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Novel transgenic medaka models to detect disruption of sex hormone signalling and gonadal development

Petra Spirhanzlova

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Petra SPIRHZANOVA

Le 4.10.2016

**NOVEL TRANSGENIC MEDAKA MODELS TO DETECT DISRUPTION
OF SEX HORMONE SIGNALLING AND GONADAL DEVELOPMENT**

Sous la direction de: Pr. Barbara Demeneix et Dr. Andrew Tindall

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à mes parents et Ludovic

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Abstract

The toxic pressure of endocrine disruptors on biodiversity and human health has increased significantly over recent decades. As a consequence tools are needed to detect and monitor endocrine disruptors in surface water and to determine the endocrine disrupting potential of newly introduced chemicals. Fish and amphibian larvae, notably the medaka (*Oryzias latipes*) and *Xenopus laevis*, offer multiple advantages in this context.

In the research carried out in the context of this thesis, different novel medaka-based transgenic models were developed. First, transgenic *ChgH-gfp* medaka model was designed and optimized for the rapid detection of estrogens and aromatase inhibitors. The model shows significant response within 24 hours with a sensitivity of 15 ng/L ethinylestradiol.

Second, a double transgenic *42sp50-gfp_ChgH-gfp* medaka which exhibits fluorescence both in the liver in response to estrogens and in developing oocytes as a function of phenotypic sex. It is therefore a suitable model for studying the link between estrogen axis signalling and aberrations of sex determination in fish.

Third, a novel *spiggin-gfp* medaka model was developed to detect androgens and anti-androgens. This model can be exploited in a 96-hour assay with a sensitivity of 1.5 µg/L 17α-methyltestosterone and 276 µg/L flutamide.

The novel transgenic medaka models developed in this thesis allow rapid, simple and reliable detection of estrogen and androgen axis disruption and aberrations in medaka sex determination. They have been successfully applied to detect endocrine disruptors in environmental surface water and to assess chemicals with unknown endocrine disrupting potential. Taken together these results demonstrate the applicability of medaka reporter larvae as biological tools in the procedure of detection and characterisation of endocrine disruptors.

Key words: *Oryzias latipes*, Endocrine disruption, Estrogens, Androgens, Sex reversal

Abstract

La pression toxique, induite par les perturbateurs endocriniens sur l'environnement ainsi que sur la santé de l'homme a augmenté de manière significative durant les dernières décennies. Il est devenu urgent de mettre en place des outils pour détecter et surveiller les perturbateurs endocriniens et pour déterminer la possibilité de cause de perturbations endocriniennes pour les produits chimiques nouvellement introduits. Le développement de nouveaux tests biologiques *in vivo* en utilisant les larves d'organismes aquatiques tels que medaka (*Oryzias latipes*) ou *Xenopus laevis* semble être une stratégie adéquate pour identifier les perturbateurs endocriniens.

Durant cette thèse, trois nouveaux modèles transgéniques à base de l'embryon de medaka ont été développés. Le modèle transgénique de medaka *ChgH-gfp* permet la détection rapide des oestrogènes et des inhibiteurs de l'aromatase en seulement 24 heures avec une sensibilité de 15 ng / L d'éthinylestradiol. Le modèle transgénique de medaka *42sp50-gfp_CgH-gfp* montre une fluorescence dans le foie en réponse aux œstrogènes, en plus de la fluorescence émise dans les œufs en développement. C'est donc un système approprié pour étudier le lien entre la signalisation de l'axe œstrogénique et des aberrations de la détermination du sexe chez les poissons. Un embryon transgénique de medaka *spiggin-GFP* a aussi été développé pour détecter les androgènes et anti-androgènes dans un test de 96 heures avec une sensibilité de 1,5 ug / L de 17 α -méthyltestostérone et 276 ug / L de flutamide.

Les nouveaux modèles de medaka transgéniques rapporteurs développés dans cette thèse permettent une détection rapide, simple et fiable des perturbateurs d'axe œstrogénique, androgénique et de l'aberration de détermination du sexe chez le medaka. Ils ont été appliqués pour détecter les perturbateurs endocriniens dans les eaux de surface de l'environnement et pour évaluer le rôle potentiel des produits chimiques dans les perturbations endocriniennes. Cela a permis de démontrer l'applicabilité de ces embryons fluorescents comme outils biologiques dans la procédure de détection et la caractérisation des perturbateurs endocriniens.

Key words: *Oryzias latipes*, Perturbateurs endocriniens, Oestrogènes, Androgènes, Reversion sexuelle

List of abbreviations

17OHP	17 α -hydroxyprogesterone
17 β -HSD	17 β hydroxysteroid-dehydrogenase
3 β -HSD	3 β -hydroxysteroid dehydrogenase
17-MT	17-methyltestosterone
AFSS	Androgenised Female Stickleback Screen
AHR	Aryl hydrocarbon receptor
AMH	Anti-muellerian hormone
AOP	Adverse outcome pathway
AP-1	Activator protein-1
AR	Androgen receptor
BBzP	Benzylbutylphthalate
BPA	Bisphenol A
cAMP	Cyclic adenosine monophosphate
CRE	cAMP response element-binding
CREB	cAMP response element-binding protein
DBP	Phthalate di(n-butyl) phthalate
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DEHP	Bis(2-ethylhexyl) phthalate
DES	Diethylstilbestrol
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
dmrt1	Doublesex and mab-3 related transcription factor 1
DMY	DM-domain gene on the Y chromosome
DNA	Dioxuribonucleic acid
dpf	Days post fertilisation
dph	Days post hatch
E2	Estradiol
EDC	Endocrine disrupting chemical
EE2	Ethinyl-oestradiol
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
ER	Estrogen receptor
ERE/ARE	Estrogen or androgen responsive elements
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
GAL	Galactosidase

GFP	Green fluorescent protein
GnRH	Gonadotropin releasing hormone
GnRHR	Gonadotropin releasing hormone receptor
GRE	Glucocorticoid response element
Grizly	Glucocorticoid Responsive <i>In vivo</i> Zebrafish Luciferase activity
Gsdf	Gonadal soma derived factor
HAT	Histone acetyltransferase
HSP	Heat-shock protein
CHG	Choriogenin
IGF-I	Insulin-like growth factor-I
IL	Interleukin
IPPC	Directive for Integrated Pollution and Prevention Control
KO	Knock-out
LDL	Low density lipoproteins
LH	Luteinizing Hormone
LHR	Luteinizing Hormone receptor
LUC	Luciferase
MAPK	Mitogen activated protein kinase
MBP	Monobutyl phthalate
MDHT	Methyldihydrotestosterone
mRNA	Messenger ribonucleic acid
NO	Nitric oxid
NSAIDs	Non-steroidal anti-inflammatory drugs
o,p DDE	o,p Dichlorodiphenyldichloroethylene
OECD	The Organisation for Economic Co-operation and Development
PAK	Phosphokinase A
PCBs	Polychlorinated biphenyls
PCDDs)	Polychlorinated dibenzo-p-dioxins
PCDFs	Polychlorinated dibenzofurans
PCOS	Polycystic ovarian syndrome
PGC	Primordial germ cells
PKC	Protein kinase C
PLC	Phospholipase C
PR	Progesterone receptor
PRL	Prolactin
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
SERM	Selective estrogen receptor modulator
SF-1	Steroidogenic factor-1
SHBG	Steroid hormone-binding globulin

Sox	Sry-related HMG box
StAR	Steroidogenic acute regulatory protein
SV40	Simian virus 40
T	Testosterone
T3	Triiodothyronine
T4	Thyroxine
TATA	5'-TATAAAA-3'
TBT	Tributyltin
TCDD	Tetrachlorodibenzo-p-dioxin
TDS	Testicular dysgenesis syndrome
TGF	Transforming growth factor
TNF α	Tumor necrosis factor
TR	Thyroid receptor
TSH	Thyroid-stimulating hormone
UV	Ultra violet
WFD	Water Framework Directive
WHO	World Health Organisation
WNT	Working Group of National Coordinators of the Test Guidelines Programme
WWTP	Wastewater treatment plant
YE(A)S	Yeast Estrogen or Androgen Screen

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1. Spirhanzlova P., De Groef B., Nicholson F.E., Grommen S.V.H, Lamiral K., Marras G., Sebillot A., Demeneix B.A., Lemkine G., Mothre S. and Du Pasquier D. Evaluation of the thyroid, estrogenic and androgenic potentials of the pesticides linuron and fenoxycarb
2. Spirhanzlova, P., Sebillot, A., Tindall AJ. Rapid fluorescence quantification in unanaesthetised, transgenic larvae
3. Spirhanzlova P., Sebillot A., Tindall A.J. Transgenic 42sp50-ChgH-GFP medaka to reveal sensitive developmental windows for sex reversal

Scientific communication- conferences

1. Spirhanzlova, P., DuPasquier D., Tindall A.J. (2015) Practical application of transgenic *Xenopus laevis* for identifying thyroid disruption in surface water. 6th annual EFOR meeting, March 9-10 2015, Paris. **Platform presentation.**
2. Spirhanzlova, P., Tindall A.J., Du Pasquier D., Lemkine G. (2015) Transgenic fluorescent tadpoles for detecting chemical disruption of the thyroid axis. 25th annual meeting of SETAC Europe, May 3-7 2015, Barcelona. **Platform presentation.**
3. Spirhanzlova, P., Tindall A.J. (2015) Disruption of European surface water identified using transgenic tadpoles and fish fry. EDA-EMERGE conference, June 29-July 1 2015, Leipzig. **Platform presentation.**

General introduction to the thesis

The research presented in this thesis was part of the EDA-EMERGE programme, which is a 4 year Marie Curie ITN project, funded by the European Commission within the 7th framework programme running from October 2011 to September 2015.

Literally hundreds of tons of endocrine disruptors such as pesticides, plasticizers or anti-fouling agents are constantly released into the environment. In addition, thousands of new chemicals are introduced into the global market every year. It is therefore necessary to establish tools to detect and monitor endocrine disruptors in surface water and to determine the endocrine disrupting potential of newly introduced chemicals. One promising approach to detect endocrine disruptors is known as Effect-Directed Analysis (EDA). EDA combines fractionation in addition to chemical and biological *in vivo* and *in vitro* tools to reduce the complexity of the chemical mixtures found in the environment. Due to the nature of endocrine hormones, which are produced in one tissue and act on a distant target, they can act via a number of mechanisms of action in addition to receptor interaction. This limits the predictability of *in vitro* tests. To increase the power and scope of EDA, establishment of new reliable, rapid and low-cost methods is necessary and therefore the goal of the research included in this thesis was to develop novel *in vivo* medaka-based transgenic bioassays to detect disruptors of estrogen and androgen axis as well as a model suitable to assess sex reversal. Another goal was to demonstrate the applicability of the newly developed models as a part of EDA bioassays battery by testing whole water samples from different areas in Europe as well as testing the endocrine potential of pure chemicals.

The first part of the introduction of this thesis is dedicated to endocrine disruptors, their history, definition, types and their effects on the wild life and humans. Several aspects of the legislation and strategy to assess endocrine disruptors in Europe are discussed as well. Because the goal of this thesis was to develop novel bioassays to detect endocrine disruptors, the second part of the introduction is reviewing existing transgenic and non-transgenic *in vivo* and *in vitro* bioassays to detect endocrine disruptors. Hormonal control of reproduction is very complex and endocrine disruptors can affect a number of steps in the mechanism, it is therefore important to understand the pathways of sex hormone synthesis. Hence the third part of the introduction describes in detail the hypothalamo-pituitary-gonadal axis and the synthesis, regulation and signalling

pathways of sex hormones. The last part of the introduction describes the medaka as a model organism, with the focus on medaka sex determination, gonadal development and sex reversal, all key elements for the three medaka models developed in this thesis.

The results part of this thesis is composed of five publications, amongst them two have already been published and three are (ready to be) submitted.

Publication one, “*Oestrogen reporter transgenic medaka for non-invasive evaluation of aromatase activity*”, describes the development and validation of the transgenic *ChgH-gfp* medaka model, which allows rapid detection of estrogens and aromatase inhibitors in just 24 hours with a sensitivity of 15 ng/L ethinylestradiol. This paper was published in the journal “Comparative Biochemistry and Physiology, part C” in 2015.

In addition, a protocol using a fluorescence plate reader was developed to facilitate and accelerate fluorescence quantification of un-anaesthetized *ChgH-gfp* medaka larvae, also allowing sequential measures whilst eliminating anaesthetic treatment as a confounder. This additional protocol is planned to be submitted as short communication in the journal “Environmental toxicology and chemistry” as **publication two** “*Rapid fluorescence quantification in unanaesthetised, transgenic larvae*”.

Publication three, “*Transgenic medaka reveal dynamic temporal sensitivity to disruption of sex determination*” describes the transgenic *42sp50-gfp_ChgH-gfp* medaka which exhibit fluorescence in the liver in response to estrogens in addition to fluorescence in developing oocytes. It is therefore a suitable model for studying the link between estrogen axis signalling and aberrations of sex determination in fish. Short pulses of E2, DHT or the anti-androgen flutamide were administered at various time points between day post fertilisation 1 and 12 to investigate the relative sensitivity of developmental windows for hormonally induced paradoxical sex determination. This publication is expected to be submitted in the journal “Environmental science and technology”.

Publication four, “*Rapid Fluorescent Detection of (Anti)androgens with spiggin-gfp medaka*” describes a novel *spiggin-gfp* medaka model to detect androgens and anti-androgens in a 96-hour assay with the sensitivity 1.5 µg/L 17α-methyltestosterone and 276 µg/L flutamide. This publication was published in the journal “Environmental science and technology” in 2014.

The applicability of the *ChgH-gfp* and *Spiggin-gfp* transgenic models for the study of endocrine disruption in surface water samples was demonstrated within the EDA-EMERGE project. In addition a transgenic *Xenopus laevis* validated for the detection of thyroid disruption (*ThbZIP-gfp*) was included in the bioassay panel. The use of this panel resulted in the identification of several water samples from Germany and Croatia showing endocrine activity and these results are shown in the supplementary data.

The same combination of assays was also used to assess thyroid, estrogen and androgen axis disruption potential of the pesticides fenoxycarb and linuron, Fenoxycarb did not exhibit activity on any of the studied endocrine axes while linuron increased thyroid and decreased estrogen and androgen signalling *in vivo*. These results have been submitted to the journal “Molecular and cellular endocrinology” as **publication five**, “*Evaluation of the endocrine disrupting capacity of the pesticides linuron and fenoxycarb: effects on the thyroidal, estrogenic and androgenic systems*”.

BIBLIOGRAPHIC INTRODUCTION

INTRODUCTION
PART I.

ENDOCRINE DISRUPTORS

PART I. Endocrine disruptors

1. History of endocrine disruptors

The endocrine system is responsible for the homeostasis, normal development and correct function of the body. There are more than 30 endocrine glands and together they form a complex system, often cooperating and forming self-regulating loops to maintain critical physiological processes. As the endocrine system works as a very complex clockwork like mechanism, which is dependent on many actors, it is possible for parts of the system to be disrupted by exogenous chemicals, pharmaceuticals or environmental stressors. Even though the major concerns regarding endocrine disruptors rose in the 1990's, the fact, that some exogenous compounds can cause adverse effect to the endocrine system was known for centuries as for example farmers were aware of the fact, that female sheep had reproductive problems after excessive consumption of red clover (*Trifolium pretense*) (Bennetts et al., 1946; Kingbury, 1964).

After the boom of the chemical industry in 1930's, many new chemicals were introduced to the market to facilitate human life, such as hormonal contraceptives, new kind of pesticides and later plastics. The excessive use of the newly developed chemicals led to some early observations of the adverse effects and first toxicological studies focusing on the negative side of their use emerged (Schueler, 1946; Walker, 1930). An example of mass use of compounds with endocrine disrupting activity is synthetic estrogen diethylstilbestrol, prescribed from 1938 to prevent miscarriages, but by the time it was banned from the market in 1971, millions of women and their offspring were affected. The use of the pesticide dichlorodiphenyltrichloroethane (DDT) is another example of massive use of a chemical with adverse effect on the endocrine system (Burlington and Lindeman, 1950). Several effects on wild populations of fish or birds were also observed (Hickey and Anderson, 1968), but the endocrine disruption topic didn't receive major attention until the book "Silent spring" was published in 1962 by Rachel Carson. In this book, the author raised an alarm, warning about the adverse effects of EDCs on wildlife.

During the second half of 20th century, endocrine disruptors played a major role in many environmental catastrophes which affected the wildlife and human population as well, leaving consequences in multiple generations. Between the years 1947 and 1977 the General Electric Company released 209,000 -1.3 million pounds of PCBs into the Hudson River resulting in a ban

on fishing in the river in 1976 (Brown et al., 1985). In 1976 the ICMESA chemical manufacturing plant in Seveso exploded, releasing 30 kg of dioxins into the environment, affecting thousands of people (Walsh, 1977). Another example of an environmental disaster is the case of Apopka lake in Florida which was highly contaminated in the 1980's by DDT and its metabolites, causing reproductive problems for wildlife, mainly alligators (Heinz et al., 1991; Semenza et al., 1997).

At the conference in Wingspread Conference Centre in Wisconsin in 1991, the term “Endocrine disruptor” was used for the first time. The goal of this conference was the evaluation of the situation in several locations in the northern hemisphere such as Great Lakes region in the north of the USA. Specialists of many fields such as toxicology, developmental and reproductive biology, endocrinology or ecology, who attended this conference, decided to publish a consensus statement summarizing the facts and their concern about the environmental and health impacts of the endocrine disruption. After World War II, several epidemiological trends were observed such as decreased sperm quality, increased appearance of congenital malformations of the male genital tract and an increased incidence of leiomyoma, testicular cancer and breast cancer. The Wingspread conference statement connected these trends with fetal exposure to endocrine disruptors. Even though experiments earlier in the 20th century suggested possible harmful effects of endocrine disruptors, it was the Wingspread conference that triggered the rapid increase in research in this area. In 1997 Colborn published a book *Our Stolen Future, Are We Threatening Our Fertility, Intelligence and Survival?—A Scientific Detective Story* and triggering panic in the public. While until then the topic of endocrine disruptors was rarely discussed, suddenly it started to fill newspaper headlines. Government and scientific organizations such as the WHO called upon endocrinologists from all over the world to address this issue which resulted in a succession of reviews and reports (IPCS, 2002).

2. Endocrine disruptors - characterization

According to the U.S. Environmental protection Agency (EPA) an endocrine disruptor is described as “an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental processes.”

The mechanisms of action of endocrine disrupting chemicals can be separated into the following categories:

- a) alteration in hormone receptor level or hormone synthesis
- b) direct or indirect reaction with endogenous hormones
- c) blocking the interactions between hormones and their receptors
- d) activation of hormone receptors and triggering their effect

Some endocrine disruptors have a similar molecular structure to naturally occurring hormones, which allows them to bind to the receptor (Fig. 1). Endocrine disruptors act via nuclear receptors, non-nuclear steroid receptors like estrogen receptors (ER), non-steroid receptors such as neurotransmitter receptors and orphan receptors (aryl hydrocarbon receptor). The mechanism of action of each individual EDC differs and many have mixed steroidal actions. One of explanations is that these compounds are degraded into several metabolites of different properties (Kelce et al., 1995).

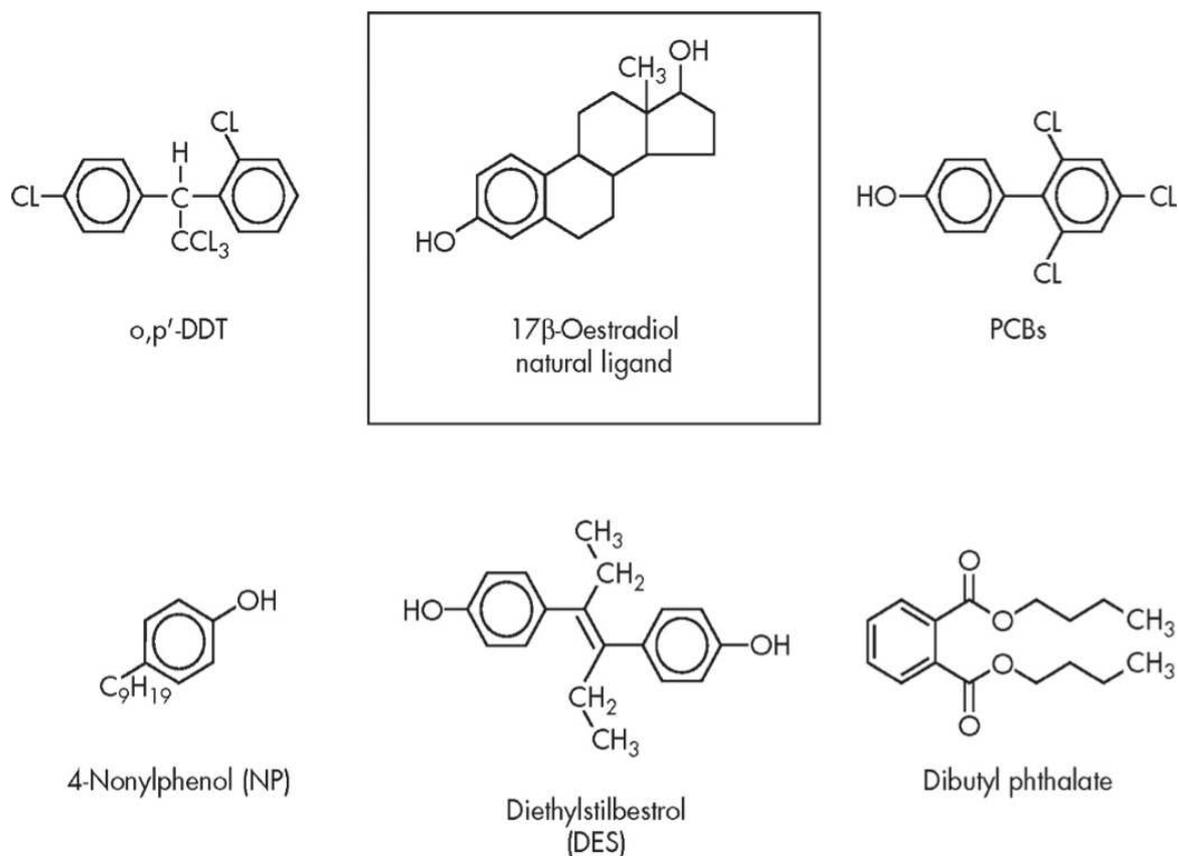


Figure 1. Molecular structure of estradiol (boxed) compared to compounds known for the ability to bind to estrogen receptor (Brown et al., 2011).

Due to the targeted action of hormones even very low concentrations (picomolar to nanomolar) of EDCs can cause harmful effect to the organism. It has also been shown, that exposure to some compounds results in U-shaped or otherwise nonmonotonic dose-response curves (Vandenberg et al., 2012; Wozniak et al., 2005) (Fig 2).

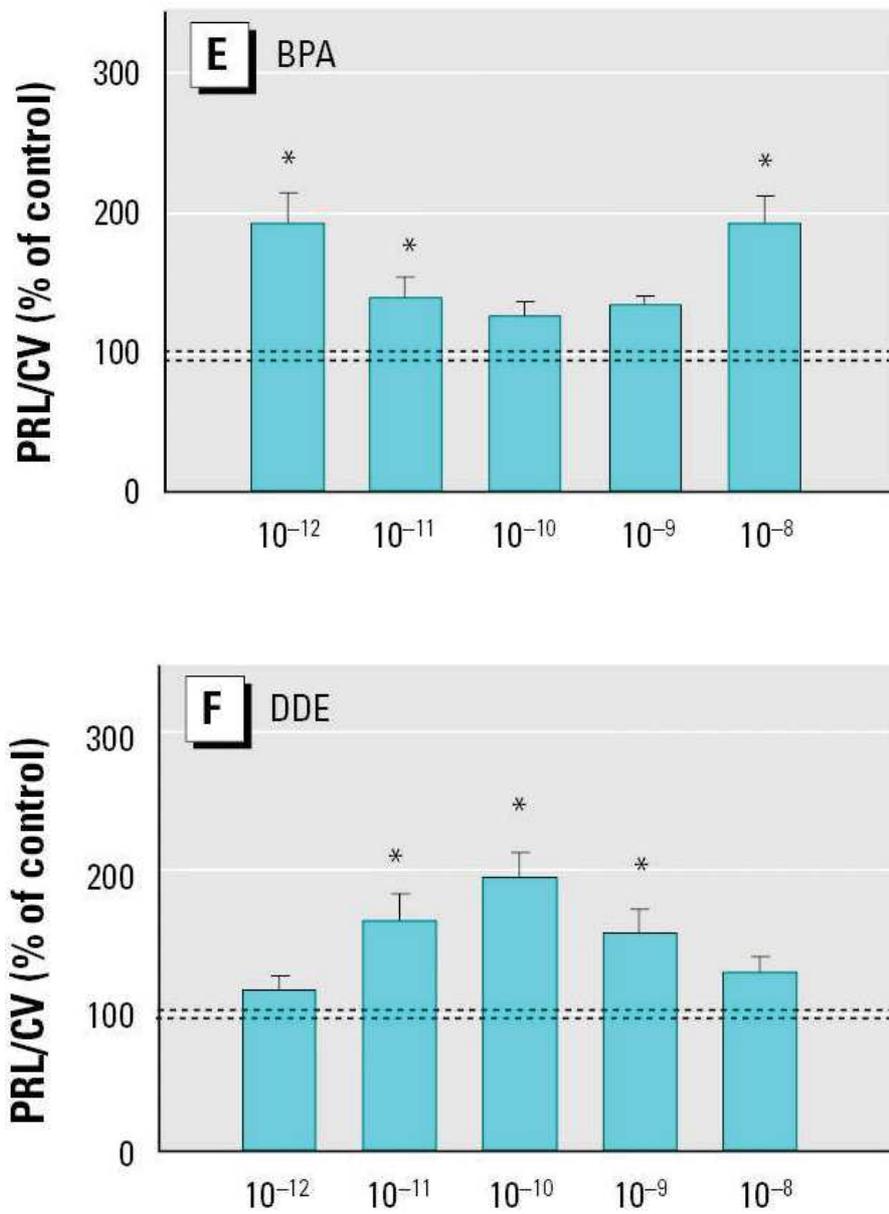


Figure 2. U and inverted U shaped nonmonotonic dose-response curves. GH3/B6/F10 cells were treated for 3 min by xenoestrogens BPA and DDE. Concentration dependent release of PRL in response to BPA and DDE shows typical U and inverted U dose-response trend respectively (Wozniak et al., 2005).

One explanation of inverted-U shaped curve can be the toxicity of the tested compound. MCF7 cells line responding to estradiol concentration between 10^{-12} to 10^{-11} M by proliferation, but concentrations greater than 10^{-6} M caused acute toxicity, therefore the dose-response curve resembles an inverted-U (Welshons et al., 2003). Another explanation of inverted-U dose-response is that cells and tissues contain different receptors and respond differently to hormone stimulation thus it is possible that a certain population of cells reacts differently than another. For example, it has been demonstrated that estrogens can induce cell proliferation and inhibit apoptosis in one type of cells, but cause the exact opposite effect in another type, which after combining, results in inverted-U shaped curve (Vandenberg et al., 2012). U-shaped curve can be explained by receptor selectivity. While several EDC's for example bisphenol A are selectively binding to estrogen receptor in low concentrations, in high concentrations they bind to other receptors as well, for example thyroid or androgen receptors (Moriyama et al., 2002; Sohoni and Sumpter, 1998). Another explanation for non-monotonous response curves is receptor down regulation and desensitization. After hormone binds to receptor and transcription of target gene occurs, the receptor has to be inactivated, which happens usually via ubiquitination or proteasome degradation. New hormone receptors are being produced in the meanwhile. When the hormonal level increases, the number of receptors to inactivate also increases until the production of new hormone receptors cannot keep up with the process anymore (Ismail and Nawaz, 2005). Desensitization is a process of biochemical inactivation of a receptor often seen in G-coupled protein receptors after long continuous exposure to ligand (Freedman and Lefkowitz, 1996). Desensitization and receptor down regulation often occur at the same receptor, which can result in non-monotonous response curve (Shankaran et al., 2007).

3. Categories of endocrine disruptors

After World War II., many man-made chemicals were introduced to increase the standard of living of humans without thinking further about the consequences. To date, there are more than 100 000 different chemicals available on the market, but toxicological tests have been performed on only a very small number. Among well characterised endocrine disrupters we can find compounds used in the chemical manufacturing industry (eg. polychlorinated biphenyls, dioxins), plasticisers (eg. bisphenol A), insecticides eg. DDT), fungicides (eg. vinclozolin) and pharmaceutical chemicals (eg. flutamide). Several natural chemicals have also been identified as endocrine disruptors, such as phytoestrogens in soya, although they have low estrogen receptor binding affinity. Some heavy metals or nanoparticles can also act as endocrine disruptors.

3.1 Compounds used in chemical industry

Phthalates are commonly found in plastic materials which are used for plastic bags, building materials, cosmetics or even toys. They are esters of phthalic acid and they can have different alkyl chain length and therefore different molecular weight resulting in different functionality. Because there is no covalent bond between the phthalates and the plastic they are included in, they are easily released into the environment (Bertelsen et al., 2013; Morton et al., 2013). The most common human exposure is through consumption, skin contact and inhalation. Examples of commonly studied phthalates are **phthalate di(n-butyl) phthalate (DBP)** and its metabolite **monobutyl phthalate (MBP)** inducing adverse effects on steroidogenesis as well as **Bis(2-ethylhexyl) phthalate (DEHP)** and **Benzylbutylphthalate (BBzP)** which act as anti-androgens (McKinnell et al., 2009; Mylchreest et al., 1999; Tanaka, 2002; Tyl et al., 2004). In the European Union no phthalates can be used in toys since 1999. Some phthalates (DEHP, BBP, and DBP) cannot be used in the toys at all, some only in case these toys can be placed in the mouth, while their amount can't exceed 0.1%. There are no other restrictions regarding the presence of phthalates in other consumer products.

Dioxins and Dioxin like compounds belong to a varied group of industrial compounds including polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs). Dioxins are produced, when an organic material is burned in the presence of chlorine, for example in municipal or medical waste incinerators or backyard barrel burning. The main source of dioxins for humans is food, especially fish and milk products as dioxins are fat soluble. Another source of dioxins is cigarette smoke. (Malisch and Kotz, 2014; Marinković et al., 2010; Muto and Takizawa, 1989). The mechanism of action of dioxins and dioxin like compounds is likely to be via aryl hydrocarbon receptor (AHR) activation (Birnbaum, 1994; Tijet et al., 2006).

One of the most potent dioxin is **Tetrachlorodibenzo-p-dioxin (TCDD)**, used in agent orange (herbicide), widely used during the Vietnam war. After a tragedy in Seveso a large amount of TCDD was released in the area and many studies were carried on the Seveso population after the exposure (Pesatori et al., 2009). It has been discovered, that TCDD has an estrogenic effect if there is lack of estrogen, however is anti-estrogenic in presence of estrogen, which also suggests, that the mechanism of action of TCDD is dependent on the life stage (Tanaka et al., 2007). **Polychlorinated biphenyls** are composed of two benzene rings (diphenyl) with attached chlorine. PCBs were used as dielectric and coolant fluids until the 1970's when they were banned. Although they cannot be used anymore, they are still present in the environment due to their high stability. They are lipophilic molecules, which allows them to remain in fat tissues and milk (McGovern, 2006). A number of studies demonstrated the estrogenicity and anti-estrogenicity of PCB's (Connor et al., 1997; Jansen et al., 1993).

Alkylphenols are created by alkylation of phenols and they are used in the production of detergents, antioxidants in plastic and rubber objects and personal care products such as hair sprays, perfumes and spermicides. The most common route of human exposure is via water and skin. Alkylphenols specifically nonylphenols were shown to act as xenoestrogens, mimicking the effect of estrogens (Laws et al., 2000; White et al., 1994). A study revealed, that exposure of mouse embryos to 4-nonylphenol results in an increased risk of mammary cancer (Recchia et al., 2004; Vivacqua et al., 2003). **Bisphenol A** is a diphenylmethane derivate and it contains two hydroxylphenyl groups. It's a compound used since 1957 in polycarbonate plastics and epoxy

resins. These plastics are very often used in food containers, plastic bottles, sports equipment or in CD's and DVD's due to their characteristics such as plasticity and transparency. Epoxy resins often coat the inner layer of metal food cans. The ester bond linking BPA monomers is easily hydrolyzed by heat, microwaves or by acid or basic conditions and released into the environment. Therefore the most common human exposure is by consumption (Maffini et al., 2006; Rubin, 2011; Vogel, 2009). Although it was long ago demonstrated that BPA acts as estrogen receptor agonist, recent studies suggests that BPA is a selective estrogen receptor modulator (SERM), based on studies showing that BPA and 17 β estradiol don't show the same effect every time and in some studies, BPA acts as E2 antagonist (Welshons et al., 2006; Wetherill et al., 2007). Many experiments were carried in order to discover the consequences of prenatal or neonatal exposure to BPA. Offspring of female rats, which were exposed to BPA showed significant increases in ER α and β expression and similar results were achieved after injecting neonatal rats with BPA (Cao et al., 2012, 2013, 2014). Another study demonstrates that BPA can also act as an anti-androgen (Sohoni and Sumpter, 1998). Anti-thyroid effect was also discovered (Fini et al., 2007). Bisphenol A suppresses the transcriptional activation of genes regulated by thyroid hormones (Gentilcore et al., 2013; Moriyama et al., 2002). To demonstrate the effect of BPA on thyroid axis, pregnant rats were fed with BPA and after the serum levels of T4 and TSH in the newborn pups were measured, serum total T4 was significantly increased, but serum TSH was not different compared to control. (Zoeller et al., 2005).

