DEVELOPMENT OF NOVEL TRANSGENIC ZEBRAFISH MODELS AND THEIR APPLICATION TO STUDIES ON ENVIRONMENTAL OESTROGENS

Submitted by Jon Marc Green

to the University of Exeter as a thesis for the degree of

Doctor of Philosophy in Biological Sciences,

September 2016

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

(Signature) ……(Jon Marc Green)

Abstract

Oestrogenic chemicals have become increasingly associated with health effects in wildlife populations and humans. Transgenic animal models have been developed to understand the mechanisms by which these oestrogenic chemicals alter hormonal signalling pathways and how these alterations can lead to chronic health effects. The use of highly informative transgenic animal models will also result in better use and potential reduction of intact animals used in animal testing in line with the principles of the 3Rs. In this thesis work, two novel oestrogen responsive transgenic zebrafish models have been generated to investigate the effects of oestrogenic chemicals, identify their tissue targets and better understand the temporal dynamics of these responses. Both models express the pigment-free 'Casper' (a mutant line lacking skin pigment) phenotype, which facilitate identification of responding target tissues in the whole fish in all fish life stages (embryos to adults).

The oestrogen response element green fluorescent (ERE-GFP)-Casper model was generated by crossing an established ERE-GFP line with the skin pigment free Casper line. The model generated is highly sensitive to oestrogenic chemicals, detecting responses to environmentally relevant concentrations of EE2, bisphenol A (BPA), genistein and nonylphenol. Use of the ERE-GFP-Casper model shows chemical type and concentration dependence for green fluorescent protein (GFP) induction and both spatial and temporal responses for different environmental oestrogens tested. A semi-automated (ArrayScan) imaging and image analysis system was also developed to quantify whole body fluorescence responses for a range of different oestrogenic chemicals in the new transgenic zebrafish model. The zebrafish model developed provides a sensitive and highly integrative system for identifying oestrogenic chemicals,

their target tissues and effect concentrations for exposures in real time and across different life stages. It thus has application for chemical screening to better direct health effects analysis of environmental oestrogens and for investigating the functional roles of oestrogens in vertebrates.

The second model generated was an ERE-Kaede-Casper line developed via crossing of the ERE-GFP-Casper line and a UAS-Kaede line and screening subsequent generations for a desired genotype and homozygous expression of the transgenes. Kaede is a photoconvertible fluorescent protein that initially fluoresces green in colour and can be permanently converted to red fluorescence upon short exposure to UV light. The model has a silenced skin pigmentation and high sensitivity to oestrogenic chemicals comparable with the previously developed ERE-GFP-Casper model. Use of this model has identified windows of tissue-specific sensitivity to ethinyloestradiol (EE2) for exposure during early-life (0-48hpf) and illustrated that exposure to oestrogen (EE2) during early life (0-48hpf) can enhance responsiveness (sensitivity) to different environmental oestrogens (EE2, genistein and bisphenol A) for subsequent exposures during development. These findings illustrate the importance of oestrogen exposure history in effects assessments and they have wider implications for the possible adverse effects associated with oestrogen exposure.

Information

This PhD thesis includes information and results that have been synthesised for three research papers, two of these papers published and the third paper is in preparation for submission for publication at the time of submitting this thesis.

"Transgenic Fish Systems and their Application in Ecotoxicology".

Lee, O; Green, JM; Tyler, CR, *Critical Reviews in Toxicology,* 2015

This is a critical review of techniques and methods used to generate transgenic zebrafish and medaka models for ecotoxicology assessments and includes a comparative analysis of the models themselves. This paper is included in the appendix in its original format.

"High-Content and Semi-Automated Quantification of Responses to Estrogenic Chemicals Using a Novel Translucent Transgenic Zebrafish"

Green, JM; Metz, J; Trznadel, M; Lee, O; Takesono, A; Kudoh, T; Owen, S; Tyler, CR, *Environmental Science and Technology*, 2016

This article documents the generation of the translucent ERE-GFP-Casper model and its application in a novel *in vivo* screening system for semi-automated identification of oestrogenic chemicals, their target tissues and relative potencies. This paper is included in its original format in Chapter 4 of the thesis.

“Early life exposure to ethinylestradiol enhances subsequent responses to environmental oestrogens measured in a novel transgenic zebrafish”

Green, JM; Lange, A; Scott, A; Wai, HA; Takesono, A; Brown, AR; Owen, SF; Kudoh, T; Tyler, CR; *in preparation for submission to Environmental Health Perspectives*, 2016

This paper describes the generation of the translucent ERE-Kaede-Casper model that expresses a photoconvertible Kaede fluorescent protein. The model has been used to identify the dynamics of fluorescence response to oestrogenic chemical exposure and assess for sensitivity to oestrogens for sequential (repeated) exposures during early life. This paper is included in its original format in Chapter 5 of the thesis.

Acknowledgements

I would like to thank my main supervisor, prof. Charles Tyler, who has been hugely supportive during my PhD whilst also instilling a large amount of belief in me to work independently. Our brainstorming meetings were genuinely one of the highlights of my PhD. I want to say a big thank you to my secondary supervisors as well: Dr Stewart Owen, Dr Tetsu Kudoh and Dr Ross Brown. They have been a huge resource of support and expertise and were always available to answer any questions I may have had. A special mention to Dr Ross Brown for helping me settle into the AstraZeneca Brixham Environmental Laboratory during my placement there, it was very much appreciated. I want to thank Dr Aya Takesono for not only teaching me a number of key skills necessary for the work, but for always making me laugh in the lab. I have been fortunate enough to have worked with a number of fantastic researchers during the project: Dr Okhyun Lee, John Moreman, Dr Jeremy Metz, Dr Maciej Trznadel, Dr Matt Winter, Dr Anke Lange and Aaron Scott. These people have made vital contributions towards this thesis and for that I am very thankful. Special mention to Dr Greg Paull who has overseen the maintenance of the zebrafish lines, has been a great friend and was always available to be nutmegged at Tuesday football.

Finally I would like to thank my friends for all their encouragement. Emma, for being so patient with my weekends of work and helping me see the light at the end of the tunnel. Most of all, I want to thank my parents for always believing in me. I hope I have done them proud.

Table of contents

Title Page and Declaration……1

Abstract and Paper Information……2

Acknowledgements……6

Contents……7

List of General Abbreviations……10

CHAPTER 1: GENERAL INTRODUCTION

1.1 THE ENDOCRINE SYSTEM……13

1.2 OESTROGEN SIGNALLING……14

1.3 EDCs AND OESTROGENIC CHEMICALS……16

1.3.1 Early Life Exposure……18

1.3.2 EE2……19

1.3.3 BPA……20

1.3.4 Nonylphenol……21

1.3.5 Genistein……22

1.3.6 Testing Chemicals for Oestrogenic Activity……23

1.4 TRANGENIC BIOSENSORS……25

1.5 TRANSGENIC MODEL GENERATION……30

1.6 SEEING THE RESPONSE – REPORTER GENES……35

1.7 TRANSGENIC FISH AND ECOTOXICOLOGY……39

1.8 ERE-GFP MODELS……43

1.9 SUMMARY……46

1.10 AIMS OF THE THESIS……48

CHAPTER 2: GENERAL METHODS

2.1 GENERAL APPROACH……...53

2.2. ANIMAL HANDLING……..…..55

2.3 EMBRYO WATER……....55

2.4 CHEMICAL PREPARATION……56

2.5 ANAESTHESIA……..56

2.6 IMAGING……...……...…..57

2.7 SCREENING……..57

CHAPTER 3: GENERATION OF TRANSGENIC ZEBRAFISH MODELS

3.1 INTRODUCTION……60

3.2 METHODS……..62

3.2.1 Generation of ERE-GFP-Casper…….62

3.2.2 Generation of ERE-Kaede-Casper……..67

3.3 RESULTS……72

3.3.1 ERE-GFP-Casper……..72

3.3.2 ERE-Kaede-Casper……...78

3.4 DISCUSSION……81

CHAPTER 4: PAPER 1 - HIGH-CONTENT AND SEMI-AUTOMATED SCREENING SYSTEM (ERE-GFP-CASPER)

4.1 ABSTRACT……87

4.2 INTRODUCTION……...88

4.3 METHODS……..91

4.4 RESULTS……96

4.5 DISCUSSION……...……100

4.6 REFERENCES……..……108

4.7 LIST OF FIGURES……..116

4.8 SUPPORTING INFORMATION……..……124

CHAPTER 5: PAPER 2 - EARLY LIFE EXPOSURE TO ETHINYLESTRADIOL ENHANCES SUBSEQUENT RESPONSES TO ENVIRONMENTAL OESTROGENS MEASURED IN A NOVEL TRANSGENIC ZEBRAFISH

5.1 ABSTRACT……..132

5.2 INTRODUCTION……133

5.3 METHODS……137

5.4 RESULTS……145

5.5 DISCUSSION……...150

5.6 REFERENCES……159

5.7 LIST OF FIGURES……..168

5.8 SUPPORTING INFORMATION……177

CHAPTER 6: THESIS DISCUSSION……188

CHAPTER 7: APPENDIX

7.1 REVIEW PAPER: Transgenic Fish Systems and their Application in Ecotoxicology……..202

CHAPTER 8: REFERENCES……..276

List of General Abbreviations

BPA	Bisphenol A
CRISPR	Clustered regularly interspaced short palindromic repeats
DES	Diethylstilbestrol
DNA	Deoxyribonucleic acid
dpf	Days post fertilisation
E2	17β-Oestradiol
EE2	17α-Ethinyloestradiol
EDCs	Endocrine disrupting chemicals
ERs	Oestrogen receptors
ERα	Oestrogen receptor α
ERβ	Oestrogen receptor β
EREs	Oestrogen response elements
GFP	Green fluorescent protein
hpf	Hours post fertilisation
L	Litre
mg	Milligrams
mM	Millimolar
mRNA	Messenger ribonucleic acid
ng	Nanograms
nM	Nanomolar
NP	Nonlyphenol
OECD	European Union and Organisation for Economic Cooperation and Development
PEC	Predicted environmental concentration
PNEC	Predicted no-effect concentration
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RFP	Red fluorescent protein
UAS	Upstream activation sequence

US-EPA	US Environmental Protection Agency
VTG	Vitellogenin
WHO	World Health Organisation
WWTP	Waste water treatment plants
YES	Yeast oestrogen screen
µg	Microgram
µM	Micromolar

Chapter 1

General Introduction

Chapter 1

General Introduction

1.1 Endocrine System

The endocrine system is an information signalling system that regulates and controls reproduction, growth, development, behaviour and metabolism. The major components of the endocrine system are reproductive tissues, the pancreas, hypothalamus, gastrointestinal tract and the pineal, pituitary, thyroid, and adrenal glands. Endocrine cells secrete hormones, usually at very low concentrations, that are then transported throughout the body via the circulatory system; eliciting responses in target cells, through nuclear and membrane bound receptor systems. The endocrine system conducts information and elicits responses at a slower rate compared with the nervous system, but the effects are often much longer lasting. In vertebrates there are two main classes of hormones: non-steroidal and steroids, the latter for which oestrogens are one example.[1] Steroid hormones are lipid based formed from cholesterol and they travel in the circulation around the body bound via attachment to carrier proteins, such as steroid-binding proteins. They then bind specifically to target receptor sites on the cell membrane or internal to the cell in the cytoplasm or to nuclear protein receptors and induce physiological changes through the release or inhibition of biochemicals, or regulation of genes. Nuclear hormone receptors are ligand-activated regulators effecting transcription of genes by interacting with specific DNA sequences upstream of their target genes.[2]

Oestrogens are primarily seen as female sex steroids due to their roles in the development and regulation of the female reproductive system and in secondary sex characteristics. However, the expression of oestrogen is not exclusive to females, nor is the role of oestrogen exclusive to the regulation of the female reproductive system.[3] Oestrogens are fundamental in the growth and development of both the female and male gonads and are now known to play roles in male fertility and testicular function.[4-6] Oestrogen has been shown to regulate growth and differentiation during development. In zebrafish (*Danio rerio)* in particular, endogenous oestrogen signalling plays a number of important roles in early stage development, including in the regulation of metabolism, transcription, tissue development and protein folding and trafficking.[7] Oestrogens have important sites of action in the central nervous system (in the pituitary, hypothalamus, and other specific brain regions) and is subsequently linked to changes in behaviour.[3] Oestrogens also exert crucial maintenance actions in bone,[8] liver,[9] immune system[10] and cardiovascular system.[11]

1.2 Oestrogen signalling

Oestrogen signalling occurs via oestrogen binding to oestrogen receptors (ERs) in the nucleus, which then dimerise and bind to oestrogen response elements (EREs) located in promoters of target genes.[12] Upon binding of the oestrogenic chemical ligand, a conformational change within the ER binding domain allows recruitment of coactivator proteins.[13] There are two receptor sub types in mammals, esr1 and esr2,[14] and three in zebrafish, esr1, esr2a and esr2b.[15-17] esr1 and esr2 subtypes in humans and zebrafish are corresponding orthologs with high amino acid sequence similarity for their respective ligand binding domains of 64% and 71% respectively.[18]

The expression of these subtypes in organs and tissues can change during development up to adolescence and the affinity of ligands for these subtypes varies for different oestrogenic chemicals. These differences influence the physiological targets and subsequent downstream effects.[19, 20] As an example, in zebrafish, expression levels of esr1, esr2a and esr2b differ and vary significantly during the first 120 hours of development.[19] Expression of esr1 and esr2b increases during the first 120 hours. At this time esr1 expression is comparatively higher. Esr2a expression remains relatively low during this period. Zebrafish ER sub-types also show tissue-specific expression; liver, brain and heart express all three ERs at relatively high levels compared with other somatic tissues at sexual maturity.[19] However, there remains insufficient information on this in comparison to human tissue-specific subtype expression, which is well characterised.[21, 22]

There is evidence that ERs can also regulate gene expression via different mechanisms. A third of genes regulated by ERs in humans do not contain ERE-like sequences,[23] and these appear to be modulated by other transcription factors.[24] Oestrogen-related receptors (ERRs) are an example of a small group of orphan nuclear receptors that appear to share some target genes with ERs.[25-28] Membrane ERs (mERs) also occur, and although features such as weight, mobility and affinity for oestradiol appear to be similar to nuclear ERs, their roles and mechanism(s) of action are still unclear.[29-31] Nevertheless, oestrogens such as 17β-oestradiol (E2) have been shown induce a rapid rise in the intracellular free Ca^{2+}concentration through membrane oestrogen receptors, thus affecting calcium-dependent cell signalling.[32-34]

Certain oestrogens have been shown to bind ERs with different affinities and the expression levels of these receptors within different tissues may be linked to

their tissue-specific effects.[35, 36] However, this is likely to be an oversimplification for explaining differences in tissue responses to oestrogens. An important aspect of the oestrogen-signalling pathway is the variable interactions of ERs with EREs and importantly the recruitment of co-regulators at the DNA-binding site. ESR1 and ESR2 differ in their abilities to recruit co-activators and different xeno-oestrogen ligands can induce distinct conformations in the receptor co-activator-binding pockets, leading to differential co-regulator recruitment.[37-39] The response-elements themselves have also been shown to influence receptor conformation and thus the subsequent recruitment of co-regulators.[39] In summary, mechanisms behind ligand-specific effects may include receptor affinity, receptor expression levels, co-regulator expression (and recruitment) and promoter site availability via chromatin remodelling; all varying across different tissues and their stages of development during growth.

1.3 EDCs and Oestrogenic Chemicals

An increasing ecological and human health concern is the prevalence of endocrine disrupting chemicals (EDCs). These chemicals interfere with hormone signalling and have been linked to a number of chronic health issues in humans, and with effects in various wildlife populations.[40, 41] Organisms endocrine systems may be affected by EDCs mimicking or antagonizing endogenous hormones or by disrupting their synthesis and metabolism.[42-44]

One group of EDCs that have gained a lot of attention is oestrogenic chemicals, due to their widespread presence in both the environment and evidence for effects in both humans and wildlife. Exposure to xeno-oestrogens includes both natural (phyto- and mycoestrogens) and synthetic oestrogens and they include

various pesticides, pharmaceuticals and industrial chemicals.[45] The main pathways of oestrogens into the environment arise through wastewater effluent discharges from municipal treatment plants, hospital effluent and livestock activities.[46] To date, over 1000 chemicals have been highlighted as being potentially endocrine active and over 200 of those have been identified as oestrogenic.[47] Key examples, and those used in this thesis work are 17α-ethinylestradiol (EE2) found in the contraceptive pill, the plasticizer bisphenol A (BPA), the surfactant breakdown product 4-nonylphenol (mixture of linear and branched forms) and the phytoestrogen genistein.[48-51]

Oestrogenic chemicals such as these are of particular interest due to their apparent potency and widespread accumulation in freshwater and marine environments. Oestrogenic chemicals have strong associations with adverse reproductive health effects in individual fish,[52, 53] for example feminization of males[52] and alteration of sexual behaviour,[54] as well as recorded population effects such as altered sexual maturation, increased incidence of intersex and overall population decline.[55-57] These adverse effects in fish populations are compounded by the multiple number of routes of chemical uptake that can occur in fish, including via skin and gills, via the diet, or via maternal transfer of EDCs that have accumulated in lipid reserves during ovarian development.[58]

Fish have been a major focus for research into the effects of oestrogenic chemicals, but other wildlife species have also been shown to be affected by exposure, including links to eggshell thinning, teratogenesis and feminisation in in wild birds,[59-62] demasculinisation of panthers (*Felis concolor coryi*) and alligators (*Alligator mississippiensis*) in Florida[63, 64] and significant declines in amphibian populations.[65] In humans too there are increasing links with exposure to environmental oestrogens and decreased semen quality/sperm

count and increased incidences of breast cancer and testicular germ cell cancer, urogenital tract malformation, heart disease and diabetes[66-69]. The effect of oestrogenic chemicals on the reproductive system and offspring development first gained major attention after the use of diethylstilbestrol (DES), a synthetic oestrogen, prescribed to many women to prevent miscarriages in the 1950s and 1960s. Adverse effects of this drug included defects in the uterus and ovaries and immune suppression in the offspring. Boys showed genital tract abnormalities and un-descended testes and girls had an unusual form of vaginal cancer.[70] A large number of studies have since focused on the effect of EDCs on the development of both humans and wildlife at early-life stages.[71, 72]

1.3.1 Early Life Exposure

Due to the important role endogenous oestrogen signalling plays in early life stage development, an increasing number of studies have investigated adverse effects linked to oestrogenic chemical exposure at this life stage. In mice, BPA exposure caused disruptions in puberty onset, regularity of oestrous cyclicity,[73] and development of polycystic ovaries.[74] Affects on sex differentiation and resultant sex ratios have been observed in zebrafish after early-life exposure to EE2.[75] As mentioned above, BPA has been consistently linked to increased risks of breast cancer and more recent findings from rodent testing have suggested that exposure in the womb may lead to altered mammary gland development and a predisposition to breast cancer.[76] A relatively recent area of study has been the role of oestrogens in brain development. Oestrogen sensitive genes such as cyp19a1b, which encodes for a brain form of aromatase (an oestrogen synthesising enzyme), have been identified in radial glial cells (RGC) that act as neuronal progenitors in both developing and adult fish.[77] This may be linked to studies that have shown that phytoestrogens, such

as genistein exposure during specific life stages, can affect zebrafish brain development.[78] These results indicate that early life exposure to oestrogenic chemicals may have significant effects on organism development and increased susceptibility to xeno-oestrogen related diseases but there requires further investigation of exposure effects during this period of life. In addition, little is known about the effect of early life exposure on organism sensitivity to repeated oestrogenic chemical exposures in later life.

In the next sections, information is included on the sources, distribution, estimated environmental concentrations, potency and ER subtype affinity of the four oestrogenic chemicals used extensively in the work reported in this thesis, which are 17α-ethinylestradiol (EE2), bisphenol A (BPA), 4-nonylphenol mix (NP), and genistein. These chemicals were chosen for use because of their well-documented oestrogenic potencies, prevalence in the environment and the fact that they are on prioritised chemical lists for international EDC screening programmes.

1.3.2 EE2

Ethinylestradiol (EE2) is a derivative from the natural oestrogen estradiol (E2) and is a component found in the majority of female contraceptive pill formulations. Globally, the female contraceptive pill is one of the most commonly used medications. Excretion of EE2 into water systems, mainly via human urine, has lead to pressure on water companies to remove EE2 from effluent in wastewater treatment plants (WWTP) before its release into the environment.[79] Concentrations of EE2 found in surface water can vary significantly depending on the site of sampling, but a review of almost twenty

different sources showed typical ranges fall within 0.2-25 ng/L range.[80] In WWTP effluent, typical measured concentrations often fall within 0.5 to 10 ng/L range but can extend higher depending on seasons or treatment techniques.[80-82] Therefore concentrations in surface waters and WWTP effluent can be much higher than the predictive non-effect concentration (PNEC) of 0.002 ng/L.[80, 83] Using *in vivo* testing, EE2 has shown to be around 30-40 times more potent than E2 whereas *in vitro* assessments had suggested only a 1.25-1.6 fold increase in potency.[48, 54, 84] Other *in vivo* studies have suggested that EE2's binding affinity for ER is much higher in living organisms and its rate of metabolism much lower than E2, perhaps explaining the 30-fold difference of potency in comparison to *in vitro* tests.[85, 86] EE2 has been found to have a high affinity for all major ER subtypes in both humans and zebrafish.[87, 88]

1.3.3. BPA

Bisphenol A (BPA) is a chemical of high-importance worldwide due to its application in the production of polycarbonate plastic, epoxy resins (used to line metal food and drink cans), and polyester resins. These materials are produced in vast quantities and widely distributed throughout the world. BPA, and more recently other bisphenolic chemicals, have become of environmental concern because they exhibit oestrogenic characteristics. In comparison to EE2, BPA is considered a weak environmental oestrogen. Nevertheless, a number of adverse effects have been associated with BPA exposure and this has resulted in the chemical being prioritised on national and international EDC screening lists. This is evidence of the importance of utilising both predictive potency data and data from *in vivo* adverse effects studies for prioritising oestrogenic chemicals for screening purposes. Notable health issues linked with BPA exposure include breast cancer, heart disease, diabetes and abnormal liver

enzymes.[69, 89] Due to their presence in a wide range of food and drinks containers, human exposure to BPA is primarily through ingestion. BPA is rapidly metabolised into water soluble compounds within the body.[90] These compounds are typically oestrogen inactive, however, recent research suggests that a particular metabolite, named MBP (4-methyl-2,4-bis(*p*-hydroxyphenyl)pent-1-ene) displays much higher oestrogenic potency compared with the parent compound BPA.[91] Studies using zebrafish models have identified that BPA has a particularly high specificity for inducing responses in heart valves in developing larvae.[49, 92] This targeted response is likely due to BPA showing a higher binding affinity for ESR1 which is believed to be highly expressed in heart valves in zebrafish larvae.[87, 92]

1.3.4 Nonylphenol

Nonylphenol (NP) as a polyethoxylate is an alkylphenol used mainly in the production of cleaning products, such as detergents (surfactants), but also in many other products such as pesticides. Surfactants have been used for over 40 years in the manufacture of plastics, elastomers, agricultural chemicals, pulping and industrial detergent formulations. NP in the environment is often found in matrices such as sewage sludge, effluents from sewage treatment works, river water and sediments, soil and groundwater. The impacts of nonylphenol in the environment include feminisation of aquatic organisms and decreased male fertility, effects that are induced in fish at concentrations as low as 8.2 µg/L.[93] NP has now been banned for use in a number of countries and the concentrations in the environment has shown as associated decrease. However it is still found at concentrations of 4.1 µg/l in river waters and 1 mg/kg in sediments.[93] As mentioned previously, potency measurements of EDC can widely vary between *in vitro* and *in vivo* tests and results from assessments of

NP potency are no exception. This is complicated by the 20 or so different isomers of NP. The linear form of NP (4n-NP) is often used as a reference for concentration and potency assessment. NP is mainly used in industry as a mixture of the isomers, most of which are branched and thus oestrogenic potency of these products can vary widely.[94] In transgenic zebrafish, short-term exposures at concentrations as low as 1 µg/L have induced reporter gene responses through the oestrogen signalling pathway.[49] According to the World Health Organisation (WHO), the estimated predicted environmental concentration (PEC) for surface water (0.6 µg/l) exceeds the aquatic PNEC of 0.33 µg/l (http://www.who.int/ipcs/methods/Nonylphenol.pdf).

1.3.5 Genistein

Genistein is a phytoestrogen found in soy products and coffee and is an oestrogenic chemical of concern. Despite this relatively few studies have focused on possible adverse effects of genistein and other phytoestrogens. Furthermore studies on the oestrogenic potency of phytoestrogens have shown varying results in different species.[95] Genistein has gained recent attention as a potential prophylaxis and therapeutic agent for cancer, obesity and diabetes as well as other chronic diseases.[96-98] The difficulties surrounding the analysis of effects linked to genistein seemingly arise from its ability to disrupt a number of different signalling pathways in both ER and ER-independent manners.[78] Hence, the risks or benefits of genistein exposure are currently unclear. It nevertheless remains an environmental oestrogenic chemical of concern due to its high affinity binding to oestrogen receptors (ERs). Furthermore, isoflavone compounds, including genistein, are discharged in water effluents and are present in agricultural runoff as a result from intensive livestock management.

Concentrations of genistein in effluents are generally in the range 7-22 ng/L and in the range of 3-7 ng/L in surface waters.[99, 100]

1.3.6 Testing Chemicals for Oestrogenic Activity

Concern over environmental oestrogens has continued to gather momentum with increasing concern about potential adverse effects from exposure to them and a desire to better understand their target tissues and the mechanisms by which these effects may take place. This concern has contributed to the establishment of national and international screening and testing programmes for EDCs. These regulatory frameworks include the European Union and Organisation for Economic Cooperation and Development's (OECD) Endocrine Disrupters Testing and Assessment (EDTA)[101] programme, the US Environmental Protection Agency's (US-EPA) Endocrine Disrupter Screening Program (EDSP)[102] and the Strategic Programmes on Endocrine Disruptors (SPEED) at the Japanese Ministry of the Environment (MOE).[103] These programs include data derived from a series of *in vitro* and *in vivo* assays that collectively attempt to make robust assessments on the chemical hazard. *In vitro* assays typically focus on a specific mechanism of oestrogen signalling demonstrated in human, rat or fish cell lines, for example oestrogen receptor binding and/or activation by a chemical ligand. *In vivo* assays are mainly focused on measuring consequent effects on reproductive development, function or output. Oestrogen's role in the development of reproductive organs in particular is well established and unsurprisingly the majority of *in vivo* oestrogenic chemical assessments have focused on potential adverse effects in these tissues. For example, based on the strong proliferative effect that oestrogens have in the rodent female genital tract, uterotrophic tests have

become commonly used by researchers for oestrogenic chemical evaluation[104, 105] and validated by international screening programmes.[106]

In vitro assays can be categorised into receptor binding assays, cell culture assays and reporter gene assays.[107] To assess the ER subtype specificity of chemicals, cell lines used for *in vitro* assays can be generated to specifically express certain oestrogen receptor subtypes. Screens have been run with fish ER receptor subtypes using both *in vitro* and *in vivo* assays to validate selectivity and further to this more recent studies have then compared oestrogenic chemical selectivity for zebrafish and human receptor subtypes. Typically, these studies show that overall potency rankings of oestrogenic chemicals are very similar across the different vertebrate phyla but that affinities and activation of the different ER subtypes can differ.[35, 36, 108, 109] These results show therefore that risk of endocrine disruption cannot necessarily be predicted for all wildlife based on simply examining receptor activation from a few selected test species.

Examples of the most adopted *in vitro* techniques for oestrogen screening are the E-Screen assay,[110] YES assay[111] and more recently the ERLUX screen.[112] These screens mainly focus on reporter gene activation via ER subtype binding, but when compared employing the same ER they can report different sensitivities to oestrogenic chemicals.[113, 114] As reported above, potency values of oestrogenic chemicals (such as EE2) using these *in vitro* techniques can also vary significantly in comparison to results from *in vivo* studies.[48, 54, 84] The variation between *in vitro* and *in vivo* results demonstrate the limitations of using *in vitro* techniques to predict potency in living organisms, as they do not account for differences in uptake, bioaccumulation or metabolic properties of oestrogenic chemicals such as EE2 that may play a crucial role during *in vivo*

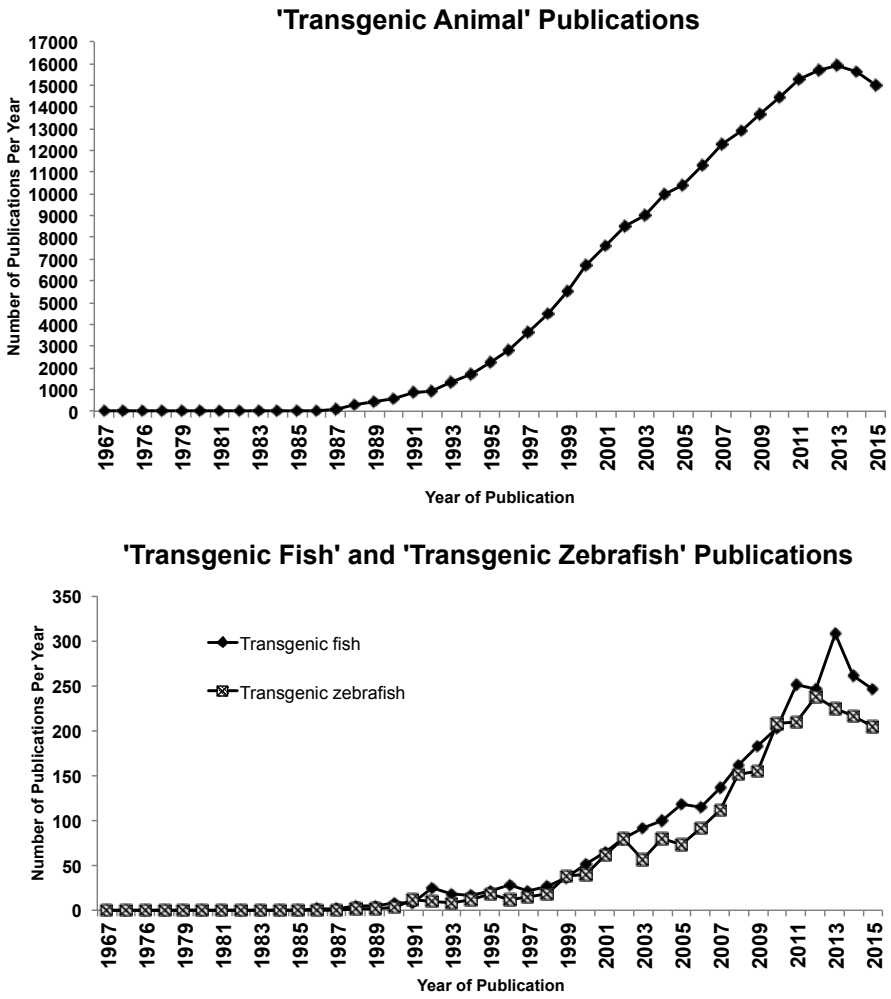
exposures. *In vitro* systems are also much less able at detect pro-oestrogenic compounds due to the fact that most cells used in these systems are limited in their capacities for chemical metabolism.[115] However, there is a current lack of *in vivo* screening tools for EDCs that can rival the high-throughput speed, cost effectiveness and ethical advantages of *in vitro* screens to improve adverse effect prediction.

Screening protocols typically consist of single chemical exposures and yet exposures in the natural environment for EDCs, including for oestrogens, occur predominantly as mixtures and studies both *in vitro*[116-118] with reporter gene assays and *in vivo* in fish[48] and mammals[119, 120] have illustrated the capacity for additive (and more than additive) effects. Furthermore, it has been shown that other EDCs, such as anti-androgens, can modulate the responses for oestrogenic chemicals when as mixture.[121] Studies on chemical mixtures have also suggested increased tissue-specific adverse effects may occur, for example as seen for responses to EDC mixtures in mammary gland development in male and female rats.[119, 120] Effects analysis for exposures representative of real world scenarios is thus extremely difficult.

1.4 **Transgenic Biosensors**

Whole-animal systems are essential tools for biological research, including their application within toxicological/safety screening studies. The development of transgenesis techniques has provided the opportunity to custom tailor whole-animal systems to answer specific biological questions, and has resulted in a vast array of highly informative transgenic animal models. Transgenic animals carry deliberately inserted foreign DNA derived from an exogenous source to express or accentuate a specific trait or feature. Transgenic animals have been

created since the early 1980s and range from mammals, birds, amphibians, fish to invertebrates.[122-131] Transgenic technology has been used in biological and medical research to find cures for cancers, diabetes, Huntington's disease and cardiovascular diseases and has contributed significantly to agriculture including for improved milk production, and both increased growth rate and disease resistance in farmed animals. [122, 123, 127, 132-136] Transgenic research applied to mammals was initiated in the 1980s and for fish somewhat after this in the late 1990s. Subsequently, there has been a rapid increase in the use of transgenic animals in research, illustrated by the number of academic papers published, increasing from around 280 in 1988 to almost 16000 in 2014 (using the search term transgenic animal- PubMed, Figure 1) and in fish from 8 in 1990 to around 300 in 2012 (using the search term fish + transgenic- PubMed, Figure 1). Interestingly the number of academic papers published per year using these terms has decreased slightly between 2013 and 2015 (the most recent available data). It may be that the rate of generation of novel transgenic animals has slowed or may perhaps be linked to laboratories recently moving towards and becoming accustomed to next generation transgenesis techniques such as CRISPR.



(Lee et. al. 2015, see appendix)

Figure 1. Number of publications between the years 1967 and 2015 that include 'transgenic animals' (1A) or transgenic (TG) fish/transgenic zebrafish (1B) as analyzed using PubMed. A search for 'transgenic (TG) zebrafish in ecotoxicology' returned only 12 publications in the last 15 years.

Transgenic fish have been developed as model systems for understanding genetic mechanisms, developmental processes and for pharmaceutical discovery, safety assessment and bio-synthesis.[137-139] Recently, transgenic fish have also been developed for studies in ecotoxicology to screen and test for chemical effects and have the potential to provision more advanced systems for integrative health impact assessments of chemicals.[49, 50, 77, 140-142] Zebrafish and medaka (*Oryzias latipes*) have become the most popular model species for transgenic manipulations. Favourable features of these species for transgenic work include their ease of breeding in the laboratory, relatively low associated maintenance costs, high fecundity, transparency of the embryos and rapid organogenesis (attractive features for studying developmental processes) and the availability of sequenced genomes providing extensive genetic resources. Additionally, for the zebrafish, the chorion of the egg is relatively soft facilitating microinjection of DNA and genetic constructs. The availability of mutant lines of these fish, such as casper (roy + nacre mutant zebrafish) that lack skin pigmentation also enable studies for observing effects in body tissues, for example tracing individual tumor (or cancer) cells as they spread through the body.[143, 144] The zebrafish has high genome (approximately 70%), structural and physiological similarities with humans.[145, 146] The zebrafish has been used to advance understanding on formation of the embryonic axis, cell lineages, and formation of the central and peripheral nervous systems. It has also proven valuable for studies on human diseases, notably carcinogenesis, wound healing, immunological diseases, behavioural abnormalities, infection and Parkinson's disease.[144, 145, 147-152]

The zebrafish and medaka have also become popular model organisms in ecotoxicological studies and especially for studies on molecular mechanisms of

chemical toxicity and analysis of behavioural outcomes.[153-155] Transgenic zebrafish and medaka employing fluorescent markers are used not only to identify chemically induced target gene activation but also to quantify relative uptake and concentration of chemicals into those tissues.

1.5 Transgenic Model Generation

Creating a transgenic fish first requires production of the gene of interest and then its introduction into the organism. The general method for generating the gene construct is shown in Figure 2.

Production of the gene construct

Several methods are now used to produce transgenic fish, but all involve a transgenic construct with a promoter and a gene. The foreign gene for transfer (transgene) is constructed using recombinant DNA techniques. Traditionally the gene of interest was most commonly expressed using *E. coli* plasmid vectors that replicate at high levels in their host cells. To clone the DNA of interest the sequence is inserted into a cloning or expression vector through the use of restriction enzymes to create compatible ends and ligase to seal the integration. This approach to cloning, however, is time-consuming and relatively inefficient, and it can be difficult to find suitable restriction enzymes in the target DNA. False positive clones (vector without insert) can be common using this method due also to universal nucleotide ligation.[156] More efficient methods for cloning the DNA target of interest now include In-Fusion cloning (Clontech) and Gateway cloning (Life Technologies) which are also considerably faster too.[157, 158]

An emerging area of genome modification for insertion of a transgene is targeted genome editing. The process involves engineering of customised nucleases to induce DNA sequence-specific double strand breaks (DSBs), which are then exploited for sequence alterations. Sequences encoding these nucleases are included in the transgene construct. The three techniques gaining most attention currently are clustered regularly interspaced short

palindromic repeats (CRISPRs), transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs), each differing in the ease of construction methods, potential off-target activities and their theoretical target range. ZFNs have been the most widely used to date but the relative difficulty and high cost of their construction have meant that CRISPRs are now gaining the most attention and show a lot of promise for future transgenic model generation as well as other genetic modification studies. For more information on these techniques see the review paper (Lee et al. 2014) in the appendix chapter.

Introduction of the gene construct into fish host:

Having produced the construct, this is then transferred into the cytoplasm of fertilized fish eggs at the one cell stage, often using microinjection or electroporation. The injected DNA undergoes replication and some cells in the embryos will subsequently carry the transgene. Some integration events occur subsequent to DNA replication giving rise to mosaic fish, however, which may, or may not, contain the transgene in the germline. Animals are then maintained to adulthood and confirmation that they carry the transgene is undertaken using polymerase chain reaction (PCR) or Southern blot analysis in an external tissue, such as fin.[159] Southern Blot is commonly used to determine transgene copy number and the number of integration sites in the transgenic founder. The identification of the integration sites and copy number is important for understanding the relationship between the integration site and the specific phenotype.

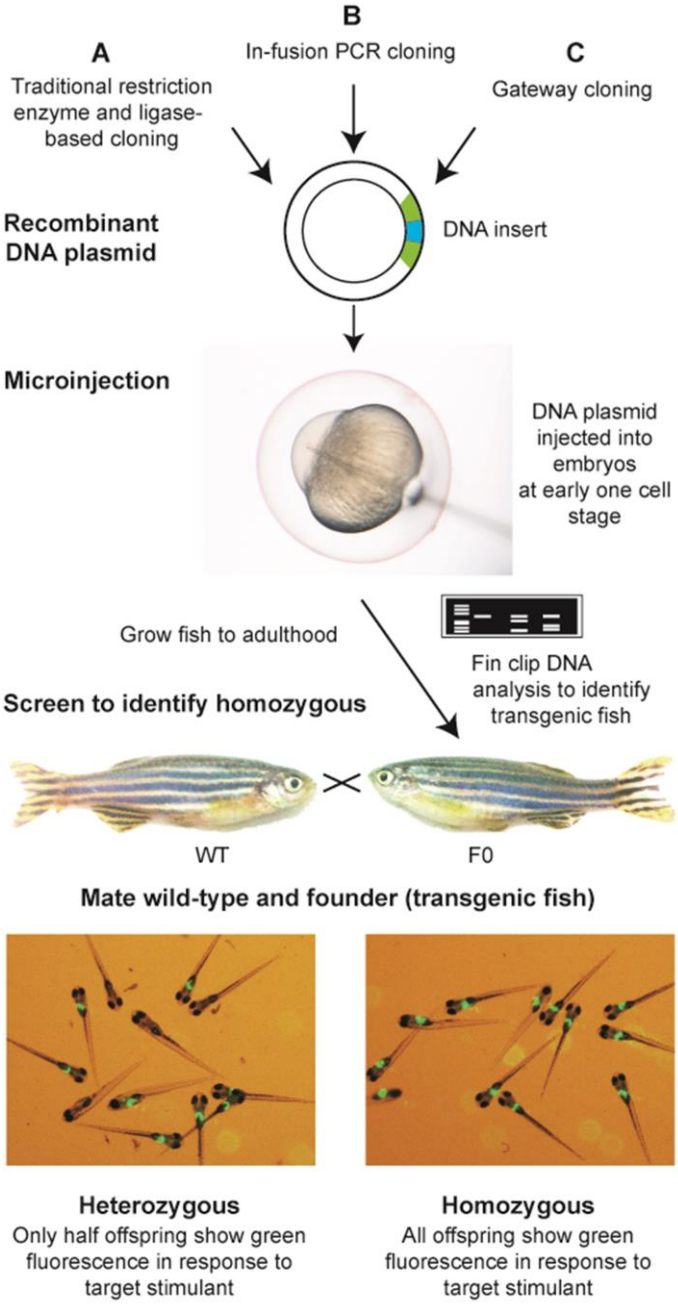
Founders containing the inherited transgene (F0) are identified by crossing founder (transgenic fish) and wild-type fish (non-transgenic fish) and assessing

whether the transgene occurs in the F1 generation. The germ-line transmission rate from the F0 generation to the F1 generation is variable because F0 are mosaic (variable cell expression) and the transgene is unevenly integrated in their gonadal tissues. Progeny derived from the F0 fish are crossed with wild-type fish, and the resulting heterozygous offspring are crossed with each other to create a homozygous fish (i.e both alleles are present in the same fish) (see Figure 2).

Crossing established transgenic lines

Once transgenic lines are established, new models can be generated via crossing lines with other transgenic or non-transgenic zebrafish lines. This follows a more traditional approach of line generation via the application of Mendelian principles to screen progeny for desired gentotypes/phenotypes. This approach negates the need for transgenesis techniques as described above but can be time consuming, requiring multiple generations to be raised and careful analysis of upwards of thousands of larvae to identify the relatively small number of F0 individuals expressing the genotype/phenotype of interest. However, the biggest attraction of this method is the potential for new models to inherit equal sensitivity and specificity of response as the model they were derived from. Transgenesis techniques are still relatively inefficient and unpredictable, even in the case of next generation technology such as CRISPRs, meaning that achieving highly sensitive and consistent transgenic lines can still require a large amount of trial and error, which can subsequently lead to extended time consummation and increased costs. Hence, the crossing of transgenic lines is becoming more and more utilised thanks in part to the creation of public transgenic zebrafish model libraries such as the ZFIN

(http://zfin.org/) that offers information on established lines and contact information for potential acquisition.



(Lee et. al. 2015, see appendix)

Figure 2. Procedure for generating transgenic fish: Once the construct is made by either restriction enzyme/ligase cloning (A), In-Fusion cloning (Clontech) (B), or Gateway cloning (Life Technologies) (C), the plasmid is microinjected into one cell stage embryos and the embryos are subsequently raised to adulthood. The presence of the transgene is confirmed by polymerase chain reaction (PCR) and/or Southern blotting on genomic DNA isolated from fin tissue. Single founder (F0) fish are mated with single wild-type (non-transgenic) fish and their offspring are mated with each other to confirm germ-line integration and to establish a homozygous transgenic line.

1.6 Seeing the Response - Reporter Genes

Reporter genes are used to quantify expression of the transgene and can also be used to rapidly determine success of gene transfer techniques and the tissue location of their expression in the host organism.[160] Most reporter genes are placed downstream of the promoter of the inserted transgene. Common reporter genes used in transgenic research include *E.coli* β-galactosidase (lacZ), chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase (NPT), luciferase (Luc), green fluorescent protein (GFP), Kaede, YFP, DsRed and mCherry.[161-167] The most common reporter genes used in transgenic fish research to date are GFP and Luc (Figure 3).

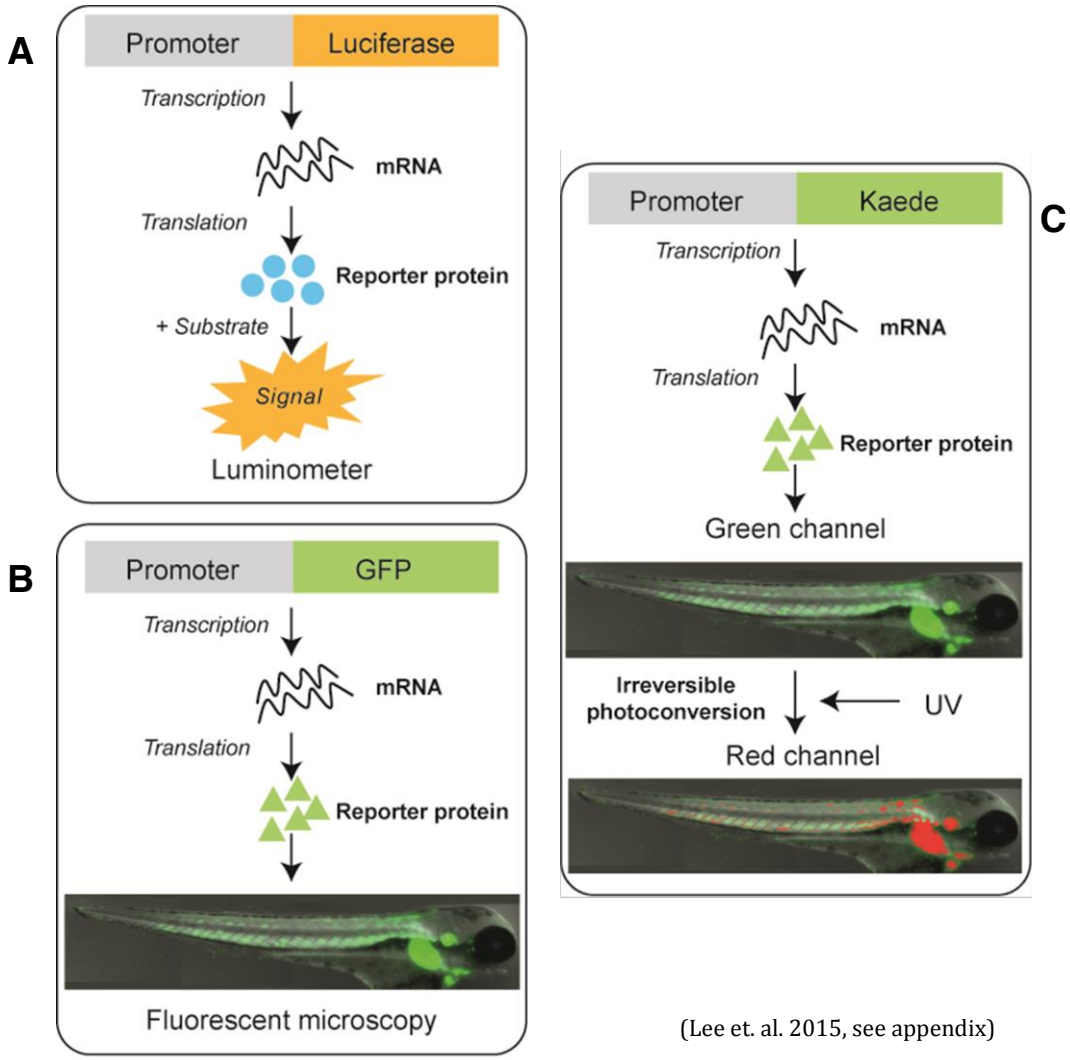
Luciferase (Luc), originates from *Photinus pyralis* (firefly) and encodes the enzyme luciferase, and causes the cell that expresses it to catalyse luciferins and produce light.[168] The luciferase gene is commonly used in cell-base reporter assays because it has high sensitivity. Furthermore, there is a high signal intensity associated with the luciferase molecule that allows for rapid measurements. It is also suitable as a reporter gene for the quantitative measurement of gene expression. The luciferase assay is widely used in pharmaceutical research (drug screening) for high-throughput analysis of gene transcription in living cells because the procedure is simple and requires only a small volume of test material. Luciferase as a method however, requires a costly substrate and is not stable (luciferase half-life varies from 1.5 to 3 hours depending on the host cell and the gene construct).[169] Furthermore, the image integration time is long to compensate for the low signal intensity.[170, 171] Recently, several groups have shown that luciferase can be detected in live zebrafish embryos, larvae and adults [172, 173].

GFP is a fluorescent protein isolated from bioluminescent jellyfish *Aequorea victoria*, and can be visualized by microscopy. It can be seen directly in living cells and intact organisms and responses can be measured in real time. Furthermore, when used in conjunction with zebrafish mutant lines that lack skin pigmentation, GFP can be detected in fish for all life stages. Advantages of GFP over luciferase include superior brightness, innate fluorescence and relatively high photostability. GFP was first used in zebrafish in 1996[174] and has since been used widely in the study of gene expression patterns, analysis of tissue-specific promoters/enhancers, tissue/organ development, cell migration and mutagenesis screening.[175-178] GFP has also been applied in the development of biosensor transgenic zebrafish to monitor chemicals such as heavy metals and oestrogens.[49-51, 77, 142] RFP (red fluorescent protein) has the advantage over GFP in that there is less background interference. DsRed, red fluorescent protein from *Discosoma sp.*, can be used together with other GFP variants for multicolor imaging.[179] mCherry is the best general-purpose red monomer due to its superior photostability.[180]

Photoconvertible fluorescent reporter sequences are starting to be used in transgenic (TG) models that can fluoresce different colours upon exposure to light in (or near) the ultraviolet (UV) wavelength region.[181] An example of a photoconvertible fluorescent protein is the Kaede protein (named after the Japanese maple where its leaves change from green to red in the autumn.[**165**]) isolated from the stony coral *Trachyphylia geoffroyi*[165] and which on exposure to UV light results in a spectral shift of the native (green) state from 508 nm (absorption) and 518 nm (emission) to longer wavelength peaks at 572 nm and 582 nm, respectively (Figure 3). Photoconversion leads to an almost complete conversion of red to green fluorescence and is irreversible and stable under

aerobic conditions.[165] Photoconvertible proteins offer the ability to track individual cells and assess effects on tissues developmentally and have been applied in studies on the development of neural networks in the brain and craniofacial skeleton in zebrafish.[182-184]

Recently, a system has been developed to manipulate further the production of incorporated fluorescent proteins, called kaloop. It involves autoactivation (self-perpetuation) of the fluorescence, and this is being applied as a powerful tool for spatiotemporal genetic fate mapping of specific cells types in zebrafish.[185]



(Lee et. al. 2015, see appendix)

Figure 3. Principles of how reporter genes (luciferase (Luc) (A), green fluorescent protein (GFP) (B) and kaede (C) enable visualizing target gene expression. The reporter genes (Luc, GFP and Kaede) are expressed when DNA is transcribed into messenger RNA (mRNA) and then translated into a reporter protein. In a reporter gene assay using luciferase, a luminescence signal is generated by the reaction of luciferase's substrate, luciferin (A). GFP-expressing cells are detected by fluorescence microscopy (B). Kaede protein, when exposed to UV or violet light, is capable of irreversible photo-conversion from a green to a red fluorescent form (C).

1.7 Transgenic Fish and Ecotoxicology

Transgenic zebrafish have considerable potential for use in aquatic ecotoxicology as biosensors and as more effective models for informing on health impacts of chemical exposure.[49, 50, 140-142] Biosensor fish work on the premise that specific genes, often enzymes or receptors, are inducible by certain chemicals/pollutants. Exposure of the fish to the pollutant of concern, or a natural water containing that pollutant, induces the activation of an inducible promoter that in turn triggers expression of the reporter (e.g. GFP) (Figure 4).

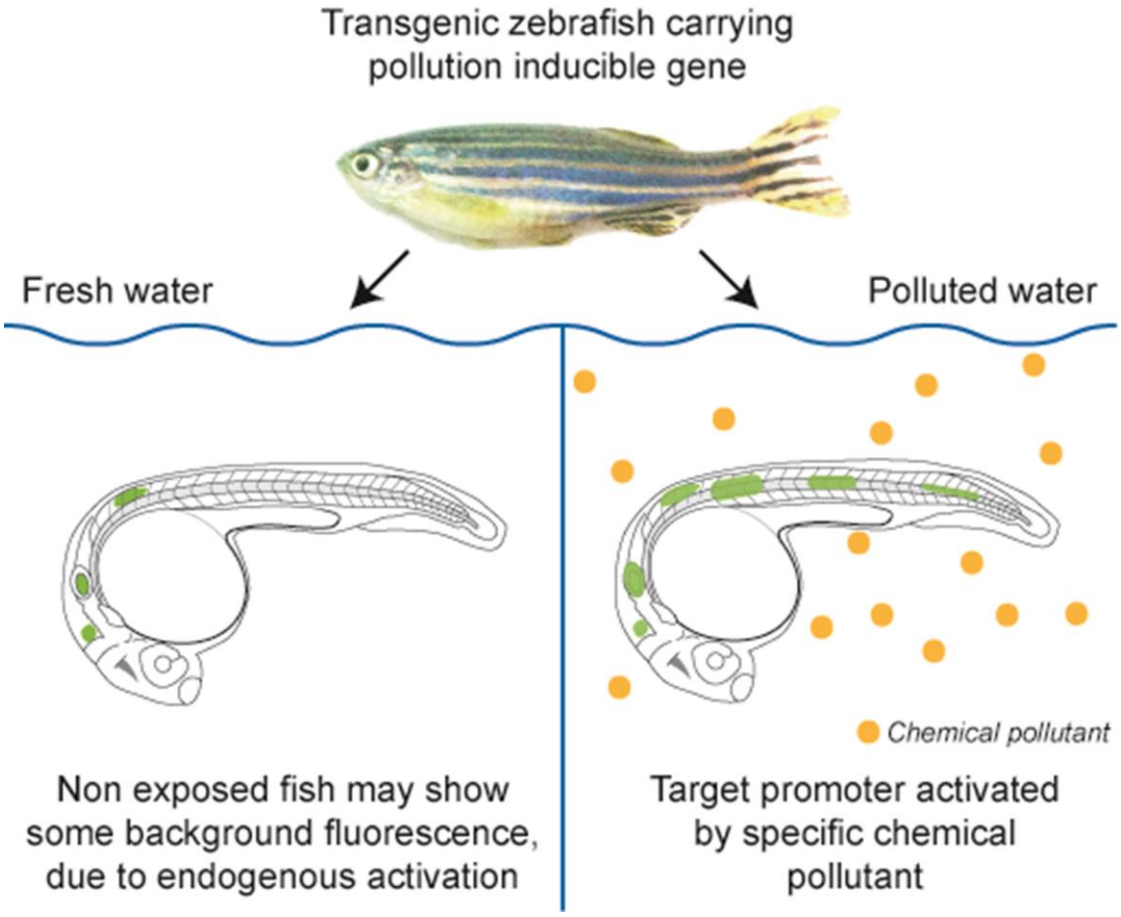
Various transgenic fish lines have been developed with an oestrogen responsible promoter derived from the *vitellogenin* (*vtg)* or *choriogenin* genes. *Vitellogenin (vtg)* is an egg yolk precursor protein, normally synthesized in the liver of females, but also inducible in males in response to oestrogen exposure, and is the most widely used biomarker for exposure to oestrogenic chemicals in aquatic ecotoxicology. *Choriogenins* are egg envelope proteins that are similarly synthesized in the liver of maturing female fish in response to oestrogens. Both *vitellogenin* and *choriogenin* respond to oestrogens in low ng/L exposure regimes and at sub-nanogramme exposure concentrations for the synthetic oestrogen ethinylestradiol.[186]

Some of the first transgenic models employing promoters for the *vitellogenin* and *choriogenin* genes with GFP reporters were developed in medaka. These models however, were not sufficiently sensitive for detecting exposure to oestrogens for environmentally relevant exposures.[187-191] A single GFP transgenic zebrafish line has been developed with an oestrogen-inducible promoter for a *vitellogein* gene (*vtg1*)[51] that was reported to detect GFP in the liver at 0.1 µg/L E_2, 1 µg/L estriol, 1 mg/L BPA, and 10 mg/L BPA. The lack of appropriate controls in this work however makes it difficult to provide a robust

evaluation of this model. The transgenic medaka and zebrafish lines developed incorporating *vitellogenin* and *choriogenin* genes in the reporter systems reported on above vary in their responsiveness to oestrogen and generally they are limited in the use for environmental monitoring as they lack the required sensitivity. Responses in all of these transgenic medaka and zebrafish for *vtg* and *choriogenin* genes are also restricted to the liver, the site of *vitellogenin* and *choriogenin* synthesis. They do nevertheless, provide systems for screening chemicals for oestrogenic activity *in vivo* in real time and can be applied to inform on cumulative responses to oestrogens.

Transgenic zebrafish have also been developed to examine the effects of oestrogenic chemical exposure on development of the brain using the promoter of a *cyp19* gene. *Cytochrome P450 aromatase* (*cyp19*) is enzyme complex that catalyses the synthesis of oestrogens, thereby controlling many different physiological processes of oestrogens and is mainly expressed in the gonad (*cyp19ala*) and brain (*cyp19a1b*).[192] These are oestrogen target genes. Tong *et al.* (2009) generated a transgenic zebrafish line that expresses GFP under the control of the brain aromatase *cyp19a1b* promoter. In this line GFP expression occurred in the radial glial cells in response to oestrogen and was associated with endogenous aromatase B expression.[77] Exposure of embryos to a variety of different oestrogenic chemical classes including natural and synthetic steroids, alkylphenolic compounds and phyto- and myco-oestrogens (for 5 days) induced strong GFP expression in the region between the anterior telecephalon and caudal hypothalmus and most of these responses were concentration dependent.[48] The effective concentrations (EC_{50}) for inducing these responses were 0.013 nM (3.9 ng/L) EE_2, 0.01 nM (2.7 ng/L) DES, 0.48 nM (130.8 ng/L) E_2, 3303 nM (0.8 mg/L) BPA, 2501 nM (0.7 mg/L) genistein.

This zebrafish line provides a very useful model for studies into the roles of natural oestrogens, and effects of environmental oestrogens, on brain development and function. Models using the *cyp19a1a* gene promoter are likely to be forthcoming in the very near future for studies into the effects of oestrogens on gonadal development.



(Lee et. al. 2015, see appendix)

Figure 4. Schematic of a biosensor transgenic zebrafish system for detecting exposure to aquatic pollutants. The transgenic zebrafish containing DNA elements responsive to a toxicant is placed in the exposure water and as the contaminants enter and reach the body tissues, activation of the response elements occurs that induces the production of GFP in that target tissue.

1.8 ERE-GFP Models

There have been a number of transgenic lines developed in zebrafish for detecting oestrogens using oestrogen response elements (ERE), which binds the oestrogen receptor–ligand complex activating oestrogen responsive genes. The first of these transgenic biosensor zebrafish developed by Legler *et al.* (2000) incorporated 3×EREs inserted upstream of TATA minimal promoter and luciferase as the reporter [140]. Juveniles of this zebrafish transgenic model were responsive to E2 at concentrations down to 0.1 nM (27.2 ng/L) (for a 96 h exposure) and the testis was the most responsive target tissue.[193] Limitations of this transgenic model include that measurement of the reporter luciferase required termination of the fish and identifying the specific responding tissues required their individual dissection and analysis. Work using this transgenic model did however, show that the main target tissues (the liver and gonad) were responsive down to exposures of EE2 (3 or 10 ng/L) and demonstrated also that responses to oestrogenic EDCs differed for different developmental stages.[141]

Two subsequent transgenic zebrafish models developed incorporated EREs with GFP. In 2011, Gorelick and Halpern (2011) reported on a transgenic zebrafish containing five-tandem consensus EREs upstream of a mouse *c-fos* minimal promoter and the GFP gene. The transgenic zebrafish embryos were exposed to a range of oestrogenic chemicals including E2, DES (diethylstilbestrol), BPA, EE2 and NP, with responses see in the heart, brain, liver, aorta and ventral fin for exposure to E_2 (with a lowest observed effect concentration (LOEC) of 100 µg/L), liver and heart for exposure to EE_2 (with a detection down to 10 ng/L). No GFP expression was detected in embryos exposed to NP. Use of this transgenic model indicated that different oestrogenic

compounds induced tissue specific differences in their activity.[50] A drawback of this model was the relatively low sensitivity-very high exposure concentrations of oestrogens (1-100 µg/L E2) were employed to induce these responses. However, in their more recent work with this model, they were able to observe tissue-specific GFP expression for exposure to wastewater effluents.[92]

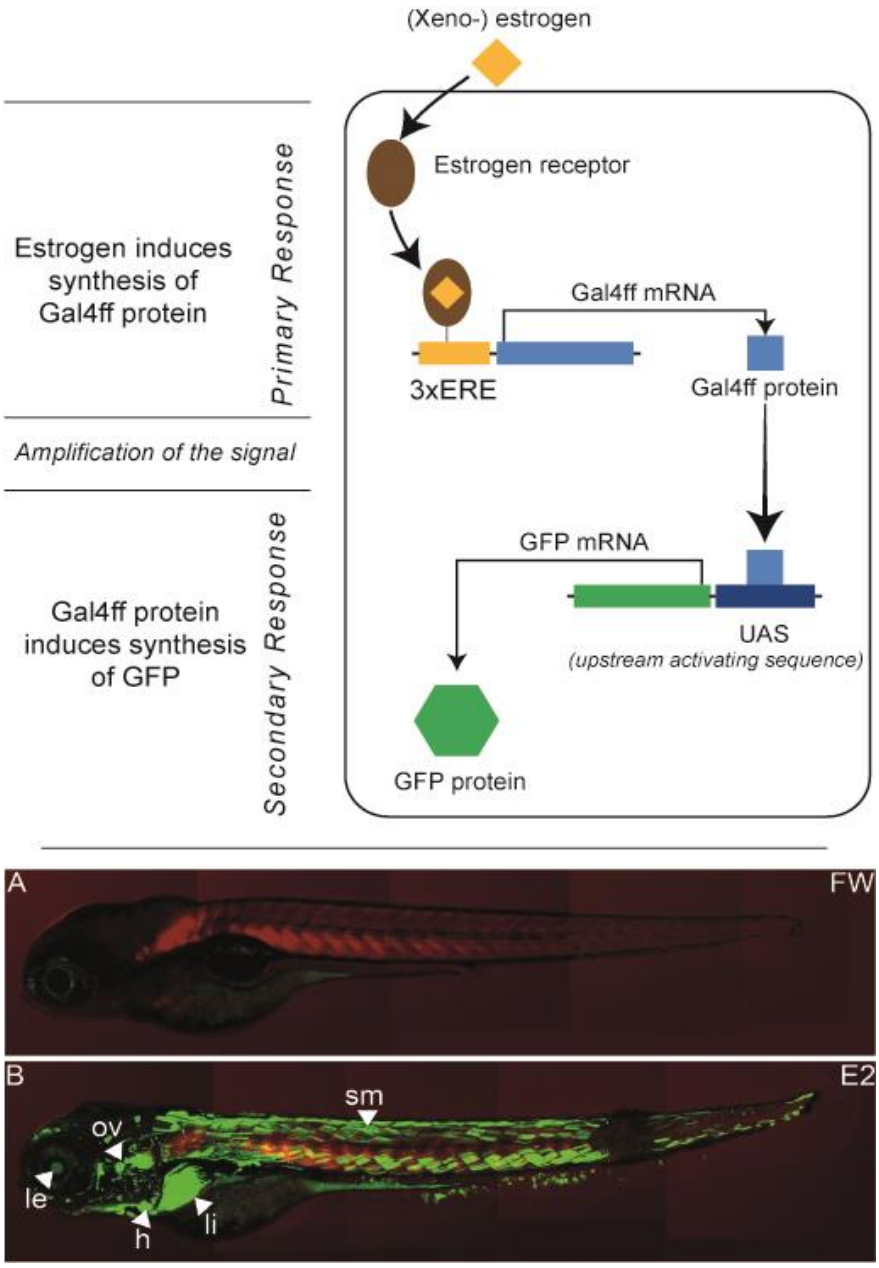
The two transgenic zebrafish models developed have both been derived from a well-established transgenic zebrafish model generated by Lee *et al.* (2012).[49] This was the most recently developed ERE transgenic zebrafish and most sensitive transgenic zebrafish system for detecting environmental oestrogens. This model includes a 3×tandem ERE and a *Tol2* mediated gal4ff-UAS system. Figure 5 illustrates how the gal4ff-UAS system helps to amplify GFP expression response upon ligand-ER binding to the ERE transgene sequence to improve the overall sensitivity of the ERE-GFP model to oestrogenic chemicals. The zebrafish embryos and larvae had response sensitivities to the EDCs tested of 1 ng/L EE_2, 5 ng/L E_2, 100 µg/L BPA and 1 µg/L NP. Responses in this model were detected in a wide variety of tissues including the liver, heart, skeletal muscle (somite and cranial), ear/eye ganglions, brain, otic vesicle, lens and neuromasts (Figure 5). Skeletal muscle cells, cranial muscle cells, heart cells and neuromast cells were especially responsive to oestrogen. There were tissue-specific expression patterns for the different environmental oestrogens indicating differences in tissues toxicities.[49] However, a limitation of this model, and other zebrafish models like it, is that beyond 3 days post fertilization skin pigmentation starts to block the fluorescence signal from internal tissues. Receptor expression, and therefore tissue response, is likely to vary throughout development of the zebrafish and hence restricts any conclusions drawn from the ERE-GFP model. This problem led groups such as White et al. to identify

various pigmentless phenotypes in zebrafish.[143] One line in particular, the Casper line, was generated through crossbreeding to carry fully silenced *roy* (dark) and *nacre* (silver) pigment genes. As demonstrated in this thesis, this line can be used effectively to produce transgenic models that can be used at all stages of zebrafish development. Very recently, a similar line named "Crystal" has been developed that also includes silenced pigmentation in the eyes of the zebrafish, further improving imaging of the brain.[194]

1.9 Summary

National and international screening programs currently rely heavily on *in vitro* assays to identify and categorise oestrogenic chemicals and other EDCs due to their time and cost effectiveness in comparison to *in vivo* tools. There is also an ethical obligation to reduce, replace and refine the use of animals in scientific testing. However, because of this reliance on *in vitro* assays, risk assessment of oestrogenic chemicals is often based on quantification of binding affinity for specific ER subtypes using a range of different cell lines and this does not allow for the more complex interactions that occur between EDCs and organisms in the environment. The development and application of transgenic fish offers huge potential for improved understanding on the effects and mechanism of effects for EDCs. Furthermore, transgenic fish models allow for more in-depth information to be derived from individual animals and therefore allow for more integrative health effects assessments. The use of highly informative transgenic fish models will result in better use and potential reduction of intact animals used in animal testing in line with the principles of the 3Rs (Replacement, Reduction and Refinement)[195]. For this reason, the zebrafish has become an attractive option for transgenic modelling due to the large amount of information that can be derived from embryos/larvae before first feeding (i.e. non-

regulated). Favourable features of this zebrafish for transgenic work also include their ease of breeding in the laboratory, relatively low associated maintenance costs, high fecundity, transparency of the embryos and rapid organogenesis (attractive features for studying developmental processes) and the availability of sequenced genomes providing extensive genetic resources.



