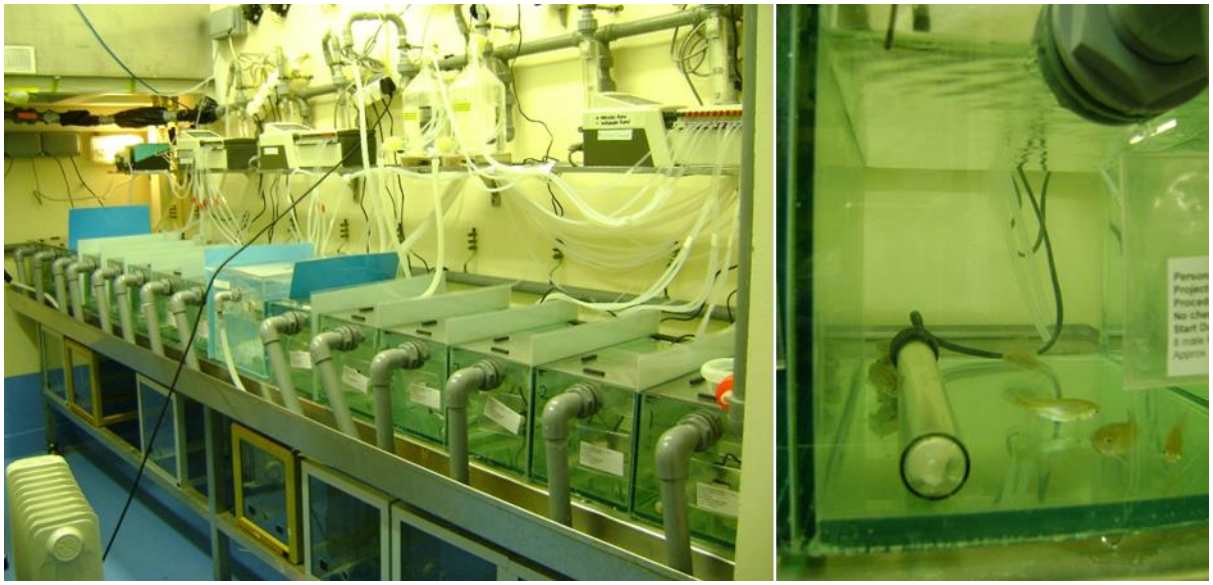

Figure A 3. VTG Test fish tank room set up with 20L tanks. Eight male fathead minnows were housed per tank.

Figure A 4. Pair Breeding Test fish tank room set up with sets of 8L tanks fed dilution water/EE2/effluent via 6- or 8-hole mixing chambers. Each tank contained a spawning chamber and housed a pair of fathead minnows.

Figure A 5. 2013 sampling – Seine netting the River Ray down stream of Swindon Waste Water Treatment Works.

Figure A 6. 2013 sampling – electrofishing upstream of the Seine net.

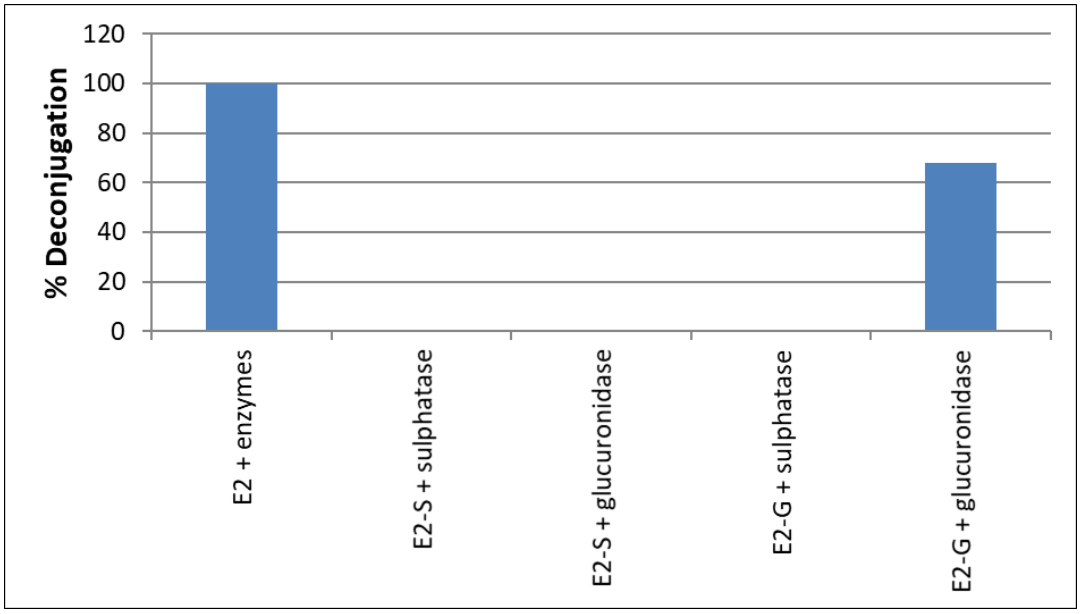

Figure A 7. Yeast oestrogen screen results showing percentage deconjugation for oestradiol (E2), oestradiol sulphate (E2-S) and oestradiol glucuronide (E2-G) with the enzymes sulphatase (type VI from *Aerobacter aerogenes*; Sigma-Aldrich) and β-glucuronidase (type VII-A from *E. coli*; Sigma-Aldrich).

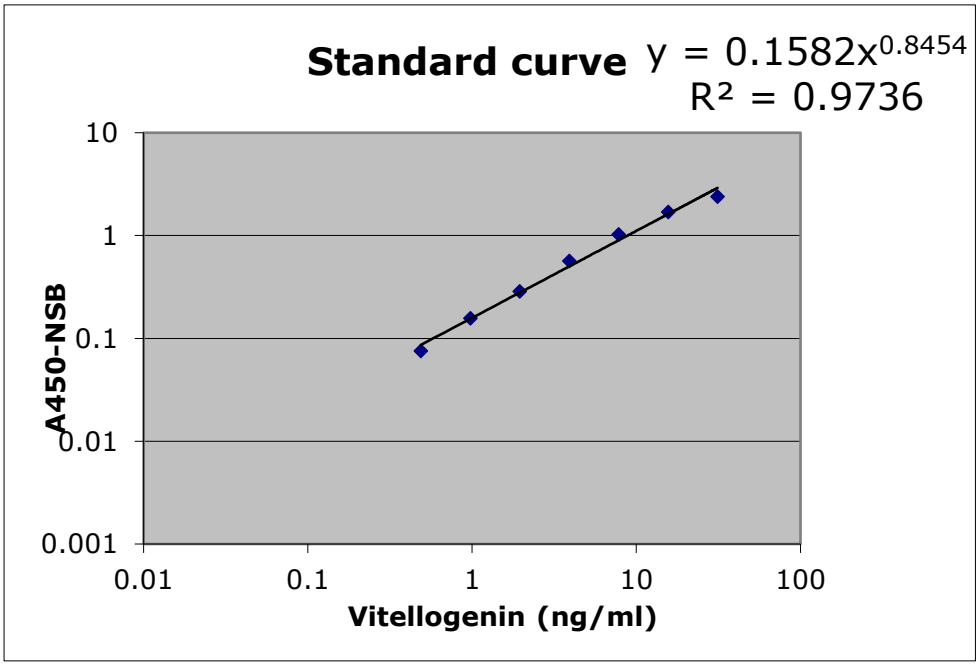

Figure A 8. An example of a standard curve using the Biosense commercial carp VTG ELISA kit for the analysis of the 2013 roach plasma samples.

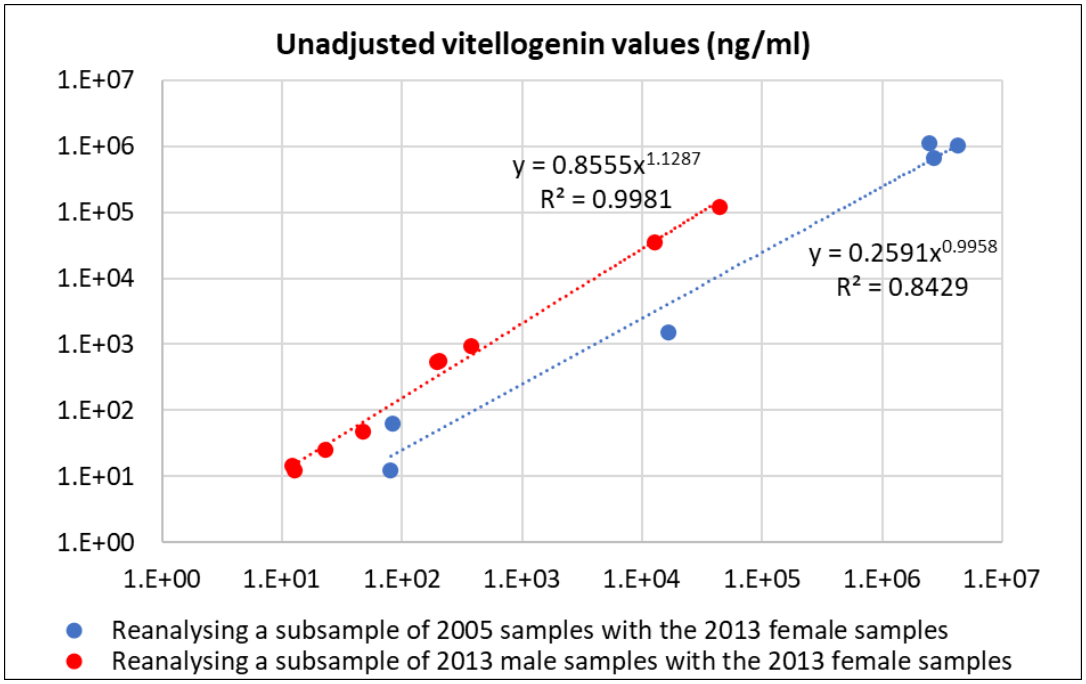

Figure A 9. The quantification of VTG in plasma samples used two different Enzyme-linked immunosorbent assay (ELISA) methods (the pre-commercialisation method by Tyler *et al*. (1999) and the Biosense commercial carp VTG ELISA kit) and took place over a number of years. To determine comparability of VTG data, subsamples of plasma from 2005 (blue circles) and male plasma from 2013 (red circles) were reanalysed in the ELISA used to determine the 2013 female plasma VTG concentrations (ng/ml). The regression models for the reanalysed 2005 and male 2013 plasma samples, had R^2 values of 0.8429 and 0.9981, respectively.

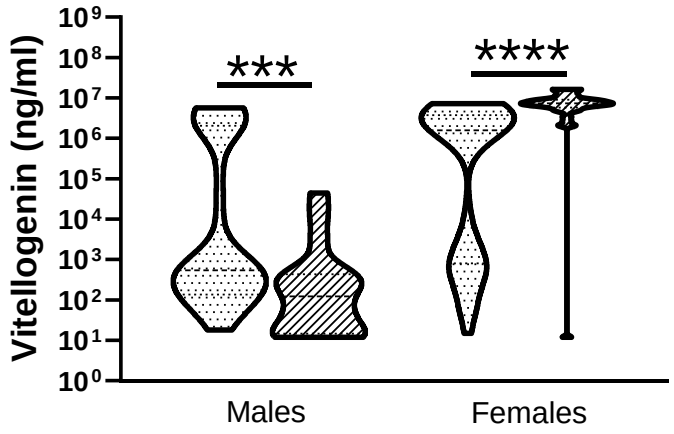

Figure A 10. Unadjusted plasma VTG concentrations in male (including intersex) and female roach sampled from the River Ray in 2005 (dotted plots; prior to GAC installation) and in 2013 (striped plots; after the GAC WwTW upgrade). The violin plots include lines at the median and quartiles. VTG values significantly different from respective 2005 values are denoted by stars; ***p<0.001 and ****p<0.0001.

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JGI_ANNO13690.fwd ANNO Pimephales promelas Whole (M) Pimephales promelas cDNA clone ANNO13690 5', mRNA sequence

Sequence ID: DT131813.1 **Length:** 722 **Number of Matches:** 1

Range 1: 78 to 269 GenBank Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
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Query  862  SRNIIL  867
            +R I L
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Figure A 11. Alignment of DT131813 with spiggin α (Accession number AAK15297) showing 36% identity.

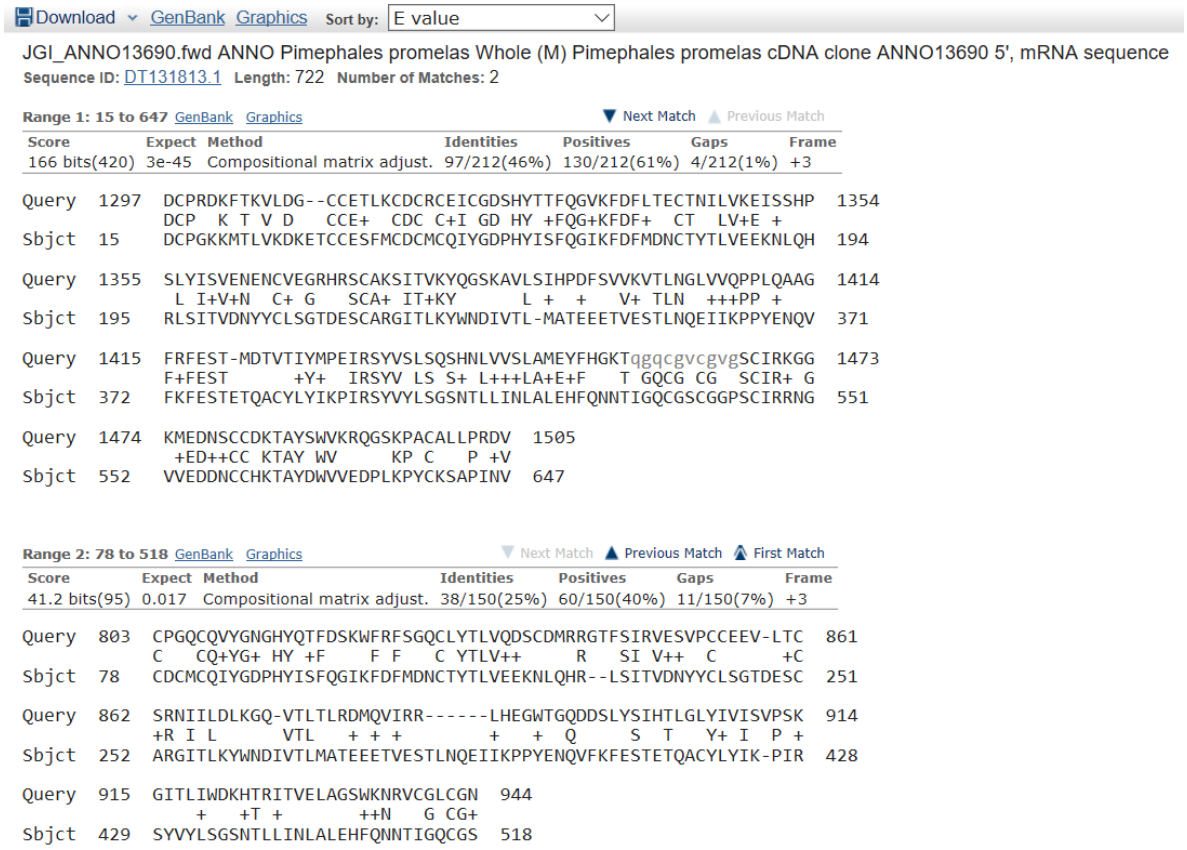

Figure A 12. Alignment of DT131813 with spiggin β (Accession number BAS02336) showing 46% and 25% identity.

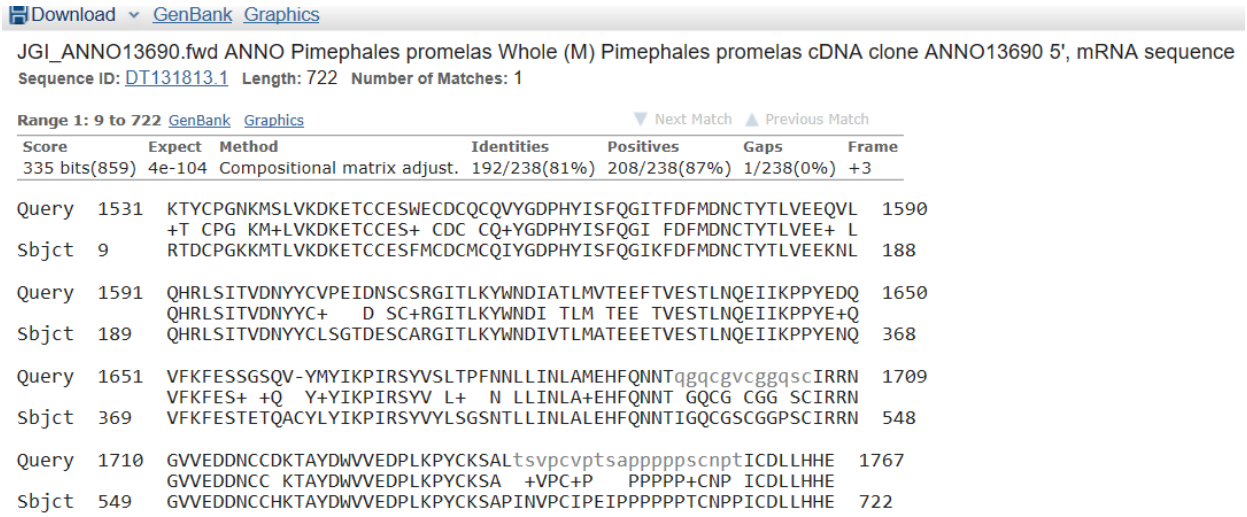

Figure A 13. Alignment of DT131813 with mucin-19-like [*Sinocyclocheilus grahami*] (Accession number XP_016126944.1) showing 81% identity.