3.2 Pesticides

After the increase in agricultural production in the 1960's "Green Revolution", higher use of pesticides was required. These compounds are designed to kill rodents, insects, weed and fungi and were meant to lead to a great increase in food quantity and quality. However, after the book "Silent spring" was released in the 1962, the benefit of the use of some pesticides started to be questioned. Soon several studies demonstrated harmful effects of some of the first generation of pesticides resulting in banning the pesticide DDT, heavily used since its discovery in 1939. Nowadays more than two million tons of pesticides are released worldwide into the environment every year with various effects on the wildlife and human health. Many epidemiological studies have focused on the harmful effects of pesticides on spermatogenesis and male reproduction.

Several studies have focused on the connection between hormone-dependent breast cancer and pesticides, for example a study published in 2010 demonstrates that 81% of reported breast cancer cases comes from areas of high pesticide use (Clementi et al., 2008; Hossain et al., 2010; Parron et al., 2010). Main source of exposure for humans are consumption of contaminated fruit, vegetable, cereals and meat. It is of note that the residue of pesticides can be detected in human breast milk and urine (Damgaard et al., 2006; Hill et al., 1995).

DDT belongs to the group of organochlorines and it was synthesized for the first time in 1874. In 1939 its pesticide action was discovered and DDT was highly used in agriculture. During World War II DDT was used extensively to fight malaria and typhus. In 1962 the book “Silent spring” was released, where the carcinogenic action of DDT was described and it started an environmental movement which resulted in the agriculture use of DDT being banned in 1972 in the USA (US EPA). In the Stockholm convention, the worldwide ban of DDT was announced, although it is still used controversially to fight disease vectors. The only country producing DDT nowadays is India. The mechanism of action of DDT as an insecticide is by opening sodium ion channels in the neural cells of insects causing their over-excitation which eventually leads to death (Perry et al., 1998). Its mechanism of action as an endocrine disruptor is competitive binding to androgen receptor, activating the proliferation of androgen dependent cells. DDT is also an estrogen and progesterone receptor agonist and it stimulates the production of estrogen receptors. Several studies showed its harmful effects on wildlife, for example eggshell thinning in various bird species and toxicity in fish and aquatic crustacean like daphnia or shrimps. The endocrine disruption effects of DDT are a subject of experimental study since the early 1950's. Fisher characterized the estrogenic effect of the DDT metabolite o,p DDE in his study in 1952 and other investigations of its estrogenicity have been carried out since (Fisher et al., 1952). One study revealed that DDT might be responsible in anti-androgenic effects in men and estrogenic effects in postmenopausal females (Freire et al., 2014).

Another pesticide which belongs to organochlorines is **Chlordecone**. Chlordecone, also known under the name kepone, is an insecticide which was used in 1980-1993, often on banana fields. Especially in the French Antilles which is still heavily polluted, the incidence of prostate cancer in Guadeloupe is the highest in the world and it is associated with the use of Chlordecone in that

area. Following the Stockholm convention, the compound was banned in the western world as was DDT. Chlordecone is estrogen receptor alpha (ER α) agonist and androgen receptor agonist (Fang et al., 2003; Hammond et al., 1979; Okubo et al., 2004).

Atrazine is a triazine herbicide used to kill broadleaf weeds in agriculture or near railways and it is one of the most widely used herbicides in the United States. Its use was banned within the European Union in 2004. Atrazine can remain in the soil for many years and it can be easily absorbed in groundwater. It is one of the most common pollutants of drinking water in the US. Several studies confirmed that atrazine is an inhibitor of androgenic signaling with a weak estrogenic effect. It induces the activity of aromatase and increases estrogen production. Atrazine also has an effect on hypothalamic control of luteinizing hormone and the level of prolactin (Cooper et al., 2000; Friedmann, 2002; Hayes et al., 2003; Sanderson et al., 2000).

The dicarboximide fungicide **Vinclozolin** is commonly used against mold in the vineyards and orchards. Humans can be exposed mainly by consumption as washing of fruit with tap water does not remove all traces of vinclozolin or its metabolites. It has been demonstrated that vinclozolin is an androgen receptor antagonist. Rats fed vinclozolin in their diet had significantly reduced prostate and sex organs weights. The effects of the exposure were transgenerational, even after three generations male offspring showed fertility disorders (Andersen et al., 2002; Anway and Skinner, 2008; Wong et al., 1995).

Another example of widely used insecticides is organophosphate **Fenithrothion**, which acts as competitive AR antagonist (Okubo et al., 2004; Sébillot et al., 2014; Tamura et al., 2003). The herbicide **Linuron** is a thyroid receptor agonist and also an AR antagonist (Fang et al., 2003; Schmutzler et al., 2007; Sébillot et al., 2014). **Fenoxycarb** is a carbamate insecticide which can interact with testosterone metabolism (Verslycke et al., 2004). The imidazole fungicide **Prochloraz** has several mode of action, it is an aromatase inhibitor and an antagonist of androgen and estrogen receptors (Grünfeld and Bonefeld-Jorgensen, 2004; Vinggaard et al., 2006).

3.3 Pharmaceutical compounds

Pharmaceuticals are man-made chemicals specifically designed to have an effect in living cells. Because many pharmaceuticals enter the body via consumption, the molecules are designed to endure degradation in the stomach. They are very persistent and they can enter the environment and have a great impact on the human health as well as the health of wildlife. A large group of well-studied pharmaceutical endocrine disruptors are synthetic estrogens widely used in female contraceptives. Traces of ethinyl- oestradiol (EE2) and other estrogens which are used as hormonal contraception can be found in wastewater treatment plant (WWTP) effluents (Mohagheghian et al., 2014; Zhou et al., 2012). Several studies demonstrate that exposure to these effluents can increase female characteristics such as plasma vitellogenin or the presence of ovarian tissue in wild male fish (D. G.J Larsson, 1999; Jobling et al., 2006). An example of a synthetic estrogen inducing pathological effects in humans is **Diethylstilbestrole (DES)**. DES is a compound widely used in the 1940's as estrogen substitute. Even though several studies demonstrated an adverse effect of DES on animals, between 1940 and 1971 millions of women were exposed to this compound. The exposure resulted in a variety of effects including premature births or breast cancer and the adverse effects were carried to future generations. Daughters of the exposed individuals were suffering from infertility, premature births or early menopause and sons from a variety of testicular abnormalities (Stillman, 1982).

A currently very controversial topic is the possible endocrine disrupting effect of NSAIDs (non-steroidal anti-inflammatory drugs) and analgesic drugs. An *in vitro* study demonstrates that **Ibuprofen** has an effect on estradiol production and aromatase activity (Han et al., 2010). It is also suggested, that ibuprofen is connected to increased risk of Luminal A and B breast cancer and increased chance of ER+/PR+ breast cancer (Brasky et al., 2011). In medaka $\mu\text{g/L}$ concentrations of ibuprofen had an effect on reproduction (increased egg production and delaying egg hatching) (Flippin et al., 2007).

Another analgesic with endocrine disrupting effect widely used by humans is **Acetaminophen**. Studies show that acetaminophen is a disruptor of estrogen axis (Miller et al., 1999). Increased vitellogenin expression was observed in medaka fish exposed to acetaminophen (Kim et al., 2012). Women taking acetaminophen for menstrual pain had altered basal hormone levels (E2 and LH) (Cramer et al., 1998). In another study acetaminophen induced proliferation of breast

cancer cells (Gadd et al., 2002; Harnagea-Theophilus et al., 1999; Takehara et al., 2011), one study demonstrates, that acetaminophen significantly reduced breast cancer risk (de Pedro et al., 2015). Other studies did not confirm any effect of acetaminophen on breast cancer (Brasky et al., 2010; Harris et al., 2006).

Another widely used analgesic **Diclofenac** is also disruptor of estrogen axis, showing that three-month exposure of fish to this compound delays hatching and increases vitellogenin expression (Gonzalez-Rey and Bebianno, 2014; Hong et al., 2007; Lee et al., 2011).

3.4 Endocrine disruptors with natural origin

Natural hormones are produced by the endocrine system such as thyroid hormones or steroid hormones. Testosterone or estradiol are not very stable and are not considered as endocrine disruptors, unlike plant-derived hormones - phyto-hormones, more precisely phytoestrogens. Phytoestrogens have a similar structure like 17 β -oestradiol and can mimic its effect and act as endocrine disruptors (Setchell, 1998). The endocrine disrupting effect of phytoestrogens was discovered in the 1940s. It was noticed that sheep feeding on meadows rich on phytoestrogen-rich red clover (*Trifolium pretense*) had altered fecundity (Kingbury, 1964).

The most commonly researched phytoestrogen is the isoflavone **genistein**, an ER β agonist which can be found in soy. The daily average genistein and genistin intake of Japanese men and women is 1.5-4.1 and 6.3-8.3 mg/person respectively, which is higher than the intake of the American and European populations (Fukutake et al., 1996). Due to its structural similarity, it has been suggested that genistein can have a positive effect on post-menopausal women suffering from osteoporosis (Atteritano et al., 2009; Miao et al., 2012). Other studies connect genistein with estrogen dependent cancer, suggesting that consumption of genistein-containing food might be dangerous for women after menopause with this kind of cancer (van Duursen et al., 2011).

Although phytoestrogens might act like oestrogens and there have been concerns about them altering male reproduction, one study revealed that after dietary intake of genistein for two months, no changes were observed in the tested group of men (Mitchell et al., 2001).

3.5 Heavy metals

The group of heavy metals includes arsenic, lead, mercury, cadmium, and nickel, which are found in nature in very small amounts. Because of recent increases of usage of some techniques like smelting and electroplating, more heavy metals are released into the environment and cause concern about their effect on human health as they are accumulating in the body. Heavy metals can create complexes with organic compounds such as nitrogen-, oxygen- or sulfur-containing groups on enzymes, which can cause errors in protein folding and enzyme inactivation (Darbre, 2006). Estrogenic effects of cadmium and nickel have been demonstrated in several studies and they are called metallo-estrogens. Due to their estrogenicity, a potential link to estrogen-dependent breast cancer has been suggested (Aquino et al., 2012). A case study measured cadmium levels in the urine of women diagnosed with estrogen dependent breast cancer (McElroy et al., 2006). The amount of cadmium positively correlated with the breast cancer risk. The cadmium level in breast cancer tissue was analyzed and its presence in cancer tissue was significantly higher than in healthy control tissue (Mohammadi et al., 2014). Similar studies show that women with diagnosed breast cancer had higher concentrations of nickel in the tumor than a control group, which suggests, that accumulation of heavy metals in breast tumor tissues can be closely related to breast cancer (Ionescu et al., 2006; Rizk and Sky-Peck, 1984).

3.6 Nanoparticles

Nanoparticles are defined as 3D nano-objects with size between 1 and 100 nm. There are two categories of nanoparticles, engineered nanoparticles, which are produced with defined properties, composition and purpose and incidental nanoparticles, which are created as side-products of welding, grinding, combustion or vaporization. Nanoparticles are used in broad spectrum of fields for example water purification, building materials or improved energy generation and conservation. Nanoparticles can easily penetrate living cells due to their small size and they are used in medical diagnosis and disease treatment (Iavicoli et al., 2013). As the increase of use of nanoparticles is exponential, many studies focused on the question whether nanoparticles have harmful effects on human health. *In vivo* studies demonstrated, that nanoparticles have negative effects on respiratory, cardiovascular and nervous systems and several studies concentrated on their endocrine disrupting effect (Warheit et al., 2008). Several *in*

in vivo studies show that carbon and metal based nanoparticles have an adverse effect on the histology of testicles in laboratory animals (Li et al., 2009; Yoshida et al., 2009). Nanoparticles also alter testosterone production in the Leydig cells, particularly by inducing the gene expression of steroidogenic acute regulatory protein, which is essential in testosterone synthesis (Komatsu et al., 2008). Nanoparticles also alter the levels of female sex hormones. In an *in vivo* study medaka embryos were treated with silver nanoparticles and increased expression of vitellogenin and choriogenin was observed (Pham et al., 2012). These proteins are involved in egg envelope formation and their expression is estrogen dependent. In another study, male zebrafish embryos were treated with carbon based nanoparticles resulting in decreased bioavailability of 17 α -estradiol (Park et al., 2010).

4. Consequences of exposure to endocrine disruptors

There are several points which have to be considered while evaluating the effect of endocrine disruptors on wildlife and human health. The effects of endocrine disruptors vary between the early embryo, fetus and adults. The effects can be more severe in the offspring than the exposed parent, and the timing of the exposure is crucial. Even though the embryo is critically exposed during its developmental phase, the effect may not show up until full maturity. Wildlife is more likely to be exposed to mixtures of environmental pollutants. Several pollutants can therefore have additive, synergistic or antagonist effects. Some studies concentrate on the trans-generational effects of endocrine disruptors. It is suggested that endocrine disruptors can affect future generation by modulation of factors regulating gene expression such as DNA methylation and histone acetylation.

4.1 Invertebrates

Probably the most studied compound causing endocrine disruption in wildlife invertebrates is tributyltin (TBT), which was used in anti-fouling paints to paint ships until the 1980's. It has been shown, that TBT is responsible for the creation of intersex organs in venus clams (Park et al., 2012). Giraud-Billoud studied the effect of TBT on the fertility of the freshwater snail *Pomacea canaliculata*. Significantly decreased copulation frequency and severely decreased

sperm storage in the seminal receptacle of TBT exposed females was observed (Giraud-Billoud et al., 2013). It was also demonstrated that TBT was causing a reduction in the number of eggs produced by periwinkles (*Littorina littorea*) (Matthiessen et al., 1995). Another study showed that not only mollusks but also water insects were affected by TBT (Hahn et al., 2002). The mechanism of action of TBT as EDC might likely be due to its ability to elevate free testosterone levels by altering the process of testosterone esterification.

4.2 Fish

Fish as aquatic organisms are very good indicators of water pollution and endocrine disruptors in wildlife and many epidemiological case studies have been carried to determine the effects of endocrine disruptors on the fish. An example of the masculinization of female fish *Gambusia affinis* was published by Howell in 1980. The fish examined in this study were caught in the proximity of a paper mill. As a part of paper waste, the plant sterol stigmasterol served as a food source for bacteria which metabolized it into androstenedione, a potent androgen (Howell et al., 1980). Anti-androgens in particular are currently of interest to environmental scientists, as several case studies on the wildlife have linked contamination of European rivers by anti-androgens (working in synergy with estrogenic compounds) to feminization of male fish (Jobling et al., 2009; Urbatzka et al., 2007). Furthermore, waste water treatment and craft pump mills effluents have been identified as sources of (anti-)androgenic activity causing masculinization of female fish (Rostkowski et al., 2011; Scott et al., 2011).

4.3 Amphibians

The larvae of amphibians live in water and therefore they are highly exposed to any endocrine disruptors present as are fish. It had been shown that the world population of amphibians is declining (Blaustein and Wake 1995). The authors of this study suggest several reasons for the decline such as UV radiation or chemicals such as pesticides. The effect of atrazine in wild amphibian populations was studied by Hayes , who describes the feminization of female frogs caused by atrazine (Hayes et al., 2002, 2010). Atrazine also caused an ovarian dysgenesis, high rates of testicular oocytes, and female-biased sex ratios in the population of frog *Lithobates blairi* collected in the wetlands in Nebraska, US. Male-biased sex ratios of cricket frogs (*Acris crepitans*) were discovered in the areas polluted with PCBs and PCDFs in Illinois, US (Reeder et al., 1998)

4.4 Reptiles

A study describing the effects of several endocrine disruptors including organochlorine pesticides or polychlorinated bisphenyls investigated their impact on the reproduction of the snapping turtle collected from the lakes in Ontario, Canada. Eggs of turtles living in this area had decreased hatching rates compared to the control group (Bishop et al., 1991). Decreased hatching was also studied by Stoker, who described the effect of organochlorine pesticides on egg shell quality and viability of the clutches in broad-snouted caiman. He suggested that organochlorine pesticides could alter hormonal actions in the oviduct causing decrease in the quality of egg shell, mainly the thickness and porosity (Stoker et al., 2013). Organochlorines were also shown to be responsible for the development alterations of alligators living in lakes in Florida, including the phallus size and gonadal morphology abnormalities. Organochlorines were detected in the blood of juvenile male alligators, whose testosterone serum levels were significantly lower compared to the control group of alligators living in organochlorines free lake, however the phallus size and the serum testosterone levels didn't correlate. Author hypothesis is, that studied alligators were exposed during embryonal development (Guillette et al., 1999). In another study, gonadal abnormalities in alligators were shown to be caused by o'p' DDE (metabolite of DDT), which have estrogenic and anti-androgenic activity (Milnes et al., 2005).

4.5 Birds

In wild birds, the exposure to EDCs has been associated with decreased fertility, hatching success and biased sex ratios, however it is difficult to demonstrate the exact relationship between EDCs and the physiological dysfunction detected in studied animals as field studies do not always support extrapolation from laboratory studies (Kubiak et al., 1989; Shugart, 1980). Thus even though correlation between the concentrations of EDCs and physiological effect had been demonstrated, EDCs can exhibit toxicity via non-endocrine mechanisms. For example, in addition to the endocrine activity of DDT, it has been shown to cause egg shell thinning in birds via disruption of calcium transport (Lundholm, 1997). Negative relationship between organochlorines and thyroid hormone serum levels was discovered in in Arctic breeding glaucous gulls (Verreault et al., 2004). A population of terns (*Sterna dougallii*) living in the PCB contaminated area in Massachusetts, US had biased sex ratios and ovo-testis were found in premature males, however the ovo-testis didn't persist until maturity (Hart et al., 2003). Another

example of feminization of male gulls was observed in St. Barbara Island in California, US. Authors suspected DDT contamination to be the cause of the effect and they successfully demonstrated that DDT injection into gull eggs induces the development of oviducts and ovotestis in male gulls (Fry and Toone, 1981).

4.6 Mammals

The effect of endocrine disruptors on wildlife mammals has been thoroughly studied since the publication of “Silent spring” by Rachel Carson in 1962. The exposure of populations of mammals to endocrine disruptors has been linked with decreased fertility in several studies; however mechanistic studies are often lacking to link these two conditions together. For example populations of seals (*Phoca vitulina*) has decreased between the years 1950 and 1975. One possible explanation of the population drop is explained by consumption of fish living in areas heavily contaminated by polychlorinated biphenyls (Reijnders, 1986). The effect of PCBs was then studied in the laboratory conditions using minx as surrogate animal to the seal and the evidence taken from the laboratory results supports the hypothesis, that the high PCB contamination and reproductive impairment seen in Baltic Seals are linked. Another hypothesis links the effects of several endocrine disruptors on polar bear populations. Blood samples from polar bears were collected in 1967 and compared to blood samples collected in 1993-1994. It has been suggested that reproductive impairment of females is linked to higher concentration of endocrine disruption in the area, however no laboratory study exposing polar bears to EDCs was carried out to confirm this theory (Derocher et al., 2003). Example of possible effect of endocrine disruptors on the Florida panthers (*Felis concolor coryi*) has been published in 1990 (Roelke, 1990).

4.7 Humans

The fact, that endocrine disruptors can have an effect on human health is nowadays widely accepted, but proving experimentally such effect is not easy due to ethical reasons. However it is possible to suggest an effect based on experiments done on animal models or human derived cell lines (Klopčič et al., 2015). Also epidemiological studies at population levels are important sources of information, giving us the possibility to study the trends in health of the populations over time. In addition, data obtained from areas of industrial accidents are very useful to understand the impact of endocrine disrupting compounds at the population level. Famous

example is the Seveso accident in 1976, when the population of Seveso was exposed to high amount of dioxins which resulted in the increase of the number of female offspring born to exposed parents as described in the chapter about Dioxins.

4.7.1 Effects of endocrine disruptors on female reproduction

Because the development and function of female reproductive organs is dependent on a series of biological processes, any factors, exogenous or endogenous, disrupting one of these processes can have serious consequences, which can predispose women to infertility, endometriosis or ectopic pregnancies. The first case of endocrine disrupting compound causing adverse effect on adult human female reproduction was documented in 1971. Daughters of women treated with DES during pregnancy suffered from severe vaginal adenocarcinoma (Herbst et al., 1971). Another example of pathology of the female reproductive tract is **polycystic ovarian syndrome (PCOS)**. This syndrome is characterized by persistent anovulation when follicles in the ova grow without selection of the dominant follicles, which causes accumulation of multiple follicles (Legro et al., 2006). The use of some personal care products containing androgenic molecules such as triclocarban were connected with PCOS (Chen et al., 2008). Several EDCs were connected to another pathology of female reproductive track **granulosa steroidogenesis** for example TCDD, DDE or BPA (Holloway et al., 2007; Kwintkiewicz and Giudice, 2008, 2009; Minegishi et al., 2003). Example of estrogen dependent disorder is **endometriosis**, causing severe pain especially during menstruation and can be responsible of infertility. Several studies suggest a connection between endometriosis and EDC, mainly DES, TCDD (data from rhesus monkeys) and phthalates (Missmer et al., 2004; Reddy et al., 2006; Rier et al., 2001).

The connection between endocrine disruptors (especially xenoestrogens) and **breast cancer** has been established (Davis et al., 1993; Ibarluzea et al., 2004). Prospective studies show a link between chemical exposure to DDT , toxaphene or some anti-inflammatory drugs like ibuprofen (Brasky et al., 2011; Cohn et al., 2007; Høyer et al., 1998).

4.7.2 Effects of endocrine disruptors on male reproduction

The male sexual differentiation is androgen and estrogen dependent and thus interaction with endocrine disrupting chemicals can have an adverse effect on male sexual axis development such as **testicular dysgenesis syndrome (TDS)** characterized by cryptorchidism, hypospadias, oligospermia, and testicular cancer (Skakkebaek, 2003). Several studies describe the effect of monobutyl phthalate on reduced sperm motility and concentration (Duty et al., 2003; Zhang et al., 2006). Also other EDC's were connected to low sperm quality and motility such as PCB's (Guo et al., 2000; Hauser et al., 2003), dioxins (Faure et al., 2014) and pesticides (Swan et al., 2003). Cryptorchidism (undescended testes) is the most common male urogenital tract malformation. It has been linked with prenatal exposure to DES (Martin et al., 2008) and pesticides (García-Rodríguez et al., 1996). **Prostate cancer** is the most common solid cancer in men and it was demonstrated that endocrine disruptors may have an effect on the prostate. The prostate contains both ER α and ER β receptors and prostate cancer has been linked with elevated estrogen levels (Modugno et al., 2001; Prins and Korach, 2008). In a large epidemiological study, the effect of pesticides on prostate of 90 000 men living in North Carolina and Iowa have been examined. This study showed a direct link between pesticides and higher prostate cancer counts (Alavanja et al., 2003).

5. Legislation on endocrine disruptors

After the Wingspread conference increased not only the interest in the research of endocrine disruptors but also the need for unified legislation. Several organizations focusing on the water quality (Water framework directive), health (World Health Organisation) or databases of chemicals (REACH) have risen. The OECD has been key in validating standard approaches to test for endocrine disruptors. The legislation is different in Europe to other parts of the world, therefore the next chapter focuses only on the European legislation.

5.1 European Water framework directive (WFD)

The so called “first wave” of early European water legislation begun in 1975-1980 by setting binding quality targets for drinking water in Europe and releasing “the Dangerous Substances Directive “. In a seminar in Frankfurt (1988), the “second wave” started and resulted in publication of several directives (The Urban Waste Water Treatment Directive, a new Drinking Water Directive, a Directive for Integrated Pollution and Prevention Control (IPPC)) between the years 1991 and 1996. The need for more global approach to water policy was evident and in 1996 the “Water Conference” was attended by number of member states representatives from water providers, industry, agriculture and environmental groups. The outcome of this conference was the **Water Framework Directive** proposal with aims of:

- expanding the scope of water protection to all waters, surface waters and groundwater
- achieving "good status" for all waters by a set deadline
- water management based on river basins
- a "combined approach" of emission limit values and quality standards
- getting the prices right
- getting the citizen involved more closely
- streamlining legislation

In 2000 the EU WFD was finally published and adopted (EU WFD, 2000).

WFD (annex X) currently monitors 11 priority hazardous substances (discharges of these compounds should be stopped within next 20 years), 14 priority substances under review and 8

priority substances (goal to significantly reduce the discharges of these compounds). Majority of the substances are (suspected) endocrine disrupters (Table 1.).

Table 1.

Number	Name of priority substance	Number	Name of priority substance
1	Alachlor	21	Mercury and its compounds*
2	Anthracene*	22	Naphthalene
3	Atrazine	23	Nickel and its compounds
4	Benzene	24	Nonylphenols*
5	Brominated diphenylether*		(4nonylphenol)*
	Pentabromodiphenylether	25	Octylphenols
6	Cadmium and its compounds*		(4(1,1',3,3'tetramethylbutyl)phenol)
7	Chloroalkanes, C1013 *	26	Pentachlorobenzene*
8	Chlorfenvinphos	27	Pentachlorophenol
9	Chlorpyrifos	28	Polyaromatic hydrocarbons*
10	1,2Dichloroethane		(Benzo(a)pyrene)*
11	Dichloromethane		(Benzo(b)fluoranthene)*
12	Di(2ethylhexyl)phthalate (DEHP)		(Benzo(g,h,i)perylene)*
13	Diuron		(Benzo(k)fluoranthene)*
14	Endosulfan*		(Indeno(1,2,3cd)pyrene)*
15	Fluoranthen	29	Simazine
16	Hexachlorobenzene*	30	Tributyltin compounds*
17	Hexachlorobutadiene*		(Tributyltincation)*
18	Hexachlorocyclohexane*	31	Trichlorobenzenes
19	Isoproturon	32	Trichloromethane (chloroform)
20	Lead and its compounds	33	Trifluralin

* Priority hazardous substances

5.2 REACH (Registration, Evaluation, Authorization and Restriction of Chemicals)

REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) is a European Union regulation established the 1 of June 2007 and it focused on the production and use of chemical substances and their impact on the environment and the health of humans and wildlife. It is a very strict collection of regulatory laws which affects chemical industry worldwide. For every compound, which is imported or produced in an amount larger than one ton per year, the chemical manufacturer has to submit a report stating the possible risk linked to their product to the European Chemicals agency to ensure that adequate safety data are available. The main goal of REACH is to register and monitor all chemical substances and address data and informational gaps. However, a chemical is required to be tested for potential endocrine disrupting activity, only if there is suspicion that it might have one. REACH requires information sharing between customers, sellers and manufacturers in order to ensure that adequate toxicological information is available (Foth and Hayes, 2008a, 2008b). The list of chemicals registered with the European Chemicals Agency is growing, up to date 13149 unique substances are registered. REACH has also generated a large amount of controversy as it has been estimated that enormous number of laboratory animals will be required in order to test all of the chemicals requiring registration. However efforts are underway to develop alternative tests (Grindon et al., 2008; Taylor, 2014).

5.3 The Organisation for Economic Co-operation and Development (OECD)

The OECD was founded in 1961 for the purpose of stimulating international trade and improving the economic progress. OECD is not involved in legislation, but it provides guidelines regarding several fields with its main focus being the trade and economics, however the mandate of the OECD also includes environmental and social sciences. Since 1996, the OECD has developed test guidelines and other tools to facilitate identification, risk assessment and management of chemicals with endocrine disrupting potential. The work of the OECD regarding endocrine disruptors is managed by Working Group of National Coordinators of the Test Guidelines Programme (WNT). The outcome of the OECD work was presented on the meeting “Workshop on OECD Countries’ Activities Regarding Testing, Assessment and Management of Endocrine

Disruptors” in Copenhagen in September 2009. The OECD test guidelines developed for testing of the endocrine disruption are described later in the introduction.

5.4 Risk assessment of Endocrine disruptors

Risk assessment of all chemicals including endocrine disruptors is a set of methodologies which focuses on the estimation of probabilities and magnitudes of adverse effects caused by endocrine disruptors. Prior to description of individual steps of the risk assessment, it is necessary to clarify, that the official definition of **hazard** is “anything what can cause harm (chemicals, electricity, working with dangerous pathogens), while **risk** is the chance, high or low, that some harm could be done by hazards, as well as indication of the seriousness of the consequences. Risk assessment is carried out in four steps which are: hazard identification, hazard characterization, exposure assessment and risk characterization (EC, 2000; WHO, 2009). During the first step, **hazard identification**, the compound of interest is identified, the database of REACH is often consulted and if there is no data available about the risk assessment of the compound of interest the second phase, **hazard characterization**, is applied. In this phase, scientific studies regarding the compound of interest are examined to find its connection to negative effects under the weight of evidence with special focus to its mode of action. It is not rare, that epidemiological studies based on the exposure of human population to the contaminant, are used in this step. The third phase, **exposure assessment**, can be carried out in parallel with the second phase. Dose-response relationships of the compound of interest and the severity and likelihood of adverse effects are studied. In exposure assessment, dose-response curves regarding human exposure to the chemical of interest rarely exist and animal testing is very common during this phase. Several OECD guidelines were validated to facilitate this step. Finally the last step of risk assessment, **risk characterization**, combines all acquired data from the three previous steps into a final report/advice for risk managers (Fig. 3) (EEA, 2011; Lammerding and Fazil, 2000; Williams and Paustenbach, 2002).

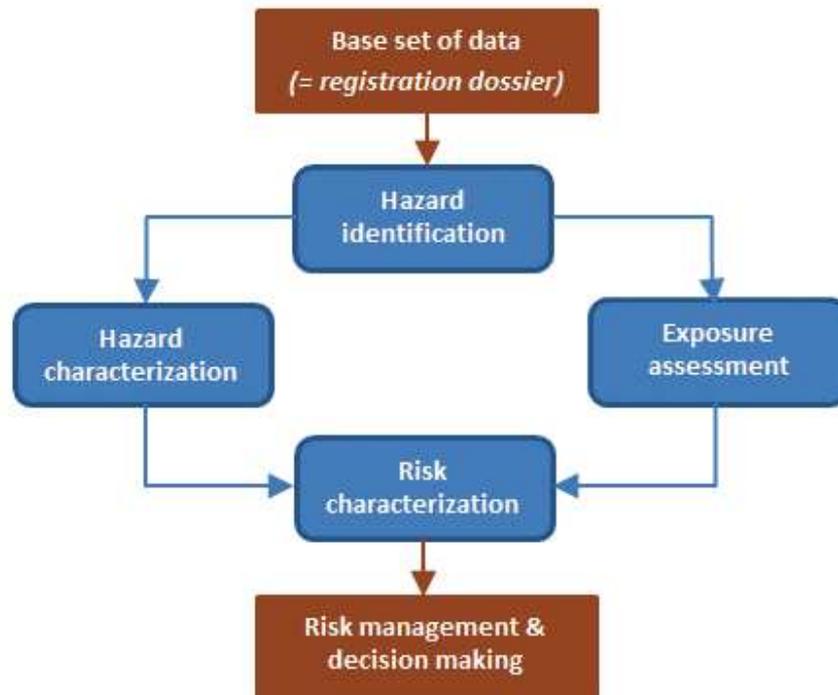


Figure 3. Ecological Risk Assessment. The four steps in Ecological risk assessment are hazard identification, Hazard characterization, Exposure assessment and Risk characterization.

INTRODUCTION

PART II.

**BIOASSAYS FOR DETECTING ENDOCRINE
DISRUPTORS**

Part II. Bioassays for detecting endocrine disruptors

Thousands of new chemicals were introduced to the market during the last decades, while the number increases every year and only a small fraction are characterized and monitored. To test the endocrine disrupting potential of these compounds, new *in vivo* and *in vitro* bioassays were developed. Bioassays are used to test the endocrine disrupting potential of chemicals, chemical mixtures or surface water samples. In order to carry out bioassays, whole intact organisms (*in vivo*) or cells and tissues are used (Fig. 4). Bioassays are often combined together to give a full spectra information about the possible endocrine activity or they serve as prescreening assays before chemical analysis in a method called Effect Directed Analysis. The bioassays can be based on cells or organisms which are genetically modified (transgenic) or wild-type. In case of transgenic bioassays developed to detect EDCs the DNA contains usually a construct composed of the promotor of a gene naturally upregulated in contact with a hormone of an axis of the interest driving the expression of a reporter gene such as gene for luciferase, green fluorescence protein or β -galactosidase. The endocrine activity is then visualized as a color change or induction of luminescence or fluorescence.