(Lee et. al. 2015, see appendix)

Figure 5. Mechanism of amplification of the oestrogenic signal in a GAL4FF–UAS transgenic zebrafish. Within the transgenic fish, oestrogen-responsive elements (EREs) respond to the oestrogenic signal to drive the first reporter, Gal4ff. Gal4ff binds to UAS in the 2nd transgene to drive the 2nd reporter, a green fluorescent protein (GFP). This two-step reporter system amplifies the signal and enhances the sensitivity of the biosensor. Induction of GFP in ERE-GFP transgenic zebrafish exposed to oestrogen. Four-day-old larvae exposed to clean water alone (control: A) or 17β-estradiol (100 ng EE2/L: B). GFP expression is observed in the heart (h), lens (le), liver (li), neuromast (n) and somite muscle (sm) by confocal microscopy (Zeiss) with a X10 objective lens.

1.10 Aims of the thesis

The overall aim of the work presented in this thesis was to develop transgenic zebrafish models with good utility for effective analysis of responses to oestrogens in zebrafish for different life stages and for application to larval screening (semi-automated). The first specific aim of this work was to generate two transgenic zebrafish models, each expressing silenced skin pigmentation to improve tissue-specific fluorescence detection throughout zebrafish development but differing in their expression of a fluorescence reporter transgene (GFP and kaede) to allow for different response assessments. This was undertaken via crossing of existing established lines that displayed silenced pigmentation, transgene stability, high sensitivity and tissue-specific responses to oestrogenic chemicals. The second specific aim was to apply the first (GFP) of these models for use in semi-automated screening of chemicals and high-content analyses to identify target tissues. This work aimed to demonstrate how the application of transgenic zebrafish models in semi-automated imaging and image-analysis systems could provide multifaceted *in vivo* screening assays for oestrogenic chemicals that are highly sensitive and highly informative for improved chemical risk assessment. The third specific aim of the work looked to identify temporal response dynamics at the early life stage of these models, after single and repeated exposures to oestrogens. The work investigated the effects of sequential exposures to oestrogens on response sensitivity in specific tissues. Further to this, the work aimed to investigate whether the sensitivity of specific tissues in the model was dependent on its stage of development.

These specific aims are described in more detail below:

Aim 1 (Chapter 3): To develop ERE-GFP-Casper and ERE-Kaede-Casper lines.

The main aim of the work carried out in this chapter was to generate new oestrogen responsive transgenic zebrafish lines for their application to larval screening (semi-automated) and effective analysis of responses to oestrogens in zebrafish for different life stages. The translucent nature of these models was intended to facilitate improved fluorescence detection and quantification in specific tissues at various body orientations and at later stages of development. Rather than using traditional transgenesis techniques, I aimed to generate these models via crossing of established lines. This approach was chosen with the intention that the new models would inherit the same sensitivity and tissue-specific responses to oestrogenic chemicals as the oestrogen responsive transgenic model they were derived from to facilitate their application in the following investigative studies. To generate the ERE-GFP-Casper model, a previously established ERE-GFP line was crossed with a Casper line. The model was compared with the ERE-GFP model and another oestrogen responsive transgenic model (cyp19a1b-GFP) to assess for sensitivity and tissue response specificities. The transmission rate of the transgenes from individual F0 ERE-GFP-Casper adults to their progeny was also analysed. Given the success of this procedure, the ERE-Kaede-Casper was subsequently developed via a similar approach crossing of the ERE-GFP-Casper model with a UAS-Kaede model. A comparative study was then run with the ERE-GFP-Casper model to assess for sensitivity and tissue response specificities.

Aim 2 (Chapter 4): To apply the ERE-GFP-Casper model in high-content analysis of potencies and target tissues of oestrogenic chemicals in addition to its application in a medium to high-throughput screening system for the semi-automated identification and quantification of oestrogenic chemicals.

The aim set out in Chapter 4 was to apply the ERE-GFP-Casper model in an *in vivo* screening assay for oestrogenic chemicals that is both highly sensitive and highly informative for improved chemical risk assessment. To achieve this aim, confocal microscopy was used to acquire high-resolution images of the larvae to provide high-content information on observable target tissue patterns of the varying oestrogenic chemicals and their concentrations. This was to highlight the unique tissue-specific "fingerprints" of each chemical and help identify commonly susceptible tissues that appeared sensitive to all chemicals. To semi-automatically screen oestrogenic chemicals, a Cellomics Array Scan system was customised to carry out fully automated image acquisition of exposed ERE-GFP-Casper larvae and (in collaboration with Jeremy Metz at the University of Exeter) a Python algorithm was designed to identify larvae within images and accurately quantify the fluorescence signal within the animal.

Aim 3 (Chapter 5): To apply the ERE-Kaede-Casper model in early-life stage analysis of tissue-specific windows of sensitivity to oestrogens and investigate for effects of repeat exposure to oestrogens on response sensitivity.

Potency analysis for EDCs typically involves single-chemical exposures with little, or no, consideration given for animal's specific stage of development or exposure history. Overall the work in this chapter aimed to improve our

understanding of the role of oestrogens during development at the early-life stage, investigate the importance of oestrogen exposure history in effects assessments and subsequently assess the implications for possible adverse effects associated with oestrogen exposure. Using the ERE-Kaede-Casper and demonstrating first the ability to fully photoconvert the kaede fluorescence signal within the model, I looked to identify windows of tissue-specific sensitivity to EE2 for exposure during early-life (0-5 days post fertilisation, dpf). Temporal dynamics of kaede response would also be assessed after removal from EE2 exposure (at 2 dpf) and incubation in embryo water (3-5 dpf) to identify any potential persistence of response. Finally, the work in this chapter looked to investigate whether exposure to oestrogen (EE2) during early life (0-48hpf) could affect responsiveness (sensitivity) to different environmental oestrogens (EE2, genistein and bisphenol A) for subsequent exposures during development. In conjunction with the repeated exposure study, qPCR analysis of ER subtype expression was carried out (in collaboration with Anke Lange at the University of Exeter) to identify any potential mechanisms of sensitivity alteration.

Chapter 2

General Methods

Chapter 2

General methods

2.1 General Approach

A major part of this PhD project involved generating transgenic zebrafish for studies into the effects of environmental oestrogens. The first line created, the ERE-GFP-Casper model, was generated via crossing of a previously established ERE-GFP line and a Casper line, that expresses silenced skin pigmentation. The second transgenic line, the ERE-Kaede-Casper, was subsequently developed via crossing of the ERE-GFP-Casper model with a UAS-Kaede model. After the initial cross, screening of subsequent generations was required to identify founder individuals carrying the desired genotype for the two models, and then further screening to identify individuals with homozygous expression of the transgenes. There was a considerable amount of work associated with the generation and subsequent maintenance, breeding and screening of zebrafish. The generation and validation of these lines is described in detail in Chapter 3 of this thesis.

The majority of zebrafish lines used for this project expressed fluorescent proteins and required fluorescence microscopy techniques to screen for desired genotypes and acquire high-quality images. A range of different fluorescence microscopes were used in this thesis work including fluorescence dissection microscopes, an inverted compound microscope, a confocal microscope and an automated “Array Scan” system. The application of these microscopes was dependent on the resolution and magnification required for screening or

imaging purposes in addition to the throughput capabilities of the microscopes to ensure timely completion of experiments. The majority of data presented in this thesis have been attained via qualitative and quantitative analysis of images attained via fluorescence microscopy. High-content analysis of tissue-specific responses was conducted by eye. This involved qualification of specific target tissues seen to be responding to oestrogenic chemicals within high-resolution images. Quantification of fluorescence intensity within images also featured heavily during the work. This was achieved using image analysis software. A semi-automated Python algorithm is presented in Chapter 4 that automatically identified larvae within images and quantified whole-body fluorescence intensity exclusively within the animal. Larval identification by the algorithm was checked by eye using a computer interface. Manual image analysis was carried out in work presented in Chapter 5 using Image J software. This was to ensure accurate quantification of tissue-specific fluorescence response in liver, heart and somite muscle. The details of microscopy and imaging techniques used for these studies are included in the methods sections of each chapter. A general method for homozygous screening is presented in this chapter and includes the use of fluorescent dissection microscopes and anaesthesia.

The preparation of chemical solutions (often oestrogenic) and exposure to zebrafish larvae was applied in a consistent manner during the work presented in this thesis. The preparation, concentrations and exposure protocols for these solutions varied for the different studies presented and details of these preparations are included in the relevant chapters. Additional information garnered from chemical exposures included measured chemical concentrations from exposure water samples via tandem liquid chromatography-mass

spectrometry (LC-MS). This was necessary to assess if the concentrations of chemicals in exposure water were nominal. In Chapter 5, qPCR analysis was used to investigate potential effects on ER subtype expression in the ERE-Kaede-Casper model after sequential oestrogenic exposures. These analyses were conducted in collaboration with colleagues within the University of Exeter with details of the methods included in the relevant chapters.

The majority of information on methods used in the work presented in this thesis are presented in Chapters 3, 4 and 5. General methods of zebrafish maintenance, chemical preparation and storage and homozygous screening are presented in this chapter that are referenced extensively throughout the thesis.

2.2 Animal Handling

All experimental procedures conducted in this research with fish were in accordance with UK Home Office regulations for the use of animals in scientific procedures and followed local ethical review guidelines ensuring their humane treatment.

2.3 Embryo Water

The term “embryo water” used throughout this thesis refers to the water solution used for larvae incubation and exposures (control, solvent control and chemical solutions). The solution comprises of four salts mixed into deionised water and is also referred to as ISO water. Individual salt solutions were first prepared using 4x1L deionised water solutions with 11.76 g calcium chloride ($CaCl_2.2H_2O$), 4.93 g magnesium sulphate ($MgSO_4.7H_2O$), 2.59 g sodium

bicarbonate ($NaHCO_3$), 0.23 g potassium chloride (KCl) added respectively and mixed thoroughly. A 1xISO “neat” stock was prepared using 25 ml of each of the neat solutions and made up to 1L using deionised water. This could be stored for up to one month at 4°C. From this stock, 1:5 ISO exposure media was prepared the day before incubation or exposure by adding 1xISO water to deionised water at a 1:5 ratio and oxygenated for three hours.

2.4 Chemical Preparation

Chemical solutions used for exposure were prepared using embryo water. Most chemicals were purchased in powder form and solvent stocks were prepared the day before exposure. Chemicals were typically dissolved in analytical grade dimethyl sulfoxide (DMSO) and stored at 4°C. On the day of exposure solvent stocks were pipetted into embryo water and incubated to 28°C to produce the required exposure solutions. More details on this preparation are included in the relevant chapters. All chemical solutions were prepared and stored in acid washed glassware.

2.5 Anaesthesia

Anaesthesia of zebrafish larvae for screening and imaging purposes was a key aspect of the work. Immobilisation of the larvae needed to be complete with minimal effects on them as they were often revived for subsequent analyses. Tricaine (MS-222, tricaine methanesulfonate) was used as the anaesthetic and was prepared by dissolving 400 mg tricaine powder in 97.9 ml embryo water and adjusting the pH to 7 by adding around 2.1 ml of 1 M Tris buffer (pH9). This gave a 0.4% tricaine stock that was stored at 4°C before use. A 0.008% tricaine working solution was then prepared for experimental use by further dilution in embryo water to be used as an anaesthetic for zebrafish larvae. For imaging,

larvae were anaesthetised for 10 minutes and immediately recovered in embryo water. For screening, larvae were anaesthetised for 20 minutes and immediately recovered in embryo water.

2.6 Imaging

For imaging, zebrafish larvae were mounted in either methylcellulose or 0.7% low-melting point agarose (for confocal microscopy only). Larvae were typically imaged at the 5 dpf life stage but exact ages are stipulated in results chapters. Methylcellulose was used more regularly for mounting as it better enabled alteration of larvae orientation during imaging. Methylcellulose was prepared by adding 1.5 g powder to 50ml embryo water (0.016% Tricaine), mixed thoroughly and placed the solution in a −20°C freezer overnight until frozen then stored in the fridge until use. Further details of microscopy and imaging techniques used for these studies are included in the methods sections of each chapter.

2.7 Screening

Transgenic zebrafish adults that are homozygous for inserted transgenes were found to produce more sensitive and consistent fluorescence responses in their larvae. These were used for chemical exposures. It was therefore important to consistently screen new generations to ensure that breeding stocks were producing offspring with similar sensitivities/responses to oestrogens over successive generations for application in this thesis work. To identify homozygous transgenic adults, transgenic individuals were screened via pairing with wild-type individuals of the opposite sex in mating tanks. Tanks were carefully labelled and egg collection chambers were then placed into tanks on the afternoon before subsequent egg collections in the following morning (after final feeding). On the morning of collection, spawning occurred between 8am

and 9am, the period of artificial dawn. Embryos were collected from chambers and washed with embryo water between 9am and 10am. Embryos were transferred into labelled petri dishes in embryo water and viewed under a dissection microscope. Unfertilised embryos were immediately removed using Pasteur pipettes. The embryos, were then exposed to 50 ng/L EE2 from 0 dpf to 3 dpf or 4 dpf (depending on transgenic model being screened) to induce strong fluorescence responses in individuals carrying oestrogen responsive transgenes. After exposure, larvae were washed and anaesthetised in embryo water containing 0.008% tricaine. Using fluorescent dissection microscopes with a GFP filter, larvae were assessed for fluorescence responses by eye and the ratio of fluorescent and non-fluorescent larvae was counted. If 100% larvae fluoresce, the transgenic adult is considered homozygous. Heterozygous adults will typically produce fluorescent larvae at 25%, 50% and 75% ratios. Non-transgenic adults will not produce any fluorescent larvae. For more detail on the analysis of these ratios see Generation of Transgenic Zebrafish Models chapter. This process of screening often took one or two months to screen a stock tank of transgenic individuals due to the large number of transgenic adults that required screening, the limited number of mating tanks available and the variable spawning habits of the zebrafish. Once identified, homozygous adults were separated from non-homozygous fish to become working stocks and breeding stocks for subsequent generations.

Chapter 3

Generation of Transgenic Zebrafish Models

Chapter 3

Generation of Transgenic Zebrafish Models

3.1 Introduction

An increasing number of oestrogenic chemicals are being identified in the environment and this has led to an increased interest in high-content biosensor models to assess both the potential short-term and long-term effects of oestrogen exposure in wildlife. The zebrafish has become a highly utilised model for genetic modification because of a combination of the availability of a sequenced genome providing the associated extensive genetic resources and an egg that is transparent with a relatively soft chorion facilitating microinjection of genetic constructs. Rapid organogenesis in zebrafish compared with in other traditional animal models also makes it an attractive option for *in vivo* modelling. This has lead to an ever-expanding library of transgenic zebrafish lines expressing different promoter and response element sequences and reporter genes. These models have been generated using a variety of transgenesis techniques. However, the current efficiency and success rate of these techniques is still limited and can be costly, even with more recently available tools such as CRISPR/Cas9.

In the work presented in this chapter, two novel transgenic zebrafish models were developed for application to the identification of oestrogenic chemicals, assessing their potencies, target tissues and investigating temporal response dynamics after single chemical and multi-chemical exposures. The first of these models, the ERE-GFP-Casper, was generated via crossing of an established ERE-GFP line and a translucent skin Casper line. Subsequent generations

were screened for desired genotypes and homozygous expression of transgene sequences. Transmission rates of these transgenes from F0 ERE-GFP-Casper adults to F1 progeny were assessed and identified a 100% transmission rate in adult females and 90-99% transmission rate in adult males deemed to be homozygous. The Casper line showed improved fluorescence response detection and quantification across all tissues and body orientations compared with the original ERE-GFP model. Comparisons of the ERE-GFP-Casper model with another oestrogen responsive transgenic model (cyp19a1b-GFP) demonstrated a similar sensitivity to ethinyloestradiol (EE2) exposure, with reported EC50 values of 0.04 nM and 0.12 nM respectively. The translucency of the ERE-GFP-Casper line enabled the model to be successfully applied in a novel medium-throughput oestrogenic chemical screening system that provides semi-automated identification of oestrogenic chemicals, quantification of their effect and high-content information on their target-tissues (Chapter 4). The model has also been used to generate the second transgenic model presented in this thesis.

The ERE-Kaede-Casper model was derived from crossing the ERE-GFP-Casper line with a UAS-Kaede line. The ERE-Kaede-Casper model expresses the photoconvertible fluorescence protein, kaede, enabling the permanent conversion of green to red fluorescence in the zebrafish upon exposure to UV light. In this chapter the use of photoconversion to screen and identify ERE-Kaede-Casper individuals is demonstrated. The model had the same oestrogenic sensitivity and tissue-specific fluorescence responses to oestrogenic chemicals as the ERE-GFP-Casper model. The ERE-Kaede-Casper model is applied in the identification of windows of tissue sensitivity to

oestrogenic chemicals and effects of exposure to EE2 during early life on subsequent responses to a series of environmental oestrogens (Chapter 5).

3.2 Methods

General Outline

The generation of the two models followed a similar method of development and validation. The models were generated via crossing of established transgenic and non-transgenic zebrafish lines rather than via transgenesis. Once working stocks of the models were established responses of the models to oestrogen were compared with established oestrogen responsive transgenic zebrafish to assess for possible model differences in tissue-specificity and sensitivity. Transmission rates of transgenes from ERE-GFP-Casper founder generation (F0) to progeny (F1) larvae were analysed and are discussed in this chapter. The photoconversion of kaede in the ERE-Kaede-Casper model is also assessed in this chapter.

3.2.1 Generation of ERE-GFP-Casper

ERE-GFP x Casper Cross

The ERE-GFP-Casper transgenic line was derived from a ERE-GFP line developed at the University of Exeter,[49] and a Casper line from University College London (Figure 1). The ERE-GFP line is sensitive to oestrogens and the tissue responses are consistent with another ERE-GFP model, developed independently by Gorelick et al.[50] The Casper line has silenced *roy* (dark) and *nacre* (silver) pigmentation genes, resulting in highly translucent skin.[143] To

increase the transmission rate of transgenic sequences to subsequent generations and to ensure consistency of oestrogen response sensitivity, the ERE-GFP model was initially screened for homozygous adults by pairing with wild-type (WIK strain) adults and assessing the ratio of fluorescent/non fluorescent offspring. Homozygous adults were then crossed with the Casper line in five mating pairs, which were rotated to promote genetic diversity. Progeny from these crosses were heterozygous for four genes of interest; the ERE:Gal4ff and UAS:GFP transgene sequences and silenced *roy* and *nacre* pigmentation genes. Crossing of progeny from distinct families produced larvae with varying combinations of the genes of interest. These larvae were screened for the homozygous ERE/Casper genotype via exposure to 50 ng/L of 17α-ethinylestradiol (EE2) from 0-3 dpf. Individuals carrying both Casper phenotype and GFP expression were raised to sexual maturity as the founder (F0) ERE-GFP-Casper generation.

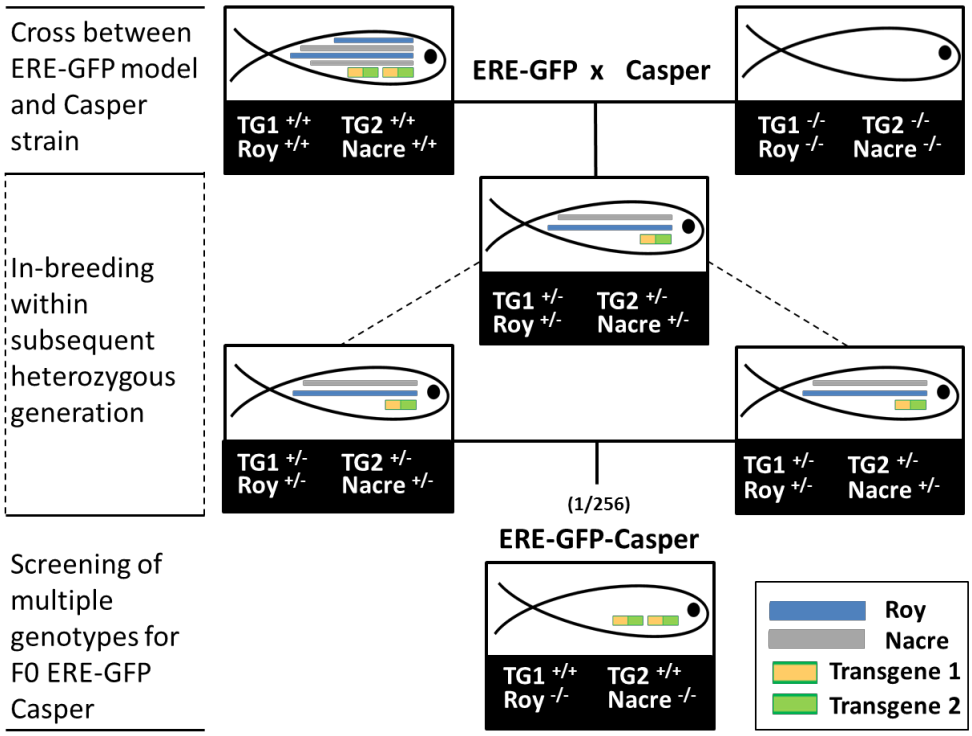


Figure 1: Generation of ERE-GFP-Casper (F0) line. TG1 indicates ERE-Gal4ff transgene sequence. TG2 indicates UAS-GFP transgene sequence. Expression of pigmentation genes *roy* (dark) and *nacre* (silver) are also shown. The ERE-GFP model, homozygous for both transgenes, and a Casper strain were initially crossed to give a heterozygous generation. In-breeding within this generation produced progeny with 256 different genotypes based on four genes of interest. One in 256 expressed F0 ERE-GFP Casper genotype of fully silenced pigmentation and homozygous transgene expression.

F0 Homozygous Screen

A total of 47 F0 individuals were raised to sexual maturity. Adult fish were visually sexed and screened for homozygous individuals using the screening protocol previously stated in the General Methods section (Chapter 2) rather than by fin clip DNA analysis. For this screen, the exact ratios of GFP and non-GFP expressing progeny were recorded to both identify the phenotype of the adult and also assess the generational transmission of the transgenes. F0 adults were categorised by the apparent copy-number of transgenes in the adult. Adults with ratios of fluorescent to non–fluorescent larvae below 60% were categorised as carrying 2 or less transgene copies, those with ratios 60-85% were categorised as carrying three transgene copies and those above 85% were classed as homozygous. Total number of progeny per adult collected varied, but at least 30 larvae were assessed per adult. For individuals that initially appeared to fall within two phenotype groups (three and four transgenes), 100-200 larvae were assessed to provide greater numerical confidence in the categorisation of homozygous adults.

Comparison of the responses to oestrogen in the ERE-GFP Casper Cross with the ERE-GFP line

Responses to EE2 in the ERE-GFP-Casper line were compared with our original ERE-GFP model to assess for sensitivity and tissue response patterning to oestrogenic chemicals. Embryos from both models were exposed to 50 ng/L EE2 from 0-5 dpf. Fluorescence response was observed on an inverted compound microscope (Zeiss Axio Observer) under GFP excitation (180 ms using filter set 38 HE: BP 470/40, FT 495, BP 525/50). Further generations have been established, and each generation screened to confirm

that only homozygous adults are used for ensuring consistently high sensitivity. F1 generation TG(ERE:GFP) Casper larvae were used in the study reported here and compared with F6 generation TG(ERE:GFP) larvae.

Comparison of the responses to oestrogen in the cyp19a1b-GFP with the ERE-GFP line

To further assess the sensitivity of the ERE-GFP-Casper model, a comparative exposure study was run with the cyp19a1b-GFP line, generated by Brion et al., which expresses GFP under control of the cyp19a1b gene that encodes brain aromatase. The comparison between the models was run using the "EASZY" assay, developed by Brion et al., which utilises their cyp19a1b-GFP model in combination with image analysis software to screen oestrogenic chemicals and quantify the resulting flourescence responses.[48] For this comparison, the ERE-GFP-Casper model was also applied in the assay. The results from this study also contributed towards a validation project (Project 2.46) in the OECD Test Guideline Programme. Embryos from both models were exposed to 0.1, 0.05, 0.025, 0.0125, 0.00625, 0.003125, 0.001525 nM EE2, a DMSO control solution and water control solution from 0-5 dpf. Fluorescence response was observed on an inverted compound microscope (Zeiss Axio Observer) under consistent GFP excitation (134 ms using filter set 38 HE: BP 470/40, FT 495, BP 525/50). Larvae were embedded in methylcellulose for imaging with Cyp19a1b-GFP larvae orientated for a dorsal view of the brain and ERE-GFP-Casper larvae orientated onto their left side for imaging of the liver, the most sensitive tissue in the model. All images were acquired using a x10 objective. Photographs were analysed using the Axiovision Imaging software and fluorescence quantification was determined using an ImageJ software macro developed specifically for

quantifying fluorescence in the cyp19a1b-GFP model. For each picture, the integrated density was measured, i.e. the sum of the gray-values of all the pixels within the region of interest. Data were expressed as a percentage of the mean response to the highest EE2 concentration (0.1nM EE2), deemed the maximum (100%) response. Standard deviation values were calculated across the dosing groups and averaged to compare variation between the two models. Concentration–response curves were modelled using the Regtox 7.5 Microsoft Excel™ macro (available at http://www.normalesup.org/~vindimian/fr_index.html), which uses the Hill equation model and allows calculation of EC values. For a given chemical, EC50 was defined as the concentration inducing 50% of its maximal effect.

3.2.1 Generation of ERE-Kaede-Casper

The ERE-Kaede-Casper transgenic line was derived from the ERE-GFP-Casper line and a UAS-Kaede line derived from the Max-Planck Institute of Neurobiology, Germany (Figure 2). The UAS-Kaede line has wild-type (WIK) pigmentation and expresses an inserted UAS-Kaede reporter transgene sequence. To enhance the transmission rate of transgenic sequences to subsequent generations and to enhance consistency in oestrogen response sensitivity, the ERE-GFP-Casper model was initially screened for homozygous adults by pair breeding individuals with wild-type (WIK strain) adults. Homozygous ERE-GFP-Casper adults were then crossed with the UAS-Kaede line in 10 mating pairs, which were rotated through different pair combinations to promote genetic diversity. Progeny from these crosses were heterozygous for five genes of interest; ERE:Gal4ff, UAS:GFP and UAS:Kaede transgene sequences and silenced *roy* and *nacre* pigmentation genes. Crossing of

progeny from distinct families produced larvae with different combinations of the genes of interest. These larvae were screened for the homozygous ERE-Fluorescence and Casper genotype via exposure to 50 ng/L EE2 from 1-3 dpf. Individuals carrying both Casper phenotype and fluorescence expression, as determined by a response to 50 ng/L EE2, were raised to sexual maturity. Screening for Kaede expression was not carried out in this first screen due to potential developmental effects of UV exposure required for distinguishing the protein from GFP. At sexual maturity these fish were paired with wild indian karyotype (WIK) line fish and larvae were screened for Kaede expression via exposure to 50 ng/L EE2 for 5 days (from 0-5 dpf) and a 2 min UV exposure to convert the Kaede excitation and emission response. Adults with progeny exclusively expressing Kaede protein (complete conversion to red fluorescence upon UV exposure) were identified as the founder generation (F0) of the ERE-Kaede-Casper line. These fish were then bred to generate an F1 generation and subsequently sexually mature F1 fish were screened for homozygous expression of the transgene sequences.

Comparison of the responses to oestrogen in the ERE-Kaede-Casper with the ERE-GFP-Casper line.

Responses to EE2 in the ERE-Kaede-Casper line were compared with our original ERE-GFP-Casper model to assess for sensitivity and tissue response patterning to oestrogenic chemicals. To do so, embryos from both models were exposed to 5 and 10 ng/L EE2 from 0-5 dpf and fluorescence response was observed using an inverted compound microscope (Zeiss Axio Observer) under consistent GFP excitation (180 ms using filter set 38 HE: BP 470/40, FT 495, BP 525/50). In addition, ERE-GFP-Casper larvae from the control group were

compared with Casper individuals incubated in embryo water to assess for auto-fluorescence. Further generations have been established for this line, and each generation screened to confirm that only homozygous adults are used to ensure consistently of responses of the line.

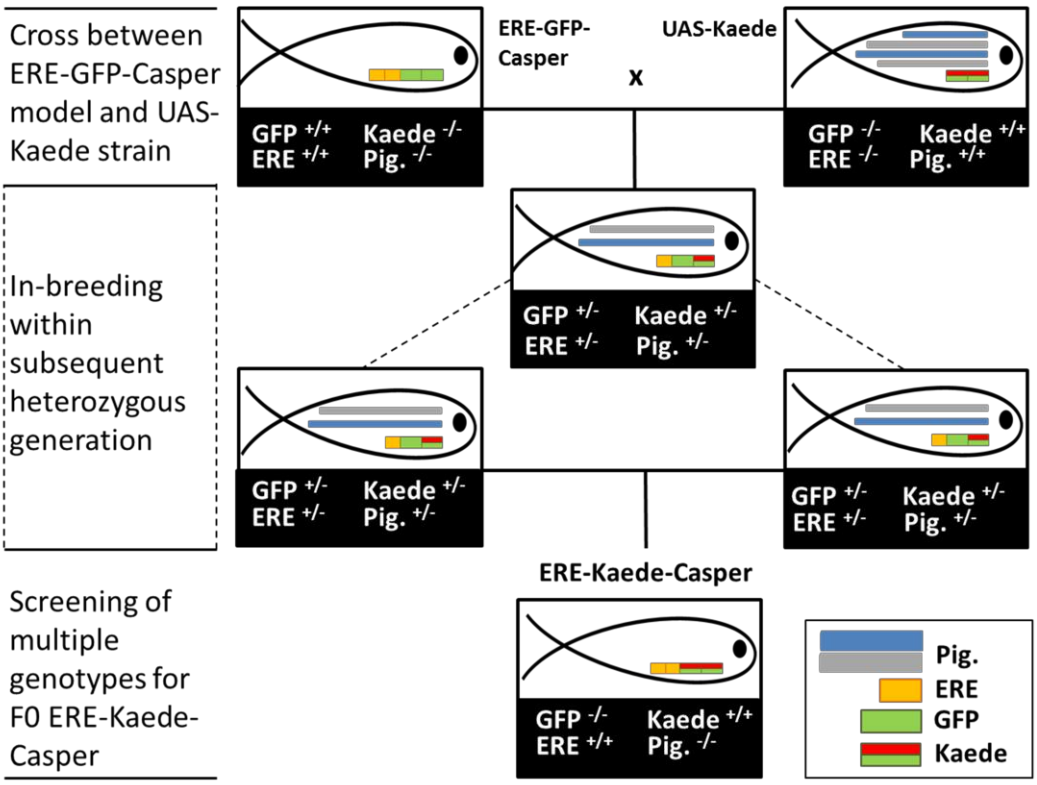


Figure 2: Generation of ERE-Kaede-Casper (F0) line. ERE denotes the ERE-Gal4ff transgene sequence, GFP denotes the UAS-GFP transgene sequence and Kaede denotes the UAS-Kaede transgene sequence. Expression of pigmentation (Pig.) genes *roy* (dark) and *nacre* (silver) are also shown. The ERE-GFP-Casper model, homozygous for both transgene sequences, and a homozygous UAS-Kaede strain were initially crossed to produce a heterozygous generation. In-breeding within this generation produced progeny with different genotypes based on four genes of interest. Larvae expressing F0 ERE-Kaede-Casper genotype with fully silenced pigmentation and TG(ERE:Gal4ff)(UAS:Kaede) expression were identified by screening for photoconvertible progeny and raised to sexual maturity.

Photoconversion of kaede in the ERE-Kaede-Casper model

To determine if full photoconversion of kaede was achievable in all tissues within the model, ERE-Kaede-Casper larvae were exposed to 100 ng/L EE2 over the period 0-5 dpf and exposed to UV light for 2 mins at the 5 dpf stage. A further group were exposed to 100 ng/L EE2 over the period 0-5 dpf with no exposure to UV light. Larvae were then subjected to imaging at 5 dpf on an inverted compound microscope (Zeiss Axio Observer). After imaging, DIC, green and red kaede fluorescence images were overlaid and the colour of individual tissue response qualified via observable ratios of green (new kaede expression), red ('old' kaede expression pre-photoconversion) and yellow (equal levels of new and old kaede expression) fluorescence.

Photoconversion during exposure in ERE-Kaede-Casper model

Photoconverting kaede in the ERE-kaede casper model allowed for assessing induction of new responses to oestrogen. In the validation process photoconversion of kaede (from green to red) was assessed in response to EE2 via imaging of the head and brain. ERE-Kaede-Casper larvae were exposed to 100 ng/L EE2 over the period 0-4 dpf and exposed to UV light for 2 mins at the 3 dpf stage. Larvae were then mounted in agarose and subjected to imaging (lateral and dorsal views) at 4 dpf on a confocal-laser scanning microscope (Zeiss LSM510), x10 objective and red and green fluorescent channels (BP 505-530 11%, LP 560 81.2%). After imaging, DIC, green and red Kaede fluorescence images were overlaid and the colour of cell or tissue response qualified by eye via observable ratios of green (new kaede expression), red ('old' kaede expression pre-photoconversion) and yellow (equal levels of new and old kaede expression) fluorescence.

3.3 Results

3.3.1 ERE-GFP-Casper model

A founder F0 generation of ERE-GFP-Casper model was successfully created and identified after phenotype screening and raised to sexual maturity (Figure 1).

F0 Homozygous Screen

Sexing of the 47 F0 adults identified 28 males and 19 females. Figure 3 shows the percentage of fluorescent larvae for the 47 individuals. In total, 21 individuals were assumed to express two or less of the four transgene copies and 20 assumed to express three of the copies due to ratios of fluorescent to non-fluorescent larvae falling below 60% and within 60-85% respectively. One individual was found to produce no fluorescent progeny (0%) and was likely included in the group in error at the larval stage. These adults were not used for generating an F1 generation. Only two individuals (both female) were found to produce 100% fluorescing larvae. Four males were found to produce 85+% fluorescing larvae. These four males were classed as homozygous and bred with the two homozygous F0 females to generate the subsequent F1 generation.

Comparing responses to oestrogen in the ERE-GFP and ERE-GFP-Casper lines

Comparison of the ERE-GFP Casper line with the original pigmented ERE-GFP model (Figure 4) demonstrated that the ERE-GFP-Casper line had a consistent tissue-specific responses and similar oestrogenic sensitivity as the original

transgenic line. There was an improved fluorescence detection signal with the ERE-GFP-Casper due to the lack of skin pigmentation compared with the original TG line. The advantages of pigment-free phenotype were most apparent from a dorsal view of the brain. Tissue-specific responses in the ERE-GFP-Casper model have been shown to be consistent in subsequent generations for which homozygous-only individuals are mainatained via regular screening. In the ERE-GFP-Casper model, auto-fluorescence occurred in the yolk sac, and was occasionally seen in the otic vesicle. Images from comparison with non-transgenic Casper (Figure 5) show yolk-sac (ys) fluorescence in both lines.

Comparing responses to oestrogen in the Cyp19a1b-GFP and ERE-GFP-Casper lines

The results from the comparison with the cyp19a1b-GFP model using the EASZY assay (Figure 6) demonstrated that the ERE-GFP-Casper model showed higher sensitivity to EE2 compared to the cyp19a1b-GFP model across a wide range of exposure concentrations. Comparison of sensitivity was based on EC50 values of 0.04nM EE2 for the ERE-GFP-Casper model and 0.12 nM EE2 for the cyp19a1b-GFP. One observable difference between the models was the high variability of response to all treatment concentrations in the cyp19a1b-GFP model compared with the ERE-GFP-Casper model, with relative standard deviation values of 25.3% and 8.2% respectively.

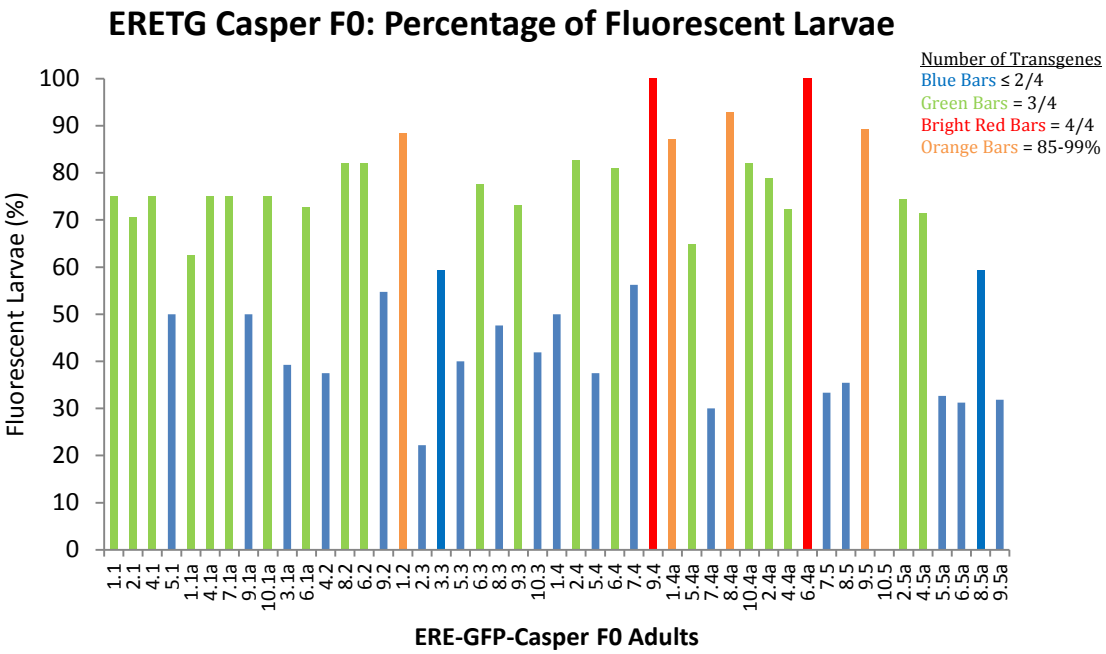


Figure 3: Screen of F0 ERE-GFP-Casper line for homozygous adults. Larvae from pairs of ERE-GFP-Casper and wildtype (WIK) adults were exposed to EE2 and assessed using fluorescent microscopy to identify percentages of fluorescing larvae from each adult ERE-GFP-Casper. ERE-GFP-Casper adults were then categorised based on suspected transgene genotype.

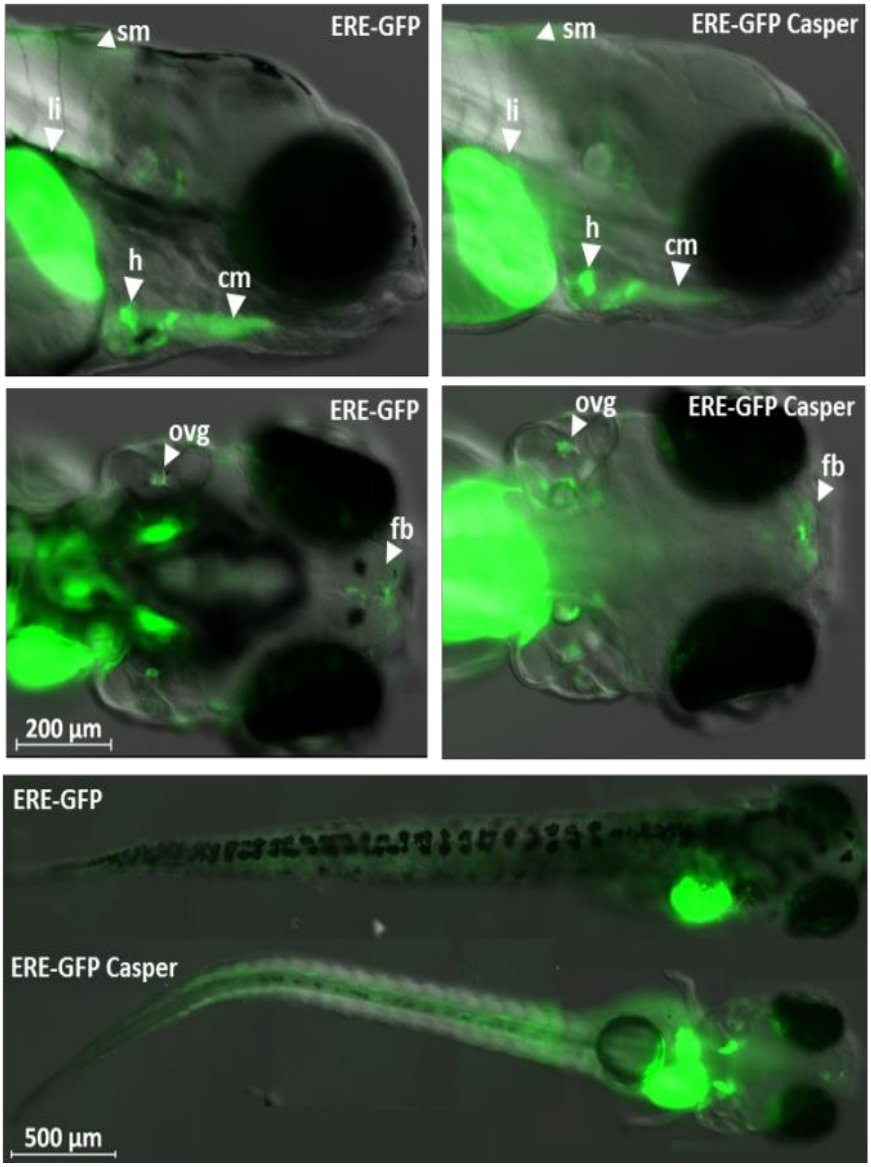


Figure 4: Comparison between ERE-GFP model and ERE-GFP-Casper models. Larvae were exposed to 50 ng/L EE2 from 0-5 dpf. Larvae were imaged at 5 dpf on an inverted compound microscope (Zeiss Axio Observer) at 10x magnification under consistent GFP excitation. Lateral and dorsal views are used for comparison. Specific tissue response in the liver (li), heart (h), cranial muscle (cm), somite muscle (sm), otic vesicle ganglions (ovg) and forebrain (fb) was consistent across the two models.

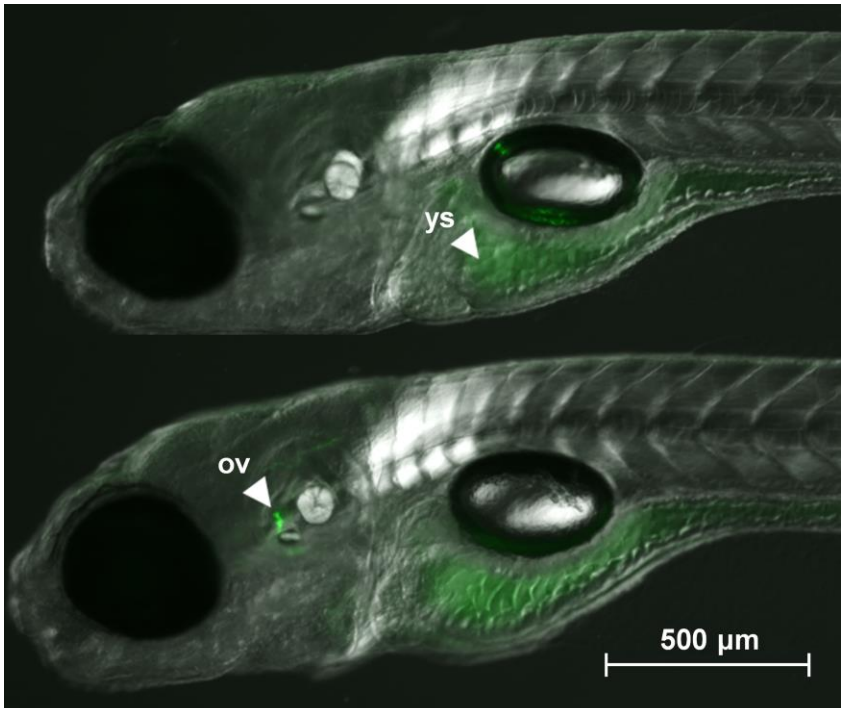


Figure 5: Auto-fluorescence in Casper and ERE-GFP-Casper lines. Caspers and ERE-GFP-Caspers were imaged at 5 dpf with no prior chemical exposure for comparison of auto-fluorescence on an inverted compound microscope (Zeiss Axio Observer). Images show yolk-sac (ys) fluorescence in both lines, which may result from naturally accumulated chemicals (such as polycyclic aromatic hydrocarbons) that fluoresce at similar excitation and emission wavelengths as GFP. GFP expression is additionally seen in the Otic vesicle (ov) of the ERE-GFP-Casper line only.

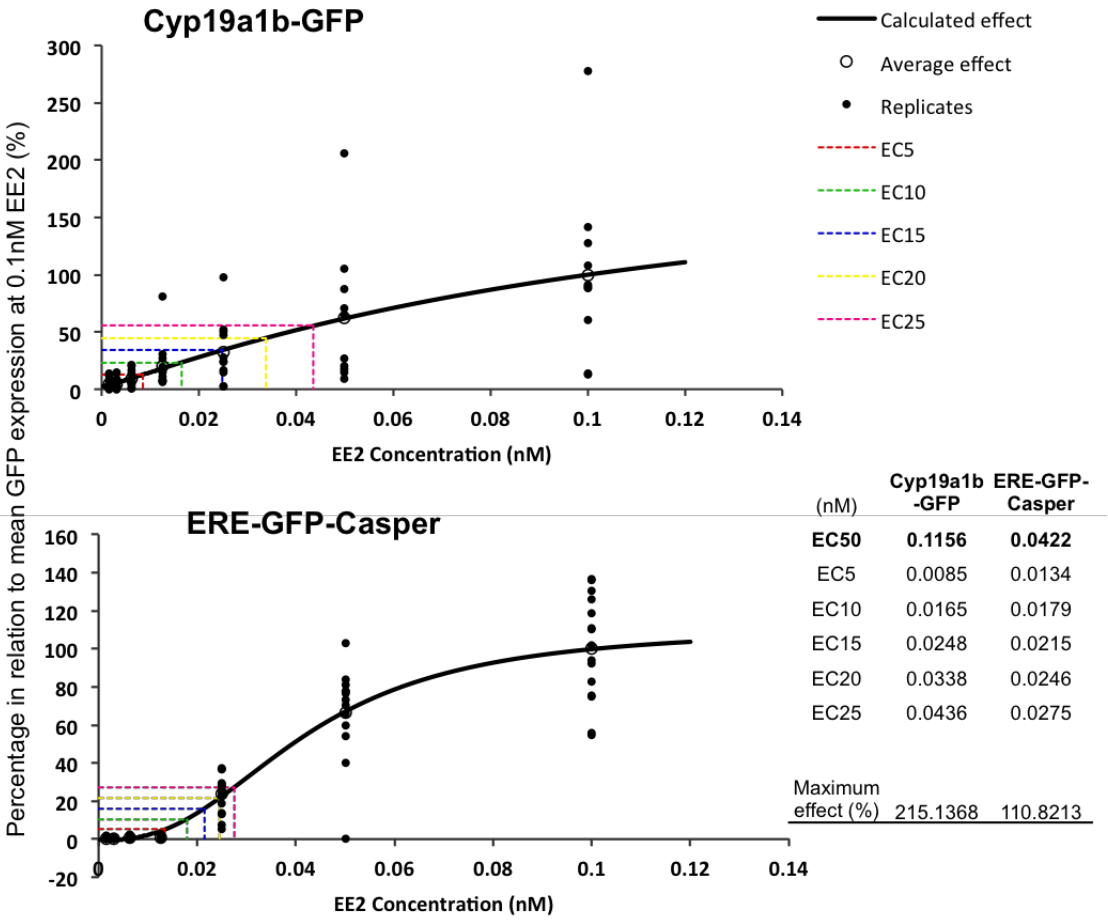


Figure 6: Comparison of ERE-GFP-Casper and cyp19a1b-GFP model using EASZY assay. Larvae from both models were exposed to a wide range of EE2 concentrations using the EASZY assay to generate dosing-curve profiles for comparison of sensitivity. Fluorescence responses in the models were quantified by image analysis (Image J) and presented as percentage in relation to the mean response at the highest dose of 0.1 nM EE2. Concentration–response curves were modelled using the Regtox 7.5 Microsoft Excel™ macro (available at http://www.normalesup.org/~vindimian/fr_index.html), which uses the Hill equation model (optimised for best fit) and allows calculation of EC values to compare relative sensitivities of the two models. Final Hill coefficient values were 1.13 for cyp19a1b-GFP and 2.57 for ERE-GFP-Casper.

3.3.2 ERE-Kaede-Casper Model

A founder F0 generation of ERE-Kaede-Casper model was identified via screening (see Figure 2) and a homozygous F1 generation generated and raised to adulthood to serve as a working stock for the exposure studies. Tissue-specific responses in the ERE-Kaede-Casper model were consistent in subsequent generations for homozygous individuals as assessed via regular screening.

Comparison with the ERE-GFP-Casper model showed there was high consistency in the response to oestrogen (tissue specificity and sensitivity) between the ERE-Kaede-Casper and the ERE-GFP-Casper models (Figure 7). Fluorescence intensity appeared slightly stronger in the muscle somites of the ERE-Kaede-Casper model. Under UV illumination kaede fluorescence was converted fully from green to red at 5 dpf (see Figure 8). Fluorescence responses in the head (lateral and dorsal views) of ERE-Kaede-Casper larvae at 4 dpf showed clearly defined temporal expression of kaede, having photoconverted kaede response at 3 dpf (Figure 9). Images show specific tissues and cells that had higher expression of kaede during the 0-3 dpf period (red), the 3-4 dpf period (green) and equal expression over the 0-4 dpf exposure period (yellow). Cells in the forebrain and midbrain had notably varied responses. Tissue-specific temporal responses are analysed in detail in Chapter 5 of this thesis, where the ERE-Kaede-Casper has been further utilised in this manner.

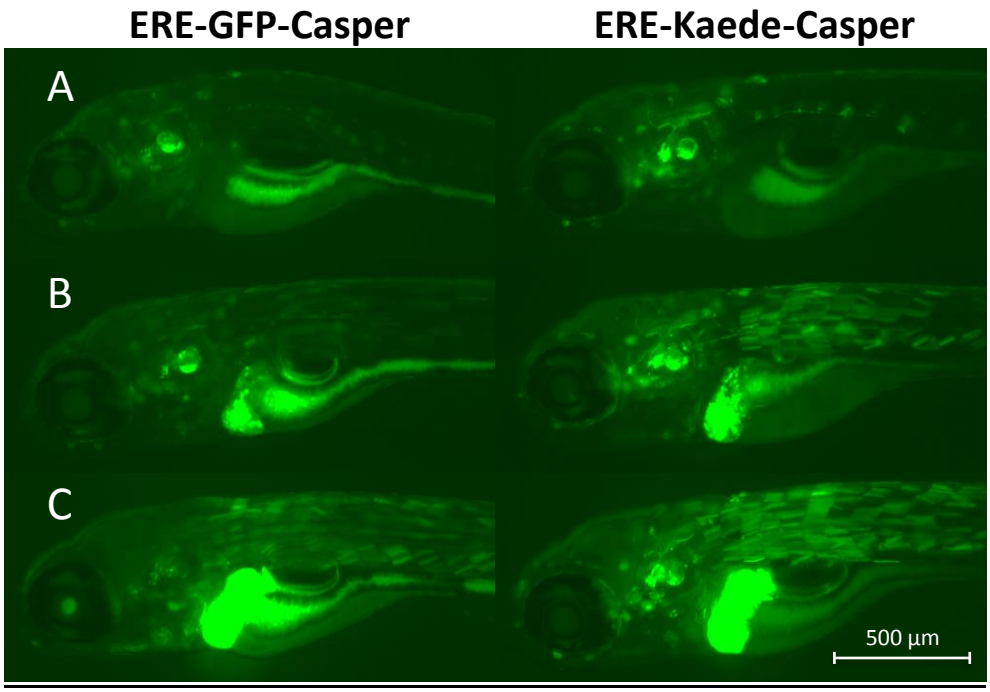


Figure 7: Comparison of ERE-GFP-Casper and ERE-Kaede-Casper models. Sensitivity and tissue response of the two models were compared via imaging after 0-5 dpf exposures to embryo water (A), 5 ng/L EE2 (B) and 10 ng/L EE2 (C). All images were acquired by inverted compound microscope (Zeiss Axio Observer) using a 5x objective. Images are presented with the GFP filter only.

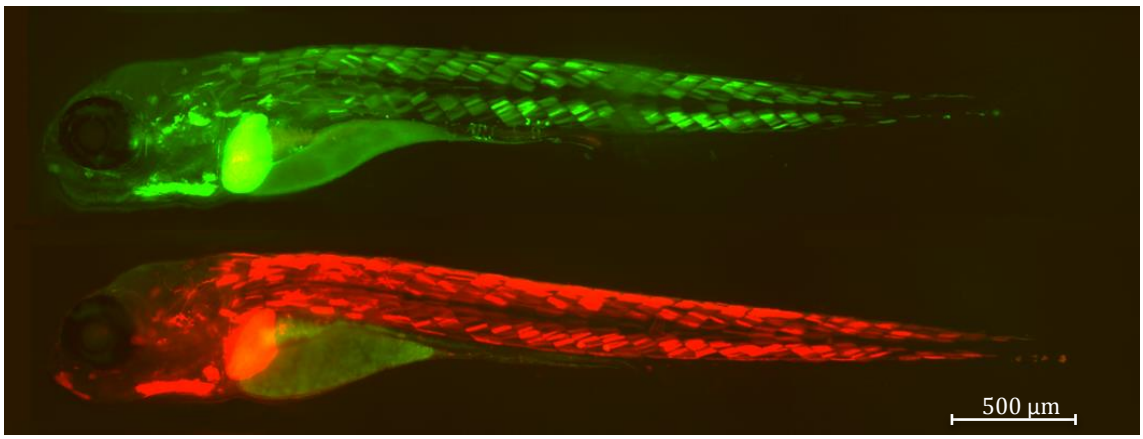


Figure 8: Kaede conversion analysis. ERE-Kaede-Casper larvae were exposed to 100 ng/L EE2 over the period 0-5dpf and imaged at 5dpf either without UV exposure (A), or after exposure to UV at 3dpf (B), 4dpf (C) and 5dpf (D) to convert Kaede fluorescence from green to red. Images acquired using a 5x objective on an inverted compound microscope (Zeiss Axio Observer).

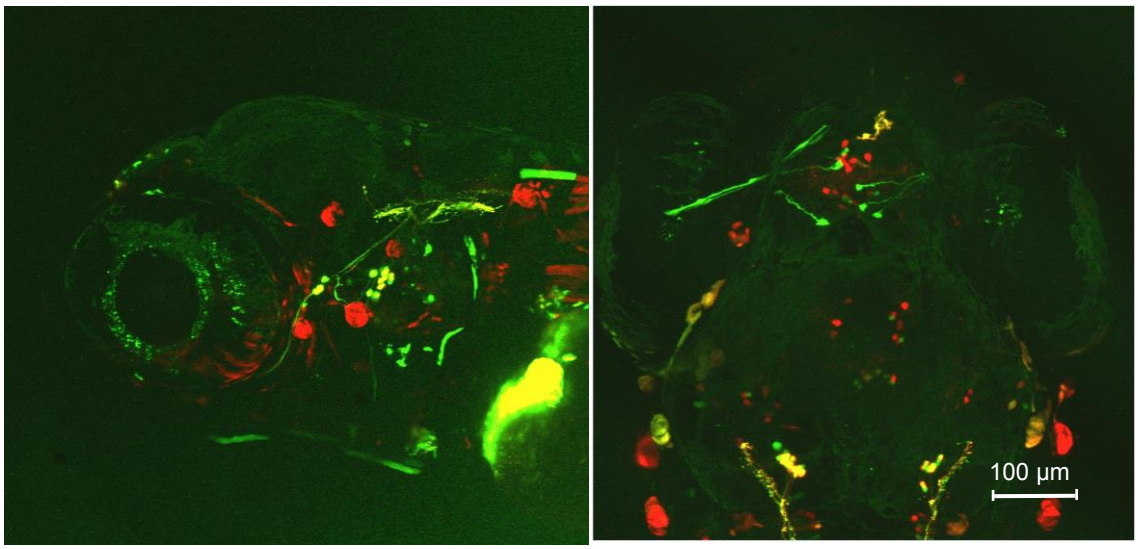


Figure 9: Kaede conversion during EE2 exposure. ERE-Kaede-Casper larvae were exposed to 100 ng/L EE2 over the period 0-4dpf, treated with UV light at 3 dpf and imaged at 4dpf. Images were acquired of the head at ventral and dorsal views a 10x objective on a confocal-laser scanning microscope (Zeiss LSM510). Red response indicates kaede expression during 0-3dpf and green response shows kaede expressed 3-4dpf. Yellow fluorescence indicates areas of continual kaede expression 0-4dpf.

3.4 Discussion

New oestrogen responsive transgenic zebrafish lines were generated and validated successfully for application to larval screening (semi-automated) and effective analysis of responses to oestrogens in zebrafish for different life stages. The translucent nature of these models with the casper gentotype facilitated improved fluorescence detection and quantification in specific tissues at various body orientations and for later stages of development compared with the original ERE-GFP model with pigmented skin. This work has resulted in models that are shown to share sensitivity and tissue-specific responses that are at least equal to the most sensitive oestrogen responsive transgenic zebrafish currently available, after qualitative and quantitative comparison of responses with the ERE-GFP and cyp19a1b-GFP models, and is further illustrated in their application in investigative studies presented in chapter 4 and 5 of this thesis.

Generation of ERE-GFP-Casper

The ERE-GFP-Casper model enables visualisation of oestrogen induced tissue-specific transcription in an intact vertebrate in real time. The ability to visualise responses to oestrogens in different body tissues simultaneously is valuable for establishing interconnections in target tissue responses for building knowledge both on the roles of oestrogens in normal function and the effect mechanisms of environmental oestrogen in vertebrates.

The required time to generate F0 ERE-GFP-Casper individuals via crossing of two separate zebrafish lines, was 5-6 months. Another 2-3 months was then required to raise the stock of F1 individuals. An alternative approach to generating the ERE-GFP-Casper would have been to use similar techniques

that were used to generate the ERE-GFP model; inserting ERE:gal4ff and UAS:GFP transgene sequences into the Casper embryos via microinjection (as opposed to the wild-type 'WIK' line). The use of transgenesis techniques offered the potential to generate the ERE-GFP-Casper model in a shorter time-span but this approach is less reliable due to varying degrees of efficiency of this approach.[196]

The ERE-GFP-Casper model had a consistent level of sensitivity and tissue specific responses to oestrogenic chemicals in comparison with the ERE-GFP model and the translucency of the ERE-GFP-Casper model appeared to reduce the effect of fluorescence signal-blocking caused by pigmentation, improving tissue response identification and potential quantification.

Screen for F0 Homozygous adults

The results from the F0 ERE-GFP-Casper screen identified a relatively small number (6 from 47) of homozygous individuals deemed suitable for producing a subsequent F1 generation. These six adults (four male, two female) were pair-bred on rotation to encourage genetic diversity in the F1 generation, however, this remains a low breeding population and risks associated inbreeding should be considered. Heavily inbred lines are found to differ in their susceptibility to chemical stressors in comparison with wild-type zebrafish lines[197] and can display altered gonadal development rates and sex ratios.[198] Future outcrossing of the line may be necessary to prevent altered sexual development or reduced sensitivity to oestrogenic chemicals.

For the majority of F0 ERE-GFP-Casper individuals, identification of genotypes was straightforward, with ratios of fluorescent to non–fluorescent larvae consistently falling within the <2/4, 3/4 and 4/4 transgene category stipulations.

However, of the six individuals categorised as homozygous (4/4 transgenes) only two females produced 100% fluorescent progeny, the other four individuals were males that produced fluorescent progeny at a 90+% transmission rate. This was consistently and significantly higher than ratios reported in adults deemed to express 3/4 transgene ratio (60-85% fluorescent progeny) and raises a question of inefficient transgene transmission to progeny from homozygous adult males in this model. Future analysis of a larger population of homozygous adults would be necessary to investigate this further. Information on average germline transmission rates using specific transgenesis techniques is well-documented[196] but transmission rates of transgenes through subsequent generations of established transgenic lines is less well known.

Comparing responses to oestrogen in the ERE-GFP-Casper lines and cyp19a-GFP

As a final element in assessing the new ERE-GFP-Casper model, the model was compared with the cyp19a1b-GFP model, a highly sensitive oestrogen biosensor and well-established transgenic line. This study showed that the ERE-GFP-Casper model displayed higher levels of sensitivity to EE2 using the EASZY assay protocol. It should be noted that the EC50 value for the cyp19a1b-GFP model reported here (0.12 nM) was significantly higher than the value reported in the published study using the EASZY assay (0.01 nM),[48] a value much closer to the EC50 result using the ERE-GFP-Casper model (0.04 nM). This is likely due to the high variation of response seen across the replicates using the cyp19a1b-GFP model (Figure 6). It is possible that this may have been caused by the presence of a large percentage of heterozygous and non-transgenic adults present in the cyp19a1b-GFP working stock at the

University of Exeter. Unlike the ERE-GFP-Casper line, this stock was not screened for homozygous individuals and these results indicate the importance of this process in maintaining high consistency in responses, as shown for the relatively low variation in responses to oestrogen seen for the ERE-GFP-Casper model.

The results from this study are based on an image analysis macro used to automatically identify and quantify fluorescence in the brain of the cyp19a1b-GFP model, but was also successfully applied to quantify fluorescence in the liver of the ERE-GFP-Casper model. Application of an image analysis macro designed specifically for the ERE-GFP-Casper may improve the overall sensitivity of fluorescence detection and quantification. Such an automated image analysis tool was generated using a Python algorithm for the ERE-GFP-Casper model and is presented in Chapter 4 of this thesis.

The results of the comparative studies conducted for the different models have demonstrated the ERE-GFP-Casper model responds to oestrogenic chemicals with high sensitivity and consistency. The model was subsequently applied in a novel chemical screening system that provides both semi-automated screening and high-content analysis of oestrogenic chemicals and their target tissues. The results from this screening system are presented in Chapter 4 of this thesis under “High-content and semi-automated screens (ERE-GFP-Casper)”.

The ERE-Kaede-Casper line

The ERE-Kaede-Casper model has a silenced skin pigmentation and high sensitivity to oestrogenic chemicals comparable with the ERE-GFP-Casper model (Figure 7). The screening process for the generation of the ERE-Kaede-Casper model differed to that of the ERE-GFP-Casper model, as two (not one)

fluorescent proteins were expressed in heterozygous progeny after the initial cross. This required the use of photoconversion of progeny in subsequent generations to identify individuals expressing the kaede transgene only, in addition to the casper phenotype. Hence, this generation of the ERE-Kaede-Casper model required an additional in-crossed heterozygous generation (2-3 months) to account for this screening step. The total time taken to establish a homozygous F1 working stock, therefore, was 11-12 months compared to the 8-9 months required for the ERE-GFP-Casper line. This demonstrates the greater challenges in crossing two transgenic lines compared with crossing with a transgenic and non-transgenic line. The requirement of photoconversion for screening also extended the time needed to generate the ERE-Kaede-Casper line.

The kaede chromophore was successfully photoconverted in living intact individuals in all responding tissues and for high levels of kaede expression (Figure 8). Translucency of the skin likely assisted efficiency of photoconversion as pigmentation normally blocks UV light penetration into the deeper tissues in larvae. Photoconversion of response in the ERE-Kaede-Casper model during exposure was used to identify windows of sensitivity to EE2 for specific tissues during early development (Figure 9). The ability to photoconvert the kaede fluorescence response in the ERE-Kaede-Casper model provides a more dynamic model for studies into temporal tissue response and assessment of sequential exposures to oestrogens compared with the ERE-GFP-Casper model. Results from these investigations using the ERE-Kaede-Casper model are provided in Chapter 5 of this thesis under “A novel transgenic model reveals increased sensitivity to repeated oestrogen exposure”.

Chapter 4

Paper 1: High-Content and Semi-Automated Quantification of Responses to Oestrogenic Chemicals Using a Novel Translucent Transgenic Zebrafish

Paper published in Environmental Science and Technology

Environ. Sci. Technol., 2016, 50 (12), pp 6536–6545

DOI: 10.1021/acs.est.6b01243

Publication Date (Web): May 26, 2016

Chapter 4

High-Content and Semi-Automated Quantification of Responses to Oestrogenic Chemicals Using a Novel Translucent Transgenic Zebrafish

Jon M. Green[1], Jeremy Metz[1], Okhyun Lee[1], Maciej Trznadel[1], Aya Takesono[1], A. Ross Brown[1], Stewart F. Owen[2], Tetsuhiro Kudoh[1], Charles R. Tyler[1*]

[1]Biosciences, College of Life and Environmental Sciences, University of Exeter, Geoffrey Pope, Stocker Road, Exeter, Devon, EX4 4QD, UK.