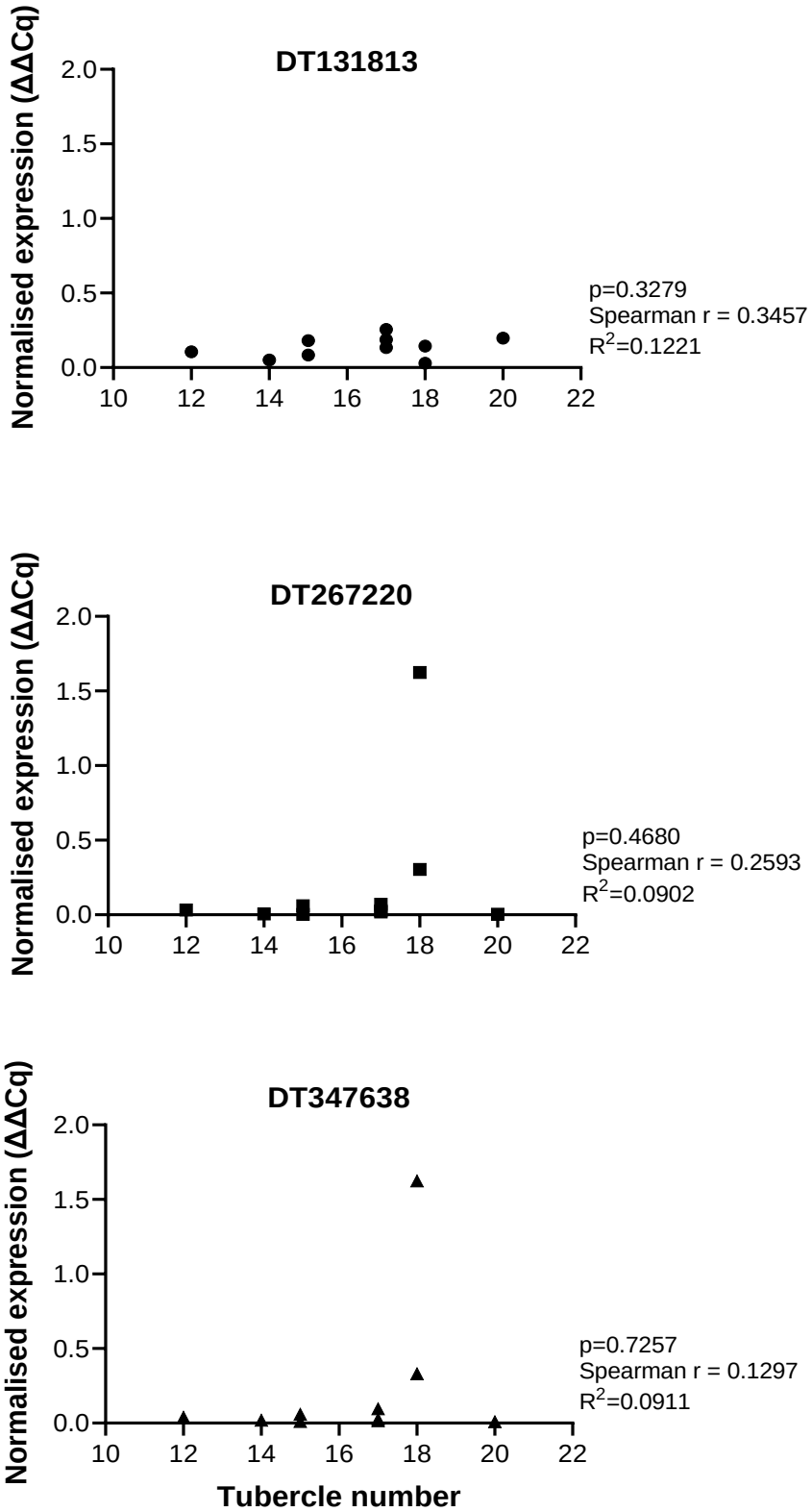

Figure A 14. Correlation analysis was carried out between the tubercle numbers and the normalised expression with the three ESTs (DT131813, p=0.3279; DT267220, p=0.4680; DT347638, p=0.7257) but no significant relationships were found.

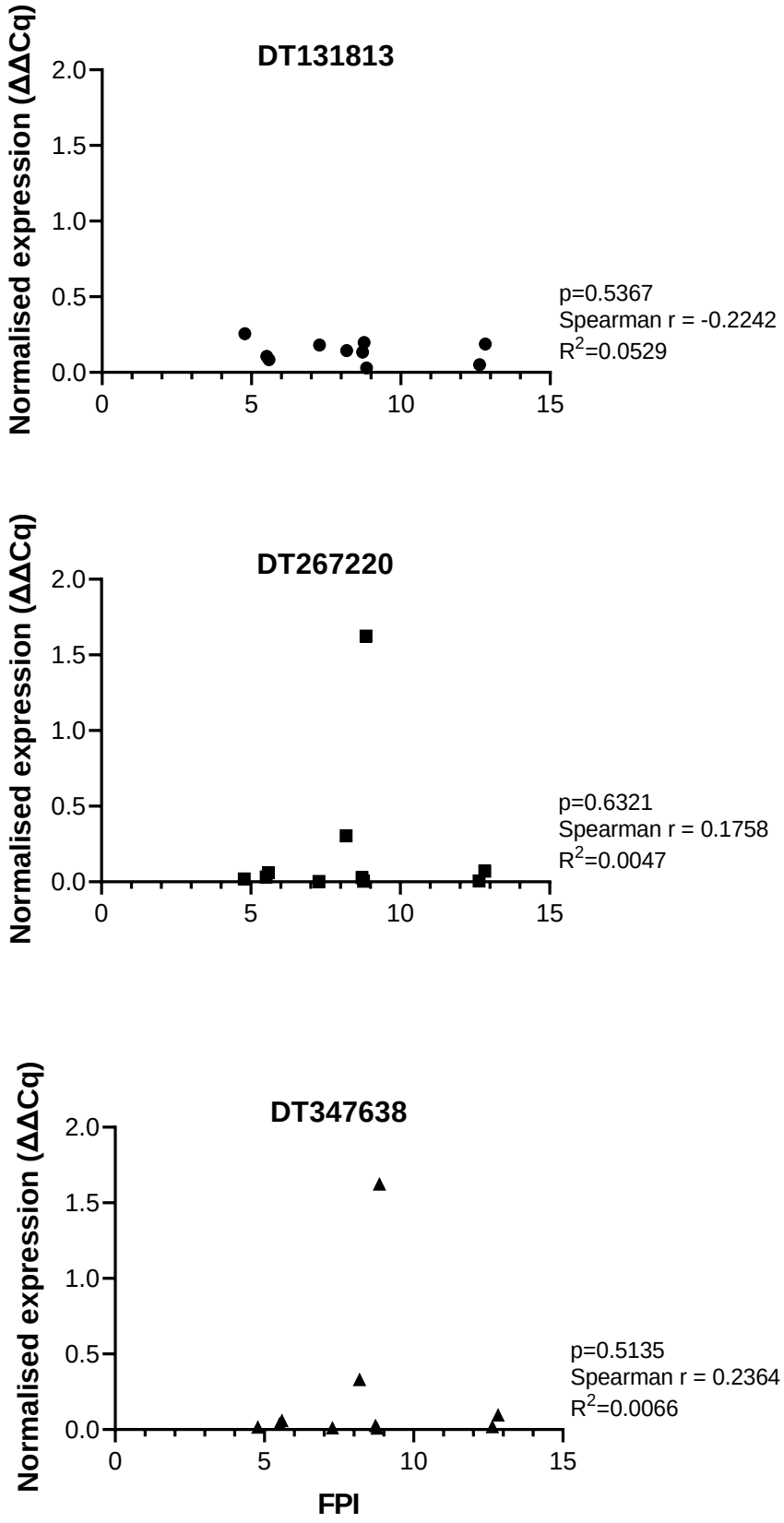

Figure A 15. Correlation analysis was carried out between the FPIs and the normalised expression with the three ESTs (DT131813, p=0.5367; DT267220, p=0.6321; DT347638, p=0.5135), but no significant relationships were found.

APPENDIX - TABLES

Table A 1. Raw fish tank water quality data from Experiment 1, VTG exposure study with standard effluent. Ammonia measurements in parts per million (ppm) taken with test strips (Precision Laboratories, Moulton, UK).

Ammonia (ppm)							
			Acclimation Period				
Date	Day	Study day	Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
21-May-09		Ac 1					
22-May-09		Ac 2	0	0	0	0	0
23-May-09		Ac 3	0.5	0.5	0.5	0.5	0.5
24-May-09		Ac 4	0.5	0.5	0	0.5	0.5
25-May-09		Ac 5	0.5	0.5	0.5	0.5	0.5
26-May-09		Ac 6	0.5	0.5	0.5	0.5	0.5
27-May-09		Ac 7	0.5	0.5	0.5	0.5	0.5
			Pre Exposure period (Weeks 1 to 3)				
Date	Day	Study day	Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
28-May-09		-21	0.5	0.5	0.5	0.5	0.5
29-May-09		-20	0	0	0	0	0
30-May-09		-19	0	0	0	0	0
31-May-09		-18	0	0	0	0	0
01-Jun-09		-17	0	0	0	0	0
02-Jun-09		-16	0	0	0	0	0.5
03-Jun-09		-15	0	0	0	0	0
04-Jun-09		-14	0	0	0	0	0
05-Jun-09		-13	0.5	0.5	0.5	0	0
06-Jun-09		-12	0	0	0.5	0.5	0.5
07-Jun-09		-11	0.5	0.5	0.5	0.5	0.5
08-Jun-09		-10	0.5	0.5	0.5	0.5	0
09-Jun-09		-9	0.5	0.5	0.5	0.5	0.5
10-Jun-09		-8	0.5	0.5	0.5	0.5	0.5
11-Jun-09		-7	0.5	0.5	0.5	0.5	0.5
12-Jun-09		-6	0.5	0.5	0.5	0	0.5
13-Jun-09		-5	0.5	0.5	0.5	0.5	0.5
14-Jun-09		-4	0	0	0	0	0
15-Jun-09		-3	0	0	0	0	0
16-Jun-09		-2	0.5	0.5	0.5	0	0.5
17-Jun-09		-1	0	0.5	0.5	0.5	0.5
18-Jun-09			0	0	0	0.5	1

Table A 2. Raw fish tank water quality data from Experiment 1, VTG exposure study with standard effluent. Nitrite measurements in parts per million (ppm) taken with test strips (Precision Laboratories, Moulton, UK).

Nitrite (ppm)							
			Acclimation Period				
Date	Day	Study day	Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
21-May-09		Ac 1					
22-May-09		Ac 2	0	0	0	0	0
23-May-09		Ac 3	0	0	0	0	0
24-May-09		Ac 4	0	0	0	0	0
25-May-09		Ac 5	0	0	0	0	0
26-May-09		Ac 6	0	0	0	0	0
27-May-09		Ac 7	0	0	0	0	0
			Pre Exposure period (Weeks 1 to 3)				
Date	Day	Study day	Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
28-May-09		-21	0	0	0	0	0
29-May-09		-20	0	0	0	0	0
30-May-09		-19	0	0	0	0	0
31-May-09		-18	0	0	0	0	0
01-Jun-09		-17	0	0	0	0	0
02-Jun-09		-16	0	0	0	0	0
03-Jun-09		-15	0	0	0	0	0
04-Jun-09		-14	0	0	0	0	0
05-Jun-09		-13	0	0	0	0	0
06-Jun-09		-12	0	0	0	0	0
07-Jun-09		-11	0	0	0	0	0
08-Jun-09		-10	0	0	0	0	0
09-Jun-09		-9	0	0	0	0	0.5
10-Jun-09		-8	0	0	0	0	0.5
11-Jun-09		-7	0	0	0	0	0
12-Jun-09		-6	0	0	0.5	0.5	0.5
13-Jun-09		-5	0	0	0.5	0.5	0.5
14-Jun-09		-4	0	0	0	0	0.5
15-Jun-09		-3	0	0	0	0	0
16-Jun-09		-2	0.5	0.5	0.5	0.5	0.5
17-Jun-09		-1	0.5	0.5	0.5	0.5	0.5
18-Jun-09			0.5	0.5	0.5	0.5	0.5

Table A 3. Raw fish tank water quality data from Experiment 1, VTG exposure study with standard effluent. Nitrate measurements in parts per million (ppm) taken with test strips (Precision Laboratories, Moulton, UK).

Nitrate (ppm)							
			Acclimation Period				
Date	Day	Study day	Control	+ve Control	25% Effluent	50% Effluent	100% Effluen
21-May-09		Ac 1					
22-May-09		Ac 2	0	10	10	10	0
23-May-09		Ac 3	10	10	10	10	10
24-May-09		Ac 4	10	25	10	10	10
25-May-09		Ac 5	10	25	25	10	25
26-May-09		Ac 6	0	10	10	10	10
27-May-09		Ac 7	0	0	0	0	0
			Pre Exposure period (Weeks 1 to 3)				
Date	Day	Study day	Control	+ve Control	25% Effluent	50% Effluent	100% Effluen
28-May-09		-21	10	10	0	10	10
29-May-09		-20	0	10	10	10	25
30-May-09		-19	0	10	10	10	10
31-May-09		-18	10	10	10	10	10
01-Jun-09		-17	0	0	10	10	25
02-Jun-09		-16	0	0	10	25	50
03-Jun-09		-15	10	10	25	25	50
04-Jun-09		-14	0	10	25	25	25
05-Jun-09		-13	10	10	25	25	50
06-Jun-09		-12	0	0	10	10	25
07-Jun-09		-11	10	10	25	25	50
08-Jun-09		-10	0	10	10	10	10
09-Jun-09		-9	10	10	25	10	25
10-Jun-09		-8	0	10	0	0	10
11-Jun-09		-7	0	10	10	25	25
12-Jun-09		-6	10	10	10	10	25
13-Jun-09		-5	10	10	10	25	25
14-Jun-09		-4	10	10	25	25	25
15-Jun-09		-3	0	10	10	10	10
16-Jun-09		-2	10	10	10	25	25
17-Jun-09		-1	10	10	10	10	25
18-Jun-09			10	10	10	10	25

Table A 4. Raw fish tank water quality data from Experiment 2, VTG exposure study with GAC effluent. Ammonia measurements in parts per million (ppm) taken with test strips (Precision Laboratories, Moulton, UK).

Ammonia (ppm)							
			Acclimation Period				
Date	Day	Study day	Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
09-Mar-09		Ac 1	0.5	0			0.5
10-Mar-09		Ac 2	0.5	0.5			0.5
11-Mar-09		Ac 3	0.5	0			0.5
12-Mar-09		Ac 4	0	0.5			0.5
13-Mar-09		Ac 5	0	0			0
14-Mar-09		Ac 6	0	0			0
15-Mar-09		Ac 7	0	0			0
			Pre Exposure period (Weeks 1 to 3)				
Date	Day	Study day	Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
16-Mar-09		-21	0	0			0
17-Mar-09		-20	0	0			0.5
18-Mar-09		-19	0	0			0
19-Mar-09		-18	0	0			0
20-Mar-09		-17	0	0.5			0
21-Mar-09		-16	0	0.5			0
22-Mar-09		-15	0.5	1			1
23-Mar-09		-14	0	0			0
24-Mar-09		-13	0	0			0
25-Mar-09		-12	0	0			0
26-Mar-09		-11	0	0			0
27-Mar-09		-10	0	0			0
28-Mar-09		-9	0	0			0
29-Mar-09		-8	0.5	0.5			0.5
30-Mar-09		-7	0	0			0
31-Mar-09		-6	0	0			0
01-Apr-09		-5	0	0			0
02-Apr-09		-4	0	0			0
03-Apr-09		-3	0	0			0
04-Apr-09		-2	0	0			0
05-Apr-09		-1	0	0			0.5
06-Apr-09							

Table A 5. Raw fish tank water quality data from Experiment 2, VTG exposure study with GAC effluent. Nitrite measurements in parts per million (ppm) taken with test strips (Precision Laboratories, Moulton, UK).

Nitrite (ppm)							
			Acclimation Period				
Date	Day	Study day	Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
09-Mar-09		Ac 1	0	0			0
10-Mar-09		Ac 2	0	0			0
11-Mar-09		Ac 3	0	0			0
12-Mar-09		Ac 4	0	0			0
13-Mar-09		Ac 5	0	0			0
14-Mar-09		Ac 6	0	0			0
15-Mar-09		Ac 7	0	0			0
			Pre Exposure period (Weeks 1 to 3)				
Date	Day	Study day	Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
16-Mar-09		-21	0	0			0
17-Mar-09		-20	0	0			0
18-Mar-09		-19	0	0			0
19-Mar-09		-18	0	0			0
20-Mar-09		-17	0	0			0
21-Mar-09		-16	0	0			0
22-Mar-09		-15	0	0			0
23-Mar-09		-14	0	0			0
24-Mar-09		-13	0	0			0
25-Mar-09		-12	0	0			0
26-Mar-09		-11	0	0			0
27-Mar-09		-10	0	0			0
28-Mar-09		-9	0	0			0
29-Mar-09		-8	0	0			0
30-Mar-09		-7	0	0			0
31-Mar-09		-6	0	0			0.5
01-Apr-09		-5	0	0.5			0.5
02-Apr-09		-4	0.5	0.5			0.5
03-Apr-09		-3	0.5	0.5			0.5
04-Apr-09		-2	0.5	0.5			0.5
05-Apr-09		-1	0	0			0.5
06-Apr-09							

Table A 6. Raw fish tank water quality data from Experiment 2, VTG exposure study with GAC effluent. Nitrate measurements in parts per million (ppm) taken with test strips (Precision Laboratories, Moulton, UK).

Nitrate (ppm)							
			Acclimation Period				
Date	Day	Study day	Control	+ve Control	25% Effluent	50% Effluent	100% Effluen
09-Mar-09		Ac 1	0	0			0
10-Mar-09		Ac 2	10	0.5			10
11-Mar-09		Ac 3	0	10			0
12-Mar-09		Ac 4	0	0			10
13-Mar-09		Ac 5	0	0			0
14-Mar-09		Ac 6	0	0			0
15-Mar-09		Ac 7	0	0			0
			Pre Exposure period (Weeks 1 to 3)				
Date	Day	Study day	Control	+ve Control	25% Effluent	50% Effluent	100% Effluen
16-Mar-09		-21	0	0			0
17-Mar-09		-20	0	0			10
18-Mar-09		-19	0	0			10
19-Mar-09		-18	0	0			10
20-Mar-09		-17	0	0			10
21-Mar-09		-16	0	0			25
22-Mar-09		-15	0	0			0
23-Mar-09		-14	0	0			0
24-Mar-09		-13	0	0			0
25-Mar-09		-12	0	0			0
26-Mar-09		-11	0	0			10
27-Mar-09		-10	0	0			10
28-Mar-09		-9	0	0			10
29-Mar-09		-8	0	0			10
30-Mar-09		-7	0	0			10
31-Mar-09		-6	0	0			10
01-Apr-09		-5	0	10			10
02-Apr-09		-4	0	10			10
03-Apr-09		-3	0	10			10
04-Apr-09		-2	0	10			10
05-Apr-09		-1	0	10			5
06-Apr-09							

Table A 7. Raw fish tank water quality data from Experiment 3, Pair Breeding exposure study with standard effluent. Ammonia measurements in parts per million (ppm) taken with test strips (Precision Laboratories, Moulton, UK).