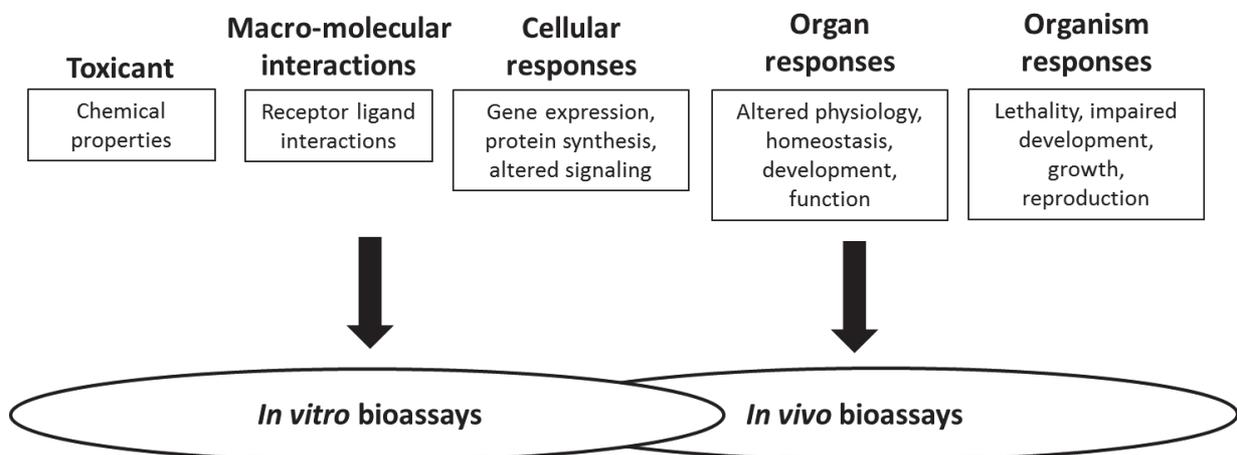


Figure 4. **Bioassays.** *In vitro* bioassays are chosen to study the effect of a toxicant on the molecular and cellular level, *in vivo* bioassays on the cellular, organ and organism level.

1. *In vitro* bioassays to detect endocrine disruption

In vitro bioassays use cells or tissues to determine biological activity of a sample of interest. Their endpoint is mostly naturally occurring signaling processes like activation of enzymatic or transcriptional processes or interaction of the ligand with its ligand binding domain. The major advantages of *in vitro* bioassays is their high throughput, low cost, rapidity and the fact that they do not use laboratory animals. However several aspects are limitations of *in vitro* tests for EDCs, in particular the fact that by definition endocrine hormones are produced in one tissue and act on another so many aspects of their synthesis, excretion, distribution, metabolism or transport are not taken into account in *in vitro* systems mimicking their target tissue. It is therefore recommended to combine *in vitro* and *in vivo* bioassays to assess the full potential of given endocrine disrupting hazard. Also non transgenic organisms can be used in bioassays, in that case physiological endpoints are measured such as mortality, growth inhibition, metabolism, gene expression, size, speed of development or behavior.

1.1 CALUX

One set of examples of *in vitro* bioassay for endocrine disruptors are the CALUX assays. These assays are cell-based reporter gene assays using transgenic cells. The cells harbor a transgenic construct consisting of synthetic promoters containing the consensus sequence of hormone response elements driving the expression of the luciferase gene. During the assay, CALUX cells are incubated in 96-well plates to form a homogenous layer and are then exposed to the compound, water sample or mixture of interest. If the promoter is activated, the production of luciferase is induced in a dose and time dependent matter. In order to induce luminescence, which can be quantified by a luminometer, the substrate of luciferase, luciferin, is added to the cells. The general principle of a CALUX assay is shown in Fig 5. To date CALUX assays for many of the endocrine pathways have been established, such as the ER-CALUX for detecting estrogens (Sonneveld et al., 2006), AR-CALUX to detect androgen disruption (van der Burg et al., 2010), GR-CALUX to detect glucocorticoid disruption, TR β -CALUX to detect thyroid disruption (Kolkman et al., 2013) or DR- CALUX specifically designed to detect dioxins and another organochlorine like compounds (Hoogenboom et al., 2006).

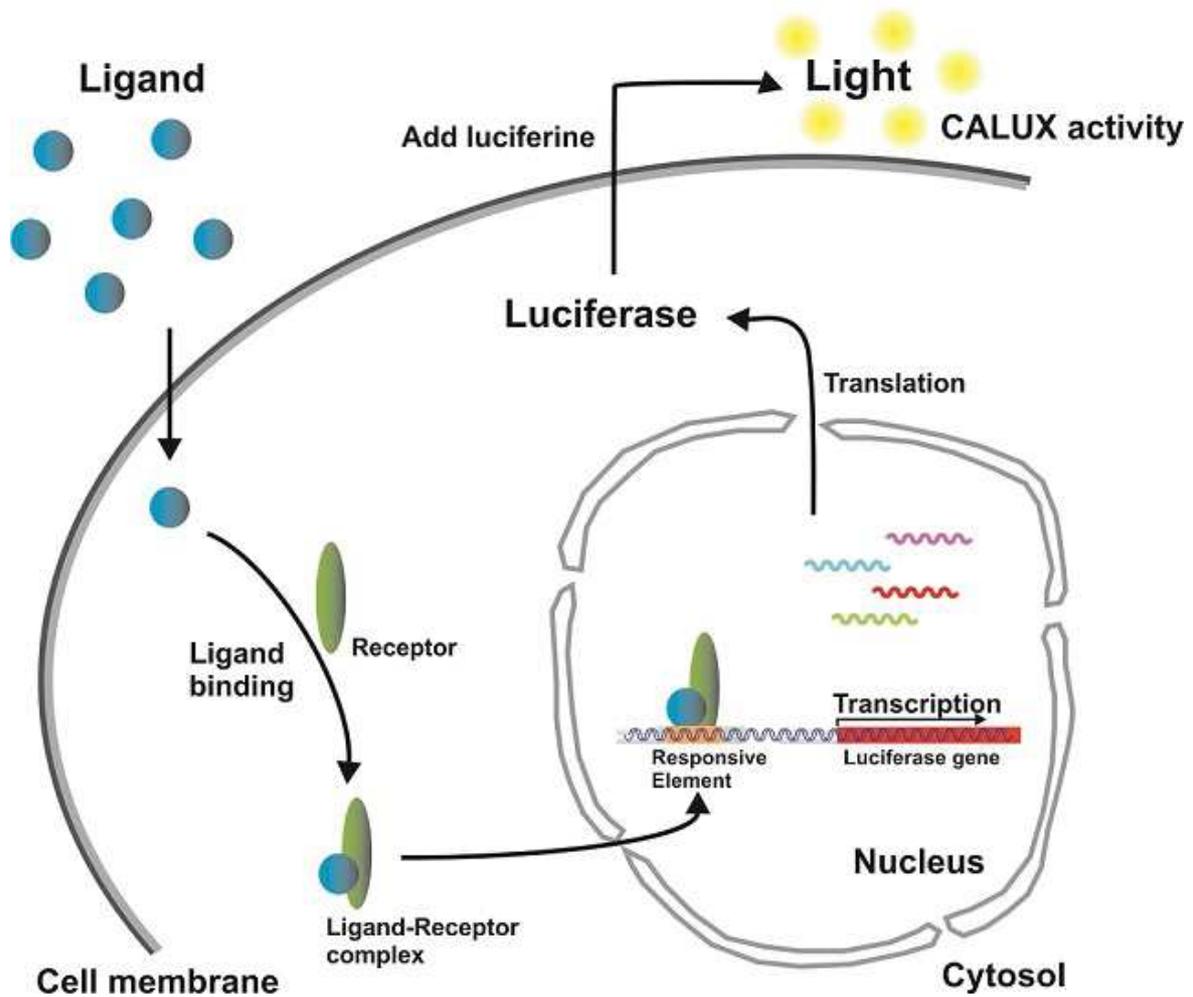


Figure 5. *CALUX assay*. The ligand-receptor complex binds to its responsive element in the nucleus and induces the transcription of luciferase gene. After the substrate of luciferase, luciferin, is added, luminescence is induced. From <http://hollandfoodpartner.com/calux/>.

1.2 YES/YAS assay

Another example of *in vitro* transcriptional bioassays used to detect endocrine disrupting activity, are the YES or YAS assays (**Y**east **E**strogen or **A**ndrogen **S**creen). The yeast used in this assay carries integrated human estrogen receptor DNA sequence and plasmids containing estrogen or androgen responsive elements (ERE/ARE) driving the expression of the lac-Z gene, which encodes the enzyme β -galactosidase. If an estrogenic or androgenic compound is present in the medium, β -galactosidase is synthesized and the medium changes color from yellow to red (Fent et al., 2006; Routledge and Sumpter, 1996).

1.3 Receptor binding assay

The ability of a test substance to interact and bind to the nuclear receptor (for example estrogen receptor) is the basis of ligand binding assays. In these assays, unlabeled test substances compete for receptor binding with radiolabeled natural ligands of the corresponding nuclear receptor (for example [H^3] 17 β -estradiol for the estrogen receptor). The receptors necessary for this assay can be obtained as a cytosolic or nuclear fraction from extracts of animal tissues by high speed centrifugation. A mix of labeled natural hormones and unlabeled test compounds are then incubated with the fraction. The unlabeled test compounds replace the labeled compound in a dose-dependent manner. The amount of unbound labeled compound can be then quantified by scintillation counting (Ankley et al., 1998; Gray Jr. et al., 1997). The disadvantage of this assay is the fact, that as it's not a cell-based assay, some chemicals can act as receptor ligands and give false positive results, although in a living organism they would not enter the cell.

1.4 Cell proliferation assay

An interesting example of a cell proliferation assay is the E-screen assay, which is performed using the MCF-7 cell line obtained from a woman suffering from a metastatic mammary carcinoma. This assay is used specifically to detect estrogenic compounds. Proliferation of MCF-7 cells is inhibited by human serum and this inhibition can be neutralized by adding estrogenic compounds, while other steroid compounds don't exhibit this effect. The cells are incubated in the presence of human serum with and without added test substance and the density (proliferation rate) of the cells is measured by spectrophotometry. As with receptor binding assays, the disadvantage of the E-screen is the possibility to obtain false positive results, as the MCF-7 cell proliferation can also be induced by various mitogens, cytokines or growth factors (Jones et al., 1997; Soto et al., 1995).

2. *In vivo* bioassays

In vivo bioassays are carried out using a variety of intact animal species, mostly transgenic or non-transgenic vertebrates such as rats, mice, frogs or fish. The use of laboratory animals is a controversial topic from the ethical point of view. As mentioned before in the chapter concerning REACH, thousands of laboratory animals are used every year to assess the risk of endocrine disruptors and there have been attempts to regulate the number. The *in vivo* bioassays can be carried out using aquatic (mostly fish and frogs) or non-aquatic organisms (mostly rats and mice). The OECD registers a number of developed test guidelines for *in vivo* screening or testing of chemicals for endocrine disruption which were published between the year 2007 and 2012. The list contains several tests carried out using aquatic organisms and also a variety of tests using non-aquatic species. Another 16 guidelines are in the progress of validation at this moment.

2.1 Non-aquatic *in vivo* bioassays

The advantage of *in vivo* bioassays which are using rodents as model organisms is the fact that rodents are mammals just like humans, so the results of the screening can be easily adapted to

humans due to similar physiology. On the other hand, the development of mammalian embryo happens in utero, which makes it impossible to observe adverse effects of chemicals during embryonal development without killing the mother. So far OECD validated three test guidelines using rodents (OECD 407, 440 and 441).

2.1.1 OECD 407

The Repeated Dose 28-day Oral Toxicity Study in Rodents (OECD 407) was adopted in 1995 and is used to determine the hazards of repeated administration of test compounds over a relative short time period of 28 days. It is not a specific *in vivo* assay for endocrine disruptors but it covers several endpoints specific for endocrine disruption. During this assay, rats are exposed to the test substance via food or water. The animals are observed every day in order to detect any signs of toxicity. Many factors are monitored and noted daily including the mortality, weight, reflexes, fur quality, hearing, sight, reaction to handling or unusual behavior. The observation usually last 14 more days after the end of 28 days lasting exposure, to detect any delayed effects. The animals are euthanized at the end of the assay and hematology and biochemistry exams are carried out.

2.1.2 OECD 440

The Uterotrophic Bioassay in rodents: A short-term Screening Assay for Oestrogenic Properties (OECD 440) was validated in 2007. During this assay, female rodents with a non-functional hypothalamic-pituitary-ovarian axis are used. This non-functionality occurs in two cases, immature females prior to puberty and adult females with removed ovaries. The animals receive a test compound daily administered dietary or by injection for three consecutive days. The test animals are euthanized 24 hours after the last dose and the increase in uterine weight or uterotrophic response is measured.

2.1.3 OECD 441

The Hershberger Bioassay in rats: A Short-Term Screening Assay for (Anti)Androgenic Properties (OECD 441) validated in 2009 is an assay use to identify androgenic and anti-androgenic substances as well as the inhibitors of 5 α reductase, enzyme responsible of conversion of testosterone to more potent androgen dihydrotestosterone. To carry out this bioassay,

castrated- peripubertal male rats are injected with test substances for consecutive 10 days. After the admission of last injection, test animals are euthanized and the changes in weight of five androgen-dependent tissues is measured: the ventral prostate, seminal vesicle (plus fluids and coagulating glands), levator ani-bulbocavernosus muscle, paired Cowper's glands and the glans penis. In the anti-androgen test, the test compound is injected together with a reference androgen.

2.2 Aquatic *in vivo* bioassays - non-transgenic

The most common model aquatic organisms used *in vivo* bioassays are fish (stickleback, minnow, medaka, zebrafish) and amphibians (*Xenopus laevis*). An important advantage of these species is their fast reproduction and the high numbers of offspring they produce. For example one medaka female can spawn up to 50 eggs a day repeatedly and one *Xenopus* female can spawn thousands of eggs in one day. This fact, in addition to the small size of the larvae, makes high scale experiments possible. Another advantage is that embryos of these species are almost transparent, which makes them excellent models to study organogenesis. The administration of the test substances is usually non-invasive, as the compound can be directly added to the water the animals are swimming in, which is also an advantage. Moreover, the legislation of the European union does not consider aquatic larvae as laboratory animals at stages where they feed on their maternal vitellus reserves (sac-fry or eleuthero-embryo stages). In the last years, several transgenic models to detect endocrine disruptors have emerged and more are currently being developed.

2.2.1 OECD 229 and OECD 230

The Fish Short Term Reproduction Assay (OECD 229) developed in 2009 is an assay originally designed for the fathead minnow (*Pimephales promelas*). During this assay, a couple is exposed to a test substance for 21 days and the effect of the test substance on the production of eggs is measured every day. At the end of the 21 day exposure, the levels of plasma vitellogenin, secondary sex characteristics and the weight of gonads is measured. A variation of the OECD 229 assay is the 21-Day Fish Assay: A Short-Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition (OECD 230). This guideline was validated for fathead minnow, zebrafish and medaka and the endpoints of this assay are also level of plasma

vitellogenin and secondary sexual characteristics. As there are no secondary sex characteristics in zebrafish, only the level of vitellogenin is considered.

2.2.2 OECD 231

The Amphibian Metamorphosis Assay (OECD 231) is an assay validated with *Xenopus laevis* and it is used to assess the disruption of the hypothalamo-pituitary-thyroid axis. Stage 51 tadpoles are exposed to the test substance for a period of 21 days, every 7 days the length of the hind-limbs of a subset of tadpoles is measured. After 21 days the developmental stage, snout-to-vent length and hind limb length are measured and the histopathology of the thyroid gland is observed.

2.2.3 AFSS assay

The AFSS assay (Androgenised Female Stickleback Screen) is a variant of OECD test guideline 230 validated for the three-spined stickleback (*Gasterosteus aculeatus*) and is used to detect anti-androgens. The assay is based on the fact, that stickleback males build nests during the breeding season using a glue like protein, Spiggin, which is produced by the kidneys. Spiggin production is androgen dependent. Stickleback adult females are exposed to test compound in co-treatment with androgen receptor agonist (17-methyltestosterone or DHT) for 21 days and after the end of exposure, Spiggin production in the kidneys of exposed individuals is measured using enzyme-linked immunosorbent assay (ELISA) (Katsiadaki et al., 2002, 2006).

2.3 Transgenic aquatic *in vivo* bioassays

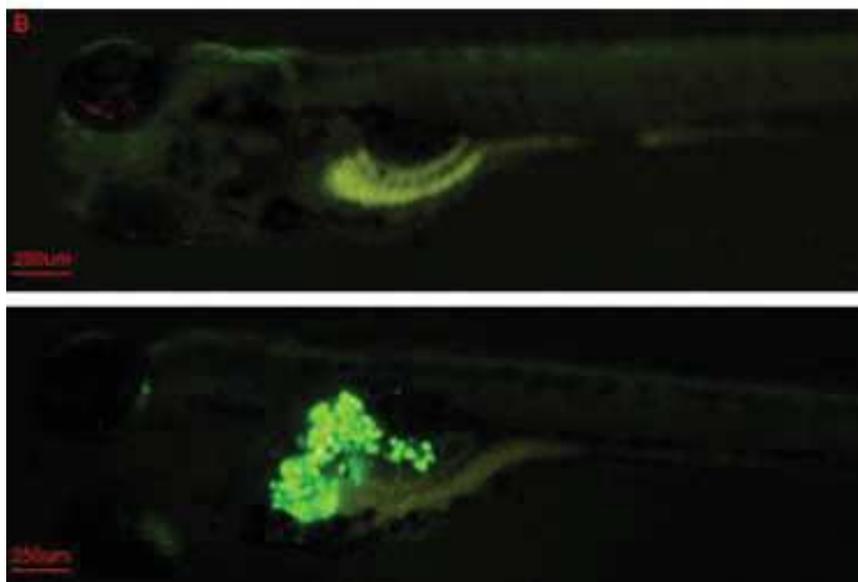
Vertebrate transgenesis is a process, when an exogenous cloned genetic material (transgene) is injected into the egg, where it integrates into the genome. As the genetic code is equal in all organisms, the same proteins will be coded by the integrated DNA regardless of the species. The subject raised from the egg then exhibits a novel feature for its species, which is transmitted to the offspring. The first *in vitro* transgenesis was performed in 1973 by Stanley N. Cohen and Herbert Boyer by introducing recombinant DNA into a bacterial cell (Cohen et al., 1973) and the first living transgenic organism (mouse) was created by Rudolf Jeanish in 1974. Jeanish and his colleague Beatrice Mintz injected simian virus 40 (SV40) viral DNA into mouse blastocysts,

which were implanted into foster females. Injected blastocysts developed into healthy adults in 40% of cases and the integration of SV40 DNA was confirmed by molecular hybridization (Jaenisch and Mintz, 1974). The fact that mouse is a mammal just like humans is a big advantage and also reason why mice are often chosen for transgenesis in human genetic diseases or cancer research, however the method is invasive and the number of offspring is limited. For environmental studies and chemical screening, fish and amphibians are more suitable model organisms (Lee et al., 2015; Winn, 2001). The most common fish species used for transgenesis are the medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*). The same traits that make these species well adapted to environmental studies also make them well adapted to transgenesis. They have transparent chorion, which allows easy injection of the transgene and the development of their embryos is very rapid. Since the 1990's a number of reporter genes have been used in the transgenesis of medaka and zebrafish, for example β galactosidase and green fluorescent protein (GFP) (Amsterdam et al., 1995; Lin et al., 1994; Peters et al., 1995). The GFP assays using the GFP gene cloned from the jellyfish *Aequorea victoria* don't require any substrate for detection, because the protein emits green fluorescence in response to excitation by blue light.

2.3.1 Estrogen responsive transgenic fish

An example of a transgenic zebrafish is the ***ERE-Luc transgenic zebrafish*** developed by Legler in 1999 (Legler et al., 1999). This stable line contains estrogen binding sequence linked to a TATA box and luciferase reporter gene and is used to detect (xeno-) estrogens. After an exogenous estrogenic substance binds to the estrogen receptor, production of luciferase is induced. After tissue lysis, the enzyme substrate luciferin is added which results in the production of luminescence. The disadvantage of this model is that the response to estrogens is not visible directly, as luciferin has to be added first to the lysate. Also the assay is invasive as the embryo has to be euthanized, thus no repeated measures are possible. An alternative reporter gene for the purpose of creating transgenic fish and amphibians is the GFP, which allows direct visualization of the signal as well as repeated measures. The larvae of fish and tadpoles are semi-transparent, allowing observation of the signal in intact embryos. An example of a model using GFP as a reporter gene is ***ere-zvtg1: gfp transgenic zebrafish*** which is used to detect (xeno-) estrogens (Chen et al., 2010). This model harbors a construct containing the zebrafish vitellogenin1 (*Zvtg1*) promoter driving the expression of the *gfp* gene. Vitellogenin is a gene produced by the liver and

then transferred to the ovaries by the blood stream where it accumulates in the eggs. The production of vitellogenin is naturally estrogen dependent and can be induced by exogenous estrogens. This model also contains ERE consensus in order to increase the sensitivity of the model. The fluorescence is present in the liver of mature adult females and can be induced in males and premature females by estrogen treatment (Fig. 6.).



*Figure 6. **Ere-zvtg1: gfp** transgenic zebrafish. The upper image shows a control **Ere-zvtg1: gfp** transgenic zebrafish, while the lower image shows an **Ere-zvtg1: gfp** transgenic zebrafish exposed to estrogen treatment. The GFP in exposed fish is expressed in the liver (Chen et al., 2010).*

A similar model to the previously described *ere-zvtg1: gfp* transgenic zebrafish is the *Mvtg1-gfp* transgenic medaka (Zeng et al., 2005). In this model the GFP coding sequence is under the control of the medaka vitellogenin 1 gene. The expression of fluorescence is in the liver of adult females and can also be induced by the treatment of estrogens in males and premature larvae. The lowest observed effect of E2 is 500ng/L in live fish and 50-100ng/L directly in the liver after dissection. Other models working on the similar principle were developed- *Chg-H* and *Chg-L GFP* transgenic medaka. Choriogenins are produced in the liver and transported to the ovaries by the blood stream where they serve as important precursors of the egg envelope (Kurauchi et al.,

2005; Ueno et al., 2004). Another example of a transgenic fish used to detect estrogenic compounds is *cyp19a1b-gfp* (aromatase B) transgenic zebrafish (Tong et al., 2009). The enzyme aromatase is responsible for conversion of testosterone to estradiol and is expressed in the brain and in gonads. In this model, aromatase *cyp19a1b* promoter is driving the expression of GFP and the fluorescence is visible in the brain of the fish (Fig. 7.) (Brion et al., 2012).

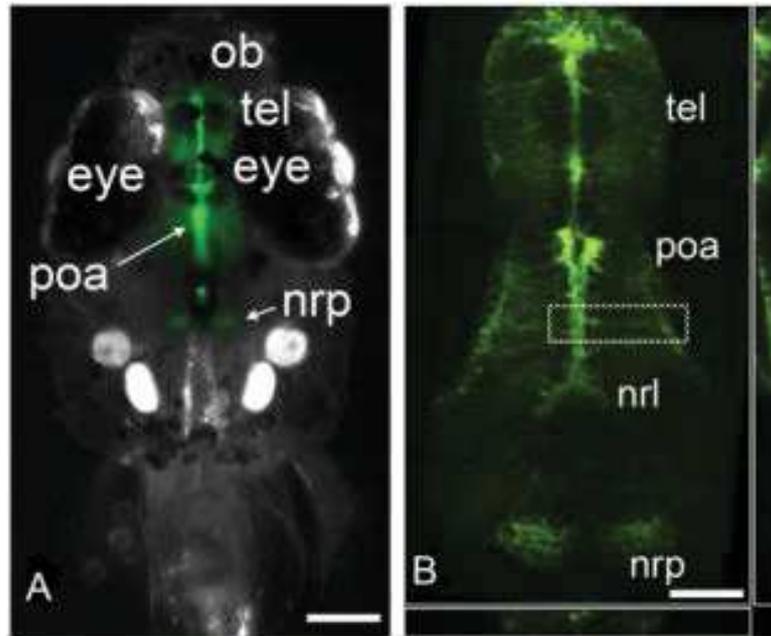


Figure 7. Cyp19a1b-gfp transgenic zebrafish. A. GFP signal visible in the brain of Cyp19a1b-gfp zebrafish exposed to 10 nM E2. B. High resolution confocal image of telencephalon of zebrafish exposed to estrogens. Telencephalon (tel), preoptic area (poa), nucleus recessus posterioris (nrp), olfactory bulb (ob), nucleus recessus lateralis (nrl) and nucleus recessus posterioris (nrp) (Brion et al., 2012)

One of the most interesting models responsive to estrogens is the double transgenic *3ERE-Gal4ff/UAS-GFP* system in zebrafish. The *ERE-Gal4ff* transgene of this line is composed of three synthetic EREs, TATA box, a *Gal4ff* reporter and two *Tol2* elements. The *UAS-GFP* transgene contains five tandem repeats of the *UAS-Gal4* binding sequence driving the expression of *EGFP* reporter. After chemical-bound ER binds to an ERE, *Gal4ff* is activated and *Gal4ff*

protein binds to the UAS response elements to induce EGFP. The fluorescence of this model can be detected in the heart, liver, eye lens, cranial muscle, somite muscles and neuromast. Several estrogenic chemicals induced different tissue patterns of GFP expression, for example fluorescence in the group of larvae exposed to BPA was enhanced in the heart and cranial muscles while in the case of nonylphenol, fluorescence was enhanced in cranial and somite muscles (Fig. 8.) (Lee et al., 2012).

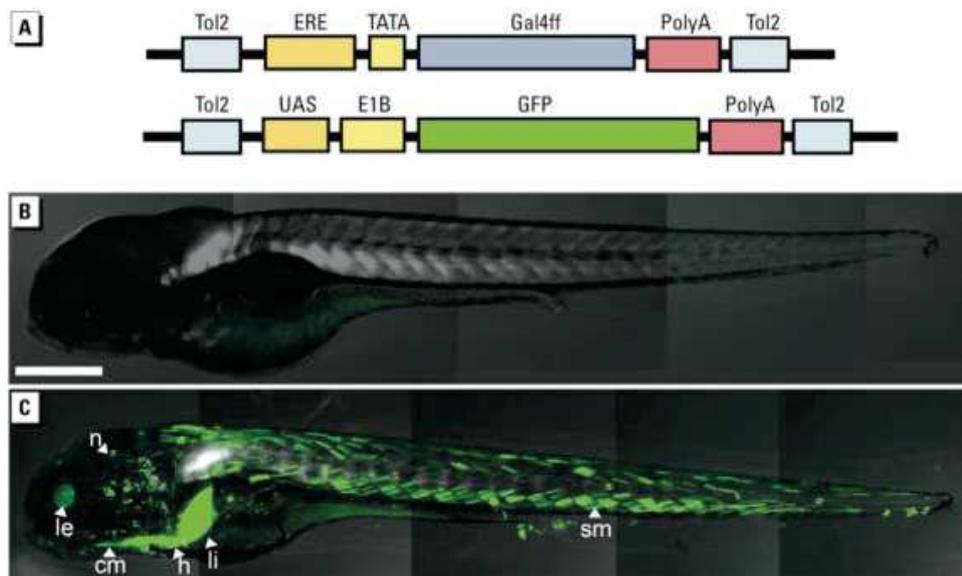


Figure 8. 3ERE-Gal4ff/UAS-GFP transgenic zebrafish. A. The ERE-Gal4ff transgene composed of three synthetic EREs, TATA box, a Gal4ff reporter and two Tol2 elements and the UAS-GFP transgene contains five tandem repeats of the UAS-Gal4 driving the expression of the GFP. B. Control 3ERE-Gal4ff/UAS-GFP transgenic zebrafish. C. 3ERE-Gal4ff/UAS-GFP transgenic zebrafish exposed to estrogens with GFP signal induced in heart (h), liver (li), eye lense (le), cranial muscle (cm), somite muscles(sm) and neuromast (nm) (Lee et al., 2012).

Also another estrogen responding model shows tissue specificity of the signal. The **5xERE:GFP** zebrafish line developed by Gorelick and Halpern in 2011 contains five consecutive elements upstream of a *c-fos* minimal promoter and green fluorescent protein (GFP). The signal is visible in the brain, liver, ventral fin near cloaca, eye lens and heart. Authors tested 11 known estrogenic substances with different expression profiles, for example BPA induced fluorescence in the liver and heart and PPT induced fluorescence in the liver and the brain (Gorelick and Halpern, 2011).

2.3.2 Glucocorticoid responsive transgenic fish

A transgenic *SR4G* zebrafish reporter line with six glucocorticoid response elements (GRE) driving the expression of GFP was developed by Krug et al., 2014 in addition to transgenic zebrafish containing nine GRE upstream of GFP coding sequence (Benato et al., 2014). The fluorescence after the exposure to glucocorticoid is visible in several organs known to be responsive to glucocorticoids, such as heart, brain or liver. Another assay to detect glucocorticoids is the **Grizly** assay (**G**lucocorticoid **R**esponsive **I**n **v**ivo **Z**ebrafish **L**uciferase **a**ctiv**Y**) (Weger et al., 2012). This model contains four GRE elements driving the expression of luciferase gene.

2.3.3 Thyroid hormone responsive transgenic tadpoles

Because thyroid hormones are a crucial factor for tadpole metamorphosis, frogs are perfect model organisms to test thyroid axis disruption. The transgenic *THbZIP-GFP Xenopus laevis* model was developed containing the promoter of the leucine zipper transcription factor THbZIP driving the expression of GFP. *THbZIP* is upregulated during tadpole metamorphosis and can be prematurely induced by exogenous thyroid hormones. The GFP signal after thyroid hormone treatment is visible in the head (mainly in the brain) (Fig. 9.) (Fini et al., 2007).

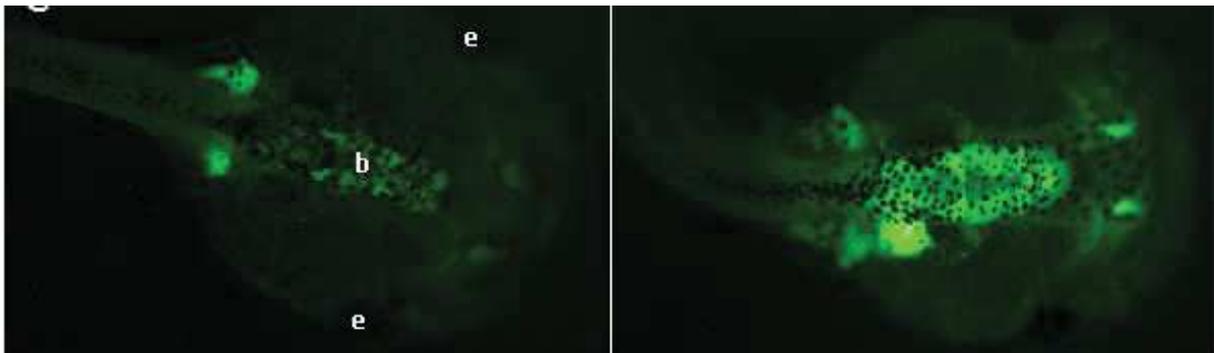


Figure 9. THbZIP-GFP transgenic Xenopus laevis. On the left TH/bZIP-GFP transgenic tadpole treated for 72 hours with hormone-free control solution, on the right TH/bZIP-GFP transgenic tadpole treated for 72 hours with 3.25 µg/L T3. b, brain; e, eye;)

INTRODUCTION

PART III.

HORMONAL CONTROL OF REPRODUCTION

PART III. Hormonal control of reproduction

Reproduction of vertebrates is dependent on the right function of the hypothalamo-pituitary-gonadal axis (HPG). In this chapter, the main factors involved in the HPG axis function such as hormones, their receptors, regulation or metabolism are reviewed as well as their interaction leading to the negative feedback loop responsible for the regulation of HPG axis (Fig. 10). Because the HPG axis is highly conserved in vertebrates, human HPG axis is described in this chapter (Ankley and Gray, 2013).

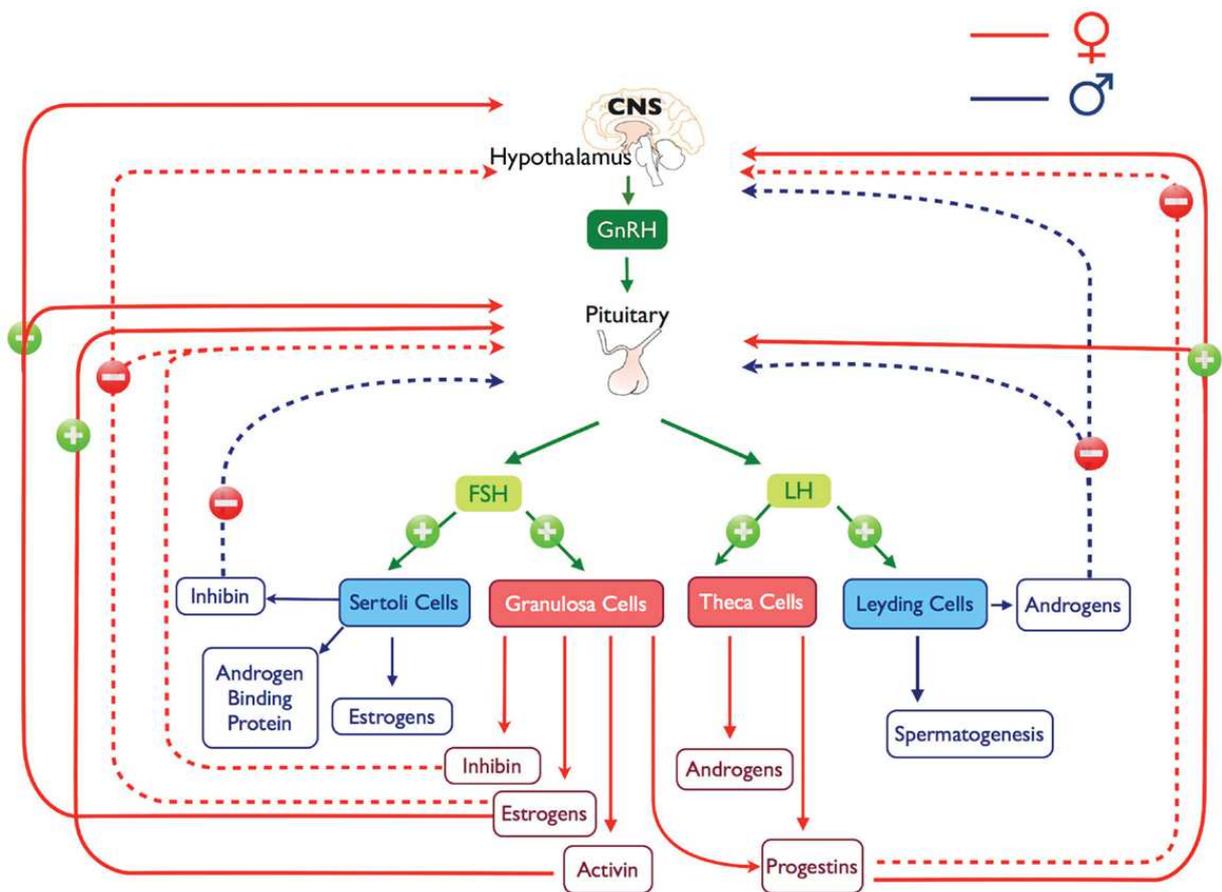


Figure 10. Hypothalamo-pituitary gonadal axis. Positive and negative feedback and products involved in the regulation of the HPG axis (Durán-Pastén and Fiordeliso, 2013).