[2]AstraZeneca, Global Environment, Alderley Park, Macclesfield, Cheshire, SK10 4TF, UK

4.1 Abstract

Rapid embryogenesis, together with genetic similarities with mammals, and the desire to reduce mammalian testing, are major incentives for using the zebrafish model in chemical screening and testing. Transgenic zebrafish, engineered for identifying target gene expression through expression of fluorophores, have considerable potential for both high-content and high-throughput testing of chemicals for endocrine activity. Here we generated an oestrogen responsive transgenic zebrafish model in a pigment-free 'Casper' phenotype, facilitating identification of target tissues and quantification of these responses in whole intact fish. Using the ERE-GFP-Casper model we show

chemical type and concentration dependence for green fluorescent protein (GFP) induction and both spatial and temporal responses for different environmental oestrogens tested. We also developed a semi-automated (ArrayScan) imaging and image analysis system that we applied to quantify whole body fluorescence responses for a range of different oestrogenic chemicals in the new transgenic zebrafish model. The zebrafish model developed provides a sensitive and highly integrative system for identifying oestrogenic chemicals, their target tissues and effect concentrations for exposures in real time and across different life stages. It thus has application for chemical screening to better direct health effects analysis of environmental oestrogens and for investigating the functional roles of oestrogens in vertebrates.

4.2 Introduction

There is increasing evidence that exposure to endocrine disrupting chemicals (EDCs) may be linked to a range of endocrine-related disorders and the need to identify and assess EDCs is crucial for safe guarding long term human and environmental health.[1, 2] EDCs shown to interact with the oestrogen signalling pathway have gained major attention over the last 20 years.[1] To date, over 200 chemicals have been identified that are oestrogenic.[3] Many EDCs enter the aquatic environment via waste discharges and for certain environmental oestrogens there are strong associations between known exposures and adverse health effects in individual fish[4, 5] and fish populations.[6, 7] In mammals, exposure to environmental oestrogens has been associated with decreases in

semen quality/sperm count,[8] heart disease and diabetes[9]. In fish there have been strong links to feminization of males[4] and alteration of sexual behaviour.[10]

Estrogen signalling occurs via oestrogen binding to oestrogen receptors (ERs) in the nucleus, which then dimerise and bind to oestrogen response elements (EREs) located in promoters of target genes.[11] There are two receptor subtypes in mammals, ESR1 and ESR2,[12] and three in zebrafish, ESR1, ESR2a and ESR2b.[13, 14] ESR1 and ESR2 subtypes in humans and zebrafish are corresponding orthologs with high amino acid sequence similarity for their respective ligand binding domains of 64% and 71%.[15] In addition, oestrogen-related receptors (ERRs), a small group of orphan nuclear receptors, share some target genes with ERs.[16] Membrane ERs (mERs) also occur, and although some of their features have been shown to be very similar to nuclear ERs, their role(s) and mechanism(s) of action are still unclear.[17, 18] The expression of these different receptors in organs and tissues can change throughout development and later life and the affinity of ligands for these subtypes varies between oestrogenic chemicals influencing the physiological targets and subsequent downstream effects.[19] Tissue-specific expression of oestrogen receptor subtypes in humans is well characterised[20, 21] but is less well established in other organisms. Gene ontology studies suggest endogenous oestrogen signalling plays a number of important roles in early stage zebrafish development, including regulation of metabolism, transcription, tissue development and protein folding and trafficking.[22] oestrogens are fundamental in the growth and development of both the female and male gonads.[23-25] In addition, oestrogens are known to play key roles in immune responses, the central nervous system, and normal somatic cell growth.[26, 27]

Concern over EDCs has resulted in the establishment of national and international screening and testing programmes, including for environmental oestrogens.[28-30] *In vitro* approaches with cell lines using reporter gene sequences typically focus on oestrogen receptor binding and/or activation by a chemical ligand and they have been used to compare oestrogenic chemical specificity for fish and human receptor subtypes.[31, 32] In some instances, however, different assays employing the same ER have been shown to have different sensitivities to the same oestrogenic chemicals.[33, 34] Furthermore, oestrogen signalling is complex and *in vitro* systems are limited in their ability to predict potency, illustrate tissue selectivity or the possible functional consequences of a chemical in an intact organism. In vitro systems are also much less able at detect pro-estrogenic compounds due to the fact that most are limited in their capacities for chemical metabolism.[35]

Transgenic zebrafish models (TG) offer potential for both high-content and high-throughput testing of chemicals for endocrine activity in EDC screening frameworks. Various TG models have been developed for studying the effects of xenoestrogens[36-39] and one is now being applied in screening systems for identifying and quantifying responses to oestrogenic chemicals. This assay, the EASZY assay, developed by Brion et al.[36] operates through aromatase induction (an oestrogenic response pathway) and is currently a validation project (Project 2.46) in the OECD Test Guideline Programme.[40]

Here we describe the development of a new TG zebrafish with a fluorescent Oestrogen Response Element (ERE) reporter[37] and silenced skin pigmentation (Casper phenotype) and its application both for high content analysis and semi-automated chemical screening with image analysis for quantifying whole body responses to oestrogenic chemicals.

4.3 Methods and Materials

Fish Husbandry and experiments

All experimental procedures conducted in this research with fish were in accordance with UK Home Office regulations for the use of animals in scientific procedures and followed local ethical review guidelines ensuring their humane treatment.

Generation of ERE-GFP-Casper

The ERE-GFP-Casper transgenic line was derived from a ERE-GFP line developed at the University of Exeter,[37] and a Casper line from University College London (Figure 1). The ERE-GFP line is sensitive to oestrogens and the tissue responses are consistent with another ERE-GFP model, developed independently by Gorelick et al.[38] The Casper line has silenced *roy* (dark) and *nacre* (silver) pigmentation genes, resulting in highly translucent skin.[41] To increase the transmission rate of transgenic sequences to subsequent generations and to ensure consistency of oestrogen response sensitivity, the ERE-GFP model was initially screened for homozygous adults by pairing with wild-type (WIK strain) adults and assessing the ratio of fluorescent/non fluorescent offspring. Homozygous adults were then crossed with the Casper line in five mating pairs, which were rotated to promote genetic diversity. Progeny from these crosses were heterozygous for four genes of interest; the ERE:Gal4ff and UAS:GFP transgene sequences and silenced *roy* and *nacre*

pigmentation genes. Crossing of progeny from distinct families produced larvae with varying combinations of the genes of interest. These larvae were screened for the homozygous ERE/Casper genotype via exposure to 50 ng/L of 17α-ethinylestradiol (EE2) from 0-3 dpf. Individuals carrying both Casper phenotype and GFP expression were raised to sexual maturity. Finally, these fish were screened for homozygous expression of the transgene sequences to produce the founder generation (F0) of the ERE-GFP-Casper line. We compared responses to EE2 in the ERE-GFP-Casper line with our original ERE-GFP model to assess for sensitivity and tissue response patterning to oestrogenic chemicals. Embryos from both models were exposed to 50 ng EE2/L from 0-5 dpf. Fluorescence response was observed on an inverted compound microscope (Zeiss Axio Observer) under consistent GFP excitation (180 ms using filter set 38 HE: BP 470/40, FT 495, BP 525/50). Further generations have been established, and each generation screened to confirm that only homozygous adults are used for ensuring consistently high sensitivity. F1 generation TG(ERE:GFP) Casper larvae were used in the study reported here and compared with F6 generation TG(ERE:GFP) larvae.

Zebrafish embryo chemical exposures

Five chemicals were chosen for testing and screening with our model; 17α-ethinylestradiol (EE2, Chemical Abstracts Service CAS no. 57-63-6) used in the contraceptive pill, bisphenol A (BPA, 80-05-7), used widely as a plasticizer, 4-nonylphenol mix (NP, 84852-15-3), a breakdown product from nonylphenolethoxylates used in industrial surfactants,[42] genistein (Gen, 446-72-0), a phytoestrogen, and ketoconazole (KCZ, 65277-42-1), a fungicide shown to be mildly anti-androgenic. EE2, NP, genistein and BPA are well documented

oestrogenic chemicals and commonly found on prioritised chemical lists for international EDC screening programmes. Ketoconazole was used as a negative control. BPA (99+%) was purchased from American Chemistry Council, all other chemicals were purchased from Sigma-Aldrich Chemical Co. (≥98%). Dosing for each chemical comprised of six to eight different concentrations ranging between mean measured environmental exposure concentrations obtained from published literature and no observed effect concentrations (NOECs) measured in this and other published studies. Stock chemicals for each concentration were dissolved in analytical grade dimethyl sulfoxide (DMSO) and stirred vigorously in glass vials 24 hours before exposure and stored at 4˚C. Required volumes of stock solution were pipetted into 40-50mL embryo culture water and stirred vigorously to give 1.25x final nominal concentration working solutions prepared on the morning of exposure. 800µl of 1.25x working solution was pipetted into each well of a 24-well plate, one plate per concentration. ERE-GFP-Casper embryos were pipetted into individual wells in 200µl embryo water, giving a final 1x nominal chemical concentration in the well and a DMSO concentration of 0.5%. The embryos were exposed from 4 to 120 hours post fertilisation (hpf) at 28˚C with no media changes. All chemicals were run on separate days and exposures were repeated three times.

Analysis of chemicals in exposure water

To assess the concentrations of chemicals in the exposure water in the 24 well plates, nominal aqueous exposure concentrations were tested at day 0 and day 5 of exposure (i.e. 0 and 5 dpf). Solvent control, minimum and maximum concentrations were sampled for each chemical from three random wells per

plate. For all chemicals, with exception of EE2, water samples were diluted in acetonitrile (ACN) before analysis by tandem liquid chromatography-mass spectrometry (LC-MS). Due to the low concentration of EE2, samples were initially run through solid phase extraction (SPE) cartridges (Sep-Pak Plus C18) into ACN, to achieve a detectable concentration for LC-MS analysis ("Water Chemistry: LC-MS Method" in Supporting Information).

Target Tissue Analysis

To resolve tissue-specific oestrogen responses, high quality images of exposed larvae were acquired for one of the three repeated chemical exposures by conducting confocal imaging during the automated ArrayScan imaging (see below). Five live larvae per concentration were washed and anaesthetised in embryo water containing 0.008% tricaine and mounted in 0.7% low melting agarose in embryo culture medium and placed into a glass bottom 35mm dish (MatTek)**.** Larvae were orientated left side down and images obtained using a Nikon A1R confocal microscope with a 4x objective under consistent 'GFP' laser excitation only. After acquisition, all images were re-orientated and combined for presentation using GIMP software (Version 2.8.14, https://www.gimp.org/). Tissue-specific GFP expression was qualified, but not quantified, for the target tissue analyses.

ArrayScan imaging

After exposures, larvae were washed and anaesthetised in embryo water containing 0.008% tricaine. Twelve larvae per concentration were pipetted into a 96-half well plate and, where possible, orientated onto their left side. An ArrayScan II high-content reader (Cellomics, Inc., Pittsburgh, PA) was used for

image acquisition. The system was customised by installation of a nonstandard ×1.25 magnification objective (Olympus) to achieve whole embryo capture in one image. A form factor program was generated for the half-well plates to ensure the objective was correctly centred above each well and the auto-focus tool used to attain clear images. A single 512x512 pixel image capturing the whole organism GFP response was acquired for each well using a 16 second fluorescent light (488 nm) exposure. With the autofocus tool selected, a full 96-well plate imaging took approximately one hour and 30 minutes.

Image analysis

We developed a custom Python pipeline to quantify larvae-specific fluorescence in the ArrayScan images ("Semi-Automated Image Analysis" in Supporting Information). The algorithm first identified the well within the image and then applied a mask (a tracing of the larvae outline) around the larvae, which was checked and confirmed by eye using a web-based interface (Figure S1). An average intensity value of the top 100 pixel intensity values within the mask was then used as a quantification of fluorescence response, as these represented the strongest response values. These steps improved the speed and sensitivity of fluorescence measurement, as well as removing influence of anomalous reflective particles in the well.

Statistical Analysis

In the ArrayScan analyses, intensity values were averaged per concentration from three repeated exposures. All values are presented as mean ± SEM. Statistical significance is indicated at the $p<0.05$(*) or <0.01(**) level and calculated using an ANOVA and Games-Howell post-hoc test. EC50 values and

their confidence intervals (±CI 95) were calculated using Regtox 7.5 Microsoft Excel™ macro (available at http://www.normalesup.org/~vindimian/fr_index.html), which uses the Hill equation model. EC50 was defined as the concentration inducing 50% of a chemicals maximal fluorescence response from a basal control response.

4.4 Results

ERE-GFP-Casper model

After crossing the ERE-GFP model and Casper line and their progeny, the founder F0 generation of ERE-GFP-Casper model was successfully identified after phenotype screening and raised to sexual maturity (Figure 1). The results of the comparison with the original pigmented ERE-GFP model (Figure 2) demonstrated that the ERE-GFP-Casper line had maintained consistent tissue-specific responses and oestrogenic sensitivity as the original transgenic line. There was, however, an improved observable fluorescent signal in certain tissues with the ERE-GFP-Casper due to the lack of skin pigmentation compared with the original TG line. The advantages of pigment-free phenotype were most apparent from a dorsal view of the brain. Tissue-specific responses in the ERE-GFP-Casper model have been shown to be consistent in subsequent generations in which we maintain homozygous-only individuals via regular screening.

Water Chemistry Analysis

In all solvent control samples chemicals were less than the limit of quantitation (LOQ). For genistein, BPA, and EE2 measured concentrations were highly

consistent, at between 99% and 133% of day 5 nominals across the concentration ranges tested. The highest measured ketoconazole concentration (nominal 5000 µg KCZ/L) was consistent over the exposure period (93-108%), but the measured concentrations for the lower nominal concentration (40 µg KCZ/L) were significantly lower on day 5 compared with day 0 (12% and 28%, respectively). Measured concentrations for NP were also somewhat lower than nominal. For the highest NP exposure concentration (nominal 2000 µg NP/L), the measured concentration was 84% of nominal on day 0, and 34% on day 5, and for the lower exposure (nominal 125 µg NP/L) the measured concentrations were 54% and 13% of nominal on days 0 and 5, respectively. Chemical analysis of the working solutions prepared in glassware for these two chemicals showed the measured concentrations were 101% of nominal (156.25 µg NP/L) and 79% of nominal (50 µg KCZ/L) for NP and ketoconazole, respectively on day 0. Exposure concentrations are reported as ng/L or µg/L in text but nM concentrations are included where direct comparisons between chemicals are made in both text and figures. Results for the full water chemistry analysis are provided in Table S3.

Target Tissue (High Content) Analysis

ERE-GFP-Casper larvae were analysed by confocal microscopy to assess the sensitivity of the model and compare tissue-specific fluorescence responses between different chemicals across a range of chemical concentrations. Confocal images, shown in figures 3A-D, demonstrate that the oestrogenic chemicals induced responses in different tissues and differed in their potencies (Figure 4). In the model auto-fluorescence occurred in the yolk sac, and occasionally in the otic vesicle.

The ERE-GFP-Casper model detected responses for exposures to environmentally relevant concentrations of EE2. In the confocal images, fluorescence responses in individual liver cells were detected for exposures as low as 1 ng EE2/L (Figure 3A). At 2.5 ng EE2/L, multiple liver cells and somite muscle tissue were seen to fluoresce strongly and faint fluorescence was also observed in the heart valves. Exposure to 5 ng EE2/L resulted in strong fluorescence in all these tissues with a response also seen in the otic vesicle. Neuromast cells in close proximity to the caudal fin and cells in the pelvic fins (thought to be erythrophores) were also seen to fluoresce. There were also faint responses in the cranial muscle and corpuscle of Stannius. Response in the eye lens and forebrain was observed at exposure concentrations of 25 ng EE2/L and above. At the highest exposure concentration adopted (100 ng EE2/L) oestrogenic responses occurred across a very wide range of tissues types with high intensity, including the gut. BPA exposure resulted in a different response profile compared with EE2. There was a very weak GFP signal detected in the heart valves at the lowest exposure concentration adopted (62.5 µg BPA/L; Figure 3B), that was more intense at higher concentrations of BPA. At an exposure of 500 µg BPA/L tissues responses were detected in somite muscle, corpuscle of Stannius and a small number of liver cells. For high concentration exposures to BPA (1000 - 2000 µg BPA/L) there was a strong hepatic response. In a similar manner to that seen for BPA, genistein induced highly localised GFP expression in the heart valves (62.5 µg genistein/L) and in the corpuscle of Stannius (Figure 3C). Responses to genistein were also detected in somite tissue and fin cells for an exposure to 125µg genistein/L, that were not seen for the BPA exposure. For exposures to genistein at between 250 – 1000 µg genistein/L liver, somite muscle, cranial muscle, eye lens and

gut cells showed strong expression of GFP. The response pattern for exposure to 1000 µg genistein/L (3700 nM) was similar overall to that for 100 ng EE2/L (0.374 nM). NP exposure induced a similar response to that seen for genistein, with an apparent higher potency based on equi-molar concentrations (Figure 3D). A notable difference between NP and genistein was the apparent lack of GFP expression in the corpuscle of Stannius for NP (even for the highest exposure concentration, 1000-2000 µg NP/L). There were teratogenic/toxicological effects for NP at exposures of 1000 and 2000 µg NP/L. Confocal images of larvae exposed to ketoconazole showed no specific responses, with some natural auto-fluorescence detected in the yolk sac and otic vesicle only.

Semi-automated ArrayScan Imaging for Responses to *Oestrogenic* Chemicals

After chemical exposure, the ArrayScan system was used to automatically acquire images of exposed larvae in a 96 half-well plate. The automated masking correctly masked 80% of larvae (as assessed by the interactive online interface) and those not masked correctly were discounted. GFP responses in masks were quantified to produce response curves (Figure 5). The semi-automated screening assay identified correctly the four oestrogenic chemicals; ketoconazole (the negative control) was the only chemical that did not show an oestrogenic response. Lowest statistically significant response (LSR) values were calculated to indicate the potency of the test chemicals to induce an oestrogenic response (Table 1). EC50 values were calculated as alternative measurements of oestrogenic potency and both values were used to compare the sensitivity of the screening assay with other *in vivo* and *in vitro* assays. The

quantified responses to the different oestrogens accurately reflected the relative potencies observed by confocal microscopy in the target tissue analyses described above. EE2 showed a >10,000 fold higher overall (whole body) potency compared with the other oestrogenic chemicals tested with substantially lower LSR and EC50 values at 0.005 µg EE2/L (0.0093nM) and 0.0096 µg EE2/L (0.0178 nM), respectively. NP and genistein had similar overall potencies, with genistein showing a lower LSR of 125 µg genistein/L (462.5 nM) to NP's 250 µg NP/L (1135 nM) but a higher EC50 of 463.4 µg genistein/L (1689 nM) to NP's 212.2 µg NP/L (1187 nM). BPA was the least potent oestrogenic chemical screened with an EC50 of 1226 µg/L (5393 nM).

The response curves for EE2 and genistein showed a similar sigmoidal response profile. The BPA response curve indicated a steeper response gradient, which is most likely due to the strong fluorescence response in the liver at 1000 µg BPA/L. At the two highest exposure concentrations for NP (1000 and 2000 µg NP/L) there were teratogenic/toxic effects (as seen in the target tissue analysis Figure 3D), and this was reflected by reduced fluorescence responses in the automated ArrayScan analyses (these toxic exposures were not included in the oestrogen response curve in Figure 5). There was a very high consistency in the responses between individual embryos for the different chemicals and exposure concentrations as illustrated by the low variance in the data (SEM across all chemical and exposure concentrations were <±14% of mean intensity).

4.5 Discussion

We have generated an oestrogen responsive transgenic model ERE-GFP-Casper for application to both chemical screening and high content analysis of

oestrogenic chemicals with high sensitivity and consistency. The model enables visualisation of oestrogen induced tissue-specific transcription in an intact vertebrate in real time. The ability to visualise responses to oestrogens in different body tissues simultaneously will help establish interconnections in target tissue responses for building knowledge both on the roles of oestrogens in normal function and the mechanisms of environmental oestrogen effects in vertebrates. In the target tissue analysis liver cell responses to EE2 were detected for exposures down to 1 ng EE2/L, showing the model can be applied to test for environmentally relevant exposure concentrations of EE2[43] and the model is equally/more sensitive than for other oestrogen responsive zebrafish models currently available,[36, 38] and the most commonly used *in vitro* assays.[44-46] A major attribute of the ERE-GFP-Casper model is the lack of skin pigmentation facilitating its application to high content tissue analysis, allowing for more effective analysis of responses in deeper body tissues, and (semi)automated screening where problems associated with body orientation on fluorescence detection and quantitation are much reduced compared with pigmented TG lines. Skin translucency in the ERE-GFP-Casper model makes it more effective for detecting oestrogen responses in older life stage fish for visualising the roles of oestrogens on gonadal sexual differentiation (that occurs between 35-42 dpf in zebrafish)[47] and in brain development (A. Takesono, pers.comm). Problems associated with fluorescence detection, however, are not overcome completely in our ERE-GFP-Casper model and in older fish (more than 30 dpf) the thickness of the body wall does reduce fluorescence detection for tissues seated deeply in the body cavity (our own unpublished data).

Responses to the chemicals tested showed the heart, liver, muscle somites, cranial muscle and fin responded to each of the selected oestrogens with

varying sensitivities. Other tissues such as brain, eye lens, gut, corpuscle of Stannius and otic vesicle responded only to certain chemicals. EE2 was by far the most potent oestrogen tested, as expected and induced responses in a very wide range of tissues, including heart, liver, somite muscle, corpuscle of Stannius, cranial muscle and forebrain, highlighting the potential for a wide range of biological effects following exposure to this synthetic oestrogen. Responses in the brain are consistent with reported adverse effects of EE2 exposure on brain tissue and effects on sexual behaviours in both mice and zebrafish.[48-50] The diverse tissue responses we observed are reflective of the wide range of known target tissues for the natural oestrogen (estradiol, E2) including sexual organs, bone, liver, brain, pancreas, adipose tissue, skeletal muscle and cardiovascular systems.[27, 51] BPA induced a different response pattern to EE2, with only the heart responding strongly, except for very high exposure concentrations where the liver and muscle somites were also shown to be responsive. Genistein and NP appeared to be similar in the tissue responses induced (and relative potencies), but even here there were clear differences, with a response in the corpuscle of Stannius and cells in the gut for genistein, but not for exposure to NP. In addition to the response seen in the brain for exposure to EE2, there were responses seen also in the brain at the highest exposure concentrations for BPA and genistein (as illustrated in Fig 3A) and this is consistent with those observed in our previously developed (pigmented) ERE-GFP model,[36] and for other studies on fish exposed to these chemicals. At higher magnification and using a dorsal orientation of the larvae, GFP expression in the brain was more clearly visible and occurred for exposures at lower exposure concentrations for BPA, genistein and NP. Nevertheless, the ERE-GFP-Casper model can provide a fingerprint for

oestrogenic compounds based on the pattern of tissue-specific responses. The high-content target tissue analysis we present may also have applications in screening selective oestrogen receptor modulators (SERMs), a diverse group of chemicals that display tissue specific oestrogenic agonist and antagonist activity.[52]

The high sensitivity of the heart to oestrogenic chemicals is consistent with responses seen in another oestrogenic responsive transgenic zebrafish line exposed to environmental water samples[53] and mounting data linking BPA to potential adverse effects on the cardiovascular system.[54] The corpuscle of Stannius in fish is an endocrine tissue thought to mimic the role of the parathyroid gland in other vertebrates in regulating calcium metabolism.[55] In humans oestrogens play a key role in regulating bone formation (density).[56] Here the high sensitivity of the corpuscle of Stannius to both genistein and EE2 suggests that exposure to these chemicals could have implications for bone formation and integrity in fish.

In the exposures studies for NP, unlike that for all the other oestrogenic chemicals tested, measured levels were considerably lower than nominals (on day 5), which may have caused us to underestimate the potency of NP in the effects analyses reported for this chemical. This finding for alkylphenolic chemicals, such as NP, is not uncommon[10] and probably relates to its absorptive properties and its tendency to stick the culture plate walls, or even to 'creep' (move out of culture well plates) in well plate culture systems.[57]

We emphasize that GFP responses seen in this study are from the inserted ERE and surrounding sequence, which may not be effective for all oestrogenic signalling systems. ERs that work through Sp1 or AP1 sites would not be

detected by this assay. The mechanisms of action for each of the selected oestrogens are still largely unclear, but different tissue-specific responses can be used to guide further work in this area. *In vivo* studies are required to further understand the interactions of oestrogenic chemicals with ERs, including the recruitment of different co-factors and/or co-opting of other receptors linked to the oestrogen signalling pathway.

Our TG model is designed to capture responses to all ER subtypes (the ERE element will bind all 3 ER subtypes in zebrafish), and thus we cannot say from the responses seen whether the different chemicals tested are mediating GFP responses via the same or different ERs or potentially ERRs and mERs. Studies combining our model with morpholinos for antagonising specific ER subtypes, or CRISPR-Cas or TALENS used to silenced ER subtype expression could help to elucidate the specific mechanisms of action for different environmental oestrogens.

We show our ERE-TG Casper zebrafish model can relatively easily be applied to a semi-automated *in vivo* screening assay. The assay is simple to carry out. The process of checking the masking was fast, with hundreds of images accepted or rejected in less than an hour, enabled by a simple visual interface. The resulting response curves derived from the image analysis showed that the screening assay correctly identified the chemicals with oestrogenic activity and reflected the responses observed in the target tissue analysis. A developmental toxicity (teratogenic) response at the higher concentrations of NP was reflected in a reduced fluorescence response detected by the system. The sensitivity of the assay system compares favourably with other *in vivo* and *in vitro oestrogen* assays. Automated high-throughput reporter quantification in zebrafish models have been hampered in previous systems due to melanophores and iridophores

that diminish the capacity to detect reporter signals and our ERE-TG Casper avoids this limitation without the need for use of additional chemical exposure to melanin synthesis inhibitors such as 1-phenyl-2-thiourea, required for pigmented zebrafsh lines.[58]

Comparing the ArrayScan analyses with the confocal images in the work presented shows that the sensitivity of the semi-automated screening assay could be improved. Challenges for generating a high throughput automated system for the detection of oestrogenic responses across the body tissues include the ability to detect responses in small focal regions in the body. As an example of this, in our Arrayscan system a statistically significant response to BPA in the heart valves was detected only for an exposure to 1000 µg BPA/L, whereas via confocal microscopy a response was seen for an exposure to 62.5 µg BPA/L (Figure 3B). This lower sensitivity relates in part to the comparatively lower quality images in the automated assay compared with confocal microscopy. Enhancement in the ArrayScan approach could be achieved also with a more consistent lateral orientation of the larvae in the microtitre plate to improve upon the current rate (80%) of successful larval masking for the python algorithm. In addition, the algorithm used could be developed further to identify, mask and measure specific organs in the zebrafish model and hence automate responses to the level of specific tissues. Auto-fluorescence generally was low with low variability between control fish and was not a major factor affecting GFP quantification in the transgenic assay (see comparison with Casper line in Figure S2). Quantification of GFP at the level of individual target tissues would remove any interference in GFP quantification that might be associated with auto-fluorescence.

Some *in vitro* EDC screening tools are limited in their predictive capabilities for potential effects on specific tissues and organs within an organism and they are highly focused on specific mechanisms of receptor interaction and activation. Despite recent concerns over reported mosaicism and random reporter insertion in the genome of certain transgenic animal models, the generational consistency in fluorescence intensity and specific tissue response to different oestrogenic chemicals has demonstrated that transgenic models such as ERE-GFP-Casper can be utilised for accurate and reliable quantification of both whole body and individual tissue responses to chemicals. Our study further highlights the potential for transgenic models, and in particular, here translucent models such as the ERE-GFP-Casper, for application in automated *in vivo* high-content, and potentially high-throughput, screens for quantitative and informative analysis of potential EDCs. In combination with exposure assessments and *in vitro* effects screening tools, *in vivo* screens such as the one reported here, allow us to more effectively and accurately identify EDCs, as well as improve our ability to predict potentially adverse outcomes in humans and wildlife populations.

Supporting Information

Information addressing LC-MS protocol for water chemistry analysis of exposure chemicals (Tables S1+S2); Coding for image analysis Python algorithm; Automated imaging of exposed ERE-GFP-Casper larvae (Figure S1);

Auto-fluorescence in Casper and ERE-GFP-Casper (Figure S2); Measured chemical concentrations in embryo incubation water (Table S3). This material is available free of charge via the Internet at http://pubs.acs.org.

Author Information

Corresponding Author

Address for correspondence: Charles R. Tyler, Biosciences, College of Life and Environmental Sciences, University of Exeter, Stocker Road, Exeter, Devon EX4 4QD, UK. Tel: 44 (0)1392 264450. Fax: 44(0)1392 263434. E-mail: C.R.Tyler@exeter.ac.uk

Notes

The authors declare no competing financial interest.

Acknowledgements

We thank the fish facility for fish husbandry, University College London for their Casper line, Imagen Biotech Ltd and Matthew Winter for their assistance in the application of the ArrayScan system. J.M.G's studentship was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) and AstraZeneca. J.M. was generously supported by a Welcome Trust Institutional Strategic Support Award (WT097835MF).

4.6 References

1. Bergman, Å.; Heindel, J.; Jobling, S.; Kidd, K.; Zoeller, R. T., State-of-the-science of endocrine disrupting chemicals, 2012. *Toxicol Lett* **2012,** *211*, S3.

2. Gore, A. C.; Chappell, V. A.; Fenton, S. E.; Flaws, J. A.; Nadal, A.; Prins, G. S.; Toppari, J.; Zoeller, R. T., EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. *Endocr Rev* **2015,** *36*, (6), E1-e150.

3. TEDX List of Potential Endocrine Disruptors. http://endocrinedisruption.org/endocrine-disruption/tedx-list-of-potential-endocrine-disruptors/overview (accessed Jan 08, 2016).

4. Lange, A.; Paull, G. C.; Coe, T. S.; Katsu, Y.; Urushitani, H.; Iguchi, T.; Tyler, C. R., Sexual reprogramming and estrogenic sensitization in wild fish exposed to ethinylestradiol. *Environ Sci Technol* **2009,** *43*, (4), 1219-25.

5. Xu, H.; Yang, J.; Wang, Y.; Jiang, Q.; Chen, H.; Song, H., Exposure to 17alpha-ethynylestradiol impairs reproductive functions of both male and female zebrafish (*Danio rerio*). *Aquat Toxicol* **2008,** *88*, (1), 1-8.

6. Jobling, S.; Beresford, N.; Nolan, M.; Rodgers-Gray, T.; Brighty, G. C.; Sumpter, J. P.; Tyler, C. R., Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. *Biol Reprod* **2002,** *66*, (2), 272-81.

7. Kidd, K. A.; Blanchfield, P. J.; Mills, K. H.; Palace, V. P.; Evans, R. E.; Lazorchak, J. M.; Flick, R. W., Collapse of a fish population after exposure to a synthetic estrogen. *Proc Natl Acad Sci U S A* **2007,** *104*, (21), 8897-901.

8. Li, D. K.; Zhou, Z.; Miao, M.; He, Y.; Wang, J.; Ferber, J.; Herrinton, L. J.; Gao, E.; Yuan, W., Urine bisphenol-A (BPA) level in relation to semen quality. *Fertil Steril* **2011,** *95*, (2), 625-30.e1-4.

9. Melzer, D.; Rice, N. E.; Lewis, C.; Henley, W. E.; Galloway, T. S., Association of urinary bisphenol a concentration with heart disease: evidence from NHANES 2003/06. *PLoS One* **2010,** *5*, (1), e8673.

10. Van den Belt, K.; Berckmans, P.; Vangenechten, C.; Verheyen, R.; Witters, H., Comparative study on the in vitro/in vivo estrogenic potencies of 17beta-estradiol, estrone, 17alpha-ethynylestradiol and nonylphenol. *Aquat Toxicol* **2004,** *66*, (2), 183-95.

11. Nilsson, S.; Makela, S.; Treuter, E.; Tujague, M.; Thomsen, J.; Andersson, G.; Enmark, E.; Pettersson, K.; Warner, M.; Gustafsson, J. A., Mechanisms of estrogen action. *Physiol Rev* **2001,** *81*, (4), 1535-65.

12. Pettersson, K.; Gustafsson, J. A., Role of estrogen receptor beta in estrogen action. *Annu Rev Physiol* **2001,** *63*, 165-92.

13. Bardet, P. L.; Horard, B.; Robinson-Rechavi, M.; Laudet, V.; Vanacker, J. M., Characterization of oestrogen receptors in zebrafish (*Danio rerio*). *J Mol Endocrinol* **2002,** *28*, (3), 153-63.

14. Bondesson, M.; Hao, R.; Lin, C. Y.; Williams, C.; Gustafsson, J. A., Estrogen receptor signaling during vertebrate development. *Biochim Biophys Acta* **2015,** *1849*, (2), 142-51.

15. Brown, A. R.; Gunnarsson, L.; Kristiansson, E.; Tyler, C. R., Assessing variation in the potential susceptibility of fish to pharmaceuticals, considering evolutionary differences in their physiology and ecology. *Philos Trans R Soc Lond B Biol Sci* **2014,** *369*, (1656).

16. Giguere, V.; Yang, N.; Segui, P.; Evans, R. M., Identification of a new class of steroid hormone receptors. *Nature* **1988,** *331*, (6151), 91-4.

17. Razandi, M.; Pedram, A.; Greene, G. L.; Levin, E. R., Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. *Mol Endocrinol* **1999,** *13*, (2), 307-19.

18. Soltysik, K.; Czekaj, P., Membrane estrogen receptors - is it an alternative way of estrogen action? *J Physiol Pharmacol* **2013,** *64*, (2), 129-42.

19. Cosnefroy, A.; Brion, F.; Maillot-Marechal, E.; Porcher, J. M.; Pakdel, F.; Balaguer, P.; Ait-Aissa, S., Selective activation of zebrafish estrogen receptor subtypes by chemicals by using stable reporter gene assay developed in a zebrafish liver cell line. *Toxicol Sci* **2012,** *125*, (2), 439-49.

20. Ascenzi, P.; Bocedi, A.; Marino, M., Structure-function relationship of estrogen receptor alpha and beta: impact on human health. *Mol Aspects Med* **2006,** *27*, (4), 299-402.

21. Koehler, K. F.; Helguero, L. A.; Haldosen, L. A.; Warner, M.; Gustafsson, J. A., Reflections on the discovery and significance of estrogen receptor beta. *Endocr Rev* **2005,** *26*, (3), 465-78.

22. Hao, R.; Bondesson, M.; Singh, A. V.; Riu, A.; McCollum, C. W.; Knudsen, T. B.; Gorelick, D. A.; Gustafsson, J. A., Identification of estrogen target genes during zebrafish embryonic development through transcriptomic analysis. *PLoS One* **2013,** *8*, (11), e79020.

23. Mahato, D.; Goulding, E. H.; Korach, K. S.; Eddy, E. M., Estrogen receptor-alpha is required by the supporting somatic cells for spermatogenesis. *Mol Cell Endocrinol* **2001,** *178*, (1-2), 57-63.

24. O'Donnell, L.; Robertson, K. M.; Jones, M. E.; Simpson, E. R., Estrogen and spermatogenesis. *Endocr Rev* **2001,** *22*, (3), 289-318.

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

26. Filby, A. L.; Tyler, C. R., Molecular characterization of estrogen receptors 1, 2a, and 2b and their tissue and ontogenic expression profiles in fathead minnow (*Pimephales promelas*). *Biol Reprod* **2005,** *73*, (4), 648-62.

27. Gustafsson, J. A., What pharmacologists can learn from recent advances in estrogen signalling. *Trends Pharmacol Sci* **2003,** *24*, (9), 479-85.

28. OECD Work Related to Endocrine Disrupters. http://www.oecd.org/env/ehs/testing/oecdworkrelatedtoendocrinedisrupters.html (accessed Jan 11, 2016).

29. US EPA, O., OSCP Endocrine Disruption Screening Program. http://www.epa.gov/endocrine-disruption (accessed Jan 11, 2016).

30. MOE Endocrine Disrupting Effects of Substances [MOE]. http://www.env.go.jp/en/chemi/ed.html (accessed Jan 11, 2016).

31. Chakraborty, T.; Katsu, Y.; Zhou, L. Y.; Miyagawa, S.; Nagahama, Y.; Iguchi, T., Estrogen receptors in medaka (*Oryzias latipes*) and estrogenic environmental contaminants: an in vitro-in vivo correlation. *J Steroid Biochem Mol Biol* **2011,** *123*, (3-5), 115-21.

32. Pinto, C.; Grimaldi, M.; Boulahtouf, A.; Pakdel, F.; Brion, F.; Ait-Aissa, S.; Cavailles, V.; Bourguet, W.; Gustafsson, J. A.; Bondesson, M.; Balaguer, P., Selectivity of natural, synthetic and environmental estrogens for zebrafish estrogen receptors. *Toxicol Appl Pharmacol* **2014,** *280*, (1), 60-9.

33. Dobbins, L. L.; Brain, R. A.; Brooks, B. W., Comparison of the sensitivities of common in vitro and in vivo assays of estrogenic activity: application of chemical toxicity distributions. *Environ Toxicol Chem* **2008,** *27*, (12), 2608-16.

34. Dreier, D. A.; Connors, K. A.; Brooks, B. W., Comparative endpoint sensitivity of in vitro estrogen agonist assays. *Regul Toxicol Pharmacol* **2015,** *72*, (2), 185-93.

35. Folmar, L. C.; Hemmer, M.; Denslow, N. D.; Kroll, K.; Chen, J.; Cheek, A.; Richman, H.; Meredith, H.; Grau, E. G., A comparison of the estrogenic potencies of estradiol, ethynylestradiol, diethylstilbestrol, nonylphenol and methoxychlor in vivo and in vitro. *Aquat Toxicol* **2002,** *60*, (1-2), 101-10.

36. Brion, F.; Le Page, Y.; Piccini, B.; Cardoso, O.; Tong, S. K.; Chung, B. C.; Kah, O., Screening estrogenic activities of chemicals or mixtures in vivo using transgenic (cyp19a1b-GFP) zebrafish embryos. *PLoS One* **2012,** *7*, (5), e36069.

37. Lee, O.; Takesono, A.; Tada, M.; Tyler, C. R.; Kudoh, T., Biosensor zebrafish provide new insights into potential health effects of environmental estrogens. *Environ Health Perspect* **2012,** *120*, (7), 990-6.

38. Gorelick, D. A.; Halpern, M. E., Visualization of estrogen receptor transcriptional activation in zebrafish. *Endocrinology* **2011,** *152*, (7), 2690-703.

39. Chen, H.; Hu, J.; Yang, J.; Wang, Y.; Xu, H.; Jiang, Q.; Gong, Y.; Gu, Y.; Song, H., Generation of a fluorescent transgenic zebrafish for detection of environmental estrogens. *Aquat Toxicol* **2010,** *96*, (1), 53-61.

40. OECD Test Guideline Programme. http://www.oecd.org/env/ehs/testing/TGP%20work%20plan_declassification_July%202015.pdf (accessed Jan 05, 2016).

41. White, R. M.; Sessa, A.; Burke, C.; Bowman, T.; LeBlanc, J.; Ceol, C.; Bourque, C.; Dovey, M.; Goessling, W.; Burns, C. E.; Zon, L. I., Transparent adult zebrafish as a tool for in vivo transplantation analysis. *Cell Stem Cell* **2008,** *2*, (2), 183-9.

42. Walker, C.; Goodhead, R.; Tyler, C., Endocrine-disrupting chemicals and their environmental impacts. In *Organic Pollutants: An Ecotoxicological Perspective, Second Edition*, CRC Press: 2008; pp 265-292.

43. Aris, A. Z.; Shamsuddin, A. S.; Praveena, S. M., Occurrence of 17alpha-ethynylestradiol (EE2) in the environment and effect on exposed biota: a review. *Environ Int* **2014,** *69*, 104-19.

44. Soto, A. M.; Sonnenschein, C.; Chung, K. L.; Fernandez, M. F.; Olea, N.; Serrano, F. O., The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ Health Perspect* **1995,** *103 Suppl 7*, 113-22.

45. Arnold, S. F.; Klotz, D. M.; Collins, B. M.; Vonier, P. M.; Guillette, L. J., Jr.; McLachlan, J. A., Synergistic activation of estrogen receptor with combinations of environmental chemicals. *Science* **1996,** *272*, (5267), 1489-92.

46. Wilson, V. S.; Bobseine, K.; Gray, L. E., Jr., Development and characterization of a cell line that stably expresses an estrogen-responsive luciferase reporter for the detection of estrogen receptor agonist and antagonists. *Toxicol Sci* **2004,** *81*, (1), 69-77.

47. Schulz, R. W.; Bogerd, J.; Male, R.; Ball, J.; Fenske, M.; Olsen, L. C.; Tyler, C. R., Estrogen-induced alterations in amh and dmrt1 expression signal for disruption in male sexual development in the zebrafish. *Environ Sci Technol* **2007,** *41*, (17), 6305-10.

48. Derouiche, L.; Keller, M.; Duittoz, A. H.; Pillon, D., Developmental exposure to Ethinylestradiol affects transgenerationally sexual behavior and neuroendocrine networks in male mice. *Sci Rep* **2015,** *5*, 17457.

49. Vosges, M.; Le Page, Y.; Chung, B. C.; Combarnous, Y.; Porcher, J. M.; Kah, O.; Brion, F., 17alpha-ethinylestradiol disrupts the ontogeny of the forebrain GnRH system and the expression of brain aromatase during early development of zebrafish. *Aquat Toxicol* **2010,** *99*, (4), 479-91.

50. Coe, T. S.; Soffker, M. K.; Filby, A. L.; Hodgson, D.; Tyler, C. R., Impacts of early life exposure to estrogen on subsequent breeding behavior and reproductive success in zebrafish. *Environ Sci Technol* **2010,** *44*, (16), 6481-7.

51. Heldring, N.; Pike, A.; Andersson, S.; Matthews, J.; Cheng, G.; Hartman, J.; Tujague, M.; Strom, A.; Treuter, E.; Warner, M.; Gustafsson, J. A., Estrogen receptors: how do they signal and what are their targets. *Physiol Rev* **2007,** *87*, (3), 905-31.

52. Martinkovich, S.; Shah, D.; Planey, S. L.; Arnott, J. A., Selective estrogen receptor modulators: tissue specificity and clinical utility. *Clin Interv Aging* **2014,** *9*, 1437-52.

53. Gorelick, D. A.; Iwanowicz, L. R.; Hung, A. L.; Blazer, V. S.; Halpern, M. E., Transgenic zebrafish reveal tissue-specific differences in estrogen signaling in response to environmental water samples. *Environ Health Perspect* **2014,** *122*, (4), 356-62.

54. Gao, X.; Wang, H. S., Impact of bisphenol a on the cardiovascular system - epidemiological and experimental evidence and molecular mechanisms. *Int J Environ Res Public Health* **2014,** *11*, (8), 8399-413.

55. Greenwood, M. P.; Flik, G.; Wagner, G. F.; Balment, R. J., The corpuscles of Stannius, calcium-sensing receptor, and stanniocalcin: responses

to calcimimetics and physiological challenges. *Endocrinology* **2009,** *150*, (7), 3002-10.

56. Klein-Nulend, J.; van Oers, R. F.; Bakker, A. D.; Bacabac, R. G., Bone cell mechanosensitivity, estrogen deficiency, and osteoporosis. *J Biomech* **2015,** *48*, (5), 855-65.

57. Beresford, N.; Routledge, E. J.; Harris, C. A.; Sumpter, J. P., Issues arising when interpreting results from an in vitro assay for estrogenic activity. *Toxicol Appl Pharmacol* **2000,** *162*, (1), 22-33.

58. Walker, S. L.; Ariga, J.; Mathias, J. R.; Coothankandaswamy, V.; Xie, X.; Distel, M.; Koster, R. W.; Parsons, M. J.; Bhalla, K. N.; Saxena, M. T.; Mumm, J. S., Automated reporter quantification in vivo: high-throughput screening method for reporter-based assays in zebrafish. *PLoS One* **2012,** *7*, (1), e29916.

4.7 List of Figures

Figure 1: Generation of ERE-GFP-Casper (F0) line. TG1 indicates ERE-Gal4ff transgene sequence. TG2 indicates UAS-GFP transgene sequence. Expression of pigmentation genes roy (dark) and nacre (silver) are also shown. The ERE-GFP model, homozygous for both transgenes, and a Casper strain were initially crossed to give a heterozygous generation. In-breeding within this generation produced progeny with 256 different genotypes based on four genes of interest. One in 256 expressed F0 ERE-GFP Casper genotype of fully silenced pigmentation and homozygous transgene expression.

Figure 2: Comparison between ERE-GFP model and ERE-GFP-Casper model. Larvae were exposed to 50 ng/L EE2 from 0-5 dpf. Images of larvae were acquired 5 dpf on an inverted compound microscope (Zeiss Axio Observer) at 10x magnification under consistent GFP excitation. Lateral and dorsal views are used for comparison. Specific tissue response in the liver (li), heart (h), cranial muscle (cm), somite muscle (sm), otic vesicle ganglions (ovg) and forebrain (fb) was consistent across the two models.

Figure 3A-D: Confocal microscopy images of ERE-GFP-Casper larvae (5 days post fertilisation) after five day chemical exposure to different concentrations of (**A)** EE2 (**B)** genistein (**C)** BPA and **(D)** Nonylphenol. Specific tissue response in the liver (li), heart (h), somite muscle (sm), otic vesicle (ov), fin (f), cardiac muscle (cm), corpuscle of Stannius (cs), brain (b), eye (e), neuromast (n) and gut (g).

Figure 4: Fluorescence in body tissues of ERE-GFP Casper zebrafish exposed to estrogens. Tissue responses were analysed using confocal

microscopy after 5 days chemical exposures. Graph shows the concentration ranges that induced a fluorescence response in a selection of different body tissues. C.o.S. = Corpuscle of Stannius, C.M. = Cranial Muscle.

Figure 5: Responses to chemicals in ERE-GFP Casper quantified using a semi-automated imaging system. Data are reported as mean ± SEM (asterisk indicate significant difference compared with the control, * $p < 0.05$ and ** $p < 0.01$).

Table 1: Lowest Significant Response (LSR), effective concentrations (EC50), EC50 confidence intervals (±CI 95) and developmental toxicity concentration (Tox) for exposures to various oestrogens. LSR is the lowest exposure concentration of a chemical that gave a fluorescence response statistically higher ($p < 0.05$) than the control response. Statistical significance values were calculated using ANOVA and Games-Howell post-hoc test. EC50 values are the concentrations inducing 50% of the maximal fluorescence response for that chemical and, in addition to their confidence intervals (±CI 95) , were based on a Hill equation model. The only chemical found to induce developmental toxicity (Tox) was 4-Nonylphenol (NP).

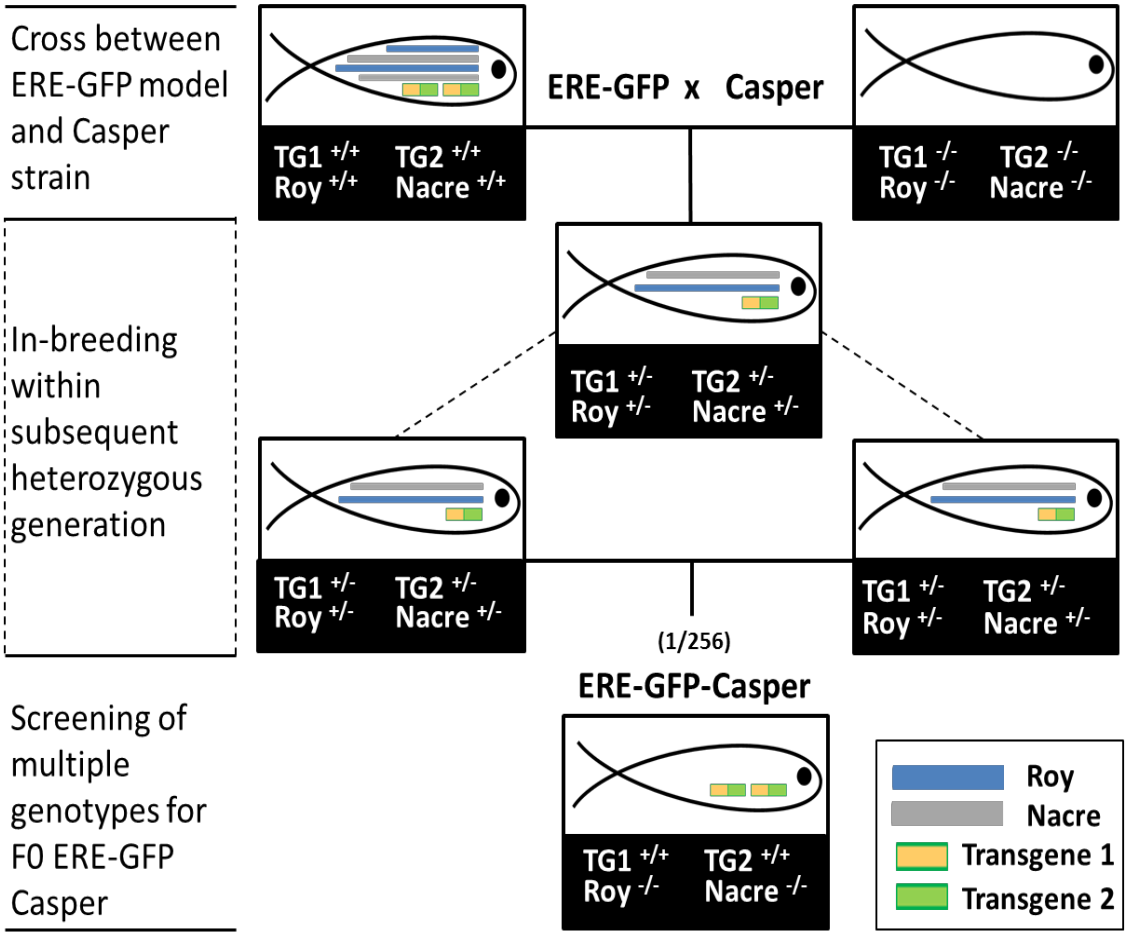


Figure 1: Generation of ERE-GFP-Casper (F0) line.

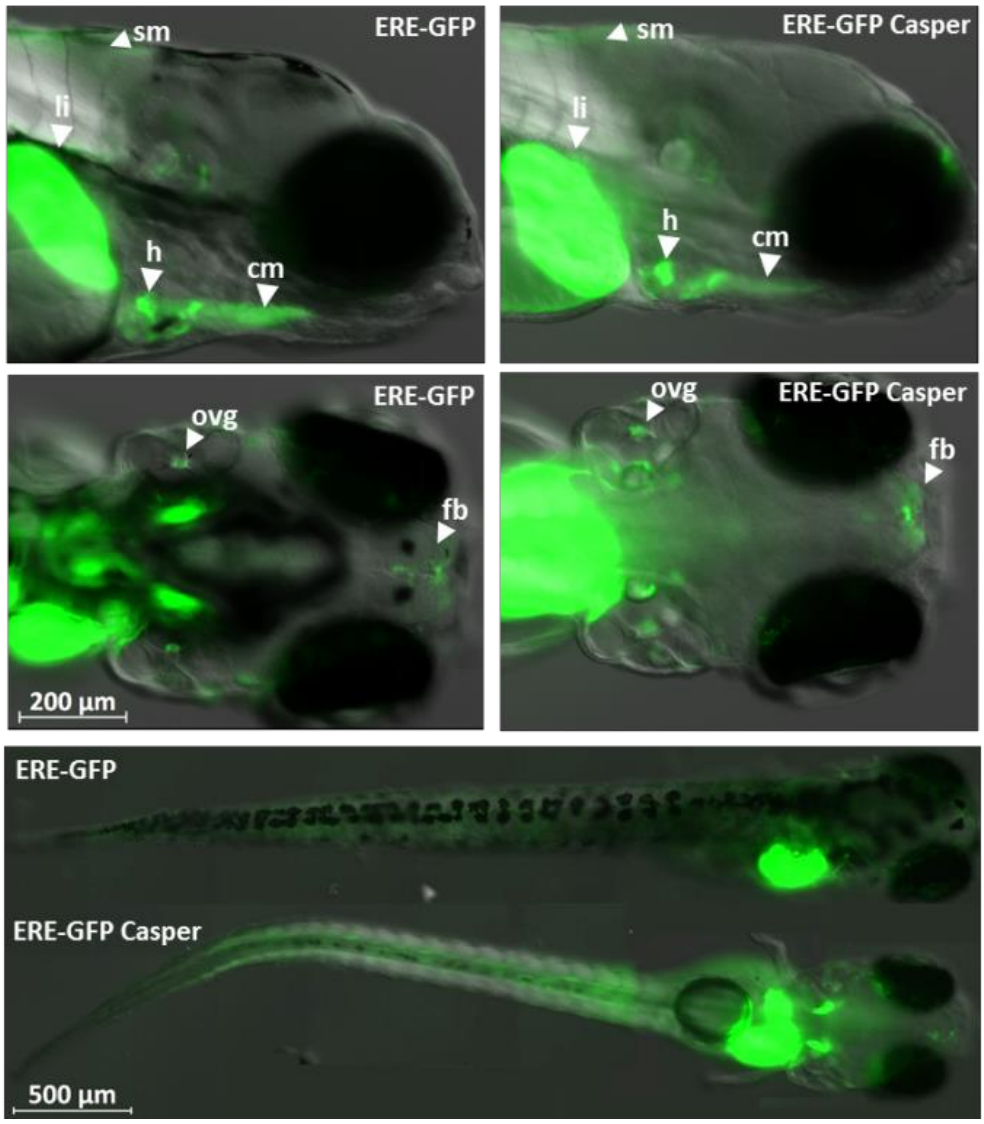


Figure 2: Comparison between ERE-GFP model and ERE-GFP-Casper model.

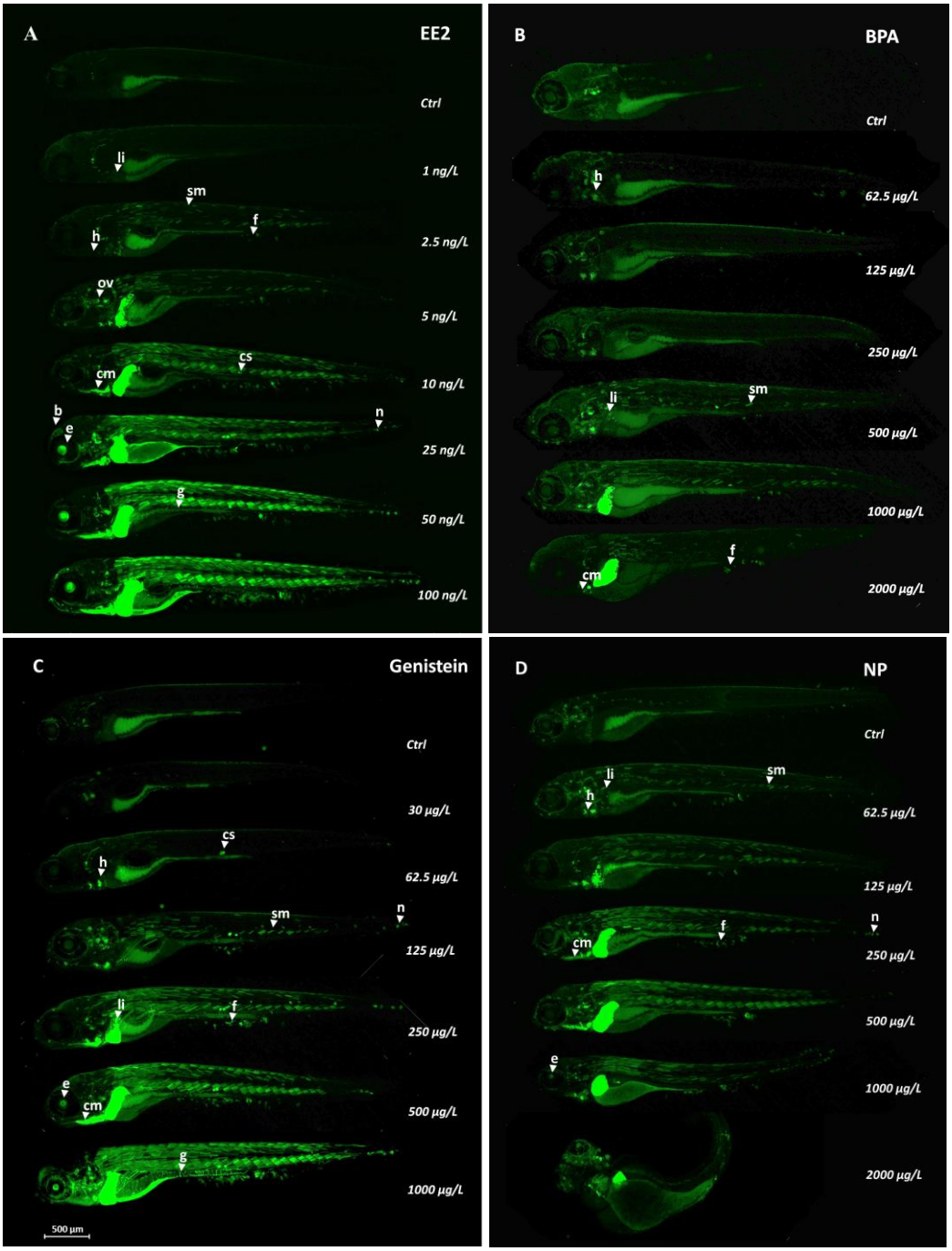

A
EE2
Ctrl
1 ng/L
2.5 ng/L
5 ng/L
10 ng/L
25 ng/L
50 ng/L
100 ng/L
li
sm
f
h
ov
cs
cm
b
e
n
g
B
BPA
Ctrl
62.5 µg/L
125 µg/L
250 µg/L
500 µg/L
1000 µg/L
2000 µg/L
h
li
sm
f
cm
C
Genistein
Ctrl
30 µg/L
62.5 µg/L
125 µg/L
250 µg/L
500 µg/L
1000 µg/L
cs
h
sm
n
li
f
e
cm
g
500 µm
D
NP
Ctrl
62.5 µg/L
125 µg/L
250 µg/L
500 µg/L
1000 µg/L
2000 µg/L
li
sm
h
cm
f
n
e

Figure 3A-D

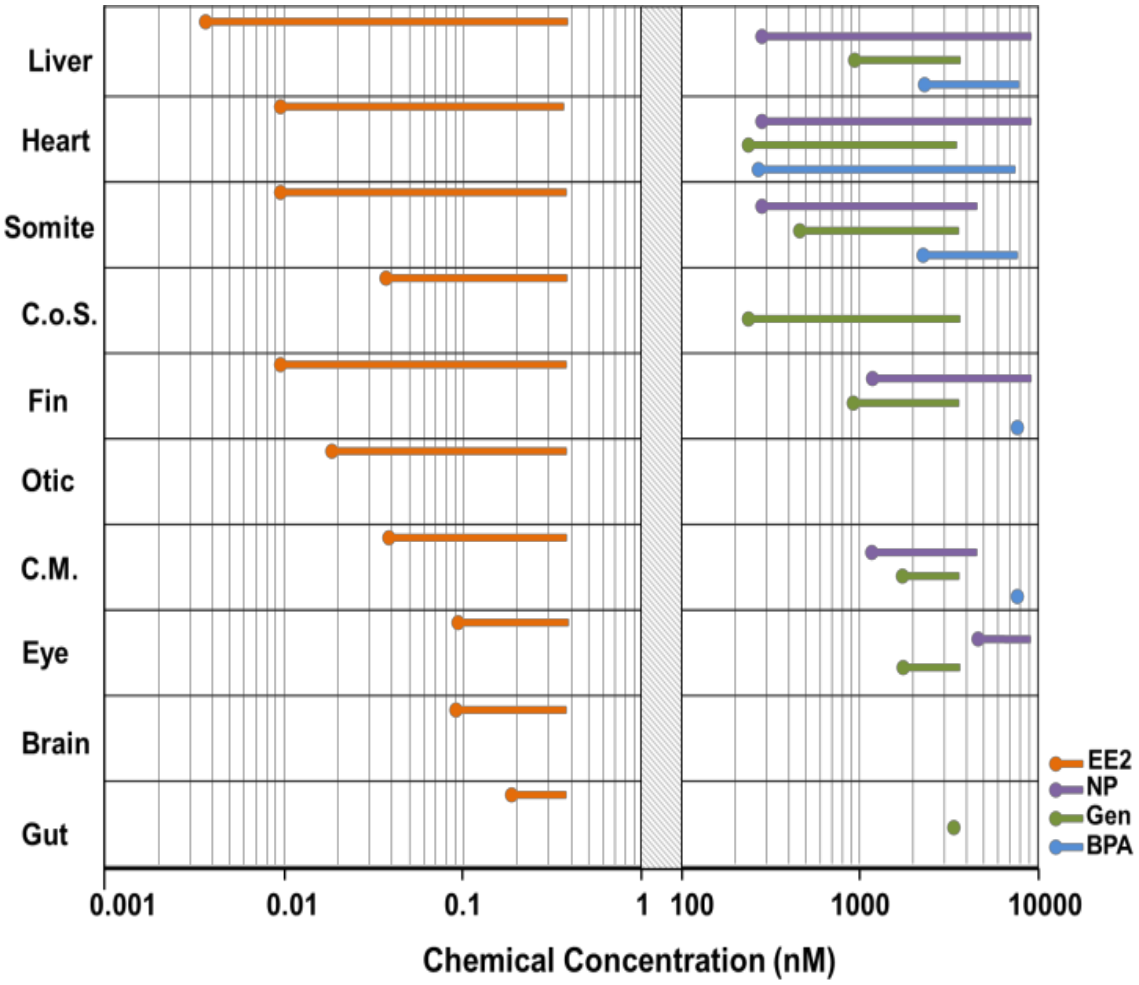


Figure 4: Fluorescence in body tissues of ERE-GFP Casper zebrafish exposed to estrogens.

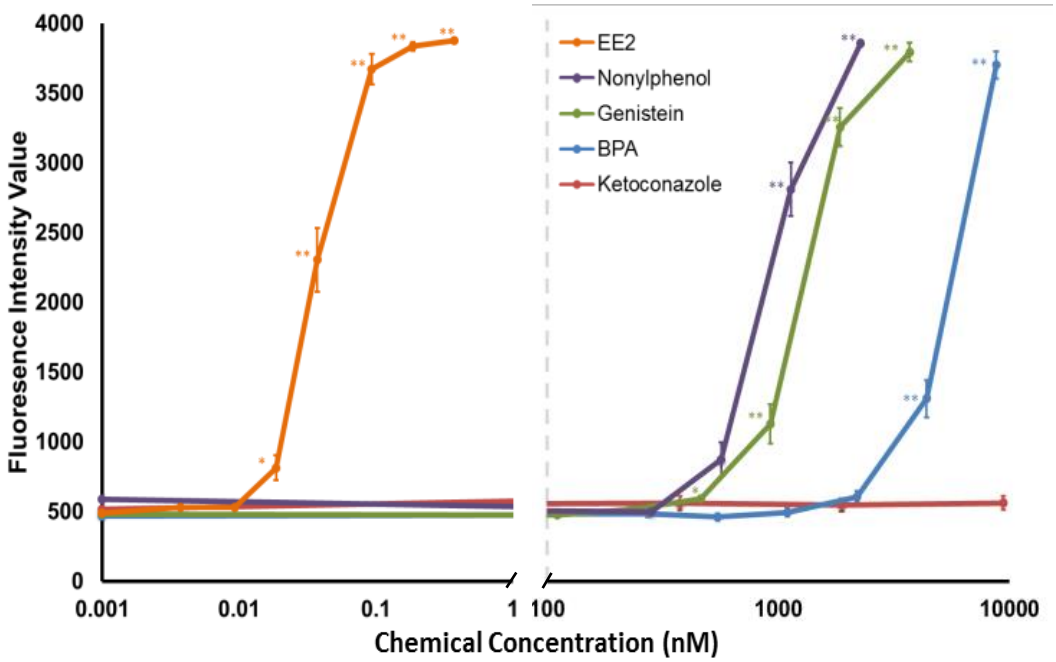


Figure 5: Responses to chemicals in ERE-GFP Casper quantified using a semi-automated imaging system.

µg/L (nM)	EE2	Gen	BPA	NP	Keto
LOEC	0.005 (0.0093)	125 (462.5)	1000 (4380.32)	250 (1134.56)	n/a
EC50	0.0096 (0.0178)	463.4 (1715)	1226 (1370)	212.2 (963.2)	n/a
±CI 95 (EC50)	0.0089 – 0.0103	455.6 – 469.7	1169 – 1310	193.6 – 229.2	n/a
Tox	n/a	n/a	n/a	1000 (4380.32)	n/a

Table 1: Lowest observed effect concentration (LOEC), effective concentrations (EC50), EC50 confidence intervals (±CI 95) and developmental toxicity concentration (Tox) for exposures to various estrogens.

4.8 Supporting Information

Water Chemistry: LC-MS Method (Include Table S1 and S2)

Analyses of water samples for exposure were performed using a Surveyor MS Pump plus a HPLC pump with an HTC PAL autosampler coupled to a TSQ Vantage triple quadrupole mass spectrometer. The mass spectrometer was equipped with a heated electrospray (HESI II) source (all ThermoFisher Scientific, Hemel Hempstead, UK).

Chromatographic separation was achieved using a reversed-phase, 3 µm particle size, C18 Hypersil GOLD column (50 mm × 2.1 mm i.d., Thermo Scientific, San Jose CA, USA).

Analytes were separated using a linear gradient of (A) aqueous phase and (B) organic solvent containing additives specific for analysed compounds and are listed together with initial conditions in the table below (Table S1). Solvent B increased to 100% in 4.5 min for BPA and 1.5 min for other analytes. This was maintained for 1 min for BPA and 1.5 min for remaining compounds, before returning to the initial condition. The flow rate was 500µL/min. Temperature of autosampler was set at 8˚C while column was kept at a room temperature.