Ammonia (ppm)							
			Acclimation Period				
Date	Day	Study day	1	+ve Control	25% Effluent	50% Effluent	100% Effluent
30-Apr-09		Ac 1	0.5	0.5	0.5	0.5	0.5
01-May-09		Ac 2	0.5	0.5	0.5	0.5	0.5
02-May-09		Ac 3	0.5	0.5	0.5	0	0.5
03-May-09		Ac 4	0	0	0	0	0
04-May-09		Ac 5	0	0	0	0	0
05-May-09		Ac 6	0.5	0	0	0.5	0
06-May-09		Ac 7	0	0	0	0	0
			Pre Exposure period (Weeks 1 to 3)				
Date	Day	Study day	Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
07-May-09		-21	0.5	0.5	0	0.5	0.5
08-May-09		-20	0.5	0.5	0	0.5	0.5
09-May-09		-19	0.5	0.5	0.5	0.5	0.5
10-May-09		-18	0.5	0.5	0.5	0.5	0.5
11-May-09		-17	0.5	0.5	0.5	0.5	0.5
12-May-09		-16	0	0	0	0	0
13-May-09		-15	0	0	0	0	0
14-May-09		-14	0.5	0	0	0	0
15-May-09		-13	0	0	0	0	0
16-May-09		-12	0	0	0.5	0	0
17-May-09		-11	0	0	0.5	0	0
18-May-09		-10	0.5	0.5	0.5	0.5	0.5
19-May-09		-9	0.5	0.5	0	0	0
20-May-09		-8	0.5	0.5	0.5	0.5	0.5
21-May-09		-7	0.5	0.5	0.5	0.5	0.5
22-May-09		-6	0.5	0.5	0.5	0.5	0.5
23-May-09		-5	0.5	0.5	0.5	0.5	0.5
24-May-09		-4	0	0	0	0	0
25-May-09		-3	0.5	0	0	0.5	0.5
26-May-09		-2	0.5	0.5	0.5	0.5	0.5
27-May-09		-1	0	0	0	0	0
			Exposure period (Weeks 4 to 6)				
Date	Day	Study day	Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
28-May-09		1	0	0	0	0	0
29-May-09		2	0	0	0	0	0
30-May-09		3	0	0	0	0	0
31-May-09		4	0	0	0	0	0
01-Jun-09		5	0	0	0	0.5	0.5
02-Jun-09		6	0	0	0	0	0
03-Jun-09		7	0	0	0	0	0
04-Jun-09		8	0	0	0	0	0
05-Jun-09		9	0	0.5	0.5	0.5	0.5
06-Jun-09		10	0	0.5	0.5	0.5	0.5
07-Jun-09		11	0	0.5	0.5	0.5	0.5
08-Jun-09		12	0	0	0.5	0.5	0.5
09-Jun-09		13	0	0	0	0	0
10-Jun-09		14	0	0	0	0	0
11-Jun-09		15	0	0	0	0	0
12-Jun-09		16	0	0.5	0	0	0.5
13-Jun-09		17	0	0	0	0	0.5
14-Jun-09		18	0	0	0	0	0.5
15-Jun-09		19	0	0	0.5	0.5	1
16-Jun-09		20	0.5	0.5	0.5	0.5	1
17-Jun-09		21	0.5	0.5	0.5	0.5	0.5

Table A 8. Raw fish tank water quality data from Experiment 3, Pair Breeding exposure study with standard effluent. Nitrite measurements in parts per million (ppm) taken with test strips (Precision Laboratories, Moulton, UK).

Nitrite (ppm)							
			Acclimation Period				
Date	Day	Study day	Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
30-Apr-09		Ac 1	0	0	0	0	0
01-May-09		Ac 2	0	0	0	0	0
02-May-09		Ac 3	0	0	0	0	0
03-May-09		Ac 4	0	0	0	0	0
04-May-09		Ac 5	0	0	0	0	0
05-May-09		Ac 6	0	0	0	0	0
06-May-09		Ac 7	0	0	0	0	0
			Pre Exposure period (Weeks 1 to 3)				
Date	Day	Study day	Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
07-May-09		-21	0	0	0	0	0
08-May-09		-20	0	0	0	0	0
09-May-09		-19	0	0	0	0	0
10-May-09		-18	0	0	0	0	0
11-May-09		-17	0	0	0	0	0
12-May-09		-16	0	0	0	0	0
13-May-09		-15	0	0	0	0	0
14-May-09		-14	0	0	0	0	0
15-May-09		-13	0	0	0	0	0
16-May-09		-12	0	0	0	0	0
17-May-09		-11	0	0	0	0	0
18-May-09		-10	0	0	0	0	0
19-May-09		-9	0	0.5	0	0	0
20-May-09		-8	0	0	0	0	0
21-May-09		-7	0	0	0	0	0
22-May-09		-6	0	0	0	0	0
23-May-09		-5	0	0	0	0	0
24-May-09		-4	0	0	0	0	0
25-May-09		-3	0	0.5	0	0	0
26-May-09		-2	0	0	0	0	0
27-May-09		-1	0	0.5	0	0	0
						.	
			Exposure period (Weeks 4 to 6)				
Date	Day	Study day	Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
28-May-09		1	0	0	0.5	0.5	0
29-May-09		2	0	0	0.5	0.5	0.5
30-May-09		3	0	0	0	0.5	0.5
31-May-09		4	0.5	0.5	0.5	0.5	0.5
01-Jun-09		5	0.5	0.5	0.5	0.5	0.5
02-Jun-09		6	0	0.5	0.5	0.5	0.5
03-Jun-09		7	0.5	0.5	0.5	0.5	0.5
04-Jun-09		8	0.5	0.5	0.5	0.5	0.5
05-Jun-09		9	0	0	0	0.5	0
06-Jun-09		10	0	0	0	0	0.5
07-Jun-09		11	0	0	0	0	0.5
08-Jun-09		12	0	0.5	0	0	0.5
09-Jun-09		13	0	0.5	0	0.5	0.5
10-Jun-09		14	0	0.5	0	0.5	0.5
11-Jun-09		15	0	0.5	0	0	0
12-Jun-09		16	0.5	0.5	0	0	0
13-Jun-09		17	0.5	0.5	0	0	0
14-Jun-09		18	0.5	0.5	0	0	0
15-Jun-09		19	0.5	0.5	0	0	0
16-Jun-09		20	0.5	0	0	0	0
17-Jun-09		21	0.5	0	0	0	0

Table A 9. Raw fish tank water quality data from Experiment 3, Pair Breeding exposure study with standard effluent. Nitrate measurements in parts per million (ppm) taken with test strips (Precision Laboratories, Moulton, UK).

Nitrate (ppm)							
			Acclimation Period				
Date	Day	Study day	Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
30-Apr-09		Ac 1	10	0	10	0	0
01-May-09		Ac 2	0	0	0	0	0
02-May-09		Ac 3	0	0	0	0	0
03-May-09		Ac 4	0	0	0	0	0
04-May-09		Ac 5	0	0	0	0	0
05-May-09		Ac 6	0	0	0	0	0
06-May-09		Ac 7	0	0	0	0	0
			Pre Exposure period (Weeks 1 to 3)				
Date	Day	Study day	Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
07-May-09		-21	10	10	10	10	10
08-May-09		-20	0	0	0	0	0
09-May-09		-19	0	0	0	0	0
10-May-09		-18	0	0	0	0	0
11-May-09		-17	0	0	0	0	0
12-May-09		-16	0	10	0	10	0
13-May-09		-15	0	10	0	10	0
14-May-09		-14	10	10	10	0	10
15-May-09		-13	0	0	0	10	10
16-May-09		-12	0	0	0	10	0
17-May-09		-11	0	0	10	10	10
18-May-09		-10	0	0	10	0	10
19-May-09		-9	0	10	10	10	10
20-May-09		-8	10	0	0	10	10
21-May-09		-7	10	10	10	10	10
22-May-09		-6	0	10	10	10	10
23-May-09		-5	0	0	10	10	0
24-May-09		-4	10	10	10	10	10
25-May-09		-3	10	10	10	10	10
26-May-09		-2	10	0	10	10	10
27-May-09		-1	0	10	10	10	10
			Exposure period (Weeks 4 to 6)				
Date	Day	Study day	Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
28-May-09		1	0	10	10	25	25
29-May-09		2	10	10	25	25	25
30-May-09		3	0	0	10	25	10
31-May-09		4	10	10	10	25	25
01-Jun-09		5	10	10	25	50	50
02-Jun-09		6	10	10	25	50	50
03-Jun-09		7	10	10	25	50	100
04-Jun-09		8	10	10	50	50	50
05-Jun-09		9	10	10	25	50	5
06-Jun-09		10	10	10	10	25	0
07-Jun-09		11	10	10	10	25	50
08-Jun-09		12	10	10	10	25	50
09-Jun-09		13	10	10	10	25	25
10-Jun-09		14	10	10	0	25	25
11-Jun-09		15	0	10	0	10	10
12-Jun-09		16	0	0	10	10	0
13-Jun-09		17	10	10	10	10	10
14-Jun-09		18	25	25	25	10	25
15-Jun-09		19	10	10	10	10	25
16-Jun-09		20	25	0	10	25	50
17-Jun-09		21	10	10	25	25	25

Table A 10. Raw fish tank water quality data from Experiment 4, Pair Breeding exposure study with GAC effluent. Ammonia measurements in parts per million (ppm) taken with test strips (Precision Laboratories, Moulton, UK).

Ammonia (ppm)							
Date	Day	Study day	Acclimation Period				
			Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
16-Feb-09		Ac 1	0.5	0.5			0.5
17-Feb-09		Ac 2	0.5	0.5			0.5
18-Feb-09		Ac 3	0.5	0.5			0.5
19-Feb-09		Ac 4	0.5	0.5			0.5
20-Feb-09		Ac 5	0.5	0.5			0.5
21-Feb-09		Ac 6	0.5	0.5			1.0
22-Feb-09		Ac 7	0.5	0.5			0.5
Date	Day	Study day	Pre Exposure period (Weeks 1 to 3)				
			Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
23-Feb-09		-21	0.5	0.5			0.5
24-Feb-09		-20	0.5	0.5			0.5
25-Feb-09		-19	0.5	0.5			0.5
26-Feb-09		-18	0.5	0.5			0.5
27-Feb-09		-17	0.5	0			0
28-Feb-09		-16	1	0.5			0.5
01-Mar-09		-15	0.5	0			0.5
02-Mar-09		-14	0	0.5			0.5
03-Mar-09		-13	0.5	0.5			0.5
04-Mar-09		-12	0.5	0.5			0.5
05-Mar-09		-11	0	0			0.5
06-Mar-09		-10	0.5	0.5			0.5
07-Mar-09		-9	0.5	0.5			0.5
08-Mar-09		-8	0.5	0			0.5
09-Mar-09		-7	0.5	0.5			0.5
10-Mar-09		-6	0.5	0.5			0.5
11-Mar-09		-5	0.5	0.5			0.5
12-Mar-09		-4	0.5	0.5			0.5
13-Mar-09		-3	0.5	0.5			0.5
14-Mar-09		-2	0.5	0.5			0.5
15-Mar-09		-1	0.5	0.5			0.5
Date	Day	Study day	Exposure period (Weeks 4 to 6)				
			Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
16-Mar-09		1	0.5	0.5			0.5
17-Mar-09		2	0.5	0			0.5
18-Mar-09		3	0.5	0.5			1
19-Mar-09		4	1	1			0.5
20-Mar-09		5	0.5	0.5			1
21-Mar-09		6	0.5	1			0.5
22-Mar-09		7	0.5	0.5			1
23-Mar-09		8	0.5	0.5			1
24-Mar-09		9	0.5	0.5			1
25-Mar-09		10	1	0.5			0.5
26-Mar-09		11	0.5	1			0.5
27-Mar-09		12	0.5	0.5			1
28-Mar-09		13	0	0.5			0.5
29-Mar-09		14	0.5	0.5			0.5
30-Mar-09		15	0	0.5			1
31-Mar-09		16	0.5	0.5			1
01-Apr-09		17	0.5	0.5			0.5
02-Apr-09		18	0.5	0.5			0.5
03-Apr-09		19	0	0.5			0.5
04-Apr-09		20	0.5	0.5			0.5
05-Apr-09		21	0.5	0.5			0.5

Table A 11. Raw fish tank water quality data from Experiment 4, Pair Breeding exposure study with GAC effluent. Nitrite measurements in parts per million (ppm) taken with test strips (Precision Laboratories, Moulton, UK).

Nitrite (ppm)							
Date	Day	Study day	Acclimation Period				
			Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
16-Feb-09		Ac 1	0	0			0
17-Feb-09		Ac 2	0	0			0
18-Feb-09		Ac 3	0	0			0
19-Feb-09		Ac 4	0	0			0
20-Feb-09		Ac 5	0	0			0
21-Feb-09		Ac 6	0	0			0
22-Feb-09		Ac 7	0	0			0
Date	Day	Study day	Pre Exposure period (Weeks 1 to 3)				
			Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
23-Feb-09		-21	0	0			0
24-Feb-09		-20	0	0			0
25-Feb-09		-19	0	0			0
26-Feb-09		-18	0	0			0
27-Feb-09		-17	0	0			0
28-Feb-09		-16	0	0			0
01-Mar-09		-15	0	0			0
02-Mar-09		-14	0	0			0
03-Mar-09		-13	0	0			0
04-Mar-09		-12	0	0			0
05-Mar-09		-11	0	0			0
06-Mar-09		-10	0	0			0
07-Mar-09		-9	0	0			0
08-Mar-09		-8	0.5	0.5			0.5
09-Mar-09		-7	0.5	0			0
10-Mar-09		-6	0	0			0
11-Mar-09		-5	0	0			0
12-Mar-09		-4	0	0			0
13-Mar-09		-3	0	0			0
14-Mar-09		-2	0	0			0
15-Mar-09		-1	0	0			0
Date	Day	Study day	Exposure period (Weeks 4 to 6)				
			Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
16-Mar-09		1	0	0			0
17-Mar-09		2	0.5	0			0.5
18-Mar-09		3	0.5	0.5			0.5
19-Mar-09		4	0	0.5			0.5
20-Mar-09		5	0	0			0
21-Mar-09		6	0	0			0
22-Mar-09		7	0	0			0.5
23-Mar-09		8	0	0			0.5
24-Mar-09		9	0	0			0
25-Mar-09		10	0	0			0
26-Mar-09		11	0	0			0
27-Mar-09		12	0	0			0
28-Mar-09		13	0	0			0
29-Mar-09		14	0	0			0.5
30-Mar-09		15	0	0			0.5
31-Mar-09		16	0	0			0
01-Apr-09		17	0	0			0.5
02-Apr-09		18	0	0			0.5
03-Apr-09		19	0	0.5			0.5
04-Apr-09		20	0	0.5			0.5
05-Apr-09		21	0	0			0.5

Table A 12. Raw fish tank water quality data from Experiment 4, Pair Breeding exposure study with GAC effluent. Nitrate measurements in parts per million (ppm) taken with test strips (Precision Laboratories, Moulton, UK).

Nitrate (ppm)							
Date	Day	Study day	Acclimation Period				
			Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
16-Feb-09		Ac 1	10	10			0
17-Feb-09		Ac 2	0	10			0
18-Feb-09		Ac 3	0	0			0
19-Feb-09		Ac 4	0	0			0
20-Feb-09		Ac 5	0	0			10
21-Feb-09		Ac 6	0	0			0
22-Feb-09		Ac 7	10	10			25
Date	Day	Study day	Pre Exposure period (Weeks 1 to 3)				
			Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
23-Feb-09		-21	25	25			25
24-Feb-09		-20	25	10			25
25-Feb-09		-19	25	25			25
26-Feb-09		-18	25	25			10
27-Feb-09		-17	10	10			10
28-Feb-09		-16	25	10			10
01-Mar-09		-15	10	10			10
02-Mar-09		-14	10	10			10
03-Mar-09		-13	10	10			10
04-Mar-09		-12	10	10			10
05-Mar-09		-11	10	10			10
06-Mar-09		-10	0	10			0
07-Mar-09		-9	10	10			0
08-Mar-09		-8	10	10			10
09-Mar-09		-7	10	10			10
10-Mar-09		-6	10	10			10
11-Mar-09		-5	10	10			10
12-Mar-09		-4	25	25			10
13-Mar-09		-3	25	25			10
14-Mar-09		-2	0	0			0
15-Mar-09		-1	10	10			10
Date	Day	Study day	Exposure period (Weeks 4 to 6)				
			Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
16-Mar-09		1	0	0			0
17-Mar-09		2	0	0			25
18-Mar-09		3	10	10			100
19-Mar-09		4	10	10			50
20-Mar-09		5	0	10			10
21-Mar-09		6	0	10			10
22-Mar-09		7	0	0			10
23-Mar-09		8	10	0			10
24-Mar-09		9	0	0			10
25-Mar-09		10	0	0			0
26-Mar-09		11	0	0			10
27-Mar-09		12	0	0			10
28-Mar-09		13	10	10			25
29-Mar-09		14	0	0			25
30-Mar-09		15	10	10			25
31-Mar-09		16	10	10			25
01-Apr-09		17	10	10			25
02-Apr-09		18	10	10			25
03-Apr-09		19	10	10			25
04-Apr-09		20	0	10			25
05-Apr-09		21	0	0			25

Table A 13. Raw physicochemistry data for fish tank water from Experiment 1, VTG exposure study with standard effluent.