1. Gonadotropin releasing hormone GnRH and its receptor

GnRH is a trophic peptide hormone discovered by Andrew Shally in 1970's and it consists of ten amino acids highly conserved for millions of years during evolution. It exists in eleven naturally occurring variants identified in various species. GnRH can be found in most of the species in two isoforms, hypothalamic form, which structure varies amongst the species and highly conserved extra-hypothalamic form having neuromodulator function (Jones et al., 1997). The *gnrhl* gene, coding for the GnRH precursor, is located on chromosome 8 in humans. Similar to other neuropeptides it is synthesized as a large prohormone consisting of 92 amino acids and it is enzymatically cleaved inside the secretory granules into the GnRH decapeptid, a 23 amino acid signaling sequence responsible for direction of intracellular packaging and secretion, a Gly-Lys-Arg proteolytic processing site and a 56 amino acid GnRH-associated protein (GAP) (Wetsel and Srinivasan, 2002).

GnRH is secreted mainly by hypothalamic neurons. Only several thousands of GnRH neurons are present in the arcuate nucleus of the medial basal hypothalamus in humans, surprisingly few considering their critical function: the regulation of synthesis of gonadotropin hormones in the anterior pituitary (Schwanzel-Fukuda and Pfaff, 1989). GnRH secretion is not continuous but pulsatile. In a set of experiments carried out on primates, pulsatile excretion was exchanged for continuous excretion, resulting in arrest of gonadotropin secretion, potentially caused by uncoupling of the GnRH receptor (Knobil, 2005).

The gonadotropin releasing hormone receptor (GnRHR) belongs to the rhodopsin family of G protein-coupled, seven-transmembrane receptors (Sealfon et al., 1997). After the ligand is bound to the GnRHR, the G protein is activated (mainly G_q and G_{11}) which continues with the activation of phospholipase $C\beta$, calcium release and thus the promotion of the activity of protein kinase C (PKC) and calcium/calmodulin kinase II. GnRHR activation is followed by the activation of several branches of MAPK (mainly JNK and ERK1/2) (FUJUN LIU, 2002; Haisenleder et al., 2003; Stanislaus et al., 1997). The activation of PKC and MAPK then stimulates the expression of gonadotropins (Fig. 11). As with *gnrh*, *gnrhr* is expressed outside the hypothalamus, in fact *gnrhr* were found in various tissues (Cheung and Wong, 2008).

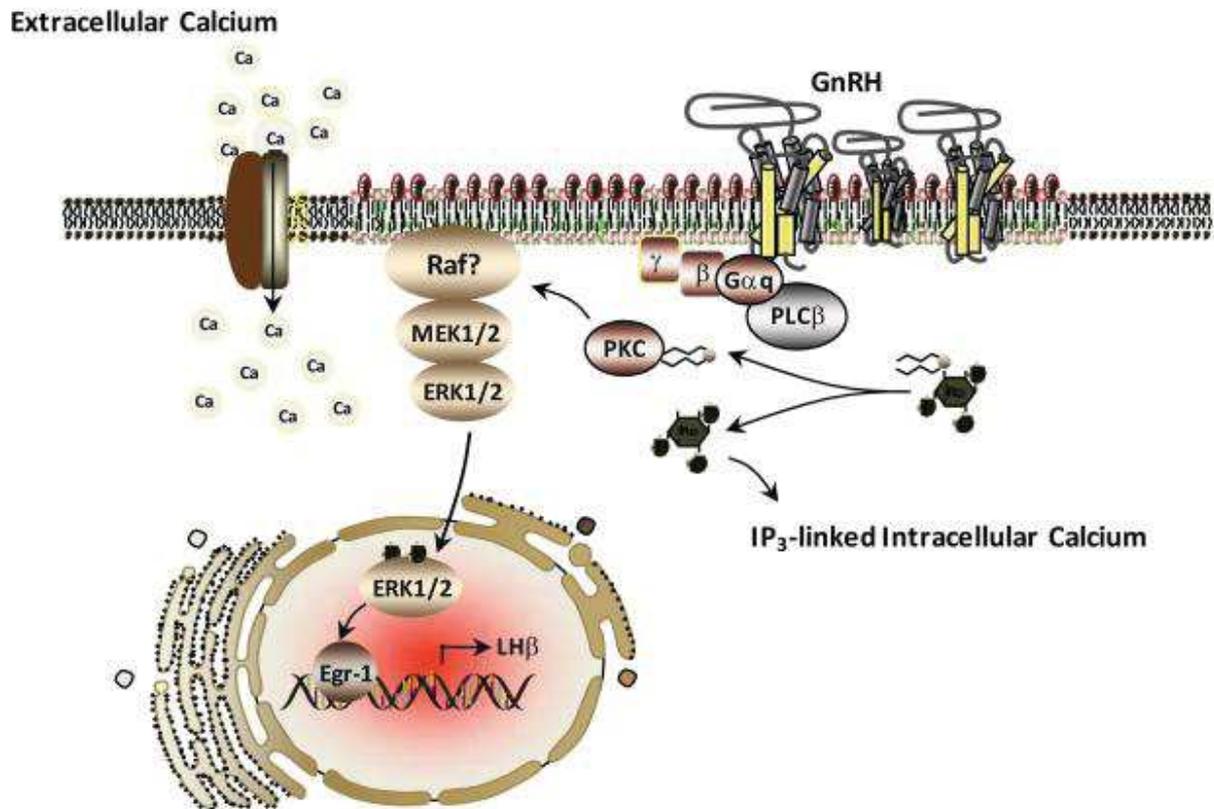


Figure 11. **GnRH signaling pathway.** Ligand activates the G protein in the GnRHR. This event is followed by PLCβ activation or calcium release. PLCβ in turn activates Protein kinase C (PKC) which then activates several branches of MAPK (mainly JNK and ERK1/2) leading to the expression of gonadotropins (Bliss et al., 2010).

1.1 Regulation of secretion of GnRH and expression of GnRHR

One of the factors regulating the secretion of GnRH is the polypeptide kisspeptin and its receptor G protein coupled 54 (GPR₅₄) (Sukhbaatar et al., 2013). Kisspeptin is secreted by neurons which can be found in the hypothalamic anteroventral periventricular nuclei and its primary function is to stimulate the secretion of GnRH and secondary function is the direct stimulation of gonadotropin secretion in the pituitary gland (Choe et al., 2013; Gutiérrez-Pascual et al., 2007). The expression of the gene for kisspeptin *kiss1* is directly regulated by estrogens and androgens and the kisspeptin neurons are part of steroid hormone negative feedback (Watanabe et al., 2014).

Several hormones directly affect the secretion of GnRH, mainly estradiol. The estrogen receptor beta (ER β) is expressed in the neurons which secrete GnRH. In studies, where females naturally lacking GnRH were treated with GnRH prior to estrogen treatment, the estradiol inhibited the induced gonadotropin synthesis (Marshall et al., 1983). The concentrations of estradiol in female body depends on the follicular stage, low concentrations of E2 have a negative feedback on the synthesis of gonadotropins, but increasing amount of E2 results in the pre-ovulatory surge of LH. The estradiol acts on the hypothalamus and pituitary glands. In the pituitary E2 increases the expression of GnRHR, which increases the sensitivity to GnRH (Ferin et al., 1979; Nakai et al., 1978; Turzillo et al., 1998).

The expression of GnRHR is under the control of its ligand GnRH but also as mentioned above by steroid hormones like estrogens, androgens and progestins (An et al., 2005; Rispoli and Nett, 2005). Pulsatile GnRH stimulates the expression of GnRHR however continuous release leads towards inhibition of GnRHR expression. It has been demonstrated that cortisol reduces the GnRH pulse frequency and therefore has an effect on GnRHR expression (Oakley et al., 2009).

2. Gonadotropins LH and FSH

Gonadotropin hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are glycoprotein polypeptide hormones produced by gonadotrophs located in the anterior pituitary. Both hormones have crucial role in the regulation of many pathways involved in the reproduction, and the disruption of these pathways can lead to reproduction problems, infertility, cancer or sex reversal (Table 2).

In female gonads gonadotropins are responsible for the growth and maturation of the ovarian follicle, ovulation and steroid synthesis. FSH is responsible for follicle growth and for the promotion of transcription of genes encoding growth factors (Sullivan et al., 1999). FSH also induces the transcription of the LH receptors gene. LH receptors are located in the membrane of granulosa cells and are crucial for maintaining the maturing dominant follicle from diminution (Zelevnik and Hillier, 1984). LH plays an important role in the offset of ovulation by restarting the meiosis in the oocyte and by induction of enzymes necessary for the rupture of the follicle followed by the luteinization of granulosa cells (Espey, 1974).

FSH and LH work in synergy to regulate the levels of steroid hormones in bloodstream. FSH stimulates the expression of aromatase, the enzyme converting testosterone to estradiol and LH

induces the secretion of 17α -hydroxylase in thecal cells leading to conversion of cholesterol and pregnenolone into androgens (Erickson et al., 1985; Hillier et al., 1994; Richards, 1994).

In male gonads, gonadotropins play a crucial role in spermatogenesis and steroidogenesis. FSH stimulates spermatogenesis by induction of androgen binding protein (ABP) in Sertoli cells and LH is responsible for the stimulation of testosterone synthesis by Leydig cells, while the main inducer of spermatogenesis is FSH. Therefore qualitatively and quantitatively normal spermatogenesis is dependent on the supply of testosterone in addition to FSH.

Table 2.

	FSH	LH
Female	<ul style="list-style-type: none"> – Follicle growth – Promotion of the transcription of growth factors – Induction of the transcription of LH receptors at the membrane of granulosa cells – Stimulation of the transcription of aromatase 	<ul style="list-style-type: none"> – Induction of ovulation by restarting of meiosis – Induction of enzymes necessary for the rupture of the follicle – induction of the secretion of 17α-hydroxylase
Male	<ul style="list-style-type: none"> – Stimulation of spermatogenesis – Stimulation of inhibin transcription 	<ul style="list-style-type: none"> – Stimulation of testosterone synthesis by the Leydig cells

2.1 Regulation of the gene expression of the gonadotropins LH and FSH

Both hormones are dipeptides with common α subunit and a unique β subunit. In addition to the gonadotrophs, the α subunit is also produced in the thyrotrophs and is crucial for the synthesis of thyroid-stimulating hormone and in the placenta, where it contributes to the synthesis of chorionic gonadotropin. It is important to note that the synthesis of LH and FSH is dependent on well-coordinated regulation of the transcription of separate genes, while the α subunit is usually

transcribed in excess, the β subunits are the limiting factor in LH and FSH production (Thackray et al., 2010).

As mentioned in previous chapter, GnRH regulates the expression of gonadotropins in a pulsatile manner. During the slow pulses, more FSH is produced while fast pulses induce the expression of LH (Tsutsumi and Webster, 2009).

After the binding of GnRH to its receptor GnRHR (which is located on the surface of gonadotroph cells) a range of signaling cascades is initiated. GnRH stimulated PKC are mediators of several MAPK cascades (ERK, JNK and p38), which initiate transcriptional activation of genes coding the gonadotrophic subunits by phosphorylating both cytoplasmic and nuclear proteins. The induction of LH β begins by induction of early gene *Egr-1* which interacts with tissue specific and ubiquitous basal factors and regulates the promoter of LH β . In the proximal promoter, two Egr-1 sites are coupled with two sites for SF-1 (steroidogenic factor-1) located at the both sides of a homeodomain element (HD), which is a target of pituitary-specific HD protein, Ptx1 and it is the synergy between SF-1, Egr-1 and Ptx1, which is plays the essential role in the LH β induction by GnRH (Shupnik, 1996; Thackray et al., 2010) (Fig. 12).

The FSH β gene is regulated by GnRH through induction of the GnRH responsive element activator protein-1 (AP-1) located within the proximal FSH β promoter, which consists of dimers of several Fos and Jun isoforms, (especially c-fos, junB, c-Jun and FosB), which bind together after the induction by MAPK and induce the FSH β promoter (Kraus et al., 2001).

Another molecule responsible for the regulation of the synthesis of gonadotropins is activin. It is a dimer composed of two β subunits and there are two types of activin receptor I and II. After binding to the receptor II, receptor I is phosphorylated and heterodimerised and the phosphorylation of Smad 2 and Smad 3 occurs. These phosphorylated smads bind and phosphorylate Smad 4 and activate transcription of target genes. Both LH β and FSH β promoters contain activin response elements (ARE) although LH β is far less dependent on activin regulation than FSH β .

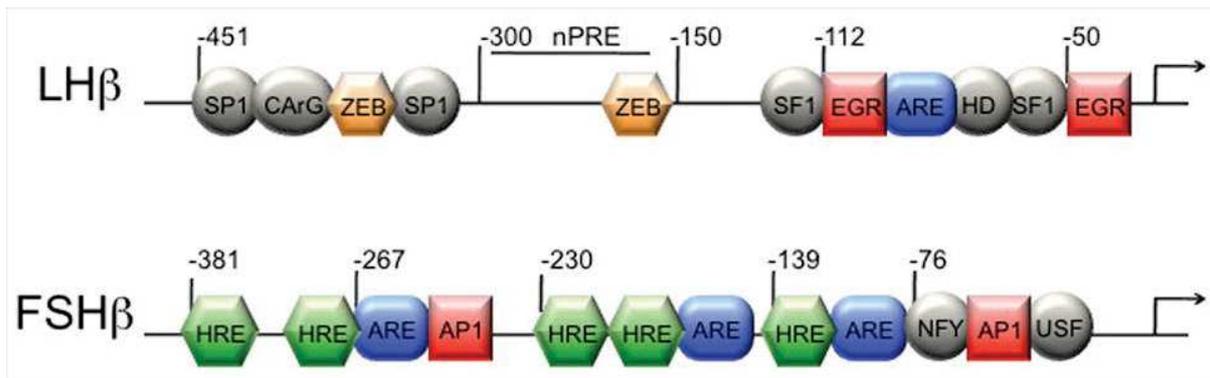


Figure 12. Promoters of LHβ and FSHβ and the binding sites of transcription factors involved in their induction. Circles and ovals illustrate binding sites for factors involved in basal expression, which interact with factors or sites involved in hormonal regulation. Factors involved in GnRH are indicated by squares. Hormone response elements, which are indicated as hexagons are sites involved in steroid hormone regulation. Round rectangles are labeled activin responsive elements (Thackray et al., 2010).

Steroid hormones, mainly estrogens and progesterone are involved in gonadotropin regulation in the hypothalamus and anterior pituitary by both positive and negative feedback. During the follicular phase of ovarian cycle, E2 is suppressing the levels of LH. Several studies demonstrated, that in animals with removed ovaries the levels of FSH and LH increased, which can be averted by exogenous administration of E2 (Ross et al., 1970; Shupnik et al., 1988). A study suggested that estrogen receptors (ER) are responsible for the LH level decrease, as knock-out of ER causes an increase in LH levels (Glidewell-Kenney et al., 2008). On the other hand, E2 is responsible for the gonadotrophin surge which leads to the rupture of Graffian follicle and ovulation followed by the creation of corpus luteum in humans. E2 directly induces the LHβ transcription by recruitment to the LHβ promotor through the interaction with SF-1 and Ptx-1 (Luo et al., 2005) or indirectly by enhancing the expression of Egr-1 (Kim et al., 2014). To date, no study demonstrated direct FSHβ induction by E2 and it is suggested, that E2 is involved in the FSH synthesis only indirectly via regulation of GnRH and activin.

Progesterone levels are higher in the estrous cycle at the same time as the transcription of LHβ decreases, which suggests that progesterone has an inhibitory effect on LHβ synthesis

directly at the transcriptional level or via alteration of GnRH synthesis. Inhibition of LH β transcription by progesterone is connected to the region -300 to -150 on the LH β promoter and that the suppression of LH β is happening via indirect binding of PR on the LH β promoter (Kanasaki et al., 2012).

Inhibins are heterodimeric glycoproteins produced by Sertoli cells in the testes and by the granulosa cells of ovary. Their primary role is the inhibition of FSH synthesis via negative feedback. Inhibins are antagonists of activin. As mentioned before, inhibin expression is induced by FSH itself and reduced by LH (Luisi et al., 2005; Ramaswamy et al., 2003).

2.2 LH and FSH receptors

The FSH and LH receptors are G protein-coupled receptors. In males they are located in the membrane of Sertoli cells and in females they are expressed in the granulosa cells. The expression of FSH receptor is strictly limited to the gonads, unlike in the case of LH receptor, which can be found in the variety of tissues and organs. The LHR is located at the cytoplasmic membrane of Leydig cells in the testis and on the granulosa, thecal and luteal cells in the ovaries. It can be also found in extra gonadal tissues like uterus, prostate, breast, thyroid or brain (Frazier et al., 1990; Meduri et al., 1997; Zhang et al., 2001). LHR is also binding chorion gonadotropin hormone (CGH) with high affinity. Both receptors consist of three domains, a transmembrane domain of seven hydrophobic helices, an intracellular domain coupled to G proteins, and an extracellular domain. After receptor activation of the extracellular domain, the intracellular domain induces a number of signaling cascades which lead to specific biological response. The receptor changes conformation once bound and couples with G proteins, which result in the induction of adenylyl cyclase and stimulation of the production of cAMP. Intracellular cAMP activates PKA (phosphokinase A), which is the leading factor in phosphorylation of structural proteins, enzymes and transcriptional activators (A R Means et al., 1980). In addition, both hormones also increase the calcium concentration in target cells and LH is not only activating the cAMP signaling pathway, but also PLC (phospholipase C) signaling pathway (Gudermann et al., 1992a, 1992b) (Fig.13).

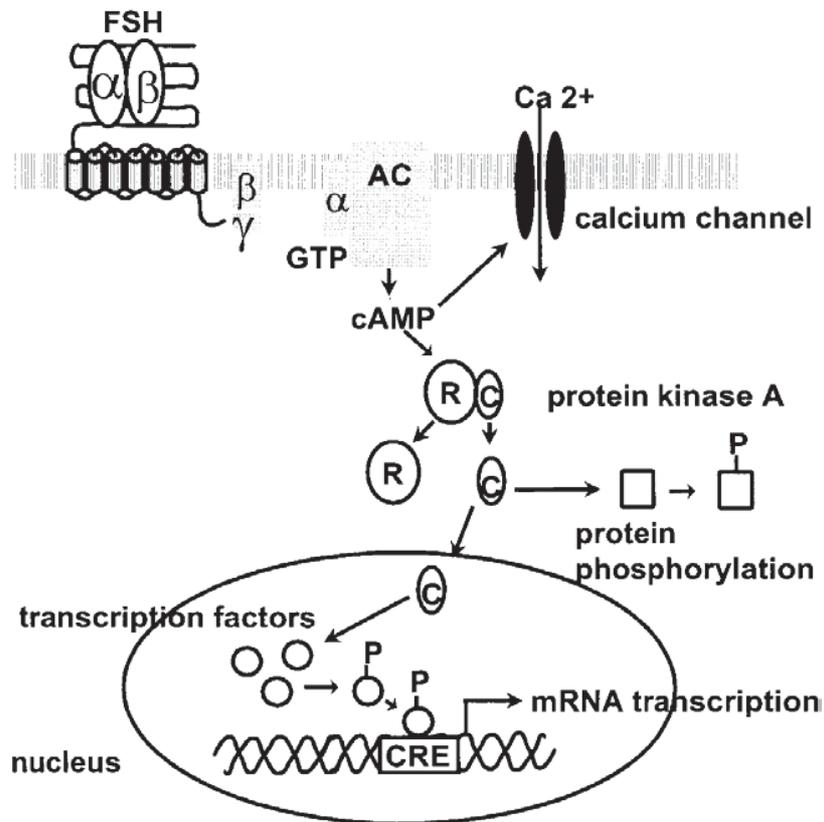


Figure 13. Signalling pathway of the FSH receptor. After FSH binds to the FSH receptor, the G subunit associates with GTP and activates adenylcyclase which leads to cAMP synthesis. This leads to dissociation of the catalytic subunit (C) from the regulatory subunit (R). Active catalytic site then activates proteins by phosphorylation or phosphorylates transcription factors in the nucleus which leads to mRNA synthesis (A R Means et al., 1980).

The expression of FSHR mRNA and receptor binding is dependent on several factors with FSH levels being the major one by downregulating the expression of FSHR gene in a time and dose-dependent manner (Themmen et al., 1991). Estrogen alone has no effect on the expression of FSHR nor receptor binding to granulosa cells directly, however it works in synergy with FSH and increases the number of FSHR probably by inducing the proliferation of granulosa cells (Richards, 1980). Activin increases the number of FSHR and prevents the downregulation of FSHR gene induced by FSH at the concentration 100 ng/mL and has the opposite effect at lower concentrations (3 – 30 ng/mL) (Nakamura et al., 1995; Tano et al., 1997). Several growth factors,

which are produced locally in granulosa cells also seem to affect FSHR levels. TGF β increases the levels of FSHR mRNA whereas TGF α and EGF reduce the number of FSHR by reducing the levels of FSHR mRNA and reducing the FSH- binding sites respectively (Findlay, 1993; L Dunkel, 1994). The expression of LHR is increased due to follicle growth induced by FSH and estrogens in the ovarian cycle (Zelevnik and Hillier, 1984). LHR is downregulated by its own ligand LH during the preovulatory LH surge by desensitization of G coupled response domain (Hunzicker-Dunn et al., 1979).

3. Steroidogenesis of androgens and estrogens

Steroidogenesis is the process of cholesterol conversion to steroid hormones. In this chapter, only the steroidogenesis of androgens and estrogens will be described, however the steroid hormone family consist of five groups which regulate various developmental and physiological processes: androgens, estrogens, progestogens, corticosteroids and mineralocorticoids. Cholesterol can be synthesized *de novo* from acetate, but most cholesterol is produced from plasma low density lipoproteins (LDL) from dietary intake which are transported in the cells via endocytosis (Miller, 1988). The first step in steroid hormone synthesis is the increased expression of steroidogenic acute regulatory protein (StAR) in response to trophic stimulation. In the case of the androgen and estrogen synthesis pathways this trophic stimulation is LH binding to LHR in Leydig and granulosa cells in the testes and ovaries respectively (Clark et al., 1994). StAR is responsible for the transport of cholesterol from the cytoplasm, through outer mitochondrial membrane to inner mitochondrial membrane, where it undergoes cleavage by enzyme P450_{ss} (cholesterol side chain cleavage enzyme) to form pregnenolone (Fig. 14).

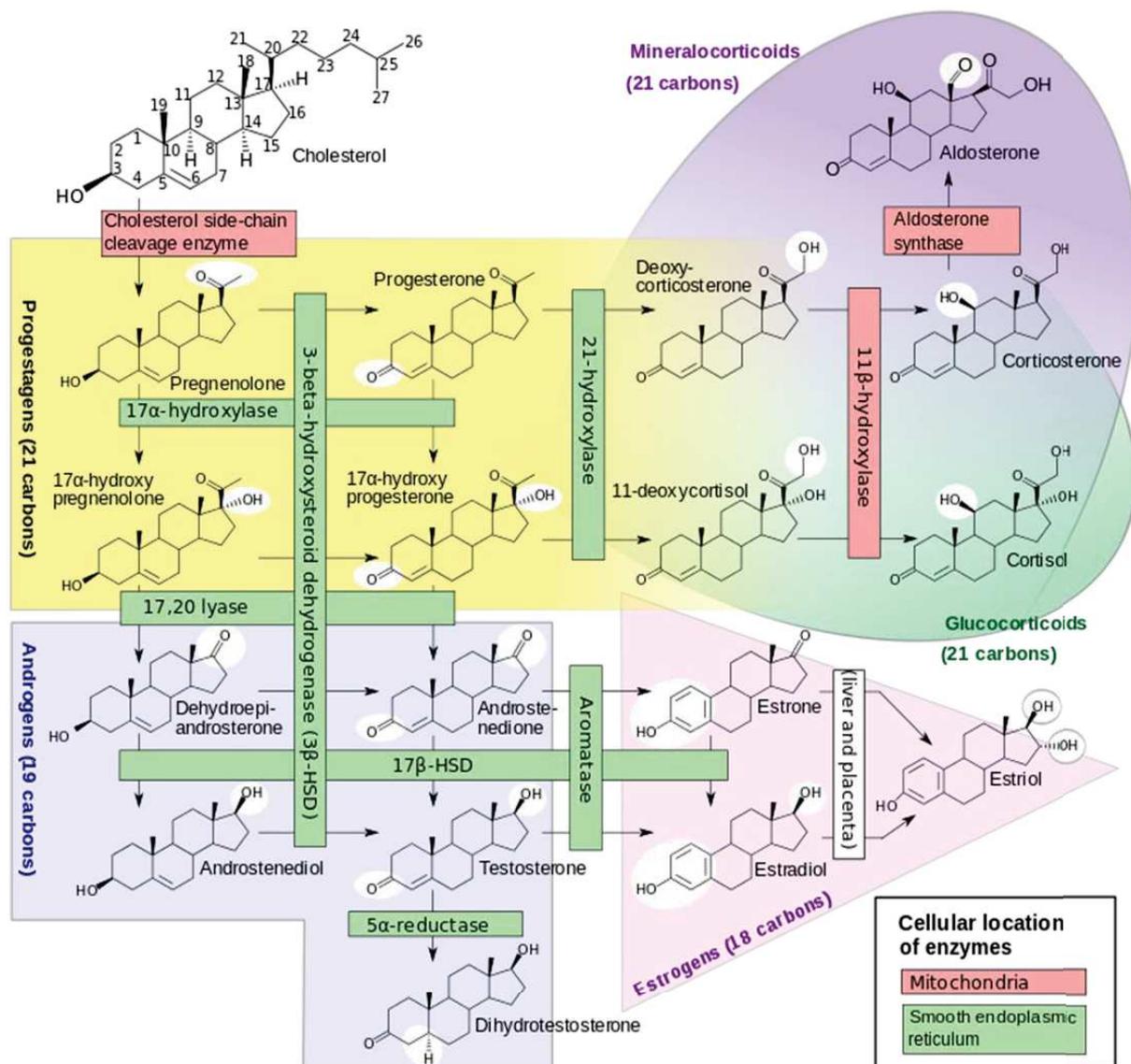


Figure 14. **Schematic of steroidogenesis.** Cholesterol is converted to pregnenolone via cholesterol side-chain cleavage enzyme. Pregnenolone is converted to progesterone by 3β-HSD or to 17α-hydroxypregnenolone by 17α-hydroxylase. 17α-hydroxypregnenolone is converted by 17,20-lyase to dehydroepiandrosterone or by 3β-HSD to 17α-hydroxyprogesterone which can be converted to androstenedione by 17,20-lyase. The enzyme 17β-HSD catalyzes the conversion of dehydroepiandrosterone to androstenediol and androstenedione to testosterone. Androstenedione and testosterone are converted by the enzyme aromatase to estrogens. David Richfield and Mikael Häggström.

One of the enzymes involved in several steps of steroidogenesis is **3 β -hydroxysteroid dehydrogenase (3 β -HSD)**, which is responsible for the conversion of pregnenolone to progesterone, 17 α -hydroxypregnenolone to 17 α -hydroxyprogesterone, dehydroepiandrosterone (DHEA) to androstenedione and androstenediol to testosterone. All reactions catalyzed by 3 β -HSD work with similar efficiency (Morel et al., 1997; Thomas et al., 1989).

The enzyme **17 α -hydroxylase** catalyzes the synthesis of 17 α -hydroxypregnenolone from pregnenolone and 17 α -hydroxyprogesterone from progesterone. This reaction is followed by the conversion of 17 α -hydroxypregnenolone to dehydroepiandrosterone (DHEA) and 17 α -hydroxyprogesterone (17OHP) to androstenedione by the enzyme **17,20-lyase**. It is important to note, that the enzymes 17 α -hydroxylase and 17,20-lyase are in fact one enzyme (**P450C17**) and the difference is purely functional, not genetic or structural (Chung et al., 1987; Nakajin and Hall, 1981).

The enzyme **17 β -hydroxysteroid-dehydrogenase (17 β -HSD)** exists in five isoforms in humans and is crucial for the final steps of sex hormone synthesis. 17 β -HSD-I is responsible for the synthesis of estradiol from estrone, while 17 β -HSD-II catalyzes the same reaction in the opposite direction. 17 β -HSD-III converts DHEA to androstenediol and androstenedione to testosterone (Luu-The et al., 1995).

In testes, namely in Leydig cells, testosterone synthesis principally happens via the DHEA pathway, the production of androstenedione from 17 α -hydroxyprogesterone contributes only minimally (Flück et al., 2003). In the ovaries, steroidogenesis is divided between granulosa cells and theca cells. After LH stimulation in granulosa cells, pregnenolone and progesterone synthesized from cholesterol cannot be converted by enzyme P450C17, because this enzyme is not produced in this cell type, therefore they diffuse into theca cells, where they undergo conversion to androstenedione. Small amounts of androstenedione serve as a substrate for testosterone production and the rest is transported back to the granulosa cells to be used for the final steps of estrogen synthesis. The enzyme catalyzing the conversion of androstenedione and testosterone to estrone and estradiol respectively is aromatase.

Aromatase (P450aro) is a microsomal member of the cytochrome P450 superfamily and the product of the *cyp19* gene (Simpson et al., 1994). Aromatase binds the C19 of androgens and catalyzes a reaction which results in the creation of the phenolic A ring of estrogens. Aromatase

is expressed in steroidogenic tissues such as granulosa cells in premenstrual women or placenta in pregnant women and in non-steroidogenic tissues, like brain, fat and bones, which are the main areas of estrogen formation in men and postmenopausal women (Grodin et al., 1973; Simpson, 2003; Simpson et al., 1994). Gonadal and extragonadal expression has different regulatory pathways. Aromatase production in the ovary is controlled by FSH. As described in figure 13, after FSH binds to its receptor, adenyl cyclase is activated which results in the formation of cAMP followed by the activation of PKA. PKA phosphorylates CREB, which binds to the CRE sequence in the aromatase promoter. Apart from the binding of CREB, SF-1 binds to its corresponding site in the promoter, leading to the expression of aromatase. Expression in adipose tissues happens in stromal mesenchymal cells and is stimulated by class I cytokines (IL-11, IL-1) and TNF α via JAK-STAT3 pathway. Interestingly, another factor necessary for aromatase expression by adipose tissue is the presence of glucocorticoids. In the promoter of the aromatase gene, an interferon γ activation site (GAS) and glucocorticoid response element (GRE) are found upstream of the transcriptional start (Zhao et al., 1995). In bones, the expression of aromatase was located mainly in osteoblasts and chondrocytes. As in adipose tissue, the expression of aromatase is regulated by class I cytokines and TNF α (Nawata et al., 1995). Aromatase is also expressed in the brain mainly in the medial preoptic/anterior hypothalamus, the medial basal hypothalamus, and the amygdala (Naftolin et al., 1975). The formation of estrogens in extragonadal tissues has probably only local paracrine or intracrine effect, which makes it rather difficult to interpret the circulating levels of estrogen in men and postmenopausal women as the blood levels of estrogen may be very low, whereas local levels can be high (Labrie et al., 1998).

3.1 Androgens

Androgens are steroid hormones responsible mainly for male sexual development and function; however they also play an important role in females. In the male fetus, the undifferentiated gonads start to form into testes approximately 7 to 12 weeks after gestation in response to the expression of the SRY region of the short arm of the Y chromosome. The developing testes express testosterone and AMH (anti-muellerian hormone). Testosterone is responsible for the formation of Wolffian ducts and epididymis, *vas deferens* and seminal vesicle, while AMH suppresses the formation of female specific Muellerian ducts. DHT (converted from testosterone by the enzyme 5 α -reductase) is essential for the development of the prostate, penis and scrotum. The production of testosterone is regulated by LH as described previously and testosterone levels prior to puberty are low. After the initiation of GnRH secretion at the onset of puberty, testosterone levels increase, secondary sexual characteristics develop and spermatogenesis is initiated in response to FSH. Testosterone is necessary for normal spermatogenesis and it prevents the apoptosis of germ cells. While testosterone is the androgen responsible for male sexual development, it is DHT which is crucial for maintaining the ability of the epididymis to sustain sperm maturation. Testosterone (and its metabolite estradiol) also inhibits GnRH and gonadotropins acting as negative feedback in the regulation of spermatogenesis.