The HESI probe was operating in both negative and positive mode; an ion-spray voltage of -2.75 kV was applied for analysis of genistein, +3.75 kV for ketoconazole and -4.0 kV for remaining compounds. The heated capillary temperature was set at 275 °C and the vaporizer temperature was 60 °C for BPA, 500°C for nonylphenol and 350°C for remaining compounds. Nitrogen was employed as a sheath and auxiliary gas at a pressure of 60 and 2 arbitrary units, respectively.

The argon CID gas was used at a pressure of 1.5 mTorr and the optimum collision energy (CE) for each transition was selected. Quantification of the target compounds was performed by monitoring two characteristic multiple reaction monitoring (MRM) transitions (Table S2).

Semi-Automated Image Analysis

We developed a custom Python pipeline to quantify larvae-specific fluorescence in the arrayscanner images. The first processing step involved detecting the well within each image using a series of circular Hough transforms to detect the location and size of the well. Once the well had been identified, the image was filtered using a median filter and the background subtracted (to reduce noise and background gradients respectively). The resulting filtered image was passed through a Sobel edge detection algorithm and median-absolute-deviation thresholding to pick out the edges of the image. After applying a distance transform to grow the edges (to close small gaps in the outline), the largest contiguous region was selected as representing the larvae outline. The full larval mask was then calculated as the convex hull of the outline image.

Figure S1: Semi-automated image analysis. Fluorescence responses to chemical were quantified using a Python macro developed in house. The macro identified the well within the image (yellow circle) and subsequently masked the larvae (green mask) for fluorescence intensity measurement. Larval masking was accepted or rejected using a interactive internet interface.

Table S3: Measured chemical concentrations in the embryo incubation water. Chemical content was measured by LC-MS in controls, and in the highest and lowest exposure concentrations for each chemical tested.

Figure S2: Auto-fluorescence in Casper and ERE-GFP-Casper lines. Caspers and ERE-GFP-Caspers were imaged at 5 dpf with no prior chemical exposure for comparison of auto-fluorescence on an inverted compound microscope (Zeiss Axio Observer). Images show yolk-sac (ys) fluorescence in both lines, likely due to naturally accumulated chemicals (such as polycyclic aromatic hydrocarbons) that fluoresce at similar excitation and emission wavelengths as GFP. GFP expression is additionally seen in the Otic vesicle (ov) of the ERE-GFP-Casper line only.

	(A)	(B)	Initial Conditions [% of B]
Genistein	Water +0.1% Formic Acid	Methanol +0.1% Formin Acid	20
Ketoconazole	Water +0.1% Ammonium Hydroxide	Acetonitrile +0.1% Ammonium Hydroxide	20
EE2	Water +0.1% Ammonium Hydroxide	Methanol + 0.1% Ammonium Hydroxide	20
Nonylphenol	Water +0.1% Ammonium Hydroxide	Acetonmitrile + 0.1% Ammonium Hydroxide	30
BPA	Water	Methanol	10

Table S1: Separation of analytes using a linear gradient of (A) aqueous phase and (B) organic solvent containing additives specific for analysed compounds (with initial conditions).

Compound	Parent ion (m/z)	Product ion (m/z)	CE (eV)
Genistein	269.0	133.0	32
		181.0	25
Ketoconazole	531.2	489.0	27
		82.0	38
EE2	295.2	188.9	22
		145.0	42
Nonylphenol	219.2	133.0	35
		147.1	27
BPA	227.1	133.1	23
		117.0	49

Table S2: Quantification of the target compounds using two characteristic multiple reaction monitoring (MRM) transitions at optimum collision energies (CE).

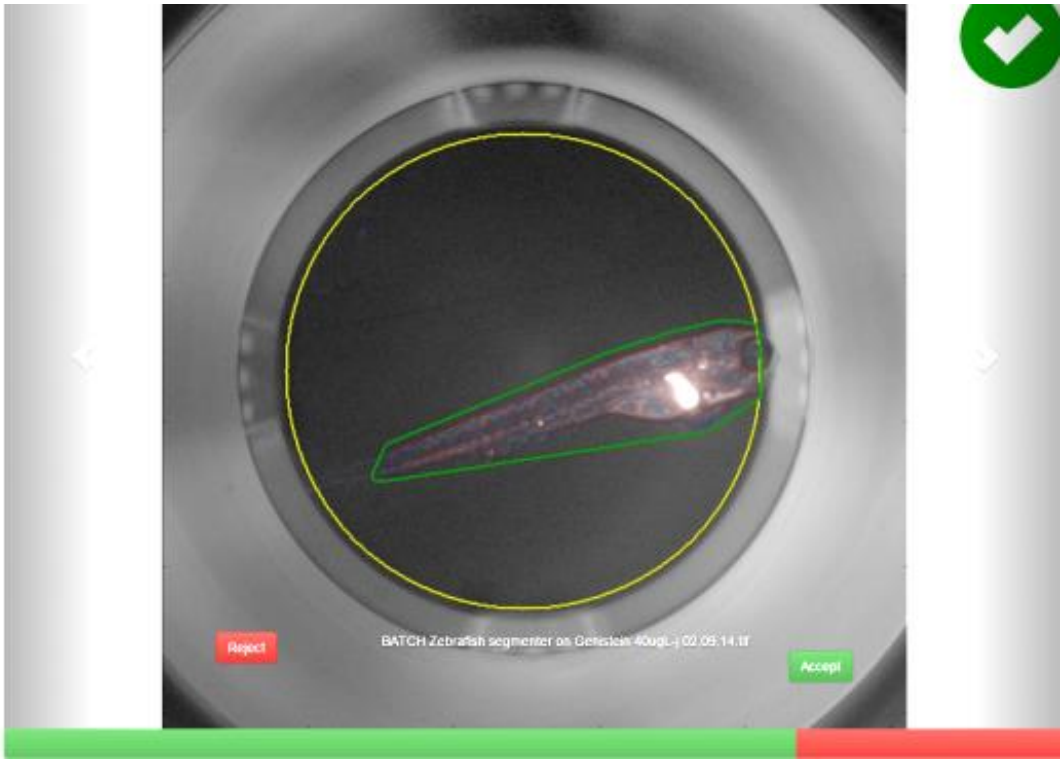


Figure S1: Semi-automated image analysis.

	EE2	Gen		BPA		NP			Keto		
Nominal (µg/L)	**0.1**	**62.5**	**1000**	**125**	**2000**	**125**	**156.25**	**2000**	**40**	**50**	**5000**
Day 0	0.14* (140%)	58.6 (94%)	1001 (100%)	137.5 (115%)	2200 (110%)	67.4 (54%)	157.4* (101%)	1688 (84%)	11.4 (28%)	39.5* (79%)	5400 (108%)
Day 5	0.099 (99%)	62.1 (99%)	956 (96%)	132.4 (103%)	2669.14 (133%)	16.6 (13%)		688 (34%)	4.7 (12%)		4700 (93%)

Table S3: Measured chemical concentrations in the embryo incubation water.

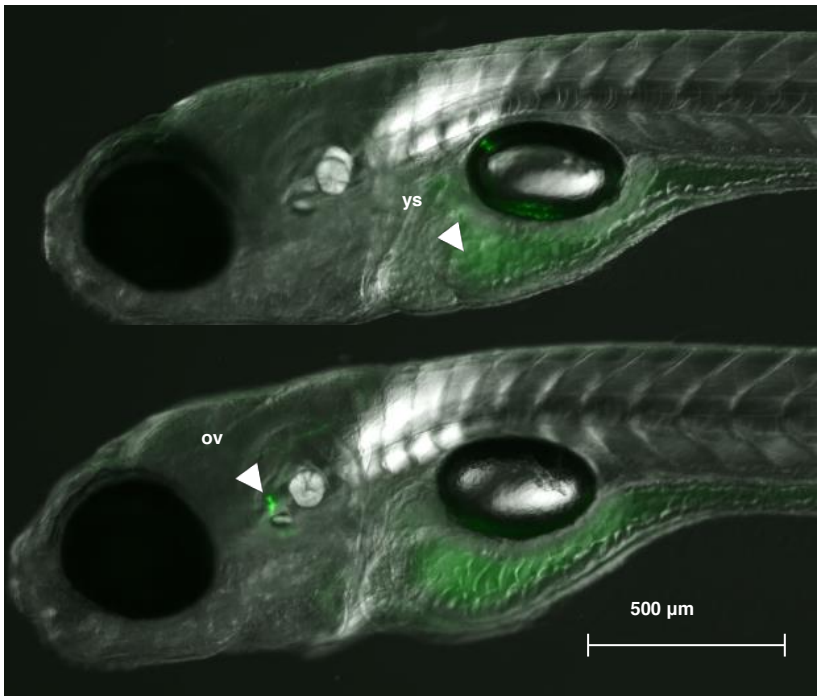


Figure S2: Auto-fluorescence in Casper and ERE-GFP-Casper lines.

Chapter 5

Paper 2: Early life exposure to ethinylestradiol enhances subsequent responses to environmental oestrogens measured in a novel transgenic zebrafish

Paper in preparation for submission to Environmental Health Perspectives

Chapter 5

Early life exposure to ethinylestradiol enhances subsequent responses to environmental oestrogens measured in a novel transgenic zebrafish

Jon M. Green[1], Anke Lange[1], Aaron Scott[1], Maciej Trznadel[1], Htoo Aung Wai[1], Aya Takesono[1], A. Ross Brown[1], Stewart F. Owen[2], Tetsuhiro Kudoh[1], Charles R. Tyler[1*]

[1]Biosciences, College of Life and Environmental Sciences, University of Exeter, Geoffrey Pope, Stocker Road, Exeter, Devon, EX4 4QD, UK.

[2]AstraZeneca, Global Environment, Alderley Park, Macclesfield, Cheshire, SK10 4TF, UK

5.1 Abstract

Background: Oestrogen plays fundamental roles in a range of developmental processes and exposure to oestrogen mimicking chemicals has been associated with various adverse health effects in both wildlife and human populations. Oestrogenic chemicals are found commonly as mixtures in the environment and can have additive effects, however risk analysis is typically conducted for single-chemicals with little, or no, consideration given for animal's exposure history.

Objectives: To develop and apply a novel oestrogen sensitive transgenic zebrafish to establish responses to environmental oestrogens for repeated exposures during early life.

Methods: We crossbred two transgenic zebrafish lines (ERE-GFP-Casper and UAS-Kaede) to create an oestrogen responsive transgenic zebrafish with a photoconvertible fluorophore (Kaede) in a skin pigment-free mutant element (ERE)-Kaede-Casper model. This was applied to quantify tissue-specific fluorescence biosensor responses for combinations of oestrogen exposures during early life using fluorescence microscopy and image analysis. Expression of oestrogen receptor subtypes (ER) in whole larvae was also quantified using qPCR analysis.

Results: Our ERE-Kaede-Casper model was sensitive to oestrogen exposure and photoconvertion of induced Kaede protein (green to red) was rapid upon exposure to UV light. Using this model we identify windows of tissue-specific sensitivity to ethinylestradiol (EE2) for exposure during early-life (0-5 dpf) and illustrate that exposure to oestrogen (EE2) during 0-48 hpf enhances responsiveness (sensitivity) to different environmental oestrogens (EE2, genistein and bisphenol A) for subsequent exposures during development.

Conclusions: Our findings illustrate the importance of oestrogen exposure history in effects assessments for oestrogens and wider implications for the possible health effects associated with oestrogen exposure.

5.2 Introduction

Endocrine Disrupting Chemicals

There is strong evidence that exposure to endocrine disrupting chemicals (EDCs) is linked with a range of adverse health disorders and further understanding of EDCs effects is crucial for safe guarding long-term human and environmental health.[1, 2] Over the last 20 years, EDCs shown to interact with the oestrogen signaling pathways have gained considerable attention and more than 200 chemicals are known to have oestrogenic activity.[1, 3] Oestrogens are fundamental for gonad growth and development in both females and males,[4-6] and they are crucial also for immune responses, central nervous system function, and normal somatic cell growth.[7, 8] In zebrafish, oestrogens also play important roles in regulating metabolism, transcription, tissue development and protein folding and trafficking during early life.[9]

Many EDCs with oestrogenic activity enter the aquatic environment via waste discharges and there are strong associations between exposures to specific environmental oestrogens (e.g. the contraceptive oestrogen, 17α-ethinylestradiol, EE2) and adverse health effects in individual fish[10, 11] and fish populations.[12, 13] Laboratory based studies on fish have illustrated associations between various environmental oestrogens and feminization of males[10, 14] and alteration of sexual behavior.[15] In mammals too, exposure to environmental oestrogens has been associated with decreases in semen quality/sperm count,[16] heart disease and diabetes.[17] Exposure to oestrogenic chemicals during early life-stages in both mammals and fish has received much recent attention with reports of significant adverse physical and behavioral effects.[18-20]

Exposures to oestrogens in the natural environment occurs predominantly as mixtures and both *in vitro* studies with reporter gene assays[21-23] and *in vivo* studies in fish[24] and mammals[25, 26] have illustrated the capacity for additive (and more than additive) effects. Furthermore, it has been shown that other EDCs,

such as anti-androgens, can modulate the responses for oestrogenic chemicals when combined as mixture *in vitro*[27] and *in vivo.*[28] Studies on chemical mixtures have also suggested enhanced tissue-specific effects may occur, for example as seen for responses to EDC mixtures in mammary gland development in male and female rats.[25, 26] Effects analysis for exposures representative of real world scenarios is therefore complicated by mixture permutations, chemical interactions and tissue-specific responses.

Estrogen Signaling

Estrogen signaling occurs via oestrogen binding to oestrogen receptors (ERs) in the nucleus, which then dimerise and bind to oestrogen response elements (EREs) located in promoters of target genes.[29] There are two ER subtypes in mammals, Esr1 and Esr2,[30] and three in zebrafish, Esr1, Esr2a and Esr2b.[31, 32] Esr1 and Esr2 subtypes in humans and zebrafish are corresponding orthologs with high amino acid sequence similarity for their respective ligand binding domains (64% and 71%, respectively).[33] Membrane ERs (mERs) and oestrogen-related receptors (ERRs), a small group of orphan nuclear receptors that share some target genes with ERs,[34] also occur but their role(s) and mechanism(s) of action are less clear than for the nuclear ERs.[35, 36] Transcriptional co-factors also play an important role in regulating the interaction of ERs with oestrogen related response elements and their downstream expression sequences.[37, 38] The expression of ER subtypes in organs and tissues can vary during development, and in later life, and the affinity of ligands for these ER subtypes also varies for different oestrogenic chemicals, influencing the physiological targets and subsequent downstream effects.[39] Tissue-specific expression of ER subtypes in humans is better

characterized than for other organisms.[40, 41] In zebrafish, expression levels of *esr1*, *esr2a* and *esr2b* differ and vary significantly during the first 120 hours of development.[42] Expression of *esr1* and *esr2b* increases during the first 120 hours and *esr1* expression is comparatively higher. *Esr2a* expression is significantly lower than the expression of *esr1* and *esr2b* during this period. At sexual maturity, these ER subtypes show tissue-specific expression also with the liver, brain and heart expressing all three ERs at relatively high levels compared with other somatic tissues.[42] Exposure to oestrogenic chemicals during early life has been shown to increase expression of ERs and EREs with tissue-specific targeting for these chemicals.[42, 43] This effect of sensitization and increased responsiveness has been shown to persist even after a prolonged phase of depuration.[44]

Transgenic Fish Models

Transgenic zebrafish models (TG models) have emerged as effective systems for investigating EDC exposure effects *in vivo*, and they have been applied to study responses in real time and to better establish chemical effect mechanisms.[45] Highly sensitive oestrogen responsive models have been developed with an inserted oestrogen response element (ERE) transgene[43, 46, 47] or brain-specific *cyp19a1b* transgene[24] and applied to study various environmental oestrogens including EE2, the plasticizer bisphenol A (BPA) and the phytoestrogen genistein. Transgenic zebrafish are typically generated by inserting a reporter fluorescent protein sequence for GFP, and the expression of this reporter sequence is driven by ligand-receptor binding to either endogenous or inserted response elements for the target of interest (e.g. via the ERE for ER TG models). Alternative fluorescent reporter sequences to GFP

used in TG models now include those that are photoconvertible upon exposure to light in (or near) the ultraviolet (UV) wavelength region.[48] An example of a photoconvertible fluorescent protein is the Kaede protein (named after the Japanese maple where, in the autumn, the leaves change from green to red)[49] which on exposure to UV light results in a spectral shift of the native (green) state from 508 nm (absorption) and 518 nm (emission) to longer wavelength peaks at 572 nm and 582 nm, respectively. Photoconversion leads to an almost complete conversion of green to red fluorescence and is irreversible and stable under aerobic conditions.[49] Photoconvertible proteins offer the ability to track individual cells and assess effects on tissue development.[50-52]

In this study we generated ERE-Kaede-Casper zebrafish, a novel oestrogen responsive transgenic zebrafish model with a Kaede photoconvertable (green to red) fluorescent protein and applied it to assess both for windows of tissue-sensitivity to oestrogen exposure during early-life and to investigate how exposure to oestrogen during early life affects responsiveness to environmental oestrogens for subsequent exposures.

5.3 Methods

Chemicals

17α-ethinylestradiol was purchased from Sigma-Alderich Chemical Co. (CAS no. 57-63-6, ≥98% pure), bisphenol A was purchased from the American Chemistry Council (BPA, 80-05-7, 99%+pure), and genistein, a phytoestrogen, was purchased from Sigma-Aldrich Chemical Co. (Gen, 446-72-0, ≥98% pure).

Animal Experiments

All experimental procedures with fish were conducted in accordance with UK Home Office regulations for the use of animals in scientific procedures and followed local ethical review guidelines ensuring their humane treatment.

Generation of the ERE-Kaede-Casper Zebrafish Model

The ERE-GFP-Casper transgenic line was derived from an ERE-GFP-Casper line previously developed at the University of Exeter (Green et al 2016), and a UAS-Kaede[53] line from Max-Planck Institute of Neurobiology, Germany (Figure 1). The ERE-GFP-Casper line is sensitive to oestrogens, with GFP expression detected in hepatocytes for an exposure to 1 ng EE2/L, and shows tissue-specific responses to different oestrogenic chemicals.[43] The ERE-GFP-Casper line has silenced *roy* (dark) and *nacre* (silver) pigmentation genes (the "Casper" phenotype),[54] resulting in a translucent phenotype and as a consequence improved GFP signal detection via fluorescence image analysis. The UAS-Kaede line has wild-type (WIK) pigmentation and expresses an inserted UAS-Kaede reporter transgene sequence. To enhance both the transmission rate of transgenic sequences to subsequent generations and consistency in oestrogen response sensitivity, the ERE-GFP-Casper model was initially screened for homozygous adults by pair breeding individuals with wild-type (WIK strain) adults and assessing the ratio of fluorescent/non-fluorescent offspring produced (homozygous ERE-GFP-Casper adults produce 100% fluorescent offspring whereas heterozygous ERE-GFP-Casper adults produce 75% or a lower proportion of fluorescent offspring). Homozygous ERE-GFP-Casper adults were then crossed with the UAS-Kaede line in 10 different mating pairs, rotating the pair combinations to promote genetic diversity. Progeny from these crosses were heterozygous for five genes of interest; ERE:Gal4ff, UAS:GFP and

UAS:Kaede transgene sequences and silenced *roy* and *nacre* pigmentation genes. Crossing of progeny from distinct families produced larvae with different combinations of the genes of interest. These larvae were screened for the homozygous ERE-Fluorescence and Casper genotype via exposure to 50 ng EE2/L from 1-3 days post fertilisation (dpf). Individuals carrying both Casper phenotype and fluorescence expression, as determined by their response to 50 ng EE2/L, were raised to sexual maturity. Screening for Kaede expression was not carried out in this first screen due to potential developmental effects of UV exposure required for distinguishing the protein from GFP. At sexual maturity, these fish were screened for Kaede expression via exposure to 50 ng EE2/L for 5 days (from 0-5 dpf) and a 2 min UV exposure to convert the Kaede excitation and emission response. Adults with progeny exclusively expressing Kaede protein (complete conversion from green to red fluorescence upon UV exposure) were identified as the founder generation (F0) of the ERE-Kaede-Casper line. These fish were then bred to generate an F1 generation and sexually mature F1 fish were screened for homozygous expression of the transgene sequences as described for F0. We compared responses to EE2 in the ERE-Kaede-Casper line with our original ERE-GFP-Casper model to assess for sensitivity and tissue response patterning to oestrogenic chemicals. To do so, embryos from both models were exposed to 10 ng EE2/L from 0-5 dpf and fluorescence response was observed using an inverted compound microscope (Zeiss Axio Observer) under consistent GFP excitation (180 ms using filter set 38 HE: BP 470/40, FT 495, BP 525/50). Further generations have been established for the ERE-Kaede-Casper line, and each generation has been screened to confirm that only homozygous adults are used for ensuring consistently high sensitivity.

Tissue responses to EE2 during early life in the ERE-Kaede-Casper model

We investigated tissue responses to EE2 (over 0-5 dpf) and the ability to photoconvert oestrogen induced green fluorescence in the Kaede-Casper model. ERE-Kaede-Casper larvae were exposed to 100 ng EE2/L over the period 0-5 dpf and exposed to UV light for 2 mins at the intervals of 3 dpf, 4 dpf and 5 dpf. A further group was exposed to 100 ng EE2/L throughout the period 0-5 dpf with no exposure to UV light. Larvae were then subjected to imaging at 5 dpf on an inverted compound microscope. After imaging, differential interference contrast (DIC), green and red Kaede fluorescence images were overlaid and the color of individual tissue response qualified via the ratios of green (new Kaede expression), red ('old' Kaede expression pre-photoconversion) and yellow (equal levels of new and old Kaede expression) fluorescence.

Development of a protocol for multiple oestrogen exposures in ERE-Kaede-Casper model

To investigate for effects of oestrogen exposure during early life on the subsequent responsiveness (sensitivity) to a further oestrogen challenge we first developed an appropriate experimental protocol to identify an appropriate exposure interval and concentration for the EE2 primary exposure. EE2 was adopted for these exposure studies because of its effects on a wide range of tissues in the ERE-GFP-Casper model, including at environmentally relevant concentrations, and strong environmental relevance. The temporal dynamics of oestrogen induced fluorescence response was investigated for exposures to

(nominal) 10 and 50ng EE2/L. Twenty larvae were exposed to each of the two test EE2 concentrations and six larvae per concentration were imaged and subjected to photoconversion every 24 hours (2-5 dpf) to compare the patterns and levels of new (green) and old (red) fluorescence induction at each time step.

Quantifying responses to EE2 in the primary exposure

The experimental protocol for the multiple exposures studies is presented in Figure 2. The initial -exposure period was for 48 hours (0-2 dpf) to an EE2 at a concentration of 10 ng/L (see results section). For the primary dosing to EE2, embryo-larvae (0-2 dpf) were cultured in embryo water either with (10 ng EE2/L, “E”) or without (DMSO solvent control group, “C”) oestrogen treatment. Using multi-well plates, each treatment comprised of 6 wells, each containing 12 embryos. After the exposure, larvae were removed from the incubation solutions, washed three times in embryo water and re-plated in their groups in oestrogen (and solvent) free embryo water for a depuration period of 24 hours to allow for complete (including any delayed) Kaede expression in the oestrogen treated larvae. At 3 dpf, 6 larvae from each of the two treatment groups were imaged and all larvae were subjected to UV illumination to photoconvert any green fluorescence. Prior to imaging and UV illumination larvae were washed and anaesthetised in embryo water containing 0.008% tricaine and mounted in methylcellulose in embryo culture medium and placed into a glass bottom 35mm dish (MatTek). Larvae were orientated to rest on their left side and images captured using an inverted compound microscope using GFP, RFP and DIC filters (1500 ms using filter set 38 HE: BP 470/40, FT 495, BP 525/50) with a 5× objective. After imaging at the 3 dpf stage, all larvae were

mounted and exposed to 2 × 1min bursts of UV light (DAPI filter) at 5× magnification to fully convert the expressed Kaede to red fluorescence excitation and emission response wavelengths.

Responses to environmental oestrogens after early life exposure to EE2

Three oestrogenic chemicals were chosen for the second exposures of the ERE-Kaede-Casper larvae, namely, EE2, BPA and genistein, all of which induce oestrogen responses in different body tissues in zebrafish and have environmental relevance.[43] Single chemical concentrations were adopted for these studies specifically: EE2 (10 ng/L), genistein (500 µg/L), BPA (2000 µg/L) and were based on activation of a low level of Kaede expression in the liver of the ERE-Kaede-Casper from initial screening trials (5 dpf larvae for a 48 h exposure) ensuring any potential increase or decrease in Kaede expression in the liver caused by EE2 pre-exposure would be both identifiable and quantifiable. Stock chemicals for each concentration were dissolved in analytical grade dimethyl sulfoxide (DMSO) and stirred vigorously in glass vials for 24 hours and stored at -20˚C. Required volumes of stock solution were pipetted into 50 mL embryo culture water and stirred vigorously to give final nominal concentration working solutions (0.1% DMSO concentration) prepared on the morning of exposure.

ERE-Kaede-Casper larvae from the initial 48 h exposure (0.1% DMSO solvent control “C”, and EE2-exposed “E”), after 24 h depuration, followed by UV photoconversion and imaging and at 3 dpf, (see Figure 2) were then exposed to EE2, BPA or genistein. They were first incubated in EDC (and solvent) free embryo medium for periods of either 0, 48 or 144 hours (embryo water changes

were conducted every 24 h) prior to the second oestrogen treatment. For these exposures, larvae were separated into four dosing groups; C-Water, C-Chemical, E-Water and E-Chemical (where Water denotes solvent control water, and Chemical is the second oestrogen treatment – either EE2, BPA or genistein). ERE-GFP-Casper embryos (in embryo water) were pipetted into six-well plates, with twelve embryos per well. Each treatment regime consisted of 3 well replicates each containing 12 larvae. The larvae were exposed to embryo water (Water) or oestrogen treatment (Chemical) for a 48 h period. The exposure regimes were: EE2 3-5 dpf, 5-7 dpf and 9-11 dpf; BPA 3-5 dpf and genistein 3-5 dpf. The imaging protocol was identical to that described for the first exposure studies (3 dpf stage for EE2) and was carried out at 5 dpf (EE2, BPA, genistein), 7 dpf (EE2), and 11 dpf (EE2). Images were also collected for specific tissues, including the liver, heart and somite muscle using a 10× objective and green fluorescent Kaede expression quantified using ImageJ™ software. These tissues of interest were masked (outlined) manually to give a specific quantifiable region of interest (ROI, see Figure S1 in Supplemental Material). The mean pixel intensity value from this ROI was used as a quantification of fluorescence response for the individual tissues.

Analytical Chemistry

To assess stock concentrations of the chemicals used in this study, two concentrations of each chemical were measured at 0 dpf and 5 dpf using tandem liquid chromatography-mass spectrometry (LC-MS), as described in Green et al 2016. For all chemicals, with the exception of EE2, water samples were diluted in acetonitrile (ACN) before analysis by LC-MS. Due to the low concentration of EE2, samples were initially run through solid phase extraction

(SPE) cartridges (Sep-Pak Plus C18) into ACN, to achieve a detectable concentration for LC-MS analysis (see Green et al., 2016, Supplemental Material for full protocol details and results).

qPCR

Relative expression levels of the three oestrogen receptor genes (*esr1*, *esr2a* and *esr2b*) in whole bodies of ERE-Kaede Casper zebrafish were analyzed using quantitative polymerase chain reaction qPCR at 5 dpf after the exposures to EE2 (primary and a secondary exposure). Efficiency-corrected relative expression levels[7] were determined by normalizing to the reference gene ribosomal protein L8 (*rpl8*), which was measured in each sample. For full details of the qPCR protocol see Supplemental Material and details on primer sequences, sizes of PCR products and PCR assay conditions are provided in Table S1.

Statistical Analysis

For the imaging data in the definitive oestrogen exposure studies tissue-specific intensity values from the four treatment groups C-Water, E-Water, C-Chemical and E-Chemical were converted to a fold- increase value over their respective controls (C-Water repeat average intensity value). Tissue specific percentage-increases for the three repeats (final n = 18) for each treatment group were then averaged to give a single fold-increase value per treatment group. All values are presented as mean ± SEM. Statistical significance between treatment groups is indicated at the $p<0.05$(*) or <0.01(**) level and calculated using an ANOVA and Games-Howell post-hoc test. Using mean fold-increase data, responses from the E-Chemical groups were compared to C-Chemical groups

and presented as percentage-increase values in the text, so as to differentiate from fold-increase over C-Water values. The two control groups (C-Water and E-Water) that were incubated in embryo water during the second exposure period were expected to produce no new (green) fluorescence response in tissues after the second exposure period. However, it could not be assumed that there would be complete Kaede photoconversion (green to red fluorescence) by UV light following the initial exposure period. Therefore, if the pre-exposed control group (E-Water) showed a statistically significant fold-increase to the equivalent C-Water control tissue value, the other pre-exposed group (E-Chemical) results were then normalized based on this fold-increase on the assumption that green fluorescence had remained after incomplete photoconversion at the 3 dpf stage.

After qPCR analysis, relative *esr* subtype expression values from the four treatment groups C-Water, E-Water, C-Chemical and E-Chemical were converted to an increase value over their respective control (C-Water repeat average value). ER subtype percentage-increases for the three repeats (final n = 3) for each treatment group were then averaged to give a single fold-increase value per treatment group. All values presented as mean ± SEM and statistical significance was calculated using an ANOVA.

5.4 Results

ERE-Kaede-Casper model

A founder F0 generation of the ERE-Kaede-Casper model was established (see Figure 1) and a homozygous F1 generation generated and raised to adulthood

for subsequent use for the exposure studies. Tissue-specific responses in the ERE-Kaede-Casper model were consistent in subsequent generations for homozygous individuals as assessed via regular screening and there was high consistency in the response to oestrogen (tissue specificity and sensitivity) between the ERE-Kaede-Casper model and the original ERE-GFP-Casper model (Figure S3).

Water Chemistry Analysis

In all water control samples chemicals were less than the limit of quantitation (LOQ). For genistein, BPA, and EE2 measured concentrations were highly consistent, at between 99% and 133% of day 5 nominals across the concentration ranges tested. Exposure concentrations are reported as ng/L or µg/L in text but nM concentrations are included where direct comparisons between chemicals are made in both text and figures. Results for the full water chemistry analysis are provided in Table S2.

Tissue responses to EE2 during early life in the ERE-Kaede-Casper model

Under UV illumination Kaede fluorescence was converted fully from green to red at the intervals tested over the life period 0-5 dpf (see Figure 3D) thus enabling visualisation and quantification of tissue responses to oestrogen for multiple time windows and for repeat (see later) exposures in the same individual.

Exposure to EE2 induced a wide range of tissues responses over early life (0-5dpf) in the ERE-Kaede-Casper model. Without photoconversion, tissues such as liver, heart, gut, brain, somite muscle, Corpuscle of Stannius and cranial

muscle all showed high levels of fluorescence when imaged at 5 dpf after EE2 exposure (Figure 3A). However, UV conversion of Kaede at 3 and 4 dpf, indicated differences in the temporal responses to EE2 stimulation for the different tissues. The heart and liver responded consistently to EE2 over the 0-5 day study period with new Kaede protein (green) expressed subsequent to UV photoconversion at 3 dpf and 4 dpf. Other tissues showed more variable temporal responses to EE2 during this period of development. Photoconversion highlighted different temporal expression of Kaede across regions of the tail. Muscle somites at the tip of the tail (caudal peduncle) showed a stronger response to EE2 between 3-5 dpf compared with the muscle somites nearer the tail head, which appeared to become less responsive by 3 dpf (Figure 3B). This difference in sensitivity can be seen more clearly after the 4 dpf photoconversion (Figure 3C). Muscle surrounding the cranium appeared to be most responsive to EE2 after 4 dpf, with little or no Kaede expression before this time (no red fluorescence). The Corpuscle of Stannius (identified in this model by Rod Wilson at the University of Exeter), a collection of cells located in the tail above the anus and involved in calcium homeostasis, responded most strongly to the EE2 treatment during 3-5 dpf. Response in the brain to EE2 also appeared to differ temporally for the early life exposures (see Takesono et al., in prep.).

Protocol for investigating multiple oestrogen exposures in the ERE-Kaede-Casper model

Tissue response patterns after the 48h exposure to 10 ng EE2/L and 50 ng EE2/L were similar, but response intensity increased with exposure concentration (Figure S4). Photoconvertion of the Kaede fluorescence and

subsequent imaging after 24 h (at 3 dpf) demonstrated further delayed Kaede expression in liver and muscle somites for the 50 ng EE2/L treatment, but not for the 10 ng EE2/L treatment. Based on these findings, the protocol we adopted for priming with EE2 prior to subsequent exposure to environmental oestrogens, was to expose embryo-larvae (0-48 hpf) to 10 ng EE2/L for 48 h followed by a 24 h incubation of the larvae in an oestrogen-free embryo culture medium followed by photoconversion of the Kaede fluorescence via treatment with UV light for 2 minutes (see Figure 2).

Responses to environmental oestrogens after early life exposure to EE2

Autofluorescence was detected in the yolk sac and otic vesicle only at 5, 7 and 11 dpf in control groups (C-Water and E-Water; Figure S5, Supplemental Material), as has been shown to occur previously for the ERE-GFP-Casper model.[43] No green fluorescence was detected for the C-Water treated groups at 3, 5, 7 or 11 dpf, or for the E-Water controls with the exception that at 5 dpf there was a 15% higher average pixel intensity in the liver (as determined quantitatively by image analysis, Figure 4A). Responses in the liver in the E-Chemical groups were thus normalized against the pixel intensity of the E-Water exposure for all time-points to account for the higher average pixel intensity in this tissue. Pixel intensity values for the heart and somite muscle in E-Water groups did not differ from the C-Water groups.

Responses to the different oestrogenic chemicals were highly consistent between individual embryo-larvae as illustrated by the low variance in the data (Figure 4). Exposure to EE2 during early life (0-48 hpf) affected subsequent responses to the exposures to EE2, BPA and genistein (3-5 dpf). In the liver at

5 dpf (3-5 dpf exposure) for exposure to EE2 (10 ng/L) and BPA (2000 µg/L) expression of GFP in E-Chemical groups was 682% and 98% higher than C-Chemical responses, respectively (Figure 4B). This was also the case for responses in heart tissue at 5 dpf (3-5 dpf exposure), where responses to genistein and BPA were 105% and 206% higher respectively in E-Chemical groups than in C-Chemical groups (Figure 4C). There was an apparent enhanced response to BPA in the somite muscle at 5 dpf, but the difference between C-B and E-B groups was not statistically significant (the data were more highly variable compared with other tissues) (Figure 4D). A small, but statistically significant difference, in somite muscle response occurred in the groups exposed to genistein (C-G and E-G) but neither of the groups' fluorescence response was significantly higher compared with the C-Water control (Figure 4D). There was higher fluorescence induction in the liver (342%) in the E-E treatment compared with the C-E groups for the exposures at 7 dpf (5-7 dpf exposure, Figure 5), but no such difference between these treatment groups for the exposure at 11 dpf (9-11 dpf exposure, Figure 5). Fluorescence images for the quantified results (Figure 5) are presented in Figure 6.

qPCR

Expression levels of the three oestrogen receptor genes (*esr1*, *esr2a* and *esr2b*) in whole bodies of ERE-Kaede-Casper zebrafish at 5 dpf after the exposures to EE2 (primary and a secondary exposures) are shown in Figure S6. For all three genes, expression appeared to be highest in the E-EE2 group, most notably for the *esr2b* gene, compared to C-Water larvae, but there were no statistically significant differences for the expression of any of the *esr*s between the different treatments.

5.5 Discussion

ERE-Kaede-Casper Model

We generated a novel oestrogen responsive transgenic model ERE-Kaede-Casper and have shown that it has great utility for studies into the effects of environmental oestrogens. Using the ERE-Kaede-Casper model we have illustrated the dynamics of tissue responses to EE2 exposure, provided new information on ontogeny of these responses and shown enhancements in sensitivity in different body tissue for exposure to environmental oestrogens following an exposure to EE2 during early life (0-2 dpf). The zebrafish model, generated by crossing two established transgenic models and phenotypic screening of subsequent generations, has a (high) sensitivity to oestrogenic chemicals, comparable with our previously developed ERE-GFP-Casper model (Figure S3)[43] and a silenced skin pigmentation that enhances fluorescence detection. We have shown that the Kaede chromophore can be successfully photoconverted in living intact individuals in all responding tissues and for high levels of Kaede expression, without any overt indication of development toxicity (Figure 3). Translucency of the skin assisted efficiency of photoconversion as pigmentation normally blocks UV light penetration into the deeper tissues in larvae. The ability to photoconvert the Kaede fluorescence response in the ERE-Kaede-Casper model provides a more dynamic model for studies into temporal dynamics and mixture responses to oestrogen compared with the ERE-GFP-Casper model. For the liver only, in some instances we found persistence of the green fluorophore of Kaede after applying two 1-minute UV

light exposures. This may have been due to an incomplete conversion of the Kaede chromophore [49] or as a consequence of the higher optical density and/or thickness of the liver, compared with some of the other responding body tissues (e.g. heart and somite muscle), that may also have limited UV penetrance and consequently inhibited the photoconversion process. However, this reduced Kaede photoconvertion efficiency in the liver of embryo-larval stages was easily accounted and adjusted for when calculating the response to oestrogens in this tissue versus controls. It is likely that photoconversion efficiency in other body tissues may be reduced with further growth and development of the fish.

Tissue responses to EE2 during early life in the ERE-Kaede-Casper model

We show windows of sensitivity to EE2 for specific tissues during early development with our ERE-Kaede-Casper model. The heart and liver responded in a consistent manner to EE2 during the life period studied, between 0-5 dpf. In contrast, other tissues, including muscle somites and the brain appeared to vary in their responses over this life period. The development of zebrafish tissues and organs have been studied extensively[55] but the role and importance of oestrogens in the development of individual somatic tissues is lacking. In mammals, oestrogen has been shown to regulate growth and differentiation of a wide range of tissues including specific regions of the brain, bone, liver, and the cardiovascular system.[56] The major expression sites of ERs in mammals during fetal development are the ovaries, testes and adrenal gland, but other tissues with notable ER expression include the brain, bone, heart, lung, kidney and intestines.[32] In mammals, oestrogen has been shown to play a key role in early development of the brain via the regulation of apoptosis and

synaptogenesis, with estradiol inhibiting these actions in some regions but promoting it in others.[57] In zebrafish, studies have shown that phytoestrogens, such as genistein, can affect brain development when exposed during the early life-stage of growth.[58] Oestrogen has recently been linked to cardiovascular maintenance and repair in zebrafish also[59] and appears to play an important role in the development of the peripheral nervous system (PNS) within skeletal muscle.[60] These roles of oestrogens are reflected in the tissue-specific responses observed in the ERE-Kaede-Casper model, and in other oestrogen responsive transgenic zebrafish lines during early life-stages.[43, 61]

Temporal dynamics of the oestrogen response to EE2 in the ERE-Kaede-Casper model

The ERE-Kaede-Casper model was used to study tissue-specific responses following 0-2 dpf exposure to EE2. Embryo-larvae were subject to photoconversion at 24 h intervals after the initial 48 h exposure to EE2. The results (Figure S4) show that fluorescence induction continued after the EE2 exposure for periods that varied depending on the exposure concentration. Kaede expression continued in the liver, heart, brain and somite muscle for 24 hours and 48 hours after exposure to 10 ng EE2/L and 50 ng EE2/L, respectively. Kaede expression was most prominent in the liver. This illustrated the ERE-Kaede-Casper model's capability for studying temporal response dynamics to oestrogenic chemicals exposures using photoconversion. The factors behind the different dynamics of response across the different responding body tissues over time are not known but they may involve differences in accumulation, metabolism and excretion of the chemical within these tissues, as well as possible differences in the number and types of ER

that are expressed and dynamics concerning the conscription of cofactors. Zebrafish have been applied successfully for *in vivo* toxicokinetic studies assessing uptake, metabolism and excretion of oestrogenic chemicals.[62] These are challenging studies however as only small amounts of plasma can be obtained for analytical chemistry measurements, placing major practical restrictions on what can be done relating to studying the uptake dynamics of the chemical. The ERE-Kaede-Casper could provide a valuable model for supporting such toxicokinetic studies. The ability to photoconvert Kaede fluorescence could potentially be applied as a proxy to assess for both the presence and persistence of the exposure chemical in the target tissues. This would operate on the assumption that the level of Kaede expression is directly correlated with the parent chemical and assumes that the products of metabolism are not biologically (estrogen) active. This may not always be the case for oestrogens, for example for BPA, where the metabolite MBP (in mammals) is more potent as an oestrogen than the parent compound. In many (most) cases however, where the parent compound only is oestrogen active the ERE-Kaede-Casper model could potentially offer an effective system to non-destructively study the toxicokinetics of oestrogenic chemicals in zebrafish in real time.

Responses to environmental oestrogens after early life exposure to EE2

There is a reliance on single chemical exposures for environmental effects assessments, but in contrast wildlife and humans are exposed intermittently, or continuously, to complex mixtures of chemicals, including EDCs. Many studies have now shown interactive (including additive) effects of oestrogens and other

EDCs.[24-26] Studies have also shown that exposure to oestrogens during early life stages can result in adverse health outcomes in later life, including learning deficits,[20] breeding and behavior effects[18] in zebrafish. Studies have also suggested tissue-specific effects may be enhanced by chemical mixtures, for example as seen for responses to EDC mixtures in mammary gland development in male and female rats.[25, 26] Almost nothing, however, is known for the effects of repeated or sequential exposures to oestrogens on tissue responses or on the health implications for these exposures, which will occur for many (most) ambient environments.[63]

Using the ERE-Kaede-Casper model, we show that exposure to EE2 during early life (0-48 hpf) has a significant bearing on the subsequent responsiveness of body tissues to further oestrogen exposure, here EE2, genistein and BPA, three oestrogens of environmental concern. Furthermore, we show that the enhancement of the response differed for these three environmental chemicals, the highest percentage-increase occurring after the second exposure to EE2. Of the three responding tissues analyzed in detail by image analysis, the liver appeared to be the most affected (sensitized) after the initial early life exposure to EE2, with secondary EE2 exposure leading to a 682% increase in fluorescence induction compared with relevant control. In the heart, fluorescence induction in response to genistein following an early life exposure to EE2 was two-fold higher compared with the relevant control. The heart appears to be especially responsive to phytoestrogens, including genistein, in comparison to other tissues [43] and this has been associated with adverse implications for cardiovascular maintenance and repair in zebrafish.[59] Oestrogenic responses to BPA were enhanced for an early life exposure to EE2

in the liver (98%) and heart (206%). BPA has been consistently linked to cardiovascular defects and abnormal liver enzymes in mammals.[17, 64]

Response to EE2 was detected strongly in the liver only at 7 dpf (5-7 dpf exposure) and 11 dpf (9-11 dpf exposure) and only weakly so, or not at all, in the heart and somatic muscle (data not presented), which concurs with the results seen for the tissue ontogeny response study (Figure 3). The sensitized response of the liver to EE2 after an initial early life stage exposure to EE2 was found to diminish at later stages of development, from 682% at 5 dpf (3-5 dpf exposure), to 342% at 7 dpf (5-7 dpf exposure), to no significant change at 11 dpf (9-11 dpf exposure). There also appeared to be an increase in variation of liver fluorescence response in the later life stages.

The mechanisms leading to the enhancement in responsiveness of certain tissues, and not others, are not clear. Nor is it clear why this sensitization effect diminishes at later stages of development, as measured specifically in the liver in this study. Changes in ER(s) number is proposed as a potential mechanism and is discussed further below. In addition, changes in response to oestrogenic chemicals may have epigenetic origins via DNA methylation or histone acetylation of gene sequences (collectively known as the epigenome) related to oestrogen signaling. Oestrogen signaling genes are regulated, in part, through DNA methylation of their promoter regions in a gender- and region-specific manner [65]. For example, exposure to E2 in adult zebrafish has been shown to increase DNA methylation levels in the 5′ flanking region of the *vitellogenin I* gene in the liver and brain, and to a greater extent in the livers of males.[66] Exposure to BPA, on the other hand, did not show any association with DNA methylation levels in the 5′ flanking region of esr1 in adult zebrafish, but altered the transcription of key enzymes involved in DNA methylation maintenance.[67]

Furthermore, DNA methylation and subsequently the transcription levels of ER genes are influenced substantially by exposure to environmental chemicals at developmental sensitive windows such as embryogenesis and early postnatal stages.[68-70] Although it is now widely accepted that chemicals affect the epigenome, epigenetic mechanisms are not yet considered in chemical risk assessment or utilized in the monitoring of the exposure and effects of chemicals and environmental change.

ER Expression

The expression of the oestrogen receptor genes *esr1*, *esr2a* and *esr2b* was quantified in whole bodies using qPCR to investigate whether changes in receptor expression occurred for the different subtypes for the different treatment regimes (Figure S6). There was no change, however, in the expression of any of the subtypes across the different exposure groups. There was an indication that expression was higher for all ER subtypes in the E-E group treatment, but this was not statistically significant. In other studies, E2 (0.1 μM) has been shown to induce a significant increase in *esr1* expression after 96 h in zebrafish, using a similar exposure protocol and qPCR analysis.[9] Collectively, the findings suggest that changes in ER(s) number may not be the major effect mechanism for the enhancement seen in the responses to environmental oestrogens after an early life exposure to EE2. However, we say this with caution as measuring responses in whole body extracts is a relatively crude approach and tissue level effects analyses are needed to provide any degree of certainty on this assumption. Furthermore, as the qPCR analysis was conducted at 5 dpf and there may have been changes in the level(s) of *esr* expression prior to this analysis time-point that we could not account for (ER

responses to oestrogen have been shown to occur within 48 h in zebrafish) [9]. In summary, even with the above caveats we did not observe a clear trend in the *esr* expression dynamics that could be directly related to the sensitized responses to environmental oestrogens caused by early life exposure to EE2.

Conclusion

Our study illustrates the need to consider exposure history of animals in environmental risk assessments and highlights the potential for interactive effects for environmental oestrogens. Applying a novel ERE-Kaede-Casper model we have both identified windows of tissue-sensitivity to oestrogens and illustrated how exposure to EE2 during early life affects responsiveness to environmental oestrogens in subsequent exposures in later stages of development. We show also, however, that the enhanced responsiveness to oestrogen may decay with time, as seen in the exposure to EE2 where at 11 dpf no enhancement in the oestrogenic response (in the liver) was seen after an initial early life (0 to 2 dpf) exposure to EE2.

Supplemental Material

Information addressing the image analysis protocol (Figure S1); primer alignment with *esr* sequences (Figure S2); the qPCR protocol (Table S1); results from water chemistry analysis (Table S2); results from model comparison (Figure S3); Results from temporal analysis of fluorescence induction (Figure S4); Kaede expression in control groups at 5, 7 and 11 dpf (Figure S5); results of ER expression from qPCR analysis (Figure S6).

Author Information

Corresponding Author

Address for correspondence: Charles R. Tyler, Biosciences, College of Life and Environmental Sciences, University of Exeter, Stocker Road, Exeter, Devon EX4 4QD, UK. Tel: 44 (0)1392 264450. Fax: 44(0)1392 263434. E-mail: C.R.Tyler@exeter.ac.uk

Acknowledgements

J.M.G's studentship was funded on a BBSRC CASE studentship with AstraZeneca (reference 620033640) and a BBSRC grant (reference BB/L01548X/1).

Notes

The authors declare no competing financial interest.

5.6 References

1. Bergman, Å.; Heindel, J.; Jobling, S.; Kidd, K.; Zoeller, R. T., State-of-the-science of endocrine disrupting chemicals, 2012. *Toxicol Lett* **2012,** *211*, S3.

2. Gore, A. C.; Chappell, V. A.; Fenton, S. E.; Flaws, J. A.; Nadal, A.; Prins, G. S.; Toppari, J.; Zoeller, R. T., EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. *Endocr Rev* **2015,** *36*, (6), E1-e150.

3. TEDX List of Potential Endocrine Disruptors. http://endocrinedisruption.org/endocrine-disruption/tedx-list-of-potential-endocrine-disruptors/overview (accessed Jan 08, 2016),

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

5. Mahato, D.; Goulding, E. H.; Korach, K. S.; Eddy, E. M., Estrogen receptor-alpha is required by the supporting somatic cells for spermatogenesis. *Mol Cell Endocrinol* **2001,** *178*, (1-2), 57-63.

6. O'Donnell, L.; Robertson, K. M.; Jones, M. E.; Simpson, E. R., Estrogen and spermatogenesis. *Endocr Rev* **2001,** *22*, (3), 289-318.

7. Filby, A. L.; Tyler, C. R., Molecular characterization of estrogen receptors 1, 2a, and 2b and their tissue and ontogenic expression profiles in fathead minnow (*Pimephales promelas*). *Biol Reprod* **2005,** *73*, (4), 648-62.

8. Gustafsson, J. A., What pharmacologists can learn from recent advances in estrogen signalling. *Trends Pharmacol Sci* **2003,** *24*, (9), 479-85.

9. Hao, R.; Bondesson, M.; Singh, A. V.; Riu, A.; McCollum, C. W.; Knudsen, T. B.; Gorelick, D. A.; Gustafsson, J. A., Identification of estrogen target genes during zebrafish embryonic development through transcriptomic analysis. *PLoS One* **2013,** *8*, (11), e79020.

10. Lange, A.; Paull, G. C.; Coe, T. S.; Katsu, Y.; Urushitani, H.; Iguchi, T.; Tyler, C. R., Sexual reprogramming and estrogenic sensitization in wild fish exposed to ethinylestradiol. *Environ Sci Technol* **2009,** *43*, (4), 1219-25.

11. Xu, H.; Yang, J.; Wang, Y.; Jiang, Q.; Chen, H.; Song, H., Exposure to 17alpha-ethynylestradiol impairs reproductive functions of both male and female zebrafish (*Danio rerio*). *Aquat Toxicol* **2008,** *88*, (1), 1-8.

12. Jobling, S.; Beresford, N.; Nolan, M.; Rodgers-Gray, T.; Brighty, G. C.; Sumpter, J. P.; Tyler, C. R., Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. *Biol Reprod* **2002,** *66*, (2), 272-81.

13. Kidd, K. A.; Blanchfield, P. J.; Mills, K. H.; Palace, V. P.; Evans, R. E.; Lazorchak, J. M.; Flick, R. W., Collapse of a fish population after exposure to a synthetic estrogen. *Proc Natl Acad Sci U S A* **2007,** *104*, (21), 8897-901.

14. Thorpe, K. I.; Hutchinson, T. H.; Hetheridge, M. J.; Sumpter, J. P.; Tyler, C. R., Development of an in vivo screening assay for estrogenic chemicals using juvenile rainbow trout (*Oncorhynchus mykiss*). *Environmental Toxicology and Chemistry* **2000,** *19*, (11), 2812-2820.

15. Van den Belt, K.; Berckmans, P.; Vangenechten, C.; Verheyen, R.; Witters, H., Comparative study on the in vitro/in vivo estrogenic potencies of 17beta-estradiol, estrone, 17alpha-ethynylestradiol and nonylphenol. *Aquat Toxicol* **2004,** *66*, (2), 183-95.

16. Li, D. K.; Zhou, Z.; Miao, M.; He, Y.; Wang, J.; Ferber, J.; Herrinton, L. J.; Gao, E.; Yuan, W., Urine bisphenol-A (BPA) level in relation to semen quality. *Fertil Steril* **2011,** *95*, (2), 625-30.e1-4.

17. Melzer, D.; Rice, N. E.; Lewis, C.; Henley, W. E.; Galloway, T. S., Association of urinary bisphenol a concentration with heart disease: evidence from NHANES 2003/06. *PLoS One* **2010,** *5*, (1), e8673.

18. Coe, T. S.; Soffker, M. K.; Filby, A. L.; Hodgson, D.; Tyler, C. R., Impacts of early life exposure to estrogen on subsequent breeding behavior and reproductive success in zebrafish. *Environ Sci Technol* **2010,** *44*, (16), 6481-7.

19. Mathieu-Denoncourt, J.; Wallace, S. J.; de Solla, S. R.; Langlois, V. S., Plasticizer endocrine disruption: Highlighting developmental and reproductive effects in mammals and non-mammalian aquatic species. *Gen Comp Endocrinol* **2015,** *219*, 74-88.

20. Saili, K. S.; Corvi, M. M.; Weber, D. N.; Patel, A. U.; Das, S. R.; Przybyla, J.; Anderson, K. A.; Tanguay, R. L., Neurodevelopmental low-dose bisphenol A exposure leads to early life-stage hyperactivity and learning deficits in adult zebrafish. *Toxicology* **2012,** *291*, (1-3), 83-92.

21. Arnold, S. F.; Klotz, D. M.; Collins, B. M.; Vonier, P. M.; Guillette, L. J., Jr.; McLachlan, J. A., Synergistic activation of estrogen receptor with combinations of environmental chemicals. *Science* **1996,** *272*, (5267), 1489-92.

22. Kortenkamp, A., Low dose mixture effects of endocrine disrupters and their implications for regulatory thresholds in chemical risk assessment. *Curr Opin Pharmacol* **2014,** *19*, 105-11.

23. Silva, E.; Rajapakse, N.; Kortenkamp, A., Something from "nothing"--eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. *Environ Sci Technol* **2002,** *36*, (8), 1751-6.

24. Brion, F.; Le Page, Y.; Piccini, B.; Cardoso, O.; Tong, S. K.; Chung, B. C.; Kah, O., Screening estrogenic activities of chemicals or mixtures in vivo using transgenic (cyp19a1b-GFP) zebrafish embryos. *PLoS One* **2012,** *7*, (5), e36069.

25. Christiansen, S.; Kortenkamp, A.; Axelstad, M.; Boberg, J.; Scholze, M.; Jacobsen, P. R.; Faust, M.; Lichtensteiger, W.; Schlumpf, M.; Burdorf, A.; Hass, U., Mixtures of endocrine disrupting contaminants modelled on human high end exposures: an exploratory study in rats. *Int J Androl* **2012,** *35*, (3), 303-16.

26. Mandrup, K. R.; Johansson, H. K.; Boberg, J.; Pedersen, A. S.; Mortensen, M. S.; Jorgensen, J. S.; Vinggaard, A. M.; Hass, U., Mixtures of environmentally relevant endocrine disrupting chemicals affect mammary gland development in female and male rats. *Reprod Toxicol* **2015,** *54*, 47-57.

27. Evans, R. M.; Scholze, M.; Kortenkamp, A., Additive mixture effects of estrogenic chemicals in human cell-based assays can be influenced by inclusion of chemicals with differing effect profiles. *PLoS One* **2012,** *7*, (8), e43606.

28. Lange, A.; Sebire, M.; Rostkowski, P.; Mizutani, T.; Miyagawa, S.; Iguchi, T.; Hill, E. M.; Tyler, C. R., Environmental chemicals active as human antiandrogens do not activate a stickleback androgen receptor but enhance a feminising effect of oestrogen in roach. *Aquat Toxicol* **2015,** *168*, 48-59.

29. Nilsson, S.; Makela, S.; Treuter, E.; Tujague, M.; Thomsen, J.; Andersson, G.; Enmark, E.; Pettersson, K.; Warner, M.; Gustafsson, J. A., Mechanisms of estrogen action. *Physiol Rev* **2001,** *81*, (4), 1535-65.

30. Pettersson, K.; Gustafsson, J. A., Role of estrogen receptor beta in estrogen action. *Annu Rev Physiol* **2001,** *63*, 165-92.

31. Bardet, P. L.; Horard, B.; Robinson-Rechavi, M.; Laudet, V.; Vanacker, J. M., Characterization of oestrogen receptors in zebrafish (*Danio rerio*). *J Mol Endocrinol* **2002,** *28*, (3), 153-63.

32. Bondesson, M.; Hao, R.; Lin, C. Y.; Williams, C.; Gustafsson, J. A., Estrogen receptor signaling during vertebrate development. *Biochim Biophys Acta* **2015,** *1849*, (2), 142-51.

33. Brown, A. R.; Gunnarsson, L.; Kristiansson, E.; Tyler, C. R., Assessing variation in the potential susceptibility of fish to pharmaceuticals, considering evolutionary differences in their physiology and ecology. *Philos Trans R Soc Lond B Biol Sci* **2014,** *369*, (1656).

34. Giguere, V.; Yang, N.; Segui, P.; Evans, R. M., Identification of a new class of steroid hormone receptors. *Nature* **1988,** *331*, (6151), 91-4.

35. Razandi, M.; Pedram, A.; Greene, G. L.; Levin, E. R., Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. *Mol Endocrinol* **1999,** *13*, (2), 307-19.

36. Soltysik, K.; Czekaj, P., Membrane estrogen receptors - is it an alternative way of estrogen action? *J Physiol Pharmacol* **2013,** *64*, (2), 129-42.

37. Leclercq, G.; Lacroix, M.; Laios, I.; Laurent, G., Estrogen receptor alpha: impact of ligands on intracellular shuttling and turnover rate in breast cancer cells. *Curr Cancer Drug Targets* **2006,** *6*, (1), 39-64.

38. Smith, C. L.; O'Malley, B. W., Coregulator function: a key to understanding tissue specificity of selective receptor modulators. *Endocr Rev* **2004,** *25*, (1), 45-71.

39. Cosnefroy, A.; Brion, F.; Maillot-Marechal, E.; Porcher, J. M.; Pakdel, F.; Balaguer, P.; Ait-Aissa, S., Selective activation of zebrafish estrogen receptor

subtypes by chemicals by using stable reporter gene assay developed in a zebrafish liver cell line. *Toxicol Sci* **2012,** *125*, (2), 439-49.

40. Ascenzi, P.; Bocedi, A.; Marino, M., Structure-function relationship of estrogen receptor alpha and beta: impact on human health. *Mol Aspects Med* **2006,** *27*, (4), 299-402.

41. Koehler, K. F.; Helguero, L. A.; Haldosen, L. A.; Warner, M.; Gustafsson, J. A., Reflections on the discovery and significance of estrogen receptor beta. *Endocr Rev* **2005,** *26*, (3), 465-78.

42. Chandrasekar, G.; Archer, A.; Gustafsson, J.-Å.; Lendahl, M. A., Levels of 17β-estradiol receptors expressed in embryonic and adult zebrafish following in vivo treatment of natural or synthetic ligands. *PloS one* **2010,** *5*, (3), e9678.

43. Green, J. M.; Metz, J.; Lee, O.; Trznadel, M.; Takesono, A.; Brown, A. R.; Owen, S. F.; Kudoh, T.; Tyler, C. R., High-Content and Semi-Automated Quantification of Responses to Estrogenic Chemicals Using a Novel Translucent Transgenic Zebrafish. *Environ Sci Technol* **2016**.

44. Lange, A.; Paull, G. C.; Coe, T. S.; Katsu, Y.; Urushitani, H.; Iguchi, T.; Tyler, C. R., Sexual Reprogramming and Estrogenic Sensitization in Wild Fish Exposed to Ethinylestradiol. **2009**.

45. Lee, O.; Green, J. M.; Tyler, C. R., Transgenic fish systems and their application in ecotoxicology. *Crit Rev Toxicol* **2015,** *45*, (2), 124-41.

46. Gorelick, D. A.; Halpern, M. E., Visualization of estrogen receptor transcriptional activation in zebrafish. *Endocrinology* **2011,** *152*, (7), 2690-703.

47. Lee, O.; Takesono, A.; Tada, M.; Tyler, C. R.; Kudoh, T., Biosensor zebrafish provide new insights into potential health effects of environmental estrogens. *Environ Health Perspect* **2012,** *120*, (7), 990-6.

48. Campbell, R. E.; Davidson, M. W., Fluorescent Reporter Proteins. *Molecular Imaging with Reporter Genes (Cambridge Univ Pr)* **2010**, 1.

49. Ando, R.; Hama, H.; Yamamoto-Hino, M.; Mizuno, H.; Miyawaki, A., An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proc Natl Acad Sci U S A* **2002,** *99*, (20), 12651-6.

50. Dougherty, M.; Kamel, G.; Shubinets, V.; Hickey, G.; Grimaldi, M.; Liao, E. C., Embryonic fate map of first pharyngeal arch structures in the sox10: kaede zebrafish transgenic model. *J Craniofac Surg* **2012,** *23*, (5), 1333-7.

51. Hatta, K.; Tsujii, H.; Omura, T., Cell tracking using a photoconvertible fluorescent protein. *Nat Protoc* **2006,** *1*, (2), 960-7.

52. Sato, T.; Takahoko, M.; Okamoto, H., HuC:Kaede, a useful tool to label neural morphologies in networks in vivo. *Genesis* **2006,** *44*, (3), 136-42.

53. Scott, E. K.; Mason, L.; Arrenberg, A. B.; Ziv, L.; Gosse, N. J.; Xiao, T.; Chi, N. C.; Asakawa, K.; Kawakami, K.; Baier, H., Targeting neural circuitry in zebrafish using GAL4 enhancer trapping. *Nat Methods* **2007,** *4*, (4), 323-6.

54. White, R. M.; Sessa, A.; Burke, C.; Bowman, T.; LeBlanc, J.; Ceol, C.; Bourque, C.; Dovey, M.; Goessling, W.; Burns, C. E.; Zon, L. I., Transparent adult zebrafish as a tool for in vivo transplantation analysis. *Cell Stem Cell* **2008,** *2*, (2), 183-9.

55. Kimmel, C. B.; Ballard, W. W.; Kimmel, S. R.; Ullmann, B.; Schilling, T. F., Stages of embryonic development of the zebrafish. *Dev Dyn* **1995,** *203*, (3), 253-310.

56. Katzenellenbogen, B. S., Estrogen receptors: bioactivities and interactions with cell signaling pathways. *Biol Reprod* **1996,** *54*, (2), 287-93.

57. McCarthy, M. M., Estradiol and the developing brain. *Physiol Rev* **2008,** *88*, (1), 91-124.

58. Sassi-Messai, S.; Gibert, Y.; Bernard, L.; Nishio, S.; Ferri Lagneau, K. F.; Molina, J.; Andersson-Lendahl, M.; Benoit, G.; Balaguer, P.; Laudet, V., The phytoestrogen genistein affects zebrafish development through two different pathways. *PLoS One* **2009,** *4*, (3), e4935.

59. Allgood, O. E., Jr.; Hamad, A.; Fox, J.; Defrank, A.; Gilley, R.; Dawson, F.; Sykes, B.; Underwood, T. J.; Naylor, R. C.; Briggs, A. A.; Lassiter, C. S.; Bell, W. E.; Turner, J. E., Estrogen prevents cardiac and vascular failure in the 'listless' zebrafish (*Danio rerio*) developmental model. *Gen Comp Endocrinol* **2013,** *189*, 33-42.

60. Houser, A.; McNair, C.; Piccinini, R.; Luxhoj, A.; Bell, W. E.; Turner, J. E., Effects of estrogen on the neuromuscular system in the embryonic zebrafish (*Danio rerio*). *Brain Res* **2011,** *1381*, 106-16.

61. Gorelick, D. A.; Iwanowicz, L. R.; Hung, A. L.; Blazer, V. S.; Halpern, M. E., Transgenic zebrafish reveal tissue-specific differences in estrogen signaling in response to environmental water samples. *Environ Health Perspect* **2014,** *122*, (4), 356-62.

62. Pery, A. R.; Devillers, J.; Brochot, C.; Mombelli, E.; Palluel, O.; Piccini, B.; Brion, F.; Beaudouin, R., A physiologically based toxicokinetic model for the zebrafish Danio rerio. *Environ Sci Technol* **2014,** *48*, (1), 781-90.

63. Kortenkamp, A.; Altenburger, R., Toxicity from combined exposure to chemicals. *Mixture Toxicity. Linking Approaches from Ecological and Human Toxicology* **2010**, 95-119.

64. Rogers, J. A.; Metz, L.; Yong, V. W., Review: Endocrine disrupting chemicals and immune responses: a focus on bisphenol-A and its potential mechanisms. *Mol Immunol* **2013,** *53*, (4), 421-30.

65. Mirbahai, L.; Chipman, J. K., Epigenetic memory of environmental organisms: a reflection of lifetime stressor exposures. *Mutat Res Genet Toxicol Environ Mutagen* **2014,** *764-765*, 10-7.

66. Stromqvist, M.; Tooke, N.; Brunstrom, B., DNA methylation levels in the 5' flanking region of the vitellogenin I gene in liver and brain of adult zebrafish (*Danio rerio*)--sex and tissue differences and effects of 17alpha-ethinylestradiol exposure. *Aquat Toxicol* **2010,** *98*, (3), 275-81.

67. Laing, L. V.; Viana, J.; Dempster, E. L.; Trznadel, M.; Trunkfield, L. A.; Uren Webster, T. M.; van Aerle, R.; Paull, G. C.; Wilson, R. J.; Mill, J.; Santos, E. M., Bisphenol A causes reproductive toxicity, decreases dnmt1 transcription, and reduces global DNA methylation in breeding zebrafish (*Danio rerio*). *Epigenetics* **2016,** *11*, (7), 526-38.

68. Wilson, M. E.; Sengoku, T., Developmental regulation of neuronal genes by DNA methylation: environmental influences. *Int J Dev Neurosci* **2013,** *31*, (6), 448-51.

69. McCarthy, M. M.; Crews, D., Epigenetics--new frontiers in neuroendocrinology. *Front Neuroendocrinol* **2008,** *29*, (3), 341-3.

70. Crews, D., Epigenetics, brain, behavior, and the environment. *Hormones (Athens)* **2010,** *9*, (1), 41-50.

5.7 Figure Legends

Figure 1: Generation of ERE-Kaede-Casper (F0) line. ERE denotes the ERE-Gal4ff transgene sequence, GFP denotes the UAS-GFP transgene sequence and Kaede denotes the UAS-Kaede transgene sequence. Expression of pigmentation (Pig.) genes *roy* (dark) and *nacre* (silver) are also shown. The ERE-GFP-Casper model, homozygous for both transgene sequences, and a homozygous UAS-Kaede strain were initially crossed to produce a heterozygous generation. In-breeding within this generation produced progeny with different genotypes based on four genes of interest. At sexual maturity, F0 ERE-Kaede-Casper adults were identified by screening for photoconvertible progeny with fully silenced pigmentation and TG(ERE:Gal4ff)(UAS:Kaede) expression.