Flow in ml per minute; temperature in ^{o}C; dissolved oxygen in mg/L

Protocol Number	EXPT 1 - VTG STANDARD	Start Date:	21-May-09		
Treatment Tank Size	20L	Number of Replicate Tanks per Treatment	2	Number of Treatments	5
Types of Treatment	Control, +ve control, 25, 50 & 100% final effluent	Photo Period	16hrs light : 8 hrs dark	Total Flow Rate	40ml/min
Replacement Water Time	38hr	Temperature of Water	25^{o}C		

Pre Exposure period (Weeks 1 to 3)

	Baseline		Control		"+ve" control		25% Effluent		50% Effluent		100% Effluent	
	Actual	SD	Actual	SD	Actual	SD	Actual	SD	Actual	SD	Actual	SD
Mean Actual effluent flow rate												
Mean Actual dilution water flow rate	21.00	0.35	20.13	0.53	20.13	1.59	19.75	0.35	20.38	0.53		
Mean Positive control flow rate												
Mean PH	8.13	0.03	8.22	0.04	8.29	0.07	8.28	0.07	8.22	0.04	8.35	0.05
Mean % Effluent												
Mean Temperature	25.05	0.14	25.35	0.21	25.33	0.11	25.08	0.18	25.23	0.04	25.25	0.07
Mean Dissolved Oxygen	7.16	0.13	7.48	0.34	7.51	0.06	7.33	0.17	7.18	0.14	7.58	0.19

Exposure period (Weeks 4 to 6)

	Baseline		Control		"+ve" control		25% Effluent		50% Effluent		100% Effluent	
	Actual	SD	Actual	SD	Actual	SD	Actual	SD	Actual	SD	Actual	SD
Mean Actual effluent flow rate							5.15	0.17	10.29	0.27	19.92	0.43
Mean Actual dilution water flow rate			20.50	0.52	20.13	0.53	15.08	0.47	10.08	0.43		
Mean Positive control flow rate					0.20	0.20						
Mean PH			8.17	0.10	8.19	0.09	8.21	0.12	8.23	0.12	8.28	0.09
Mean % Effluent							25.44		50.51		100.00	
Mean Temperature			25.06	0.60	25.26	0.21	24.96	0.21	25.08	0.17	24.84	0.09
Mean Dissolved Oxygen			7.42	0.35	7.14	0.30	7.28	0.38	7.29	0.40	7.40	0.30

Table A 14. Raw physicochemistry data for fish tank water from Experiment 2, VTG exposure study with GAC effluent.

Flow in ml per minute; temperature in ^{o}C; dissolved oxygen in mg/L

Protocol Number	EXPT 2 - VTG GAC	Start Date:	9-Mar-09

Treatment Tank Size	20L	Number of Replicate Tanks per Treatment	2	Number of Treatments	3

Types of Treatment	Control, +ve control & 100% GAC effluent	Photo Period	16hrs light : 8 hrs dark	Total Flow Rate	40ml/min

Replacement Water Time	38hr	Temperature of Water	25^{o}C

Pre Exposure period (Weeks 1 to 3)

	Baseline		Control		"+ve" control		25% Effluent		50% Effluent		100% Effluent	
	Actual	SD	Actual	SD	Actual	SD	Actual	SD	Actual	SD	Actual	SD
Mean Actual effluent flow rate												
Mean Actual dilution water flow rate	41.13	1.24	41.75	1.06	41.50	0.71						
Mean Positive control flow rate												
Mean PH	8.22	0.02	8.18	0.01	8.31	0.01					8.27	0.01
Mean % Effluent												
Mean Temperature	24.95	0.21	25.13	0.04	24.98	0.04					25.43	0.04
Mean Dissolved Oxygen	7.00	0.12	6.83	0.21	7.16	0.19					7.01	0.03

Exposure period (Weeks 4 to 6)

	Day Zero		Control		"+ve" control		25% Effluent		50% Effluent		100% Effluent	
	Actual	SD	Actual	SD	Actual	SD	Actual	SD	Actual	SD	Actual	SD
Mean Actual effluent flow rate											43.67	0.75
Mean Actual dilution water flow rate			42.67	1.66	40.54	1.14						
Mean Positive control flow rate					0.40	0.41						
Mean PH			8.31	0.07	8.33	0.03					8.16	0.07
Mean % Effluent											100.00	
Mean Temperature			25.00	0.09	24.93	0.17					25.12	0.22
Mean Dissolved Oxygen			7.46	0.42	7.42	0.30					7.52	0.30

Table A 15. Raw physicochemistry data for fish tank water from Experiment 3, Pair Breeding exposure study with standard effluent.

Flow in ml per minute; temperature in ^{o}C; dissolved oxygen in mg/L

Protocol Number	EXPT 3 - PAIR STANDARD	Start Date:	30-Apr-09

Treatment Tank Size	8.5L	Number of Replicate Tanks per Treatment	8	Number of Treatments	5

Types of Treatment	Control, +ve control, 25%, 50% & 100% final effluent	Photo Period	16 hrs light : 8 hrs dark	Total Flow Rate	20ml/min

Replacement Water Time	32hr	Temperature of Water	25^{o}C

Pre Exposure period (Weeks 1 to 3)

	Control		"+ve" control		25% Effluent		50% Effluent		100% Effluent	
	Flow rate	SD	Flow rate	SD	Flow rate	SD	Flow rate	SD	Flow rate	SD
Mean Actual effluent flow rate										
Mean Actual dilution water flow rate	21.40	0.88	19.89	1.30	20.24	1.48	19.82	1.28	19.99	1.41
Mean Positive control flow rate										
Mean PH	8.30	0.06	8.21	0.08	8.23	0.05	8.36	0.05	8.39	0.06
Mean % Effluent										
Mean Temperature	25.15	0.36	25.07	0.39	24.95	0.44	25.06	0.38	25.43	0.40
Mean Dissolved Oxygen	7.71	0.18	7.65	0.19	7.71	0.15	7.71	0.14	7.65	0.15

Exposure period (Weeks 4 to 6)

	Control		"+ve" control		25% Effluent		50% Effluent		100% Effluent	
	Flow rate	SD	Flow rate	SD	Flow rate	SD	Flow rate	SD	Flow rate	SD
Mean Actual effluent flow rate					5.36	0.10	9.98	0.06	20.54	0.45
Mean Actual dilution water flow rate	20.06	1.44	19.47	1.39	15.46	1.43	10.00	0.64		
Mean Positive control flow rate			0.20	0.02						
Mean PH	8.26	0.10	8.13	0.10	8.16	0.08	8.32	0.06	8.31	0.08
Mean % Effluent					25.76		49.95		100.00	
Mean Temperature	25.20	0.23	25.43	0.14	24.87	0.22	24.62	0.19	24.86	0.19
Mean Dissolved Oxygen	7.68	0.27	7.48	0.18	7.54	0.30	7.67	0.22	7.66	0.17

Table A 16. Raw physicochemistry data for fish tank water from Experiment 4, Pair Breeding exposure study with GAC effluent.

Flow in ml per minute; temperature in ^{o}C; dissolved oxygen in mg/L

Protocol Number	EXPT 4 - PAIR GAC	Start Date:	16-Feb-09			
Treatment Tank Size	8.5L	Number of Replicate Tanks per Treatmer	8	Number of Treatments	3	
Types of Treatment	Control, +ve control & 100% final effluent	Photo Period	16 hrs light : 8 hrs dark	Total Flow Rate	20ml/min	
Replacement Water Time	32hr	Temperature of Water	25^{o}C			

Pre Exposure period (Weeks 1 to 3)

	Control		"+ve" control		25% Effluent		50% Effluent		100% Effluent	
	Flow rate	SD	Flow rate	SD	Flow rate	SD	Flow rate	SD	Flow rate	SD
Mean Actual effluent flow rate									22.71	0.78
Mean Actual dilution water flow rate	23.05	1.77	23.20	1.86						
Mean Positive control flow rate										
Mean PH	8.19	0.11	8.20	0.09					8.37	0.09
Mean % Effluent									100.00	
Mean Temperature	24.84	0.24	25.32	0.28					25.29	0.23
Mean Dissolved Oxygen	7.54	0.20	7.52	0.21					7.76	1.09

Exposure period (Weeks 4 to 6)

	Control		"+ve" control		25% Effluent		50% Effluent		100% Effluent	
	Flow rate	SD	Flow rate	SD	Flow rate	SD	Flow rate	SD	Flow rate	SD
Mean Actual effluent flow rate									21.49	0.45
Mean Actual dilution water flow rate	22.00	1.45	21.35	1.46						
Mean Positive control flow rate			0.21	0.01						
Mean PH	8.30	0.05	8.26	0.04					8.34	0.09
Mean % Effluent									100.00	
Mean Temperature	24.42	0.34	24.71	0.27					24.73	0.35
Mean Dissolved Oxygen	8.20	0.35	8.11	0.31					8.11	0.35

Table A 17. Raw male fish data from Experiment 1, VTG exposure study with standard effluent.

Treatment	Fish no.	Sex	Wet weight (g)	Total length (mm)	Gonad weight (mg)	Condition factor	GSI	Tubercles - stage	Tubercles - number	Fatpad weight (mg)	FPI	Vitellogenin (ng/ml)
Baseline	1	M	2.931	57	23	1.583	0.785	0	0	0	0	5
Baseline	2	M	1.935	54	14	1.229	0.724	2	8	0	0	38
Baseline	3	M	2.128	48	22	1.924	1.034	1	10	0	0	1,075
Baseline	4	M	1.831	51	22	1.380	1.202	3	10	0	0	512
Baseline	5	M	2.209	54	20	1.403	0.905	2	12	22	0.996	116
Baseline	6	M	2.645	58	43	1.356	1.626	2	12	0	0	103
Baseline	7	M	2.259	57	31	1.220	1.372	3	14	20	0.885	1,161
Baseline	8	M	1.636	51	13	1.233	0.795	1	10	0	0	11
Baseline	9	M	3.599	65	35	1.311	0.972	1	8	0	0	29
Baseline	10	M	1.583	51	10	1.193	0.632	0	0	0	0	13
Baseline	11	M	2.037	54	23	1.294	1.129	3	14	0	0	216
Baseline	12	M	2.116	56	18	1.205	0.851	1	11	0	0	10
Baseline	13	M	3.051	59	43	1.486	1.409	2	12	77	2.524	169
Baseline	14	M	2.519	57	13	1.360	0.516	0	0	0	0	10
Baseline	15	M	2.661	59	50	1.296	1.879	2	12	0	0	12
Baseline	16	M	1.899	54	13	1.206	0.685	0	0	0	0	8
Control	17	M	2.06	52	52	1.465	2.524	4	17	84	4.078	9
Control	18	M	2.72	51	48	2.050	1.765	4	11	83	3.051	67
Control	19	M	2.12	54	16	1.346	0.755	3	11	42	1.981	793
Control	20	M	2.57	58	36	1.317	1.401	2	9	32	1.245	41
Control	21	M	1.7	53	28	1.142	1.647	2	8	18	1.059	89
Control	22	M	2.79	59	47	1.358	1.685	4	13	38	1.362	16
Control	23	M	2.75	60	44	1.273	1.600	1	2	40	1.455	44
Control	24	M	1.88	51	49	1.417	2.606	4	12	26	1.383	32
Control	25	M	1.92	53	34	1.290	1.771	2	10	48	2.500	11
Control	26	M	2.52	56	54	1.435	2.143	2	12	112	4.444	13
Control	27	M	2.22	52	63	1.579	2.838	4	14	24	1.081	34
Control	28	M	3.06	60	54	1.417	1.765	1	12	20	0.654	5
Control	29	M	2.82	57	76	1.523	2.695	4	12	72	2.553	785
Control	30	M	2.54	55	57	1.527	2.244	4	15	64	2.520	62
Control	31	M	2.46	56	59	1.401	2.398	4	15	42	1.707	30
Control	32	M	2.28	53	57	1.531	2.500	3	12	155	6.798	49

Treatment	Fish no.	Sex	Wet weight (g)	Total length (mm)	Gonad weight (mg)	Condition factor	GSI	Tubercles - stage	Tubercles - number	Fatpad weight (mg)	FPI	Vitellogenin (ng/ml)
+ve Control	33	M	2.64	58	80	1.353	3.030	4	15	48	1.818	57,543,617
+ve Control	34	M	3.89	62	77	1.632	1.979	2	9	98	2.519	59,064,708
+ve Control	35	M	2.03	54	36	1.289	1.773	1	7	45	2.217	41,781,339
+ve Control	36	M	2.41	57	27	1.301	1.120	0	0	39	1.618	44,387,158
+ve Control	37	M	2.39	54	50	1.518	2.092	2	14	39	1.632	55,047,387
+ve Control	38	M	2.69	57	67	1.453	2.491	4	15	59	2.193	31,109,061
+ve Control	39	M	2.4	55	37	1.443	1.542	1	8	40	1.667	87,930,275
+ve Control	40	M	1.78	51	27	1.342	1.517	2	13	128	7.191	72,626,635
+ve Control	41	M	2.9	61	46	1.278	1.586	4	13	170	5.862	41,478,646
+ve Control	42	M	2.73	58	39	1.399	1.429	1	1	58	2.125	33,664,747
+ve Control	43	M	1.72	52	22	1.223	1.279	0	0	0	0	47,178,078
+ve Control	44	M	2.4	55	55	1.443	2.292	2	13	68	2.833	42,328,885
+ve Control	45	M	3.18	55	53	1.911	1.667	1	6	0	0	45,478,549
+ve Control	46	M	2.25	56	27	1.281	1.200	0	0	20	0.889	69,584,531
+ve Control	47	M	1.74	52	27	1.237	1.552	4	15	77	4.425	63,078,799
+ve Control	48	M	2.52	57	45	1.361	1.786	2	14	56	2.222	72,776,270
25% Effluent	49	M	1.82	50	38	1.456	2.088	2	10	39	2.143	462
25% Effluent	50	M	2.78	59	48	1.354	1.727	4	12	17	0.612	656
25% Effluent	51	M	3.19	60	72	1.477	2.257	4	13	50	1.567	532
25% Effluent	52	M	2.52	59	36	1.227	1.429	1	7	18	0.714	795
25% Effluent	53	M	2.99	59	73	1.456	2.441	4	15	82	2.742	93
25% Effluent	54	M	2.53	54	50	1.607	1.976	4	17	132	5.217	653
25% Effluent	55	M	1.98	54	26	1.257	1.313	1	2	10	0.505	328
25% Effluent	56	M	2.35	53	68	1.578	2.894	4	12	54	2.298	74
25% Effluent	57	M	3.24	60	70	1.500	2.160	4	13	120	3.704	9,270
25% Effluent	58	M	2.43	60	19	1.125	0.782	0	0	0	0	26
25% Effluent	59	M	2.5	58	45	1.281	1.800	4	15	30	1.200	27
25% Effluent	60	M	2.27	56	17	1.293	0.749	0	0	13	0.573	6
25% Effluent	61	M	3.23	60	69	1.495	2.136	4	12	75	2.322	270
25% Effluent	62	M	2.27	58	46	1.163	2.026	4	16	33	1.454	941
25% Effluent	63	M	2.14	56	32	1.219	1.495	1	9	0	0	28
25% Effluent	64	M	2.8	59	44	1.363	1.571	4	16	57	2.036	41

Treatment	Fish no.	Sex	Wet weight (g)	Total length (mm)	Gonad weight (mg)	Condition factor	GSI	Tubercles - stage	Tubercles - number	Fatpad weight (mg)	FPI	Vitellogenin (ng/ml)
50% Effluent	65	M	3.69	65	47	1.344	1.274	2	17	26	0.705	4,822
50% Effluent	66	M	3.37	59	62	1.641	1.840	4	15	36	1.068	6
50% Effluent	67	M	2.68	60	34	1.241	1.269	0	0	0	0	12
50% Effluent	68	M	1.97	51	46	1.485	2.335	1	5	0	0	79
50% Effluent	69	M	2.62	55	48	1.575	1.832	4	16	68	2.595	18
50% Effluent	70	M	1.84	51	31	1.387	1.685	4	13	19	1.033	597
50% Effluent	71	M	2.23	57	24	1.204	1.076	0	0	0	0	3
50% Effluent	72	M	2.3	52	62	1.636	2.696	3	11	58	2.522	8
50% Effluent	73	M	2.49	57	31	1.345	1.245	0	0	0	0	38
50% Effluent	74	M	1.99	53	48	1.337	2.412	3	11	44	2.211	152
50% Effluent	75	M	2.2	54	35	1.397	1.591	3	14	22	1	12
50% Effluent	76	M	2.12	54	20	1.346	0.943	0	0	0	0	14
50% Effluent	77	M	3.79	64	90	1.446	2.375	4	15	83	2.190	150
50% Effluent	78	M	2.47	58	33	1.266	1.336	0	0	0	0	10
50% Effluent	79	M	2.48	56	38	1.412	1.532	4	15	28	1.129	73
50% Effluent	80	M	2.69	60	46	1.245	1.710	2	13	9	0.335	28
100% Effluent	81	M	1.99	54	30	1.264	1.508	0	0	0	0	771
100% Effluent	82	M	2.5	57	48	1.350	1.920	3	17	28	1.120	8
100% Effluent	83	M	3.06	60	38	1.417	1.242	2	13	31	1.013	1,430
100% Effluent	84	M	2.3	57	29	1.242	1.261	0	0	0	0	9
100% Effluent	85	M	1.88	53	40	1.263	2.128	2	13	14	0.745	374
100% Effluent	86	M	2.08	53	36	1.397	1.731	2	12	20	0.962	35
100% Effluent	87	M	2.74	58	49	1.404	1.788	3	13	0	0	34
100% Effluent	88	M	2.53	58	40	1.297	1.581	0	0	0	0	3
100% Effluent	89	M	2.4	55	29	1.443	1.208	0	0	0	0	43
100% Effluent	90	M	2.47	57	34	1.334	1.377	3	16	25	1.012	13
100% Effluent	91	M	3.76	65	66	1.369	1.755	1	6	25	0.665	8
100% Effluent	92	M	2.4	55	57	1.443	2.375	2	14	25	1.042	56
100% Effluent	93	M	3.82	63	60	1.528	1.571	2	16	68	1.780	66
100% Effluent	94	M	3.06	62	45	1.284	1.471	1	8	27	0.882	43
100% Effluent	95	M	1.76	53	12	1.182	0.682	0	0	0	0	21
100% Effluent	96	M	2.62	59	44	1.276	1.679	1	11	0	0	75

Table A 18. Raw male fish data from Experiment 2, VTG exposure study with GAC effluent.