In females, androgens are the source of estrogen production. For a long time it was unclear whether androgens play any important role in the physiology of females other than being precursors for estrogens. Several studies have demonstrated the capability of androgens to stimulate follicle growth and development as well as ovulation. (Wang et al., 2001; Yang and Fortune, 2006) The stimulating role of androgens on the female libido has been extensively studied as well (Cappelletti and Wallen, 2016; Wylie et al., 2010). Not only do androgens act directly on the hypothalamo-pituitary gonadal axis, but during development they also play a role in the formation of other organs, e.g. the lungs and kidneys (Ballard, 1989; Bardin and Catterall, 1981).

The precursors of androgens in females and males are synthesized in the *zona fasciculata/reticularis* of the adrenal gland. In males, testosterone is synthesized in the Leydig cells of the testis interstitial compartment. In mature men, the production of testosterone by the testis is 5000–7000 mg/day. In females, about 50% of testosterone is produced in the theca cells of the

ovaries and 50% from circulating adrenal precursors and the amount of testosterone produced in premenopausal women is approximately 300 µmg/day (Palacios, 2007). Only approximately 2% of all testosterone present in the body is in the free (biologically active) form. The rest of the testosterone is bound to sex steroid hormone-binding globulin (SHBG) and with lower affinity to albumin (Hammond, 2011).

3.1.1 Androgen receptor

The androgen receptor belongs to the nuclear receptor superfamily and its ligand is testosterone or DHT and it is key transcription factor in androgen signaling. Androgen receptors not only mediate the development of male sexual organs, they are also involved in cell proliferation, differentiation, apoptosis and metabolism of several other tissues. Immunohistochemistry has revealed the presence of AR for example in the mammary gland, the cervix, hair follicles, cardiac muscle and the sweat glands (Winter et al., 1991). AR is composed of three domains: an N-terminal domain and a DNA binding domain, which is connected by a hinge region to the C-terminal ligand binding domain (Jenster et al., 1991). When the AR is inactive, it is located in the cytoplasm and it is associated with heat-shock proteins (HSP). The whole complex is anchored in the cytoplasm by cytoskeletal proteins, for example filamin-A. After a ligand is bound to the ligand binding domain, conformational change allows the receptor to be dissociated from the heat-shock proteins. The whole complex then migrates to the nucleus where it is recognized by androgen response elements located in the promotor of androgen sensitive genes, which leads to recruitment of coactivators of transcription, for example histone acetyltransferase (HAT) enzymes CBP/p300, P/CAF, via SRC/p160 co-activator family members and the beginning of transcription by RNAPolIII (RNA polymerase III). AR is transferred back to the cytoplasm and recycled or undergoes proteosomal degradation by E3 ubiquitin ligase (Jenster, 1998) (Fig. 15).

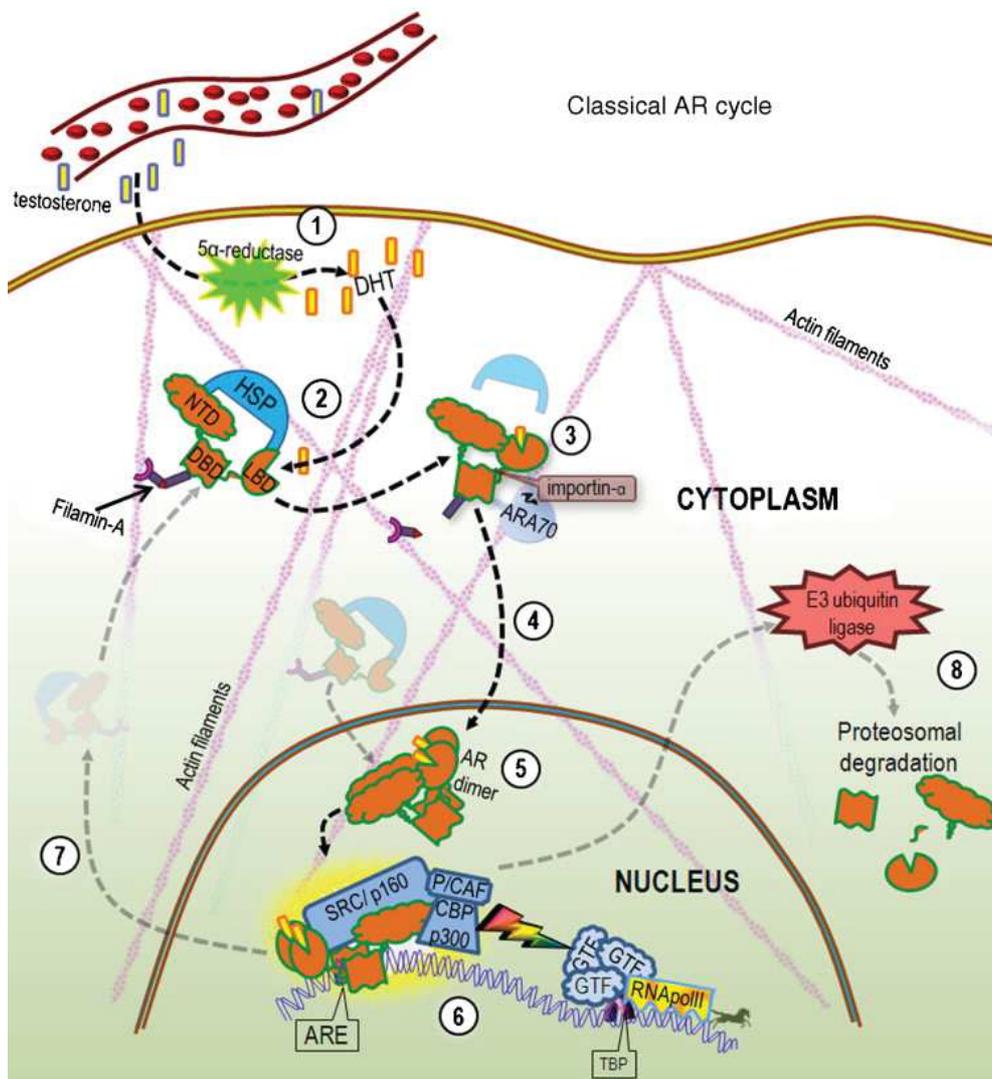


Figure 15. **The androgen receptor cycle.** Androgen receptor (orange) is composed of NTD (N-terminal), DBD (DNA binding) and LBD (ligand binding) domains. (1) Testosterone is reduced to DHT by 5- α reductase in some cases. (2-3) Testosterone binds to the AR complex which causes conformational changes and the release of AR from HSP. (4-5) The AR complex is then translocated into the nucleus where it dimerizes. (6) Several co-factors bind to AR and the DBD binds to androgen response elements (ARE) which results in chromatin remodeling followed by binding of general transcription factors (GTF) and the onset of transcription by RNAPolIII. (7-8) AR is transferred back to the cytoplasm and re-cycled or undergoes proteosomal degradation by E3 ubiquitin ligase (Bennett et al., 2010).

AR regulation occurs mainly via autoregulation. In all target tissues examined, the autoregulation by androgens played an important role by downregulation of AR mRNA transcription, however in a few studies, AR had the opposite effect. Studies using castrated male rats revealed, that levels of AR mRNA were increased in kidney, brain, epididymis, and anterior prostate of castrated animals compared to intact animals. Endogenous administration of testosterone to castrated animals reduced the AR mRNA to lower levels or AR mRNA measured in non-castrated male rats. Estradiol didn't have any effect on the levels of AR mRNA, demonstrating, that the androgen dependent down regulation is not due to testosterone conversion to estradiol by aromatase (Quarmby et al., 1990). Cell culture studies revealed that the autoregulation of AR is transcriptional. Four androgen response elements were found in D and E exon of the AR promoter and silencing of these AREs resulted in functional AR not affected by androgen mediated regulation (Grad et al., 1999).

3.2 Estrogens

Estrogens - C18 steroids play many roles in women and men. They are involved in physiological processes such as the development of secondary sexual characteristics, regulation of gonadotropin secretion and thus ovulation, maintenance of bone mass, prevention of urogenital atrophy and maintenance of cognitive function (Gruber et al., 2002; Nelson and Bulun, 2001).

The effect of estrogens on breast tissue had been well demonstrated in studies of aromatase deficient female patients, whose breast tissue does not undergo normal pre and post-menopausal development but was re-established after administration of estrogen therapy (MacGillivray et al., 1998). Also estrogen ER knock-out mice have been created which do not have the capability to develop mammary glands (Couse and Korach, 1999). Estrogens have been also connected with the incidence of breast cancer, as nearly 70% of all breast cancer cases are ER positive, which allows this type of tumors to be treated by anti-estrogens (Lumachi et al., 2013). The effects of estrogen deficiency on the urogenital tract of females are characterized by increased levels of gonadotropins as the estrogen negative feedback is absent, which is the cause of polycystic ovaries. Female patients with low estrogen levels also exhibit ambiguous genitalia, which is however caused by the deficiency of aromatase, therefore higher levels of androgens in the blood stream. The negative effects can be averted by treatment with estrogen (MacGillivray et al.,

1998). Strains of mice with knocked-out ER receptors exhibited partial (ER β KO) or full (ER α and Double KO) infertility (Couse and Korach, 1999).

Estrogens are involved in bone development and homeostasis. They prevent bone loss by maintaining the balance between bone resorption and bone formation. The presence of ER α was detected in the bone forming osteoblasts and in osteoclasts which are responsible for bone resorption. It has been suggested as well, that there is mutual dependence between estrogens and growth hormones in bone, which makes estrogen indirectly responsible for bone growth and mineral density. Studies on mice and rats demonstrated, that estrogen administration increased the levels of growth hormones (Chen et al., 1995).

It is a well-known fact, that men are affected by cardiovascular diseases at an earlier age and with higher incidence than premenopausal women, which might be caused by higher levels of estrogen in pre-menopausal women, especially as woman older than 65 have the same risk of developing cardiovascular diseases as men of the same age. Hormone replacement therapy in post-menopausal women prevents atherosclerosis and other cardiovascular diseases (Mendelsohn, 2009; Mendelsohn and Karas, 1999). ER α and ER β can be found expressed in smooth muscle cells, myocardial cells and vascular endothelial cells (Grohé et al., 1998; Register and Adams, 1998). Estrogen have been demonstrated to cause non-genomic vasodilatation by affecting ion-channel function, nitric oxid (NO) synthesis, modulation of prostaglandin and NO synthase and the expression of endothelin (Nuedling et al., 2001; Saitta et al., 2001).

As mentioned before, estrogens play various roles on cognitive function. It has been reported, that estrogens influence memory, learning, fine motor skills and mood. Several brain structures and related neurotransmitter systems are connected with the action of estrogens (McEwen and Alves, 1999), for example the serotonin system which is responsible for mood, reproduction and sleep (Amin et al., 2005). Estrogen has been demonstrated to upregulate the expression of tryptophan hydroxylase (an enzyme crucial for serotonin synthesis) as well as downregulating the expression of serotonin transporters (Hiroi et al., 2006; Pecins-Thompson et al., 1998). Estrogen also affects the dopaminergic system, which is involved in the control of the hypothalamic-pituitary axis and motor functions by direct potentiation of dopamine release, increasing the expression of dopamine transporters and dopamine receptors (Chavez et al., 2010; Jacobs and D'Esposito, 2011). The central cholinergic system, which is involved in learning and memory, is

also influenced by estrogen which induces the activity of choline acetyl transferase (McEwen and Alves, 1999).

3.2.1 Estrogen receptors

The stimulation or inhibition of estrogen signaling pathways in target tissues is dependent on the balance of the activity of the estrogen receptors. ERs have a role of transcription factors which affect transcription of a number of genes, hence the level of ER transcription is crucial determinant of growth and metabolism of estrogen responsive tissues. Estrogen receptor α and β are nuclear receptors as are androgen receptors and they are encoded on the chromosome 6 and 14 respectively in human and they have different biological function. The structure and activation of ERs is also very similar to AR as described previously. N-terminal domains of ER α and β share only 30% sequence identity and the ligand binding domains 53% sequence identity, which contributes to the selectivity of ERs to control transcription of specific target genes (Kong et al., 2003). While ER α is expressed mainly in the breast, liver, uterus, theca cells of the ovaries, testicular Leydig cells as well as prostate stroma and epididymis, ER β can be found in the brain, granulosa cells of the ovaries, bone marrow, testes and prostate epithelium (Pelletier, 2000). As mentioned earlier, several knock-out mice models were developed to determine the role of estrogen receptors in morphogenesis and fertility and their phenotype is summarized in a Table 3., adapted from (Walker and Korach, 2004).

Table 3.

Tissue	α ERKO	β ERKO	$\alpha\beta$ ERKO
<i>Fertility</i>			
	Both sexes are infertile	Males are fertile; Subfertile females; infrequent pregnancies and reduced litter size	Both sexes are infertile

Tissue	αERKO	βERKO	$\alpha\beta$ERKO
<i>Female Reproductive System</i>			
Uterus	Hypoplastic uterus; insensitive to estradiol; no implantation	Normal response to estradiol; supports normal pregnancy	Resembles α ERKO phenotype; insensitive to estradiol
Ovary	No ovulation; immature follicles; hemorrhagic cysts developing at puberty due to chronic elevated LH; elevated levels of estrogen and testosterone	Normal appearance; reduced ovulation	Granulosa cells undergo transdifferentiation into Sertoli-like cells
Mammary Gland	Immature; only a ductal rudiment present	Normal structure; normal lactation	Immature; resembles α ERKO phenotype
<i>Male Reproductive System</i>			
Testes	Normal development; testes weight decreases with age; fluid retention and dilation of seminiferous tubules; sperm have poor motility	Normal	Resembles α ERKO phenotype
Mating Behavior	Decreased aggression; disrupted mating behavior	Normal sexual behavior	Males do not mount; disrupted mating behavior
<i>Nonreproductive Phenotypes</i>			
Bone	Both sexes are shorter than wild-type; females have decreased bone diameter; males have decreased density	Females have increased density; normal in males	Both sexes are shorter than wild-type
Cardiovascular	Estrogen protection retained in vascular injury study	Estrogen protection retained in vascular injury study	Estrogen protection lost in vascular injury study

The regulation of ER expression has been widely studied using several estrogen responsive cell lines derived from breast cancer cells. Study of the MCF-7 cell line suggests that estrogen itself downregulates the expression of estrogen binding sites and contributes to the decrease of the

level of steady state ER proteins, but its downregulation of ER gene transcription is transient, which suggests, that the mechanism of ER regulation is post-transcriptional (Pink and Jordan, 1996; Saceda et al., 1988). In the T47D cell line however, estrogen exposure increased levels of ER mRNA and ER protein. The authors of the study suggest two different models of ER regulation. In the case of model I in MCF-7, ZR-75 and PEO4 cell lines, the response to estrogen exposure is the decrease of steady state ER mRNA levels which is rapidly reversed at the moment of estrogen deprivation. This model of ER regulation is typical for tissues with high cell proliferation such as in the uterus (Pink and Jordan, 1996). The model II of ER regulation, typical for the T47D cell line requires longer exposure to estradiol to induce maximal ER response and synthesis and is typical for vitellogenin synthesis in avian or *Xenopus* liver (Pink and Jordan, 1996). Other studies using MCF-7 cell line suggest that epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I) play a role in ER α regulation. EGF (0.4 ng/ml) and IGF-I (40 ng/L) treatment decreased the ER α protein levels and estradiol binding sites by 60% and 50% respectively in their separate studies (Stoica et al., 2000a, 2000b). Mitogen activated protein kinase (MAPK), which is a downstream effector of the signaling of growth factors, was also discovered to be crucial in ER α regulation. Hyper-activation of MAPK in MCF-7 cells reduced the levels of ER and inhibition of MAPK by its inhibitor U0126 resulted in massive up-regulation (Oh et al., 2001).

INTRODUCTION

PART IV

MEDAKA SEX DETERMINATION

Part IV.: Medaka sex determination

1. Medaka as model organism

Medaka (*Oryzias latipes*) belongs to the beloniformes atherinomorpha taxon. It is a freshwater oviparous fish. Medaka originate from East Asia mainly in Japan, China, Taiwan and Korea. The first description of medaka was given in Siebold's Fauna Japonica in 1846 and since then medaka has become a favorite model in developmental biology. There are several advantages to using medaka as a model organism to study embryonic development: (i) medaka is an egg laying fish with almost transparent embryos, (ii) some strains are transparent even after they reach maturity (iii), medaka is very tolerant to temperature variability which allows them to survive temperatures between 4°C and 40°C (iv), females spawn up to 50 eggs every day, (v) the larvae hatch after nine days and (vi) generation time is seven weeks. Under current legislation in European Union, medaka fry are not considered to be laboratory animals whilst they ingest egg yolk and are not independent feeders, i.e. until approximately five days post hatch. A major advantage of medaka compared to zebrafish (*Danio rerio*- another fish species used as model organism) is that at maturation, medaka males and females are sexually dimorphic, which allows gender distinction without genotyping. Adult males have a convex anal fin with tubercle-like processes at the rays and a dorsal fin notch.

2. Medaka sex determination

All vertebrates, if they develop normally, have either female or male gonads. In most vertebrates the gonads develop from a precursor organ common for both ovaries and testis and it is dependent on the sex determining gene or the environment (temperature, social interactions), which developmental pathway the bipotential gonad will undergo. In mammals, the sex determining gene *sry* is located within the Y chromosome (Sinclair et al., 1990), while in non-mammalian vertebrates the ortholog of *Sry* gene has not been found. The mechanism of sex determination of most of fish species remain to be discovered, although several studies have demonstrated the close relationship between fish sex determination and sex steroids (Yamamoto, 1975). Fish sex determining systems that have been elucidated seem to be very heterogeneous and seem to have evolved from a number of different genes independently, for example *amhy* might be the sex determining gene in the Patagonian pejerrey, *Gsdf* in *Oryzias luzonensis*, *Amhr2*

in fugu and *sdY* in rainbow trout (Kikuchi and Hamaguchi, 2013). The genus medaka has a male heterogametic sex determining system (XX/XY) (Aida, 1921), and the two chromosomes have different morphology (Uwa and Ojima, 1981). It has been discovered that the master sex-determining gene in medaka is *DMY* a paralogue of *dmrt1* (Matsuda, 2003; Nanda et al., 2002).

3. Medaka gonadal formation differentiation

In medaka, gonads develop from primordial germ cells (PGCs), which will later form the gametes and somatic cells which will derive into additional gonadal tissue. It is suggested, that the relationship between those two cell lines is crucial in establishing gonadal sexual dimorphism (Tanaka et al., 2008). In medaka, PGCs are first discernible by the expression of *nanos* during early gastrulation (Kurokawa et al., 2006). After the beginning of gastrulation, around 10 PGCs are located at the animal pole of the egg and around stage 12 (10h 20 min), they start to migrate. The migrating PGCs express *cxcr4*, which interacts with chemokine SDF-1a expressed in the lateral plate mesoderm. As *sdf-1a* expression is strictly around gonadal somatic precursors, PGCs are directed to their final destination in the gonadal region (marginal zone) (Kurokawa et al., 2006). During the migration towards the marginal zone, the number of PGCs increases to 30-40 and they reach the area of somatic gonadal precursors, located around the eleventh to thirteenth somites at stage 26 (2 days 6 hours). Around stages 31 to 33 (3 days 23hours- 4 days 10 hours), PGCs and gonadal somatic cells merge and form gonadal primordium which is later separated into left and right lobes (Nakamura et al., 2006). Starting at stage 36 (5 days 12 hours), *dmy* can be detected in the somatic cells forming male gonads and from now on, the proliferation of germ cells undergoes sexual differentiation. Until stage 39 (9 days- hatching period) male and female gonads are not distinguishable. At the time of hatching, female gonadal germ cells start proliferate intensively and a fraction of them enters meiosis, while male germ cells remain less active.

3.1 Differences between male and female gonadal formation

Two kind of proliferation occurs in medaka, slow intermittent type (type I), ensuring germ line cells continuum and fast continuous type (type II), proliferating to cyst-like formation creating the foundation for future gametes. Male and female germ cells both undergo type I proliferation, while a fraction of female germ cells also initiate type II proliferation. Therefore XY gonads are composed of large germ cells surrounded by somatic cells, while in XX gonads two types of germ cells appear, large germ cells similar to XY germ cells and smaller, dense germ cells forming cysts, which as mentioned above enter meiosis at the time of hatching. After meiosis some of these cells start to form follicles (Saito et al., 2007).

Somatic cells in female gonads start to differentiate around age 10 days post hatch (dph). Somatic cells in proximity to growing oocytes form granulosa cells expressing *foxl2* while somatic cells surrounding the growing follicles are foundation for future theca cells and express aromatase. A third type of differentiated somatic cells express *P450c17* and appears all over the gonad as another component of theca. At 30 dph, the formation of oviducts and ovarian cavities begins (Kanamori et al., 1985; Matsuda, 2008; Suzuki and Shibata, 2004).

Unlike in female gonads, the differentiation of male somatic and germ cells does not occur until 10 dph even though *dmy* is expressed by the somatic cells starting around stage 34 (Matsuda, 2005). At 18-20 dph, somatic cells start to express *dmrt1* which is later involved in spermatogonial differentiation (Kobayashi et al., 2004; Ohmuro-Matsuyama et al., 2003). Just like in female gonad, somatic cells expressing *P450c17* are present around the entire male gonad. Testis development is then followed by formation of the efferent ducts around dph 20 and around 40-50 dph spermatogenesis begins as germ cells undergo meiotic division (Suzuki and Shibata, 2004).

3.2 Gene expression during normal sex differentiation- testicular development

3.2.1 DMY

The *dmy* gene, also named *dmrt1b(Y)*, is a gene specific to the Y chromosome of medaka which consists of 6 exons and encodes a putative protein of 267 amino acids including a DNA binding domain named a DM-domain (Doublesex and Mab related domain). This gene is believed to have arisen from a duplication of the *dmrt1* gene, which is an autosomal transcription factor located on linkage group 9 (Nanda et al., 2002). *Dmy* mRNA expression had been detected at specific stages of development in the somatic cells surrounding the primordial germ cells (PGCs) in the early gonadal primordium and later in the precursors of Sertoli cells (Kobayashi et al., 2004). To investigate the function of DMY on male gonad development, Paul-Prasanth carried out a study with *dmy* knocked-down medaka. *Dmy* knock-down resulted in expression of *Scp3* (meiosis specific marker) in gonads of XY males at dph 0, even though normally the male germ cells don't undergo meiosis before dph 40-50. Expressing *Scp3* from dph0 is female-specific, hence in absence of *dmy*, the XY gonads follow the path of female development (Paul-Prasanth et al., 2006). In another study, *dmy* was cloned into XX females which resulted into testis development (Matsuda et al., 2007). DMY plays an important role in mediating the cell cycle of cells expressing it and inducing its arrest in the G2 check-point may be the mechanism by which female germ cells undergo meiosis earlier in development (Herpin et al., 2007). The *dmy* gene has an autoregulation loop controlled by the Izanagi element in the promoter, where it can bind and induce its own transcription (Herpin et al., 2010). A recent study suggests, that DMY determines male characteristics through the activation of *gsdf* and *dmrt1* without participating itself in developmental processes of sex initiation and maintenance (Zhang et al., 2016).

3.2.2 Dmrt1

DMRT1 is a putative transcription factor involved in testicular formation not only specific for male differentiation in medaka, but also in other species like mouse, chicken or frog (Raymond et al., 1999; Shibata et al., 2002). In medaka, the expression of *dmrt1* in the somatic cells surrounding germ cells is first detectable around 18-20 dph in the same quantity both in female

and male gonad. After 20 dph the expression of *dmrt1* increases many fold in the XY gonads. At around 40 dph, the expression of *dmy* and *dmrt1* in XY gonads is at similar levels and in later stages, the expression of *dmrt1* is significantly increased compared to *dmy*. Concomitant with the initiation of *dmrt1* expression, type A spermatogonia start to proliferate and it is suggested that DMRT1 is involved in spermatogonial proliferation (Kobayashi et al., 2004). Several functional studies were carried out to investigate the function of DMRT1. Masuyama and coworkers showed that XY *dmrt1* missense mutants developed testes (until 5 dph) before undergoing gonadal differentiation into ovaries and becoming egg-laying females. Transgenesis of the non-mutated *dmrt1* region rescued the male phenotype, hence it is suggested that DMY triggers male gonadal differentiation, but DMRT1 is necessary for maintenance of testicular differentiation. The mechanism of this action seems to be DMRT1 restraining the germ cells type II proliferation (Masuyama et al., 2011). *Dmrt1* expression is induced and maintained by GSDF, without *gsdf* expression *dmrt1* expression is abolished (Zhang et al., 2016). At the same time a study demonstrated, that DMRT1 induces the expression of *gsdf* (Jiang et al., 2016).

3.2.3 Gsdf

Gonadal soma derived factor (GSDF) is a transcription factor that starts to be expressed in somatic cells surrounding germ cells in XY gonads around dpf 6, similar to the expression of *dmy*, while in female gonad *gsdf* it is barely detectable. In later stages, around 30dph, GSDF was present in somatic cells surrounding other male-specific gonadal structures, such as efferent and sperm duct, which suggests the involvement of GSDF in their formation. In XX female gonads, GSDF can be detected around 30 dph, however at 60dph, no further *gsdf* expression was detected. It was confirmed that *gsdf* expression is co-localised both spatially and temporally with that of *dmy* and its expression therefore correlates with early testicular differentiation (Shibata et al., 2010). A recent study using *gsdf* deletions and additions confirmed, that GSDF is necessary and sufficient for the initiation of testicular formation (Zhang et al., 2016).

3.2.4 Sox9b

Sox9b, an orthologue of the sex determining region Y-box 9, it is a gene involved in proliferation and survival of germ cells in gonads. In mammals and many other vertebrate species *sox9* is an important gene in testis differentiation. It has been demonstrated that a *sox9* overexpressing transgenic female mouse developed testes (Vidal et al., 2001). In medaka, it is expressed in the germ cells of female gonads and the *sox9b* mutation caused decrease in germ cells count and masculinisation of females. It is suggested that SOX9b in medaka maintains germ cells but is not responsible for testicular formation (Nakamura et al., 2008, 2012a; Yokoi et al., 2002).

3.2.5 AMH

Anti-Müllerian hormone (AMH) and its receptor gene *amhrII* are expressed in somatic cells in the developing gonads of both sexes and are involved in the normal development of both male and female gonads and somatic cells as well as sex-dependent regulation of germ-cell proliferation Nakamura demonstrated in his study, that defective AMH hormone signaling causes hyper-proliferation in mitotic active germ cells and feminisation of male medaka (Nakamura et al., 2012b). Also Morinaga reported sex reversal in XY medaka carrying a mutation of the *amhrII* receptor (Morinaga et al., 2007).

3.3 Gene expression during normal sex differentiation- ovarian development

3.3.1 Cyp19a1a- gonadal aromatase and the role of E2

Cyp19 is the gene encoding aromatase, a key enzyme involved the conversion of testosterone into estrogen which plays a major role in maintaining the hormonal balance in medaka and other vertebrates. Two versions of this gene have been described in medaka, one of them mainly expressed in the brain (*cyp19alb*) (Diotel et al., 2010) while the other is found in the gonads (*cyp19a1a*) (Guiguen et al., 2010). Both are expressed in male and female medaka fish and are highly regulated. No maternal aromatase is present in the non-fertilized eggs, and both types of aromatase are first detectable at stage 39 (hatching). Gonadal aromatase expression in females is 1.4- 2.7x higher than in the males at this stage, however the amount of gonadal aromatase decreases in females just after hatch until 5 dph, which is not the case in males. Starting at 8 dph,

the level of expression of gonadal aromatase increases dramatically in the females again (Patil and Gunasekera, 2008). The role of E2 in medaka ovarian development is not entirely understood yet, however studies were carried out using another teleost fish (salmon). When XY males are exposed to exogenous E2, ovaries are developed and while the inhibition of aromatase in salmon caused masculinization of genetically female fish, no such trend was observed in medaka embryo (Kawahara and Yamashita, 2000; Piferrer et al., 1994). In 2013 however, Paul-Prasanth demonstrated that E2 is responsible for the adult female phenotype by exposing adult egg-laying females to the aromatase inhibitor exemestane. The lack of E2 caused development of secondary male characteristics and the development of a male gonadal phenotype (Paul-Prasanth et al., 2013).

3.3.2 Foxl2, FtzF1 and Dax1 – regulators of aromatase

The main role of the transcription factor FOXL2, present in the nuclei of all granulosa cells in the ovary, is responsibility for the gonadal differentiation and maintenance by direct transcriptional up-regulation of aromatase (Herpin et al., 2013; Pannetier et al., 2006; Uhlenhaut et al., 2009). *Foxl2* starts to be expressed around stage 38 in the cells which eventually differentiate to granulosa cells. *Foxl2* knock-out is not sufficient to induce reversal to a male phenotype in genetic females and the morphological differences between male and female gonad appear prior to the start of *foxl2* expression, thus it is expected that *foxl2* is not an ovarian determining gene, although it is possible, that it may have a role in the onset of mitosis in the ovarian germ cells (Nakamoto et al., 2006). Transcription factor FTZF1 is a medaka homolog of mammalian FTZF1 transcription factor which is expressed since the early gonadal development in gonadal somatic cells (Morinaga et al., 2004). In tilapia, FTZF1 interacts with previously described FOXL2 which enhances aromatase expression (Yoshiura et al., 2003). In a study by Nakamura two populations of somatic gonadal precursors were identified, one expressing *ftzF1* and the second one expressing *sox9b*, which suggests that the role in gonadal differentiation even before gonadal somatic precursors reach gonadal primordium (Nakamura et al., 2006). Dax1 is a nuclear receptor known for its role in the regulation of the aromatase enzyme by inhibiting *Foxl2* and *FtzF1* expression (Nakamoto et al., 2007).

4. Molecular and hormonal interactions- maintaining the phenotype

The level of expression of both *dmrt1* and *cyp19a1a* are crucial sex determining factors, however a great number of genes are involved. A low *dmrt1* expression in addition to a high aromatase level would lead to an ovarian differentiation while a high level of *dmrt1* and a low level of aromatase would lead to differentiation of primordial cells into testis. The male phenotype, once initiated by the presence of *dmy*, down-regulates the estrogenic pathway as *dmrt1* and *dax1* repress activity of aromatase.

4.1 Maintenance of normal male (XY) and female (XX) phenotype

In XY medaka male DMY induces the expression of *dmrt1* which, in addition to *dax1*, represses *cyp19a1a* (aromatase) expression through direct inhibition as well as inhibition of *foxl2* and *FtzF1*-mediated expression, leading to a decrease in E2 level and thus increase of the level of androgens (Chakraborty et al., 2016; Li et al., 2013; Nakamoto et al., 2006, 2007; Yoshiura et al., 2003; Zhang et al., 2016). *DMY* directly induces the expression of *gdsf* and *sox9a*, genes involved in the formation of the male phenotype (Chakraborty et al., 2016). It has been suggested, that GSDF also plays a role in the decreasing level of estrogens, which plays a role in testicular formation and maintaining of the male phenotype (Jiang et al., 2016).

In female medaka, the *dmy* gene is not present, thus can't upregulate the expression of *dmrt1* which inhibits the expression of *foxl2* (aromatase stimulator). FOXL2 then stimulates aromatase transcription both directly and indirectly via *ftzF1* expression. Expression of *foxl2* also represses *dmrt1* expression and thus the male phenotype (Herpin et al., 2013). High levels of aromatase allow a high conversion of testosterone into estrogen in the gonads. E2 increase is involved in the female-differentiation process and maintenance of the female phenotype by inducing oocyte maturation and vitellogenin and choriogenin production by binding to estrogen receptors in target tissues as described earlier in the introduction of this thesis. FOXL2 also induces the activation of the *rspo1* pathway which in turn activates the *wtn4* and *wnt* pathways which have been shown to promote ovarian development and suppress the formation of the testis cord (Chassot et al., 2008). Follistatin, FST, is also expressed during female differentiation and contributes to cell proliferation and meiosis as well as inducing E2 production.

4.2 Sex-reversed male (XX) phenotype and sex-reversed female (XY) phenotype

During the primary stage of development, the addition of exogenous androgens to the medium of genetic females has been shown to induce a sex reversal from a female to male phenotype. A possible explanation for this phenomenon is that androgenic substances stimulate *dmrt1* expression and induce an indirect repression of the *foxl2* gene expression via the following pathway in medaka as it has been demonstrated in rainbow trout, flounder and tilapia (Baron et al., 2004; Jo et al., 2007; Li et al., 2013; Rolland et al., 2013). *Dmrt1* expression would both promote transcription of male-phenotype-promoting substances such as *gsfd* or *dax1*, thus stimulating the underlying pathway leading to the male phenotype (Jiang et al., 2016). In addition, inhibition of *foxl2* greatly reduces the transcription of aromatase and the production of E2 from testosterone in favor of DHT conversion.