Figure 2: Exposure Protocol Outline. ERE-Kaede-Casper embryos were initially separated into 48h control (C) and EE2 (10 ng/L) initial-exposure (E) groups. After a subsequent 24h non-exposure period larvae were imaged and Kaede expression underwent photoconvertion (green to red fluorescence, 3 dpf). Various intervals of non-exposure were then adopted before a second oestrogen exposure was conducted. Larvae from the two initial treatments (C and E) were each divided into two groups; one control exposure (C-Water and E-Water) and the second an oestrogenic chemical exposure (C-Chemical and E-Chemical). Imaging was carried out at the final time point with subsequent image analysis for quantification of Kaede expression. The expression of the three nuclear oestrogen receptor subtypes was also quantified at the final time point using qPCR.

Figure 3: Kaede conversion analysis. ERE-Kaede-Casper larvae were exposed to 100 ng EE2/L over the period 0-5 dpf and imaged at 5 dpf either without UV exposure (A), or after exposure to UV at 3dpf (B), 4dpf (C) and 5dpf (D) to convert Kaede fluorescence from green to red. Specific tissue response in the liver (li), heart (h), somite muscle (sm), otic vesicle (ov), cardiac muscle (cm), corpuscle of Stannius (cs), brain (b), neuromast (n), and gut (g).

Figure 4: Quantification of target tissue responses in ERE-Kaede-Casper transgenic zebrafish exposed to oestrogens during early life, as determined by fluorescence induction. Green fluorescence intensity was quantified in liver, heart and somite muscle (S.M.) in controls (A) at 5 dpf. Control (non-exposed) larvae and larvae exposed initially to 10 ng EE2/L over the period of 48h (0-2 dpf) and green fluorescence intensity in liver (B), heart (C) and S.M. (D) were quantified after EE2 (10 ng/L), genistein (500 µg/L) and BPA (2000 µg/L) exposures for 3-5 dpf. Quantification of liver responses in the E-Chemical (E-E, E-G or E-B, respectively) treatment groups were normalized against their respective C-W controls (A), which were set to a value of 1. Data are reported as mean fold induction ± SEM (n=18). Statistical significance values were calculated using ANOVA and Games-Howell post-hoc test (* $p < 0.05$ and ** $p < 0.01$).

Figure 5: Quantification of liver responses in ERE-Kaede-Casper transgenic zebrafish exposed to EE2 at different stages of development, as determined by fluorescence induction. Responses in the liver were quantified after EE2 exposure at 3-5 dpf, 5-7 dpf and 9-11 dpf. Quantification of liver responses in the E-Chemical treatment groups were normalised against their respective controls. Data are reported as mean fold induction ± SEM

(n=18). Statistical significance values were calculated using ANOVA and Games-Howell post-hoc test (* $p < 0.05$ and ** $p < 0.01$).

Figure 6: Sensitivity to ethinylestradiol for repeated exposures. Control (non-exposed) larvae and larvae exposed initially to 10 ng EE2/L over the period of 48h (0-2 dpf) were imaged at 3 dpf (A) and the Kaede response was then converted fully from green to red fluorescence via UV exposure (B). Both groups of photoconverted larvae (control and EE2-exposed) were then exposed to 10 ng EE2/L over the period 3-5 dpf (C), 5-7 dpf (D) or 9-11 dpf (E) and imaged on final day of exposure (n=18). Newly generated Kaede expression (green fluorescence) in liver, heart and somite muscle green was quantified by image analysis. All images were acquired by inverted compound microscope using a 5× objective. A and B images were acquired using GFP, RFP and DIC filters. C, D, and E are presented with the GFP filter only. Specific tissue response in the liver (li), heart (h), somite muscle (sm), otic vesicle (ov), cardiac muscle (cm) and neuromast (n).

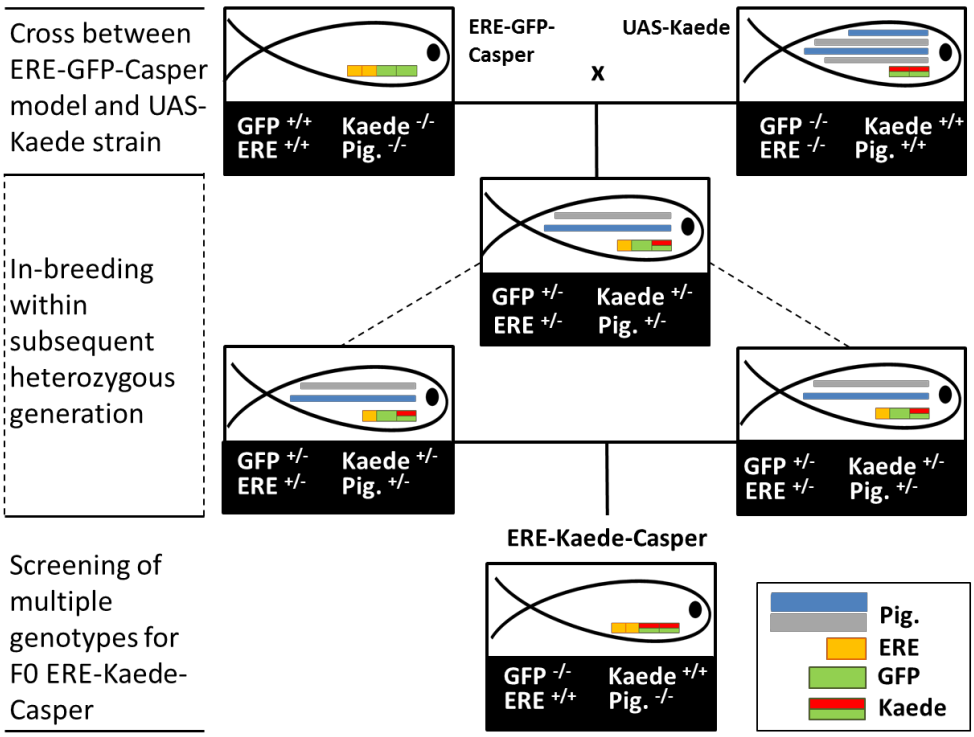


Figure 1: Generation of ERE-Kaede-Casper (F0) line.

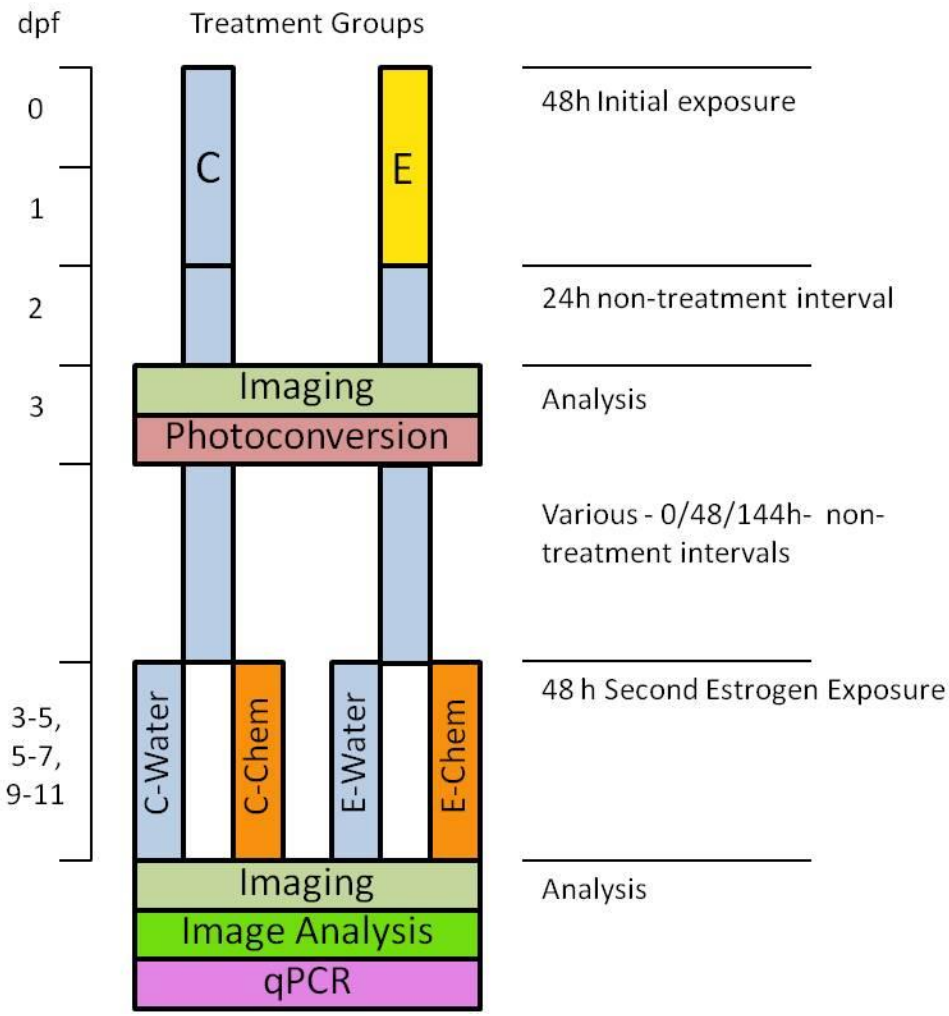


Figure 2: Exposure Protocol Outline.

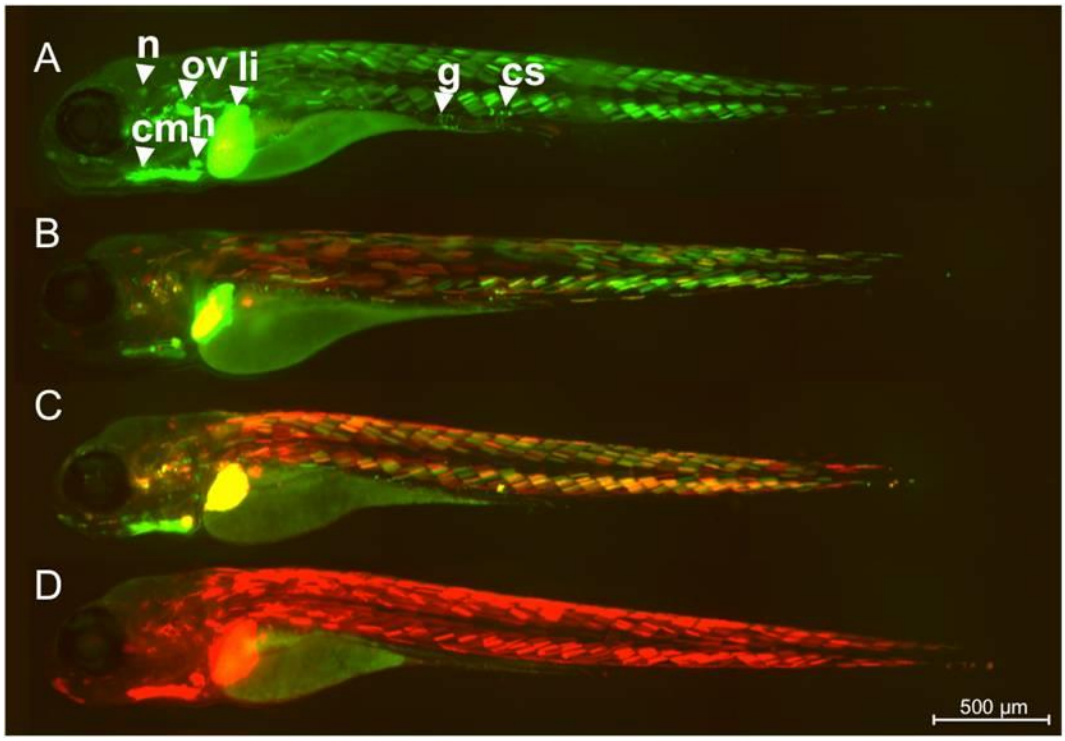


Figure 3: Kaede conversion analysis.

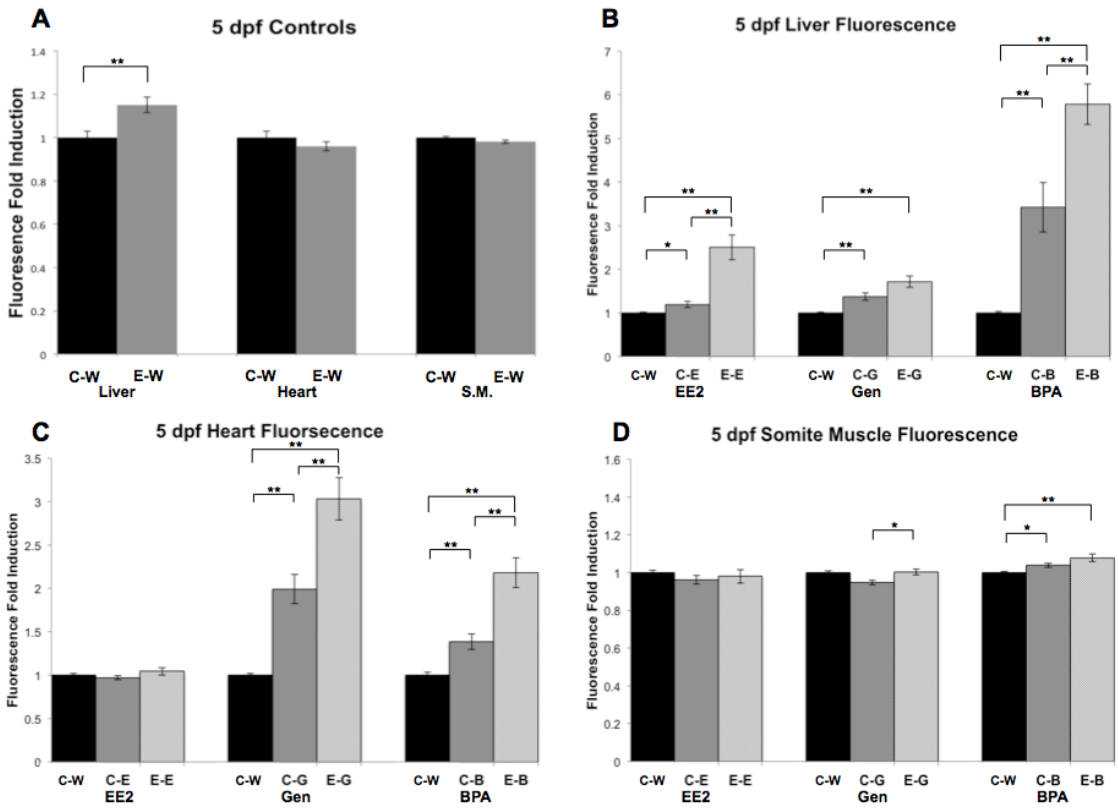


Figure 4: Quantification of target tissue responses in ERE-Kaede-Casper transgenic zebrafish exposed to estrogen treatment, as determined by fluorescence induction.

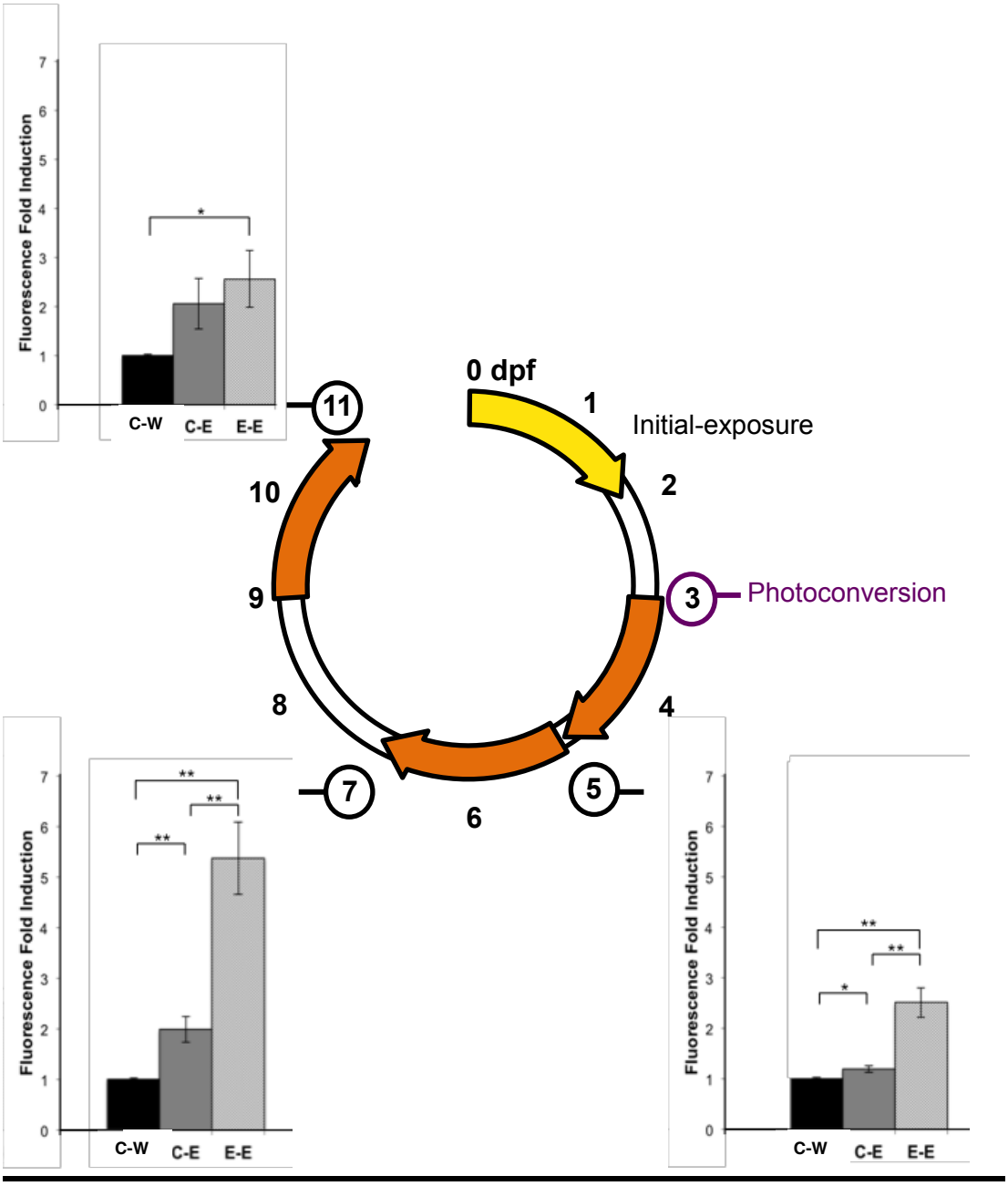


Figure 5: Quantification of liver responses in ERE-Kaede-Casper transgenic zebrafish exposed to EE2 treatment at different stages of development, as determined by fluorescence induction.

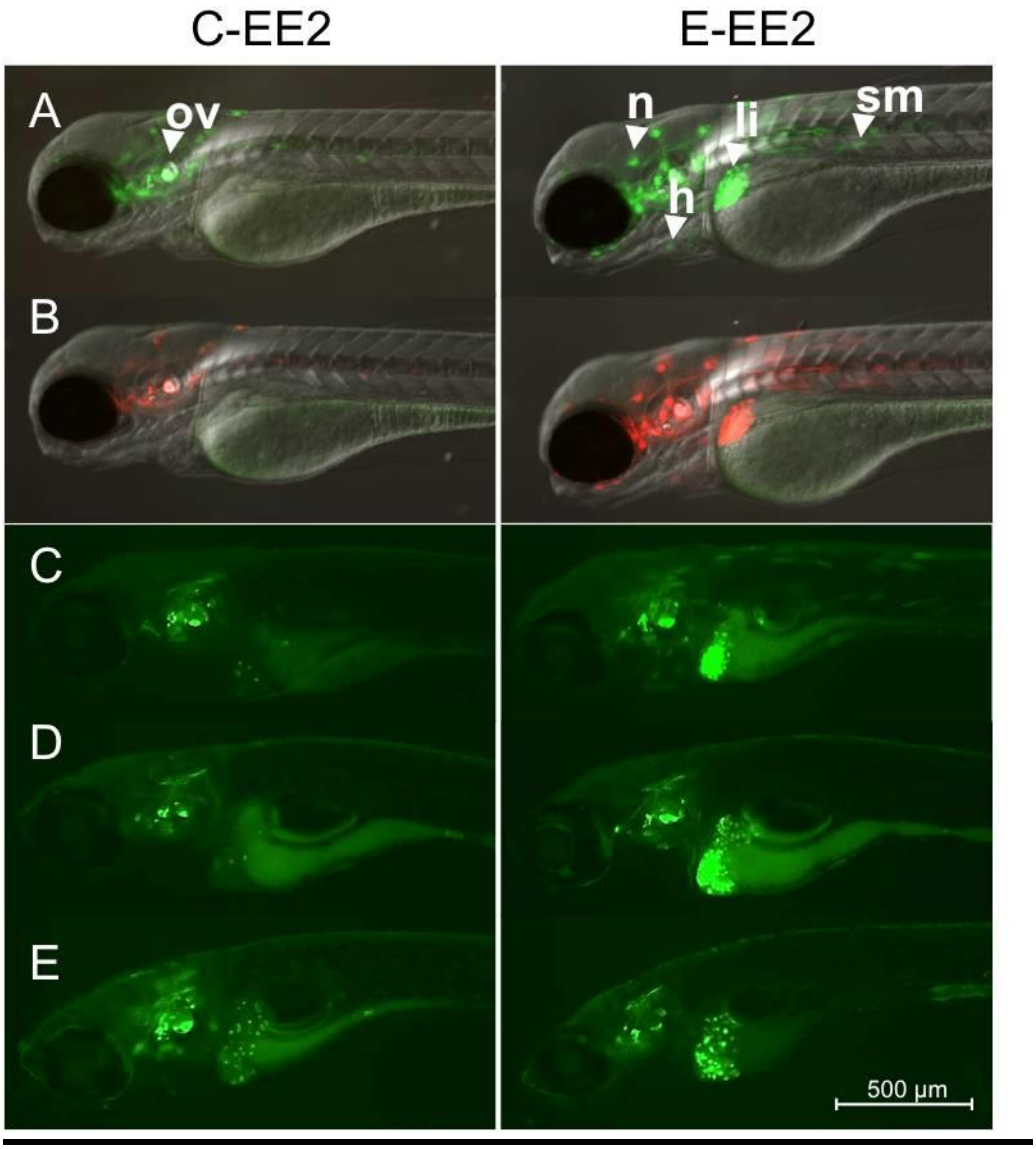


Figure 6: Sensitivity to ethinyloestradiol is enhanced for repeated exposures.

5.8 Supporting Information

Figure S1: Image analysis. Fluorescence induction for specific tissues was quantified via initially masking manually that tissue (see: yellow outline for liver (A), heart (B) and somite muscle (C)) and quantified using ImageJ™ software.

qPCR Protocol

Effects of the initial exposure to EE2 were assessed on the expression of *esr1*, *esr2a* and *esr2b* for the secondary EE2 exposure via qPCR. Three pools of 15 embryos from the secondary exposure to EE2 exposure (at the 3-5 dpf stage) were collected from each treatment regime after imaging at 5 dpf, snap frozen in liquid nitrogen and stored at -80 °C until analysis. Total RNA was extracted using Tri reagent (Sigma-Aldrich, UK) according to the manufacturer's protocol. The quantity and quality of the RNA was assessed spectrometrically using a NanoDrop 1000 Spectrophotometer. Two µg total RNA were treated with RQ1 RNase-Free DNase (Promega, Southampton, UK) and subsequently reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Promega) and random hexamers (Eurofins Genomics), following manufacturer's instructions. The expression of target mRNA was subsequently determined by qPCR using target-specific SybrGreen assays on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories Ltd., Hercules, CA, US) followed by melt curve analysis to validate amplification of a single PCR product. Efficiency-corrected relative expression levels were determined by normalising to the reference gene ribosomal protein L8 (*rpl8*), which was measured in each sample. Details on primer sequences, sizes of PCR products and PCR assay conditions are provided in Table S1.

Figure S2: qPCR Alignment. Alignments of qPCR primers to their respective ESR sequences.

Table S1: Target genes, primer sequences [(1)], amplicon size, annealing temperature and PCR efficiency for the RT-QPCR assays used in this study.

Figure S3: Comparison of ERE-GFP-Casper and ERE-Kaede-Casper models. Sensitivity and tissue response of the two models were compared via imaging after 0-5 dpf exposures to embryo water (A), 5 ng/L EE2 (B) and 10 ng/L EE2 (C). All images were acquired by inverted compound microscope (Zeiss Axio Observer) using a 5x objective. Images are presented with the GFP filter only.

Figure S4: Temporal analysis of fluorescence induction after a single 48h exposure exposure to EE2. ERE-Kaede-Casper embryos were exposed to 10 ng/L and 50ng/L EE2 for 48 hours then washed and incubated in embryo water up to 5 dpf. At intervening 24 hour time-points larvae were imaged to assess new Kaede expression, then immediately exposed to UV light to photoconvert Kaede fluorescence from green to red. All images were acquired by inverted compound microscope (Zeiss Axio Observer) using a 5x objective. Images are presented with the GFP filter only and therefore only 24 hours worth of Kaede expression is shown in each image.

Figure S5: Kaede expression in control groups C-Water and E-Water. Control (non exposed) larvae and larvae that were exposed initially to 10 ng EE2/L over the period 0-2 dpf were imaged at 3 dpf (A) and the Kaede response then converted fully from green to red fluorescence via UV exposure (B). Both groups of larvae (control and EE2-exposed) were then exposed to embryo water over the period 3-5 dpf (C), 5-7 dpf (D) or 9-11 dpf (E) and imaged on final day of exposure (n=18). Newly generated Kaede expression (green fluorescence) was not observed (there was some auto-fluorescence). All images were acquired by inverted compound microscope (Zeiss Axio Observer) using a 5x objective. A and B images were acquired using GFP, RFP and DIC filters. C, D, and E are presented with the GFP filter only.

Figure S6: qPCR quantification of esr1, esr2a and esr2b gene expression. Results from qPCR analysis of esr gene expression in the four treatment groups from the 3-5 dpf second EE2 exposure treatment. Larvae were sampled at 5 dpf. Expression of the individual esr genes were compared for each treatment group. Data are reported as mean fold-induction ± SEM (n=3).

References

Filby, A. L.; Paull, G. C.; Hickmore, T. F.; Tyler, C. R., Unravelling the neurophysiological basis of aggression in a fish model*, BMC Genomics*, **2010,** 11:498.

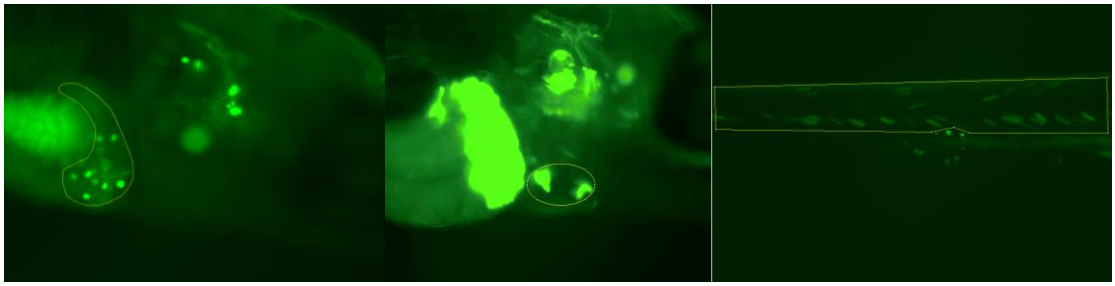


Figure S1: Image analysis.

Alignment of the zebrafish esr1 sequence (NM_174862.3 *Danio rerio* estrogen receptor 1 (esr1), mRNA) with the qPCR primers (underlined) used.

NM_152959.1	GATACATCAGTGAGAGAGAGAAAGCATCCAGCCTGTAATGGGACTCAAGT	50
NM_152959.1	AAAACATCAGAGGAGTGAACATTTTGACCAGCGGAGAGGCTGTTGGTGCC	100
NM_152959.1	AGGCAGCGACGCAGGACCAGCCCGATTCCTGAGAGAGAAGCCCTGGAGGA	150
NM_152959.1	ACCTTCCTCGCCACCTGCCGCCCACAAACTCTCACCCATGTACCCTAAGG	200
NM_152959.1	AGGAGCACAGCGCGGGGGGCATCAGCTCCTCTGTCAACTACCTAGATGGA	250
NM_152959.1	GCTTATGAGTACCCGAACCCCACACAGACCTTCGGCACCTCGTCGCCTGC	300
NM_152959.1	AGAGCCTGCTTCAGTGGGATACTACCCGGCTCCCCCAGACCCCCACGAGG	350
NM_152959.1	AACATCTACAGACGCTTGGCGGTGGATCAAGCAGCCCCCTCATGTTCGCA	400
NM_152959.1	CCCTCCAGCCCACAGCTGTCCCCGTATCTGAGCCATCACGGAGGACACCA	450
NM_152959.1	CACCACCCCTCATCAGGTGTCCTACTACCTGGATTCCTCGTCCAGCACCG	500
NM_152959.1	TCTACAGGTCCAGTGTGGTGTCCTCTCAGCAGGCAGCCGTGGGCCTGTGT	550
NM_152959.1	GAGGAGCTGTGCAGTGCCACTGACAGGCAGGAGTTGTACACTGGATCCAG	600
NM_152959.1	AGCGGCTGGAGGCTTTGATTCAGGGAAAGAGACTCGCTTCTGTGCGGTGT	650
NM_152959.1	GCAGTGACTACGCCTCTGGATATCATTACGGAGTCTGGTCGTGTGAGGGA	700
NM_152959.1	TGCAAAGCTTTCTTCAAGAGAAGCATTCAAGGTCACAATGACTATGTTTG	750
NM_152959.1	TCCAGCGACCAACCAGTGCACTATTGACAGAAACCGTCGAAAGAGCTGCC	800
NM_152959.1	AAGCATGCAGACTGCGCAAGTGTTATGAAGTAGGCATGATGAAAGGAGGT	850
NM_152959.1	ATTCGTAAAGATCGCGGAGGGCGTTCTGTCAGGCGTGAGAGAAGGAGAAG	900
NM_152959.1	CAGTAATGAAGATCGAGACAAGAGCAGCAGTGATCAGTGCAGCCGTGCTG	950
NM_152959.1	GCGTGAGGACGACTGGCCCACAGGACAAGAGGAAGAAGCGCAGTGGTGGG	1000
NM_152959.1	GTGGTCAGCACTTTATGCATGTCGCCTGACCAGGTGCTGCTGCTGCTGCT	1050

```
NM_152959.1             GGGGGCTGAGCCACCCGCTGTCTGCTCACGACAGAAACACAGCCGGCCCT  1100

NM_152959.1             ACACAGAGATCACCATGATGTCCCTGCTCACCAACATGGCTGACAAAGAA  1150

NM_152959.1             CTCGTCCACATGATCGCCTGGGCCAAGAAAGTGCCAGGATTCCAGGATCT  1200

NM_152959.1             GTCTCTGCATGACCAGGTTCAGTTGTTGGAGAGCTCTTGGCTGGAAGTGT  1250

NM_152959.1             TGATGATTGGCCTCATATGGAGGTCCATTCATTCCCCTGGAAAACTCATC  1300

NM_152959.1             TTTGCTCAGGATCTCATCCTGGATAGGAGTGAAGGAGAATGTGTTGAGGG  1350

NM_152959.1             CATGGCTGAGATTTTCGACATGCTCTTAGCAACTGTGGCTCGATTTCGGA  1400

NM_152959.1             GTCTCAAGCTCAAGCTGGAGGAATTTGTGTGTCTCAAAGCCATCATACTC  1450

NM_152959.1             ATCAATTCTGGTGCATTTTCATTCTGCTCCAGTCCAGTGGAGCCGCTGAT  1500

NM_152959.1             GGACAACTTCATGGTGCAGTGCATGCTGGACAACATCACTGATGCCCTCA  1550

NM_152959.1             TTTACTGCATCAGTAAATCAGGAGCGTCGCTGCAGCTGCAGTCCCGTCGC  1600

NM_152959.1             CAGGCCCAGCTCCTGCTGCTGCTCTCACACATCAGACACATGAGCAACAA  1650

qPCR_esr1 _sense                                          CGAGTGCCGCTGT
NM_152959.1             AGGAATGGAGCACTTATACCGAATGAAATGTAAGAATCGAGTGCCGCTGT  1700

qPCR_esr1_sense         ATGAC
NM_152959.1             ATGACCTGTTGCTGGAGATGCTGGACGCTCAGCGGTTCCAGTCTTCAGGG  1750

NM_152959.1             AAGGTGCAGCGAGTGTGGTCTCAGAGCGAGAAAAACCCTCCATCTACACC  1800

NM_152959.1             CACAACCAGCAGCAGCAGCAACAACAGTCCAAGAGGAGGAGCTGCAG     1850
qPCR_esr1_antisense     CACAACCAGCAGCAGCA

NM_152959.1             CCATTCAGTCCAACGGCGCCTGTCACAGCCATAGCCCTGACCCCTGACCT  1900

NM_152959.1             GTGCACTGCCACCATATACACATTATACACAATTTGAAGAGCAGAAATGA  1950

NM_152959.1             GGACTAGGACTTCAGTCTCTTCTTGTGCCTTACGAGAGTCTACTATTGAA  2000

NM_152959.1             ACCGAGACATTCGAAAGAGAATTTGTTTATAGAAAAAAAAGTCTGTAAAT  2050

NM_152959.1             AAGAATAAGCCTCCCAAACACTATATATTGAGGATCTTTTTACGGTTGAT  2100

NM_152959.1             TTCGCTCGGTTTTTGTGGAAGCTGATTACAGGAATTTGTTTTTCCCATAC  2150

NM_152959.1             ACTTGAGAGAAACAGAGAGAGATGCTTAACTCATCTTTTATTCTTTTACA  2200

NM_152959.1             ATTTGTGACCGTTTGACATCACAAACTCACTTAAAGCTCTCATACTGATC  2250

NM_152959.1             ATATGGCAGTCAAAAAATATTGATTTAATTTTCAATAATTAATATATTAT  2300

NM_152959.1             AATAGTATATTAGTGTTAAAATAGTATTTTGTATTTTTAGAGTATTTTAT  2350

NM_152959.1             TGTCATAAATAATGTTAATGCAATAGTAATTATCCATTTATGACCAGATA  2400

NM_152959.1             TTACAAAGTTTTAAGAAACAGATCTGAAAATGAAATTTCCAACTTTTAAA  2450

NM_152959.1             TGTTGAATGCAGAAATGAAAAAGTTCCACATCCACACAGTAGGCTACCAA  2500

NM_152959.1             AAAACAAGTAAATTAAGTTTAAACGCTGCTCTGATTTCCTCATCTACATT  2550

NM_152959.1             CTTTATAGTATAGCTTATGTTTGCATTGCAAAAAAAGAAAAAAAAGAATG  2600

NM_152959.1             AAAGAAGGGATAATTGTTTTTTTAACTACTCAAGTTCTTCTAGTTGACGC  2650

NM_152959.1             ATGTGCCAACTTTTTCTTGCCAATCTTAAGCCAATTGTAATGTTTCGTTT  2700

NM_152959.1             TTAGTTCAATTTGGAATTTCCACAACTTTGGCAGAGATCGGGGGATCAGA  2750

NM_152959.1             GTGAGCGCTCCTACTTGGCCCCACCCTGATCAAGAAAGGCATAAATTAAA  2800

NM_152959.1             GCGCTCTGACTGAATTTTATGATTGGTCCAGTATTATTACTGACATGCTT  2850

NM_152959.1             AAATATTTTTTGTTTGTTTTTTGCTTTTACTTATTTTTATTGAATTCTTA  2900

NM_152959.1             ATTTGTTAAAATATTAGCCTGTTTTGCTTTTCTTTCTTTTTTTTTTTTAA  2950
```

NM_152959.1	AGATCCTGTTTACACACAATTCCCTAAACAGATTACGGACGTTGCTATTA	3000
NM_152959.1	AATAACAGTTTTTCATGTTAATAAGCTTTTTATCTAAAATAACATGAGCT	3050
NM_152959.1	CTGCGATATAAAGTGTGAACAGACAGAAAACAGCCCAGGAGAAAAGTTAC	3100
NM_152959.1	TCTTAGAACAAACGGGGAAAAACCTGATTACTTGTAAAAGGGGCTTTATG	3150
NM_152959.1	TTAATGAAGTGAGTCTTTTCATTGCAATCAGGGATATATTATGGATCTAA	3200
NM_152959.1	ATCCCTCTTCTATTTTAAACGTTGATATGTAATTTAATGGGGAAAAACTG	3250
NM_152959.1	CTCAGTGATCAGTGGTAACATTATAGCTGCATCTTATTTGNGCAAAAATG	3300
NM_152959.1	TTTACTTGTTGGTTAATATGAAATGATTTTTTTAATTGCTGTTCTTGTTA	3350
NM_152959.1	CAGTGCTGTCTGTCATTGTTGATTTTATTGCTATCTTAACAGATTACTGC	3400
NM_152959.1	TTGAAAAGAAAGCCCAATTTCAACATTGTGAAGGTCGAA 3439	

Alignment of the zebrafish esr2a sequence (NM_180966 *Danio rerio* estrogen receptor 2a (esr2a), mRNA) with the qPCR primers (underlined) used.

NM_180966.2	AGAGACAGTGTGGTATACCGCCTTGCTCACTGTCTTGCTACAACACTGAG	50
NM_180966.2	GAGTATCGAGGACTGAGAAGTTCACCATGACACACATCAACAGTCCCCCG	100
NM_180966.2	TTACTGATGCGACAGCTCTGAACTCATCCGCCTTCACCATGTCCGAGTAT	150
NM_180966.2	CCCGAAGGAGACAGTCCTCTTCTTCAGCTGCAGGAAGTGGACTCAGGCCG	200
NM_180966.2	TGTGGGAGGTCACATCCTCTCCCCCATCTTCAACTCATCCTCTCCATCTC	250
NM_180966.2	TGCCAGTGGAGAATCACCCCATCTGCATCCCATCGCCCTACACAGACCTT	300
NM_180966.2	GGCCACGACTTCAGCACTCTGCCCTTTTACAGTCCCGCTCTGCTGGGGTA	350
NM_180966.2	CAGCACATCGCCTTTATCGGACTGCTCGTCTGTGCGCCAGTCGCTAAGCC	400
NM_180966.2	CGACTTTATTTTGGCCACCTCACAGCCATGTTTCCTCACTCACATTGCAA	450
NM_180966.2	CAACAGAGTCGACTTCAACAGAACCATGCTACTAGTGGGACTTGGACAGA	500
NM_180966.2	ACATACACCACATGATCATGTTGAAGAGGAAAACAGCAAACCACTGGTGA	550
NM_180966.2	AGCGGGTAGCAGATACAGAGGAGACGTCTGTGTCTCTGAGAGGTAAAGCT	600
NM_180966.2	GACATGCACTACTGTGCCGTCTGCAGTGATTACGCCTCTGGATATCATTA	650
NM_180966.2	TGGAGTGTGGTCGTGTGAAGGCTGCAAAGCCTTTTTCAAGAGGAGTATAC	700
NM_180966.2	AAGGACACAATGACTACATCTGTCCTGCCACAAACCAATGCACTATCGAC	750
NM_180966.2	AAGAACCGCCGCAAAAGCTGCCAGGCCTGCCGACTCCGAAAGTGCTATGA	800
NM_180966.2	AGTTGGAATGATGAAATGTGGGTTGCGGCGAGATCGTAGCAGCTACCAAC	850
NM_180966.2	AAAGAGGAGCACAGCAGAAACGACTGGTCCGATTCTCTGGCAGAATGAGA	900
NM_180966.2	ATGACTGGTCCGAGGTCTCAAGAGATAAAGAGTATCCCGCGTCCTCTCAG	950
NM_180966.2	TGGGAATGAGGTGGTGCGCATATCACTGAGCCCAGAGGAGCTAATCTCCC	1000
NM_180966.2	GGATCATGGAAGCAGAGCCGCCGGAGATTTATCTCATGAAAGACATGAAG	1050
NM_180966.2	AAGCCCTTCACTGAGGCAAACGTCATGATGTCACTGACCAACCTGGCTGA	1100
NM_180966.2	CAAAGAGCTTGTGCACATGATCAGCTGGGCCAAGAAGATCCCAGGTTTTG	1150
NM_180966.2	TGGAGCTCAGTCTTTTCGATCAGGTGCATTTGTTGGAGTGCTGCTGGTTA	1200
NM_180966.2	GAGGTGCTTATGCTGGGATTGATGTGGCGCTCCGTTAATCACCCTGGAAA	1250
NM_180966.2	GCTCATTTTCTCTCCAGACCTCTGTCTCAGCAGGGATGAAAGCAGCTGTG	1300
NM_180966.2	TGCAGGGGTTGGTGGAGATCTTTGATATGCTGCTGGCAGCCACGTCCAGA	1350

```
NM_180966.2               TTCAGAGAGTTGAAGTTACAGAGAGAAGAGTACGTGTGTCTCAAAGCCAT      1400

NM_180966.2               GATCCTCCTGAACTCCAACATGTGTCTGGGCTCGTCTGAGGGAGGAGAAG      1450

NM_180966.2               ATCTGCAGAGCCGCTCCAAGCTGCTGTGTCTGCTGGACTCGGTGACTGAC      1500

NM_180966.2               GCTCTGGTTTGGGCCATCTCGAAGACCGGCCTTAGCTTCCAGCAGCGCTC      1550

NM_180966.2               CACCAGACTGGCCCATCTGCTCATGCTGCTCTCACACATACGCCACGTCA      1600

NM_180966.2               GTAACAAAGGCATGGACCACCTGCACTGCATGAAGATGAAGAAGATGGTT      1650

NM_180966.2               CCTCTGTATGACCTGCTCCTGGAGATGCTGGACGCTCACATCATGCACAG      1700

NM_180966.2               CTCCCGTTTGTCTCACTCCGGCCCTCGAGCCCCTGCTGCTCACAAAGACA      1750

NM_180966.2               ACAAGAGTGTCCAGGAGGCCTTTCCCTGCAGCTCACAGCACGGACCCTAA      1800

NM_180966.2               ACACGGCCCTCACAGTCACACAAACACAGTACTTAAGGATGATCTGTAGT      1850

NM_180966.2               TTCGGGAGGATGGACAACCAAAACACCCACATAAAAGCAAGGAGTAGGAT      1900

NM_180966.2               TTCACAAGACGAAAAGCATCATGGATATCTTGAATGTTTTTCCTGTTTTT      1950

NM_180966.2               AAATGTTGGCAATTGCTGTGAAATCTCTTTATTGTTTCCACACAACTCTG      2000

NM_180966.2               ACAACTTTTTAATGATTTTATGTAGCACAACTGCAAAAGAAAAGAAAAAA      2050

NM_180966.2               AGAAATGAATACCCACATTGGATTTCAAGAGATTTTTGGAAACTTCATTA      2100

NM_180966.2               CACGGTGAACTATAATTTCTGGCATTATATGCATAATGGAGGCCATCTAG      2150

qPCR_esr2a_sense           AGGAGAAAACCAAGTAAACCAATC
NM_180966.2               TAGGAGAAAACCAAGTAAACCAATCATGCCCACCTTCTTTTTTAAAAGAA      2200
                           ************************

NM_180966.2               CCAGTTCAAACTCAATTGAGAGAGTGCATTGGTGCCATGACCCGGATGAC      2250

NM_180966.2               AGTAGGTTTTATTAAATCTGTCATCTCTGGCCTTGAGAGACTGTGGCCTT      2300

NM_180966.2               TAGCATTAGCCTTGTTAGCAGCCTGCCTCTCAAGTACTCACCAGTTCAGA      2350
qPCR_esr2a_antisense         CATTAGCCTTGTTAGCAGCCT
                             *********************

NM_180966.2               TCCCGCTGTAAGTAGGACGGGGTTATAATGTCCATGAAAGTTTTGGCCTC      2400

NM_180966.2               ATTTTCATGACATCCGTGACAACATGGACAAGAGCAGTGTGCGATCTCTA      2450

NM_180966.2               CTTTTTCTCTTTCTTACATAAGCTTGCTGCTTTCACATTTTGTAAAATGA      2500

NM_180966.2               AACAAAATATCGTAAACTCATTACTTTCGACAATACTGGCAGTAAAATCA      2550

NM_180966.2               AATTTGATTTAGTCTGTTGTAAATGGACAGAATCATTCCTGATAGATGAG      2600

NM_180966.2               ACAAAGCATTGTATGCTGAGTGAGCAAACCATATTCTGGACTAAGGGCAA      2650

NM_180966.2               GCAGTATTTTAGACTTACAAAACAAAAAACCTGAATGATTGAATGAATGT      2700

NM_180966.2               CAGCTGGATGTTTCATTTACATCTGTTGTTATTGAGAAATGGAACCATTG      2750

NM_180966.2               CAAAATGGCCTCGTGCACATTTTAGTGATTAATTCTTTTTTGCAAACAAA      2800

NM_180966.2               CATTTATGTACAGGAGTGACAGCCGCATTTTACAGCCATCGCCGTGACCA      2850

NM_180966.2               TATCTTGAACATTGGAGGGTTTGTGTTAGAAATTATAAAGTAGGACATTT      2900

NM_180966.2               CAGGCCTAATTCTAGTGGAAAGGTATTTGCAAAAAAAAAATTACAAAATC      2950

NM_180966.2               AATTTGTAAACCTAATATGTTTCACATTCAGTTAGAAAAGACTATAATTA      3000

NM_180966.2               GGTAACATCTCAGTTCACAATGATGAAATCTGAGTTAAAAAGCATCATTA      3050

NM_180966.2               CTGCTGGCTATTGAATATTTCTACCATAAAACCATTTGTATAACAAAAAA      3100

NM_180966.2               AAAAAAAAAAA        3111
```

Alignment of zebrafish esr2b sequences (NM_174862.3 *Danio rerio* estrogen receptor 2b

(esr2b), mRNA and AJ414566.1 *Danio rerio* mRNA for estrogen receptor beta1 protein) and alignment of qPCR primers (underlined) used. Asterisks indicate nucleotides in agreement between both published sequences. NM_174862.3 is the provisional NCBI reference sequence (accessed 31.01.2017) whereas AJ414566 is the original database used to design the primers used in the study.

```
NM_174862.3      --------------------ACAGTTGGGCCTGAGATGCAGTAGTGTGTCAGGCCAGGGT     40
AJ414566.1       CACATCACCTGCTGCCTGCTAACGTTGGGCCTGAGATGCAGTAGTGTGTCAGGCCAGGGT     60
                                     *  **********************************

NM_174862.3      CTCTCTTGTGTTCTCCAGCATGAGCTCCTCCCCTGGACCTGCCCCTGTCCTGGACTCCAG     100
AJ414566.1       CTCTCTTGTGTTCTCCAGCATGAGCTCCTCCCCTGGACCTGCCCCTGTCCTGGACTCCAG     120
                 ************************************************************

NM_174862.3      CAAGGCCGATCGAGGGGCCTCGCCTGCCCTCTTACCCCGGCTCTACGCCTCCCCGCTCGG     160
AJ414566.1       CAAGGCCGATCGAGGGGCCTCGCCTGCCCTCTTACCCCGGCTCTACGCCTCCCCGCTCGG     180
                 ************************************************************

NM_174862.3      CATGGACAACCAGACCGTCTGCATTCCTTCCCCATATGTGGAGGCCTGTCAGGATTACTC     220
AJ414566.1       CATGGACAACCAGACCGTCTGCATTCCTTCCCCATATGTGGAGGCCTGTCAGGATTACTC     240
                 ************************************************************

NM_174862.3      TCCACCGCATGGGGGAGAGTTCAACCACGGAGCCCTAACGCTCTACAGCCCCGTCTCCTC     280
AJ414566.1       TCCACCGCATGGGGGAGAGTTCAACCACGGAGCCCTAACGCTCTACAGCCCCGTCTCCTC     300
                 ************************************************************

NM_174862.3      TGCAGTGCTCGGCTTCCATCGTCCTCCTGTGTCCGAAAGCCTCGTCCCGCTTAGTCCCAC     340
AJ414566.1       TGCAGTGCTCGGCTTCCATCGTCCTCCTGTGTCCGAAAGCCTCGTCCCGCTTAGTCCCAC     360
                 ************************************************************

NM_174862.3      AATCCTGTGGCCGCCTCACTCTCTGCACTACCCGCCTCCGCTGGCCTACAGCGAAACACG     400
AJ414566.1       AATCCTGTGGCCGCCTCACTCTCTGCACTGCCCGCCTCCGCTGGCCTACAGCGAAACACG     420
                 ***************************** ******************************

NM_174862.3      TTCACACAGCGCCTGGGAGGAGGCCAAGACACACACGCTCAGCCAGAGCAGTTCTGTCCT     460
AJ414566.1       TTCACACAGCGCCTGGGAGGAGGCCAAGACACACACGCTCAGCCAGAGCAGTTCTGTCCT     480
                 ************************************************************

NM_174862.3      TAGTCATACAAAGCTGTTAGGACAGCAGCTAGAAGGTGACAATGGCTTGAATCCCTCCGC     520
AJ414566.1       TAGTCATACAAAGCTGTTAGGACAGCAGCTAGAAGGTGACAATGGCTTGAATCCCTCCGC     540
                 ************************************************************

NM_174862.3      GAGCATTGTGGGTAAAGGAGACACTCACTTCTGCGCCGTGTGCCATGACTACGCTTCTGG     580
AJ414566.1       GAGCATTGTGGGTAAAGGAGACACTCACTTCTGCGCCGTGTGCCATGACTACGCTTCTGG     600
                 ************************************************************

NM_174862.3      ATATCACTATGGTGTCTGGTCATGTGAAGGGTGCAAGGCTTTCTTCAAGCGTAGCATTCA     640
AJ414566.1       ATATCACTATGGTGTCTGGTCATGTGAAGGGTGCAAGGCTTTCTTCAAGCGCAGCATTCA     660
                 ************************************************* ********

NM_174862.3      AGGTCACAATGACTATATTTGTCCAGCCACCAACCAGTGCACTATTGACAAGAGCAGACG     700
AJ414566.1       AGGTCACAATGACTATATTTGTCCAGCCACCAACCAGTGCACTATTGACAAGAGCAGACG     720
                 ************************************************************

NM_174862.3      CAAGAGCTGTCAGGCCTGTCGACTCCGCAAGTGCTATGAAGTGGGCATGATGAAGTGTGG     760
AJ414566.1       CAAGAGCTGTCAGGCCTGTCGACTCCGCAAGTGCTATGAAGTGGGCATGATGAAGTGTGG     780
                 ************************************************************

NM_174862.3      TGTGAGGAGGGAGCGCTGCAGTTATCGAGGTGCTCGACATCGTCGTAACCCCCAAATCAG     820
AJ414566.1       TGTGAGGAGGGAGCGCTGCAGTTATCGAGGTGCTCGACATCGTCGTAACCCCCAAATCAG     840
                 ************************************************************

NM_174862.3      AGACAGCTCTGGCGGGGTGGTAGGGCTCAGAGGTCAATCCCAGCAGCATCTAGAGTTCCC     880
AJ414566.1       AGACAGCTCTGGCGGGGTTGTAGGACTCAGAGGTCAATCCCAGCAGCATCTAGAGTTCCC     900
                 ****************** ***** **********************************

NM_174862.3      CCTCAGTCCCTCTCAGCACCTCTTTCCTTCAGGGGGCAGAGCCGAGGGACGGGGCCTGAA     940
AJ414566.1       CCTCAGTCCCTCTCAGCACCTCTTTCCTTCAGGGGGCAGAGCCGAGGGACGGGCCCTGAA     960
                 ************************************************* ******

NM_174862.3          CTATTCCCCTGAACAATTGGTTAGCTGTATTCTAGAGGCGGAGCCACCTCAAATTTACCT  1000
```

```
AJ414566.1          CTATTCCCCTGAACAATTGGTTAGCTGTATTCTAGAGGCGGAGCCACCTCAAATTTACCT 1020
                    ************************************************************

NM_174862.3         GAGAGAGCCGGTGAAAAAGCCATACACTGAGGCTAGCATGATGATGTCACTAACAAGCCT 1060
AJ414566.1          GAGAGAGCCGGTGAAAAAGCCGTACACTGAGGCTAGCATGATGATGTCACTAACAAGCCT 1080
                    ********************* ****************************************

NM_174862.3         CGCCGACAAGGAGCTAGTGCTCATGATTAGCTGGGCGAAGAAGATACCAGGTTTTGTAGA 1120
AJ414566.1          CGCCGACAAGGAGCTGGTGCTCATGATTAGCTGGGCGAAGAAGATACCAGGTTTTGTAGA 1140
                    *************** ********************************************

NM_174862.3         GTTGACTTTGTCAGATCAGGTGCATTTGCTGGAATGCTGCTGGCTGGATATTCTGATGTT 1180
AJ414566.1          GTTGACTTTGTCAGATCAGGTGCATTTGCTGGAATGCTGCTGGCTGGATATTCTGATGTT 1200
                    ************************************************************

NM_174862.3         AGGATTGATGTGGAGATCTGTGGATCATCCTGGGAAACTCATCTTCACCCCTGACCTCAA 1240
AJ414566.1          AGGATTGATGTGGAGATCTGTGGATCATCCTGGGAAACTCATCTTCACCCCTGACCTCAA 1260
                    ************************************************************

NM_174862.3         GCTCAACAGGGAGGAAGGGAATTGTGTTGAAGGCATCATGGAGATTTTCGACATGCTGCT 1300
AJ414566.1          GCTCAACAGGGAGGAAGGGAATTGTGTTGAAGGCATCATGGAGATTTTTGACATGCTGCT 1320
                    ********************************************** ***********

NM_174862.3         GGCCACCACCTCTCGATTCAGAGAGCTGAAGCTGCAGAGAGAGGAATACGTCTGTCTCAA 1360
AJ414566.1          GGCCACCACCTCTCGATTCAGAGAGCTGAAGCTGCAGAGAGAGGAATACGTCTGTCTCAA 1380
                    ************************************************************

NM_174862.3         AGCCATGATCCTGCTCAACTCTAATAACTGTTCGAGTTTGCCACAGACTCCTGAGGATGT 1420
AJ414566.1          AGCCATGATCCTGCTCAACTCTAATAACTGTTCGAGTTTGCCACAGACTCCTGAGGATGT 1440
                    ************************************************************

NM_174862.3         GGAGAGTCGCGGGAAGGTGCTGAATCTGCTGGACTCAGTGACCGATGCTCTGGTGTGGAT 1480
AJ414566.1          GGAGAGTCGCGGGAAGGTGCTGAATCTGCTGGACTCAGTGACCGATGCTCTGGTGTGGAT 1500
                    ************************************************************

NM_174862.3         CATCTCCAGAACGGGTCTGTCCTCACAACAACAGTCCATCCGGCTCGCTCATCTGCTAAT 1540
AJ414566.1          CATCTCCAGAACGGGTCTGTCCTCACAACAACAGTCCATCCGGCTAGCTCATCTGCTAAT 1560
                    ********************************************** **************

NM_174862.3         GCTGCTCTCACACATCCGACACCTCAGCAACAAAGGCATCGAGCATCTGTCAAACATGAA 1600
AJ414566.1          GCTGCTCTCACACATCCGACACCTCAGCAACAAGGGCATCGAGCATCTATCTAACATGAA 1620
                    ********************************** ************** ** ********

NM_174862.3         GAGGAAAAACGTGGTGCTGCTGTACGATCTTCTGCTAGAGATGCTGGACGCAAACACGTC 1660
AJ414566.1          GAGGAAAAACGTGGTGCTACTGTACGATCTTCTGCTAGAGATGCTGGACGCAAACGCGTC 1680
                    ****************** ************************************ ****

NM_174862.3         TCAGAGCAGCCGGATGCTGGAAGACAGACAACAGAGCCCAGAAAACCTTCACACATCCAG 1720
AJ414566.1          TCAGAGCAGCCGGATGCTGGAAGACAGACAACAGAGCCCAGAAAACCTTCACACATCCAG 1740
                    ************************************************************

qPCR_esr2b_sense                                                        TGAGGA
NM_174862.3         ACCGCAGCCTGACCTGAAAGACAGCGACCAGGAGACCCCACACAGTCCACGGGCTGAGGA 1780
AJ414566.1          ACCGCAGCCTGACCTGAAAGACAGCGACCAGGAGACCCCACACAGTCCACGGGCTGAGGA 1800
                    ************************************************************

qPCR_esr2b_sense    GATGGTGAACAAGAC
NM_174862.3         GACGGTGAACAAGACATTGCATTCTAGTCTGCTTCGAGAGGACATGGACACAAACTGACA 1840
AJ414566.1          GATGGTGAACAAGACATTGCATTCTAGTCTGCTTCGAGAGGACATGGACACAAACTGACA 1860
                    ** ********************************************************

NM_174862.3         GTCACAGACTTTTGTGGGGGGGGGGGGGGACTCTGGATCTGGCATTTCATTCATCCATCC 1900
AJ414566.1          GTCACAGACTTTTGTGGGGGGGG-GGGGGACTCTGGATCTGGCATTTCATTCATCCATCC 1919
                    ********************** ***********************************
qPCR_esr2b_antisense                                        CATTTCATTCATCCATCC

NM_174862.3         ATCAGTTTTCCAGTCTGCATTTTTTGCATGTGGGATTAAGACATGAAAAAAGGAACATCA 1960
AJ414566.1          ATCAGT------------------------------------------------------ 1925
                    ******
qPCR_esr2b_antisense ATCAGT

NM_174862.3      GGTGATCGACGACAACCTGAACCAAACAGGGCTTTCAAACAGCACTCAGTCCTGCTTCAA     2020
AJ414566.1       ------------------------------------------------------------     1925


NM_174862.3      CACAATGCTACTACAACACTACATTGTTTTCCTTTAACAGAGGAATCATTCTCATCTCAT     2080
AJ414566.1       ------------------------------------------------------------     1925


NM_174862.3      TTGTTCAAGAAAATCTTCAATGAAACATTTTATGGCTCTATCTGGTTCTGTATGTGGTAA     2140
```

```
AJ414566.1      ------------------------------------------------------------     1925

NM_174862.3     AGGTGAAGTGTGTCATTTCTGTGCCGCTAGAAGAATGAAAACAACAACAACAACAACAAC     2200
AJ414566.1      ------------------------------------------------------------     1925

NM_174862.3     AACAACAAGATTCAAACACTCCCAAAGTCTGCCATTGCAACCAGGAAGCCCAGCCCCAAA     2260
AJ414566.1      ------------------------------------------------------------     1925

NM_174862.3     AATCACACCATTGGCTGAGACAGTTACATAGCGTTTATTTTGTTTTAATCAAACTGTAAA     2320
AJ414566.1      ------------------------------------------------------------     1925

NM_174862.3     TGTTTCTTTTGCCATCCAAGATTTCAGACTGAACTATTGATTAACGAAAACGGTGCGCAC     2380
AJ414566.1      ------------------------------------------------------------     1925

NM_174862.3     ACTTCTAATAGTTTATGCATTTTGTACTTTGTGTTTCTGTAAGTCGTTGGAATGTCATTT     2440
AJ414566.1      ------------------------------------------------------------     1925

NM_174862.3     GCTGTCCTGTAGTGCTTTTCGTGGTTTATAAAGTACTTGTCATTGACAGTAAATATGTCT     2500
AJ414566.1      ------------------------------------------------------------     1925

NM_174862.3     TTGTGAGTTGATGCCTCTTTTGGCTCCAAAACCCAAATGATACACACAAAAGTGTGTAGC     2560
AJ414566.1      ------------------------------------------------------------     1925

NM_174862.3     TCTCACTCTCAGAAACACACTTGTTCCTAAAGGGTCTATAATGGTAGCCTAAAAATGCTA     2620
AJ414566.1      ------------------------------------------------------------     1925

NM_174862.3     ATTAGAATTTTGAAAGCTATGTACTAATATTTACCTTGTGGTATTAATATGGACTCTTTA     2680
AJ414566.1      ------------------------------------------------------------     1925

NM_174862.3     AATACAAATGTGTATGTTTTGAAAAAAAAAAAAAAAAA     2718
AJ414566.1      ----------------------------------------     1925
```

Figure S2: qPCR Alignment.

Gene name	gene symbol	sense primer	antisense primer	Product size (bp)	Ta (°C)	E[2]
estrogen receptor 1	*esr1*	CGAGTGCCGCTGTATGAC	TGCTGCTGCTGGTTGTG	130	59.5	1.97
estrogen receptor 2a	*esr2a*	AGGAGAAAACCAAGTAAACCAATC	AGGCTGCTAACAAGGCTAATG	173	59.0	1.91
estrogen receptor 2b	*esr2b*	TGAGGAGATGGTGAACAAGAC	ACTGATGGATGGATGAATGAAATG	132	57.8	2.03
ribosomal protein L8	*rpl8*	CCGAGACCAAGAAATCCAGAG	CCAGCAACAACACCAACAAC	91	59.5	2.09

[1] primers sequences from Filby et al. (2010) [2] E = PCR efficiency

Table S1

	EE2	Gen		BPA	
Nominal (µg/L)	**0.1**	**62.5**	**1000**	**125**	**2000**
Day 0	0.14* (140%)	58.6 (94%)	1001 (100%)	137.5 (115%)	2200 (110%)
Day 5	0.099 (99%)	62.1 (99%)	956 (96%)	132.4 (103%)	2669.14 (133%)

Table S2: Measured chemical concentrations in the embryo incubation water.

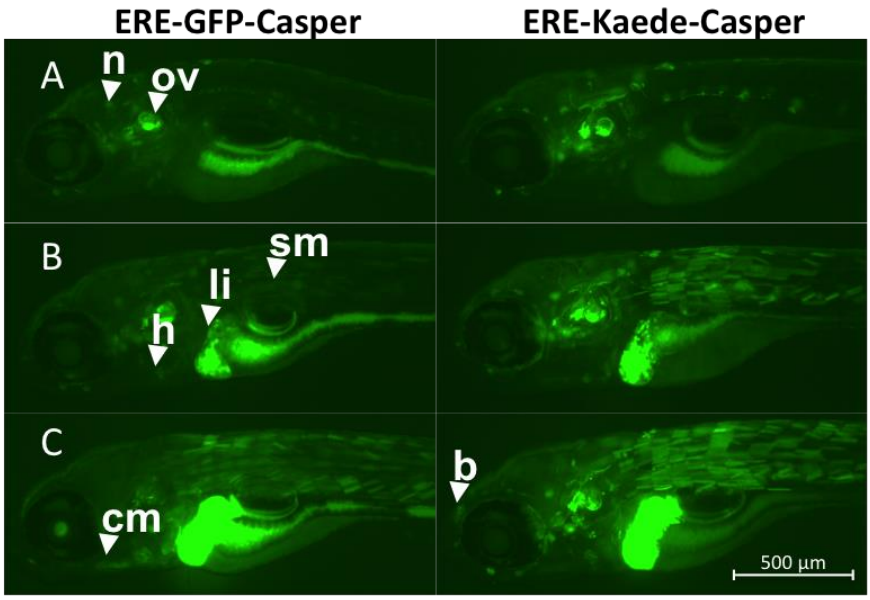


Figure S3: Comparison of ERE-GFP-Casper and ERE-Kaede-Casper models.

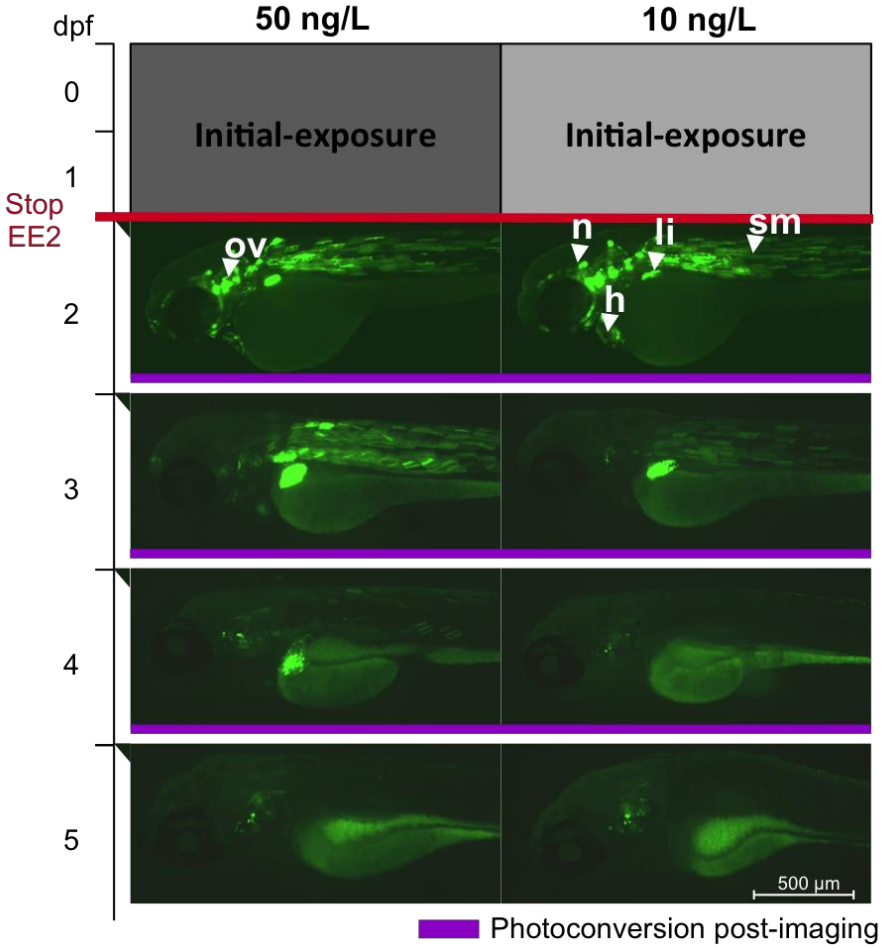


Figure S4: Temporal analysis of fluorescence induction after a single 48h exposure to EE2.