Treatment	Fish no.	Sex	Wet weight (g)	Total length (mm)	Gonad weight (mg)	Condition factor	GSI	Tubercles - stage	Tubercles - number	Fatpad weight (mg)	FPI	Vitellogenin (ng/ml)
Baseline	1	M	2.435	60	27	1.127	1.109	0	0	0	0	52
Baseline	2	M	3.616	64	53	1.379	1.466	2	14	100	2.765	56
Baseline	3	M	2.829	59	63	1.377	2.227	3	12	152	5.373	554
Baseline	4	M	2.846	54	46	1.807	1.616	0	0	0	0.000	68
Baseline	5	M	2.098	55	27	1.261	1.287	1	6	20	0.953	130
Baseline	6	M	4.005	66	49	1.393	1.223	1	8	22	0.549	144
Baseline	7	M	2.025	54	50	1.286	2.469	0	0	0	0	7
Baseline	8	M	3.089	64	60	1.178	1.942	0	0	0	0	18
Baseline	9	M	3.529	65	33	1.285	0.935	0	0	0	0	39
Baseline	10	M	2.735	60	36	1.266	1.316	0	0	0	0	289
Baseline	11	M	3.368	58	63	1.726	1.871	2	14	124	3.682	1,569
Baseline	12	M	2.448	57	16	1.322	0.654	0	0	0	0	12
Baseline	13	M	3.719	65	52	1.354	1.398	2	8	24	0.645	27,176
Baseline	14	M	2.618	61	25	1.153	0.955	0	0	0	0	773
Baseline	15	M	2.457	60	25	1.138	1.018	0	0	0	0	100
Baseline	16	M	1.899	54	23	1.206	1.211	0	0	0	0	202
Control	17	M	2.337	58	36	1.198	1.540	2	10	0	0	28
Control	18	M	2.384	60	15	1.104	0.629	0	0	0	0	21
Control	19	M	3.482	65	52	1.268	1.493	4	12	75	2.154	2,373
Control	20	M	4.495	69	69	1.368	1.535	2	12	0	0	114
Control	21	M	3.195	58	81	1.638	2.535	3	16	99	3.099	865
Control	22	M	2.773	61	71	1.222	2.560	0	0	0	0	12
Control	23	M	2.433	58	18	1.247	0.740	0	0	0	0	86
Control	24	M	2.493	58	41	1.278	1.645	1	10	0	0	85
Control	25	M	2.462	59	62	1.199	2.518	2	15	32	1.300	82
Control	26	M	2.179	55	34	1.310	1.560	4	16	20	0.918	226
Control	27	M	3.045	58	65	1.561	2.135	3	20	9	0.296	1,941
Control	28	M	3.792	67	77	1.261	2.031	3	16	26	0.686	2,216
Control	29	M	2.488	59	14	1.211	0.563	0	0	0	0	69
Control	30	M	3.582	63	64	1.433	1.787	4	16	70	1.954	23,963
Control	31	M	3.519	63	74	1.407	2.103	2	26	24	0.682	1,440
Control	32	M	2.165	52	38	1.540	1.755	2	11	0	0	19

Treatment	Fish no.	Sex	Wet weight (g)	Total length (mm)	Gonad weight (mg)	Condition factor	GSI	Tubercles - stage	Tubercles - number	Fatpad weight (mg)	FPI	Vitellogenin (ng/ml)
+ve Control	33	M	3.259	64	27	1.243	0.828	0	0	0	0	59,635,414
+ve Control	34	M	2.138	57	29	1.154	1.356	0	0	0	0	68,930,469
+ve Control	35	M	2.318	57	30	1.252	1.294	1	4	0	0	157,586,483
+ve Control	36	M	1.534	48	27	1.387	1.760	2	8	38	2.477	76,558,716
+ve Control	37	M	2.816	63	23	1.126	0.817	0	0	0	0	108,667,416
+ve Control	38	M	2.877	62	33	1.207	1.147	0	0	0	0	81,137,478
+ve Control	39	M	1.993	54	23	1.266	1.154	0	0	0	0	92,357,497
+ve Control	40	M	2.717	58	51	1.393	1.877	1	4	0	0	126,152,125
+ve Control	41	M	2.101	58	19	1.077	0.904	1	6	0	0	54,989,002
+ve Control	42	M	3.315	59	50	1.614	1.508	1	12	0	0	81,324,693
+ve Control	43	M	3.332	61	40	1.468	1.200	2	14	50	1.501	12,944,459
+ve Control	44	M	3.468	65	51	1.263	1.471	0	0	0	0	102,704,225
+ve Control	45	M	2.295	58	28	1.176	1.220	1	10	0	0	34,490,268
+ve Control	46	M	2.568	59	41	1.250	1.597	0	0	0	0	212,986,175
+ve Control	47	M	3.718	65	60	1.354	1.614	0	0	0	0	135,599,654
+ve Control	48	M	3.033	63	29	1.213	0.956	1	8	62	2.044	52,390,732
100% Effluent	81	M	3.388	64	80	1.292	2.361	3	16	36	1.063	4,366
100% Effluent	82	M	3.169	64	66	1.209	2.083	4	16	14	0.442	169
100% Effluent	83	M	3.881	68	62	1.234	1.598	2	18	0	0	85
100% Effluent	84	M	2.187	54	12	1.389	0.549	1	6	0	0	182
100% Effluent	85	M	4.638	67	64	1.542	1.380	2	16	0	0	136
100% Effluent	86	M	3.575	64	71	1.364	1.986	3	14	65	1.818	525
100% Effluent	87	M	2.937	61	52	1.294	1.771	3	12	29	0.987	781
100% Effluent	88	M	3.118	62	77	1.308	2.470	4	16	42	1.347	593
100% Effluent	89	M	3.82	64	87	1.457	2.277	3	20	0	0	119
100% Effluent	90	M	2.888	63	31	1.155	1.073	1	12	0	0	92
100% Effluent	91	M	4.319	69	71	1.315	1.644	2	16	0	0	82
100% Effluent	92	M	2.336	58	39	1.197	1.670	1	4	0	0	112
100% Effluent	93	M	2.553	57	44	1.379	1.723	2	10	0	0	501
100% Effluent	94	M	3.205	63	34	1.282	1.061	2	14	0	0	21
100% Effluent	95	M	2.915	61	59	1.284	2.024	0	0	0	0	273
100% Effluent	96	M	3.234	61	51	1.425	1.577	3	12	31	0.959	54

Table A 19. Raw male fish data from Experiment 3, Pair Breeding exposure study with standard effluent.

Treatment	Fish no.	Sex	Wet weight (g)	Total length (mm)	Gonad weight (mg)	Condition factor	GSI	Tubercles - stage	Tubercles - number	Fatpad weight	FPI	Vitellogenin (mg/ml)
Control	1	M	3.5	59	39	1.704	1.114	3	15	172	4.914	222
Control	2	M	4.9	63	106	1.960	2.163	4	17	366	7.469	4,198
Control	3	M	3.35	56	79	1.908	2.358	3	15	208	6.209	397
Control	4	M	3.96	58	78	2.030	1.970	3	12	554	13.990	678
Control	5	M	4.45	64	87	1.698	1.955	4	17	311	6.989	1,085
Control	6	M										
Control	7	M	3.5	63	47	1.400	1.343	4	18	366	10.457	792
Control	8	M	3.95	64	66	1.507	1.671	3	16	120	3.038	3,001
+ve Control	9	M	2.83	53	58	1.901	2.049	3	11	194	6.855	47,958,541
+ve Control	10	M	3.37	60	61	1.560	1.810	3	15	173	5.134	50,048,316
+ve Control	11	M	2.81	54	52	1.785	1.851	2	14	160	5.694	31,364,653
+ve Control	12	M	4.62	61	69	2.035	1.494	1	12	253	5.476	56,265,761
+ve Control	13	M	2.63	57	28	1.420	1.065	1	9	141	5.361	42,326,354
+ve Control	14	M	3.97	61	61	1.749	1.537	1	14	177	4.458	99,993,245
+ve Control	15	M	4.48	63	60	1.792	1.339	2	17	239	5.335	44,488,688
+ve Control	16	M										
25% Effluent	17	M	3.64	58	97	1.866	2.665	4	18	172	4.725	21,311
25% Effluent	18	M	3.63	57	57	1.960	1.570	3	13	411	11.322	541
25% Effluent	19	M	5.32	67	86	1.769	1.617	4	17	278	5.226	6,784
25% Effluent	20	M	3.68	64	50	1.404	1.359	3	15	54	1.467	3,877
25% Effluent	21	M	3.17	60	48	1.468	1.514	4	15	72	2.271	42,187
25% Effluent	22	M	4.05	65	99	1.475	2.444	3	13	71	1.753	941
25% Effluent	23	M	4.03	62	63	1.691	1.563	4	20	228	5.658	821
25% Effluent	24	M	3.89	60	83	1.801	2.134	3	9	162	4.165	4,900
50% Effluent	25	M	3.99	60	85	1.847	2.130	4	15	93	2.331	6,660
50% Effluent	26	M	3.49	60	74	1.616	2.120	4	14	133	3.811	775
50% Effluent	27	M	3.39	56	48	1.930	1.416	4	16	264	7.788	582
50% Effluent	28	M	3.14	60	65	1.454	2.070	4	15	137	4.363	5,414
50% Effluent	29	M	3.53	61	69	1.555	1.955	3	14	154	4.363	910
50% Effluent	30	M	4.06	62	86	1.704	2.118	4	16	157	3.867	10,271
50% Effluent	31	M										
50% Effluent	32	M	2.36	52	41	1.678	1.737	3	14	157	6.653	11,166

Treatment	Fish no.	Sex	Wet weight (g)	Total length (mm)	Gonad weight (mg)	Condition factor	GSI	Tubercles - stage	Tubercles - number	Fatpad weight	FPI	Vitellogenin (mg/ml)
100% Effluent	33	M	3.87	64	73	1.476	1.886	3	15	185	4.780	371
100% Effluent	34	M	2.23	50	37	1.784	1.659	3	13	114	5.112	3,871
100% Effluent	35	M	2.81	57	62	1.517	2.206	3	13	176	6.263	999
100% Effluent	36	M	3.29	55	73	1.977	2.219	3	14	181	5.502	32,709
100% Effluent	37	M	3.03	60	54	1.403	1.782	3	13	93	3.069	311
100% Effluent	38	M	3.67	58	77	1.881	2.098	4	15	131	3.569	698
100% Effluent	39	M	2.73	53	96	1.834	3.516	2	10	74	2.711	1,202
100% Effluent	40	M	4.07	65	83	1.482	2.039	2	14	111	2.727	93,073

Table A 20. Raw female fish data from Experiment 3, Pair Breeding exposure study with standard effluent.

Treatment	Fish no.	Sex	Wet weight (g)	Total length (mm)	Gonad weight (mg)	Condition factor	GSI	Vitellogenin (mg/ml)
Control	1	F	1.44	47	150	1.387	10.417	8,056,983
Control	2	F	1.05	41	201	1.523	19.143	3,203,821
Control	3	F	1.79	50	205	1.432	11.453	3,269,313
Control	4	F	2.7	57	297	1.458	11.000	7,372,679
Control	5	F	1.37	46	142	1.407	10.365	5,645,720
Control	6	F						
Control	7	F	1.89	48	310	1.709	16.402	7,863,811
Control	8	F	1.47	47	176	1.416	11.973	6,514,843
+ve Control	9	F						
+ve Control	10	F	1.86	49	235	1.581	12.634	26,881,449
+ve Control	11	F	1.42	49	196	1.207	13.803	2,980,511
+ve Control	12	F						
+ve Control	13	F	1.91	52	205	1.358	10.733	8,528,242
+ve Control	14	F	1.18	45	135	1.295	11.441	5,288,824
+ve Control	15	F	1.42	46	116	1.459	8.169	9,853,673
+ve Control	16	F	1.19	49	347	1.011	29.160	4,313,757
25% Effluent	17	F	1.66	50	147	1.328	8.855	5,884,171
25% Effluent	18	F	1.21	47	132	1.165	10.909	4,616,504
25% Effluent	19	F	1.89	53	218	1.270	11.534	4,152,276
25% Effluent	20	F	1.78	50	198	1.424	11.124	6,300,294
25% Effluent	21	F	1.58	50	181	1.264	11.456	4,519,199
25% Effluent	22	F	1.74	49	235	1.479	13.506	1,351,816
25% Effluent	23	F	1.8	49	228	1.530	12.667	6,715,822
25% Effluent	24	F	1.45	46	147	1.490	10.138	4,662,980
50% Effluent	25	F	1.38	49	147	1.173	10.652	2,760,326
50% Effluent	26	F	1.76	53	170	1.182	9.659	5,913,898
50% Effluent	27	F	1.77	52	163	1.259	9.209	7,487,300
50% Effluent	28	F	1.68	44	292	1.972	17.381	1,835,315
50% Effluent	29	F	2.14	57	282	1.156	13.178	6,911,821
50% Effluent	30	F	1.98	50	304	1.584	15.354	6,784,438
50% Effluent	31	F						
50% Effluent	32	F	1.24	45	155	1.361	12.500	5,753,703

Treatment	Fish no.	Sex	Wet weight (g)	Total length (mm)	Gonad weight (mg)	Condition factor	GSI	Vitellogenin (mg/ml)
100% Effluent	33	F	1.59	49	145	1.351	9.119	8,226,767
100% Effluent	34	F	1.45	47	205	1.397	14.138	6,615,049
100% Effluent	35	F	1.27	42	186	1.714	14.646	6,032,801
100% Effluent	36	F	1.58	47	200	1.522	12.658	5,450,322
100% Effluent	37	F	1.95	52	286	1.387	14.667	6,830,483
100% Effluent	38	F	1.66	50	106	1.328	6.386	313,309
100% Effluent	39	F	1.71	48	299	1.546	17.485	3,822,243
100% Effluent	40	F	1.61	48	225	1.456	13.975	5,230,163

Table A 21. Raw male fish data from Experiment 4, Pair Breeding exposure study with GAC effluent.

Treatment	Fish no.	Sex	Wet weight (g)	Total length (mm)	Gonad weight (mg)	Condition factor	GSI	Tubercles - stage	Tubercles - number	Fatpad weight (mg)	FPI	Vitellogenin (mg/ml)
Control	1	M	3.64	60	55	1.685	1.511	3	12	313	8.599	277
Control	2	M	5.558	70	66	1.620	1.187	3	18	192	3.454	101
Control	3	M	4.723	63	81	1.889	1.715	2	14	368	7.792	349
Control	4	M	2.541	59	31	1.237	1.220	2	20	82	3.227	116
Control	5	M	4.138	64	68	1.579	1.643	3	20	210	5.075	970
Control	6	M	3.429	63	32	1.371	0.933	3	14	186	5.424	95
Control	7	M	5.32	69	109	1.619	2.049	4	12	172	3.233	4,963
Control	8	M	3.257	61	59	1.435	1.811	3	12	241	7.399	207
+ve Control	9	M	3.091	61	48	1.362	1.553	2	14	132	4.270	194,626,684
+ve Control	10	M	2.476	58	30	1.269	1.212	1	14	99	3.998	157,051,897
+ve Control	11	M	4.194	63	88	1.677	2.098	2	12	283	6.748	101,689,057
+ve Control	12	M	1.996	51	32	1.505	1.603	2	12	25	1.253	176,257,134
+ve Control	13	M	3.165	61	33	1.394	1.043	2	16	197	6.224	100,487,199
+ve Control	14	M	2.866	59	38	1.395	1.326	2	10	113	3.943	143,697,115
+ve Control	15	M	1.899	54	21	1.206	1.106	2	10	165	8.689	81,199,884
+ve Control	16	M	3.342	64	106	1.275	3.172	1	6	36	1.077	149,399,636
100% Effluent	33	M	3.666	61	59	1.615	1.609	3	14	85	2.319	35,521
100% Effluent	34	M	4.355	64	82	1.661	1.883	2	12	125	2.870	903
100% Effluent	35	M	2.426	53	42	1.630	1.731	2	16	66	2.721	269
100% Effluent	36	M	3.015	59	59	1.468	1.957	2	16	185	6.136	336
100% Effluent	37	M	5.348	72	96	1.433	1.795	2	12	156	2.917	278
100% Effluent	38	M	3.379	62	38	1.418	1.125	3	10	220	6.511	118
100% Effluent	39	M	3.707	64	63	1.414	1.699	4	16	107	2.886	1,054
100% Effluent	40	M	2.896	60	52	1.341	1.796	2	14	135	4.662	1,770

Table A 22. Raw female fish data from Experiment 4, Pair Breeding exposure study with GAC effluent.

Treatment	Fish no.	Sex	Wet weight (g)	Total length (mm)	Gonad weight (mg)	Condition factor	GSI	VTG
Control	1	F	1.46	51	179	1.101	12.260	4,511,492
Control	2	F	1.302	42	149	1.757	11.444	5,343,656
Control	3	F	1.337	47	132	1.288	9.873	4,377,164
Control	4	F	1.191	42	193	1.608	16.205	4,271,379
Control	5	F	1.348	46	245	1.385	18.175	8,091,481
Control	6	F	1.54	50	150	1.232	9.740	4,089,753
Control	7	F	1.894	53	118	1.272	6.230	8,152,686
Control	8	F	1.361	49	140	1.157	10.287	6,563,039
+ve Control	9	F	1.995	55	123	1.199	6.165	91,552,710
+ve Control	10	F	1.479	50	40	1.183	2.705	102,240,129
+ve Control	11	F	1.737	54	118	1.103	6.793	153,576,805
+ve Control	12	F	1.367	48	139	1.236	10.168	7,476,980
+ve Control	13	F	1.44	48	191	1.302	13.264	4,476,343
+ve Control	14	F	0.989	43	107	1.244	10.819	14,510,280
+ve Control	15	F	1.296	49	42	1.102	3.241	101,042,185
+ve Control	16	F	1.095	44	113	1.285	10.320	15,198,074
100% Effluent	33	F	1.144	47	111	1.102	9.703	4,779,665
100% Effluent	34	F	1.078	43	130	1.356	12.059	3,457,706
100% Effluent	35	F	1.71	52	175	1.216	10.234	3,143,121
100% Effluent	36	F	1.837	53	276	1.234	15.024	19,897,363
100% Effluent	37	F	1.201	46	113	1.234	9.409	7,873,889
100% Effluent	38	F	2.307	54	173	1.465	7.499	8,154,752
100% Effluent	39	F	1.002	43	87	1.260	8.683	1,767,562
100% Effluent	40	F	1.546	51	218	1.165	14.101	8,105,935

Table A 23. Raw egg production data from Experiment 3, Pair Breeding exposure study with standard effluent. Data collected over a 6 week period; 3 weeks pre-exposure and 3 weeks exposure to control (dilution water), positive control (+ve control; 20 ng/L EE2), or to 100%, 50% or 25% standard effluent.