Male to female sex reversal has been induced by the treatment with exogenous E2. E2 is a female-phenotype promoting substance whose addition to the medium both increases *foxl2* expression and represses *dmrt1* transcription in other fish species (*Gobiocypris rarus*, zebrafish) however similar studies with medaka have not yet been carried out. By promoting cell proliferation and upregulating aromatase production, estrogens lead the gonads to differentiate into ovarian tissue. (Filby et al., 2007; Jiang et al., 2011; Schulz et al., 2007).

Results

Publication n° 1

Presentation of publication n°1

Estrogen reporter transgenic medaka for non-invasive evaluation of aromatase activity

This publication was published in the journal “Comparative Biochemistry and Physiology, part C” in 2015.

Normal functioning of the estrogen axis is not only crucial for correct development and functioning of the sexual organs, but also as reviewed earlier in the introduction of this thesis, for correct development of brain, bones and other tissues. It has been demonstrated, that some chemicals disrupt estrogen signaling, which is in some cases via disruption of aromatase activity (Piferrer et al., 1994b).

The purpose of this study was to create a stable ChgH-gfp medaka line to detect estrogenic activity and aromatase inhibition and develop a rapid, non-invasive protocol using pre-feeding larvae of this line. The germinal transgenesis resulted in a stable line.

Before using this line to evaluate the endocrine activity of chemicals or water samples it was important to characterize the response of the line to known endocrine active compounds acting via different mechanisms of action as well as supposed inert compounds. To determine the sensitivity of our assay, newly hatched (dph0) fry were exposed to a dose range of ethinyl-estradiol. Aromatase inhibitors were tested in co-treatment with testosterone to demonstrate the applicability of the ChgH-gfp model to detect aromatase disruption. The specificity of the model was determined by exposure of the test fry with or without the testosterone co-treatment to a number of inert compounds- chemicals which weren't characterized in literature as active on the reproductive axis as well as known agonists of related hormonal axis (corticosteroid, mineralo-corticosteroid and thyroid).

We demonstrated in this publication, that our protocol using ChgH-gfp pre feeding fry is reliable, rapid and simple tool to assess potential endocrine disruptors of estrogen axis whilst complying with the three R's principle of animal replacement - reduction, replacement and refinement of animal experiments (Fenwick et al., 2009).



Oestrogen reporter transgenic medaka for non-invasive evaluation of aromatase activity



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ABSTRACT

Vertebrate reproduction involves complex steroid hormone interplay and inter-conversion. A critical element in maintaining sex steroid levels is the enzyme aromatase (cytochrome P450 19A1) which converts androgens to oestrogens. In turn oestrogen signalling is targeted by numerous chemicals, from pharmaceuticals to agricultural chemicals, both frequent sources of contamination in waste waters and consequently rivers. Although many models are now available to address disruption of oestrogen signalling, there are currently no published protocols allowing discrimination between alterations in testosterone metabolism and in oestrogenic signalling. It was with this limitation in mind that we optimised this protocol. We show using a 48 h protocol that pre-feeding fry of the *choriogenin h-gfp* (*chgh-gfp*) medaka line are sensitive to 0.05 nM EE2 (15 ng/L), within the range of the lowest published observable physiological effect concentrations for medaka. In addition, co-treatment with testosterone can reveal potential effects of test substances on aromatase enzymatic activity. As the measurements are visualised in real-time without affecting embryo viability, repeated measures are possible. We demonstrate the ability of this model to detect oestrogen receptor agonists, aromatisable androgens, P450 aromatase activity modulators and selective oestrogen response modulators. Importantly, the range of this assay is physiologically relevant.

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1. Introduction

A major issue in environmental biology is modification of sex ratios in wildlife, as observed in animals ranging from invertebrates to amphibians, reptiles and mammals (Stoker et al., 2003; Brande-Lavridsen et al., 2008; Steinberg et al., 2008; Gagné et al., 2011). Such changes in sex ratios have been shown to be linked to the increasing presence of man-made chemicals in surface water (Larsson and Förllin, 2002; Hayes et al., 2010).

These findings underline the urgent need for physiological screening methods to identify endocrine disrupting chemicals acting on different

levels of steroid signalling. To date the oestrogen axis has been the focus of most attention in this field due to its critical importance in cancer, reproduction, foetal development and sexual identity.

A diverse array of chemicals have been identified as affecting the oestrogen axis, ranging from phytochemicals to agricultural chemicals, pharmaceuticals, plasticisers, flame retardants, UV screens and industrial waste (Hokanson et al., 2007; Schmitt et al., 2008; Gyllenhammar et al., 2009; Kamata et al., 2011; Zhao and Mu, 2011; Edlow et al., 2012; Ezechiáš et al., 2012). In addition, population level effects of many of these substances have been proven, inducing effects as serious as intersex gonads in entire populations of male fish followed by a rapid decline in the population size (Jobling et al., 1998; Kidd et al., 2007).

However, in addition to identifying oestrogen axis disrupting chemicals active at the receptor level, it is also a priority to develop tests capable of detecting endocrine disrupting chemicals (EDCs) interfering with different aspects of steroid metabolism.

The enzyme aromatase (cytochrome P450 19A1) is responsible for the production of oestrogens from androgenic precursor molecules. It is therefore crucial in determining the relative levels of androgens and oestrogens in target tissues. It is now becoming clear that a number of environmentally relevant chemicals alter the activity of aromatase (Vinggaard et al., 2000; Hinfray et al., 2006). Recently developed

Abbreviations: Tam, Tamoxifen; Fad, Fadrozole; Dex, Dexamethasone; T3, 3,3',5'-triiodo-L-thyronine; EE2, 17 α -ethinyl oestradiol; E2, 17 β -oestradiol; EC₅₀, Effective concentration 50 (half maximal effective concentration); T, Testosterone; SEM, Standard error of the mean; EDCs, Endocrine disrupting chemicals; ChgH, Choriogenin H; GFP, Green fluorescent protein; DMSO, Dimethyl sulfoxide; MS222, Ethyl 3-aminobenzoate methanesulfonate; dph, Day post hatch; ER, Oestrogen receptor; SERM, Selective oestrogen response modulator; LOEC, Lowest observed effect concentration; REACH, Registration, evaluation, authorisation & restriction of chemicals; OECD, Organisation for economic co-operation and development.

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in vitro tests for aromatase disruption, such as the human placental microsomal assay and the choriocarcinoma cell line assay, have proved to be useful screening tools for identifying aromatase disrupting chemicals (Snider and Brueggemeier, 1987; Brueggemeier and Katlic, 1990; Brueggemeier et al., 1997, 1992; Vinggaard et al., 2000). However, the lack of a simple and rapid *in vivo* test has made it difficult to determine the efficiency of these assays, which due to their *in vitro* nature fail to adequately take into account the complex nature of the reproductive axis, a system that involves interplay between numerous organs and crosstalk with other endocrine axes. In order to correctly evaluate exposure risks to humans and wildlife, it is important to distinguish between chemicals interacting with the oestrogen receptor and those altering the activity of aromatase.

The reaction of the European Union to the danger posed by EDCs has been to impose testing of all current and new chemicals produced in a quantity greater than one ton per year and suspected of being endocrine disruptors (REACH legislation). Considering the number of chemicals requiring testing, a screening test of at least medium throughput capacity is evidently required.

In this respect aquatic species such as medaka, zebrafish or premetamorphic *Xenopus* offer great promise. Their aquatic nature facilitates dosing, as the test substance can be added directly to the culture medium. Furthermore, the identification of the master sex determining gene in medaka (*dmy*) and more recently in *Xenopus laevis* (*dmw*) provides the additional advantage of allowing determination of the genetic sex of the test organism (Paul-Prasanth et al., 2006; Matsuda et al., 2007, 2002; Patil and Hinze, 2008; Yoshimoto et al., 2008). Coupled with the monitoring of gonadal status *via* biomarkers or histology, this allows cases of complete or partial sex reversal to be identified. At the time of writing, the master sex determining gene has recently been identified in zebrafish, although the study demonstrated that the gene is present on chromosome four in wild zebrafish, but is absent in laboratory strains (Wilson et al., 2014).

Many satisfactory oestrogenic biomarker genes have been identified (Trudeau et al., 2005; Kim et al., 2009; Chung et al., 2011; Gorelick and Halpern, 2011; Brion et al., 2012; O. Lee et al., 2012a, 2012b; W. Lee et al., 2012). In order to reveal the net effect over the entire oestrogenic pathway, and provide physiologically relevant information, the biomarker should be an endogenous gene relating to a physiological endpoint. Of this subset of identified genes, many are related to egg production, including *choriogenin h* (*chgh*) which codes for a structural protein of the egg envelope, produced in the liver (Ueno et al., 2004; Beck et al., 2005; Scholz et al., 2005; Zeng et al., 2005; Kurauchi et al., 2008, 2005; Salam et al., 2008; Chen et al., 2010). The OECD has acknowledged this viewpoint in a scientific review stating that the oestrogen sensitive protein *chgh-gfp* medaka embryo overcomes traditional limitations with the use of laborious whole organism tests, such as sensitivity and rapidity, as well as eliminating many ethical concerns (OECD, 2010).

Therefore, in order to fall in line with the three R's principal of reduction, replacement and refinement of animal experiments, these huge screening projects necessitate the use of non-regulated, early stage, pre-feeding fish fry or tadpoles. In particular, tests need to be ratified for their capacity to respond to a wide range of oestrogenic substances within a reduced time frame, ideally 24–48 h, and provide a sufficiently robust signal for robotised readout.

Here, we present an optimised, rapid 48 h protocol using pre-feeding *chgh-gfp* medaka for monitoring net oestrogen axis activity as well as the activity of the key enzyme P450 aromatase which controls the conversion of testosterone into 17 β -oestradiol (E2). The activity of the oestrogen axis in the presence of test compounds was determined by the quantification of green fluorescent protein (GFP). Due to the low endogenous levels of testosterone in pre-feeding medaka fry, the readout of aromatase activity was achieved by co-treating fry with a known concentration of testosterone. Increases or decreases in the rate of conversion of testosterone to E2 resulted in a respective increase

or decrease in the production of GFP under the control of the *chgh* promoter (Fig. 1).

Finally we present results generated with a number of reference compounds, selected in order to cover a range of mechanisms of action. We show that pre-feeding *chgh-gfp* medaka fry are a sensitive indicator of disruption of the steroid axes *via* a variety of mechanisms.

2. Materials and methods

2.1. Reagents

Ethyl 3-aminobenzoate methanesulfonate (MS222, CAS no. 886-86-2), dimethyl sulfoxide (DMSO, CAS no. 67-68-5), testosterone (CAS no. 58-22-0), 17 α -ethinyl oestradiol (EE2, CAS no. 57-63-6), anastrozole (CAS no. 120511-73-1), 17 β -oestradiol (E2, CAS no. 50-28-2), cromolyn (CAS no. 15826-37-6), saccharin (CAS no. 82385-42-0), cefuroxime (CAS no. 56238-63-2), arabinose (CAS no. 5328-37-0), amantadine (CAS no. 665-66-7), atenolol (CAS no. 29122-68-7), fadrozole (CAS no. 102676-31-3), aldosterone (CAS no. 52-39-1), progesterone (CAS no. 57-83-0), 3,3',5'-triiodo-L-thyronine (T3, CAS no. 55-06-1), dexamethasone (CAS no. 50-02-2) and tamoxifen (CAS no. 10540-29-1). Medaka medium contained the following quantities of salts per litre: 10 g NaCl, 0.4 g CaCl₂·2H₂O, 0.4 g MgSO₄·7H₂O, 0.3 g KCl, 0.02 g methylene blue and its pH was adjusted to 7.2–8.0. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Transgenic line

All experiments were carried out with fry from generation F6 or greater of the WatchFrog *chgh-gfp* line. This line harbours a genetic construct consisting of 2047 bp of the medaka *chgh* gene immediately upstream from the translation start site driving expression of *gfp*, as initially described by Kurauchi et al. (2005). The line has a CarBio genetic background and displays a non-inducible basal fluorescence in cardiac muscle fibres and in certain cells around the mouth and eyes, allowing transgenic fry to be selected prior to exposure to potential EDCs.

2.3. Exposure studies

Stocks of test solutions were made up in DMSO and 8 μ L of stock solution was added directly to 8 mL of medaka medium in 6-well plates. In addition either 8 μ L of DMSO or 8 μ L of testosterone in DMSO was added to each well. Final DMSO concentration was 0.2% in all wells. Five newly hatched (day post hatch zero; dph 0) medaka fry were added to each well and four wells were used for each exposure group giving a total of $n = 20$ fry per exposure group. All compounds were tested in the presence and absence of testosterone (28.57 μ g/L). Three replicate experiments were carried out giving similar results, a representative replicate is shown. Solutions were renewed every 24 h and the experiments were read at 48 h (dph 2) with the exception of the experiment shown in Fig. 3. This experiment, to demonstrate the presence or absence of aromatase activity immediately after hatch was read after 24 h (dph 1).

2.4. Image capture

Chgh-gfp fry were anaesthetised with 200 mg/L MS222 in medaka medium and positioned dorsally on a black plastic plate for imaging. Images of the ventral region of the abdomen of each fry were captured with a 0.3 s exposure time at 8 \times magnification using an Infinity 1-3C camera (Lumenera Corporation, Ottawa, ON, Canada) fitted to a Leica MZ10F stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany) and were stored as 8-bit colour jpg files. Larvae were illuminated with a 120 W fluorescence source and ET-GFP long-pass

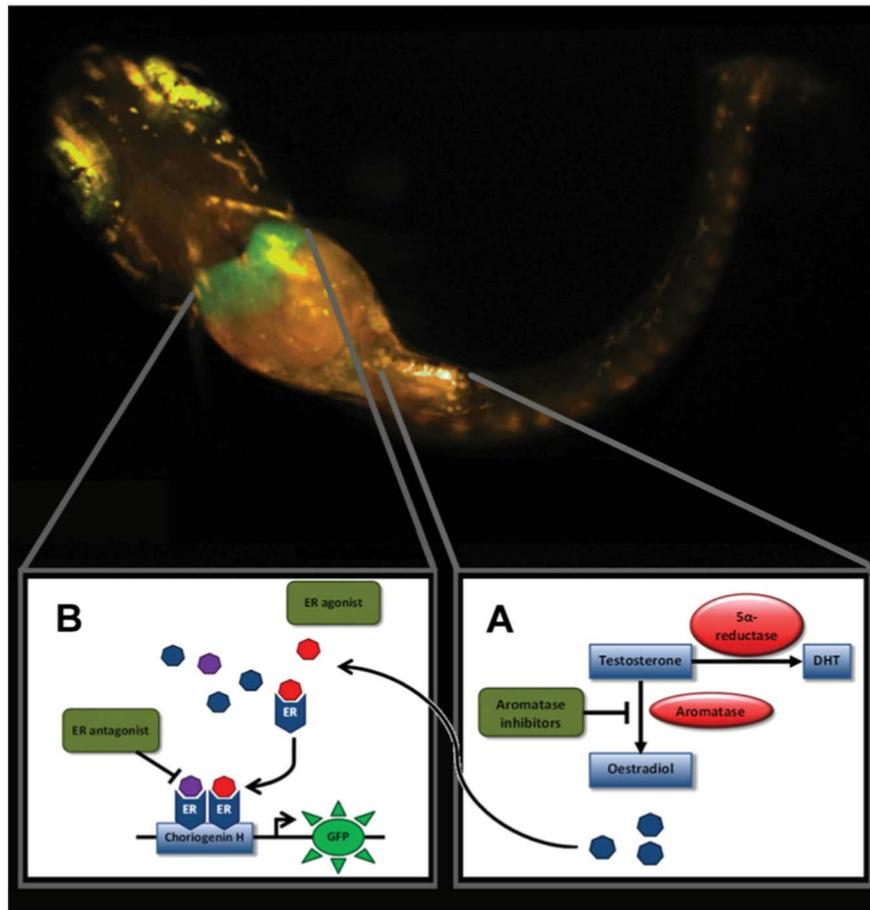


Fig. 1. Schematic diagram of the induction of GFP via aromatase in choriogenin H-GFP medaka. A) Testosterone in the exposure medium is converted to oestradiol via aromatase and DHT via 5 α -reductase principally in the gonads. B) Oestradiol in turn binds to oestrogen receptors. Ligand-receptor complexes then bind to oestrogen response elements (EREs) within the choriogenin H promoter driving expression of GFP in the liver.

filters (excitation 480/40, emission 510LP, Leica Microsystems GmbH, Germany).

2.5. Image analysis

An ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA) macro was used to carry out the following analysis. Colour images of medaka fry were separated into red, green and blue layers. In order to remove background fluorescence resulting from endogenous pigments present in the medaka fry, the intensity of each pixel in the red layer was doubled and subtracted from the green layer. A threshold intensity of 10–255 was applied to the resulting images. The thresholded images showed only the fluorescence resulting from the GFP signal in the heart and liver. The region containing the liver was manually selected and the intensity of all pixels within this region was summed (Supplementary Fig. 1).

2.6. Data analysis and statistics

Total fluorescence values for each fry within a replicate experiment were normalised to the mean of the 28.57 $\mu\text{g/L}$ testosterone control group where present. A D'Agostino and Pearson omnibus normality test was carried out on all exposure groups followed by analysis by Student's T-test, Mann–Whitney test or Wilcoxon test. Groups not exposed to testosterone were compared to the vehicle control and groups co-treated with testosterone were compared to the testosterone control. All statistical tests were carried out with GraphPad Prism version 5.04 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Oestrogen response

Germline transgenesis resulted in the creation of a stable transgenic line. To verify that this *chgh-gfp* line responds to oestrogens as demonstrated for the previously published line (Kurauchi et al., 2005; Scholz et al., 2005), groups of fry were exposed to a concentration range of the synthetic oestrogen ethinyl oestradiol (EE2). Fig. 2 shows a concentration dependent increase in fluorescence when fry were exposed to EE2 in the low ng/L range. Under these assay conditions the response begins to plateau between 135 and 270 ng/L of EE2 and the EC_{50} is 71.9 ng/L with a 95% confidence interval of 63.8 to 80.9 ng/L.

3.2. Functionality of aromatase

To ascertain whether the fry were capable of metabolising androgens into oestrogens immediately after hatch, dph 0 *chgh-gfp* fry were exposed to a range of concentrations of testosterone from 285.7 ng/L to 28.57 $\mu\text{g/L}$. A fluorescent signal was observed in the liver 48 h after exposure to testosterone at 2.86 $\mu\text{g/L}$ and above, whereas only the highest concentration of 28.57 $\mu\text{g/L}$ was able to induce fluorescence following 24 h of treatment (data not shown). The concentration of 28.57 $\mu\text{g/L}$ was selected for the following experiments.

This fluorescence response was predicted to be due to conversion of the testosterone to E2 via aromatase, rather than weak affinity of testosterone for the oestrogen receptor (ER). To test this hypothesis dph 0 *chgh-gfp* fry were exposed to testosterone (28.57 $\mu\text{g/L}$) plus or minus

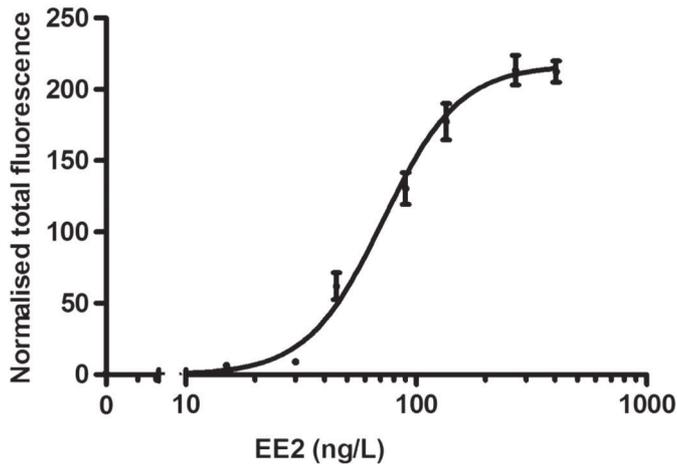


Fig. 2. The *chgh-gfp* line described here responds to the synthetic oestrogen, EE2 in a concentration dependent manner. The response of the fry to 15 ng/L was statistically different to 0 ng/L ($P < 0.05$, *) for all other exposure groups $P < 0.001$ (***) compared to 0 ng/L. $N = 20$ per group, mean \pm SEM are given.

the pharmaceutical aromatase inhibitor anastrozole (2.93 mg/L) (Fig. 3). No signal was observed in vehicle controls (0.2% DMSO) indicating a lack of oestrogenic signalling. A statistically significant induction of fluorescence was observed in the testosterone-treated group. Co-treatment with anastrozole completely blocked oestrogenic signalling suggesting that the signal observed following testosterone treatment was due to conversion of the testosterone to E2 via the enzyme aromatase. To confirm that anastrozole was not acting as an ER antagonist *chgh-gfp* fry were challenged with E2 alone or in the presence of anastrozole. No significant difference in fluorescent signal was observed.

3.3. Reproducibility of the response to testosterone

To ensure that the level of fluorescence induced by testosterone is homogenous between groups of fry, six groups of $n = 20$ *chgh-gfp*

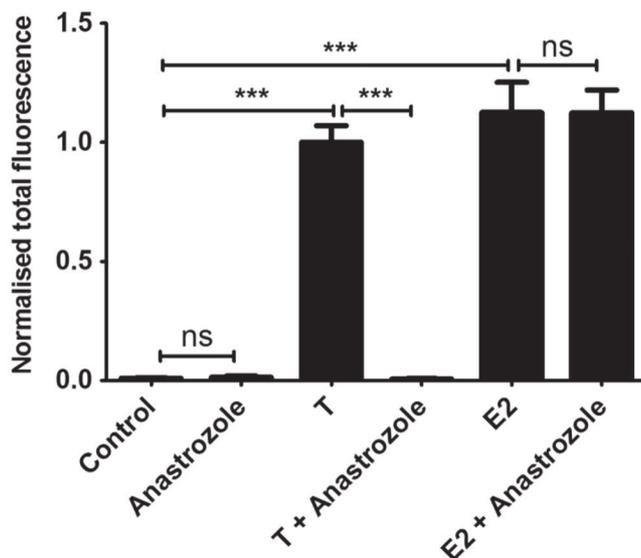


Fig. 3. Aromatase activity is required for a testosterone induced increase in oestrogenic signalling. Exposure to 28.57 $\mu\text{g/L}$ testosterone induces a statistically significant increase in fluorescent signal after 24 h. This increase in fluorescence is completely abolished by co-treatment with 2.93 mg/L anastrozole. The induction of fluorescence in response to an ER agonist (E2, 180 ng/L) cannot be inhibited by cotreatment with anastrozole. *** indicates $P < 0.001$ and ns indicates not statistically significant. $N = 20$ per group, mean \pm SEM are given.

dph 0 embryos were exposed to 28.57 $\mu\text{g/L}$ testosterone for 48 h. In parallel two groups of embryos were treated with carrier solvent alone (Supplementary Fig. 2). The solvent control groups exhibited no fluorescent signal (zero arbitrary units) whereas the minimum level observed in the testosterone treated groups was over 1.5 million arbitrary units. Although mean fluorescence values for the testosterone-treated groups varied, no statistically significant difference was observed between any of these groups.

3.4. Assay characterisation – inert compounds

It has previously been published that *chgh-gfp* medaka embryos can be used to detect ER agonists and aromatisable androgens (Kurauchi et al., 2005). However, to determine whether the 48 h protocol described in this article is sufficiently sensitive to be used for screening substances with unknown mechanisms of action for oestrogen axis disruption, several well characterised substances with different modes of action were tested alone and in co-treatment with testosterone (28.57 $\mu\text{g/L}$). The test is based on the principle that addition of testosterone together with test substance and readout of ER transactivation reveals effects of the test substance on the kinetics of testosterone conversion to E2 via aromatase.

Prior to testing known aromatase modulating chemicals, six chemicals were tested which have not been described as active on the reproductive axes (Fig. 4A). These six chemicals were cromolyn, saccharine, cefuroxime, arabinose, atenolol and amantadine. In the absence of testosterone, all six compounds failed to induce fluorescence in the fry. When tested in the presence of testosterone, none of the six compounds provoked a significant difference in fluorescence signal compared to testosterone alone.

3.5. Assay characterisation – active compounds

The selective oestrogen response modulator (SERM) tamoxifen was used to determine the ability of this protocol to identify ER antagonism (Fig. 4B). In the absence of testosterone, tamoxifen increased oestrogenic signalling with a LOEC of 483 $\mu\text{g/L}$. Inversely, in co-treatment with testosterone, a different profile was observed, with a statistically significant decrease in fluorescence observed for the 483 $\mu\text{g/L}$ group.

To investigate the ability of this assay to identify inhibition of aromatase activity, the fry were exposed to the pharmacological aromatase inhibitor fadrozole (Fig. 4C). Fadrozole did not induce fluorescence in the absence of testosterone. However in the presence of testosterone, a concentration dependent decrease in fluorescence was observed with increasing concentrations of fadrozole becoming statistically significant at concentrations of 10 $\mu\text{g/L}$ and above.

3.6. Assay characterisation – hormonal crosstalk

Once the ability of this assay to correctly identify inert and active compounds had been established, the ability of strong agonists for related hormonal axes to interfere with the results was investigated. The assay was challenged with a high concentration (10^{-7} M, 31–67 $\mu\text{g/L}$ according to the molecular weight) of reference agonists for the mineralocorticoid, progesterone and thyroid hormone receptors (aldosterone, progesterone and T3, respectively). In the absence of testosterone no GFP signal was observed. In the presence of testosterone T3 but not aldosterone or progesterone inhibited the generation of fluorescent signal (Fig. 5A).

Due to a number of recent publications (Hayashi et al., 2010; Kitano et al., 2012) indicating that corticosteroids inhibit expression of aromatase in medaka a concentration range of the powerful synthetic corticosteroid, dexamethasone, was tested (Fig. 5B). Once again, in the absence of testosterone, no statistically significant difference in fluorescence was observed. In the presence of testosterone, the highest concentration

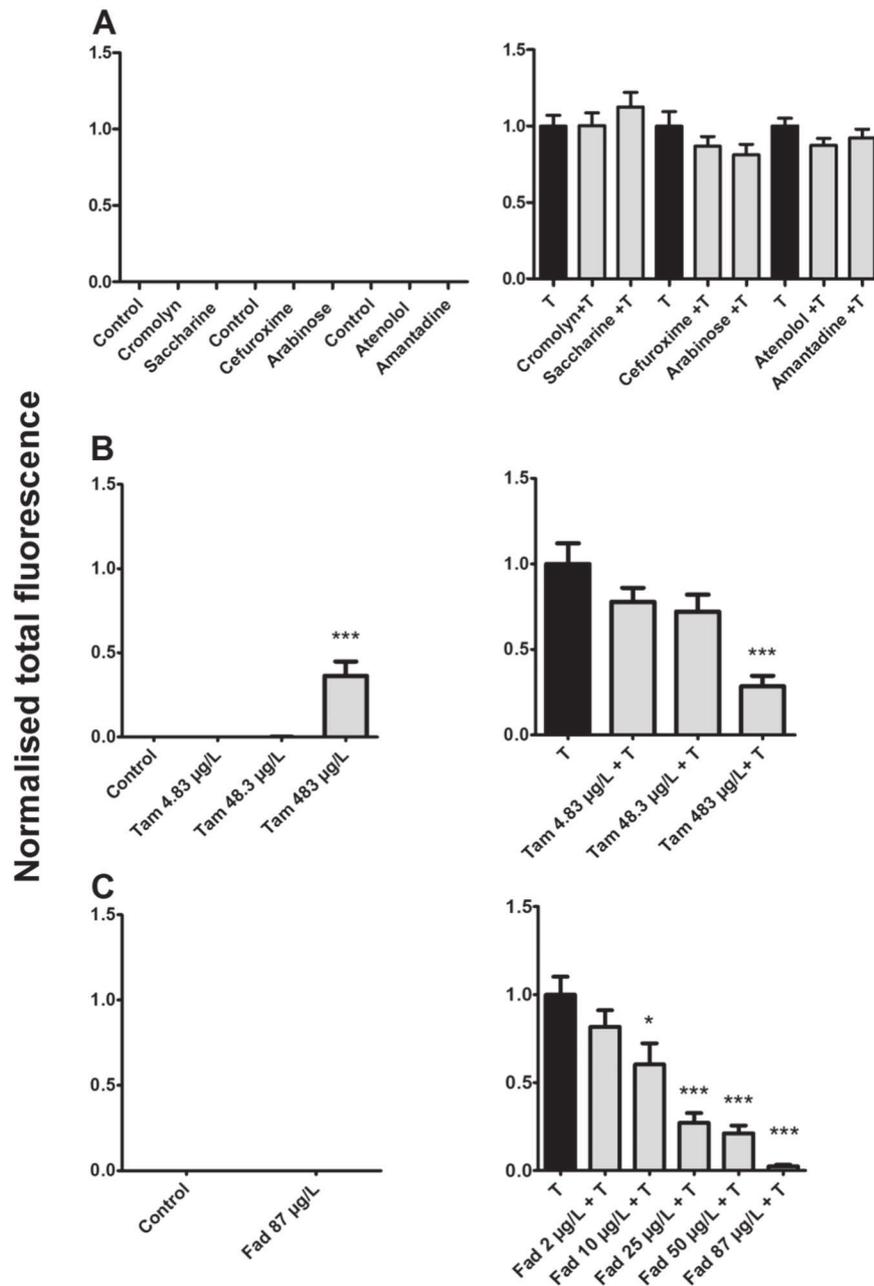


Fig. 4. A) A series of compounds without published effects on the oestrogenic axis are identified as inert. B) Tamoxifen (Tam, an SERM) and C) Fadzole (Fad, an aromatase inhibitor), both well-characterised as oestrogen axis disruptors are correctly identified as active. *** indicates $P < 0.001$ and * indicates $P < 0.05$. $N = 20$ per group, mean \pm SEM are given.

tested (3.92 mg/L) resulted in a significant reduction in GFP signal. Increasing the number of fry per group to 60 increased the assay sensitivity to dexamethasone to 39.2 µg/L (data not shown).

4. Discussion

We characterised a new line of *chgh-gfp* transgenic medaka which exhibits a basal fluorescent signal in cardiac muscle fibres as well as cells around the mouth and eyes. This line allows non-transgenic embryos to be removed from the test pool prior to initiating an experiment. This sorting step reduces the incidence of false negative and false positive results by ensuring that all fry used in the experiments are transgenic, therefore, increasing the robustness of the assay.

Using this medaka line we have developed a sensitive readout protocol employing an imagery system which allows efficient removal of

innate fluorescent signal generated by the medaka fry. This contributes to the high dynamic range observed for this assay. Fluorescence signal induction compared to control was in the range of 6.5-fold to 212-fold for an EE2 concentration range from 15 to 405 ng/L (Fig. 2), therefore, fitting the requirements of a standardised test for EDCs.

A recently published review identifies a concentration range of 1 to 488 ng/L EE2 as altering physiological endpoints in fish depending on the length of the test, the species studied and the endpoint measured (Caldwell et al., 2008). We have demonstrated that *chgh-gfp* medaka are capable of detecting EE2 equivalents in the range of 15 ng/L to 64 ng/L with a linear concentration response relationship (R^2 0.999 from 15–64 ng/L; R^2 0.960 from 0–64 ng/L).

Previously, aromatase gene expression had been observed by *in situ* hybridisation in developing medaka between 4 and 10 dph (Suzuki et al., 2004). In addition, a previously reported *chgh-gfp* line was

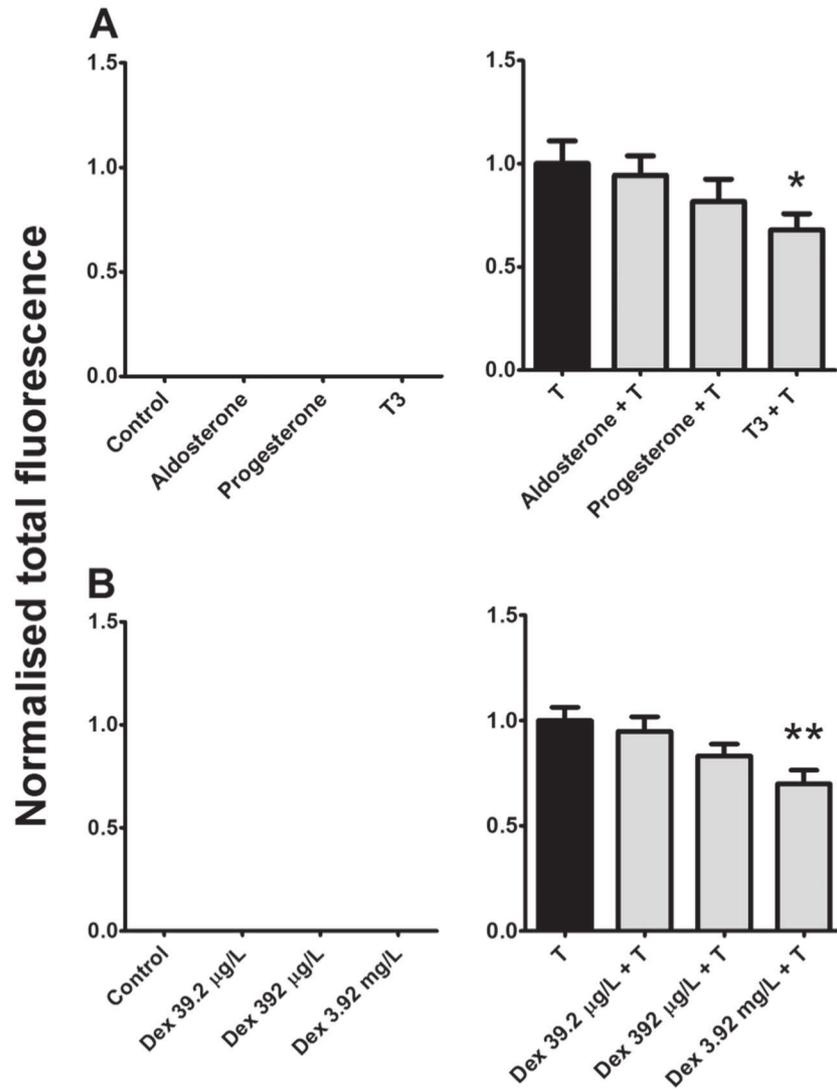


Fig. 5. A) High concentrations (10^{-7} M) of reference agonists for the mineralocorticoid, progestin and thyroid hormone receptors do not induce fluorescence in *chgh-gfp* fry. However, induction of fluorescence by testosterone is inhibited by T3. B) The reference corticosteroid dexamethasone (Dex) inhibits fluorescence signal induced by testosterone in a dose dependent manner. ** indicates $P < 0.01$ and * indicates $P < 0.05$. $N = 20$ per group, mean \pm SEM are given.

shown to respond to testosterone, although this was not clearly demonstrated to be due to biotransformation by aromatase (Kurauchi et al., 2005). In this study, we have demonstrated that the aromatisable androgen testosterone induced expression of GFP *via* the oestrogen responsive *chgh* promoter and that this was due to the presence of functional aromatase protein prior to dph 1 when the experiment was read.