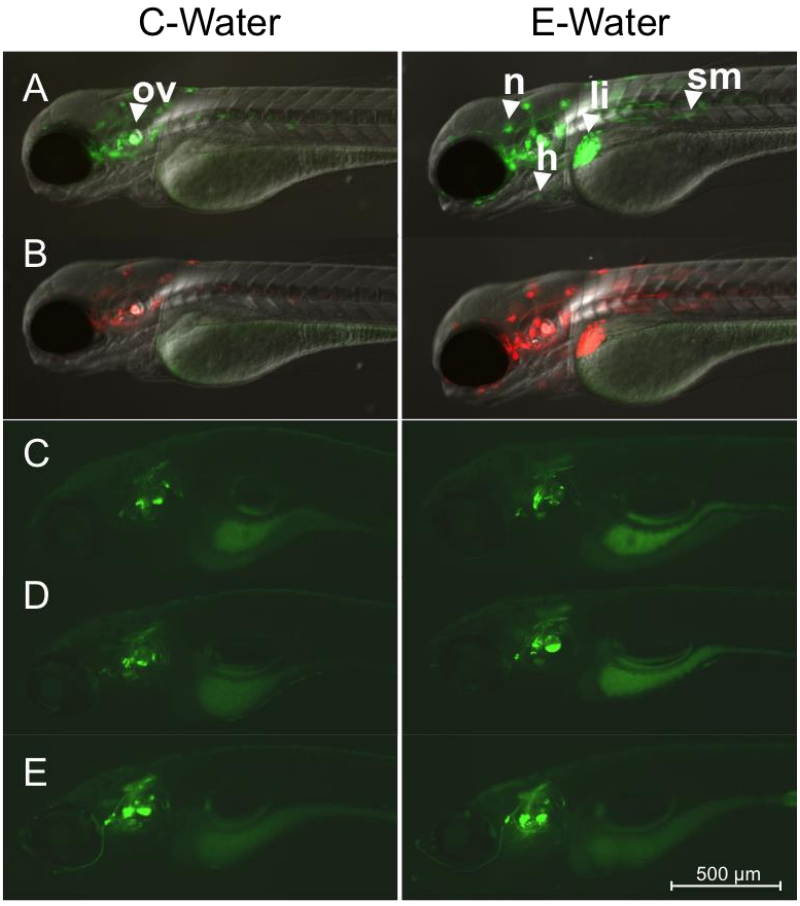


Figure S5: Kaede expression in control groups C-Water and E-Water.

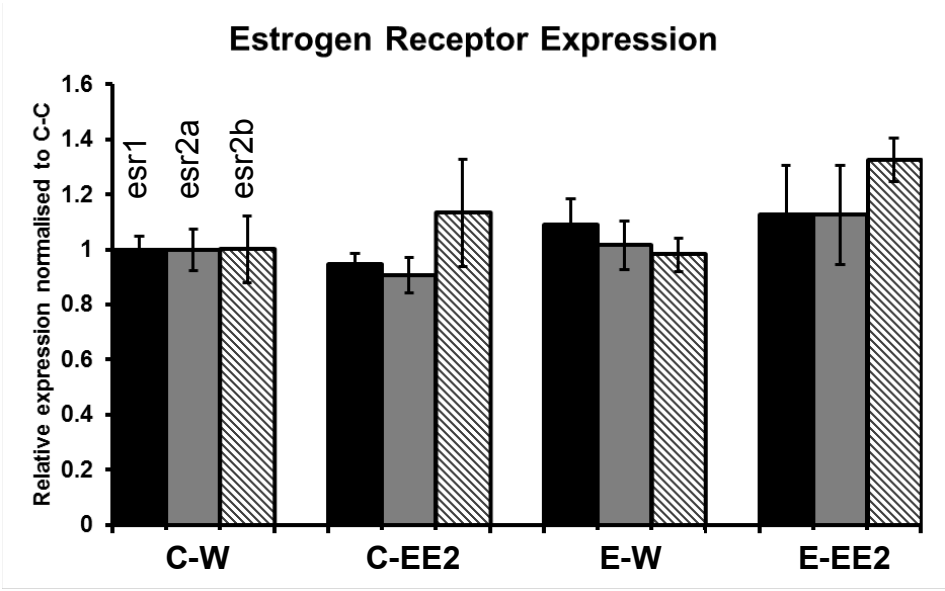


Figure S6: qPCR quantification of *esr1*, *esr2a* and *esr2b* gene expression.

Chapter 6

Thesis Discussion

Chapter 6

Thesis Discussion

The work presented in this thesis demonstrates that transgenic zebrafish models can be developed with good utility for effective analysis of responses to oestrogens for different early-life stages and for application to larval screening (semi-automated). National and international screening programs currently rely heavily on *in vitro* assays, meaning risk assessment of oestrogenic chemicals is often based on quantification of binding affinity for specific ER subtypes using a range of different cell lines and this does not allow for the more complex interactions that occur between EDCs and organisms in the environment. This work further highlights that the development and application of transgenic fish offer huge potential for improved understanding on the effects and mechanism of effects for EDCs and therefore allow for more integrative health effects assessments. The use of informative transgenic fish models can also result in better use and potential reduction of intact animals used in animal testing in line with the principles of the 3Rs (Replacement, Reduction and Refinement).[195]

The ERE-GFP-Casper and ERE-Kaede-Casper Models

Over the last ten years, multiple transgenic zebrafish models have been generated in an attempt to better understand the potency and effects of oestrogenic chemicals in living organisms. However, these models have consistently been limited in their sensitivity or tissue specificity of response, as highlighted in the literature review included in the appendix of this thesis. The ERE-GFP model, previously developed at the University of Exeter using a modified reporter system, displayed considerably enhanced sensitivity in a

variety of tissues compared to previously established models, allowing the detection of responses to chemical exposures at environmentally relevant concentrations. A limitation however of this model and others, was the development of skin pigmentation in the animal, causing fluorescence signal-blocking and restricting accurate response assessment to the early life stage using non-dorsal orientations for microscopy and imaging.

The work presented has demonstrated the development and application of transgenic systems using skin pigment-free lines. The translucent phenotype present in both transgenic zebrafish lines created was crucial for improved signal quantification in the screening system and high-content analysis presented in Chapter 4. The transgenic casper models have also been applied to a range of other studies in the Exeter laboratory including charting the development and role of oestrogens in the brain in zebrafish (Takesono et al., in prep), assessing effects of chronic exposure to Bisphenol A on heart development and function (Brown et al., in prep) and an analysis of effects of exposure to oestrogenic wastewater treatment works across different life stages on sexual development in zebrafish (Cooper et al., in prep). Thus the casper models (ERE-GFP and ERE-Kaede) I have created in my thesis work are being exploited widely. There are imitations with the casper models relating to fluorescence detection however as in post-larval stages (more than 30 dpf) the thickness of the body wall reduces fluorescence detection (quenching of the signal) for tissues seated deeply in the body cavity. Very recently, a similar line named “Crystal” has been developed that also includes silenced pigmentation in the eyes of the zebrafish, further improving imaging of the brain and may provide improved translucency at later stages of development than the casper phenotype.[194]

The results from Chapter 5 of this thesis have demonstrated that by utilising the simple mechanism of fluorescence photoconversion, the ERE-Kaede-Casper model provides a more dynamic model for studies into temporal tissue response and assessment of sequential exposures to oestrogens compared with the ERE-GFP-Casper model. Photoconvertible proteins have been previously used to track individual cells and assess effects on tissues developmentally, [67, 182, 184] and in this model kaede was used to identify dynamics of tissue-specific response during exposure to oestrogenic chemicals. Importantly, photoconversion of green fluorescence to red at multiple time-points allowed for continual analysis of individuals during exposure without the need of termination or multiple groups of larvae.

The transgenic models were generated via crossing of established lines rather than the use of transgenesis techniques and this resulted in models that inherited the same high sensitivity and tissue-specificity of response as the original ERE-GFP model. The models presented in this thesis are comparable to the most sensitive *in vivo* and *in vitro* oestrogen responsive systems currently available.[48, 107, 113, 114] One initial concern with generating transgenic lines via crossing was the possibility of eventual silencing of the transgene response over multiple generations, which would have resulted in individuals with either mosaic or fully silenced progeny.[199] Screening of the ERE-GFP-Casper line identified a 90-99% transmission rate of transgenes from homozygous F0 males to F1 progeny that was lower than the 100% (expected) rate observed using homozygous F0 females but this was inconclusive due to the small sample size. Analysis of response consistency and sensitivity in subsequent generations suggested that the transgene sequences are very stable within the three separate models (ERE-GFP, ERE-GFP-Casper and ERE-Kaede-Casper).

To produce stable transgenic fish lines via crossing requires careful planning and genotype screening is labour intensive as assessment is required of thousands of larvae using fluorescent microscopy. Inbreeding within heterozygous stocks is also required for this process and therefore subsequent generations need to be tested on a regular basis through routine screening of their responses to ensure consistency. This avoids potential problems associated with inbreeding, such as issues with sexual development[198] and altered susceptibility to chemical stressors in comparison with wild-type zebrafish lines.[197] Consistency in responses of specific transgenic lines is crucial for any standardized chemical testing. Thus homozygous working stocks need to be maintained, as demonstrated in Chapter 3 (p73-74) by the high variation of response seen in the unscreened cyp19a1b-GFP line in comparison to the more consistent responses seen in the homozygous ERE-GFP-Casper line. However, the development and maintenance of transgenic fish lines is not a trivial exercise and more easily undertaken in large, well-funded laboratories. This being the case, transgenic lines created should be made available, on request, to other research laboratories to maximize their utility and model uptake and independent validation. The work presented in this thesis was only made possible thanks to this policy of collaboration.

In summary, transgenic zebrafish have been applied widely in studies in developmental biology and medicine but to date only a few models have been developed for studies in ecotoxicology that have both the required specificity and sensitivity for application to understand environmental health effects. The development of translucent lines such as the ERE-GFP-Casper and ERE-Kaede-Casper have opened the door for further applications of transgenic zebrafish within ecotoxicology assessment, as discussed below. Transgenic

zebrafish lines that can be utilised during a wider range of life stages may also prove particularly beneficial in elucidating of adverse outcome pathways of chemicals.

In vivo **Screening**

Large-scale screening assays conducted by national and international governing bodies have helped ensure that potential oestrogenic chemicals in the environment (and other EDCs) are continually assessed and identified. The use of high-throughput *in vitro* screening tools has made this process both time- and cost-effective in comparison to the use of traditional *in vivo* screening systems. However, these *in vitro* screening assays are very limited in their ability to accurately predict potency, tissue-specificity and potential adverse effects of these chemicals in living organisms. As discussed in the introduction to this thesis in the examples of EE2, genistein and BPA, potency and ER receptor subtype affinity values attributed to oestrogenic chemicals via *in vitro* screens can underrepresent the equivalent values and associated adverse effects reported using *in vivo* screens and environmental and clinical data.

The work presented in this thesis demonstrate that transgenic zebrafish, when applied in suitable screening and assay systems, can provide advantages characteristic of both *in vitro* and *in vivo* systems for testing oestrogenic chemicals or other EDCs. The screening assays presented in Chapter 4 showed high-sensitivity identification and quantification of oestrogenic chemicals and their tissue-specific potencies. The results showed unique tissue-specific affinities of individual oestrogenic chemicals and also identified tissues that were consistently sensitive to exposure from all oestrogenic chemicals. The high sensitivity seen in the heart is consistent with responses

seen in another oestrogenic responsive transgenic zebrafish line exposed to environmental water samples[200] and mounting data linking BPA to potential adverse effects on the cardiovascular system.[201]

As embryos/larvae, the models presented can be utilised as reasonably rapid screening systems with high sensitivity and good repeatability. They can also be relatively cheap to use, once the models have been established, and especially so when compared with similar mammalian models. Equally however, there are reasonably high costs, most notably in skilled time, to develop novel transgenic models using transgenic techniques and then in maintaining the lines. Nevertheless, these costs can be justified if screening systems such as the one presented in Chapter 4 are further improved upon to become fully automated, high-content, high-throughput *in vivo* chemical screens. Such systems can offer chemical throughput and detection sensitivity to rival *in vitro* screens whilst also providing highly informative data on target tissues and potential adverse effects giving a more reliable and efficient tool for chemical risk assessment. Other novel automated systems are now being developed to scan and analyse responses in transgenic zebrafish larvae in medium throughput systems[202] and new technology such as the VAST™ system shows potential for further improvement upon the work presented here.

A further major benefit of transgenic fish and their application in such screens is that they could lead to a significant replacement and reduction in animals for chemical testing (in line with the 3Rs principle),[195] as they allow for better effects targeting and analysis across multiple organ systems in individual organisms in real time. Use of transgenic embryos rather than adult fish could also substantially reduce the numbers of fish in chemical testing.

As shown in the Generation of Transgenic Zebrafish Models chapter (3), it has to be recognized that substantial validation of these transgenic models is required before their application in these screening systems. Results from Chapter 5 highlight that life stage dependent effects may occur for specific chemicals and thus responses in embryos may not always be representative of responses in later life stages. In the short term, this will demand use of more intact animals for the validation process. Furthermore, to date there has been little attempt to compare directly responses in transgenic animals with wild type lines, embryos or otherwise, and this warrants further study. As an example, although a wide array of target tissues for oestrogens are identified in this work, the models express only one type of transgenic ERE and surrounding sequence, which will not be effective for all oestrogenic signalling systems. Confidence that transgenic fish are truly representative of the more standard lines of fish currently used in chemical testing will be required before they are taken up widely in standardised chemical testing. Indeed it could be argued that a standardised validation process needs to be established for transgenic lines before they are accepted into standardised regulatory guideline tests for chemicals.

Sequential Exposures and Temporal Tissue Response

Oestrogenic chemicals are commonly found as mixtures in the environment and there is increasing evidence to suggest EDCs can have interactive (e.g. additive) effects. Potency analysis for EDCs, however, typically involves single-chemical exposures and with little, or no, consideration given for animal exposure history. Unlike combined chemical mixture exposure, the application of sequential exposure is a much simpler approach to assessing chemical

interactions in living organisms but is one that is currently significantly underutilised in EDC mixture analysis.

In Chapter 5 (p171-172), the ERE-Kaede-Casper model was applied to assess the effects of sequential exposures and identified an increased sensitivity of response to oestrogenic chemicals after an initial (0-48 hpf) exposure to an environmentally relevant concentration of EE2. The driving factors behind this sensitisation effect were unclear. No significant change in ER expression was identified by qPCR analysis but this may have been related to issues of timing or sensitivity. The sensitisation effect also appeared to be reduced after longer periods of incubation in embryo water. Nevertheless, these results highlight the importance of chemical mixture assessment (sequential or combined) to better predict potential oestrogenic chemical effects in wildlife and human populations.

The application of the ERE-GFP-Casper in Chapter 4 (p120-121) had previously highlighted different target tissue affinities characteristic of the individual chemicals. However, it was suspected that the sensitivity of tissues is also likely to be dependent on the stage of growth and development of the zebrafish. As demonstrated using the ERE-Kaede-Casper line in Chapter 5 (p170), photoconversion of fluorescence responses at particular time-points offers the possibility to identify windows of sensitivity for specific tissues in response to oestrogenic chemicals. As an example, non-photoconvertion of ERE-Kaede-Casper larvae imaged at 5 dpf after exposure suggested that EE2 had consistently induced high levels of kaede expression in a wide range of tissues during the 5-day exposure. This result mirrored those that were seen using the ERE-GFP-Casper model in Chapter 4. However, photoconversion at 3 dpf and 4 dpf stages (during the exposure) revealed that sensitivity of the individual tissues varied significantly during the 5-day period. These results on

temporal tissue sensitivity to EE2 give us a better understanding of the highly dynamic roles that oestrogen may play during the early stages of life in developing organisms such as the zebrafish. This work also suggests that the risk of tissue-specific adverse effects caused by exposure to these chemicals may be strongly linked to the stage of development of an organism.

Finally, the ERE-Kaede-Casper model was used to study tissue responses to oestrogen in individual tissues at 24-hour periods after an initial 0-2 dpf exposure to EE2. The results in Chapter 5 (p178) show that fluorescence induction continued after the EE2 exposure for periods that varied depending on the exposure dose. The driving factors behind the dynamics of response are not clear from this study but could likely involve accumulation, metabolism and excretion of the chemical within these tissues. Zebrafish have been applied successfully as *in vivo* toxicokinetic models for assessing uptake, metabolism and excretion of oestrogenic chemicals.[57] Models such as the ERE-Kaede-Casper may offer useful non-destructive tools for toxicodynamics assessment as proposed below.

Final Thoughts and Future Prospects

1) Oestrogen Exposures

Additive effects previously seen using EDC mixtures and the sensitisation effect identified using the ERE-Kaede-Casper model in sequential exposures have highlighted the need for further study of complex oestroen exposures. These models provide the opportunity for others to investigate the effects of many aspects of oestrogen exposure. The effects of multiple compounds in mixture

(combined or sequential), windows of exposure and concentrations offer a complexity that previously was not readily examinable.

2) Toxicodynamics

The ERE-Kaede-Casper could provide a valuable model for supporting toxicokinetic studies. The ability to photoconvert kaede fluorescence could potentially be applied to assess for both the presence and persistence of the exposure chemical in the target tissues. In many (most) cases where the parent compound only is oestrogen active the ERE-Kaede-Casper could potentially offer an effective system to non-destructively study the toxicodynamics of oestrogenic chemicals in zebrafish.

3) High-throughput, high-content *in vivo* screening system

The screening system presented in this thesis is currently limited in its capacity to analyse chemicals at high-throughput levels achieved via *in vitro* assays. The sensitivity of the overall system is also limited by measurement of whole-body fluorescence response rather than tissue-specific response quantification. Both of these issues can be improved upon significantly in the near future via the use of more modern automated dosing and imaging systems such as VAST™ to achieve higher throughput of chemicals (and their mixtures) and improved automated tissue-specific quantification using current image analysis programs. Using dynamic models such as the ERE-Kaede-Casper, such a system could additionally undertake the proposed sequential exposure and toxicodynamics assessments. In doing so, this system would act as a multifaceted screening

tool for improved predictions of potency and adverse effects of oestrogenic chemicals in wildlife and human populations.

4) Effect mechanisms and signalling pathways

Transgenic fish systems such as the models presented in this work also offer the potential for studying detailed effect mechanisms and signalling pathways. As an example, application of morpholinos/CRISPRs could be applied in combination with chemical exposures to ablate specific receptors and to identify roles of receptor subtypes in mediating the toxicant response. The transgenic models published to date are also relatively simple and combining different reporter systems in the same animals (and using different fluorescent markers) in the future should allow for multiple toxicological responses (linked to multiple signalling pathways) in the same animal.

Chapter 7

Appendix

Chapter 7

Appendix

The appendix in this thesis contains:

“Transgenic Fish Systems and their Application in Ecotoxicology”

Lee, O; Green, JM; Tyler, CR, *Critical Reviews in Toxicology,* 2015

This is a critical review of techniques and methods used to generate transgenic zebrafish and medaka models for ecotoxicology assessments and includes a comparative analysis of the models themselves. This paper is included in its original format.

7.1 Transgenic Fish Systems and their Application in Ecotoxicology

Okhyun Lee, Jon M. Green, Charles R. Tyler*

Critical Reviews in Toxicology, 2015

Crit Rev Toxicol. 2015 Feb;45(2):124-41. doi: 10.3109/10408444.2014.965805. Epub 2014 Nov 14.

University of Exeter, Biosciences, College of Life & Environmental Sciences,

Exeter EX4 4QD, United Kingdom

Abstract

The use of transgenics in fish is a relatively recent development for advancing understanding of genetic mechanisms, developmental processes, aquaculture, and in pharmaceutical discovery. Transgenic fish have also been applied in ecotoxicology where they have the potential to provide more advanced and integrated systems for assessing health impacts of chemicals. The zebrafish (*Danio rerio*) is the most popular fish for transgenic models, for reasons including their high fecundity, transparency of their embryos, rapid organogenesis and availability of extensive genetic resources. The most commonly used technique for producing transgenic zebrafish is via microinjection of transgenes into fertilized eggs. Transposon and meganuclease have become the most reliable methods for insertion of the genetic construct in the production of stable transgenic fish lines. The Gal4-UAS system, where GAL4 is placed under the control of a desired promoter and UAS is fused with a

fluorescent marker, has greatly enhanced model development for studies in ecotoxicology. Transgenic fish have been developed to study for the effects of heavy metal toxicity (via heat-shock protein genes), oxidative stress (via an electrophile responsive element), for various organic chemicals acting through the aryl hydrocarbon receptor, thyroid and glucocorticoid response pathways, and estrogenicity. These models vary in their sensitivity with only very few able to detect responses for environmentally relevant exposures. Nevertheless, the potential of these systems for analyses of chemical effects in real time and across multiple targets in intact organisms is considerable. Here we illustrate the techniques used for generating transgenic zebrafish and assess progress in the development and application of transgenic fish (principally zebrafish) for studies in environmental toxicology. We further provide a viewpoint on future development opportunities.

Introduction

Since the early 1980, transgenic animals – those that carry foreign DNA derived from an exogenous source and deliberately inserted into their genome - have been created in a variety of animals including mammals, birds, amphibians, fish and invertebrates (Donovan, Kerr, and Wall, 2005; Fan and Watanabe, 2003; Hamra et al., 2002; Hong et al., 2009; Hrytsenko, Pohajdak, and Wright, 2010; Huss, Poynter, and Lansford, 2008; Mozdziak and Petitte, 2004; Sinzelle et al., 2006; Tsai, Lai, and Yang, 1997; Wang et al., 1995). Transgenic technology has been used in biological and medical research to find cures for cancers, diabetes, Huntington's disease and cardiovascular diseases and has contributed significantly to agriculture including for improved milk production,

and both increased growth rate and disease resistance in farmed animals (Bader et al., 2000; Donovan, Kerr, and Wall, 2005; Fan and Watanabe, 2003; Hansson et al., 1999; Houdebine, 1995; Mozdziak and Petitte, 2004; Reventos and Munell, 1997; Thomas et al., 2001). Transgenic research applied to mammals was initiated in the 1980s and for fish somewhat after this in the late 1990s. Subsequently, there has been a rapid increase in the use of transgenic animals in research, illustrated by the number of academic papers published, increasing from around 280 in 1988 to almost 16000 in 2012 (using the search term transgenic animal- PubMed, Figure 1a) and in fish from 8 in 1990 to around 250 in 2012 (using the search term fish + transgenic- PubMed, Figure 1).

Transgenic fish have been developed as model systems for understanding genetics mechanisms, developmental processes and for pharmaceutical discovery (target validation), safety assessment and bio-synthesis (Dunham, 2009; Moro et al., 2012; Nguyen et al., 2012). They have also been created to study specific processes in fish including cold tolerance, and for enhancing both disease resistance and growth rate in aquaculture species (Devlin et al., 2004; Du et al., 1992; Dunham, 2009; Wang et al., 1995). Recently, transgenic fish have also been developed for studies in ecotoxicology to screen and test chemical where they can inform on tissue targets and effect mechanisms (Blechinger et al., 2002; Bogers et al., 2006; Gorelick and Halpern, 2011; Lee et al., 2012; Legler et al., 2000; Tong et al., 2009). Transgenic fish have been created in divergent species including the loach *Misgurnus anguillicaudtis*, mud loach *Misgurnus mizolepis*, goldfish *Carassius auratus,* common carp *Cyprinus carpio*, rainbow trout *Oncorhynchus mykiss*, atlantic salmon *Salmo salar*, coho salmon *Oncorhynchus kisutch,* channel catfish *Ictalurus punctatus,* and nile

tilapia *Oreochromis niloticus,* zebrafish *Danio rerio* and medaka *Oryzias latipes* (Devlin et al., 2004; Dunham et al., 2002; Dunham et al., 2002; Kinoshita, 2004; Kobayashi et al., 2007; Lawson and Weinstein, 2002; Maclean, Penman, and Zhu, 1987; Nam et al., 2001; Saunders, Fletcher, and Hew, 1998; Uzbekova et al., 2000; Wang et al., 1995).

Zebrafish and medaka have become the most popular model species for transgenic manipulations. Favorable features of these species for transgenic work include their ease of breeding in the laboratory, relatively low associated maintenance costs, high fecundity, transparency of the embryos and rapid organogenesis (attractive features for studying developmental processes) and the availability of sequenced genomes providing extensive genetic resources. Additionally, for the zebrafish, the chorion of the egg is relatively soft facilitating microinjection of DNA and genetic constructs. The availability of mutant lines for this species, such as casper (roy + nacre mutant zebrafish) that lack skin pigmentation also enable studies for observing effects in body tissues, for example tracing individual cancer cells as they spread through the body (Feitsma and Cuppen, 2008; White et al., 2008). The zebrafish has high genome (approximately 70%), structural and physiological similarities with humans (Blader and Strahle, 2000; Briggs, 2002).. High quality genomic resources are also available for the medaka (Matsumoto et al., 2009), which has a large within species genetic diversity. The medaka has smaller genome compared with the zebrafish (about half the size) and human (one-third the size) (Naruse et al., 2004).

The zebrafish has been used to advance understanding on formation of the embryonic axis, cell lineages, and formation of the central and peripheral nervous systems. It has also proven valuable for studies on human diseases,

notably carcinogenesis, wound healing, immunological diseases, behavioural abnormalities, infection and Parkinson's disease (Blader and Strahle, 2000; Bretaud, Lee, and Guo, 2004; Burgess and Granato, 2008; Feitsma and Cuppen, 2008; Kelly, Erezyilmaz, and Moon, 1995; Kimmel and Warga, 1987; Lieschke and Currie, 2007; Martin and Feng, 2009). The zebrafish and medaka have both become popular model organisms for studies on molecular mechanisms of chemical toxicity and analysis of behavioural outcomes (Hill et al., 2005; Nagel, 2002; Puiseux – Dao and Edery, 2006). Transgenic zebrafish and medaka employing fluorescent markers are used not only to identify chemically induced target gene activation but also to quantify relative uptake and accumulation of chemicals in those tissues. An advantage of the medaka over the zebrafish for studies into the effects of chemicals on sex, is that it has an established XY/XX genetic sex determination mechanism, whereas the sex determining mechanism(s) in the zebrafish are not determined.

In this review, we first present a critical overview on the methods for producing transgenic fish, the recent progress made in different gene transfer methods, and illustrate their utility and applications. In latter part of this review we detail progress in the development of transgenic zebrafish and medaka for studies in environmental toxicology. Finally, we set out some thoughts on future prospects for the use of these small transgenic fish in environmental toxicology.

Methods for producing transgenic fish

Creating a transgenic fish first requires production of the gene of interest and then its introduction into the organism. The general method for generating the gene construct is shown in Figure 2.

Production of the gene construct

Several methods are now used to produce transgenic fish, but all involve a transgenic construct with a promoter and a gene. The foreign gene for transfer (transgene) is constructed using recombinant DNA techniques. Traditionally the gene of interest was most commonly expressed using *E. coli* plasmid vectors that replicate at high levels in their host cells. To clone the DNA of interest the sequence is inserted into a cloning or expression vector through the use of restriction enzymes to create compatible ends and ligase to seal the integration. This approach to cloning however, is time-consuming and relatively inefficient, and it can be difficult to find suitable restriction enzymes in the target DNA. False positive clones (vector without insert) can be common using this method due also to universal nucleotide ligation (Liu, 2013). More efficient methods for cloning the DNA target of interest now include In-Fusion cloning (Clontech) and Gateway cloning (Life Technologies) which are also considerably faster too. In-Fusion cloning can ligate a DNA fragment with 15 bases of homology at their linear ends and a linearized vector with the use of their proprietary In-Fusion enzyme, a poxvirus DNA polymerase with 3′–5′ exonuclease activity. The Gateway cloning system is a site-specific recombination method and uses a set of the components of the λ (lambda) system for *in vitro* transfer of DNA, the λ integrase protein (Int), the λ excisionase protein (Xis), the *E.coli* protein IHF and the *att* recombination sequences embedded in the DNA to be recombined (Marsischky and LaBaer, 2004). There are many other cloning systems available from commercial suppliers, but In-Fusion and Gateway cloning systems have become especially popular for gene construct generation due to their ease of use, fewer number of cloning steps, and good reliability(Xiao et al., 2010).

Introduction of the gene construct into fish host:

Having produced the construct, this is then transferred into the cytoplasm of fertilized fish eggs at the one cell stage, often using microinjection or electroporation (see next section). The injected DNA undergoes replication and some cells in the embryos will subsequently carry the transgene. Some integration events occur subsequent to DNA replication giving rise to mosaic fish, however, which may, or may not, contain the transgene in the germline. Animals are then maintained to adulthood and confirmation that they carry the transgene is undertaken using polymerase chain reaction (PCR) or Southern blot analysis in an external tissue, such as fin (Horvat et al., 1993). Southern Blot is commonly used to determine transgene copy number and the number of integration sites in the transgenic founder. The identification of the integration sites and copy number is important for understanding the relationship between the integration site and the specific phenotype.

Founders containing the inherited transgene (F0) are identified by crossing founder (transgenic fish) and wild-type fish (non-transgenic fish) and assessing whether the transgene occurs in the F1 generation. The germ-line transmission rate from the F0 generation to the F1 generation is variable because F0 are mosaic (variable cell expression) and the transgene is unevenly integrated in their gonadal tissues. Progeny derived from the F0 fish are crossed with wild-type fish, and the resulting heterozygous offspring are crossed with each other to create a homozygous fish (i.e both alleles are present in the same fish) (see Figure 2).

Gene transfer methods:

Microinjection

Transgenes can be introduced into the fertilized eggs, via microinjection or electroporation, or via the use of primordial germ cells. Gene transfer by microinjection is the most popular method. In mammals, the gene is injected into the pronuclei of the embryo, but in fish the pronuclei cannot be seen after fertilization and some fish eggs are opaque (Chen and Powers, 1990; Palmiter et al., 1982). Consequently, in fish linearized DNA is microinjected into the *cytoplasm* of fertilized eggs. This approach in fish has been shown to induce high, albeit variable (35% to 80%), rates of mortality and relatively low integration rates (2-17%) (Hayat et al., 1991). Differences in success rates between the studies in fish probably include differences in the timing of the injections (embryo stage). The amount of DNA injected depends on the egg size of the fish species, but often includes up to 10^6 copies (Culp, Nusslein-Volhard, and Hopkins, 1991; Stuart, McMurray, and Westerfield, 1988). Amplification of the injected DNA occurs in the embryo between the initiation of gastrulation and onset of somitogenesis (Stuart, McMurray, and Westerfield, 1988). Introducing DNA into the egg of fish species that produce eggs with a hard outer surface (chorion) is difficult and more likely induces significant damage. Embryos in some fish species also develop very rapidly, passing through the first cell stage in a very short time interval and making it extremely difficult to microinject a large compliment of eggs.

Electroporation

Electroporation the application of short electrical pulses that result in an enhanced permeability of the cell membrane - is another method for introducing

the DNA insert into the host embryo(Cerda et al., 2006; Chen and Powers, 1990; Dunham, 2003). *In vivo* electroporation has proved to be an efficient method for delivering expression plasmids to large numbers of cells in different regions of the developing nervous system in zebrafish embryos and adults and has also been used to study gain or loss of function in various tissues (Cerda et al., 2006; Kera, Agerwala, and Horne, 2010; Rao, Rambabu, and Rao, 2008; Teh C, Chong SW, and Korzh V, 2003).

Voltage, buffer choice, quantity of DNA, and the manner in which the voltage is applied (bursts and frequency of the current) can all affect the efficiency of the electroporation. A major drawback of electroporation as a technique is the considerable variation in the uptake efficiency of the DNA into the embryo, making standardization(s) difficult (Powers et al., 1995). Electroporation, however, can be used to target specific tissues and this has been shown for embryonic neural tube, retinal, and somatic tissue (Tawk et al., 2002; Teh C, Chong SW, and Korzh V, 2003; Vergara and Canto-Soler, 2012).

Primordial germ cells

The use of primordial germ cells (PGCs) is the most recently used method for gene transfer. PGCs are the precursors of the germ cell lineage which are committed to differentiate into either spermatogonia or oogonia after gonadal sex differentiation (Yoshizaki et al., 2003). PGC-mediated gene transfer is used to deliver a foreign gene into the genome of cultured cells and the transformants are isolated by drug selection before they are introduced into individual fish (Takeuchi et al., 2002). Germ-line contribution can be performed by microinjection into the peritoneal cavity of hatched embryos with cultured PGC (Matsui, Zsebo, and Hogan, 1992). PCG-mediated gene transfer offers the

potential for producing transgenic fish of commercially valuable species that have a long generation time and produce low numbers of offspring. This method also allows for the generation of gene-targeted fish via homologous recombination. A method for the culture of PGCs obtained from zebrafish embryos has been established by Fan *et al.* (2008), but this method is limited because it is difficult to isolate PGCs in high quantities from early stage embryos. Furthermore, the information on the required *in vitro* culture conditions for enabling proliferation and survival of PGCs in fish is extremely limited.

Transgenesis Vectors and Systems

A number of different vectors have been used for the delivery and insertion of transgenes into the genome of zebrafish (and medaka) embryos. Commercially available *E. coli* plasmid constructs have most commonly been used due to their wide availability, ease of use and suitable size for carrying most transgene sequences. There are, however, other vectors that hold certain advantages over traditional *E. coli* plasmid constructs, which we now consider.

BAC Clone and Fosmids

Bacterial Artificial Chromosomes (BAC) have been used widely to study zebrafish gene function and regulation. BAC clones are able to hold large DNA insertions (up to 300kb) and therefore have the potential to carry a complete gene structure, including any endogenous promoters associated with cell-type specificity or temporal expression of the gene. Another benefit of BACs is their apparent resistance to positional effects in comparison with smaller plasmid vectors (Suster et al., 2011). Positional effects are a category of transgene silencing incurred by undesirable chromosomal location and flanking DNA., An

example of the application of BAC transgenesis is for a reporter system for oestrogen like substances in the medaka described in more detail below under *Transgenic Fish in Ecotoxicology* (Kurauchi et al., 2005).

An alternative but similar technique to BAC recombination is the use of fosmids. Fosmids are single-copy plasmids that act as a vector for large DNA insertions. This system has been applied successfully for the production of a transgenic *Cyp1a* reporter zebrafish investigating dioxin target tissues (Kim et al., 2013), also described below in the section *Transgenic Fish in Ecotoxicology*. The large size of BAC clones and fosmids however, tend to suffer from low efficiency rates in producing transgenic lines. The use of *Tol2* transposon assisted insertions (see *Transposons* section), however, in particular, an inverted *Tol2* cassette (*iTol2* cassette) is improving success rates considerably (Suster et al., 2011).

Retroviruses

The Moloney murine leukemia virus (MLV) is a viral vector that can be adapted (pseudotyped) to infect a range of different host cells, including zebrafish cells, for transgene delivery. Retroviral insertion has been used in the application of gene traps (GT), enhancer traps (ET) and protein traps (PT) in conjunction with typical transgenic reporter sequences (Trinh le and Fraser, 2013). These traps are an effective means for the identification of transcriptionally active genes and analysis of their function (reverse genetic screening). The MLV retrovirus has been used in a large scale enhancer detection screen to insert an enhancer trap vector into zebrafish (Ellingsen et al., 2005). This technique involves the insertion of a zebrafish promoter and reporter sequence (such as GFP) into the

genome via the MLV vector for the identification and characterization of regulatory gene activity during development.

The pseudotyped MLV system has proven to be one of the most efficient transgene insertion techniques available and can result in successful germ line transmission of insertions to almost all F1 progeny, with an average of 10 copies per cell (Chen et al., 2002; Wang et al., 2007). Due to the difficulties associated with producing high-titer viruses and limitations in cargo sizes, however, transposable elements are the more popular approach (Trinh le and Fraser, 2013). Nevertheless, the technique holds significant promise for use in reverse genetic screens in ecotoxicology and provides a strong alternative to genetic knockout technologies (Bedell, Westcot, and Ekker, 2011; Meng et al., 2008).

Transposons

The frequency of germ line transmission for conventional DNA microinjection is very low and results in mosaic expression in F0. Furthermore, gene silencing can occur over generations using conventional DNA microinjection methods. In the light of these problems, transposons have become popular as methods for gene transmission. Transposons are sequences of DNA that are able to move directly from one site to another site within the chromosome or onto extra chromosomol DNA within the same cell. There are two types of transposons, autonomous and non-autonomous. An autonomous transposon encodes its own enzyme (transposase) and can excise and transpose. In contrast, a non-autonomous transposon does not encode its own transposition proteins and requires transposase activity, which can be supplied as mRNA, in order to enable its relocation. Only non-autonomous transposons that requires artificially

provided transposase can be utilised to produce stage transgenics (Kawakami et al., 2004; Ryder and Russell, 2003). Use of transposons requires transposase introduction into early one cell stage eggs by microinjection together with transposase mRNA. *Tol2* transposon and *Sleeping Beauty* (*SB*) are the main transposons used currently in creating transgenic zebrafish. Both these methods use enzymes to facilitate the integration of foreign DNA into the host genome. The *Tol2* transposon system, derived from medaka and belonging to the hAT(hobo/Ac/Tam3) family of transposons, is now widely used in zebrafish, and in other vertebrates, due to its high efficiency for gene delivery and expression (Hamlet et al., 2006; Kawakami, 2005; Kawakami, Shima, and Kawakami, 2000; Sato et al., 2007). Using a *Tol2* construct co-injected with transposase mRNA, germ line transmission frequency success rates can be as high as 50% (Kawakami et al., 2004). Furthermore the use of the *Tol2* transposon system tends to avoid gene silencing effects that can occur with linear plasmid injection (Kawakami, 2005). This transposon vector system is useful for the generation of a stable transgenic fish or analyses of activation of a promoter, an enhancer, or a gene of interest in transient expression assay (Asakawa and Kawakami, 2009; Asakawa et al., 2008; Lee et al., 2012; Lee, Tyler, and Kudoh, 2012). The *Tol2* transposon is derived from the medaka so this system cannot not been used in the medaka due to an endogenous activity.

Sleeping beauty is a reactivated transposon from the Tc1/*mariner* superfamily of transposable elements isolated from fish and is used regularly in the production of transgenic zebrafish models (Ivics et al., 1997; Miskey et al., 2005). The transgenesis rate using *sleeping beauty* , however, tends to lower than that for *Tol2* (at around 30% (Davidson et al., 2003; Thermes et al., 2002)),

and so the latter has become the most favoured transposon system (Balciunas et al., 2006).

Meganucleases

The traditional approach of microinjecting circular or linearized plasmid DNA into fertilized embryos can often result in concatemeric copies of transgenic DNA. This can lead to mosaicism or silencing in F0 generations, as well as reduced germ line integration (Culp, Nusslein-Volhard, and Hopkins, 1991; Stuart, McMurray, and Westerfield, 1988; Westerfield et al., 1992; Winkler, Vielkind, and Schartl, 1991). To improve the specificity and rate of stable transgenesis, transposase and meganuclease *I-SceI* can be co-injected with plasmid DNA (Grabher, Joly, and Wittbrodt, 2004; Kawakami, 2007; Rembold et al., 2006; Thermes et al., 2002). Meganuclease is used to mediate a single insertion at a low copy number whereas transposase is used to produce numerous single-copy insertions (Kawakami et al., 2004; Ogino, McConnell, and Grainger, 2006; Rembold et al., 2006; Thermes et al., 2002).

Meganuclease *I-SceI* is an endonuclease that prevents concatamerization when co-injected with a plasmid containing *I-SceI* sites flanking the transgene (Jacquier and Dujon, 1985). It is thought that meganulcease *I-SceI* remains bound to the flanking recognition sites, which may be how concatamerization is prevented, but in general the mechanism of meganuclease's success is still unclear (Soroldoni, Hogan, and Oates, 2009). For comparative studies on *cis*-regulatory sequences in a fish genome, a consistent and unique locus of transgene insertion is extremely important. Having meganuclease sites artificially engineered into the genome gives high specificity of a single insertion, and is therefore particularly useful in these studies (Grabher and

Wittbrodt, 2008). However, *Tol2* assisted insertion remains the most reliable and efficient systems in producing stable transgenic lines and hence remains a much more popular technique for mediating transgenesis.

PhiC31 integrase-based targeting method

Most established methods for transgenesis are not able to control copy number and integration sites leading to variable transgene expression caused by so-called position effects (Kirchmaier et al., 2013). The position effects refer to varying transcription of a transgene inserted into different chromosomal regions. An exception to this is the recently developed *PhiC31* integrase-based targeting method, developed in zebrafish and medaka, which enables pre-selection of successfully targeted integrations early on in the injected generation and provides highly reproducible patterns of transgene activity (Kirchmaier et al., 2013; Roberts et al., 2014).

Targeted Genome Editing

An emerging area of genome modification for insertion of a transgene is targeted genome editing. The process involves engineering of customised nucleases to induce DNA sequence specific double strand breaks (DSBs) which are then exploited for sequence alterations. The three techniques gaining most attention currently are (Miller et al., 2010) clustered regularly interspaced short palindromic repeats (CRISPRs), transcription activator-like effector nucleases (TALENs) (Miller et al., 2010) and Zinc Finger Nucleases (ZFNs), each differing in the ease of construction methods, potential off-target activities and their theoretical target range. ZFNs have been the most widely used to date but the relative difficulty and high cost of their construction, has meant that CRISPRs and TALENs are now gaining the most attention.

CRISPRs

The highly adaptive CRISPR–Cas (clustered regularly interspaced short palindromic repeats–CRISPR-associated proteins) immune system occurs in bacteria and archaea to provide protection against viruses, plasmid DNAs and other disruptive mobile genetic elements (MGEs). Once exposed to a new MGE, spacer sequences derived from the MGE are incorporated into the CRISPR loci, separating a series of repetitive DNA motifs. Transcription leads to small CRISPR RNAs that are displayed on Cas protein complexes to provide RNA-guided targeting and degradation of foreign DNA by Cas nucleases.

Hwang et al. have shown that site-specific nucleotide deletions or substitutions in the zebrafish genome can be achieved using RNA-guided Cas9 nuclease (RGN) with germ line transmission of the mutations reaching up to 100% (Hwang et al., 2013). Auer and co-workers have since demonstrated the use of the CRISPR-Cas9 RGNs to mediate the loci-specific insertion (or 'knock-in') of DNA cassettes (Auer et al., 2014). Using previously established transgenic zebrafish lines, Auer et al. (2014) were able to target GFP reporter sequences and knock-in a KalTA4 sequence (an alternate version of Gal4, (Distel, Wullimann, and Koster, 2009)) resulting in the expression of KalTA4 in formally GFP positive cells. The accuracy and efficiency of the CRISPR-Cas9 targeting system has made RGNs a promising tool as an alternative to the use of morpholinos for gene silencing as well as for creating transgenic zebrafish reporter lines. CRISPR RGNs are also relatively easy to construct (Joung and Sander, 2013).

TALENs

TALENs comprise a non-specific *Fok*I nuclease fused to a customisable DNA-binding domain composed of highly conserved repeats derived from TALEs; proteins which are secreted by *Zanthomonas* spp. bacteria to alter gene transcription in host plant cells (Boch and Bonas, 2010; Miller et al., 2010). Huang and co-wrokers first demonstrated heritable mutations in zebrafish target genes could be achieved using TALENs, having successfully introduced indel mutations in *tnikb* and *dip2a* genes (expressed in blood cells and vasculature of zebrafish embryos) (Huang et al., 2011). Use of TALENs has also been extended to the knock-in of genes via homologous recombination (Hwang, Peterson, and Yeh, 2014; Zu et al., 2013). Although slightly more complex to construct than CRISPRs, TALENs has a broader targeting range with almost no restrictions in target sequence (Reyon et al., 2012).

The GAL4-UAS Transgene Expression System

Various manipulated systems have been introduced to improve efficiency, sensitivity, tissue specificity and ease of generation transgenic fish, as described above. A system that has greatly enhanced transgenic models in zebrafish (and *Xenopus*), and is now being employed for studies in ecotoxicology, is the GAL4/UAS system (Hartley, Nutt, and Amaya, 2002; Scheer and Campos-Ortega J. A., 1999). This is comprised of a two-part expression system using a yeast *(Saccharomyces cerevisiae)* transcription activator protein GAL4 and its target sequence UAS (Upstream Activated Sequence) (Duffy, 2002; Hartley, Nutt, and Amaya, 2002). The GAL4 gene encodes a protein of 881 amino acid and in the yeast is a regulator of the

galactose-inducible genes (Johnston and Hopper, 1982; Scott et al., 2007). The UAS element is analogous to an enhancer element defined in multicellular eukaryotes, which is essential for the transcriptional activation of Gal4-regulated genes (Brand and Perrimon, 1993; Duffy, 2002).

The GAL4-UAS system was first introduced into *Drosophila* by Brand and Perrimon (1993) (Brand and Perrimon, 1993) to analyse the function of developmental genes (Brand and Dormand, 1995; Brand, Manoukian, and Perrimon, 1994). GAL4/UAS introduced spatial and temporal control of transgene expression using two transgenic lines, one activator line and one effector line, that were combined (Brand and Perrimon, 1993; Fischer et al., 1988). In an activator line the gene for the yeast transcriptional activator GAL4 is placed under the control of a desired promoter whereas the effector lines contain DNA-binding motif of GAL4-UAS linked the gene of interest (Scheer and Campos-Ortega J. A., 1999). UAS is fused to an effector gene which is silent if the GAL4 activator is absent. GAL4 can be expressed in many different patterns through placement under the control of various '*Drosophila melanogaster*' tissue-specific promoter sequences (Kramer and Staveley, 2003). Many GAL4 lines have been developed and widely used for ectopic expression (the expression of a gene in an abnormal place in an organism) for genes of interest. When the GAL4 activator line and UAS effector line are crossed, the offspring express a GAL4-dependent transgene in a tissues-specific manner (Distel, Wullimann, and Koster, 2009).

The GAL4-UAS system was first applied in zebrafish by Scheer and Campos-Ortega (1999) and has been used since in the generation of various transgenic zebrafish models including for studies into cell type-specific ablation and in the mapping of neuronal circuits and neuronal activity (Asakawa et al., 2008;

Davison et al., 2007; Distel, Wullimann, and Koster, 2009; Scott et al., 2007). GAL4FF is an engineered transcriptional activator consisting of the DNA-binding domain from GAL4 fused to a duplicated portion of the VP16 transcriptional activation domain. GAL4FF is reported to be less toxic to zebrafish embryos (Asakawa et al., 2008) and a GAL4FF-UAS system (Figure 5) has been successfully applied for chemicals effects studies in ecotoxicology (Lee et al., 2012).

A drawback in the generation of transgenic lines using the UAS systems is that these sequences can be prone to CpG methylation and thus silencing, especially with high UAS copy number (Goll et al., 2009; Subedi et al., 2013). Attempts have been made to solve this problem by modifying the UAS to mitigate for silencing (Akitake et al., 2011). Recently, alternative bipartite reporter systems have developed in the zebrafish that are not prone to transcriptional silencing, including the Q transcriptional regulatory system and tryptophan repressor (Subedi et al., 2013; Suli et al., 2014). The Q transcriptional regulatory system, derived from genes in *Neurospora crassa*, is similar to GAL4/UAS system. The transcriptional activator QF binds to the QUAS upstream regulatory sequence and induces the expression of target genes. Transcriptional silencing of Q system does not occur because the QF binding site does not carry essential CpG dinucleotide sequences that are subject to DNA methylation (Subedi et al., 2013).

Recently, the *Escherichia coil* (*E. coli*) tryptophan repressor was used in stable zebrafish transgenic lines and the tryptophan repressor system showed no transcriptional silencing in subsequent generations of zebrafish (Suli et al., 2014). A regulatory protein called a repressor can bind to the operator site of the tryptophan operon and becomes active only when it is associated with

tryptophan. The binding of tryptophan to the tryptophan repressor protein causes a change in conformation in the repressor.

Seeing the Response - Reporter Genes

Reporter genes are used to quantify expression of the transgene and can also be used to rapidly determine success of gene transfer techniques and the tissue location of their expression in the host organism (Alam and Cook, 1990). Most reporter genes are placed downstream of the promoter of the inserted transgene. Common reporter genes used in transgenic research include *E.coli* *β*-galactosidase (lacZ), chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase (NPT), luciferase (Luc), green fluorescent protein (GFP), Kaede, YFP, DsRed and mCherry (Ando et al., 2002; Culp, Nusslein-Volhard, and Hopkins, 1991; Dunham et al., 1999; Leclerc et al., 2000; Nagai et al., 2002; Razak et al., 1999; Uzbekova et al., 2003). The most common reporter genes used in transgenic fish research to date are GFP and Luc (Figure 3).

Luciferase (Luc), originates from *Photinus pyralis* (firefly) and encodes the enzyme luciferase, and causes the cell that expresses it to catalyse luciferins and produce light (Contag and Bachmann, 2002). Furthermore, there is a high signal intensity associated with the luciferase molecule which allows for rapid measurements. It is also suitable as a reporter gene for the quantitative measurement of gene expression. The luciferase assay is widely used in pharmaceutical research (drug screening) for high-throughput analysis of gene transcription in living cells because the procedure is simple and requires only a small volume of test material. Luciferase as a method however, requires a costly substrate and is not stable (luciferase half-life varies from 1.5 to 3 hours

depending on the host cell and the gene construct) (Goodman and Gao, 1999). Furthermore, the image integration time is long to compensate for the low signal intensity (Vooijs et al., 2002; Xiong et al., 1999). Recently, several research groups have shown that luciferase can be detected in live zebrafish embryos, larvae and adults (Chen et al., 2013; Weger et al., 2012).

GFP is a fluorescent protein isolated from bioluminescent jellyfish *Aequorea victoria*, and can be visualized by microscopy. It can be seen directly in living cells and intact organisms and responses can be measured in real time. Furthermore, when used in conjunction with zebrafish mutant lines that lack skin pigmentation, GFP can be detected in fish for all life stages. Advantages of GFP over luciferase include superior brightness, innate fluorescence and relatively high photostability. GFP was first used in zebrafish in 1996 (Amsterdam et al., 1996) and has since been used widely in the study of gene expression patterns, analysis of tissue-specific promoters/enhancers, tissue/organ development, cell migration and mutagenesis screening (Driever et al., 1996; Gong, Ju, and Wan, 2001; Haffter et al., 1996; Udvadia and Linney, 2003). GFP has also been applied in the development of biosensor transgenic zebrafish to monitor chemicals such as heavy metals and oestrogens (Blechinger et al., 2002; Chen et al., 2010; Gorelick and Halpern, 2011; Lee et al., 2012; Tong et al., 2009). RFP (red fluorescent protein) has the advantage over GFP in that there is less background interference. DsRed, red fluorescent protein from *Discosoma sp.*, can be used together with other GFP variants for multicolor imaging (Dietrich and Maiss, 2002). mCherry is the best general-purpose red monomer due to its superior photostability (Shaner, Steinbach, and Tsien, 2005).

Kaede is another fluorescent protein applied as a reporter in transgenic animals. The encoding gene for this protein was isolated from the stony coral

Trachyphylia geoffroyi (Ando et al., 2002). The kaede protein is capable of irreversible photoconversion from green to red fluorescence under UV or violet light illumination (Figure 3). This lends itself, for example, to studies on assessing how stimulation of a gene response subsequently affects its response for a subsequent stimulation of that gene as the events can be separated by the different colour patterning in the organism (Ando et al., 2002). Kaede is also a useful tool for sequentially tracking selectively labeled cells in fish embryos (Sato, Takahoko, and Okamoto, 2006).

Recently, a system has been developed to manipulate further the production of incorporated fluorescent proteins, called kaloop. It involves autoactivation (self-perpetuation) of the fluorescence, and this is being applied as a powerful tool for spatiotemporal genetic fate mapping of specific cells types in zebrafish (Distel, Wullimann, and Koster, 2009).

Transgenic Fish and Ecotoxicology

Transgenic zebrafish have considerable potential for use in aquatic ecotoxicology as biosensors and as more effective models for identifying molecular mechanisms that underlie signalling pathways, and for understanding the physiological and pathological impacts of chemical exposure (Blechinger et al., 2002; Bogers et al., 2006; Gorelick and Halpern, 2011; Lee et al., 2012; Legler et al., 2000). Biosensor fish work on the premise that specific genes, often enzymes or receptors, are inducible by certain chemicals/pollutants. Exposure of the fish to the pollutant of concern, or a natural water containing that pollutant, induces the activation of an inducible promoter that in turn triggers expression of the reporter (e.g. GFP) (Figure 4). Transgenic fish

developed to study contaminant and other environmental stressors include for cadmium and copper toxicity via induction of heat-shock protein gene, oxidative stress - through the induction of an electrophile responsive element (EpRE), for various organic chemicals acting interacting with the aryl hydrocarbon receptor-mediated toxicity (measured via *cyp1a1*), thyroid and glucocorticoid response pathways, and estrogenicity (*vitellogenin*, *choriogenins*, oestrogen receptor responsive elements) generally employing either luciferase or GFP as reporter genes (Almeida et al., 2010; Blechinger et al., 2007; Blechinger et al., 2002; Bogers et al., 2006; Chen et al., 2010; Gorelick and Halpern, 2011; Ji et al., 2012; Kurauchi, Hirata, and Kinoshita, 2008; Kurauchi et al., 2005; Kusik, Carvan, and Udvadia, 2008; Lee et al., 2012; Legler et al., 2000; Legler et al., 2002; Mattingly, McLachlan, and Toscano, 2001; Petersen et al., 2013; Salam et al., 2008; Tong et al., 2009; Zeng et al., 2005) (See Table 1 for a summary of the different transgenic fish lines developed).

Heat-shock protein

Transgenic biosensor fish have been developed that employ the heat shock protein (*hsp*) promoters, for 70 (*hsp 70*) and 27 (*hsp 27*), that are readily induced by various environmental stressors including increased temperature and heavy metals. In a transgenic zebrafish created using eGFP (enhanced green fluorescent protein) as the reporter under the control of the *hsp 70* gene promoter, exposure to cadmium (Cd, for 96 hours) found a response at exposure concentrations as low as 0.2 μM (22.5 μg/L) (Blechinger et al., 2002). This is a more sensitive response system than for Cd induction of *hsp 70* expression in cultured cells (0.5~50 μM , 0.06~5.6 mg/L)) (Ait-Aissa et al., 2000; Braeckman et al., 1999). In the *hsp 70-eGFP* transgenic zebrafish cadmium treatment induced GFP expression in tissues in a dose-dependent manner with

a tissue sensitivity order of gill> skin> olfactory organ> digestive tract> liver> pronephros. Subsequent studies identified responses in olfactory sensory neurons (OSNs) for a brief (3 h) 5 μM Cd (562.1 μg/L) exposure and responses in the lateral line neuromasts after 24 h for a 0.2 μM Cd (22.5 μg/L) exposure (Blechinger et al., 2007). In addition, cell death in the olfactory placode was observed following a 3 h 125 μM Cd (14.1 mg/L) exposure.

Transgenic zebrafish developed with GFP driven by the regulatory region o*f hsp 27 (*also known as HspB1- one of the most widely expressed and distributed small heat shock proteins) have also been shown to be responsive to Cd exposure with GFP expression occurring in heart, skin and muscle (Wu et al., 2008) after a 24 h heat shock treatment. This model (hsp 27-GFP transgenic zebrafish), however, was far less sensitive to Cd compared with the hsp 70-eGFP transgenic zebrafish (Blechinger et al., 2002), with GFP expression detected in embryos only at high Cd exposure 320 μM Cd *(36 mg/L)*, but not for exposures between 1.35~135 μM *(0.2~15.2 mg/L)*).

Cyp1a1 promoter

The promoter of the cyp1a1 gene has been used to drive a *GFP reporter gene* for measuring exposure to organic chemicals in both transgenic zebrafish and medaka (Hung et al., 2012; Kim et al., 2013; Ng and Gong, 2013). Cyp1a1 is a cytochrome P450 enzyme involved in the oxidative metabolism of various organic substances including, polycyclic aromatic hydrocarbon (PAHs*)* and polychlorinated biphenyls (PCBs). Cyp1a1 also acts as a marker of the activation of the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor known to mediate the toxic effects of several classes of environmental contaminants including polychlorinated biphenyls (PCBs),

polycyclic or halogenated aromatic hydrocarbons (PAHs) and dioxins (e.g. 2,3,7,8-Tetrachlorodibenzo-p-dioxin, TCDD) (Mattingly, McLachlan, and Toscano, 2001).

Transgenic Cyp1a-GFP medaka embryos (72 hpf) exposed to TCDD (for 24 h) showed GFP expression in kidney, liver and gut at an exposure concentration of only 0.005 nM (1.6 ng/L) (Ng and Gong, 2013). Other responding tissues at a higher TCDD exposure concentration (0.5 nM (161 ng/L)) included olfactory pits, caudal fin, gills, neuromast cells. This transgenic medaka was also shown to be highly responsive to other PAHs including, *3*-methylcholanthrene (3-MC) and benzo[a]pyrene (BaP) with expression in the liver and kidney. A similar transgenic Cyp1a-*GFP* zebrafish was shown to be responsive to PCBs in liver and intestine at exposure concentrations down to 0.04 µg/ml *(*40 µg*/L)*(Hung et al., 2012). In the most recently developed transgenic cyp1a reporter zebrafish, incorporating eGFP, responses to TCDD were detected in brain vessel, heart, gut, retinal bipolar cells, otic vesicle, lateral line, cloaca and pectoral fin bud down to 1 nM TCDD (322 ng/L) (Kim et al., 2013). The transgenic cyp1a-eGFP zebrafish embryos was also responsive to the AhR agonists benzo[a]pyrene (B[a]P), 3-methylcholanthrene (3-MC) and β-naphthoflavone (β-NF) at exposure concentrations of 1 µM (268.4 µg/L) and 10 µM (2.7 mg/L), respectively.

Electrophile responsive element

Transgenic zebrafish models developed for studying oxidative stress, where chemical modification of DNA, proteins and lipids can alter their biological functions, have incorporated an electrophile-responsive element (EpRE), with luciferase and GFP as reporters (Kusik, Carvan, and Udvadia, 2008). In the first zebrafish model developed of this kind *EpRE-LUC-GFP,* a Luc-GFP protein fusion protein was used (Kusik, Carvan, and Udvadia, 2008). This combination of GFP and luciferase allowed for both visualizing the location of the tissue responses (GFP) and quantifying transgene expression (via luciferase) in the same fish. In the transient assay for this model (embryos injected at the one cell stage with a plasmid pEpRE-LUC-GFP), GFP expression was observed in the skin for exposure to 0.1 µM (27.2 µg/L) mercuric chloride ($HgCl_2$) and in many other cells, including neurons, muscle and the notochord, for exposure to 0.3 µM (81.45 µg/L) $HgCl_2$. In the stable transgenic *EpRE-LUC-GFP* zebrafish line that was created there was a $HgCl_2$ concentration dependent induction of luciferase activity in embryos (LOEC 0.2 µM (54.3 µg/L)) but no GFP expression, the reason for which was unclear. In a similar *EpRE* transgenic zebrafish developed with luciferase only as a reporter embryos were not found to be especially responsive when challenged with copper sulphate limiting their use for environmental studies. There are other responsive elements activated directly or indirectly by oxidative stress such the Nrf2 (nuclear factor E2-related factor 2) -antioxidant response element (ARE) and metal response elements (MREs) (Chu et al., 1999; Nguyen, Nioi, and Pickett, 2009), but these have not yet been applied in transgenic fish models.

Endocrine Disrupting Chemicals

There is increasing concern about the impacts of chemicals in the environment that have ability to disrupt the endocrine system which can result in adverse effects on developmental, reproduction, neurological and/or immune function in both humans and wildlife (Chia et al., 2010; Goodhead and Tyler, 2009; Li et al., 2011). These chemicals have been referred to as endocrine disrupting chemicals (EDCs) and they are derived from both natural and anthropogenic sources. Transgenic zebrafish models have been developed for studying the structure or function of thyroid, corticosteroid, androgen and oestrogen systems.

A single zebrafish transgenic model has been developed for studying thyroid hormone function by Ji et al., 2012. They generated a transgenic zebrafish with eGFP driven by the *TSHβ* (glycoprotein hormone - thyrotropin) β) promoter. Exposure to thyroid hormones, thyroxine (T4) and tri-iodothyronine (T3) decreased GFP expression in the pituitary gland (20 nM, 13 µg/L) for T3, 90 nM (69.8 µg/L) for T4) for a 3 day exposure. Exposure to goitrogens (3.96 mM, 548.9 mg/L) $KClO_4$ and 1.3 mM (221.3 mg/L) PTuracil) for 3 days induced activation of *TSHβ* expression and enhanced GFP expression in the pituitary gland. This transgenic *TSHβ-eGFP* zebrafish could usefully be applied for studies on pituitary TSHβmRNA expression and thyroid function, but not as an effective biosensor for assessing the effects of thyroid disrupting chemicals due to the high levels of GFP expression resulting from endogenous TSHβ.

Transgenic zebrafish lines have been developed for measuring responses to glucocorticoids. Glucocorticoids are important modulators of energy metabolism and a number of studies have showed that some EDCs might be able to stimulate glucocorticoid signaling through effects on the glucocorticoid receptor (GR) or by disturbing glucocorticoid synthesis or function (Atanasov et al., 2005; Sargis et al., 2010). The first transgenic zebrafish model developed for

glucocorticoid receptors contained four glucocorticoid response elements (GRE) upstream of TATA minimal promoter and luciferase as the reporter (Weger et al., 2012). The *GRE-LUC* transgenic zebrafish larvae were shown to respond in a concentration -dependent manner to dexamethasone (DEX) treatment (2.5-10 μM (1~3.9 mg/L)) and glucocorticoid signaling was blocked by exposure to the GR antagonist, mifepristone (Weger et al., 2012). Exposure of these transgenic zebrafish larvae to tributyltin (TBT), an antifoulant used to prevented growth of marine organisms, inhibited ligand binding to GR and blocked GR activation. The metabolite of TBT, dibutyltin (DBT) is known to be responsible for this GR interaction. GC signaling in GRE-LUC larvae was inhibited with environmentally relevant exposure concentrations for TBT (20 nM). Very recently, two further GRE transgenic zebrafish lines have developed by different research groups (Benato et al., 2014; Krug et al., 2014). One of these models (Krug et al., 2014) contains six glucocorticoid response elements and a short half-life green fluorescent protein (6 X GRE:d4EGFP). For acute and chronic exogenous glucocorticoid treatment strong GFP was observed in a variety of tissues, including the brain after exposure to 10 μM (3.6 mg/L) hydrocortisone and 10 μM (5mg/L) fluticasone propionate (Krug et al., 2014). This model was also shown to be applicable for studies into the stress response with elevated cortisol. In the GRE model developed by Benato et al (2014) nine GRE tandem repeats were included to drive eGFP expression (Benato et al., 2014). In untreated transgenic zebrafish there was ubiquitous expression of eGFP in the head, posterior trunk and tail bud at 14 hpf and subsequently in the head and caudal trunk around the yolk sac and its extension at 24 hpf. GFP intensity was then reduced in the head and tail regions at 72 hpf and become more localized in internal organs at 6 dpf. Exposure of 48 hpf embryos to DEX induced a

concentration related responses with a LOEC of 100 nM (39.3 µg/L). At an exposure of 10 µM (3.9 mg/L) DEX eGFP signal was enhanced in pectoral fins, dermal mesenchymal-like cell, muscle fibers, pituitary, pineal gland and blood vessels and blood cells. Reporter activity in this transgenic line was decreased by GR knockdown and RU486 treatment. GFP expression was also observed in several organs in untreated (natural expression) adult males and females. After 10 µM (3.9 mg/L) DEX treatment for 24 h, GFP signal was increased in the brain, liver, intestinal muscosa, kidney, splanchnocranium, spinal cord, eye and skin in the both male and female in this transgenic line. This model therefore looks to have good utility for studies into GC functions in both early and adult life. Various transgenic fish lines have been developed with an oestrogen responsible promoter derived from the *vitellogenin* (*vtg)* or *choriogenin* genes. *Vitellogenin (vtg)* is an egg yolk precursor protein, normally synthesized in the liver of females, but also inducible in males in response to oestrogen exposure, and is the most widely used biomarker for exposure to oestrogenic chemicals in aquatic ecotoxicology. *Choriogenins* are egg envelope proteins that are similarly synthesized in the liver of maturing female fish in response to estrogens. Both *vitellogenin* and *choriogenin* respond to oestrogens in low ng/L exposure regimes and at sub-nanogramme exposure concentrations for the synthetic oestrogen ethinylestradiol (Thomas-Jones et al., 2003).