Mean egg production/day						
Date	Study day	Pre Exposure period (Weeks 1 to 3)				
		Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
07-May-09	-21	45	63	76	200	167
08-May-09	-20	49	90	6	55	112
09-May-09	-19	48	0	136	3	0
10-May-09	-18	95	127	0	0	115
11-May-09	-17	0	75	43	20	31
12-May-09	-16	83	56	60	238	0
13-May-09	-15	7	49	70	0	57
14-May-09	-14	82	30	67	3	106
15-May-09	-13	73	89	50	110	77
16-May-09	-12	52	134	26	64	24
17-May-09	-11	27	36	26	43	61
18-May-09	-10	34	75	112	38	24
19-May-09	-9	0	0	94	78	106
20-May-09	-8	126	129	0	48	72
21-May-09	-7	69	9	32	71	0
22-May-09	-6	0	71	30	16	20
23-May-09	-5	38	92	133	81	115
24-May-09	-4	20	66	85	54	59
25-May-09	-3	47	0	0	40	89
26-May-09	-2	12	41	26	0	0
27-May-09	-1	89	57	75	31	52

Cumulative egg production						
Date	Study day	Pre Exposure period (Weeks 1 to 3)				
		Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
07-May-09	-21	45	63	76	200	167
08-May-09	-20	95	153	81	255	279
09-May-09	-19	142	153	217	258	279
10-May-09	-18	238	280	217	258	394
11-May-09	-17	238	355	260	278	425
12-May-09	-16	321	410	320	515	425
13-May-09	-15	328	459	390	515	482
14-May-09	-14	409	489	457	518	588
15-May-09	-13	483	578	506	629	665
16-May-09	-12	535	711	532	693	689
17-May-09	-11	561	748	558	736	750
18-May-09	-10	596	823	669	774	774
19-May-09	-9	596	823	763	852	879
20-May-09	-8	721	952	763	900	951
21-May-09	-7	790	961	795	971	951
22-May-09	-6	790	1032	825	987	971
23-May-09	-5	828	1124	958	1068	1086
24-May-09	-4	848	1190	1042	1121	1145
25-May-09	-3	895	1190	1042	1162	1234
26-May-09	-2	907	1231	1069	1162	1234
27-May-09	-1	996	1287	1143	1192	1285

Date	Study day	Exposure period (Weeks 4 to 6)				
		Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
28-May-09	1	22	47	68	77	36
29-May-09	2	28	95	0	46	49
30-May-09	3	0	36	77	49	89
31-May-09	4	36	0	75	12	50
01-Jun-09	5	79	0	97	126	0
02-Jun-09	6	0	76	28	0	0
03-Jun-09	7	11	39	48	0	140
04-Jun-09	8	84	0	71	47	61
05-Jun-09	9	0	4	53	118	36
06-Jun-09	10	57	6	56	50	12
07-Jun-09	11	4	56	0	51	9
08-Jun-09	12	25	1	111	0	0
09-Jun-09	13	6	0	18	0	0
10-Jun-09	14	32	0	45	3	122
11-Jun-09	15	52	53	104	107	6
12-Jun-09	16	32	5	38	0	21
13-Jun-09	17	0	2	17	43	23
14-Jun-09	18	67	0	35	70	46
15-Jun-09	19	23	18	99	0	63
16-Jun-09	20	29	0	98	121	29
17-Jun-09	21	53	0	38	5	64

Date	Study day	Exposure period (Weeks 4 to 6)				
		Control	+ve Control	25% Effluent	50% Effluent	100% Effluent
28-May-09	1	22	47	68	77	36
29-May-09	2	50	142	68	123	85
30-May-09	3	50	178	146	173	174
31-May-09	4	86	178	220	185	224
01-Jun-09	5	165	178	317	311	224
02-Jun-09	6	165	254	345	311	224
03-Jun-09	7	176	293	393	311	363
04-Jun-09	8	260	293	465	358	424
05-Jun-09	9	260	297	517	476	460
06-Jun-09	10	316	302	573	526	472
07-Jun-09	11	320	358	573	577	481
08-Jun-09	12	346	359	684	577	481
09-Jun-09	13	352	359	702	577	481
10-Jun-09	14	383	359	747	580	603
11-Jun-09	15	435	412	851	687	609
12-Jun-09	16	467	417	888	687	630
13-Jun-09	17	467	420	905	730	653
14-Jun-09	18	535	420	941	800	699
15-Jun-09	19	558	438	1040	800	761
16-Jun-09	20	587		1137	921	790
17-Jun-09	21	640		1175	926	854

Table A 24. Raw egg production data from Experiment 4, Pair Breeding exposure study with GAC effluent. Data collected over a 6 week period; 3 weeks pre-exposure and 3 weeks exposure to control (dilution water), positive control (+ve control; 20 ng/L EE2) or to 100% GAC effluent.

Mean egg production/day				
Date	Study day	Pre Exposure period (Weeks 1 to 3)		
		Control	+ve Control	100% Effluent
23-Feb-09	-21	35	52	32
24-Feb-09	-20	63	61	44
25-Feb-09	-19	32	0	87
26-Feb-09	-18	0	0	16
27-Feb-09	-17	67	66	35
28-Feb-09	-16	51	0	20
01-Mar-09	-15	13	48	50
02-Mar-09	-14	47	63	20
03-Mar-09	-13	24	11	106
04-Mar-09	-12	0	11	20
05-Mar-09	-11	29	31	25
06-Mar-09	-10	18	44	0
07-Mar-09	-9	35	0	56
08-Mar-09	-8	28	39	22
09-Mar-09	-7	25	38	22
10-Mar-09	-6	48	43	42
11-Mar-09	-5	15	26	34
12-Mar-09	-4	37	24	34
13-Mar-09	-3	19	7	9
14-Mar-09	-2	27	0	18
15-Mar-09	-1	5	9	8

Cumulative egg production				
Date	Study day	Pre Exposure period (Weeks 1 to 3)		
		Control	+ve Control	100% Effluent
23-Feb-09	-21	35	52	32
24-Feb-09	-20	99	113	76
25-Feb-09	-19	130	113	163
26-Feb-09	-18	130	113	179
27-Feb-09	-17	197	179	214
28-Feb-09	-16	249	179	234
01-Mar-09	-15	262	227	283
02-Mar-09	-14	309	290	303
03-Mar-09	-13	333	301	409
04-Mar-09	-12	333	312	429
05-Mar-09	-11	362	343	454
06-Mar-09	-10	380	387	454
07-Mar-09	-9	415	387	509
08-Mar-09	-8	443	426	532
09-Mar-09	-7	468	464	554
10-Mar-09	-6	516	507	596
11-Mar-09	-5	531	533	629
12-Mar-09	-4	568	557	663
13-Mar-09	-3	587	564	672
14-Mar-09	-2	613	564	690
15-Mar-09	-1	618	573	698

Date	Study day	Exposure period (Weeks 4 to 6)		
		Control	+ve Control	100% Effluent
16-Mar-09	1	0	13	28
17-Mar-09	2	18	0	21
18-Mar-09	3	9	0	0
19-Mar-09	4	45	9	0
20-Mar-09	5	20	38	22
21-Mar-09	6	0	27	3
22-Mar-09	7	21	0	52
23-Mar-09	8	26	54	42
24-Mar-09	9	22	0	0
25-Mar-09	10	23	0	17
26-Mar-09	11	0	0	63
27-Mar-09	12	47	15	28
28-Mar-09	13	0	31	0
29-Mar-09	14	39	0	3
30-Mar-09	15	6	18	41
31-Mar-09	16	37	16	17
01-Apr-09	17	9	25	0
02-Apr-09	18	0	0	21
03-Apr-09	19	17	0	37
04-Apr-09	20	67	24	54
05-Apr-09	21	5	38	0

Date	Study day	Exposure period (Weeks 4 to 6)		
		Control	+ve Control	100% Effluent
16-Mar-09	1	0	13	28
17-Mar-09	2	18	13	49
18-Mar-09	3	27	13	49
19-Mar-09	4	71	22	49
20-Mar-09	5	91	60	71
21-Mar-09	6	91	87	74
22-Mar-09	7	112	87	125
23-Mar-09	8	138	141	167
24-Mar-09	9	160	141	167
25-Mar-09	10	184	141	184
26-Mar-09	11	184	141	247
27-Mar-09	12	231	156	275
28-Mar-09	13	231	187	275
29-Mar-09	14	269	187	278
30-Mar-09	15	275	204	319
31-Mar-09	16	312	220	335
01-Apr-09	17	321	245	335
02-Apr-09	18	321	245	357
03-Apr-09	19	338	245	394
04-Apr-09	20	404	269	448
05-Apr-09	21	410	307	448

Table A 25. Roach sampled from the River Ray in April 2005. Of the 145 fish sampled, only 140 fish were included in analyses as it was not possible to sex five fish due to missing gonads.

Fish no.	Sex	Age	Year class	Wet weight (g)	Fork length (mm)	CF	Gonad weight (g)	GSI	Ovarian cavity	No. of oocytes	Intersex Index	Microscopic sex	Histological score	E2EQ ng/ml bile	FLUTEQ mg/ml bile	VTG (ng/ml)
1	F	5	2000	168	218	1.62	11.9	7.62	y			arrested/ inhibited female	IV			600
2	F	5	2000	132.9	195	1.79	19.8	17.51	y			female	V			2,600,000
3	F	5	2000	164.6	210	1.78	37.2	29.20	y			female	V			2,575,000
4		4	2001	43.4	135	1.76	1.4	3.33	y			Missing	Missing			73
5	F	1	2004		63				y			immature female	II			67
6	F	5	2000	134.8	197	1.76	22.5	20.04	y			female	V			3,020,000
7	F	5	2000	117.9	198	1.52	16.5	16.27	y			female	V			3,410,000
8	F	4	2001	166.9	207	1.88	40.3	31.83	y			female	V			1,150
9	F	4	2001	122	196	1.62	23	23.23	y			female	V			2,300,070
10	M	3	2002	65.8	164	1.49	4	6.47	n	1,0,0,0,1,0	0.333	intersex				4,300,000
11	F	4	2001	95.4	178	1.69	16.4	20.76	y			female	V			800
12	F	8	-	347.1	286	1.48	7	2.06	y			arrested/ inhibited female	II	4.00	<300	54
13	F	5	2000	186.1	214	1.90	48.8	35.54	y			female	V			530
14	F	4	2001	111.3	184	1.79	22.5	25.34	y			female	V			750
15	F	5	2000	152	205	1.76	30.2	24.79	y			female	V			1,105,000
16	F	6	1999	321.7	249	2.08	74.7	30.24	y			female	V			no blood
17	M	4	2001	82.6	180	1.42	2.3	2.86	n	0,0,0,0,0,0	0	male	VI	60.00	N/A	81
18	F	4	2001	126.1	205	1.46	1.8	1.45	y			Arrested /inhibited female	II	21.00	<300	2,840,000
19	F	7	-	250.3	258	1.46	6.3	2.58	y			arrested/ inhibited female	I	26.00	710.00	1,125,000
20	M	5	2000	113.5	198	1.46	6.7	6.27	n	2,0,2,3,2,2	0.833	intersex		80.00	<300	4,350,000
21	M	3	2002	47.1	145	1.54	1.5	3.29	n	0,0,0,0,0,0	0	male	V	110.00	<300	328
22	F	5	2000	165.5	217	1.62	3.1	1.91	y			arrested/ inhibited female	II	27.00	1138.00	205
23	F	4	2001	111.5	190	1.63	2.3	2.11	y			arrested/ inhibited female	II	19.00	1785.00	3,750,000
24	M	5	2000	94.1	184	1.51	4.2	4.67	n	0,0,0,0,0,0	0	male	V	26.00	<300	2,500,000
25	M	4	2001	114.4	185	1.81	5.7	5.24	n	0,0,2,1,3,0	0.5	intersex		47.00	<300	591

Fish no.	Sex	Age	Year class	Wet weight (g)	Fork length (mm)	CF	Gonad weight (g)	GSI	Ovarian cavity	No. of oocytes	Intersex Index	Microscopic sex	Histological score	E2EQ ng/ml bile	FLUTEQ mg/ml bile	VTG (ng/ml)
26	M	5	2000	79	172	1.55	3.4	4.50	n	0,0,0,0,0,0	0	male	V	47.00	<300	597
27	F	4	2001	111.4	191	1.60	5.1	4.80	y			arrested/ inhibited female	II			590
28	M	5	2000	132.5	210	1.43	6.5	5.16	n	0,0,0,0,0,0	0	male	V	50.00	<300	447
29	M	3	2002	35.6	130	1.62	1.5	4.40	n	0,0,0,0,0,0	0	male	V	59.00	<300	no blood
30	M	4	2001	99.6	188	1.50	3.8	3.97	n	0,0,0,0,0,0	0	male	V	60.00	2454.00	901
31	F	2	2003	39.6	135	1.61	0.2	0.51	y			immature female	II			2,400
32	F	5	2000	201.3	220	1.89	41.5	25.97	y			female	V			980
33	F	5	2000	203.7	219	1.94	45.4	28.68	y			female	V			650,000
34	F	3	2002	70.9	160	1.73	15	26.83	y			female	V			3,355,000
35	F	5	2000	124.2	195	1.68	26.1	26.61	y			female	V			no blood
36	F	4	2001	105.3	182	1.75	19.5	22.73	y			female	V			2,250
37	F	4	2001	167.9	215	1.69	3.1	1.88	y			arrested/ inhibited female	II	20.00	2363.00	3,950,000
38	F	3	2002	73.5	165	1.64	14.9	25.43	y			female	V			610
32	F	5	2000	201.3	220	1.89	41.5	25.97	y			female	V			980
33	F	5	2000	203.7	219	1.94	45.4	28.68	y			female	V			650,000
34	F	3	2002	70.9	160	1.73	15	26.83	y			female	V	8.00	N/A	3,355,000
35	F	5	2000	124.2	195	1.68	26.1	26.61	y			female	V			no blood
36	F	4	2001	105.3	182	1.75	19.5	22.73	y			female	V			2,250
37	F	4	2001	167.9	215	1.69	3.1	1.88	y			arrested/ inhibited female	II			3,950,000
38	F	3	2002	73.5	165	1.64	14.9	25.43	y			female	V	14.00	1675.00	610
39	F			163	211	1.74	28.4	21.10	y			female	V	4.00	6987.00	5,510,000
40	F	4	2001	158.5	206	1.81	34.5	27.82	y			female	V	24.00	N/A	2,050
41	M	4	2001	86.4	182	1.43	4	4.85	n	0,0,0,0,0,0	0	male	V	47.00	<300	118
42	F	5	2000	179.2	215	1.80	38.4	27.27	y			female	V			3,200
43	F	5	2000	182.9	219	1.74	39	27.10	y			female	V	50.00	<300	590
44	F	5	2000	130.6	195	1.76	25.9	24.74	y			female	V	59.00	<300	500
45	F	5	2000	151.1	211	1.61	3.4	2.30	y			arrested/ inhibited female	II	60.00	2454.00	43
46	F	5	2000	118.9	204	1.40	4.4	3.84	y			arrested/ inhibited female	II			2,800
47	M	4	2001	88.5	176	1.62	4.6	5.48	n	0,0,0,0,0,0	0	male	V			30

Fish no.	Sex	Age	Year class	Wet weight (g)	Fork length (mm)	CF	Gonad weight (g)	GSI	Ovarian cavity	No. of oocytes	Intersex Index	Microscopic sex	Histological score	E2EQ ng/ml bile	FLUTEQ mg/ml bile	VTG (ng/ml)
48	F	4	2001	124	194	1.70	24.7	24.87	y			female	V			300
49	M	4	2001	70.5	169	1.46	3.5	5.22	n	0,0,0,0,0,0	0	male	V	73.00	<300	2,755,000
50	F	3	2002	75.2	168	1.59	12.6	20.13	y			female	V			1,500,000
51	F	6	1999	348.9	254	2.13	84.6	32.01	y			female	V			3,655,000
52	F	4	2001	146.3	198	1.88	31	26.89	y			female	V			300
53	F	5	2000	228	225	2.00	52	29.55	y			female	V			1,360,000
54	F	4	2001	170.6	215	1.72	37.6	28.27	y			female	V			2,690,000
55	M	6	1999	206	230	1.69	7.7	3.88	n	2,3,4,1,11,1	1.1667	intersex		35.00	<300	no blood
56	M	3	2002	78.3	175	1.46	3.2	4.26	n	0,1,0,2,0,0,	0.333	intersex		65.00	<300	3,590,000
57	M	5	2000	94.9	193	1.32	2.7	2.93	y	4,0,0,0,0,0	0.1667	intersex		37.00	<300	4,030,000
58	F	3	2002	89	175	1.66	18.3	25.88	y			female	V			735,000
59	F	4	2001	77.7	171	1.55	12.2	18.63	y			female	V			2,020,000
60	F	4	2001	99.1	172	1.95	18.8	23.41	y			female	V			3,760,000
61	F	3	2002	115.3	186	1.79	30.6	36.13	y			female	V			3,860,000
62	F	4	2001	112.3	194	1.54	21.2	23.27	y			female	V			3,610,000
63	M	5	2000	100.3	191	1.44	3	3.08	y	0,0,0,0,0,0	0	intersex		54.00	<300	743
64	M	4	2001	66.2	169	1.37	3.4	5.41	n	0,0,0,0,0,0	0	male	V	49.00	<300	489
65	M	4	2001	71.8	165	1.60	4.1	6.06	n	0,0,0,0,0,0	0	male	IV	143.00	<300	103
66	M	4	2001	82.7	180	1.42	1.6	1.97	n	6,8,1,8,21,9	2	intersex		64.00	<300	3,080,000
67	M	4	2001	104.5	189	1.55	4.8	4.81	n	0,0,0,0,0,0	0	male	V	61.00	<300	2,320
68	M	4	2001	128.2	198	1.65	7.7	6.39	n	0,0,0,0,0,0	0	male	IV	Not enough	N/A	1,905,000
69	F	2	2003	19.6	108	1.56	0.4	2.08	y			immature female	II	55.00	1262.00	1,640,000
70	F	5	2000	152.5	205	1.77	23.3	18.03	y			female	V			no blood
71	F	2	2003	28.7	124	1.51	0.7	2.50	y			immature female	III	13.00	1946.00	4,750,000
72	F	2	2003	28.2	117	1.76	0.6	2.17	y			immature female	II	20.00	2063.00	450
73	F	2	2003	22.2	109	1.71	1.7	8.29	y			female	V			no blood
74	F	4	2001	80	177	1.44	1.3	1.65	y			arrested/ inhibited female	II	56.00	1195.00	no blood
75	F	2	2003	41.2	134	1.71	3.5	9.28	y			immature female	IV			7,325,000
76	F	4	2001	123.4	192	1.74	27.6	28.81	y			female	V			2,500,000