We have tested a range of chemicals with the protocol described within this article and have demonstrated that the model can detect oestrogen axis disruption caused by EDCs *via* at least four different mechanisms. ER agonists (EE2, E2), aromatisable androgens (testosterone), aromatase inhibitors (anastrozole, fadrozole) and SERMs (tamoxifen). All gave a statistically significant modulation of fluorescence in either spiked (plus testosterone) or unspiked mode.

An interesting profile was observed with the SERM tamoxifen (Fig. 4B). Tamoxifen can act as an agonist or antagonist of the ER depending on the species and the tissue tested. It has previously been shown to increase oestrogenic signalling in male medaka, resulting in increased serum vitellogenin (Sun et al., 2007). This was confirmed by our studies which showed that, in the absence of testosterone, tamoxifen increased oestrogenic signalling whilst in co-treatment with testosterone it caused a decrease in fluorescence. This decrease was observed

in all three replicate experiments. These observations could potentially be due to competition for ER binding between tamoxifen and E2 generated by the fry in response to the testosterone challenge. For this to be the case, tamoxifen would have to be less effective at activating transcription of ER dependent genes than E2. This theory is supported by a study by Kawamura et al. (2002) who rescued E2 induced inhibition of yolk veins in medaka embryos by co-treatment with tamoxifen, thus demonstrating an inhibition of E2 action by tamoxifen in medaka *in vivo*. Further support comes from Sun et al. (2011) who showed that tamoxifen exposed male medaka showed an increase in hepatic vitellogenin mRNA expression whereas tamoxifen exposed female medaka showed a decrease. The difference between the two genders may be due to an increased circulating E2 load present in the female fish.

In addition, we have demonstrated the ability of this model to correctly identify indirect effects *via* hormonal crosstalk. Fig. 5 shows that a high concentration (10^{-7} M, 67 µg/L) of the natural thyroid receptor agonist T3 (triiodothyronine) results in a statistically significant reduction in fluorescence generated by a testosterone challenge. This suggests that T3 may be inhibiting aromatase activity, and is in line with published studies in rat Sertoli cells and porcine thecal cells, that show T3 inhibits aromatase activity, but not its expression (Ulisse et al., 1994; Gregoraszcuk et al., 1998; Andò et al., 2001). Similarly, evidence exists

for an inhibitory effect of progesterone on aromatase activity (Pasqualini and Chetrite, 2008). Although a reduction in fluorescence was observed with high dose progesterone, this did not achieve statistical significance, despite being observable in all three replicate experiments. A literature search failed to find evidence for modulation of aromatase activity by aldosterone.

This transgenic line and the protocol described here provide a reliable tool to screen potential EDCs acting on the oestrogen axis via a variety of mechanisms. Furthermore, this protocol provides information on steroid signalling from an entire intact organism whilst complying with the three R's principle of animal replacement. This will allow the much needed, rapid screening of compounds. The previous century was marked by a continuing increase in human developmental defects of the reproductive tract, as exposure to increasing numbers of chemicals has intensified (de Mouzon et al., 1996; Toppari et al., 1996; Toppari and Skakkebaek, 1998; Virtanen and Toppari, 2008). The method presented here is a step along the path towards prevention of these disorders which pose a risk not only to human health, but have wide impacts on biodiversity. As habitats for many animals continue to shrink, reducing population sizes, even small changes in fecundity may result in loss of species, underlining the urgency to exploit rapid screening approaches to identify and eliminate or control the use of chemicals negatively impacting the reproductive health of humans or wildlife (Western, 2001).

Author contributions

The manuscript was written by Dr Andrew Tindall with contributions from the other authors. All authors have given approval to the final version of the manuscript.

Conflict of interest

The authors declare the following competing financial interest(s). Two of the authors (G.L. and B.D.) have financial interests in WatchFrog, which commercializes the test described here. A.S., P.S., A.T. and G.L. are currently employed by WatchFrog. M.L. was employed by WatchFrog whilst she was involved in this study. T.I. declares no competing interests.

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Manuscript n°2

Presentation of manuscript n°2

Rapid fluorescence detection of unanaesthetised transgenic larvae

This manuscript is planned to be submitted as a short communication to the journal “Environmental toxicology and chemistry”

Previously, in publication n°1 of this thesis we describe the ChgH-gfp transgenic medaka. This model, together with the other transgenic GFP-harbouring models developed to detect endocrine disruptors shares common steps in the process of fluorescence detection and quantification-anaesthesia and manual positioning (Brion et al., 2012; Fini et al., 2007). This step is not only time consuming and thus costly, but also may interfere with the physiology of the organism, which might affect the final results of the experiment when multiple time-points are analysed (Laitinen et al., 1981). To our knowledge, no study has been published describing the fluorescence acquisition in multi-well plate readers without the use of anaesthesia. Therefore the purpose of this publication was to describe a novel protocol for simple, rapid and low cost fluorescence detection of un-anaesthetised transgenic larvae using a fluorescence plate reader and 96-well plates and thus facilitate the transfer of the already existing protocol using ChgH-gfp transgenic medaka and ThbZIP-gfp transgenic *Xenopus laevis* (Fini et al., 2007; Spirhanzlova et al., 2015). Protocols for reading these two lines using a fluorescent plate reader have since been published as French Standards (AFNOR XP_T90-716-1 XP_T90-716-2).

To validate the protocol, ChgH-gfp medaka fry and ThbZIP-gfp *Xenopus laevis* were exposed to a dose range of estradiol and T3 respectively. Prior to fluorescence quantification, both fry and tadpoles were placed unanaesthetised in clear bottom 96-well plates. Fluorescence was measured in a fluorescence plate reader, using custom read settings and parameters, which are described in this manuscript. To compare effectiveness of the newly developed protocol, fluorescence was also quantified for comparison by processing the images of the same fry and tadpoles anaesthetised and manually positioned. The sensitivity using fluorescence measurement of anaesthetised larvae in the plate reader was identical to the sensitivity of imagery, however the time and the number of persons needed to perform such fluorescence quantification decreased dramatically. Here we provide a reliable, rapid, low cost and simple protocol to quantify fluorescence of free swimming larvae, which is suitable especially with respect to the current

push towards automation and increased throughput of screening platforms for endocrine disruptors and which greatly facilitates the already existing protocols using ChgH-gfp medaka and ThbZIP-gfp tadpoles.

Rapid fluorescence quantification in unanaesthetised, transgenic larvae

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Abstract

Here we describe a new automated approach to fluorescence quantification of free-swimming larvae. We demonstrate cross-species applicability using transgenic medaka and *Xenopus laevis* larvae expressing green fluorescence protein. This method is as sensitive as quantification by imagery, but is less time consuming and lower cost. Using this technique, repeated measures can be performed without manipulating or anaesthetising the larvae, thus facilitating time course experiments and eliminating confounders.

1. Introduction

Today many laboratories use transgenic organisms harbouring genetic constructs driving expression of fluorescent proteins to link gene expression with physiological effects. Common model organisms used for this purpose are the medaka (*Oryzias latipes*)¹⁻⁴ as well as *Xenopus laevis*⁵. Transgenic embryos from these species are small, semi-transparent, easily maintained and produce large numbers of eggs, making these models very useful for rapid, large scale *in vivo* experiments. In addition, early developmental stages of fish, which still feed from their reserves of vitellus, are not considered as laboratory animals under European Union regulations (Directive 2010/63/EU).

The most common method to quantify fluorescence in medaka or xenopus embryos is by imagery^{6,7}. The need for anaesthesia followed by manual positioning and image capture of each individual fry makes this method time consuming and expensive, and therefore limits research productivity. As the embryos are often euthanized directly after fluorescence quantification, repeated measures of the same embryo are impossible to carry out. If repeated measures are attempted, the use of anaesthetics may also have an effect on the physiology of the embryos, confounding experimental results.

Recently, there have been varied attempts to facilitate the manipulation of larvae and embryos using several automated platforms⁸⁻¹¹, however a simple low-cost alternative solution has yet to be proposed.

An alternative option for reading fluorescence intensity is the use of microplate readers, which are often used in laboratories performing *in vitro* experiments. Microplate readers are devices designed to measure and quantify the fluorescence, luminescence or absorbance of samples placed in microplates. The most common microplates are 96-well with a working volume of up to 300µl per well. The main advantages of microplate readers compared to fluorescence microscopy are ease of use, full automation, temperature control and reduced cost.

Microplate readers have been used for *in vitro* bioassays for many years ^{12,13}. However, examples of their use in *in vivo* experiments have been rarely reported ⁶ and to our knowledge have always involved anaesthesia and manual positioning of each embryo. There have been attempts to use fluorescence plate readers to measure the fluorescence of homogenized embryos, although this method was unsuccessful ⁷. To our knowledge, no protocols using plate readers have been developed for measuring the fluorescence of live, free swimming aquatic larvae.

To simplify and improve the method of measuring the fluorescence, we developed a rapid and inexpensive protocol for reading non-anaesthetised medaka and xenopus embryos in a fluorescence plate reader. Here we describe our newly developed protocol and compare the results to those obtained by fluorescence imagery of the same tadpoles or fry.

2. Materials and Methods

2.1. Solutions

Evian water used in this study was purchased in glass bottles. Medaka medium contained 4.02 mM KCl, 1.62 mM MgSO₄ · 7H₂O, 1.36 mM CaCl₂ · 2H₂O, 0.17 M NaCl and 62.5 µM methylene blue. The pH was adjusted to 7.2-8.0. Ethyl 3-aminobenzoate methanesulfonate salt (MS222) was prepared at 1g/L, the pH was adjusted to 7.5-8.0 and it was diluted as necessary in medaka medium or Evian water. All salts and test substances were purchased from Sigma-Aldrich.

2.2. Exposure of fish fry

To evaluate the fluorescence plate reader protocol described here, we compared its performance with a previously described imagery based protocol. A simple experiment was carried out using a transgenic medaka line capable of detecting estrogen axis activity. This line harbours a genetic construct containing 2047bp of the upstream region of the medaka *choriogenin h (chgh)* gene driving expression of the coding sequence of *gfp*. This transgene expresses *gfp* in the liver of the fry in response to estrogen axis activation (Figure 1A). In the absence of exogenous estrogen treatment no GFP is observable in the liver. However, basal fluorescence in the heart allows sorting of transgenic and non-transgenic embryos.

Three experiments were carried out with transgenic day post hatch zero (dph0) medaka embryos (n=20 per group) which were treated with a series of concentrations of ethinyl estradiol (EE2). The stock solutions of hormones were prepared in DMSO and the final concentration of DMSO was 0.1%. Five embryos were placed in each well resulting in total of 4 wells per group.

The embryos were maintained for 48 hours in an incubator at 26°C with a 10 h dark/ 14 h light photoperiod. The test solutions were renewed once after 24 hours. Shortly before the read, embryos were rinsed in fresh medaka medium.

2.3. Fluorescence quantification of fish fry by imagery

Each fry was anaesthetized with 200mg/L buffered MS222 in medaka medium and manually positioned to allow imaging of the ventral region. An Infinity 1-3C camera (Lumenera Corporation, Ottawa, ON, Canada) fitted to a Leica MZ10F stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany) was used to image each embryo's abdomen. Embryos were illuminated with an Excite 120Q fluorescence source (Lumen Dynamics Group Inc., Mississauga, ON, Canada) and a GFP longpass filter set (Excitation 470/40, Emission 515LP). Images were captured with a 300ms exposure and 8x magnification and saved as 8-bit colour jpg files.

The images of the fry were analysed using an ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA) macro as previously described ¹⁴. A 10-255 threshold was applied to the resulting images to eliminate residual autofluorescence from endogenous pigments, and the liver was manually selected. The sum of all pixel intensities within the selected region was calculated.

2.4. Exposure of tadpoles

To determine the cross-species applicability of our protocol, we performed a similar experiment using a transgenic *Xenopus laevis* line capable of detecting thyroid axis disruption. This line harbours the leucine zipper transcription factor (TH/bZIP) promoter driving expression of *gfp* coding sequence. The *gfp* expression is principally in the brain ⁵ (Figure 1B). The TH/bZIP *Xenopus* line is homozygous, therefore sorting of transgenic and non-transgenic tadpoles was not required.

We exposed groups of 20 stage 45 tadpoles ¹⁵ to a range of concentrations of triiodothyronine (T3) diluted in Evian water. The tadpoles were incubated in six-well plates containing 8 ml of exposure solution per well and 10 tadpoles per well. The exposure time was 72 hours, and the exposure solution was changed every 24 hours. The tadpoles were maintained in the dark in an incubator at 21°C. At the end of the exposure, tadpoles were rinsed in fresh Evian water.

2.5. Fluorescence quantification of tadpoles by imagery

Tadpoles were rinsed in 50 ml Evian medium and anaesthetized in 50ml MS222 200 mg/L for 5 minutes. Tadpoles were placed separately in the wells of a black, conical based-96 well plate (Greiner Bio-one), with the head in the centre of the well and the dorsal surface in contact with the plate so as to limit interference with the GFP signal by melanocytes present on the dorsal surface of the skin. Each group of 20 tadpoles was placed on two consecutive lines, ten tadpoles per line, leaving the last two wells empty for the quantification of background fluorescence. Four groups were placed on each plate. An image of each well was automatically captured using an imagery system based on a macrofluor stereomicroscope (Leica), a robotized platform

(Marzhauser Wetzlar GmbH & Co. Germany) and a high resolution ORCA-AG camera (Hamamatsu). The entire imagery system was controlled by Simple PCI software (Hamamatsu). The system was equipped for fluorescence with band pass GFP filters (excitation 475-495 nm, emission 495-545 nm) (Leica). Quantification of the mean pixel intensity was carried out for each image using Simple PCI software. Background normalization was carried out on a plate by plate basis by subtracting the mean pixel intensity of the empty wells from all of the values acquired on the same plate.

2.6. Rapid fluorescence quantification of fish fry and tadpoles

The same fry or tadpoles read using the two imagery systems described above were also read using a fluorescence plate reader (Infinite F200, Tecan) without the use of anaesthesia. Due to the localisation of the fluorescence expression in the liver of the medaka fish (localized ventrally) and *Xenopus* brain (more easily visualised ventrally), 96-well transparent bottomed micro-plates (Greiner) were used, allowing fluorescence measurement from below. Non-anaesthetized tadpoles or fry were placed one per well in approximately 300µl of Evian or medaka medium respectively. Ten embryos were placed per row with last two wells empty for the quantification of background fluorescence. During the fluorescence measurement, the device scanned each well divided across a 49 (7x7) point grid, resulting in 49 values from each well (Figure 2). The highest value for each well was assumed to correspond to the area expressing fluorescence and was selected (Fmax). The mean Fmax value for the empty wells on each plate was calculated and subtracted from the Fmax values of wells containing fry or tadpoles. To increase the accuracy of the data, each plate was read three times consecutively and the mean of the three values obtained for each well was used for further analysis.

2.7. Time course experiment

An advantage of reading tadpoles or fry without the stress of manipulation or anaesthetics is the opportunity to carry out time-course experiments. Newly hatched medaka fry (n=20 per group) were treated in Petri dishes containing 100 ml of medaka medium plus or minus 500µg/L 17β-

oestradiol (E2). Fry were kept at 26°C for six hours before placing them into a 96-well plate. The fry were maintained in the 96-well plate in 300µl of the relevant exposure solution at 26°C until 28 hours after the start of the exposure. The plate-reader was programmed to read the plate every two hours and the data were acquired with the same protocol described in section 2.6.

2.8. Statistical Analysis of Results

All results are expressed as mean and SEM. Data were tested for normality using the D'Agostino and Pearson test. Significance was assessed using a one-way ANOVA followed by Dunnett's test (normal data) or Dunn's Test (non-normal data). All groups were compared to the control group. Differences were considered significant at $p < 0.05$. Statistics were carried out with Prism v5.04 (GraphPad). All graphs show mean \pm SEM, *** indicates $P < 0.001$, ** indicates $P < 0.01$, * indicates $P < 0.05$.

3. Results

3.1. Comparison of sensitivity of fluorescence quantification by imagery and by fluorescence plate reader

In the case of the experiment with the *ChgH-GFP* medaka fry, the imagery data acquired by fluorescence microscope were taken prior to the read in the fluorescence plate reader. Readouts with both sets of equipment were performed within one hour of each other. With our newly developed protocol, we obtained a detection limit of 45ng/l EE2 which is equal to the detection limit obtained by fluorescence microscopy (Figure 3A).

Conversely to the protocol used for the medaka experiment described in section 1.3, the fluorescence measurement of the experiment with the *TH/bZIP-GFP* tadpoles was carried out with the fluorescence plate reader followed by image capture, also in the time frame of one hour. This reading order was changed between the two lines to reduce bias towards one readout system. The limit of sensitivity of the imagery read was 2µg/L T3 which is identical to the sensitivity obtained using the fluorescence plate reader (Figure 3B).

3.2. Time-course experiment

In the time course experiment, GFP induction attained statistical significance 20 hours after exposure of the fry to 500µg/L 17β-oestradiol (E2). The experiment was repeated three times and all three replicates were pooled (Figure 4). Even after 28 hours of exposure of the fry, of which 22 hours were in a 96-well plate, no mortality was observed, demonstrating that our method is at least suitable for subchronic exposures (Figure 4).

4. Conclusion

We developed a protocol describing fluorescence quantification in live non-anaesthetised larvae using a fluorescence plate reader. We compared our newly developed method with classic imagery, requiring anaesthesia and manual positioning of each individual larvae. A time course experiment was performed to demonstrate that our newly developed method is applicable for experiments, where the multiple measures are advantageous.

When imagery is used for the experimental readout, the results are in the form of images of each embryo. This is advantageous as it allows direct observation of the signal location, intensity and area. In addition, the presence or absence of signal can be confirmed visually, reducing the chance of misinterpretation of data. However, this method can be highly time consuming and costly. Our goal was to decrease the time and cost of an experimental readout and facilitate the procedure of fluorescence quantification greatly facilitating inter-laboratory technology transfer.

The method we developed shows a number of advantages. The price of fluorescence microscope, camera and software is many fold higher than the cost of a fluorescence plate reader. Technically, the use of fluorescence plate reader is simple, and does not require specialised training. Furthermore, most laboratories already possess this type of equipment.

Another advantage of our protocol is the reduction in time as it takes only 21 minutes to read one 96-well plate in three consecutive reads. As the readout is fully automated, human assistance isn't required during the reads, allowing time points to be measured easily during inconvenient periods.

The sensitivity of this protocol is identical to the sensitivity obtained by imagery, and so, importantly, this is not a case of compromising between speed/cost and quality considerations, but is instead an alternative method of comparable quality. Considering all described advantages,

our protocol for fluorescence quantification using a fluorescence plate reader is a rapid, low cost and simple alternative to imagery suitable for inter-laboratory cooperation.

Figures

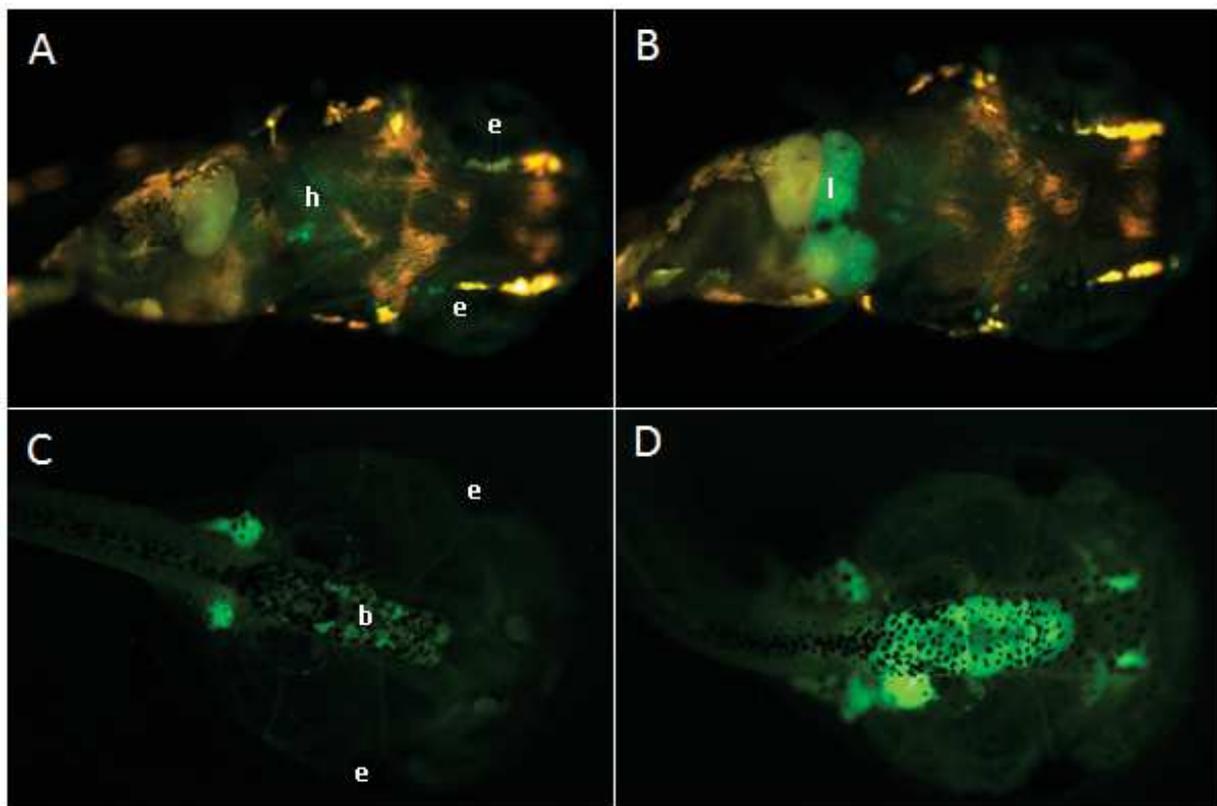


Figure 1. Fluorescence induction of transgenic fry. (A) ChgH-GFP transgenic medaka fry treated for 48 hours with a hormone-free control solution. (B) ChgH-GFP transgenic medaka fry treated for 48 hours with 90 ng/L EE2 visualised under blue light. (C) TH/bZIP-GFP transgenic tadpole treated for 72 hours with hormone-free control solution. (D) TH/bZIP-GFP transgenic tadpole treated for 72 hours with 3.25 $\mu\text{g/L}$ T3. b, brain; e, eye; h, heart and l, liver.

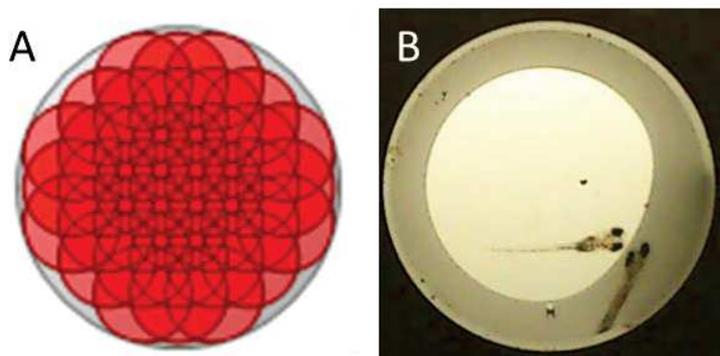


Figure 2. Image of one well of a 96-well plate. (A) Representation of the 49 (7x7) point grid pattern used for fluorescent measurement of each well. (B) Medaka fry swimming freely in a well.

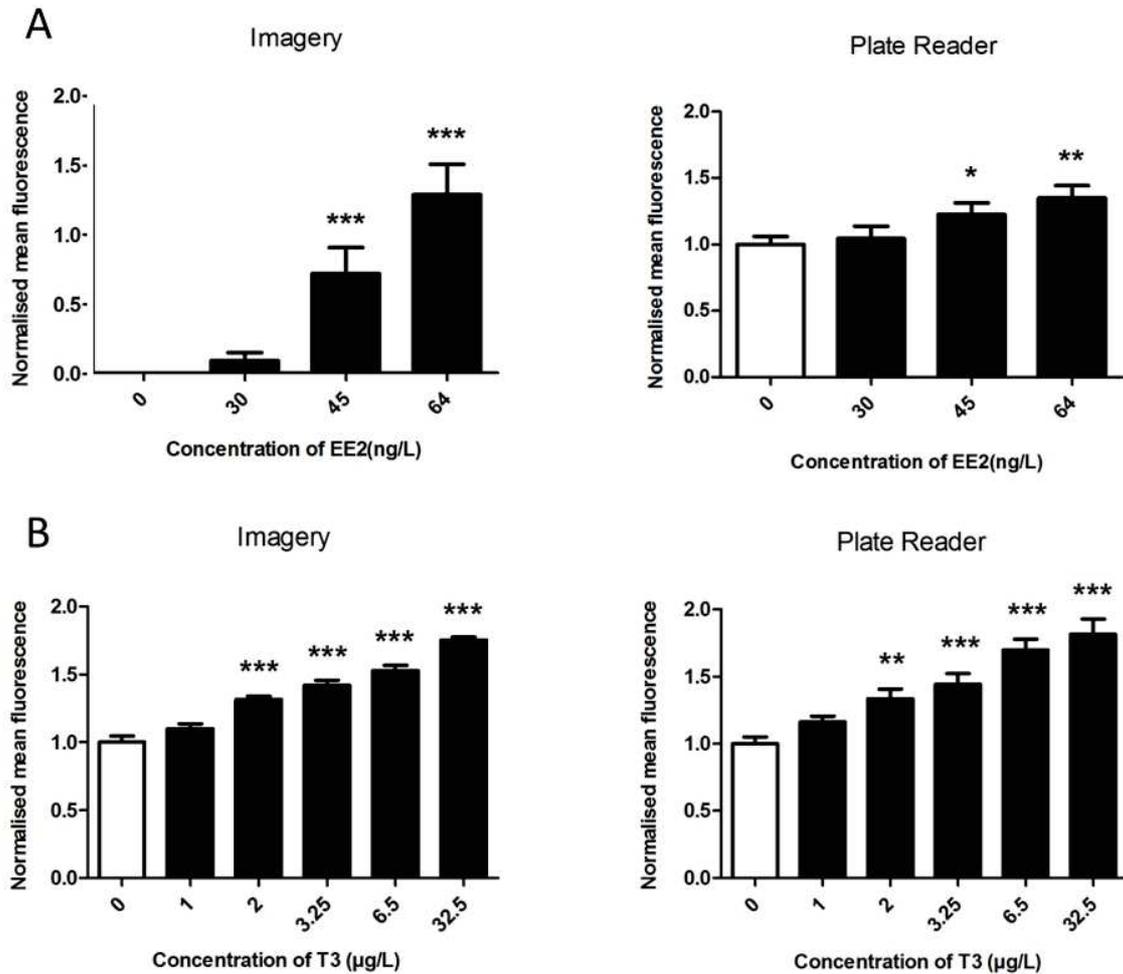


Figure 3. Comparison of imagery and plate reader fluorescence quantification protocols. (A) Dose-response of EE2 measured by imagery followed by fluorescence plate reader. (B) Dose response of T3 measured by fluorescence plate reader followed by imagery.

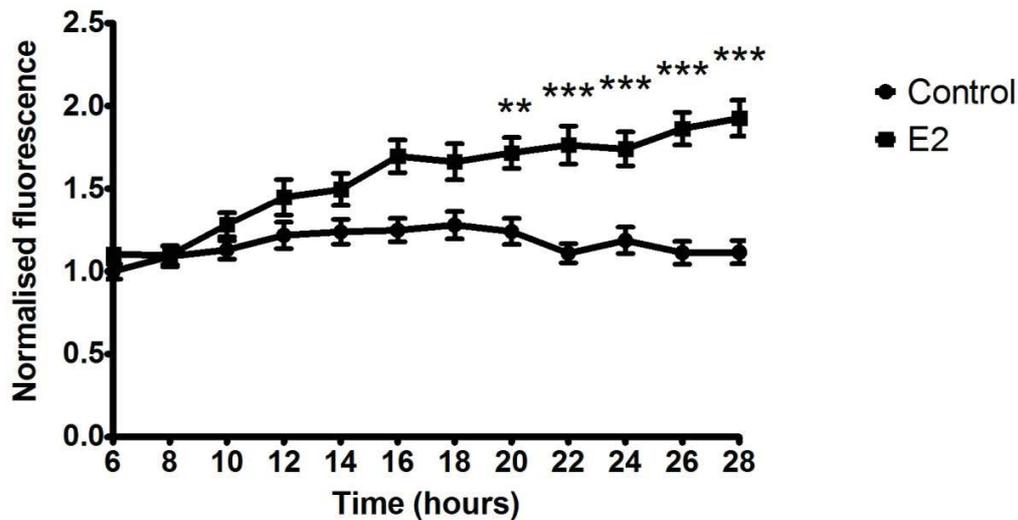


Figure 4. Evolution of fluorescent signal during a 28 h exposure to 500µg/L E2. The ChgH-GFP medaka fry were exposed in 96-well plates incubated within the fluorescent plate reader for the entire period shown (6-28 hours).

5. Author contribution

The manuscript was written by Petra Spirhanzlova with contribution from the other authors. All authors have given approval to the final version of the manuscript.

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Manuscript n°3

Presentation of manuscript n°3

Transgenic medaka reveal dynamic temporal sensitivity to disruption of sex determination

Previously in publication n°1 we described a transgenic ChgH-gfp medaka used to detect (anti-) estrogens and aromatase inhibitors. Several definitions of an endocrine disruptor require the identification of endocrine activity leading to an adverse physiological effect (Zoeller et al., 2014). With this in mind we set out to develop a transgenic model to facilitate the identification of the (anti-)estrogenic potential of endocrine disruptors in addition to their impact on the feminisation of the gonads. We created a double transgenic medaka harbouring biomarkers for both oestrogen axis signalling (ChgH-gfp) and the presence of developing oocytes (42sp50-gfp). The overall aim was to develop protocol using the ChgH-gfp_42sp50-gfp medaka line to screen potential endocrine disruptors in a short time frame, which would confirm or deny the impact of the given chemical to alter estrogen signalling and/or induce physiological changes in the gonads.

Ovo-testis formation can be induced in the laboratory by exposing fish to estrogens or androgens, but it can also be induced by other environmental stressors for example hypoxia or high temperature. The germinal transgenesis resulted in stable ChgH-gfp_42sp50-gfp line expressing GFP in the liver in response to estrogen treatment and in developing oocytes. Medaka fish are in particular suitable for creation of such models, as the identification of a male specific gene – *dmy* allows clear identification of the genetic sex.

We document in this study the first step of the protocol development- ChgH-gfp_42sp50-gfp transgenic medaka creation and identification of the most sensitive embryonic periods for disruption of sex determination, by administration of 24 hours long pulses of high doses of estradiol, dihydrotestosterone or the pharmacological anti-androgen flutamide.

Transgenic medaka reveal dynamic temporal sensitivity to disruption of sex determination

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Abstract

Gonadal development in medaka (*Oryzias latipes*) is dependent on the synergy between oestrogens and androgens. Disruption of steroid hormone levels can induce formation of ovo-testis, a clear indicator of abnormal development of germ cells. To determine the sensitive windows for hormonally induced sex reversal in medaka, we developed a novel 42sp50-GFP_ChgH-GFP transgenic medaka line. This line allows the identification of female gonadal tissue by fluorescence (GFP) present in developing oocytes and in parallel allows quantification of oestrogen axis activity via GFP in the liver. Germinal transgenesis resulted in a stable line exhibiting strong GFP signal in the ovaries and in the liver in response to oestrogens. To identify the sensitive developmental period to exogenous agents, fry were exposed to 24 hour pulses of oestradiol (E2; 500µg/L), dihydrotestosterone (DHT; 2.5µg/L or 500µg/L) or flutamide (5mg/L) at various time points between day post fertilisation (dpf) 0 and 12. Evaluation of phenotype followed by genotyping at dpf 16, revealed sensitivity to E2 between dpf 1-11 and two periods of increased susceptibility to DHT or flutamide between dpf 0-1 and dpf 5-8. No phenotypic sex reversal was detected after exposure to DHT or E2 on dpf 11-12 or 12 respectively. We show that early developmental stages are sensitive to sexual steroids responding with the induction of ovo-testis in XY and XX medaka. The model is thus making this an excellent tool for studying disruption of gonadal development.