Some of the first transgenic models employing promoters for the *vitellogenin* and *choriogenin* genes with GFP reporters were developed in medaka. These models however, were not sufficiently sensitive for detecting exposure to oestrogens for environmentally relevant exposures. Examples of this include for a *vitellogenin*1 (*vtg*1) gene promoter Zeng et al., 2005 that required 500 ng E_2/L (17β-*estradiol)*, 50 ng EE_2/L (17α-ethinylestradiol) or 1 mg BPA/L (bisphenol A)

exposures to induce detectable levels of GFP. Similarly, a transgenic medaka developed with gene regulatory elements of *choriogenin H* detected responses in the liver for exposure concentrations of 0.63 nM for E_2(171 ng E_2/L), 0.34 nM for EE_2 (100 ng EE_2/L) and 14.8 nM for estrone (E_1, 4 µg E_1/L) after a 24h exposure (Kurauchi, Hirata, and Kinoshita, 2008). Kinoshita et al., 2010 applied this model to detect levels of estrogen-like substances in waters in Thailand and Malaysia, but its use in this regard would be for sites extremely heavily polluted with oestrogen only. A further transgenic medaka incorporating a *choriogenin H* gene promoter and a red fluorescent protein reporter (RFP) gene was similarly shown to have a relatively low detection sensitivity for estrogens, with responses to 1 µg E_1/L, 200 ng E_2/L, 1 µg DES/L and 10 mg BPA/L, for 7 day exposures (Cho, Kim, and Nam, 2013). Although not sufficiently sensitive to detect oestrogens that occur for most environments, the species of medaka used was euryhaline allowing for comparative effects analysis on chemicals in aqueous environments of differing salinity. The most sensitive transgenic medaka produced using the *choriogenin L* gene with a GFP reporter was responsive to 25 ng E_2/L (for a 6 day exposure) (Salam et al., 2008).. A single GFP transgenic zebrafish line has been developed with an estrogen-inducible promoter for a *vitellogein* gene (*vtg1*) (Chen et al., 2010) that was reported to detect GFP in the liver at 0.1 µg E_2/L, 1 µg estriol /L, 1 mg BPA/L, and 10 mg nonylphenol (NP)/L. The lack of appropriate controls in this work however makes it difficult to provide a robust evaluation of this model.

The transgenic medaka and zebrafish lines developed incorporating *vitellogenin* and *choriogenin* genes in the reporter systems reported on above vary in their responsiveness to oestrogen and generally they are limited in the use for environmental monitoring as they lack the required sensitivity. Responses in all

of these transgenic medaka and zebrafish for *vtg* and *choriogenin* genes are also restricted to the liver, the site of *vitellogenin* and *choriogenin* synthesis. They do nevertheless, provide systems for screening chemicals for oestrogenic activity *in vivo* in real time and can be applied to inform on cumulative responses to estrogens.

Of a slightly different nature, but nevertheless relevant for informing on the feminizing effects of oestrogens in fish, transgenic medaka containing the regulatory promoter of the *42Sp50* gene driving GFP or RFP have been developed for detecting testis ova, a condition induced in response to oestrogen exposure in males. *42Sp50* is abundantly expressed in oocytes (Kinoshita et al., 2009). In these studies induction of testis ova occurred for a 2 week exposure to 830 ng estradiol-benzoate/L. Although this model was not sufficiently sensitive for detecting effects of oestrogens on germ cell development for environmentally relevant exposures it has utility for studying the mechanisms controlling oogenesis and germ cell differentiation.

There have been a number of transgenic lines developed in zebrafish for detecting oestrogens using oestrogen response elements (ERE), which binds the oestrogen receptor–ligand complex activating oestrogen responsive genes. The first of these transgenic biosensor zebrafish developed by Legler *et al.* (2000) incorporated *3*×EREs inserted upstream of TATA minimal promoter and luciferase as the reporter (Legler et al., 2000). Juveniles of this zebrafish transgenic model were responsive to E_2 at concentrations down to 0.1 nM (27.2 ng/L) (for a 96 h exposure) and the testis was the most responsive target tissue. This transgenic zebrafish, was subsequently applied to show antiestrogenic effects of the polycyclic musks (such as 6-acetyl-1,1,2,4,4,7-hexamethyltetraline (AHTN) and 1,2,4,6,7,8-hexahydro- 4,6,6,7,8,8-

hexamethylcyclopenta-ç-2-benzopyran (HHCB)), commonly used in the fragrance industry) (Schreurs et al., 2004). Limitations of this transgenic model include that measurement of the reporter luciferase required termination of the fish and identifying the specific responding tissues required their individual dissection and analysis. Work using this transgenic model did however, show that the main target tissues (the liver and gonad) were responsive down to exposures of EE_2 (3 or 10 ng/L) and demonstrated also that responses to oestrogenic EDCs differed for different developmental stages (Bogers et al., 2006).

Two subsequent transgenic zebrafish models developed incorporated EREs with GFP. In 2011, Gorelick and Halpern (2011) reported on a transgenic zebrafish containing five tandem consensus EREs upstream of a mouse *c-fos* minimal promoter and the GFP gene. The transgenic zebrafish embryos were exposed to a range of oestrogenic chemicals including E_2, DES (diethylstilbestrol), BPA, EE_2 and NP, with responses see in the heart, brain, liver, aorta and ventral fin for exposure to E_2 (with a LOEC of 100 µg/L), liver and heart for exposure to EE_2 (with a detection down to 10 ng/L). No GFP expression was detected in embryos exposed to NP. Use of this transgenic model indicated that different oestrogenic compounds induced tissue specific differences in their activity. A drawback of this model was the relatively low sensitivity- very high exposure concentrations of oestrogens (1-100 µg E_2/L) were employed to induce these responses. However, in their more recent work with this model, they were able to observe tissue-specific GFP expression for exposure to wastewater effluents (Gorelick et al., 2014).

The most recently developed ERE transgenic zebrafish developed by Lee *et al.* (2012) is currently the most sensitive transgenic zebrafish system developed for

detecting environmental estrogens. This model includes a 3×tandem ERE and a *Tol2* mediated GAL4FF-UAS system. The zebrafish embryos and larvae had response sensitivities to the EDCs tested of 1 ng EE_2/L, 5 ng E_2/L, 100 µg BPA/L and 1 µg NP/L. Responses in this model were detected in a wide variety of tissues including the liver, heart, skeletal muscle (somite and cranial), ear/eye ganglions, brain, otic vesicle, lens and neuromasts (Figure 5). Skeletal muscle cells, cranial muscle cells, heart cells and neuromast cells were especially responsive to estrogen. There were tissue-specific expression patterns for the different environmental oestrogens indicating differences in tissues toxicities (Lee et al., 2012).

Life stage differences in responses to oestrogens and differences in health effects for different developmental stages, most likely relate to the expression patterns of the different oestrogen receptor (esr) subtypes. However, it is not yet known how the oestrogenic response pattern observed (via GFP expression) relates to the expression of ERs, although this could now be studied in this model using morpholino knockdown or CRISPR of the different oestrogen receptor sub-types in organisms and in real time.

Transgenic zebrafish have also been developed to examine the effects of oestrogenic chemical exposure on development of the brain using the promoter of a *cyp19* gene. *Cytochrome P450 aromatase* (*cyp19*) is enzyme complex that catalyses the synthesis of estrogens, thereby controlling many different physiological processes of oestrogens and is mainly expressed in the gonad (*cyp19ala*) and brain (*cyp19a1b*) (Chiang et al., 2001). These are oestrogen sensitive target genes. Tong *et al.* (2009) generated a transgenic zebrafish line that expresses GFP under the control of the brain aromatase *cyp19a1b* promoter. In this line GFP expression occurred in the radial glial cells in

response to oestrogen and was associated with endogenous aromatase B expression (Tong et al., 2009). Exposure of embryos to a variety of different oestrogenic chemical classes including natural and synthetic steroids, alkylphenolic compounds and phyto- and myco-estrogens (for 5 days) induced strong GFP expression in the region between the anterior telecephalon and caudal hypothalmus and most of these responses were concentration dependent (Brion et al., 2012). The effective concentrations (EC_{50}) for inducing these responses were 0.013 nM (3.9 ng/L) EE_2, 0.01 nM (2.7 ng/L) DES, 0.48 nM (130.8 ng/L) E_2, 3303 nM (0.8 mg/L) BPA, 2501 nM (0.7 mg/L) genistein. This zebrafish line provides a very useful model for studies into the roles of natural estrogens, and effects of environmental estrogens, on brain development and function. Models using the *cyp19a1a* gene promoter are likely to be forthcoming in the very near future for studies into the effects of oestrogens on gonadal development.

Benefits, Limitations and Future Application of Transgenic Fish in Ecotoxicology

Transgenic fish systems provide the advantages of both *in vitro* and *in vivo* systems for testing chemicals. As embryos/larvae they offer as reasonably rapid screening systems with high sensitivity (for some models) and good repeatability. They can also be relatively cheap to use, once the models have been established, and especially so when compared with similar mammalian models. Equally however, there are reasonably high costs, most notably in skilled time, to develop fish transgenic models and then in maintaining the lines. Transgenic fish allow for effective toxicokinetic studies for assessing uptake,

distribution and accumulation of chemical ligands/activators in the tissues of live fish and this can be combined with biological effects analyses in a targeted manner. The ability to visualize tissues responsive to chemicals for exposures during early life offers the possibility also to track subsequent effects in those specific tissues in later life. Furthermore, transgenic fish could be usefully applied to study how different chemicals (single chemicals or chemical mixtures) interact within the body to affect different body tissues to assess the effects of sequential exposures, and investigate effects of real world mixtures (e.g. effluent discharges) on developmental processes and health. The availability of fluorescent markers such as kaede offer exciting opportunities to help identify responses and effects for sequential exposures to the same, or to different chemicals. Transgenic fish systems also offer the potential for studying detailed effect mechanisms and signaling pathways. As an example application of morpholinos/CRISPRs could be applied in combination with chemical exposures to ablate specific receptors and to identify roles of receptor subtypes in mediating the toxicant response. The transgenic models published to date are relatively simple and combining different reporter systems in the same animals (and using different fluorescent markers) in the future should allow for multiple toxicological responses in the same animal. As an example incorporating both the ERE and cyp19 gene promoters that are linked with different fluorescent proteins in the same transgenic fish will more fully inform on the roles and effects of oestrogens and oestrogen mimicking chemicals in fish. Novel automated systems are now being developed to scan and analyze responses in transgenic zebrafish larvae in medium throughput systems (Chang et al., 2012) and this will enhance further small fish transgenic for chemicals screening and testing. A further major benefit of transgenic fish systems is they

could lead to a significant replacement and reduction in animal in chemical testing, as they allow for better effects targeting and analysis across multiple organ systems in individual organisms in real time. Use of transgenic embryos rather than adult fish could also reduce substantially the numbers of fish in chemical testing. Equally however, it has to be recognized that substantial validation of these transgenic models is required before this is possible. Life stage dependent effects may occur for specific chemicals and thus responses in embryos may not always be representative of responses in later life stages. This in turn will, in the short term, demand use of more intact animals for the validation process. Furthermore, to date there has been little attempt to compare directly responses in transgenic animals with wild type lines, embryos of otherwise, and this warrants further study. Confidence that transgenic fish are truly representative of the more standard lines of fish currently used in chemical testing will be required before they are taken up widely in standardized chemical testing. Indeed, it could be argued that a standardized validation process needs to be established for transgenic lines before they are accepted into standardized regulatory guideline tests for chemicals.

Although transgenic fish have been developed and applied successfully for detecting responses to a range of chemicals, there are also a number of inherent difficulties/limitations of the models. Most of these, however, should be relatively easy to resolve, in the near future with scientific endeavor and the appropriate resources to do so. It is clear from the transgenic fish models reported upon in the previous section that considerable differences occur in their sensitivity and efficiency. Some of these differences in transgene expression may be due to differences in the test chemicals/ chemical formulations used from different manufactures in the different laboratories

(rarely are details given in this regard). This however is unlikely to result in the orders of magnitude differences seen in sensitivity between some experimental models for the same promoter. More likely, these differences are due to differences in the targeting vector, the number of responsive sequences used, promoters adopted, and/or reporter gene and the site of integration. Differences in the exposure duration and fish life stages exposed may also account for some of the differences in sensitivity reported. It is difficult to reconcile these differences for recommending an 'optimized approach' and in fact it is unlikely that one approach will be optimal for all genes of interest. Nevertheless, there are some general features that will help in creating both a stable and responsive transgenic fish. From the published information *Tol2* transposon and *I-SceI* meganuclease-mediated transgenesis protocols, appear to be the more reliable (and stable) systems for creating transgenic biosensor fish as they have been successfully applied to a wide range of molecular sequences and both are suited to microinjection methods. The insertion of multiple response elements, rather than a single element, also appears to result a more sensitive transgenic model. An area of research that could provide fruitful dividends is into the efficacies of the different types (i.e. natural vs synthetic) and the nature of promoter sequences.

Pigmentation in skin has been a major limitation for using juvenile and adult transgenic fish. Embryos and early life stages of zebrafish, and many other fish species, lack significant skin pigmentation and induction of GFP and other fluorescent markers is easily observed even in relative deeply seated tissues. In juveniles and adults, however, this is not the case and animals have to be dissected to view responses in internal organs. A way to circumvent this problem is to develop the transgenic systems in skin pigment free lines (e.g.

casper, as one example for zebrafish). It is also the case that for models developed for understanding chemicals interacting with endogenous hormone receptors the endogenous hormones will affect which life stages and possibly which sex can be usefully applied. As an example for studies into the effects of environmental estrogens, sexually maturing females will contain varying (but significant) levels of endogenous circulating estrogen, making any chemical effects analyses extremely difficult, and in adults such studies may only be practicable with males.

It should be recognized that to produce and maintain stable transgenic fish lines is a considerable undertaking and careful planning is required to ensure valuable lines can be maintained and secured for future studies. Separate lines of transgenic zebrafish should be bred and subsequent generations tested on a regular basis through routine screening of their responses to ensure consistency and also to avoid potential problems associated with inbreeding, such as a reduction of reproductive fitness. Consistency in responses of specific transgenic lines is crucial for any standardized chemical testing. Thus the development and maintenance of transgenic fish lines is not a trivial exercise and more easily undertaken in large, well funded laboratories. This being the case, transgenic lines created should be made available, on request, to other research laboratories to maximize their utility and model uptake and independent validation.

In conclusion, transgenic fish (principally zebrafish) have been applied widely in studies in developmental biology and medicine, and provided systems for exploring many biological questions. Recent developments show that transgenic zebrafish offer promise as biosensors in ecotoxicology, but to date only a few models have been developed that have both the required specificity

and sensitivity for application to understand environmental health effects. Most of the fish models developed, however, have good utility for investigating effect mechanisms of chemicals and in a highly integrative manner. Transgenic fish line may prove particularly beneficial in elucidating of adverse outcome pathways. With the technology established for the creation of stable and sensitive transgenic fish for studying chemical effects and in real time there are exciting possibilities for ecotoxicologists.

Acknowledgements

We wish to thank Drs. Ross Brown and Jason Snape (AstraZeneca) and Dr. Tetsuhiro Kudoh (University of Exeter) for their constructive comments on the manuscript. We also thank Susan Rouillard (Drawing Service, University of Exeter) for supporting in the production of the figures.

Declaration of interest:

The author's affiliation is as shown on the cover page and the authors have sole responsibility for the writing and content of the paper. Okhyun Lee was funded on a NERC grant (reference NE/I014470/1) and Jon Green on a BBSRC CASE studentship with AstraZeneneca (reference 620033640). AstraZeneca was provided with the opportunity to comment on the paper. The literature review conducted, the interpretations made and the conclusions drawn are exclusively those of the authors. None of the authors has any actual or potential competing financial interests.

Figure and table legends:

Table 1. Summary of the different transgenic zebrafish and transgenic medaka lines developed for ecotoxicology. The table includes the transgene sequences and insertion techniques used to generate the individual transgenic lines as well as the chemicals used in exposures.

Figure 1. Number of publications between the years 1967-2013 that include "transgenic animals' (1a) or transgenic (TG) fish/transgenic zebrafish as analyzed using PubMed. A search for 'transgenic (TG) zebrafish in ecotoxicology' returned only12 publications in the last 15 years.

Figure 2. Procedure for generating transgenic fish. Once the construct is made by either restriction enzyme/ligase cloning (a), In-Fusion cloning (Clontech) (b), or Gateway cloning (Life Technologies) (c), the plasmid is microinjected into one cell stage embryos and the embryos are the subsequently raised to adulthood. The presence of the transgene is confirmed by polymerase chain reaction (PCR) and/or Southern blotting on genomic DNA isolated from fin tissue. Single founder (F0) fish are mated with single wild-type (non-transgenic) fish and their offspring are mated with each other to confirm germ-line integration and to establish a homozygous transgenic line.

Figure 3. Principles of how reporter genes (luciferase (Luc) (A), green fluorescent protein (GFP) (B) and kaede (C) enable visualizing target gene

expression. The reporter genes (Luc, GFP and Kaede) are expressed when DNA is transcribed into messenger RNA (mRNA) and then translated into a reporter protein. In a reporter gene assay using luciferase, a luminescence signal is generated by the reaction of luciferase's substrate, luciferin (A). GFP expressing cells are detected by fluorescence microscopy (B). Kaede protein, when exposed to UV or violet light, is capable of irreversible photo-conversion from a green to a red fluorescent form (C).

.

Figure 4. Schematic of a biosensor transgenic zebrafish system for detecting exposure to aquatic pollutants. The transgenic zebrafish containing DNA elements responsive to a toxicant is placed in the exposure water and as the contaminants enter and reach the body tissues, activation of the response elements occurs that induces the production of GFP in that target tissue.

Figure 5. Mechanism of amplification of the oestrogenic signal in a GAL4FF-UAS transgenic zebrafish. Within the transgenic fish, oestrogen responsive elements (EREs) respond to the oestrogenic signal to drive the first reporter, Gal4ff. Gal4ff binds to UAS in the 2nd transgene to drive the 2nd reporter, a green fluorescent protein (GFP). This two step reporter system amplifies the signal and enhances the sensitivity of the biosensor.

Induction of GFP in *3×ERE:GAL4FF/UAS:GFP* transgenic zebrafish exposed to estrogen. Four day old larvae exposed to clean water alone (control:

A) or 17β-estradiol (100 ng E_2/L: B). GFP expression is observed in the cranial muscle (cm), heart (h), lens (le), liver (li), neuromast (n) and somite muscle (sm) by confocal microscopy (Zeiss) with a ×10 objective lens.

Table 1

Table 1. Summary of the different transgenic zebrafish and transgenic medaka lines developed for ecotoxicology. The table includes the transgene sequences and insertion techniques used to generate the individual transgenic lines as well as the chemicals used in exposures.

Signaling pathway	Chemicals	Fish	Reporter transgene	Insertion	Vector	Transposon	References
Estrogen Anti-Estrogen	E_1, E_2, EE_2, NP (Nonylphenol) AHTN and HHCB	Zebrafish	3×ERE:Luc	Microinjection	Linearized DNA	None	Bogers et al. (2006), Legler et al. (2000), Legler et al. (2002), Schreurs et al. (2004)
Estrogen	E_2, Environmental water samples	Zebrafish	*5×ERE-GFP*	Microinjection	Circular plasmid	*Tol2*	Gorelick and Halpern (2011), Gorelick et al. (2014)
Estrogen	EE_2, E_2, BPA (Bisphenol A), NP	Zebrafish	*3×ERE:Gal4ff-UAS:GFP*	Microinjection	Circular plasmid	*Tol2*	Lee et al. (2012a)
Estrogen + *Vitellogenin*	EE_2, E_2, $CdCl_2$ (Cadmium chloride), zearalenone, E_1, DES, BPA	Zebrafish	*ERE-zvtg1-GFP*	Microinjection	Linearized plasmid	None	Chen et al. (2010)
Vitellogenin	EE_2, E_2 and BPA	Medaka	*Mvtg1-GFP*	Microinjection	Linearized plasmid	None	Zeng et al. (2005)
Choriogenin	E_2	Medaka	*ChgL:GFP-emgh:RFP*	Microinjection	Plasmid	None	Salam et al. (2008), Ueno et al. (2004)
Choriogenin	EE_2, E_2 and E_1	Medaka	*ChgH-GFP*	Microinjection	BAC clone	None	Kurauchi et al. (2008), Kurauchi et al. (2005)
Choriogenin	E_1, EE_2, E_1, BPA and DES	Medaka	*ChgH-RFP*	Microinjection	Linearized plasmid	None	Cho et al. (2013)
42Sp51	EB (estradiol-benzoate)	Medaka	*42Sp50-GFP/RFP*	Microinjection	Circular plasmid	None	Kinoshita et al. (2010)
Cyp19a1b	E_2 More than 30 different estrogenic chemicals	Zebrafish	*Cyp19a1b-GFP*	Microinjection	Linearized plasmid	None	Brion et al. (2012), Tong et al. (2009)
Cyp1a1	Polychlorinated biphenyl (PCB)	Zebrafish	*Cyp1a-GFP*	Microinjection	Circular plasmid	None	Hung et al. (2012)
Cyp1a1	TCDD (2,3,7,8-Tetrachlorodibenzo-p-dioxin)	Zebrafish	*Cyp1a:nls-EGFP*	Microinjection	Fosmid	None	Kim et al. (2013)
Cyp1a2	TCDD, 3-MC (3-methylcholanthrene) and BaP (benzo[a]pyrene)	Medaka	*Cyp1a-GFP*	Microinjection	Circular plasmid	None	Ng and Gong (2013)
TSHβ	T3 (Triiodothyronine) and T4 (Thyroxine)	Zebrafish	*TSHβ:EGFP*	Microinjection	Linearized plasmid	None	Ji et al. (2012)
Hsp70	$CdCl_2$	Zebrafish	*Hsp70:EGFP*	Microinjection	Linearized plasmid	None	Blechinger et al. (2007), Blechinger et al. (2002)
Hsp27	$Na_2HAsO_4 \bullet 7H_2O$ (Sodium arsenate) and $CdCl_2$	Zebrafish	*Hsp27:GFP*	Microinjection	Linearized plasmid	None	Wu et al. (2008)
EpRE	$HgCl_2$	Zebrafish	*EPRE:LUC-GFP*	Microinjection	Linearized plasmid	*I-Sce I* meganuclease	Kusik et al. (2008)
EpRE	$CuSO_4$	Zebrafish	*EPRE:LUC*	Microinjection	Circular plasmid	None	Almeida et al. (2010)
GRE	DEX (Dexamethasone), HC(Hydrocortisone), BM(Betamethasone) and etc.	Zebrafish	*4×GRE:LUC*	Microinjection	Circular plasmid	*Tol2*	Weger et al. (2012)
GRE	HC Fluticasone propionate	Zebrafish	*6×GRE:dHEGFP*	Microinjection	Circular plasmid	*Tol2*	Krug et al. (2014)
GRE	DEX	Zebrafish	*9×GCRE-HSV. UI23:EGFP*	Microinjection	Circular plasmid	*Tol2*	Benato et al. (2014)
Ospl	EE_2, E_2, NP	Medaka	*Ospl-EGFP*	Microinjection	Linearized plasmid	None	Yanbin et al. (2014)

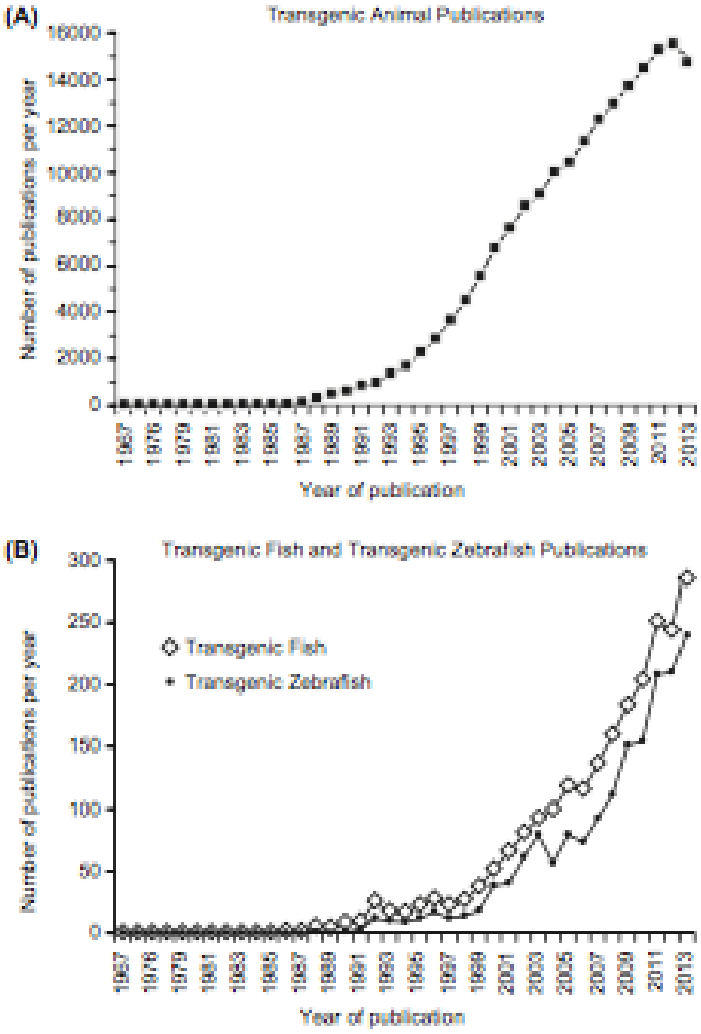

(A)
Transgenic Animal Publications
Number of publications per year
16000
14000
12000
10000
8000
6000
4000
2000
0
1957
1976
1979
1981
1983
1985
1987
1989
1991
1993
1995
1997
1999
2001
2003
2005
2007
2009
2011
2013
Year of publication
(B)
Transgenic Fish and Transgenic Zebrafish Publications
Number of publications per year
300
250
200
150
100
50
0
Transgenic Fish
Transgenic Zebrafish
1957
1976
1979
1981
1983
1985
1987
1989
1991
1993
1995
1997
1999
2001
2003
2005
2007
2009
2011
2013
Year of publication

Figure 1

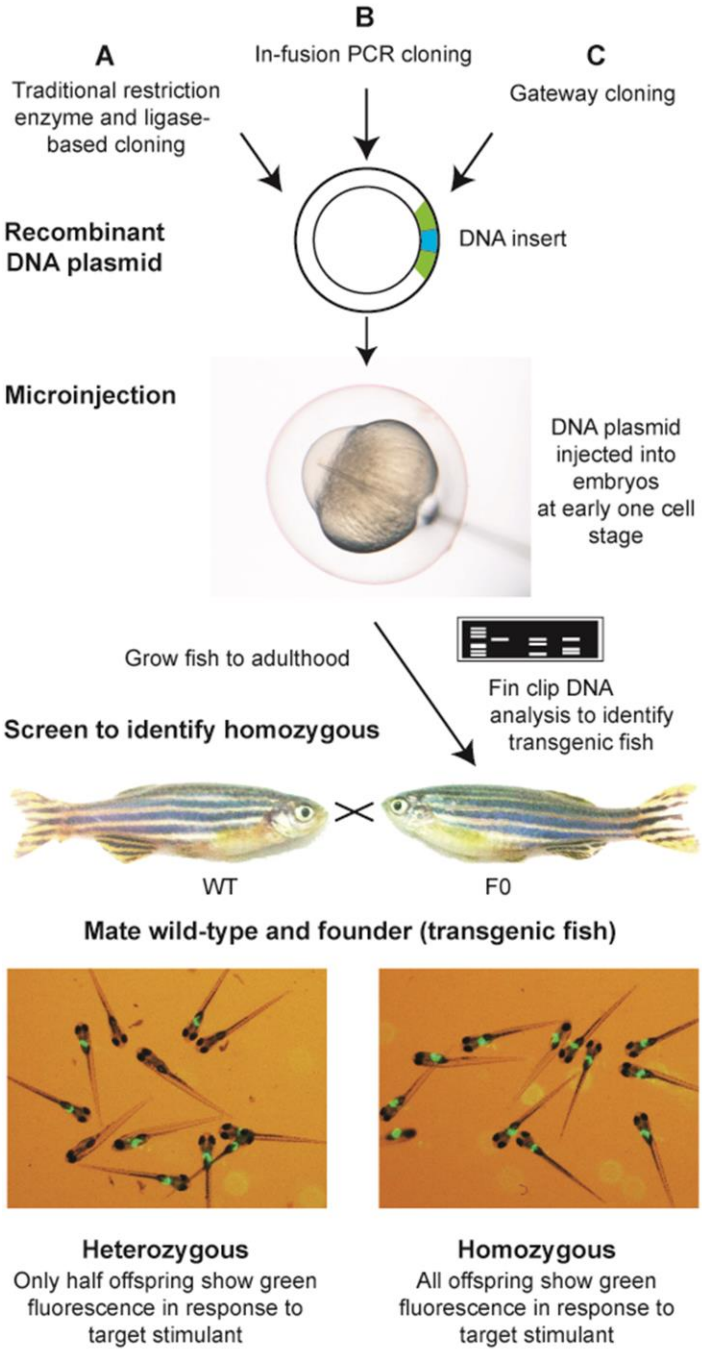


Figure 2. Procedure for generating transgenic fish

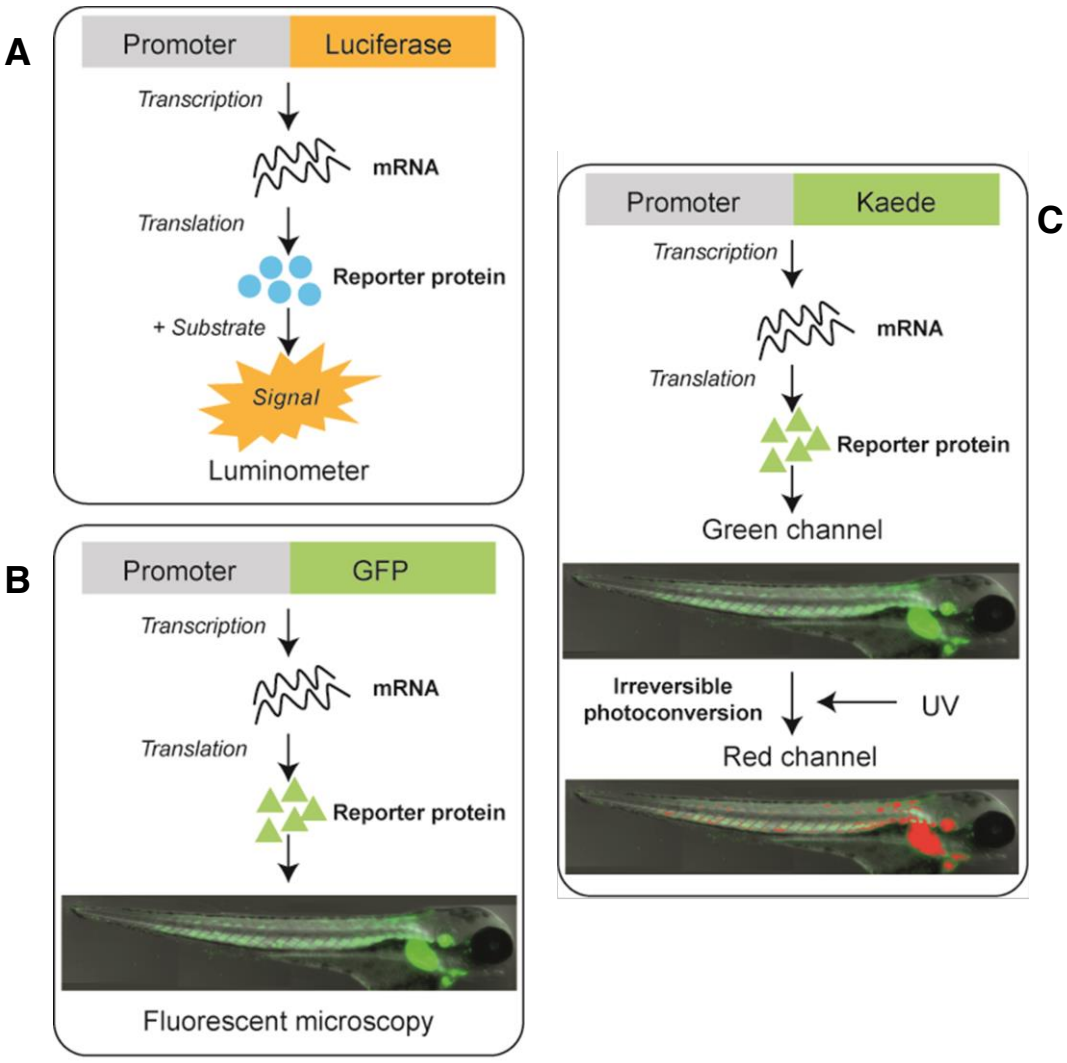


Figure 3. Principles of how reporter genes (luciferase (Luc) (A), green fluorescent protein (GFP) (B) and kaede (C) enable visualizing target gene expression.

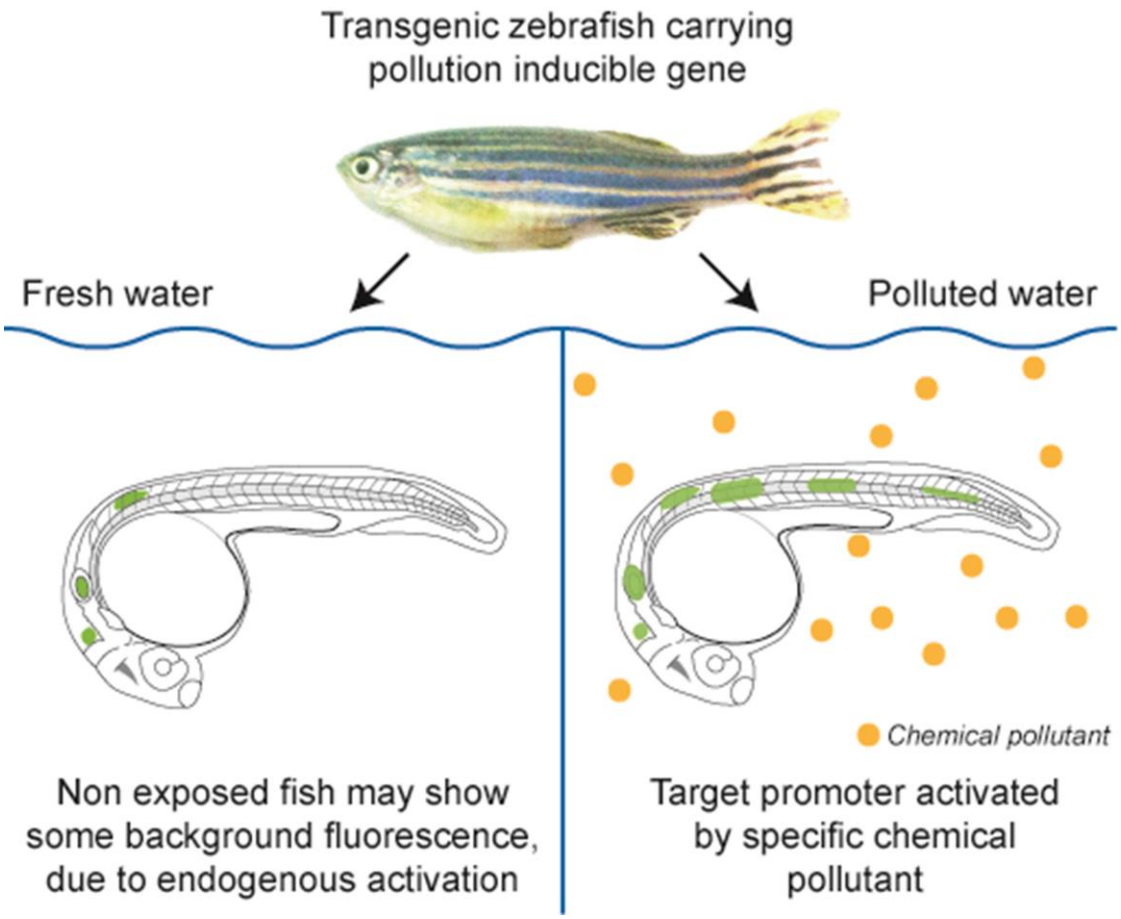


Figure 4. Schematic of a biosensor transgenic zebrafish system for detecting exposure to aquatic pollutants.

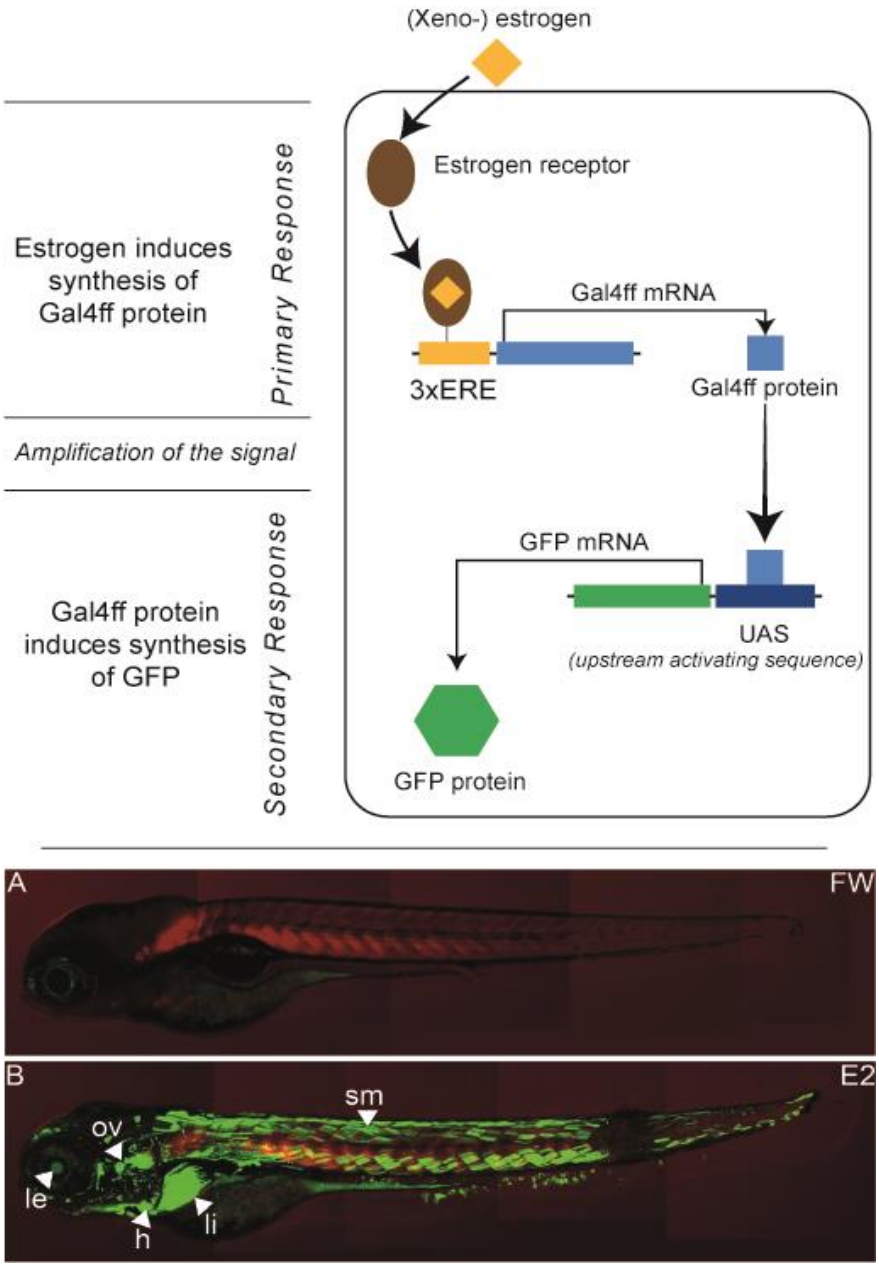


Figure 5. Mechanism of amplification of the oestrogenic signal in a GAL4FF–UAS transgenic zebrafish.

References

1. Ait-Aissa S, Porcher J, Arrigo A, Lambre C. (2000).Activation of the hsp70 promoter by environmental inorganic and organic chemicals: relationships with cytotoxicity and lipophilicity. Toxicology, 145, 147-57

2. Akitake CM, Macurak M, Halpern ME, Goll MG. (2011).Transgenerational analysis of transcriptional silencing in zebrafish. Dev Biol, 352, 191-201

3. Alam J, Cook JL. (1990).Reporter genes: application to the study of mammalian gene transcription. Analytical biochemistry, 188, 245-54

4. Almeida DV, Nornberg BF, Geracitano LA, Barros DM, Monserrat JM, Marins LF. (2010).Induction of phase II enzymes and hsp70 genes by copper sulfate through the electrophile-responsive element (EpRE): insights obtained from a transgenic zebrafish model carrying an orthologous EpRE sequence of mammalian origin. Fish Physiol Biochem, 36, 347-53

5. Amsterdam A, Lin S, Moss LG, Hopkins N. (1996).Requirements for green fluorescent protein detection in transgenic zebrafish embryos. Gene, 173, 99-103

6. Ando R, Hama H, Yamamoto-Hino M, Mizuno H, Miyawaki A. (2002).An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. Proceedings of the National Academy of Sciences of the United States of America, 99, 12651-6

7. Asakawa K, Kawakami K. (2009).The Tol2-mediated Gal4-UAS method for gene and enhancer trapping in zebrafish. Methods, 49, 275-81

8. Asakawa K, Suster ML, Mizusawa K, Nagayoshi S, Kotani T, Urasaki A, Kishimoto Y, Hibi M, Kawakami K. (2008).Genetic dissection of neural circuits by Tol2 transposon-mediated Gal4 gene and enhancer trapping in zebrafish. Proceedings of the National Academy of Sciences of the United States of America, 105, 1255-60

9. Atanasov AG, Nashev LG, Tam S, Baker ME, Odermatt A. (2005).Organotins disrupt the 11beta-hydroxysteroid dehydrogenase type 2-dependent local inactivation of glucocorticoids. Environmental health perspectives, 113, 1600-6

10. Auer TO, Duroure K, Cian AD, Concordet J-P, Bene FD. (2014).Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair.

11. Bader M, Bohnemeier H, Zollmann FS, Lockley-Jones OE, Ganten D. (2000).Transgenic animals in cardiovascular disease research. Experimental physiology, 85, 713-31

12. Balciunas D, Wangensteen K, Wilber A, Bell J, Geurts A, Sivasubbu S, Wang X, Hackett P, Largaespada D, McIvor R. (2006).Harnessing a high cargo-capacity transposon for genetic applications in vertebrates. PLoS Genet, 2, e169

13. Bedell VM, Westcot SE, Ekker SC. (2011).Lessons from morpholino-based screening in zebrafish. Briefings in functional genomics, 10, 181-8

14. Benato F, Colletti E, Skobo T, Moro E, Colombo L, Argenton F, Dalla Valle L. (2014).A living biosensor model to dynamically trace glucocorticoid

transcriptional activity during development and adult life in zebrafish. Molecular and cellular endocrinology, 392, 60-72

15. Bikard D, Marraffini LA. (2013).Control of gene expression by CRISPR-Cas systems. F1000prime reports, 5, 47

16. Blader P, Strahle U. (2000).Zebrafish developmental genetics and central nervous system development. Human molecular genetics, 9, 945-51

17. Blechinger SR, Kusch RC, Haugo K, Matz C, Chivers DP, Krone PH. (2007).Brief embryonic cadmium exposure induces a stress response and cell death in the developing olfactory system followed by long-term olfactory deficits in juvenile zebrafish. Toxicology and applied pharmacology, 224, 72-80

18. Blechinger SR, Warren JT, Jr., Kuwada JY, Krone PH. (2002).Developmental toxicology of cadmium in living embryos of a stable transgenic zebrafish line. Environmental health perspectives, 110, 1041-6

19. Boch J, Bonas U. (2010).Xanthomonas AvrBs3 Family-Type III Effectors: Discovery and Function. http://dxdoiorg/101146/annurev-phyto-080508-081936

20. Bogers R, Mutsaerds E, Druke J, De Roode DF, Murk AJ, Van Der Burg B, Legler J. (2006).Estrogenic endpoints in fish early life-stage tests: luciferase and vitellogenin induction in estrogen-responsive transgenic zebrafish. Environmental toxicology and chemistry / SETAC, 25, 241-7

21. Braeckman B, Smagghe G, Brutsaert N, Cornelis R, Raes H. (1999).Cadmium uptake and defense mechanism in insect cells. Environmental research, 80, 231-43

22. Brand AH, Dormand E-L. (1995).The GAL4 system as a tool for unravelling the mysteries of the Drosophila nervous system. Current Opinion in Neurobiology, 5, 572-78

23. Brand AH, Manoukian AS, Perrimon N. (1994).Ectopic expression in Drosophila. Methods in cell biology, 44, 635-54

24. Brand AH, Perrimon N. (1993).Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development, 118, 401-15

25. Bretaud S, Lee S, Guo S. (2004).Sensitivity of zebrafish to environmental toxins implicated in Parkinson's disease. Neurotoxicology and teratology, 26, 857-64

26. Briggs JP. (2002).The zebrafish: a new model organism for integrative physiology. American journal of physiology Regulatory, integrative and comparative physiology, 282, R3-9

27. Brion F, Le Page Y, Piccini B, Cardoso O, Tong SK, Chung BC, Kah O. (2012).Screening estrogenic activities of chemicals or mixtures in vivo using transgenic (cyp19a1b-GFP) zebrafish embryos. PloS one, 7, e36069

28. Burgess HA, Granato M. (2008).The neurogenetic frontier--lessons from misbehaving zebrafish. Briefings in functional genomics & proteomics, 7, 474-82

29. Cerda GA, Thomas JE, Allende ML, Karlstrom RO, Palma V. (2006).Electroporation of DNA, RNA, and morpholinos into zebrafish embryos. Methods, 39, 207-11

30. Chang TY, Pardo-Martin C, Allalou A, Wahlby C, Yanik MF. (2012).Fully automated cellular-resolution vertebrate screening platform with parallel animal processing. Lab on a chip, 12, 711-6

31. Chen CH, Durand E, Wang J, Zon LI, Poss KD. (2013).zebraflash transgenic lines for in vivo bioluminescence imaging of stem cells and regeneration in adult zebrafish. Development, 140, 4988-97

32. Chen H, Hu J, Yang J, Wang Y, Xu H, Jiang Q, Gong Y, Gu Y, Song H. (2010).Generation of a fluorescent transgenic zebrafish for detection of environmental estrogens. Aquat Toxicol, 96, 53-61

33. Chen TT, Powers DA. (1990).Transgenic fish. Trends in biotechnology, 8, 209-15

34. Chen W, Burgess S, Golling G, Amsterdam A, Hopkins N. (2002).High-throughput selection of retrovirus producer cell lines leads to markedly improved efficiency of germ line-transmissible insertions in zebra fish. Journal of virology, 76, 2192-8

35. Chia VM, Li Y, Quraishi SM, Graubard BI, Figueroa JD, Weber JP, Chanock SJ, Rubertone MV, Erickson RL, McGlynn KA. (2010).Effect modification of endocrine disruptors and testicular germ cell tumour risk by hormone-metabolizing genes. International journal of andrology, 33, 588-96

36. Chiang EF, Yan YL, Guiguen Y, Postlethwait J, Chung B. (2001).Two Cyp19 (P450 aromatase) genes on duplicated zebrafish chromosomes are expressed in ovary or brain. Molecular biology and evolution, 18, 542-50

37. Cho YS, Kim DS, Nam YK. (2013).Characterization of estrogen-responsive transgenic marine medaka Oryzias dancena germlines harboring

red fluorescent protein gene under the control by endogenous choriogenin H promoter. Transgenic research, 22, 501-17

38. Chu WA, Moehlenkamp JD, Bittel D, Andrews GK, Johnson JA. (1999).Cadmium-mediated activation of the metal response element in human neuroblastoma cells lacking functional metal response element-binding transcription factor-1. The Journal of biological chemistry, 274, 5279-84

39. Contag CH, Bachmann MH. (2002).Advances in in vivo bioluminescence imaging of gene expression. Annual review of biomedical engineering, 4, 235-60

40. Culp P, Nusslein-Volhard C, Hopkins N. (1991).High-frequency germ-line transmission of plasmid DNA sequences injected into fertilized zebrafish eggs. Proc Natl Acad Sci U S A, 88, 7953-7

41. Davidson A, Balciunas D, Mohn D, Shaffer J, Hermanson S, Sivasubbu S, Cliff M, Hackett P, Ekker S. (2003).Efficient gene delivery and gene expression in zebrafish using the Sleeping Beauty transposon. Developmental biology, 263, 191 - 202

42. Davison JM, Akitake CM, Goll MG, Rhee JM, Gosse N, Baier H, Halpern ME, Leach SD, Parsons MJ. (2007).Transactivation from Gal4-VP16 transgenic insertions for tissue-specific cell labeling and ablation in zebrafish. Developmental biology, 304, 811-24

43. Devlin RH, D'Andrade M, Uh M, Biagi CA. (2004).Population effects of growth hormone transgenic coho salmon depend on food availability and genotype by environment interactions. Proceedings of the National Academy of Sciences of the United States of America, 101, 9303-8

44. Dietrich C, Maiss E. (2002).Red fluorescent protein DsRed from Discosoma sp. as a reporter protein in higher plants. Biotechniques, 32, 286, 88-90, 92-3

45. Distel M, Wullimann MF, Koster RW. (2009).Optimized Gal4 genetics for permanent gene expression mapping in zebrafish. Proceedings of the National Academy of Sciences of the United States of America, 106, 13365-70

46. Donovan DM, Kerr DE, Wall RJ. (2005).Engineering disease resistant cattle. Transgenic research, 14, 563-7

47. Driever W, Solnica-Krezel L, Schier AF, Neuhauss SC, Malicki J, Stemple DL, Stainier DY, Zwartkruis F, Abdelilah S, Rangini Z, Belak J, Boggs C. (1996).A genetic screen for mutations affecting embryogenesis in zebrafish. Development, 123, 37-46

48. Du SJ, Gong ZY, Fletcher GL, Shears MA, King MJ, Idler DR, Hew CL. (1992).Growth enhancement in transgenic Atlantic salmon by the use of an "all fish" chimeric growth hormone gene construct. Biotechnology (N Y), 10, 176-81

49. Duffy JB. (2002).GAL4 system in Drosophila: a fly geneticist's Swiss army knife. Genesis, 34, 1-15

50. Dunham RA. (2003).Status of genetically modified (transgenic) fish: research and application. Food and Agriculture Organization/World Health Organization expert hearings on biotechnology and food safety ftp://ftpfaoorg/es/esn/food/GMtopic2pdf

51. Dunham RA. (2009).Transgenic fish resistant to infectious diseases, their risk and prevention of escape into the environment and future candidate genes

for disease transgene manipulation. Comparative immunology, microbiology and infectious diseases, 32, 139-61

52. Dunham RA, Chatakondi N, Nichols A, Chen TT, Powers DA, Kucuktas H. (2002).Survival of F2 transgenic common carp (Cyprinus carpio) containing pRSVrtGH1 complementary DNA when subjected to low dissolved oxygen. Mar Biotechnol (NY), 4, 323-7

53. Dunham RA, Chitmanat C, Nichols A, Argue B, Powers DA, Chen TT. (1999).Predator Avoidance of Transgenic Channel Catfish Containing Salmonid Growth Hormone Genes. Mar Biotechnol (NY), 1, 545-51

54. Dunham RA, Warr GW, Nichols A, Duncan PL, Argue B, Middleton D, Kucuktas H. (2002).Enhanced bacterial disease resistance of transgenic channel catfish Ictalurus punctatus possessing cecropin genes. Mar Biotechnol (NY), 4, 338-44

55. Ellingsen S, Laplante M, Konig M, Kikuta H, Furmanek T, Hoivik E, Becker T. (2005).Large-scale enhancer detection in the zebrafish genome. Development, 132, 3799 - 811

56. Fadool J, Hartl D, Dowling J. (1998).Transposition of the mariner element from Drosophila mauritiana in zebrafish. Proc Natl Acad Sci USA, 95, 5182 - 86

57. Fan J, Watanabe T. (2003).Transgenic rabbits as therapeutic protein bioreactors and human disease models. Pharmacol Ther, 99, 261-82

58. Feitsma H, Cuppen E. (2008).Zebrafish as a cancer model. Molecular cancer research : MCR, 6, 685-94

59. Fischer JA, Giniger E, Maniatis T, Ptashne M. (1988).GAL4 activates transcription in Drosophila. Nature, 332, 853-6

60. Goll MG, Anderson R, Stainier DY, Spradling AC, Halpern ME. (2009).Transcriptional silencing and reactivation in transgenic zebrafish. Genetics, 182, 747-55

61. Gong Z, Ju B, Wan H. (2001).Green fluorescent protein (GFP) transgenic fish and their applications. Genetica, 111, 213-25

62. Goodhead RM, Tyler CR. (2009). Endocrine-Disrupting Chemicals and Their Environmental Impacts. In Walker CH, ed.^eds. Organic Pollutants - An Ecotoxicological Perspective, Taylor & Francis Group, 265-92

63. Goodman SD, Gao Q. (1999).Firefly Luciferase as a Reporter to Study Gene Expression in Streptococcus mutans. Plasmid, 42, 154-57

64. Gorelick DA, Halpern ME. (2011).Visualization of estrogen receptor transcriptional activation in zebrafish. Endocrinology, 152, 2690-703

65. Gorelick DA, Iwanowicz LR, Hung AL, Blazer VS, Halpern ME. (2014).Transgenic Zebrafish Reveal Tissue-Specific Differences in Estrogen Signaling in Response to Environmental Water Samples. Environmental health perspectives

66. Grabher C, Joly JS, Wittbrodt J. (2004).Highly efficient zebrafish transgenesis mediated by the meganuclease I-SceI. Methods in cell biology, 77, 381-401

67. Grabher C, Wittbrodt J. (2008).Recent advances in meganuclease-and transposon-mediated transgenesis of medaka and zebrafish. Methods Mol Biol, 461, 521-39

68. Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, Kane DA, Odenthal J, van Eeden FJ, Jiang YJ, Heisenberg CP, Kelsh RN, Furutani-

Seiki M, Vogelsang E, Beuchle D, Schach U, Fabian C, Nusslein-Volhard C. (1996).The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. Development, 123, 1-36

69. Hamlet MR, Yergeau DA, Kuliyev E, Takeda M, Taira M, Kawakami K, Mead PE. (2006).Tol2 transposon-mediated transgenesis in Xenopus tropicalis. Genesis, 44, 438-45

70. Hamra FK, Gatlin J, Chapman KM, Grellhesl DM, Garcia JV, Hammer RE, Garbers DL. (2002).Production of transgenic rats by lentiviral transduction of male germ-line stem cells. Proceedings of the National Academy of Sciences of the United States of America, 99, 14931-6

71. Hansson O, Petersen A, Leist M, Nicotera P, Castilho RF, Brundin P. (1999).Transgenic mice expressing a Huntington's disease mutation are resistant to quinolinic acid-induced striatal excitotoxicity. Proceedings of the National Academy of Sciences of the United States of America, 96, 8727-32

72. Hartley KO, Nutt SL, Amaya E. (2002).Targeted gene expression in transgenic Xenopus using the binary Gal4-UAS system. Proceedings of the National Academy of Sciences of the United States of America, 99, 1377-82

73. Hayat M, Joyce CP, Townes TM, Chen TT, Powers DA, Dunham RA. (1991).Survival and integration rate of channel catfish and common carp embryos microinjected with DNA at various developmental stages. Aquaculture, 99, 249-55

74. Higashijima S-i, Okamoto H, Ueno N, Hotta Y, Eguchi G. (1997).High-Frequency Generation of Transgenic Zebrafish Which Reliably Express GFP in

Whole Muscles or the Whole Body by Using Promoters of Zebrafish Origin. Developmental biology, 192, 289-99

75. Hill AJ, Teraoka H, Heideman W, Peterson RE. (2005).Zebrafish as a Model Vertebrate for Investigating Chemical Toxicity. Toxicological Sciences, 86, 6-19

76. Hong SG, Kim MK, Jang G, Oh HJ, Park JE, Kang JT, Koo OJ, Kim T, Kwon MS, Koo BC, Ra JC, Kim DY, Ko C, Lee BC. (2009).Generation of red fluorescent protein transgenic dogs. Genesis, 47, 314-22

77. Horvat S, Medrano JF, Behboodi E, Anderson GB, Murray JD. (1993).Sexing and detection of gene construct in microinjected bovine blastocysts using the polymerase chain reaction. Transgenic research, 2, 134-40

78. Houdebine LM. (1995).The production of pharmaceutical proteins from the milk of transgenic animals. Reproduction, nutrition, development, 35, 609-17

79. Hrytsenko O, Pohajdak B, Wright JR, Jr. (2010).Production of transgenic tilapia homozygous for a humanized insulin gene. Transgenic research, 19, 305-6

80. Huang P, Xiao A, Zhou M, Zhu Z, Lin S, Zhang B. (2011).Heritable gene targeting in zebrafish using customized TALENs. Nature biotechnology, 29, 699-700

81. Hung KWY, Suen MFK, Chen YF, Cai HB, Mo ZX, Yung KKL. (2012).Detection of water toxicity using cytochrome P450 transgenic zebrafish

as live biosensor: For polychlorinated biphenyls toxicity. Biosensors and Bioelectronics, 31, 548-53

82. Huss D, Poynter G, Lansford R. (2008).Japanese quail (Coturnix japonica) as a laboratory animal model. Lab Anim (NY), 37, 513-9

83. Hwang WY, Fu Y, Reyon D, Maeder ML, Kaini P, Sander JD, Joung JK, Peterson RT, Yeh J-RJ. (2013).Heritable and Precise Zebrafish Genome Editing Using a CRISPR-Cas System.