Fish no.	Sex	Age	Year class	Wet weight (g)	Fork length (mm)	CF	Gonad weight (g)	GSI	Ovarian cavity	No. of oocytes	Intersex Index	Microscopic sex	Histological score	E2EQ ng/ml bile	FLUTEQ mg/ml bile	VTG (ng/ml)
77	F	4	2001	95.3	180	1.63	17.8	22.97	y			female	V			3,100,000
78	F	5	2000	107	190	1.56	17.4	19.42	y			female	V			6,700
79	F	4	2001	126	200	1.58	18.5	17.21	y			female	V			1,685,000
80	F	5	2000	167.7	210	1.81	29.5	21.35	y			female	V			no blood
81	F	5	2000	263.4	234	2.06	54.8	26.27	y			female	V			825
82	F	3	2002	61.8	157	1.60	9	17.05	y			female	V			no blood
83	M	3	2002	49.6	155	1.33	1.5	3.12	n	0,0,0,0,0,0	0	male	V	71.00	<300	366
84	M	5	2000	104	194	1.42	3.3	3.28	n	0,0,0,0,0,0	0	male	V	57.00	2351.00	465
85	M	3	2002	41	144	1.37	1.6	4.06	n	0,0,0,0,0,0	0	male	V	54.00	<300	3,625,000
86	F	2	2003	26.3	122	1.45	4	17.94	y			female	V			5,950,000
87	M	3	2002	55.4	162	1.30	2.6	4.92	n	0,0,0,0,0,0	0	male	V	49.00	<300	1,255,000
88	F	3	2002	53.7	153	1.50	9.1	20.40	y			female	V			3,575,000
89	M	6	1999	158.9	214	1.62	3.3	2.12	n	0,0,1,1,6	0.666	intersex		Not enough		18
90	M	2	2003	50.1	146	1.61	0.9	1.83	n	0,0,0,0,0,0	0	male	V	27.00	<300	5,810,000
91	M	3	2002	30.2	130	1.37	0.8	2.72	n	0,0,0,0,0,0	0	male	V			no blood
92	F	2	2003	23	115	1.51	0.9	4.07	y			immature female	II			21,000
93	F	4	2001	107.4	194	1.47	3	2.87	y			arrested/ inhibited female	II	6.00	<300	15
94	M	3	2002	42.5	147	1.34	1.7	4.17	n	0,0,0,0,0,0	0	male	V	74.00	<300	408
95	M	3	2002	16.1	106	1.35	0.6	3.87	n	0,0,0,0,0,0	0	male	V	Not enough		4,400,000
96	M	3	2002	35.4	138	1.35	1.4	4.12	n	0,0,0,0,0,0	0	male	V	49.00	<300	629
97	F	2	2003	38.5	136	1.53	0.8	2.12	y			immature female	II	109.00	5759.00	100
98	F	4	2001	92.7	184	1.49	0.6	0.65	y			spent female	VI	18.00	583.00	62
99	F	2	2003	34.3	135	1.39	1.2	3.63	y			immature female	II			3,750,000
100	M	3	2002	65.5	163	1.51	2.8	4.47	n	0,0,0,0,0,0	0	male	V			106,000
101	M	2	2003	32.2	130	1.47	1.4	4.55	n	0,0,0,0,0,0	0	male	V			591
102	M	3	2002	44.4	144	1.49	2.2	5.21	n	0,0,0,0,0,0	0	male	V			234
103	F	3	2002	69.7	172	1.37	1.5	2.20	y			arrested/ inhibited female	II	19.00	1361.00	4,225,000

Fish no.	Sex	Age	Year class	Wet weight (g)	Fork length (mm)	CF	Gonad weight (g)	GSI	Ovarian cavity	No. of oocytes	Intersex Index	Microscopic sex	Histological score	E2EQ ng/ml bile	FLUTEQ mg/ml bile	VTG (ng/ml)
104	M	3	2002	39.3	139	1.46	1.8	4.80	n		0	male	V	11.00	<300	no blood
105	F	5	2000	185.8	229	1.55	4.7	2.60	y			arrested/ inhibited female	II	21.00	4105.00	no blood
106	F	2	2003	32.4	130	1.47	0.7	2.21	y			immature female	II	40.00	848.00	119
107	M	4	2001	74.6	177	1.35	4	5.67	n	0,0,0,0,0,0	0	male	V	Not enough	N/A	141
108	M	2	2003	31.4	129	1.46	1.8	6.08	y	0,0,0,0,0,0	0	intersex		79.00	<300	no blood
109	F	2	2003	27.6	123	1.48	0.4	1.47	y			immature female	II	47.00	1116.00	no blood
110	M	2	2003	32.1	130	1.46	0.6	1.90	n	0,0,0,0,0,0	0	male	V	Missing	Missing	254
111	M	3	2002	57.4	154	1.57	3.8	7.09	n	0,0,0,0,0,0	0	male	IV	21.00	<300	16,794
112	M	3	2002	40.8	142	1.42	1.3	3.29	n	0,0,0,0,0,0	0	male	V	75.00	<300	273
113	M	4	2001	63.6	167	1.37	2.5	4.09	n	2,1,0,0,0,0	0.333	intersex		56.00	<300	71
114	M	3	2002	46	149	1.39	1.6	3.60	n	1,0,0,0,0,0	0.1667	intersex		95.00	<300	68
115	M	3	2002	42.7	144	1.43	1.3	3.14	n	0,0,0,2,0,0	0.1667	intersex		69.00	<300	107
116	M	2	2003	23.4	121	1.32	1.3	5.88	n		0	male	V	Not enough	Not enough	119
117	M	4	2001	81.8	174	1.55	1.8	2.25	n		0	male	V	43.00	<300	84
118	M	5	2000	94.2	185	1.49	4.3	4.78	n		0	male	V	Not enough		495
119	F	3	2002	51.6	154	1.41	7.9	18.08	y			female	V			2,580,000
120	M	2	2003	22.1	111	1.62	0.3	1.38	n	0,0,0,0,0,0	0	male	I	43.00	1538.00	no blood
121	F	3	2002	68	162	1.60	1.09	1.63	y			female	V			6,800,000
122	F	3	2002	102.2	178	1.81	21.8	27.11	y			female	V			5,750,000
123	F	2	2003	40	125	2.05	3.7	10.19	y			immature female	IV			no blood
124	F	3	2002	70.9	159	1.76	14.6	25.93	y			female	V			6,040,000
125	F	5	2000	181.6	220	1.71	28.1	18.31	y			female	V			7,250,000
126		2	2003	31.3	125	1.60	0.6	1.95				Missing	Missing	45.00	2226.00	no blood
127		2	2003	35.9	129	1.67	4.8	15.43	y			Missing	Missing			no blood
128	F	3	2002	73.4	164	1.66	16.8	29.68	y			female	V			1,105,000
129	F	3	2002	76	165	1.69	12.7	20.06	y			female	V			4,710,000
130	F	3	2002	79	168	1.67	15.6	24.61	y			female	V			4,065,000
131	F	2	2003	38.1	137	1.48	1	2.70	y			immature female	II	33.00	4475.00	no blood
132	F	3	2002	17.6	103	1.61	0.3	1.73	y			arrested/ inhibited female	II	40.00	2576.00	no blood
133	F	2	2003	42.4	138	1.61	1.4	3.41	y			female	V			4,990,000

Fish no.	Sex	Age	Year class	Wet weight (g)	Fork length (mm)	CF	Gonad weight (g)	GSI	Ovarian cavity	No. of oocytes	Intersex Index	Microscopic sex	Histological score	E2EQ ng/ml bile	FLUTEQ mg/ml bile	VTG (ng/ml)
134	F	3	2002	63.6	158	1.61	11.4	21.84	y			female	V			5,960,000
135	F	2	2003	31.1	128	1.48	0.7	2.30	y			immature female	II	62.00	1751.00	no blood
136	F	2	2003	23.4	119	1.39	0.5	2.18	y			immature female	II	Not enough		no blood
137	F	2	2003	33	126	1.65	6.5	24.53	y			female	V			no blood
138	F	2	2003	38.5	134	1.60	6.3	19.57	y			female	V			no blood
139	F	2	2003	38.6	134	1.60	3.9	11.24	y			immature female	IV			940,000
140	F	3	2002	74.2	164	1.68	13.1	21.44	y			female	V			5,075,000
141	F	5	2000	147.4	207	1.66	4.5	3.15	y			arrested/ inhibited female	II	Not enough		4,320,000
142	F	4	2001	132.8	192	1.88	14	11.78	y			female	V			3,175,000
143	F	5	2000	183.6	211	1.95	42.1	29.75	y			female	V			1,040,000
144		6	1999	191.3	218	1.85	39	25.61	y			Missing	Missing			9,025,000
145		3	2002	94.3	172	1.85	12.3	15.00	y			Missing	Missing			3,575,000

Table A 26. Roach sampled from the River Ray in November 2013.

Fish no.	Sex	Age	Year class	Wet weight (g)	Fork length (mm)	CF	Gonad weight (g)	GSI	Ovarian cavity	Number of oocytes	Intersex index	Microscopic sex	Histological scoring	E2EQ ng/ml bile	FLUTEQ mg/ml bile	VTG ng/ml
1	M	4	2010	67.6	168	1.43	3.18	4.93	n	0,0,0,0,0,0	0	male	II	66.14	<DL	23
2	F	6	2008	169.8	212	1.78	22.00	14.89	y			female	V	287.55	<DL	9,086,221
3	M	3	2011	47.4	148	1.46	2.30	5.10	n	0,0,0,0,0,0	0	male	II	57.64	<DL	208
4	F	5	2009	156.5	212	1.64	15.79	11.22	y			female	V	779.38	0.46	2,057,824
5	F	4	2010	64.5	165	1.44	4.26	7.08	y			female	V	378.62	<DL	2,128,342
6	F	5	2009	113.2	192	1.60	12.99	12.96	y			female	V	217.21	<DL	16,369,566
7	F	5	2009	169.7	208	1.89	22.58	15.35	y			female	V	200.17	1.00	7,827,507
8	M	4	2010	53.6	158	1.36	1.91	3.69	n	0,0,0,0,0,0	0	male	II	109.87	0.859	385
9	F	3	2011	51.6	153	1.44	4.32	9.15	y			female	V	188.66	<DL	6,780,351
10	F	5	2009	188.4	220	1.77	21.34	12.78	y			female	V	536.01	<DL	5,645,739
11	M	3	2011	23.7	118	1.44	1.12	4.96	n	0,0,0,0,0,0	0	male	II	61.54	<DL	12
12	F	3	2011	18.9	113	1.31	0.36	1.94	y			female	II	Not enough	Not enough	12
13	F	4	2010	222.9	228	1.88	28.04	14.39	y			female	V	735.61	<DL	8,894,592
14	F	4	2010	109.4	193	1.52	10.95	11.12	y			female	V	519.60	<DL	7,395,979
15	F	5	2009	207.3	233	1.64	21.76	11.73	y			female	V	795.01	<DL	6,042,602
16	M	5	2009	130.7	203	1.56	7.03	5.68	n	0,0,0,0,0,0	0	male	II	177.74	<DL	45,131
17	F	5	2009	212.3	227	1.81	25.29	13.53	y			female	V	776.08	<DL	12,315,044
18	F	5	2009	190.3	222	1.74	26.31	16.05	y			female	V	945.55	<DL	11,686,033
19	F	4	2010	224	232	1.79	28.63	14.66	y			female	V	546.75	<DL	7,271,278
20	F	3	2011	105	193	1.46	9.09	9.48	y			female	V	134.39	<DL	3,411,269
21	F	4	2010	187.1	223	1.69	20.56	12.35	y			female	V	386.99	<DL	7,794,625
22	F	4	2010	188.3	220	1.77	21.13	12.64	y			female	V	197.23	<DL	6,998,899
23	F	5	2009	207.2	227	1.77	27.02	14.99	y			female	V	1975.80	<DL	7,781,379
24	F	3	2011	111.8	187	1.71	14.15	14.48	y			female	V	1460.16	<DL	4,925,665
25	M	4	2010	101	188	1.52	5.10	5.32	n	0,0,0,0,0,0	0	male	II	113.66	<DL	198
26	M	4	2010	91.5	178	1.62	4.81	5.55	n	0,0,0,0,0,0	0	male	II	96.73	<DL	12,801
27	M	4	2010	72.1	172	1.42	2.93	4.24	n	0,0,0,0,0,0	0	male	II	Not enough	<DL	12
28	F	4	2010	103.5	193	1.44	10.44	11.22	y			female	V	632.73	<DL	7,700,488

Fish no.	Sex	Age	Year class	Wet weight (g)	Fork length (mm)	CF	Gonad weight (g)	GSI	Ovarian cavity	Number of oocytes	Intersex index	Microscopic sex	Histological scoring	E2EQ ng/ml bile	FLUTEQ mg/ml bile	VTG ng/ml
29	M	4	2010	130.1	203	1.56	7.73	6.32	n	0,0,0,0,0,0	0	male	II	141.27	<DL	48
30	M	5	2009	106.2	183	1.73	6.96	7.02	n	0,0,0,0,0,0	0	male	II	94.44	<DL	76
31	F	6	2008	231.7	237	1.74	41.10	21.56	y			female	V	1108.24	0.87	6,252,489
32	F	4	2010	87	178	1.54	8.73	11.15	y			female	V	1048.53	<DL	2,709,333
33	M	4	2010	112.4	192	1.59	5.42	5.07	n	0,0,0,0,0,0	0	male	II	190.26	<DL	31
34	M	4	2010	89.8	182	1.49	4.38	5.12	n	0,0,0,0,0,0	0	male	II	Not enough	<DL	12
35	F	5	2009	184.8	223	1.67	20.30	12.34	y			female	V	690.31	<DL	8,888,113
36	M	6	2008	178.3	217	1.74	8.84	5.22	n	0,0,0,0,0,0	0	male	II	163.86	<DL	451
37	F	5	2009	144	216	1.43	13.98	10.75	y			female	V	534.08	0.36	4,589,736
38	F		2010	218.1	236	1.66	22.24	11.36	y			female	V	1636.40	0.39	6,818,972
39	F	5	2009	92	182	1.53	10.86	13.39	y			female	V	605.79	<DL	6,636,719
40	M	5	2009	183.8	218	1.77	9.30	5.33	n	0,0,0,0,0,0	0	male	II	93.55	0.35	168
41	F	3	2011	96.5	180	1.65	9.25	10.60	y			female	V	372.69	<DL	10,630,607
42	F	6	2008	155.7	206	1.78	16.04	11.48	y			female	V	527.21	<DL	13,889,598
43	M	4	2010	115.7	193	1.61	6.28	5.74	n	2,0,0,0,0,0	0.167	intersex		77.05	0.421	3,092
44	F	4	2010	150.5	213	1.56	19.69	15.05	y			female	V	1213.07	<DL	15,877,763
45	F	5	2009	207.9	227	1.78	28.63	15.97	y			female	V	1218.42	0.45	8,668,414
46	F	5	2009	186.9	225	1.64	28.44	17.94	y			female	V	1014.61	<DL	5,200,926
47	M	4	2010	85.2	182	1.41	4.13	5.10	n	0,1,0,0,0,0	0.167	intersex		129.07	<DL	64
48	F	4	2010	127.8	198	1.65	11.19	9.59	y			female	V	621.73	<DL	16,548,915
49	F	4	2010	83	174	1.58	9.69	13.22	y			female	V	550.07	<DL	7,103,383
50	M	3	2011	64.2	166	1.40	3.44	5.66	n	0,0,0,0,0,0	0	male	II	220.98	<DL	323
51	M	4	2010	110.8	192	1.57	6.11	5.84	n	0,0,0,0,0,0	0	male	II	79.60	<DL	12
52	M	3	2011	48.1	156	1.27	2.04	4.42	n	2,1,0,0,2,0	0.500	intersex		102.95	<DL	12
53	F	3	2011	126.8	203	1.52	13.90	12.31	y			female	V	497.17	<DL	9,373,810
54	M	5	2009	100.3	188	1.51	4.80	5.03	n	0,0,0,0,0,0	0	male	II	140.63	<DL	635
55	F	3	2011	78.1	176	1.43	9.69	14.17	y			female	V	1507.28	0.67	8,474,891
56	F	3	2011	66.1	166	1.45	6.02	10.02	y			female	V	680.59	0.45	6,486,493

Table A 27. Standard curves were produced using SoftMax Pro version 5.0.1 (Molecular Devices Limited, Wokingham, UK) and were used to calculate EC50s and IC50s. The response of the standard yeast androgen screen (YAS) to dihydrotestosterone (DHT) positive control gave a mean EC50 of $9.49x10^{-10}$M ± $4.87x10^{-11}$M (n=13) and the response of the standard yeast anti-androgen screen (AYAS) to flutamide positive control gave a mean IC50 of $3.78x10^{-6}$M ±$3.13x10^{-7}$M (n=16).

Standard YAS	Standard AYAS
DHT EC50 values (M)	FLUT IC50 values (M)
8.43E-10	3.75E-06
1.11E-09	4.53E-06
1.10E-09	6.46E-06
1.23E-09	4.92E-06
1.10E-09	3.64E-06
9.30E-10	3.87E-06
9.37E-10	3.92E-06
6.22E-10	3.42E-06
6.52E-10	3.42E-06
9.02E-10	2.59E-06
1.01E-09	1.97E-06
8.96E-10	3.02E-06
1.01E-09	4.65E-06
	2.02E-06
	5.67E-06
	2.60E-06
Mean 9.49E-10	3.78E-06
SE 4.87E-11	3.13E-07
No. of experiments 13	16

Table A 28. The response of the standard yeast androgen screen (YAS) to dihydrotestosterone (DHT) positive control (final concentrations from $5x10^{-8}$M to $2.44x10^{-11}$M; mean ± SE; n=13) and the response of the standard yeast anti-androgen screen (AYAS) to flutamide positive control (final concentrations of 5×10^{-5}M to 2.44×10^{-8}M; mean ± SE; n=16). Absorbance readings were taken using a plate reader at 540nm and corrected for yeast turbidity at 620nm (Spectramax 340PC microplate reader (Molecular Devices Limited, Wokingham, UK)).

DHT conc (M)	Standard YAS (n=13)		Flutamide conc (M)	Standard AYAS (n=16)	
	Mean corrected absorbance (540nm)	DHT SEM		Mean corrected absorbance (540nm)	Flutamide SEM
5.00E-08	2.5852	0.0371	5.00E-05	1.1972	0.0528
2.50E-08	2.5675	0.0400	2.50E-05	1.1749	0.0512
1.25E-08	2.5465	0.0404	1.25E-05	1.2254	0.0595
6.25E-09	2.5325	0.0403	6.25E-06	1.4092	0.0656
3.13E-09	2.5163	0.0417	3.13E-06	1.6633	0.0657
1.56E-09	2.2627	0.0653	1.56E-06	1.8314	0.0631
7.81E-10	1.6827	0.0640	7.81E-07	1.9465	0.0607
3.91E-10	1.2761	0.0424	3.91E-07	2.0049	0.0565
1.95E-10	1.1605	0.0330	1.95E-07	2.0375	0.0590
9.77E-11	1.1433	0.0342	9.77E-08	2.0424	0.0561
4.88E-11	1.1440	0.0347	4.88E-08	2.0198	0.0560
2.44E-11	1.1116	0.0438	2.44E-08	1.8630	0.0744

Table A 29. Standard curves were produced using SoftMax Pro version 5.0.1 (Molecular Devices Limited, Wokingham, UK) and were used to calculate EC50s and IC50s. The response of the modified yeast androgen screen (YAS) to dihydrotestosterone (DHT) positive control gave an EC50 of $8.89x10^{-10}$M ± $4.51x10^{-11}$M (mean ± SE; n=6) and the of the modified standard yeast anti-androgen screen (AYAS) to flutamide positive control gave an IC50 of $1.03x10^{-5}$M ±$8.59x10^{-7}$M (mean ± SE; n=11). Included in the table are the results from the standard assays for comparison.

DHT EC50 values (M)		FLUT IC50 values (M)	
Standard YAS	Modified YAS	Standard AYAS	Modified AYAS
8.43E-10	9.08E-10	3.75E-06	9.45E-06
1.11E-09	7.57E-10	4.53E-06	9.54E-06
1.10E-09	8.55E-10	6.46E-06	9.49E-06
1.23E-09	7.97E-10	4.92E-06	7.18E-06
1.10E-09	9.56E-10	3.64E-06	1.04E-05
9.30E-10	1.06E-09	3.87E-06	8.81E-06
9.37E-10		3.92E-06	1.11E-05
6.22E-10		3.42E-06	8.81E-06
6.52E-10		3.42E-06	1.09E-05
9.02E-10		2.59E-06	9.00E-06
1.01E-09		1.97E-06	1.82E-05
8.96E-10		3.02E-06	
1.01E-09		4.65E-06	
		2.02E-06	
		5.67E-06	
		2.60E-06	
Mean	9.49E-10	8.89E-10	3.78E-06	1.03E-05
SE	4.87E-11	4.51E-11	3.13E-07	8.59E-07
No. of experiment	13	6	16	11

Table A 30. The response of the modified yeast androgen screen (YAS) to dihydrotestosterone (DHT) positive control (final concentrations from $5x10^{-8}$M to $2.44x10^{-11}$M; mean ± SE; n=6) and the response of the modified yeast anti-androgen screen (AYAS) to flutamide positive (final concentrations of 5×10^{-5}M to 2.44×10^{-8}M; mean ± SE; n=11). Absorbance readings were taken using a plate reader at 420nm and corrected for yeast turbidity at 620nm (Spectramax 340PC microplate reader (Molecular Devices Limited, Wokingham, UK)).

DHT conc (M)	Modified YAS (n=6)		Modified AYAS (n=11)	
	Mean corrected absorbance (420nm)	DHT SEM	Mean corrected absorbance (420nm)	Flut SEM
5.00E-08	2.4509	0.0372	1.1769	0.0339
2.50E-08	2.3859	0.0387	1.2300	0.0276
1.25E-08	2.4471	0.0245	1.4935	0.0311
6.25E-09	2.3669	0.0372	1.7736	0.0279
3.13E-09	2.2095	0.0546	1.9270	0.0218
1.56E-09	1.9983	0.0538	2.0297	0.0248
7.81E-10	1.5195	0.0524	2.0444	0.0274
3.91E-10	0.9795	0.0292	2.0335	0.0265
1.95E-10	0.7946	0.0259	2.0521	0.0268
9.77E-11	0.7559	0.0270	2.0858	0.0242
4.88E-11	0.7300	0.0152	2.0558	0.0151
2.44E-11	0.7355	0.0268	2.0205	0.0353

Table A 31. Potency values for the 20 anti-androgenic chemicals in the standard yeast anti-androgen screen (AYAS; mean ± SE). Potencies were calculated by dividing the flutamide IC50 by the chemical IC50, and the higher the number the more potent the chemical relative to flutamide.

Chemical number	Chemical name	Chemical CAS number	Potency relative to flutamide in the standard AYAS							Mean potency	Potency SEM	Number of times tested
8	Benzophenone	119-61-9	0.013	0.021	0.007	0.032	0.025			0.020	0.004	5
10	N-Butylbenzene sulfonamide	3622-84-2	0.419	0.078	0.020					0.172	0.124	3
11	Myristic acid	544-63-8	0.155	0.179	0.019	0.055	0.040	0.130	0.021	0.086	0.025	7
15	Dodecanoic acid	143-07-7	0.203	0.183	0.034	0.065	0.009	0.072	0.001	0.081	0.031	7
16	Fluoranthene	206-44-0	9.370	8.098	1.739	1.245				5.113	2.109	4
17	Triclosan	3380-34-5	13.192	12.313	11.163	2.790				9.865	2.394	4
18	Triphenyl phosphate	115-86-6	0.858	1.193	0.015	0.000				0.517	0.302	4
19	Pyrene	129-00-0	2.847	4.230	2.726					3.268	0.482	3
20	Methyl triclosan	4640-01-1	8.675	6.274	7.669	11.332				8.488	1.068	4
41	Chlorophene	204-385-8	14.330	19.765	8.305	8.246				12.662	2.765	4
51	1-[4-(hydroxy-1-methylethyl)phenyl] ethanone	54549-72-3	0.012	0.019						0.016	0.004	2
56	2-(methylthio)benzothiazole	615-22-5	0.013	0.073	0.102					0.063	0.026	3
78	9,12-Octadecadienoic acid methyl ester	2566-97-4	0.063	0.022	0.011					0.032	0.016	3
79	9H-Fluorene	86-73-7	0.065	0.426	0.641	0.447	0.845			0.485	0.129	5
81	Acetylcedrene	32388-55-9	0.239	0.190	0.230	0.046	0.052			0.151	0.043	5
82	alpha cedrol	77-53-2	0.038	0.021						0.030	0.009	2
84	Benzeneacetaldehyde	122-78-1	0.030	0.037						0.034	0.004	2
88	Dehydroabietic acid	1740-19-8	2.255	0.060						1.158	1.098	2
91	Diphenylacetic acid methyl ester	3469-00-9	0.250	0.160	0.072					0.161	0.051	3
92	Hexamethylbenzene	87-85-4	0.003	0.032						0.018	0.015	2

Table A 32. Potency values for the 20 anti-androgenic chemicals retested in the modified yeast anti-androgen screen (AYAS; mean ± SE). Potencies were calculated by dividing the flutamide IC50 by the chemical IC50, and the higher the number the more potent the chemical relative to flutamide.

Chemical number	Chemical name	Chemical CAS number	Potency relative to flutamide in the modified AYAS			Mean potency	Potency SEM	Number of times tested
8	Benzophenone	119-61-9	0.099	0.108		0.104	0.0045	2
10	N-Butylbenzene sulfonamide	3622-84-2	0.144	0.090		0.117	0.0270	2
11	Myristic acid	544-63-8	0.000	0.000		0.000	0.0000	2
15	Dodecanoic acid	143-07-7	0.044	0.160		0.102	0.0580	2
16	Fluoranthene	206-44-0	4.921	3.923	11.459	6.768	2.3633	3
17	Triclosan	3380-34-5	18.685	9.808	18.715	15.736	2.9640	3
18	Triphenyl phosphate	115-86-6	0.000	0.000		0.000	0.0000	2
19	Pyrene	129-00-0	0.782	2.257		1.520	0.7375	2
20	Methyl triclosan	4640-01-1	8.601	9.117		8.859	0.2580	2
41	Chlorophene	204-385-8	13.752	6.580		10.166	3.5860	2
51	1-[4-(hydroxy-1-methylethyl)phenyl] ethanone	54549-72-3	0.090	0.051		0.071	0.0195	2
56	2-(methylthio)benzothiazole	615-22-5	0.133	0.124		0.129	0.0045	2
78	9,12-Octadecadienoic acid methyl ester	2566-97-4	0.090	0.054		0.072	0.0180	2
79	9H-Fluorene	86-73-7	0.706	0.243		0.475	0.2315	2
81	Acetylcedrene	32388-55-9	0.358	0.230		0.294	0.0640	2
82	alpha cedrol	77-53-2	0.171	0.116		0.144	0.0275	2
84	Benzeneacetaldehyde	122-78-1	0.028	0.24		0.134	0.1060	2
88	Dehydroabietic acid	1740-19-8	1.865	2.522		2.194	0.3285	2
91	Diphenylacetic acid methyl ester	3469-00-9	0.281	0.192		0.237	0.0445	2
92	Hexamethylbenzene	87-85-4	0.010	0.017		0.014	0.0035	2

Table A 33. qPCR expression analysis using positive control targets (ff1d and vtg) with different tissues (dorsal fin, brain, kidney, liver, mouth, snout, brain) from one male and one female FHM. Expression levels were normalised to three reference genes (*rpl8, hprt I and tbp*).

Target	Sample	Expression	Expression SEM	Mean Cq	Cq SEM
ff1d	Female dorsal fin	0.00947	0.00504	37.15	0.76641
ff1d	Female brain	0.90796	0.05273	29.88	0.05489
ff1d	Female kidney	0.01409	0.00035	34.89	0.00000
ff1d	Female liver	0.20210	0.01928	32.58	0.13460
ff1d	Female mouth	0.01800	0.00220	35.21	0.17511
ff1d	Female snout	0.01971	0.00581	35.26	0.42422
ff1d	Male brain	1.30033	0.03331	28.15	0.01333
ff1d	Male dorsal fin	0.03194	0.00075	35.01	0.00000
ff1d	Male fatpad	0.01875	0.00832	36.05	0.63986
ff1d	Male kidney	0.00239	0.00170	37.22	1.02900
ff1d	Male liver	0.01973	0.00093	37.77	0.04498
ff1d	Male mouth	0.71620	0.06663	30.73	0.12794
ff1d	Male snout	0.00742	0.00105	37.43	0.20263
vtg	Female dorsal fin	0.00000	0.00000	36.50	1.13013
vtg	Female brain	0.00000	0.00000	32.65	0.29402
vtg	Female kidney	0.00162	0.00005	22.97	0.04379
vtg	Female liver	4.34537	0.11164	13.11	0.02331
vtg	Female mouth	0.00000	0.00000	35.27	0.51828
vtg	Female snout	0.00000	0.00000	36.23	0.44308
vtg	Male brain	0.00000	0.00000	33.06	1.37545
vtg	Male dorsal fin	0.00005	0.00000	29.30	0.08782
vtg	Male fatpad	0.00000	0.00000	34.17	0.51282
vtg	Male kidney	0.00001	0.00000	30.44	0.11085
vtg	Male liver	0.00002	0.00000	32.78	0.21776
vtg	Male mouth	0.00000	0.00000	35.70	0.74822
vtg	Male snout	0.00000	0.00000	36.37	0.93835

Table A 34. qPCR expression analysis using three spiggin-like targets (DT131813, DT267220 and DT347638) with different tissues (dorsal fin, brain, kidney, liver, mouth, snout, brain) from one male and one female FHM. Expression levels were normalised to three reference genes (*rpl8, hprt I and tbp*).

Target	Sample	Expression	Expression SEM	Cq SEM
DT131813	female brain	0.11108	0.01319	0.16460
DT131813	female dorsal fin	0.09943	0.00539	0.07126
DT131813	female kidney	1.49912	0.06373	0.05296
DT131813	female liver	0.03991	0.00372	0.13210
DT131813	female mouth	0.02644	0.00413	0.22439
DT131813	female snout	0.79400	0.02230	0.01184
DT131813	male brain	0.16800	0.01527	0.12135
DT131813	male fatpad	0.04481	0.00356	0.11105
DT131813	male dorsal fin	0.06200	0.00892	0.20562
DT131813	male kidney	0.14003	0.00650	0.06089
DT131813	male liver	0.12308	0.01365	0.15147
DT131813	male mouth	0.04982	0.00220	0.05168
DT131813	male snout	1.25998	0.04477	0.03212
DT267220	female brain	0.23023	0.07544	0.47034
DT267220	female dorsal fin	0.05831	0.01238	0.30457
DT267220	female kidney	0.01677	0.00122	0.09991
DT267220	female liver	0.04195	0.01440	0.49474
DT267220	female mouth	0.02144	0.01075	0.72346
DT267220	female snout	0.03104	0.00304	0.13603
DT267220	male brain	0.18739	0.03310	0.24992
DT267220	male fatpad	0.05608	0.01733	0.44492
DT267220	Male dorsal fin	0.08240	0.02095	0.36578
DT267220	male kidney	0.04275	0.00875	0.29390
DT267220	male liver	0.02185	0.00219	0.13488
DT267220	male mouth	0.04005	0.01280	0.45948
DT267220	male snout	3.23179	0.17763	0.06850
DT347638	female brain	0.70485	0.05320	0.09807
DT347638	female dorsal fin	0.12321	0.01782	0.20609
DT347638	female kidney	0.06280	0.00367	0.07831
DT347638	female liver	0.24103	0.02390	0.14081
DT347638	female mouth	0.21583	0.03224	0.21457
DT347638	female snout	0.19951	0.03238	0.23089
DT347638	male brain	1.11835	0.08526	0.09814
DT347638	male fatpad	1.04255	0.06223	0.08111
DT347638	male dorsal fin	0.13092	0.00836	0.08754
DT347638	male kidney	0.23033	0.02121	0.12990
DT347638	male liver	0.27145	0.05817	0.30482
DT347638	male mouth	0.48105	0.11001	0.32781
DT347638	male snout	3.23179	0.45866	0.20081

Table A 35. qPCR expression analysis using spiggin-like targets (DT131813, DT267220 and DT347638) with snout tissues from ten male and seven female FHM. Expression levels were normalised to three reference genes (*rpl8, hprt I and tbp*).

Target	Sample	Expression	Expression SEM	Mean Cq	Cq SEM
DT131813	Snout F1	0.49725	0.02351	22.62	0.05679
DT131813	Snout F2	0.62864	0.01607	22.07	0.02630
DT131813	Snout F6	1.49682	0.08089	20.84	0.07161
DT131813	Snout F7	0.54625	0.00690	22.06	0.01391
DT131813	Snout F8	0.45893	0.01368	22.28	0.03891
DT131813	Snout F9	0.48240	0.01130	22.40	0.02216
DT131813	Snout F10	0.70964	0.01088	22.08	0.01883
DT131813	Snout M1	0.13387	0.00309	24.03	0.00725
DT131813	Snout M2	0.25582	0.00713	24.11	0.02842
DT131813	Snout M3	0.18036	0.00592	24.02	0.03443
DT131813	Snout M4	0.08363	0.00387	24.85	0.04401
DT131813	Snout M5	0.05122	0.00183	26.28	0.04127
DT131813	Snout M6	0.10515	0.00303	24.63	0.03324
DT131813	Snout M7	0.14343	0.01501	24.46	0.14618
DT131813	Snout M8	0.18779	0.00474	23.82	0.01216
DT131813	Snout M9	0.02951	0.00116	26.62	0.05315
DT131813	Snout M10	0.19706	0.00477	23.74	0.02300
DT267220	Snout F1	0.00382	0.00090	33.78	0.33740
DT267220	Snout F2	0.00419	0.00171	33.43	0.58737
DT267220	Snout F6	0.00310	0.00100	33.89	0.46558
DT267220	Snout F7	0.00317	0.00066	33.62	0.30036
DT267220	Snout F8	0.08431	0.00422	28.86	0.06976
DT267220	Snout F9	0.05654	0.00471	29.62	0.11743
DT267220	Snout F10	0.00614	0.00007	33.07	0.01040
DT267220	Snout M1	0.02792	0.00101	30.43	0.04074
DT267220	Snout M2	0.01982	0.00403	31.93	0.29223
DT267220	Snout M3	0.00214	0.00035	34.55	0.23024
DT267220	Snout M4	0.06123	0.00719	29.43	0.16176
DT267220	Snout M5	0.00574	0.00261	33.57	0.65650
DT267220	Snout M6	0.03224	0.00174	30.47	0.07364
DT267220	Snout M7	0.30499	0.01553	27.50	0.06313
DT267220	Snout M8	0.07161	0.00499	29.34	0.09451
DT267220	Snout M9	1.62473	0.07396	24.97	0.06276
DT267220	Snout M10	0.00393	0.00017	33.52	0.05482

Target	Sample	Expression	Expression SEM	Mean Cq	Cq SEM
DT347638	Snout F1	0.03283	0.00599	31.51	0.26036
DT347638	Snout F2	0.03757	0.00271	31.10	0.10099
DT347638	Snout F6	0.01916	0.00350	32.09	0.26147
DT347638	Snout F7	0.01498	0.00319	32.21	0.30715
DT347638	Snout F8	0.13411	0.01111	29.02	0.11808
DT347638	Snout F9	0.07096	0.01190	30.13	0.24061
DT347638	Snout F10	0.03059	0.00280	31.59	0.13176
DT347638	Snout M1	0.02561	0.00186	31.38	0.09980
DT347638	Snout M2	0.01587	0.00298	33.08	0.26984
DT347638	Snout M3	0.01003	0.00053	33.16	0.06879
DT347638	Snout M4	0.05912	0.00370	30.32	0.07491
DT347638	Snout M5	0.01866	0.00258	32.70	0.19726
DT347638	Snout M6	0.03939	0.00155	31.01	0.05088
DT347638	Snout M7	0.33068	0.01333	28.22	0.04438
DT347638	Snout M8	0.09596	0.00372	29.75	0.04418
DT347638	Snout M9	1.62473	0.02854	25.80	0.01638
DT347638	Snout M10	0.00920	0.00122	33.12	0.18983