84. Hwang WY, Peterson RT, Yeh JR. (2014).Methods for targeted mutagenesis in zebrafish using TALENs. Methods

85. Ivics Z, Hackett P, Plasterk R, Izsvak Z. (1997).Molecular reconstruction of Sleeping Beauty, a Tc1-like transposon from fish, and its transposition in human cells. Cell, 91, 501 - 10

86. Jacquier A, Dujon B. (1985).An intron-encoded protein is active in a gene conversion process that spreads an intron into a mitochondrial gene. Cell, 41, 383-94

87. Ji C, Jin X, He J, Yin Z. (2012).Use of TSHβ:EGFP transgenic zebrafish as a rapid in vivo model for assessing thyroid-disrupting chemicals. Toxicology and applied pharmacology, 262, 149-55

88. Johnston SA, Hopper JE. (1982).Isolation of the yeast regulatory gene GAL4 and analysis of its dosage effects on the galactose/melibiose regulon. Proceedings of the National Academy of Sciences of the United States of America, 79, 6971-5

89. Joung JK, Sander JD. (2013).TALENs: a widely applicable technology for targeted genome editing. Nature reviews Molecular cell biology, 14, 49-55

90. Kawakami K. (2005).Transposon tools and methods in zebrafish. Developmental dynamics : an official publication of the American Association of Anatomists, 234, 244 - 54

91. Kawakami K. (2007).Tol2: a versatile gene transfer vector in vertebrates. Genome biology, 8 Suppl 1, S7

92. Kawakami K, Shima A, Kawakami N. (2000).Identification of a functional transposase of the Tol2 element, an Ac-like element from the Japanese medaka fish, and its transposition in the zebrafish germ lineage. Proceedings of the National Academy of Sciences of the United States of America, 97, 11403-8

93. Kawakami K, Takeda H, Kawakami N, Kobayashi M, Matsuda N, Mishina M. (2004).A transposon-mediated gene trap approach identifies developmentally regulated genes in zebrafish. Developmental cell, 7, 133-44

94. Kelly GM, Erezyilmaz DF, Moon RT. (1995).Induction of a secondary embryonic axis in zebrafish occurs following the overexpression of beta-catenin. Mechanisms of development, 53, 261-73

95. Kera SA, Agerwala SM, Horne JH. (2010).The temporal resolution of in vivo electroporation in zebrafish: a method for time-resolved loss of function. Zebrafish, 7, 97-108

96. Kim K-H, Park H-J, Kim JH, Kim S, Williams DR, Kim M-K, Jung YD, Teraoka H, Park H-C, Choy HE, Shin BA, Choi S-Y. (2013).Cyp1a reporter zebrafish reveals target tissues for dioxin. Aquatic Toxicology, 134–135, 57-65

97. Kimmel CB, Warga RM. (1987).Indeterminate cell lineage of the zebrafish embryo. Developmental biology, 124, 269-80

98. Kinoshita M. (2004).Transgenic medaka with brilliant fluorescence in skeletal muscle under normal light. Fisheries Science, 70, 645-49

99. Kinoshita M, Okamoto G, Hirata T, Shinomiya A, Kobayashi T, Kubo Y, Hori H, Kanamori A. (2009).Transgenic medaka enables easy oocytes detection in live fish. Molecular reproduction and development, 76, 202-7

100. Kinoshita M, Zakaria MP, Ismail A, Yusof S, Boonphakdee C, Boonphakdee T, Inoue K. (2010).An attempt to detect contamination with estrogenic compounds in river water of urban area in Thailand and Malaysia using transgenic medaka. Coast Mar Sci, 34, 216-22

101. Kirchmaier S, Hockendorf B, Moller EK, Bornhorst D, Spitz F, Wittbrodt J. (2013).Efficient site-specific transgenesis and enhancer activity tests in medaka using PhiC31 integrase. Development, 140, 4287-95

102. Kobayashi S-i, Alimuddin, Morita T, Miwa M, Lu J, Endo M, Takeuchi T, Yoshizaki G. (2007).Transgenic Nile tilapia (Oreochromis niloticus) over-expressing growth hormone show reduced ammonia excretion. Aquaculture, 270, 427-35

103. Korzh V. (2007).Transposons as tools for enhancer trap screens in vertebrates. Genome biology, 8, S8

104. Kramer JM, Staveley BE. (2003).GAL4 causes developmental defects and apoptosis when expressed in the developing eye of Drosophila melanogaster. Genetics and molecular research : GMR, 2, 43-7

105. Krug RG, 2nd, Poshusta TL, Skuster KJ, Berg MR, Gardner SL, Clark KJ. (2014).A transgenic zebrafish model for monitoring glucocorticoid receptor activity. Genes Brain Behav

106. Kurauchi K, Hirata T, Kinoshita M. (2008).Characteristics of ChgH-GFP transgenic medaka lines, an in vivo estrogenic compound detection system. Marine pollution bulletin, 57, 441-4

107. Kurauchi K, Nakaguchi Y, Tsutsumi M, Hori H, Kurihara R, Hashimoto S, Ohnuma R, Yamamoto Y, Matsuoka S, Kawai S, Hirata T, Kinoshita M. (2005).In vivo visual reporter system for detection of estrogen-like substances by transgenic medaka. Environmental science & technology, 39, 2762-8

108. Kusik BW, Carvan MJ, 3rd, Udvadia AJ. (2008).Detection of mercury in aquatic environments using EPRE reporter zebrafish. Mar Biotechnol (NY), 10, 750-7

109. Lawson ND, Weinstein BM. (2002).In Vivo Imaging of Embryonic Vascular Development Using Transgenic Zebrafish. Developmental biology, 248, 307-18

110. Leclerc GM, Boockfor FR, Faught WJ, Frawley LS. (2000).Development of a destabilized firefly luciferase enzyme for measurement of gene expression. Biotechniques, 29, 590-98

111. Lee O, Takesono A, Tada M, Tyler CR, Kudoh T. (2012).Biosensor zebrafish provide new insights into potential health effects of environmental estrogens. Environmental health perspectives, 120, 990-6

112. Lee O, Tyler CR, Kudoh T. (2012).Development of a transient expression assay for detecting environmental oestrogens in zebrafish and medaka embryos. BMC biotechnology, 12, 32

113. Legler J, Broekhof JLM, Brouwer A, Lanser PH, Murk AJ, Van Der Saag PT, Dick Vethaak A, Wester P, Zivkovic D, Van Der Burg B. (2000).A novel in

vivo bioassay for (xeno-)estrogens using transgenic zebrafish. Environmental Science and Technology, 34, 4439-44

114. Legler J, Zeinstra LM, Schuitemaker F, Lanser PH, Bogerd J, Brouwer A, Vethaak AD, De Voogt P, Murk AJ, Van der Burg B. (2002).Comparison of in vivo and in vitro reporter gene assays for short-term screening of estrogenic activity. Environmental science & technology, 36, 4410-5

115. Li DK, Zhou Z, Miao M, He Y, Wang J, Ferber J, Herrinton LJ, Gao E, Yuan W. (2011).Urine bisphenol-A (BPA) level in relation to semen quality. Fertil Steril, 95, 625-30 e1-4

116. Lieschke GJ, Currie PD. (2007).Animal models of human disease: zebrafish swim into view. Nature reviews Genetics, 8, 353-67

117. Lin S, Gaiano N, Culp P, Burns JC, Friedmann T, Yee JK, Hopkins N. (1994).Integration and germ-line transmission of a pseudotyped retroviral vector in zebrafish. Science (New York, NY), 265, 666-9

118. Liu Y. (2013).Ligation independent cloning: an efficient tool for high-throughput cloning and modular cDNA assembly. . OA Biotechnology, 2, 8

119. Maclean N, Penman D, Zhu Z. (1987).Introduction of Novel Genes into Fish. Nat Biotech, 5

120. Marsischky G, LaBaer J. (2004).Many paths to many clones: a comparative look at high-throughput cloning methods. Genome research, 14, 2020-8

121. Martin P, Feng Y. (2009).Inflammation: Wound healing in zebrafish. Nature, 459, 921-3

122. Matsui Y, Zsebo K, Hogan BL. (1992).Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell, 70, 841-7

123. Matsumoto Y, Oota H, Asaoka Y, Nishina H, Watanabe K, Bujnicki JM, Oda S, Kawamura S, Mitani H. (2009).Medaka: a promising model animal for comparative population genomics. BMC research notes, 2, 88

124. Mattingly CJ, McLachlan JA, Toscano WA, Jr. (2001).Green fluorescent protein (GFP) as a marker of aryl hydrocarbon receptor (AhR) function in developing zebrafish (Danio rerio). Environmental health perspectives, 109, 845-9

125. Meng X, Noyes MB, Zhu LJ, Lawson ND, Wolfe SA. (2008).Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. Nature biotechnology, 26, 695-701

126. Miller JC, Tan S, Qiao G, Barlow KA, Wang J, Xia DF, Meng X, Paschon DE, Leung E, Hinkley SJ, Dulay GP, Hua KL, Ankoudinova I, Cost GJ, Urnov FD, Zhang HS, Holmes MC, Zhang L, Gregory PD, Rebar EJ. (2010).A TALE nuclease architecture for efficient genome editing. Nature biotechnology, 29, 143-48

127. Miskey C, Izsvak Z, Kawakami K, Ivics I. (2005).DNA transposons in vertebrate functional genomics. Cellular and molecular life sciences : CMLS, 62, 629 - 41

128. Moro E, Ozhan-Kizil G, Mongera A, Beis D, Wierzbicki C, Young RM, Bournele D, Domenichini A, Valdivia LE, Lum L, Chen C, Amatruda JF, Tiso N, Weidinger G, Argenton F. (2012).In vivo Wnt signaling tracing through a

transgenic biosensor fish reveals novel activity domains. Developmental biology, 366, 327-40

129. Mozdziak P, Petitte J. (2004).Status of transgenic chicken models for developmental biology. Developmental dynamics : an official publication of the American Association of Anatomists, 229, 414 - 21

130. Nagai T, Ibata K, Park ES, Kubota M, Mikoshiba K, Miyawaki A. (2002).A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nature biotechnology, 20, 87-90

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

132. Nam YK, Noh JK, Cho YS, Cho HJ, Cho KN, Kim CG, Kim DS. (2001).Dramatically accelerated growth and extraordinary gigantism of transgenic mud loach Misgurnus mizolepis. Transgenic research, 10, 353-62

133. Naruse K, Hori H, Shimizu N, Kohara Y, Takeda H. (2004).Medaka genomics: a bridge between mutant phenotype and gene function. Mechanisms of development, 121, 619-28

134. Ng GH, Gong Z. (2013).GFP transgenic medaka (Oryzias latipes) under the inducible cyp1a promoter provide a sensitive and convenient biological indicator for the presence of TCDD and other persistent organic chemicals. PloS one, 8, e64334

135. Nguyen AT, Emelyanov A, Koh CH, Spitsbergen JM, Parinov S, Gong Z. (2012).An inducible kras(V12) transgenic zebrafish model for liver tumorigenesis and chemical drug screening. Disease models & mechanisms, 5, 63-72

136. Nguyen T, Nioi P, Pickett CB. (2009).The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. The Journal of biological chemistry, 284, 13291-5

137. Ogino H, McConnell WB, Grainger RM. (2006).Highly efficient transgenesis in Xenopus tropicalis using I-SceI meganuclease. Mechanisms of development, 123, 103-13

138. Palmiter RD, Brinster RL, Hammer RE, Trumbauer ME, Rosenfeld MG, Birnberg NC, Evans RM. (1982).Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature, 300, 611-5

139. Petersen K, Fetter E, Kah O, Brion F, Scholz S, Tollefsen KE. (2013).Transgenic (cyp19a1b-GFP) zebrafish embryos as a tool for assessing combined effects of oestrogenic chemicals. Aquatic Toxicology, 138–139, 88-97

140. Powers DA, Kirby VL, Cole T, L. H. (1995).Electroporation as an effective means of introducing DNA into abalone (Haliotis rufescens) embryos. Mol Mar Biol Biotechnol, 4, 369-75

141. Puiseux – Dao S, Edery M. (2006). THE MEDAKA FISH: AN EXPERIMENTAL MODEL IN ENVIRONMENTAL TOXICOLOGY ITS USE FOR THE SURVEY OF MICROALGAL TOXINS: PHYCOTOXINS AND CYANOTOXINS. In Arapis G, Goncharova N, Baveye P, ed.^eds. Ecotoxicology, Ecological Risk Assessment and Multiple Stressors, Springer Netherlands, 227-41

142. Rao NM, Rambabu KM, Rao SH. (2008).Electroporation of adult zebrafish. Methods Mol Biol, 423, 289-98

143. Razak SA, Hwang GL, Rahman MA, Maclean N. (1999).Growth Performance and Gonadal Development of Growth Enhanced Transgenic Tilapia Oreochromis niloticus (L.) Following Heat-Shock-Induced Triploidy. Mar Biotechnol (NY), 1, 533-44

144. Rembold M, Lahiri K, Foulkes NS, Wittbrodt J. (2006).Transgenesis in fish: efficient selection of transgenic fish by co-injection with a fluorescent reporter construct. Nature protocols, 1, 1133-9

145. Reventos J, Munell F. (1997).Transgenic animal models in reproductive endocrine research. European journal of endocrinology / European Federation of Endocrine Societies, 136, 566-80

146. Reyon D, Tsai SQ, Khayter C, Foden JA, Sander JD, Joung JK. (2012).FLASH assembly of TALENs for high-throughput genome editing. Nature biotechnology, 30, 460-5

147. Roberts JA, Miguel-Escalada I, Slovik KJ, Walsh KT, Hadzhiev Y, Sanges R, Stupka E, Marsh EK, Balciuniene J, Balciunas D, Muller F. (2014).Targeted transgene integration overcomes variability of position effects in zebrafish. Development, 141, 715-24

148. Ryder E, Russell S. (2003).Transposable elements as tools for genomics and genetics in Drosophila. Briefings in functional genomics & proteomics, 2, 57-71

149. Salam MA, Sawada T, Ohya T, Ninomiya K, Hayashi S. (2008).Detection of environmental estrogenicity using transgenic medaka hatchlings (Oryzias latipes) expressing the GFP-tagged choriogenin L gene. Journal of

environmental science and health Part A, Toxic/hazardous substances & environmental engineering, 43, 272-7

150. Sargis RM, Johnson DN, Choudhury RA, Brady MJ. (2010).Environmental endocrine disruptors promote adipogenesis in the 3T3-L1 cell line through glucocorticoid receptor activation. Obesity (Silver Spring), 18, 1283-8

151. Sato T, Takahoko M, Okamoto H. (2006).HuC:Kaede, a useful tool to label neural morphologies in networks in vivo. Genesis, 44, 136-42

152. Sato Y, Kasai T, Nakagawa S, Tanabe K, Watanabe T, Kawakami K, Takahashi Y. (2007).Stable integration and conditional expression of electroporated transgenes in chicken embryos. Developmental biology, 305, 616 - 24

153. Saunders RL, Fletcher GL, Hew CL. (1998).Smolt development in growth hormone transgenic Atlantic salmon. Aquaculture, 168, 177-93

154. Scheer N, Campos-Ortega J. A. (1999).Use of the Gal4-UAS technique for targeted gene in the zebrafish. Mech Dev, 86, 153-58

155. Schreurs RH, Legler J, Artola-Garicano E, Sinnige TL, Lanser PH, Seinen W, Van der Burg B. (2004).In vitro and in vivo antiestrogenic effects of polycyclic musks in zebrafish. Environmental science & technology, 38, 997-1002

156. Scott EK, Mason L, Arrenberg AB, Ziv L, Gosse NJ, Xiao T, Chi NC, Asakawa K, Kawakami K, Baier H. (2007).Targeting neural circuitry in zebrafish using GAL4 enhancer trapping. Nature methods, 4, 323-6

157. Shaner NC, Steinbach PA, Tsien RY. (2005).A guide to choosing fluorescent proteins. Nature methods, 2, 905-9

158. Sinzelle L, Vallin J, Coen L, Chesneau A, Du Pasquier D, Pollet N, Demeneix B, Mazabraud A. (2006).Generation of trangenic Xenopus laevis using the Sleeping Beauty transposon system. Transgenic research, 15, 751-60

159. Soroldoni D, Hogan BM, Oates AC. (2009).Simple and efficient transgenesis with meganuclease constructs in zebrafish. Methods Mol Biol, 546, 117-30

160. Stuart GW, McMurray JV, Westerfield M. (1988).Replication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. Development, 103, 403-12

161. Subedi A, Macurak M, Gee ST, Monge E, Goll MG, Potter CJ, Parsons MJ, Halpern ME. (2013).Adoption of the Q transcriptional regulatory system for zebrafish transgenesis. Methods, 66, 433-40

162. Suli A, Guler AD, Raible DW, Kimelman D. (2014).A targeted gene expression system using the tryptophan repressor in zebrafish shows no silencing in subsequent generations. Development, 141, 1167-74

163. Suster ML, Abe G, Schouw A, Kawakami K. (2011).Transposon-mediated BAC transgenesis in zebrafish. Nature protocols, 6, 1998-2021

164. Takeuchi Y, Yoshizaki G, Kobayashi T, Takeuchi T. (2002).Mass isolation of primordial germ cells from transgenic rainbow trout carrying the green fluorescent protein gene driven by the vasa gene promoter. Biology of reproduction, 67, 1087-92

165. Tawk M, Tuil D, Torrente Y, Vriz S, Paulin D. (2002).High-efficiency gene transfer into adult fish: A new tool to study fin regeneration. Genesis, 32, 27-31

166. Teh C, Chong SW, Korzh V. (2003).DNA delivery into anterior neural tube of zebrafish embryos by electroporation. Biotechniques, 35, 950-4

167. Thermes V, Grabher C, Ristoratore F, Bourrat F, Choulika A, Wittbrodt J, Joly J-S. (2002).I-SceI meganuclease mediates highly efficient transgenesis in fish. Mechanisms of development, 118, 91-98

168. Thomas-Jones E, Thorpe K, Harrison N, Thomas G, Morris C, Hutchinson T, Woodhead S, Tyler C. (2003).Dynamics of estrogen biomarker responses in rainbow trout exposed to 17beta-estradiol and 17alpha-ethinylestradiol. Environmental toxicology and chemistry / SETAC, 22, 3001-8

169. Thomas MK, Devon ON, Lee JH, Peter A, Schlosser DA, Tenser MS, Habener JF. (2001).Development of diabetes mellitus in aging transgenic mice following suppression of pancreatic homeoprotein IDX-1. The Journal of clinical investigation, 108, 319-29

170. Tong S-K, Mouriec K, Kuo M-W, Pellegrini E, Gueguen M-M, Brion F, Kah O, Chung B-c. (2009).A cyp19a1b-gfp (aromatase B) transgenic zebrafish line that expresses GFP in radial glial cells. Genesis, 47, 67-73

171. Trinh le A, Fraser SE. (2013).Enhancer and gene traps for molecular imaging and genetic analysis in zebrafish. Dev Growth Differ, 55, 434-45

172. Tsai HJ, Lai CH, Yang HS. (1997).Sperm as a carrier to introduce an exogenous DNA fragment into the oocyte of Japanese abalone (Haliotis divorsicolor suportexta). Transgenic research, 6, 85-95

173. Tucker NR, Ustyugov A, Bryantsev AL, Konkel ME, Shelden EA. (2009).Hsp27 is persistently expressed in zebrafish skeletal and cardiac muscle tissues but dispensable for their morphogenesis. Cell stress & chaperones, 14, 521-33

174. Udvadia AJ, Linney E. (2003).Windows into development: historic, current, and future perspectives on transgenic zebrafish. Developmental biology, 256, 1-17

175. Uzbekova S, Amoros C, Cauty C, Mambrini M, Perrot E, Hew CL, Chourrout D, Prunet P. (2003).Analysis of cell-specificity and variegation of transgene expression driven by salmon prolactin promoter in stable lines of transgenic rainbow trout. Transgenic research, 12, 213-27

176. Uzbekova S, Chyb J, Ferriere F, Bailhache T, Prunet P, Alestrom P, Breton B. (2000).Transgenic rainbow trout expressed sGnRH-antisense RNA under the control of sGnRH promoter of Atlantic salmon. Journal of molecular endocrinology, 25, 337-50

177. Vergara MN, Canto-Soler MV. (2012).Rediscovering the chick embryo as a model to study retinal development. Neural Development, 7, 22

178. Vooijs M, Jonkers J, Lyons S, Berns A. (2002).Noninvasive Imaging of Spontaneous Retinoblastoma Pathway-dependent Tumors in Mice. Cancer research, 62, 1862-67

179. Wang D, Jao LE, Zheng N, Dolan K, Ivey J, Zonies S, Wu X, Wu K, Yang H, Meng Q, Zhu Z, Zhang B, Lin S, Burgess SM. (2007).Efficient genome-wide mutagenesis of zebrafish genes by retroviral insertions. Proceedings of the National Academy of Sciences of the United States of America, 104, 12428-33

180. Wang R, Zhang P, Gong Z, Hew CL. (1995).Expression of the antifreeze protein gene in transgenic goldfish (Carassius auratus) and its implication in cold adaptation. Mol Mar Biol Biotechnol, 4, 20-6

181. Weger BD, Weger M, Nusser M, Brenner-Weiss G, Dickmeis T. (2012).A chemical screening system for glucocorticoid stress hormone signaling in an intact vertebrate. ACS chemical biology, 7, 1178-83

182. Westerfield M, Wegner J, Jegalian BG, DeRobertis EM, Puschel AW. (1992).Specific activation of mammalian Hox promoters in mosaic transgenic zebrafish. Genes & development, 6, 591-8

183. White RM, Sessa A, Burke C, Bowman T, LeBlanc J, Ceol C, Bourque C, Dovey M, Goessling W, Burns CE, Zon LI. (2008).Transparent adult zebrafish as a tool for in vivo transplantation analysis. Cell stem cell, 2, 183-9

184. Winkler C, Vielkind JR, Schartl M. (1991).Transient expression of foreign DNA during embryonic and larval development of the medaka fish (Oryzias latipes). Molecular & general genetics : MGG, 226, 129-40

185. Wu YL, Pan X, Mudumana SP, Wang H, Kee PW, Gong Z. (2008).Development of a heat shock inducible gfp transgenic zebrafish line by using the zebrafish hsp27 promoter. Gene, 408, 85-94

186. Xiao R, Anderson S, Aramini J, Belote R, Buchwald WA, Ciccosanti C, Conover K, Everett JK, Hamilton K, Huang YJ, Janjua H, Jiang M, Kornhaber GJ, Lee DY, Locke JY, Ma LC, Maglaqui M, Mao L, Mitra S, Patel D, Rossi P, Sahdev S, Sharma S, Shastry R, Swapna GV, Tong SN, Wang D, Wang H, Zhao L, Montelione GT, Acton TB. (2010).The high-throughput protein sample

production platform of the Northeast Structural Genomics Consortium. J Struct Biol, 172, 21-33

187. Xiong L, David L, Stevenson B, Zhu JK. (1999).High Throughput Screening of Signal Transduction Mutants With Luciferase Imaging. Plant Molecular Biology Reporter, 17, 159-70

188. Yoshizaki G, Takeuchi Y, Kobayashi T, Takeuchi T. (2003).Primordial germ cell: a novel tool for fish bioengineering. Fish Physiology and Biochemistry, 28, 453-57

189. Zeng Z, Shan T, Tong Y, Lam SH, Gong Z. (2005).Development of estrogen-responsive transgenic medaka for environmental monitoring of endocrine disrupters. Environmental science & technology, 39, 9001-8

190. Zu Y, Tong X, Wang Z, Liu D, Pan R, Li Z, Hu Y, Luo Z, Huang P, Wu Q, Zhu Z, Zhang B, Lin S. (2013).TALEN-mediated precise genome modification by homologous recombination in zebrafish. Nature methods, 10, 329-31

Chapter 8

References

1. Tsai, M. J.; O'Malley, B. W., Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* **1994,** *63*, 451-86.

2. Katzenellenbogen, B. S., Estrogen receptors: bioactivities and interactions with cell signaling pathways. *Biol Reprod* **1996,** *54*, (2), 287-93.

3. Gustafsson, J. A., What pharmacologists can learn from recent advances in estrogen signalling. *Trends Pharmacol Sci* **2003,** *24*, (9), 479-85.

4. Mahato, D.; Goulding, E. H.; Korach, K. S.; Eddy, E. M., Estrogen receptor-alpha is required by the supporting somatic cells for spermatogenesis. *Mol Cell Endocrinol* **2001,** *178*, (1-2), 57-63.

5. O'Donnell, L.; Robertson, K. M.; Jones, M. E.; Simpson, E. R., Estrogen and spermatogenesis. *Endocr Rev* **2001,** *22*, (3), 289-318.

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

7. Hao, R.; Bondesson, M.; Singh, A. V.; Riu, A.; McCollum, C. W.; Knudsen, T. B.; Gorelick, D. A.; Gustafsson, J. A., Identification of estrogen target genes during zebrafish embryonic development through transcriptomic analysis. *PLoS One* **2013,** *8*, (11), e79020.

8. Manolagas, S. C.; O'Brien, C. A.; Almeida, M., The role of estrogen and androgen receptors in bone health and disease. *Nat Rev Endocrinol* **2013,** *9*, (12), 699-712.

9. Yu, H. P.; Chaudry, I. H., The role of estrogen and receptor agonists in maintaining organ function after trauma-hemorrhage. *Shock* **2009,** *31*, (3), 227-37.

10. Khan, D.; Ansar Ahmed, S., The Immune System Is a Natural Target for Estrogen Action: Opposing Effects of Estrogen in Two Prototypical Autoimmune Diseases. *Front Immunol* **2015,** *6*, 635.

11. Knowlton, A. A.; Lee, A. R., Estrogen and the cardiovascular system. *Pharmacol Ther* **2012,** *135*, (1), 54-70.

12. Nilsson, S.; Makela, S.; Treuter, E.; Tujague, M.; Thomsen, J.; Andersson, G.; Enmark, E.; Pettersson, K.; Warner, M.; Gustafsson, J. A., Mechanisms of estrogen action. *Physiol Rev* **2001,** *81*, (4), 1535-65.

13. Rosenfeld, M. G.; Glass, C. K., Coregulator Codes of Transcriptional Regulation by Nuclear Receptors. **2001**.

14. Pettersson, K.; Gustafsson, J. A., Role of estrogen receptor beta in estrogen action. *Annu Rev Physiol* **2001,** *63*, 165-92.

15. Filby, A. L.; Tyler, C. R., Molecular characterization of estrogen receptors 1, 2a, and 2b and their tissue and ontogenic expression profiles in fathead minnow (*Pimephales promelas*). *Biol Reprod* **2005,** *73*, (4), 648-62.

16. Ma, C. H.; Dong, K. W.; Yu, K. L., cDNA cloning and expression of a novel estrogen receptor beta-subtype in goldfish (Carassius auratus). *Biochim Biophys Acta* **2000,** *1490*, (1-2), 145-52.

17. Socorro, S.; Power, D. M.; Olsson, P. E.; Canario, A. V., Two estrogen receptors expressed in the teleost fish, Sparus aurata: cDNA cloning, characterization and tissue distribution. *J Endocrinol* **2000,** *166*, (2), 293-306.

18. Brown, A. R.; Gunnarsson, L.; Kristiansson, E.; Tyler, C. R., Assessing variation in the potential susceptibility of fish to pharmaceuticals, considering evolutionary differences in their physiology and ecology. *Philos Trans R Soc Lond B Biol Sci* **2014,** *369*, (1656).

19. Chandrasekar, G.; Archer, A.; Gustafsson, J.-Å.; Lendahl, M. A., Levels of 17β-estradiol receptors expressed in embryonic and adult zebrafish following in vivo treatment of natural or synthetic ligands. *PloS one* **2010,** *5*, (3), e9678.

20. Tingaud-Sequeira, A.; Andre, M.; Forgue, J.; Barthe, C.; Babin, P. J., Expression patterns of three estrogen receptor genes during zebrafish (Danio rerio) development: evidence for high expression in neuromasts. *Gene Expr Patterns* **2004,** *4*, (5), 561-8.

21. Ascenzi, P.; Bocedi, A.; Marino, M., Structure-function relationship of estrogen receptor alpha and beta: impact on human health. *Mol Aspects Med* **2006,** *27*, (4), 299-402.

22. Koehler, K. F.; Helguero, L. A.; Haldosen, L. A.; Warner, M.; Gustafsson, J. A., Reflections on the discovery and significance of estrogen receptor beta. *Endocr Rev* **2005,** *26*, (3), 465-78.

23. O'Lone, R.; Frith, M. C.; Karlsson, E. K.; Hansen, U., Genomic targets of nuclear estrogen receptors. *Mol Endocrinol* **2004,** *18*, (8), 1859-75.

24. Gottlicher, M.; Heck, S.; Herrlich, P., Transcriptional cross-talk, the second mode of steroid hormone receptor action. *J Mol Med (Berl)* **1998,** *76*, (7), 480-9.

25. Giguere, V.; Yang, N.; Segui, P.; Evans, R. M., Identification of a new class of steroid hormone receptors. *Nature* **1988,** *331*, (6151), 91-4.

26. Giguere, V., To ERR in the estrogen pathway. *Trends Endocrinol Metab* **2002,** *13*, (5), 220-5.

27. Kraus, R. J.; Ariazi, E. A.; Farrell, M. L.; Mertz, J. E., Estrogen-related receptor alpha 1 actively antagonizes estrogen receptor-regulated transcription in MCF-7 mammary cells. *J Biol Chem* **2002,** *277*, (27), 24826-34.

28. Zhang, Y.; Ma, K.; Sadana, P.; Chowdhury, F.; Gaillard, S.; Wang, F.; McDonnell, D. P.; Unterman, T. G.; Elam, M. B.; Park, E. A., Estrogen-related receptors stimulate pyruvate dehydrogenase kinase isoform 4 gene expression. *J Biol Chem* **2006,** *281*, (52), 39897-906.

29. Razandi, M.; Pedram, A.; Greene, G. L.; Levin, E. R., Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. *Mol Endocrinol* **1999,** *13*, (2), 307-19.

30. Razandi, M.; Pedram, A.; Park, S. T.; Levin, E. R., Proximal events in signaling by plasma membrane estrogen receptors. *J Biol Chem* **2003,** *278*, (4), 2701-12.

31. Soltysik, K.; Czekaj, P., Membrane estrogen receptors - is it an alternative way of estrogen action? *J Physiol Pharmacol* **2013,** *64*, (2), 129-42.

32. Audy, M. C.; Vacher, P.; Duly, B., 17 beta-estradiol stimulates a rapid Ca2+ influx in LNCaP human prostate cancer cells. *Eur J Endocrinol* **1996,** *135*, (3), 367-73.

33. Lieberherr, M.; Grosse, B.; Kachkache, M.; Balsan, S., Cell signaling and estrogens in female rat osteoblasts: a possible involvement of unconventional nonnuclear receptors. *J Bone Miner Res* **1993,** *8*, (11), 1365-76.

34. Benten, W. P.; Stephan, C.; Lieberherr, M.; Wunderlich, F., Estradiol signaling via sequestrable surface receptors. *Endocrinology* **2001,** *142*, (4), 1669-77.

35. Chakraborty, T.; Katsu, Y.; Zhou, L. Y.; Miyagawa, S.; Nagahama, Y.; Iguchi, T., Estrogen receptors in medaka (Oryzias latipes) and estrogenic environmental contaminants: an in vitro-in vivo correlation. *J Steroid Biochem Mol Biol* **2011,** *123*, (3-5), 115-21.

36. Pinto, C.; Grimaldi, M.; Boulahtouf, A.; Pakdel, F.; Brion, F.; Ait-Aissa, S.; Cavailles, V.; Bourguet, W.; Gustafsson, J. A.; Bondesson, M.; Balaguer, P., Selectivity of natural, synthetic and environmental estrogens for zebrafish estrogen receptors. *Toxicol Appl Pharmacol* **2014,** *280*, (1), 60-9.

37. Routledge, E. J.; White, R.; Parker, M. G.; Sumpter, J. P., Differential effects of xenoestrogens on coactivator recruitment by estrogen receptor (ER) alpha and ERbeta. *J Biol Chem* **2000,** *275*, (46), 35986-93.

38. An, J.; Tzagarakis-Foster, C.; Scharschmidt, T. C.; Lomri, N.; Leitman, D. C., Estrogen receptor beta-selective transcriptional activity and recruitment of coregulators by phytoestrogens. *J Biol Chem* **2001,** *276*, (21), 17808-14.

39. Hall, J. M.; Korach, K. S., Analysis of the molecular mechanisms of human estrogen receptors alpha and beta reveals differential specificity in target promoter regulation by xenoestrogens. *J Biol Chem* **2002,** *277*, (46), 44455-61.

40. Bergman, Å.; Heindel, J.; Jobling, S.; Kidd, K.; Zoeller, R. T., State-of-the-science of endocrine disrupting chemicals, 2012. *Toxicol Lett* **2012,** *211*, S3.

41. Gore, A. C.; Chappell, V. A.; Fenton, S. E.; Flaws, J. A.; Nadal, A.; Prins, G. S.; Toppari, J.; Zoeller, R. T., EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. *Endocr Rev* **2015,** *36*, (6), E1-e150.

42. Diamanti-Kandarakis, E.; Bourguignon, J. P.; Giudice, L. C.; Hauser, R.; Prins, G. S.; Soto, A. M.; Zoeller, R. T.; Gore, A. C., Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocr Rev* **2009,** *30*, (4), 293-342.

43. Mills, L. J.; Chichester, C., Review of evidence: are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? *Sci Total Environ* **2005,** *343*, (1-3), 1-34.

44. Tyler, C. R.; Jobling, S.; Sumpter, J. P., Endocrine disruption in wildlife: a critical review of the evidence. *Crit Rev Toxicol* **1998,** *28*, (4), 319-61.

45. Singleton, D. W.; Khan, S. A., Xenoestrogen exposure and mechanisms of endocrine disruption. *Front Biosci* **2003,** *8*, s110-8.

46. Yin, G. G.; Kookana, R. S.; Ru, Y. J., Occurrence and fate of hormone steroids in the environment. *Environ Int* **2002,** *28*, (6), 545-51.

47. TEDX List of Potential Endocrine Disruptors. http://endocrinedisruption.org/endocrine-disruption/tedx-list-of-potential-endocrine-disruptors/overview (accessed Jan 08, 2016),

48. Brion, F.; Le Page, Y.; Piccini, B.; Cardoso, O.; Tong, S. K.; Chung, B. C.; Kah, O., Screening estrogenic activities of chemicals or mixtures in vivo using transgenic (cyp19a1b-GFP) zebrafish embryos. *PLoS One* **2012,** *7*, (5), e36069.

49. Lee, O.; Takesono, A.; Tada, M.; Tyler, C. R.; Kudoh, T., Biosensor zebrafish provide new insights into potential health effects of environmental estrogens. *Environmental health perspectives* **2012,** *120*, (7), 990-6.

50. Gorelick, D. A.; Halpern, M. E., Visualization of estrogen receptor transcriptional activation in zebrafish. *Endocrinology* **2011,** *152*, (7), 2690-703.

51. Chen, H.; Hu, J.; Yang, J.; Wang, Y.; Xu, H.; Jiang, Q.; Gong, Y.; Gu, Y.; Song, H., Generation of a fluorescent transgenic zebrafish for detection of environmental estrogens. *Aquat Toxicol* **2010,** *96*, (1), 53-61.

52. Lange, A.; Paull, G. C.; Coe, T. S.; Katsu, Y.; Urushitani, H.; Iguchi, T.; Tyler, C. R., Sexual reprogramming and estrogenic sensitization in wild fish exposed to ethinylestradiol. *Environ Sci Technol* **2009,** *43*, (4), 1219-25.

53. Xu, H.; Yang, J.; Wang, Y.; Jiang, Q.; Chen, H.; Song, H., Exposure to 17alpha-ethynylestradiol impairs reproductive functions of both male and female zebrafish (Danio rerio). *Aquat Toxicol* **2008,** *88*, (1), 1-8.

54. Van den Belt, K.; Berckmans, P.; Vangenechten, C.; Verheyen, R.; Witters, H., Comparative study on the in vitro/in vivo estrogenic potencies of 17beta-estradiol, estrone, 17alpha-ethynylestradiol and nonylphenol. *Aquat Toxicol* **2004,** *66*, (2), 183-95.

55. Jobling, S.; Beresford, N.; Nolan, M.; Rodgers-Gray, T.; Brighty, G. C.; Sumpter, J. P.; Tyler, C. R., Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. *Biol Reprod* **2002,** *66*, (2), 272-81.

56. Kidd, K. A.; Blanchfield, P. J.; Mills, K. H.; Palace, V. P.; Evans, R. E.; Lazorchak, J. M.; Flick, R. W., Collapse of a fish population after exposure to a synthetic estrogen. *Proc Natl Acad Sci U S A* **2007,** *104*, (21), 8897-901.

57. Jobling, S.; Coey, S.; Whitmore, J. G.; Kime, D. E.; Van Look, K. J.; McAllister, B. G.; Beresford, N.; Henshaw, A. C.; Brighty, G.; Tyler, C. R.; Sumpter, J. P., Wild intersex roach (Rutilus rutilus) have reduced fertility. *Biol Reprod* **2002,** *67*, (2), 515-24.

58. Jobling, S.; Tyler, C. R., Endocrine disruption, parasites and pollutants in wild freshwater fish. *Parasitology* **2003,** *126 Suppl*, S103-8.

59. Kannan, K.; Senthilkumar, K.; Elliott, J. E.; Feyk, L. A.; Giesy, J. P., Occurrence of butyltin compounds in tissues of water birds and seaducks from the united states and canada. *Arch Environ Contam Toxicol* **1998,** *35*, (1), 64-9.

60. Sheffield, S. R.; Matter, J. M.; Rattner, B.; Guiney, P. D., Fish and wildlife species as sentinels of environmental endocrine disruption. In *Principles and Processes for Evaluating Endocrine Disruption in Wildlife*, SETAC Press: 1998, pp369-430

61. Stephens, T. D.; Bunde, C. J.; Fillmore, B. J., Mechanism of action in thalidomide teratogenesis. *Biochem Pharmacol* **2000,** *59*, (12), 1489-99.

62. Fry, D. M., Reproductive effects in birds exposed to pesticides and industrial chemicals. *Environ Health Perspect* **1995,** *103 Suppl 7*, 165-71.

63. Facemire, C. F.; Gross, T. S.; Guillette, L. J., Jr., Reproductive impairment in the Florida panther: nature or nurture? *Environ Health Perspect* **1995,** *103 Suppl 4*, 79-86.

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

65. Crisp, T. M.; Clegg, E. D.; Cooper, R. L.; Anderson, D. G., Special report on environmental endocrine disruption: An effects assessment and analysis. *Washington DC: US Environmental Protection Agency* **1997**.

66. Chia, V. M.; Li, Y.; Quraishi, S. M.; Graubard, B. I.; Figueroa, J. D.; Weber, J. P.; Chanock, S. J.; Rubertone, M. V.; Erickson, R. L.; McGlynn, K. A., Effect modification of endocrine disruptors and testicular germ cell tumour risk by hormone-metabolizing genes. *International journal of andrology* **2010,** *33*, (4), 588-96.

67. Doherty, L. F.; Bromer, J. G.; Zhou, Y.; Aldad, T. S.; Taylor, H. S., In utero exposure to diethylstilbestrol (DES) or bisphenol-A (BPA) increases EZH2

expression in the mammary gland: an epigenetic mechanism linking endocrine disruptors to breast cancer. *Horm Cancer* **2010,** *1*, (3), 146-55.

68. Li, D. K.; Zhou, Z.; Miao, M.; He, Y.; Wang, J.; Ferber, J.; Herrinton, L. J.; Gao, E.; Yuan, W., Urine bisphenol-A (BPA) level in relation to semen quality. *Fertil Steril* **2011,** *95*, (2), 625-30 e1-4.

69. Melzer, D.; Rice, N. E.; Lewis, C.; Henley, W. E.; Galloway, T. S., Association of urinary bisphenol a concentration with heart disease: evidence from NHANES 2003/06. *PLoS One* **2010,** *5*, (1), e8673.

70. Giusti, R. M.; Iwamoto, K.; Hatch, E. E., Diethylstilbestrol revisited: a review of the long-term health effects. *Ann Intern Med* **1995,** *122*, (10), 778-88.

71. Colborn, T.; vom Saal, F. S.; Soto, A. M., Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ Health Perspect* **1993,** *101*, (5), 378-84.

72. Kerdivel, G.; Habauzit, D.; Pakdel, F., Assessment and molecular actions of endocrine-disrupting chemicals that interfere with estrogen receptor pathways. *Int J Endocrinol* **2013,** *2013*, 501851.

73. Markey, C. M.; Luque, E. H.; Munoz De Toro, M.; Sonnenschein, C.; Soto, A. M., In utero exposure to bisphenol A alters the development and tissue organization of the mouse mammary gland. *Biol Reprod* **2001,** *65*, (4), 1215-23.

74. Kato, H.; Ota, T.; Furuhashi, T.; Ohta, Y.; Iguchi, T., Changes in reproductive organs of female rats treated with bisphenol A during the neonatal period. *Reprod Toxicol* **2003,** *17*, (3), 283-8.

75. Andersen, L.; Holbech, H.; Gessbo, A.; Norrgren, L.; Petersen, G. I., Effects of exposure to 17alpha-ethinylestradiol during early development on sexual differentiation and induction of vitellogenin in zebrafish (Danio rerio). *Comp Biochem Physiol C Toxicol Pharmacol* **2003,** *134*, (3), 365-74.

76. Soto, A. M.; Brisken, C.; Schaeberle, C.; Sonnenschein, C., Does cancer start in the womb? altered mammary gland development and predisposition to breast cancer due to in utero exposure to endocrine disruptors. *J Mammary Gland Biol Neoplasia* **2013,** *18*, (2), 199-208.

77. Tong, S.-K.; Mouriec, K.; Kuo, M.-W.; Pellegrini, E.; Gueguen, M.-M.; Brion, F.; Kah, O.; Chung, B.-c., A cyp19a1b-gfp (aromatase B) transgenic zebrafish line that expresses GFP in radial glial cells. *Genesis* **2009,** *47*, (2), 67-73.

78. Sassi-Messai, S.; Gibert, Y.; Bernard, L.; Nishio, S.; Ferri Lagneau, K. F.; Molina, J.; Andersson-Lendahl, M.; Benoit, G.; Balaguer, P.; Laudet, V., The phytoestrogen genistein affects zebrafish development through two different pathways. *PLoS One* **2009,** *4*, (3), e4935.

79. Vethaak, A. D.; Lahr, J.; Schrap, S. M.; Belfroid, A. C.; Rijs, G. B.; Gerritsen, A.; de Boer, J.; Bulder, A. S.; Grinwis, G. C.; Kuiper, R. V.; Legler, J.; Murk, T. A.; Peijnenburg, W.; Verhaar, H. J.; de Voogt, P., An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands. *Chemosphere* **2005,** *59*, (4), 511-24.

80. Aris, A. Z.; Shamsuddin, A. S.; Praveena, S. M., Occurrence of 17alpha-ethynylestradiol (EE2) in the environment and effect on exposed biota: a review. *Environ Int* **2014,** *69*, 104-19.

81. Bertin, A.; Inostroza, P. A.; Quinones, R. A., A theoretical estimation of the concentration of steroid estrogens in effluents released from municipal sewage treatment plants into aquatic ecosystems of central-southern Chile. *Sci Total Environ* **2009,** *407*, (17), 4965-71.

82. Hinteman, T.; Schneider, C.; Scholer, H. F.; Schneider, R. J., Field study using two immunoassays for the determination of estradiol and ethinylestradiol in the aquatic environment. *Water Res* **2006,** *40*, (12), 2287-94.

83. Atkinson, S. K.; Marlatt, V. L.; Kimpe, L. E.; Lean, D. R.; Trudeau, V. L.; Blais, J. M., The occurrence of steroidal estrogens in south-eastern Ontario wastewater treatment plants. *Sci Total Environ* **2012,** *430*, 119-25.

84. Beck, I. C.; Bruhn, R.; Gandrass, J., Analysis of estrogenic activity in coastal surface waters of the Baltic Sea using the yeast estrogen screen. *Chemosphere* **2006,** *63*, (11), 1870-8.

85. Nimrod, A. C.; Benson, W. H., Xenobiotic interaction with and alteration of channel catfish estrogen receptor. *Toxicol Appl Pharmacol* **1997,** *147*, (2), 381-90.

86. Guengerich, F. P., Metabolism of 17 alpha-ethynylestradiol in humans. *Life Sci* **1990,** *47*, (22), 1981-8.

87. Cosnefroy, A.; Brion, F.; Maillot-Marechal, E.; Porcher, J. M.; Pakdel, F.; Balaguer, P.; Ait-Aissa, S., Selective activation of zebrafish estrogen receptor subtypes by chemicals by using stable reporter gene assay developed in a zebrafish liver cell line. *Toxicol Sci* **2012,** *125*, (2), 439-49.

88. Escande, A.; Pillon, A.; Servant, N.; Cravedi, J. P.; Larrea, F.; Muhn, P.; Nicolas, J. C.; Cavailles, V.; Balaguer, P., Evaluation of ligand selectivity using reporter cell lines stably expressing estrogen receptor alpha or beta. *Biochem Pharmacol* **2006,** *71*, (10), 1459-69.

89. Rogers, J. A.; Metz, L.; Yong, V. W., Review: Endocrine disrupting chemicals and immune responses: a focus on bisphenol-A and its potential mechanisms. *Mol Immunol* **2013,** *53*, (4), 421-30.

90. Klecka, G. M.; Gonsior, S. J.; West, R. J.; Goodwin, P. A.; Markham, D. A., Biodegradation of bisphenol A in aquatic environments: river die-away. *Environ Toxicol Chem* **2001,** *20*, (12), 2725-35.

91. Yamaguchi, A.; Ishibashi, H.; Kohra, S.; Arizono, K.; Tominaga, N., Short-term effects of endocrine-disrupting chemicals on the expression of estrogen-responsive genes in male medaka (Oryzias latipes). *Aquat Toxicol* **2005,** *72*, (3), 239-49.

92. Gorelick, D. A.; Iwanowicz, L. R.; Hung, A. L.; Blazer, V. S.; Halpern, M. E., Transgenic Zebrafish Reveal Tissue-Specific Differences in Estrogen Signaling in Response to Environmental Water Samples. *Environmental health perspectives* **2014**.

93. Soares, A.; Guieysse, B.; Jefferson, B.; Cartmell, E.; Lester, J. N., Nonylphenol in the environment: a critical review on occurrence, fate, toxicity and treatment in wastewaters. *Environ Int* **2008,** *34*, (7), 1033-49.

94. Preuss, T. G.; Gehrhardt, J.; Schirmer, K.; Coors, A.; Rubach, M.; Russ, A.; Jones, P. D.; Giesy, J. P.; Ratte, H. T., Nonylphenol isomers differ in estrogenic activity. *Environ Sci Technol* **2006,** *40*, (16), 5147-53.

95. Cederroth, C. R.; Zimmermann, C.; Nef, S., Soy, phytoestrogens and their impact on reproductive health. *Molecular and cellular endocrinology* **2012,** *355*, (2), 192-200.

96. Ganai, A. A.; Farooqi, H., Bioactivity of genistein: A review of in vitro and in vivo studies. *Biomed Pharmacother* **2015,** *76*, 30-8.

97. Behloul, N.; Wu, G., Genistein: a promising therapeutic agent for obesity and diabetes treatment. *Eur J Pharmacol* **2013,** *698*, (1-3), 31-8.

98. Russo, M.; Russo, G. L.; Daglia, M.; Kasi, P. D.; Ravi, S.; Nabavi, S. F.; Nabavi, S. M., Understanding genistein in cancer: The "good" and the "bad" effects: A review. *Food Chem* **2016,** *196*, 589-600.

99. Liu, Z. H.; Kanjo, Y.; Mizutani, S., A review of phytoestrogens: their occurrence and fate in the environment. *Water Res* **2010,** *44*, (2), 567-77.

100. Jarosova, B.; Javurek, J.; Adamovsky, O.; Hilscherova, K., Phytoestrogens and mycoestrogens in surface waters--Their sources, occurrence, and potential contribution to estrogenic activity. *Environ Int* **2015,** *81*, 26-44.

101. OECD Work Related to Endocrine Disrupters. http://www.oecd.org/env/ehs/testing/oecdworkrelatedtoendocrinedisrupters.htm (accessed Jan 11, 2016),

102. US EPA, O., OSCP Endocrine Disruption Screening Program. http://www.epa.gov/endocrine-disruption (accessed Jan 11, 2016),

103. MOE Endocrine Disrupting Effects of Substances [MOE]. http://www.env.go.jp/en/chemi/ed.html (accessed Jan 11, 2016),

104. Isenhower, W. D., Jr.; Newbold, R. R.; Cefalo, R. C.; Korach, K. S.; McLachlan, J. A., Absence of estrogenic activity in some drugs commonly used during pregnancy. *Biol Res Pregnancy Perinatol* **1986,** *7*, (1), 6-10.

105. Padilla-Banks, E.; Jefferson, W. N.; Newbold, R. R., The immature mouse is a suitable model for detection of estrogenicity in the uterotropic bioassay. *Environ Health Perspect* **2001,** *109*, (8), 821-6.

106. Kanno, J.; Onyon, L.; Haseman, J.; Fenner-Crisp, P.; Ashby, J.; Owens, W., The OECD program to validate the rat uterotrophic bioassay to screen compounds for in vivo estrogenic responses: phase 1. *Environ Health Perspect* **2001,** *109*, (8), 785-94.

107. Soto, A. M.; Maffini, M. V.; Schaeberle, C. M.; Sonnenschein, C., Strengths and weaknesses of in vitro assays for estrogenic and androgenic activity. *Best Pract Res Clin Endocrinol Metab* **2006,** *20*, (1), 15-33.

108. Lange, A.; Katsu, Y.; Miyagawa, S.; Ogino, Y.; Urushitani, H.; Kobayashi, T.; Hirai, T.; Shears, J. A.; Nagae, M.; Yamamoto, J.; Ohnishi, Y.; Oka, T.; Tatarazako, N.; Ohta, Y.; Tyler, C. R.; Iguchi, T., Comparative responsiveness to natural and synthetic estrogens of fish species commonly used in the laboratory and field monitoring. *Aquat Toxicol* **2012,** *109*, 250-8.

109. Miyagawa, S.; Lange, A.; Hirakawa, I.; Tohyama, S.; Ogino, Y.; Mizutani, T.; Kagami, Y.; Kusano, T.; Ihara, M.; Tanaka, H.; Tatarazako, N.; Ohta, Y.; Katsu, Y.; Tyler, C. R.; Iguchi, T., Differing species responsiveness of estrogenic contaminants in fish is conferred by the ligand binding domain of the estrogen receptor. *Environ Sci Technol* **2014,** *48*, (9), 5254-63.

110. Soto, A. M.; Sonnenschein, C.; Chung, K. L.; Fernandez, M. F.; Olea, N.; Serrano, F. O., The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ Health Perspect* **1995,** *103 Suppl 7*, 113-22.

111. Arnold, S. F.; Klotz, D. M.; Collins, B. M.; Vonier, P. M.; Jr., L. J. G.; McLachlan, J. A., Synergistic Activation of Estrogen Receptor with Combinations of Environmental Chemicals. **1996**.

112. Wilson, V. S.; Bobseine, K.; Gray, L. E., Jr., Development and characterization of a cell line that stably expresses an estrogen-responsive luciferase reporter for the detection of estrogen receptor agonist and antagonists. *Toxicol Sci* **2004,** *81*, (1), 69-77.

113. Dobbins, L. L.; Brain, R. A.; Brooks, B. W., Comparison of the sensitivities of common in vitro and in vivo assays of estrogenic activity:

application of chemical toxicity distributions. *Environ Toxicol Chem* **2008,** *27*, (12), 2608-16.

114. Dreier, D. A.; Connors, K. A.; Brooks, B. W., Comparative endpoint sensitivity of in vitro estrogen agonist assays. *Regul Toxicol Pharmacol* **2015,** *72*, (2), 185-93.

115. Folmar, L. C.; Hemmer, M.; Denslow, N. D.; Kroll, K.; Chen, J.; Cheek, A.; Richman, H.; Meredith, H.; Grau, E. G., A comparison of the estrogenic potencies of estradiol, ethynylestradiol, diethylstilbestrol, nonylphenol and methoxychlor in vivo and in vitro. *Aquat Toxicol* **2002,** *60*, (1-2), 101-10.

116. Arnold, S. F.; Klotz, D. M.; Collins, B. M.; Vonier, P. M.; Guillette, L. J., Jr.; McLachlan, J. A., Synergistic activation of estrogen receptor with combinations of environmental chemicals. *Science* **1996,** *272*, (5267), 1489-92.

117. Silva, E.; Rajapakse, N.; Kortenkamp, A., Something from "nothing"--eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. *Environ Sci Technol* **2002,** *36*, (8), 1751-6.

118. Kortenkamp, A., Low dose mixture effects of endocrine disrupters and their implications for regulatory thresholds in chemical risk assessment. *Curr Opin Pharmacol* **2014,** *19*, 105-11.

119. Christiansen, S.; Kortenkamp, A.; Axelstad, M.; Boberg, J.; Scholze, M.; Jacobsen, P. R.; Faust, M.; Lichtensteiger, W.; Schlumpf, M.; Burdorf, A.; Hass, U., Mixtures of endocrine disrupting contaminants modelled on human high end exposures: an exploratory study in rats. *Int J Androl* **2012,** *35*, (3), 303-16.

120. Mandrup, K. R.; Johansson, H. K.; Boberg, J.; Pedersen, A. S.; Mortensen, M. S.; Jorgensen, J. S.; Vinggaard, A. M.; Hass, U., Mixtures of environmentally relevant endocrine disrupting chemicals affect mammary gland development in female and male rats. *Reprod Toxicol* **2015,** *54*, 47-57.

121. Evans, R. M.; Scholze, M.; Kortenkamp, A., Additive mixture effects of estrogenic chemicals in human cell-based assays can be influenced by inclusion of chemicals with differing effect profiles. *PLoS One* **2012,** *7*, (8), e43606.

122. Donovan, D. M.; Kerr, D. E.; Wall, R. J., Engineering disease resistant cattle. *Transgenic research* **2005,** *14*, (5), 563-7.

123. Fan, J.; Watanabe, T., Transgenic rabbits as therapeutic protein bioreactors and human disease models. *Pharmacol Ther* **2003,** *99*, (3), 261-82.

124. Hamra, F. K.; Gatlin, J.; Chapman, K. M.; Grellhesl, D. M.; Garcia, J. V.; Hammer, R. E.; Garbers, D. L., Production of transgenic rats by lentiviral transduction of male germ-line stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **2002,** *99*, (23), 14931-6.

125. Hong, S. G.; Kim, M. K.; Jang, G.; Oh, H. J.; Park, J. E.; Kang, J. T.; Koo, O. J.; Kim, T.; Kwon, M. S.; Koo, B. C.; Ra, J. C.; Kim, D. Y.; Ko, C.; Lee, B. C., Generation of red fluorescent protein transgenic dogs. *Genesis* **2009,** *47*, (5), 314-22.

126. Huss, D.; Poynter, G.; Lansford, R., Japanese quail (Coturnix japonica) as a laboratory animal model. *Lab Anim (NY)* **2008,** *37*, (11), 513-9.

127. Mozdziak, P.; Petitte, J., Status of transgenic chicken models for developmental biology. *Developmental dynamics : an official publication of the American Association of Anatomists* **2004,** *229*, 414 - 421.

128. Sinzelle, L.; Vallin, J.; Coen, L.; Chesneau, A.; Du Pasquier, D.; Pollet, N.; Demeneix, B.; Mazabraud, A., Generation of trangenic Xenopus laevis using the Sleeping Beauty transposon system. *Transgenic research* **2006,** *15*, (6), 751-60.

129. Wang, R.; Zhang, P.; Gong, Z.; Hew, C. L., Expression of the antifreeze protein gene in transgenic goldfish (Carassius auratus) and its implication in cold adaptation. *Mol Mar Biol Biotechnol* **1995,** *4*, (1), 20-6.

130. Hrytsenko, O.; Pohajdak, B.; Wright, J. R., Jr., Production of transgenic tilapia homozygous for a humanized insulin gene. *Transgenic research* **2010,** *19*, (2), 305-6.

131. Tsai, H. J.; Lai, C. H.; Yang, H. S., Sperm as a carrier to introduce an exogenous DNA fragment into the oocyte of Japanese abalone (Haliotis divorsicolor suportexta). *Transgenic research* **1997,** *6*, (1), 85-95.

132. Bader, M.; Bohnemeier, H.; Zollmann, F. S.; Lockley-Jones, O. E.; Ganten, D., Transgenic animals in cardiovascular disease research. *Experimental physiology* **2000,** *85*, (6), 713-31.

133. Hansson, O.; Petersen, A.; Leist, M.; Nicotera, P.; Castilho, R. F.; Brundin, P., Transgenic mice expressing a Huntington's disease mutation are resistant to quinolinic acid-induced striatal excitotoxicity. *Proceedings of the National Academy of Sciences of the United States of America* **1999,** *96*, (15), 8727-32.

134. Houdebine, L. M., The production of pharmaceutical proteins from the milk of transgenic animals. *Reproduction, nutrition, development* **1995,** *35*, (6), 609-17.

135. Thomas, M. K.; Devon, O. N.; Lee, J. H.; Peter, A.; Schlosser, D. A.; Tenser, M. S.; Habener, J. F., Development of diabetes mellitus in aging transgenic mice following suppression of pancreatic homeoprotein IDX-1. *The Journal of clinical investigation* **2001,** *108*, (2), 319-29.

136. Reventos, J.; Munell, F., Transgenic animal models in reproductive endocrine research. *European journal of endocrinology / European Federation of Endocrine Societies* **1997,** *136*, (6), 566-80.

137. Dunham, R. A., Transgenic fish resistant to infectious diseases, their risk and prevention of escape into the environment and future candidate genes for disease transgene manipulation. *Comparative immunology, microbiology and infectious diseases* **2009,** *32*, (2), 139-61.

138. Nguyen, A. T.; Emelyanov, A.; Koh, C. H.; Spitsbergen, J. M.; Parinov, S.; Gong, Z., An inducible kras(V12) transgenic zebrafish model for liver tumorigenesis and chemical drug screening. *Disease models & mechanisms* **2012,** *5*, (1), 63-72.

139. Moro, E.; Ozhan-Kizil, G.; Mongera, A.; Beis, D.; Wierzbicki, C.; Young, R. M.; Bournele, D.; Domenichini, A.; Valdivia, L. E.; Lum, L.; Chen, C.; Amatruda, J. F.; Tiso, N.; Weidinger, G.; Argenton, F., In vivo Wnt signaling tracing through a transgenic biosensor fish reveals novel activity domains. *Developmental biology* **2012,** *366*, (2), 327-340.

140. Legler, J.; Broekhof, J. L. M.; Brouwer, A.; Lanser, P. H.; Murk, A. J.; Van Der Saag, P. T.; Dick Vethaak, A.; Wester, P.; Zivkovic, D.; Van Der Burg, B., A novel in vivo bioassay for (xeno-)estrogens using transgenic zebrafish. *Environmental Science and Technology* **2000,** *34*, (20), 4439-4444.

141. Bogers, R.; Mutsaerds, E.; Druke, J.; De Roode, D. F.; Murk, A. J.; Van Der Burg, B.; Legler, J., Estrogenic endpoints in fish early life-stage tests: luciferase and vitellogenin induction in estrogen-responsive transgenic zebrafish. *Environmental toxicology and chemistry / SETAC* **2006,** *25*, (1), 241-7.

142. Blechinger, S. R.; Warren, J. T., Jr.; Kuwada, J. Y.; Krone, P. H., Developmental toxicology of cadmium in living embryos of a stable transgenic zebrafish line. *Environmental health perspectives* **2002,** *110*, (10), 1041-6.

143. White, R. M.; Sessa, A.; Burke, C.; Bowman, T.; LeBlanc, J.; Ceol, C.; Bourque, C.; Dovey, M.; Goessling, W.; Burns, C. E.; Zon, L. I., Transparent adult zebrafish as a tool for in vivo transplantation analysis. *Cell stem cell* **2008,** *2*, (2), 183-9.

144. Feitsma, H.; Cuppen, E., Zebrafish as a cancer model. *Molecular cancer research : MCR* **2008,** *6*, (5), 685-94.

145. Blader, P.; Strahle, U., Zebrafish developmental genetics and central nervous system development. *Human molecular genetics* **2000,** *9*, (6), 945-51.

146. Briggs, J. P., The zebrafish: a new model organism for integrative physiology. *American journal of physiology. Regulatory, integrative and comparative physiology* **2002,** *282*, (1), R3-9.

147. Kelly, G. M.; Erezyilmaz, D. F.; Moon, R. T., Induction of a secondary embryonic axis in zebrafish occurs following the overexpression of beta-catenin. *Mechanisms of development* **1995,** *53*, (2), 261-73.

148. Kimmel, C. B.; Warga, R. M., Indeterminate cell lineage of the zebrafish embryo. *Developmental biology* **1987,** *124*, (1), 269-80.

149. Bretaud, S.; Lee, S.; Guo, S., Sensitivity of zebrafish to environmental toxins implicated in Parkinson's disease. *Neurotoxicology and teratology* **2004,** *26*, (6), 857-64.

150. Lieschke, G. J.; Currie, P. D., Animal models of human disease: zebrafish swim into view. *Nature reviews. Genetics* **2007,** *8*, (5), 353-67.

151. Martin, P.; Feng, Y., Inflammation: Wound healing in zebrafish. *Nature* **2009,** *459*, (7249), 921-3.

152. Burgess, H. A.; Granato, M., The neurogenetic frontier--lessons from misbehaving zebrafish. *Briefings in functional genomics & proteomics* **2008,** *7*, (6), 474-82.

153. Nagel, R., DarT: The embryo test with the Zebrafish Danio rerio--a general model in ecotoxicology and toxicology. *Altex* **2002,** *19 Suppl 1*, 38-48.

154. Hill, A. J.; Teraoka, H.; Heideman, W.; Peterson, R. E., Zebrafish as a Model Vertebrate for Investigating Chemical Toxicity. *Toxicological Sciences* **2005,** *86*, (1), 6-19.

155. Puiseux – Dao, S.; Edery, M., The Medaka Fish: An Experimental Model In Environmental Toxicology Its Use For The Survey Of Microalgal Toxins: Phycotoxins And Cyanotoxins. In *Ecotoxicology, Ecological Risk Assessment and Multiple Stressors*, Arapis, G.; Goncharova, N.; Baveye, P., Eds. Springer Netherlands: 2006; Vol. 6, pp 227-241.

156. Liu, Y., Ligation independent cloning: an efficient tool for high-throughput cloning and modular cDNA assembly. . *OA Biotechnology* **2013,** *2*, (1), 8.

157. Marsischky, G.; LaBaer, J., Many paths to many clones: a comparative look at high-throughput cloning methods. *Genome Res* **2004,** *14*, (10B), 2020-8.

158. Xiao, R.; Anderson, S.; Aramini, J.; Belote, R.; Buchwald, W. A.; Ciccosanti, C.; Conover, K.; Everett, J. K.; Hamilton, K.; Huang, Y. J.; Janjua, H.; Jiang, M.; Kornhaber, G. J.; Lee, D. Y.; Locke, J. Y.; Ma, L. C.; Maglaqui, M.; Mao, L.; Mitra, S.; Patel, D.; Rossi, P.; Sahdev, S.; Sharma, S.; Shastry, R.; Swapna, G. V.; Tong, S. N.; Wang, D.; Wang, H.; Zhao, L.; Montelione, G. T.; Acton, T. B., The high-throughput protein sample production platform of the Northeast Structural Genomics Consortium. *J Struct Biol* **2010,** *172*, (1), 21-33.

159. Horvat, S.; Medrano, J. F.; Behboodi, E.; Anderson, G. B.; Murray, J. D., Sexing and detection of gene construct in microinjected bovine blastocysts using the polymerase chain reaction. *Transgenic research* **1993,** *2*, (3), 134-40.

160. Alam, J.; Cook, J. L., Reporter genes: application to the study of mammalian gene transcription. *Analytical biochemistry* **1990,** *188*, (2), 245-54.

161. Culp, P.; Nusslein-Volhard, C.; Hopkins, N., High-frequency germ-line transmission of plasmid DNA sequences injected into fertilized zebrafish eggs. *Proc Natl Acad Sci U S A* **1991,** *88*, (18), 7953-7.

162. Razak, S. A.; Hwang, G. L.; Rahman, M. A.; Maclean, N., Growth Performance and Gonadal Development of Growth Enhanced Transgenic Tilapia Oreochromis niloticus (L.) Following Heat-Shock-Induced Triploidy. *Mar Biotechnol (NY)* **1999,** *1*, (6), 533-544.

163. Uzbekova, S.; Amoros, C.; Cauty, C.; Mambrini, M.; Perrot, E.; Hew, C. L.; Chourrout, D.; Prunet, P., Analysis of cell-specificity and variegation of transgene expression driven by salmon prolactin promoter in stable lines of transgenic rainbow trout. *Transgenic research* **2003,** *12*, (2), 213-27.

164. Dunham, R. A.; Chitmanat, C.; Nichols, A.; Argue, B.; Powers, D. A.; Chen, T. T., Predator Avoidance of Transgenic Channel Catfish Containing Salmonid Growth Hormone Genes. *Mar Biotechnol (NY)* **1999,** *1*, (6), 545-551.

165. Ando, R.; Hama, H.; Yamamoto-Hino, M.; Mizuno, H.; Miyawaki, A., An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proc Natl Acad Sci U S A* **2002,** *99*, (20), 12651-6.

166. Leclerc, G. M.; Boockfor, F. R.; Faught, W. J.; Frawley, L. S., Development of a destabilized firefly luciferase enzyme for measurement of gene expression. *Biotechniques* **2000,** *29*, 590-598.

167. Nagai, T.; Ibata, K.; Park, E. S.; Kubota, M.; Mikoshiba, K.; Miyawaki, A., A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nature biotechnology* **2002,** *20*, (1), 87-90.

168. Contag, C. H.; Bachmann, M. H., Advances in in vivo bioluminescence imaging of gene expression. *Annual review of biomedical engineering* **2002,** *4*, 235-60.

169. Goodman, S. D.; Gao, Q., Firefly Luciferase as a Reporter to Study Gene Expression in Streptococcus mutans. *Plasmid* **1999,** *42*, (2), 154-157.

170. Vooijs, M.; Jonkers, J.; Lyons, S.; Berns, A., Noninvasive Imaging of Spontaneous Retinoblastoma Pathway-dependent Tumors in Mice. *Cancer research* **2002,** *62*, (6), 1862-1867.

171. Xiong, L.; David, L.; Stevenson, B.; Zhu, J. K., High Throughput Screening of Signal Transduction Mutants With Luciferase Imaging. *Plant Molecular Biology Reporter* **1999,** *17*, (2), 159-170.

172. Weger, B. D.; Weger, M.; Nusser, M.; Brenner-Weiss, G.; Dickmeis, T., A chemical screening system for glucocorticoid stress hormone signaling in an intact vertebrate. *ACS chemical biology* **2012,** *7*, (7), 1178-83.

173. Chen, C. H.; Durand, E.; Wang, J.; Zon, L. I.; Poss, K. D., zebraflash transgenic lines for in vivo bioluminescence imaging of stem cells and regeneration in adult zebrafish. *Development* **2013,** *140*, (24), 4988-97.

174. Amsterdam, A.; Lin, S.; Moss, L. G.; Hopkins, N., Requirements for green fluorescent protein detection in transgenic zebrafish embryos. *Gene* **1996,** *173*, (1 Spec No), 99-103.

175. Driever, W.; Solnica-Krezel, L.; Schier, A. F.; Neuhauss, S. C.; Malicki, J.; Stemple, D. L.; Stainier, D. Y.; Zwartkruis, F.; Abdelilah, S.; Rangini, Z.;

Belak, J.; Boggs, C., A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* **1996,** *123*, 37-46.

176. Haffter, P.; Granato, M.; Brand, M.; Mullins, M. C.; Hammerschmidt, M.; Kane, D. A.; Odenthal, J.; van Eeden, F. J.; Jiang, Y. J.; Heisenberg, C. P.; Kelsh, R. N.; Furutani-Seiki, M.; Vogelsang, E.; Beuchle, D.; Schach, U.; Fabian, C.; Nusslein-Volhard, C., The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. *Development* **1996,** *123*, 1-36.

177. Gong, Z.; Ju, B.; Wan, H., Green fluorescent protein (GFP) transgenic fish and their applications. *Genetica* **2001,** *111*, (1-3), 213-25.

178. Udvadia, A. J.; Linney, E., Windows into development: historic, current, and future perspectives on transgenic zebrafish. *Developmental biology* **2003,** *256*, (1), 1-17.

179. Dietrich, C.; Maiss, E., Red fluorescent protein DsRed from Discosoma sp. as a reporter protein in higher plants. *Biotechniques* **2002,** *32*, (2), 286, 288-90, 292-3.

180. Shaner, N. C.; Steinbach, P. A.; Tsien, R. Y., A guide to choosing fluorescent proteins. *Nature methods* **2005,** *2*, (12), 905-9.

181. Campbell, R. E.; Davidson, M. W., Fluorescent Reporter Proteins. *Molecular Imaging with Reporter Genes (Cambridge Univ Pr)* **2010**, 1.

182. Hatta, K.; Tsujii, H.; Omura, T., Cell tracking using a photoconvertible fluorescent protein. *Nat Protoc* **2006,** *1*, (2), 960-7.

183. Dougherty, M.; Kamel, G.; Shubinets, V.; Hickey, G.; Grimaldi, M.; Liao, E. C., Embryonic fate map of first pharyngeal arch structures in the sox10: kaede zebrafish transgenic model. *J Craniofac Surg* **2012,** *23*, (5), 1333-7.

184. Sato, T.; Takahoko, M.; Okamoto, H., HuC:Kaede, a useful tool to label neural morphologies in networks in vivo. *Genesis* **2006,** *44*, (3), 136-42.

185. Distel, M.; Wullimann, M. F.; Koster, R. W., Optimized Gal4 genetics for permanent gene expression mapping in zebrafish. *Proceedings of the National Academy of Sciences of the United States of America* **2009,** *106*, (32), 13365-70.

186. Thomas-Jones, E.; Thorpe, K.; Harrison, N.; Thomas, G.; Morris, C.; Hutchinson, T.; Woodhead, S.; Tyler, C., Dynamics of estrogen biomarker responses in rainbow trout exposed to 17beta-estradiol and 17alpha-ethinylestradiol. *Environmental toxicology and chemistry / SETAC* **2003,** *22*, (12), 3001-8.

187. Zeng, Z.; Shan, T.; Tong, Y.; Lam, S. H.; Gong, Z., Development of estrogen-responsive transgenic medaka for environmental monitoring of endocrine disrupters. *Environmental science & technology* **2005,** *39*, (22), 9001-8.

188. Kurauchi, K.; Hirata, T.; Kinoshita, M., Characteristics of ChgH-GFP transgenic medaka lines, an in vivo estrogenic compound detection system. *Marine pollution bulletin* **2008,** *57*, (6-12), 441-4.

189. Kinoshita, M.; Zakaria, M. P.; Ismail, A.; Yusof, S.; Boonphakdee, C.; Boonphakdee, T.; Inoue, K., An attempt to detect contamination with estrogenic compounds in river water of urban area in Thailand and Malaysia using transgenic medaka. *Coast. Mar. Sci.* **2010,** *34*, (1), 216-222.

190. Cho, Y. S.; Kim, D. S.; Nam, Y. K., Characterization of estrogen-responsive transgenic marine medaka Oryzias dancena germlines harboring red fluorescent protein gene under the control by endogenous choriogenin H promoter. *Transgenic research* **2013,** *22*, (3), 501-17.

191. Salam, M. A.; Sawada, T.; Ohya, T.; Ninomiya, K.; Hayashi, S., Detection of environmental estrogenicity using transgenic medaka hatchlings (Oryzias latipes) expressing the GFP-tagged choriogenin L gene. *Journal of environmental science and health. Part A, Toxic/hazardous substances & environmental engineering* **2008,** *43*, (3), 272-7.

192. Chiang, E. F.; Yan, Y. L.; Guiguen, Y.; Postlethwait, J.; Chung, B., Two Cyp19 (P450 aromatase) genes on duplicated zebrafish chromosomes are expressed in ovary or brain. *Molecular biology and evolution* **2001,** *18*, (4), 542-50.

193. Schreurs, R. H.; Legler, J.; Artola-Garicano, E.; Sinnige, T. L.; Lanser, P. H.; Seinen, W.; Van der Burg, B., In vitro and in vivo antiestrogenic effects of polycyclic musks in zebrafish. *Environmental science & technology* **2004,** *38*, (4), 997-1002.

194. Antinucci, P.; Hindges, R., A crystal-clear zebrafish for in vivo imaging. *Sci Rep* **2016,** *6*, 29490.

195. Russell, W.; Burch, R., The Principles of Humane Experimental Technique. **1959**

196. Lee, O.; Green, J. M.; Tyler, C. R., Transgenic fish systems and their application in ecotoxicology. *Crit Rev Toxicol* **2015,** *45*, (2), 124-41.

197. Brown, A. R.; Bickley, L. K.; Le Page, G.; Hosken, D. J.; Paull, G. C.; Hamilton, P. B.; Owen, S. F.; Robinson, J.; Sharpe, A. D.; Tyler, C. R., Are toxicological responses in laboratory (inbred) zebrafish representative of those in outbred (wild) populations? - A case study with an endocrine disrupting chemical. *Environ Sci Technol* **2011,** *45*, (9), 4166-72.

198. Brown, A. R.; Bickley, L. K.; Ryan, T. A.; Paull, G. C.; Hamilton, P. B.; Owen, S. F.; Sharpe, A. D.; Tyler, C. R., Differences in sexual development in

inbred and outbred zebrafish (Danio rerio) and implications for chemical testing. *Aquat Toxicol* **2012,** *112-113*, 27-38.

199. Goll, M. G.; Anderson, R.; Stainier, D. Y.; Spradling, A. C.; Halpern, M. E., Transcriptional silencing and reactivation in transgenic zebrafish. *Genetics* **2009,** *182*, (3), 747-55.

200. Gorelick, D. A.; Iwanowicz, L. R.; Hung, A. L.; Blazer, V. S.; Halpern, M. E., Transgenic zebrafish reveal tissue-specific differences in estrogen signaling in response to environmental water samples. *Environ Health Perspect* **2014,** *122*, (4), 356-62.

201. Gao, X.; Wang, H. S., Impact of bisphenol a on the cardiovascular system - epidemiological and experimental evidence and molecular mechanisms. *Int J Environ Res Public Health* **2014,** *11*, (8), 8399-413.

202. Chang, T. Y.; Pardo-Martin, C.; Allalou, A.; Wahlby, C.; Yanik, M. F., Fully automated cellular-resolution vertebrate screening platform with parallel animal processing. *Lab on a chip* **2012,** *12*, (4), 711-6.