Introduction

Numerous studies have reported the presence of abnormal gonadal morphology in wild fish (Hinck et al., 2009; Kavanagh et al., 2004; Tetreault et al., 2011). The most commonly observed change in gonad structure is the formation of ovo-testis, defined as a gonad containing both testicular and ovarian tissue (Miller and Keane, 2003). Both laboratory and field studies have demonstrated that this condition can be induced by exposure to endocrine disrupting chemicals (EDCs) (Kavanagh et al., 2004). Interestingly, the recent elucidation of genetic sex determinants for several fish species [*Oryzias latipes* (Matsuda et al., 2002), *Danio rerio* (Wilson et al., 2014), *Odontesthes hatcheri* (Hattori et al., 2012), *Takifugu rubripes* (Kamiya et al., 2012), *Oryzias luzonensis* (Myosho et al., 2012), *Oncorhynchus mykiss* (Yano et al., 2012)] has revealed that

ovo-testis can be induced in fish possessing male or female genotypes (eg. Kobayashi et al., 2011; Urushitani et al., 2007).

In the case of genetically male fish, several case studies on wild teleost fish document the presence of oocytes within the testes of XY males, in addition to feminisation of reproductive tracts, elevated levels of hormones characteristic for females and abnormal levels of plasma vitellogenin (Bahamonde et al., 2015; Pollock et al., 2010; Sanchez et al., 2011). Analogous studies of masculinised female fish, very often exposed to pulp and paper mill effluents show equivalent effects. The genetically female fish exhibited ovo-testis, male secondary sex characteristics such as elongated dorsal fins and increased concentrations of hormones typical for males (Bortone and Cody, 1999; Parks et al., 2001).

In addition to the clear links with EDCs, environmental factors such as high temperature or hypoxia have also been linked to the intersex condition (Cheung et al., 2014; Sato et al., 2005). However, it now seems that at least some environmental stressors causing intersex may be acting through disruption of endocrine signalling. Hypoxia causes masculinization of female Atlantic croaker by reducing the expression of aromatase thus inducing the formation of testis-ova (Thomas and Rahman, 2012). High temperature treatment can also cause female to male sex reversal under laboratory conditions. In medaka, this has been shown to be due to elevation of cortisol levels, which in turn suppresses the expression of aromatase (Hayashi et al., 2010a; Sato et al., 2005). Masculinisation induced by high temperature or cortisol can be rescued by concomitant E2 treatment (Kitano et al., 2012).

Under the current non-regulatory definitions of an EDC given by the EU, the Environment Agency and the WHO/IPCS, it is required that the substance provokes an adverse physiological effect secondary to a modification of endocrine signalling (reviewed in Zoeller et al., 2015). It is the confirmation of an adverse physiological effect which increases the time required to perform most assays and therefore their cost. Despite the identification of a number of biomarkers capable of rapidly identifying changes in endocrine signalling, confirmation of adverse physiological effects often requires raising the animals to adult life stages and even breeding them to obtain the next generation.

A recent study explored the link between marker gene responses and the later apparition of physiological changes (Ankley and Villeneuve, 2015). One biomarker tested in this study,

vitellogenin, showed a strong correlation. Perhaps what differentiates vitellogenin from the other biomarkers studied is its essential role in egg production, one of the aforementioned physiological criteria, whereas the other biomarkers were involved in generation of an endocrine signal (plasma steroid levels and expression of steroidogenic enzymes). It is with this in mind that we set out to identify the predictability of another protein required for egg production.

Choriogenin H (ChgH) protein is produced in the liver and is transported via the hepatic-ovarian portal vein to the ovaries, where it is actively taken up and integrated into the chorion of developing eggs. Like vitellogenin, the promoter of the ChgH gene contains a number of oestrogen response elements (ERE), therefore in addition to its expression being linked to a well-recognised adverse effect, insufficient viable egg production; it is also a marker for oestrogen axis signalling. One advantage of ChgH over vitellogenin is that it has been shown to be more dynamically expressed in response to oestrogens and is therefore a more sensitive biomarker (Scholz et al., 2005; Yu et al., 2006).

The first ChgH-GFP transgenic medaka line was developed by Karauchi et al. (2005). To facilitate exploration of the predictability of the ChgH-GFP transgene for identifying endocrine disruptors we set out to develop a double transgenic line harbouring biomarkers for both oestrogen axis signalling (ChgH-GFP) and the presence of developing oocytes (42sp50-GFP). Kinoshita et al. first demonstrated the ability of the 42sp50-GFP transgene to induce a strong fluorescent signal in developing oocytes, and therefore its ability to identify females as well as intersex fish (Kinoshita et al., 2009). Our aim was also to develop a model capable of screening potential endocrine disruptors in a reduced time frame, confirming or denying the ability of the given chemical to alter endocrine signalling and to induce physiological changes.

As a first step in developing this model, we report here, the use of 24 h acute exposures to high doses of an oestrogen, an androgen and an androgen receptor (AR) antagonist to identify the most sensitive embryonic periods for disruption of sex determination.

1. Materials and methods

1.1. Reagents

Medaka medium contained 4.02 mM KCl, 1.62 mM MgSO₄ · 7H₂O, 1.36 mM CaCl₂ · 2H₂O, 0.17 M NaCl and 62.5 μM methylene blue. The pH was adjusted to 7.2-8.0. Ethyl 3-aminobenzoate methanesulfonate salt (MS222) was prepared at 1g/L, the pH was adjusted to 7.5-8.0 and it was diluted as necessary in medaka medium. All test substances were purchased from Sigma-Aldrich: 17β-oestradiol (E2, CAS: 50-28-2), 5α-androstan-17β-ol-3-one (DHT, CAS: 521-18-6) and Flutamide (CAS: 13311-84-7). Romeiod (iodophor solution 0.5% active iodine) was purchased from Cofa and diluted in medaka medium to a final concentration of 2%.

1.2. Genetic construct

A genetic construct was synthesized (GeneCust) comprising 1283 bp of the medaka *42sp50* gene immediately upstream from the translation start site, followed by the coding sequence of *gfp* and terminated by 1641 bp of the medaka *42sp50* gene immediately downstream from the stop codon. This construct was previously described by Kinoshita et al. (2009). In addition, XhoI sites were synthesized on both sides of the construct and were used to insert it into a plasmid containing an additional, previously described cassette comprising of a portion of the medaka *chgh* gene driving expression of *gfp* coding sequence (Kurauchi et al., 2005; Spirhanzlova et al., 2015). A single plasmidic clone was selected containing both genetic cassettes facing in opposite directions in order to limit interactions between the two cassettes (Figure 1C).

1.3. Germ line transgenesis

Germ line transgenesis was carried out according a previously described protocol in the absence of meganuclease by injecting a linearized construct into fertilized one cell stage Carbio red-orange strain medaka eggs (Kinoshita et al., 1996).

1.4. Exposure studies

All stocks were prepared in DMSO. For each developmental time point to be studied, 42sp50-GFP_ChgH-GFP medaka eggs were collected within two hours of fertilisation, disinfected with 2% romeiod for 10 minutes and divided into three Petri dishes containing 100 mL of medaka medium. The three groups of eggs were then randomly assigned exposure conditions. Medium was changed three times per week and dead eggs were removed every 24 hours.

The first series of exposures was carried out by adding 50 µl of 17β-oestradiol (E2, 1g/L), dihydrotestosterone (DHT, 5 mg/L) or solvent alone to the corresponding Petri dishes at the developmental time point of interest. Final concentration of DMSO was 0.05% in all groups. The second series of experiments was carried out by adding 100 µl of DHT (500 mg/L), flutamide (5 g/L) or solvent alone. Final concentration of DMSO was 0.1% in all groups.

For both sets of experiments, precisely 24 hours post-exposure, the fry were rinsed three times with fresh medaka medium and placed into clean media. After hatch, transgenic fry were identified by a basal GFP signal in the gills and were retained. Non-transgenic fry were excluded from the experiment. Hatched fry were fed with TetraMin baby (Tetra). Exposure studies were repeated and the results were pooled as necessary to obtain a minimum of 50 transgenic fry per group at the end of the exposure.

1.5. Image capture

Six days after hatching fry were anaesthetised with 200 mg/L MS222 and manually positioned to expose the ventral region. A Leica MZ10F stereo-microscope (Leica Microsystems GmbH) equipped with ET-GFP long-pass filters (excitation 480/40, emission 510LP, Leica Microsystems GmbH) and a 200W fluorescence source (Prior Scientific) was used to image the fry. Images of the genital region of the fry were taken with an exposure time of 0.75 s at 8x magnification using an Infinity 1-3C camera (Lumenera Corporation). Each fry was then euthanised with MS222, rinsed with fresh medaka medium and placed separately into 1.5ml tubes. After the removal of all liquid, the tubes were immediately frozen at -80°C for subsequent genotyping.

1.6. Genotyping

Extraction and purification of genomic DNA was carried out using the Pure Link Genomic DNA Mini Kit (Invitrogen) according to the provided protocol. PCR amplification of a region of both *dmrt1* and *dmy* genes was carried out using a single primer pair as previously described (Patil and Hinze, 2008). The PCR amplification was carried out using a BioRad MyCycler with a program consisting of one cycle of 94°C for 9 minutes followed by 42 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 2 minutes and terminated with one cycle of 72°C for 5 minutes. The DNA bands were then visualized by gel electrophoresis using 0.5x TBE and 1% agarose stained with SYBR® Safe DNA Gel Stain (Invitrogen).

1.7. Statistical Analysis

The percentage of fry in each group displaying gonadal fluorescence was compared to the percentage observed in the corresponding control group. The comparison was carried out using the binomial ratio (phenotypic sex) with the probability of success set to the ratio observed in the control group. Results which were significantly different to the null hypothesis are indicated as: *** ($P < 0.001$), ** ($P < 0.01$) and * ($P < 0.05$).

2. Results and Discussion

2.1 The 42sp50-GFP_ChgH-GFP transgenic line

Germline transgenesis generated one stable line. As with the previously described *42sp50-GFP* line, fluorescence was first visible in the gonadal region of certain fry one or two days post hatch (dph). Developing oocytes became highly fluorescent around dph 5 or 6 (Figure 1A). Phenotypically male fry display a complete absence of green fluorescence in the gonads (Kanamori and Toyama, 2013; Kinoshita et al., 2009). Due to the extremely strong fluorescence in the vitellus of the eggs of this line (Figure 1B), eleuthero-embryos from crosses with transgenic females also present strong fluorescence in their maternal reserves of vitellus. As this passes through their gastro-intestinal tract it continues to fluoresce, confounding correct identification of fluorescence in the gonads. Therefore, all experiments were carried out by crossing heterozygotic transgenic males with wild-type (red-orange strain) females.

Ectopic fluorescence, possibly due to the genomic integration site of the transgenes, is observable in the gills of males and females. This faint fluorescence allows transgenic and non-transgenic fry to be identified shortly after hatching.

As previously reported in a number of publications the ChgH-GFP transgene was expressed in the liver in response to E2 and ethinyl-oestradiol (EE2) (Kurauchi et al., 2005, 2008; Scholz et al., 2005; Spirhanzlova et al., 2015). During an embryonic chronic exposure to E2, GFP was first visible in the liver of unhatched embryos at dpf 6 (data not shown).

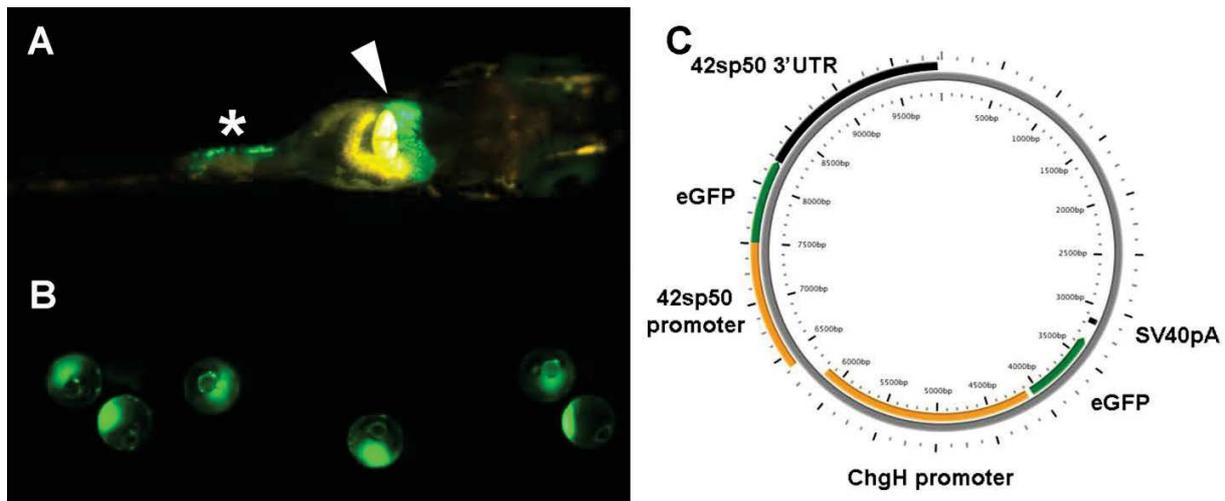


Figure 1: A) A phenotypically female fry of the 42sp50-GFP_ChgH-GFP line expressing fluorescence in the ovary and liver following exposure to EE2. B) Newly fertilised eggs from a transgenic female expressing GFP. C) The ChgH-GFP_42sp50-GFP bicistronic plasmid.

2.2 Exposure Series 1

2.2.1 Effects of DMSO treatment on sex reversal

A preliminary verification of the sex ratio in untreated embryos raised in the same manner as the treated fry demonstrated that 53% of the fry developed as phenotypic females and 47% as phenotypic males (Figure 2). No disagreement between phenotypic and genotypic sex was observed (Table 1A).

In order to aid the solubility and dispersion of test compounds, in particular those with low water solubility, DMSO was chosen as the solvent to be used in all exposure groups (except the water control). Low concentrations of DMSO have previously been shown not to interfere with the sexual differentiation of fish (Hutchinson et al., 2006). Treatment of fry with solvent alone (DMSO 0.05%) did not significantly alter the phenotypic sex of the fry compared to the non-DMSO treated control group except at dpf 2 and 4 ($P=0.04$ and $P=0.03$ respectively, Figure 2). An increased percentage of fry displayed oocytes at dpf 2 and a decreased percentage at dpf 4. This however was due to an unusual repartitioning of males and females in this batch of embryos, as confirmed by genotyping. Genotyping confirmed that for the DMSO control group treated at dpf 4, the phenotypic sex corresponded to the genotypic sex in all cases (Table 1B). Fry exposed

at dpf 2 were not genotyped.

However, when the solvent alone treatment was carried out at other time points a low percentage (0-17%) of disagreement between phenotypic and genotypic sex was observed. Prior to dpf 6 these figures are in line with those observed when rearing medaka in standard laboratory conditions: 12% XX males for Carbio strain medaka compared to 6% maximum XX fish exhibiting a male phenotype and an absence of XY females in this study (Nanda et al., 2003). These cases have previously been documented and are believed to be due to spontaneous sexual development of XX fish towards a male phenotype under the direct control of *dmrt1* rather than *dmy* (Nanda et al., 2003). It has been shown that the number of these cases increases under conditions of stress via the inhibitory action of cortisol on aromatase expression (Hayashi et al., 2010a; Kitano et al., 2012).

From dpf 6 onwards XY phenotypic females are observed (17% dpf 6 and 14% dpf 12, Table 1B). This appears to be stage specific, not observed in the dilution water control group, and may be an effect of the solvent and to our knowledge has not been previously reported.

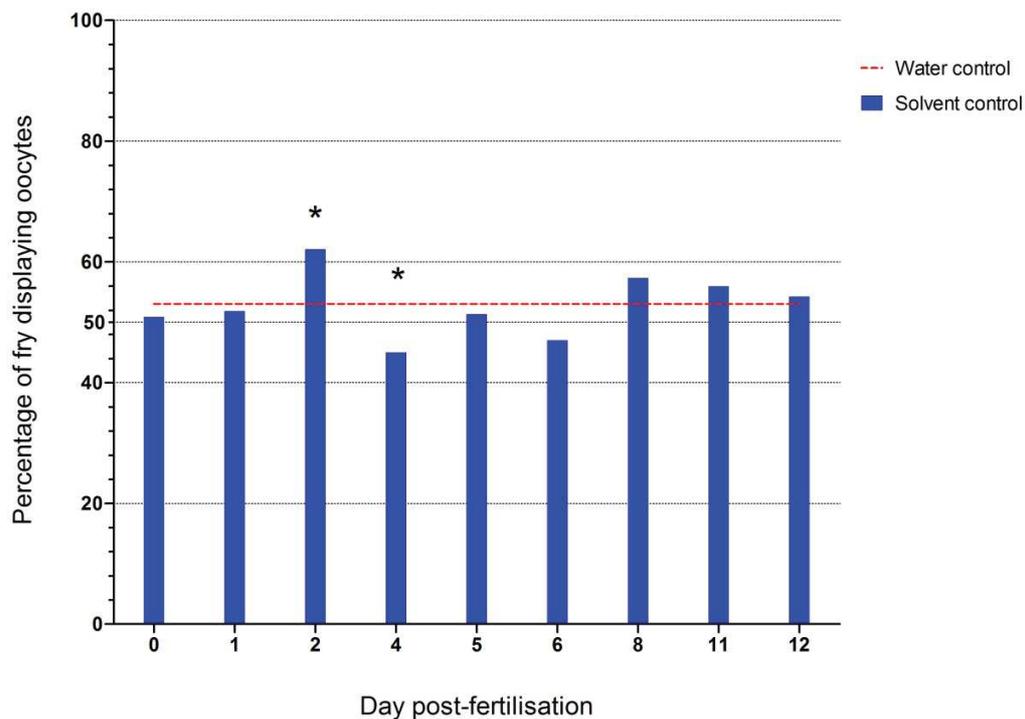


Figure 2: Percentage of fry exhibiting gonadal fluorescence at DPF16. The fry were treated with a 24 h pulse of DMSO (0.05%) at different developmental time points (x-axis). Following this treatment they were returned to clean media. The percentage of untreated control fry displaying GFP+ cells in their gonads at DPF16 is shown by the red line.

A		XY	XX
Male phenotype		16	0
		100%	0%
Female phenotype		0	15
		0%	100%
n		31	

B		0		4		5		6		12	
Day post fertilisation		XY	XX	XY	XX	XY	XX	XY	XX	XY	XX
Male phenotype		16	1	18	0	15	1	19	1	12	0
		100%	6%	100%	0%	100%	6%	83%	11%	86%	0%
Female phenotype		0	16	0	14	0	16	4	8	2	16
		0%	94%	0%	100%	0%	94%	17%	89%	14%	100%
n		33		32		33		32		30	

Table 1: Genotyping results of a subset of fry from the water control group (A) and the DMSO exposure groups (B). Phenotype is as determined by the presence or absence of fluorescence in the gonads. Percentages indicate relative numbers of fry developing normally or abnormally by genotype.

2.2.2 Dihydrotestosterone treatment

In order to determine the relative sensitivity of different developmental time points, a high concentration of DHT was used to perform 24 h acute exposures of developing medaka embryos. The concentration of DHT chosen for the first series of experiments (2.5 µg/L) was the same as the concentration of methyl-DHT previously identified as the minimum concentration producing maximum effect (Kobayashi et al., 2011). However, the percentage of fry treated with this concentration of DHT at dpf 0 and exhibiting oocytes at dpf 16 was not statistically different to the solvent alone group (Figure 3). Genotyping showed that despite the near identical sex ratio to the control group, 30% of XX fish exposed to DHT at dpf 0 failed to display oocytes (Table 2). This effect was masked in the phenotypic ratio because an equivalent number of fish (39%)

displayed oocytes despite possessing a Y-chromosome. Kobayashi et al. described this as paradoxical sex reversal which they did not observe with 2.5 µg/L of methyl-DHT but they did observe with higher concentrations of the same molecule (Kobayashi et al., 2011).

Statistically significant decreases in the number of fry displaying oocytes were however observed following treatment with DHT at dpf 2, 5 or 8 (Figure 3). Genotyping of a number of these fry confirmed an increased number of XX males over solvent control values at dpf 0, 4 and 5 (24%, 11% and 11% increases respectively) but not at dpf 6 or 12 (Table 2).

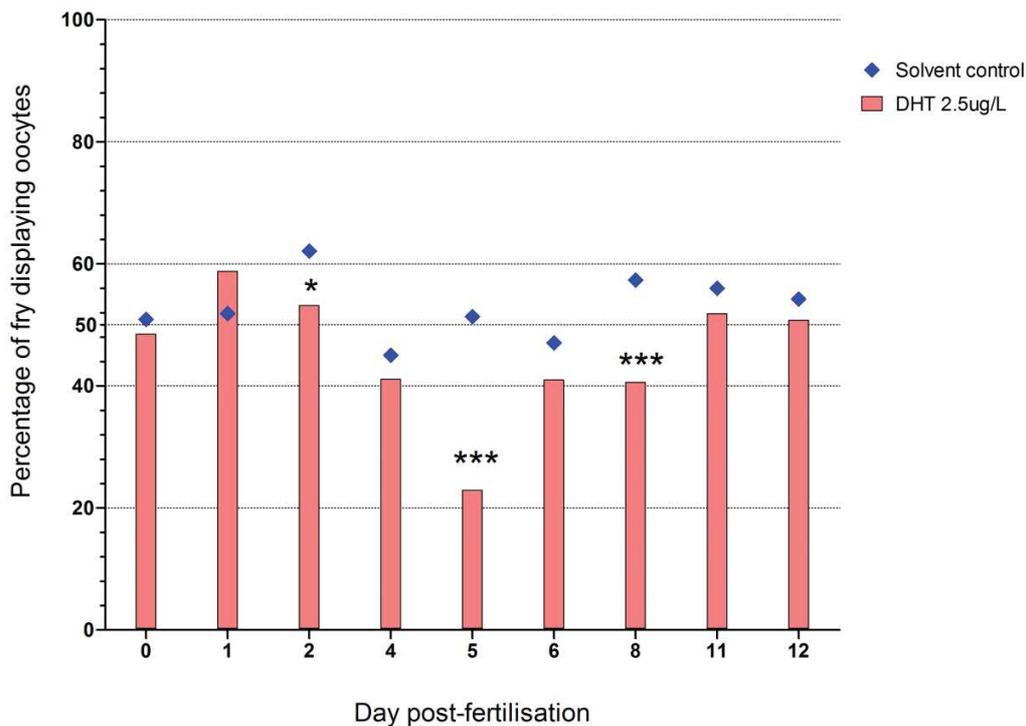


Figure 3: Percentage of fry exhibiting gonadal fluorescence at DPF16. The fry were treated with a 24 h pulse of DHT (2.5 µg/L) at different developmental time points (x-axis). Following this treatment they were returned to clean media. The percentage of fry displaying GFP+ cells in their gonads at DPF16 following 24 h treatment with a solvent control solution at different developmental time points are shown in blue.

Day post fertilisation	0		4		5		6		12	
	XY	XX	XY	XX	XY	XX	XY	XX	XY	XX
Male phenotype	11 61%	6 30%	20 87%	1 11%	21 100%	2 17%	18 82%	1 10%	18 90%	0 0%
Female phenotype	7 39%	14 70%	3 13%	8 89%	0 0%	10 83%	4 18%	9 90%	2 10%	14 100%
n	38		32		33		32		34	

Table 2: Genotyping results of a subset of fry from each exposure group. Phenotype is as determined by the presence or absence of fluorescence in the gonads. Percentages indicate relative numbers of fry developing normally or abnormally by genotype.

2.2.3 Oestradiol treatment

As with the DHT exposure, the concentration chosen for the acute (24 h) exposures to E2 was that determined by Kobayashi et al. as the minimum dose inducing a maximum effect (500 µg/L Kobayashi et al., 2011). Contrary to DHT, Kobayashi et al. did not report any paradoxical sex reversal when dpf 0 medaka embryos were exposed to E2.

In this study we confirm that treatment with this concentration of E2 caused a statistically significant increase in oocyte bearing fry at dpf 16 compared to the solvent alone group when the 24 h treatment was carried out at dpf 0-11 but not at dpf 12 (Figure 4). The greatest percentage of phenotypic females was obtained when the treatment was carried out at dpf 1, 2, 4 or 5, dpf 3 was not tested. Following dpf 5 the effect diminished in an age dependent manner until dpf 12.

Genotyping of a random set of fry from some of the exposure time points indicated that a large number (80%) of XY embryos treated at dpf 0 developed oocytes (Table 3). This peaked at 94% at dpf 5 and then dropped to 79% at dpf 6, falling to 6% at dpf 12. Paradoxical effects on sexual development were observed for most of the E2 exposed groups with 6 to 22% of XX embryos failing to exhibit oocytes. This may be due to stress induced inhibition of aromatase expression, as previously reported, due to the experimental conditions (Hayashi et al., 2010b).

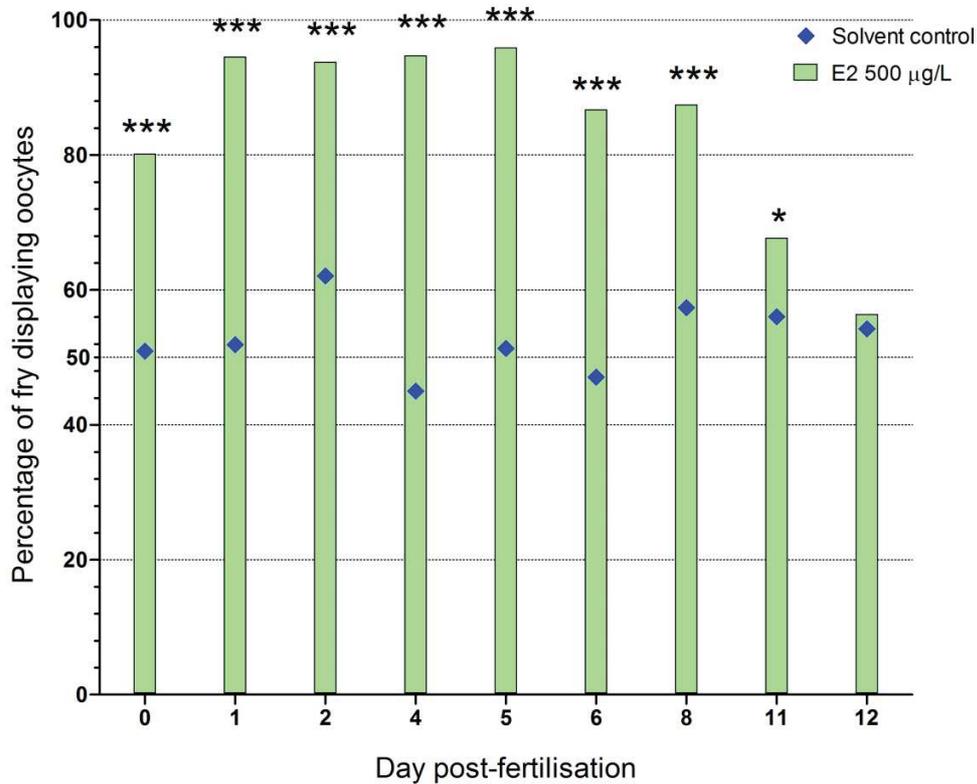


Figure 4: Percentage of fry exhibiting gonadal fluorescence at DPF16. The fry were treated with a 24 h pulse of E2 (500 µg/L) at different developmental time points (x-axis). Following this treatment they were returned to clean media. The percentage of fry displaying GFP+ cells in their gonads at DPF16 following 24 h treatment with a solvent control solution at different developmental time points are shown in blue.

Day post fertilisation	0		4		5		6		12	
	XY	XX	XY	XX	XY	XX	XY	XX	XY	XX
Male phenotype	4 20%	4 22%	1 9%	3 14%	1 6%	1 6%	4 21%	0 0%	16 94%	2 11%
Female phenotype	16 80%	14 78%	10 91%	19 86%	15 94%	15 94%	15 79%	13 100%	1 6%	16 89%
n	38		33		32		32		35	

Table 3: Genotyping results of a subset of fry from each exposure group. Phenotype is as determined by the presence or absence of fluorescence in the gonads. Percentages indicate relative numbers of fry developing normally or abnormally by genotype.

2.3 Exposure Series 2

2.3.1 Effects of DMSO treatment on sex reversal

The second series of experiments used a higher concentration of DMSO as the carrier solvent (0.1% cf. 0.05%). A 24 h acute exposure to this concentration of DMSO did not induce a significant difference in the phenotypic sex ratio compared to non-exposed medaka at any developmental time point (Figure 5), therefore genotyping was not carried out.

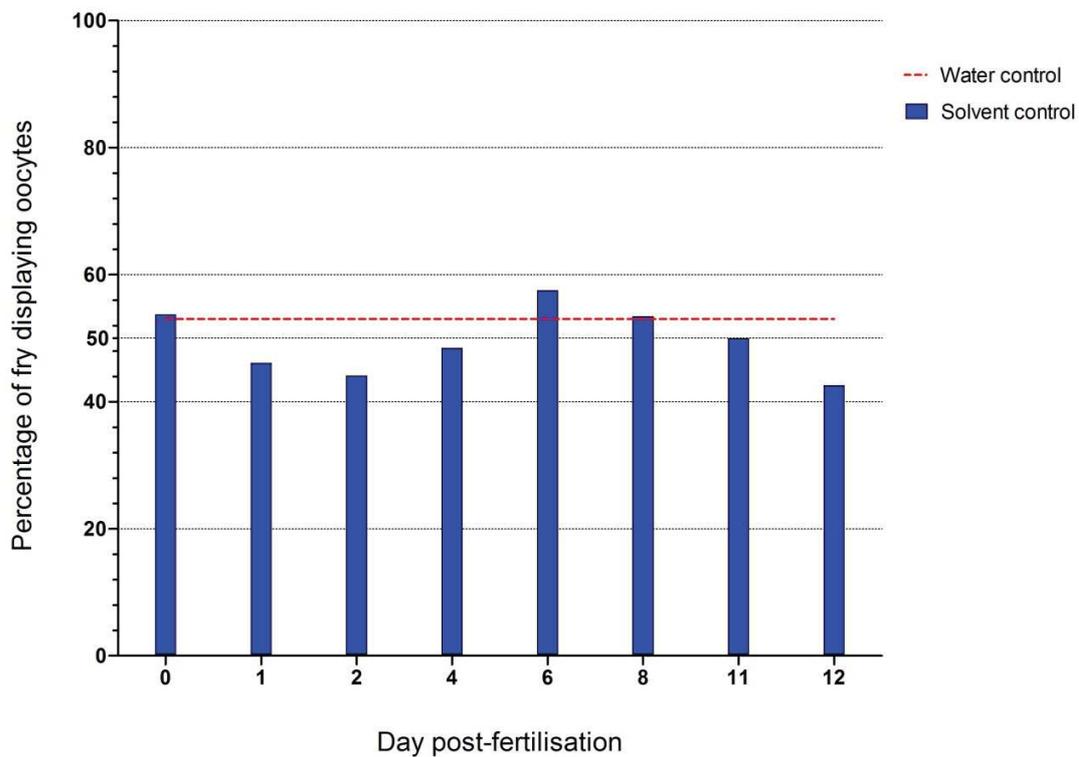


Figure 5: Percentage of fry exhibiting gonadal fluorescence at DPF16. The fry were treated with a 24 h pulse of DMSO (0.1%) at different developmental time points (x-axis). Following this treatment they were returned to clean media. The percentage of untreated control fry displaying GFP+ cells in their gonads at DPF16 is shown by the red line.

2.3.2 Dihydrotestosterone treatment

A higher concentration of DHT was used in the second set of exposures. This concentration, 500 µg/L, was chosen based on a range-finder experiment (data not shown). The aim of this second set of exposures with DHT was to induce a higher level of disruption of the sexual axes to that observed with the lower DHT concentration used in the first series of experiments. It was hoped that this would facilitate identification of the developmental time points most sensitive to disruption of sex determination by a model androgen.

This higher concentration of DHT caused a significant reduction in the number of fry displaying ovaries at dpf 16 for all developmental time points tested from dpf 0-8 (Figure 6). At dpf 11, no effect was observed and at dpf 12, a slight increase in phenotypic females was observed. There appear to be two windows showing the greatest sensitivity to this molecule, from dpf 0-1 and from dpf 6-8. Intriguingly, the period of relative insensitivity between dpf 2 and 4 may coincide with the cessation of maternal expression of AR and the beginning of zygotic expression.

Genotyping confirmed the observed result with high numbers of XX males from dpf 0-6 (76-86%) dropping to 0% at dpf 12 (Table 4). At two time points XY females were observed, which may have been due to the paradoxical effects of this molecule that have been previously observed (Kobayashi et al., 2011). However, the numbers of XY females (15% at dpf 4 and 5% at dpf 6) were too low to draw any conclusions as to the causative agent as 17% were observed in the solvent control group from the first exposure series at dpf 6.

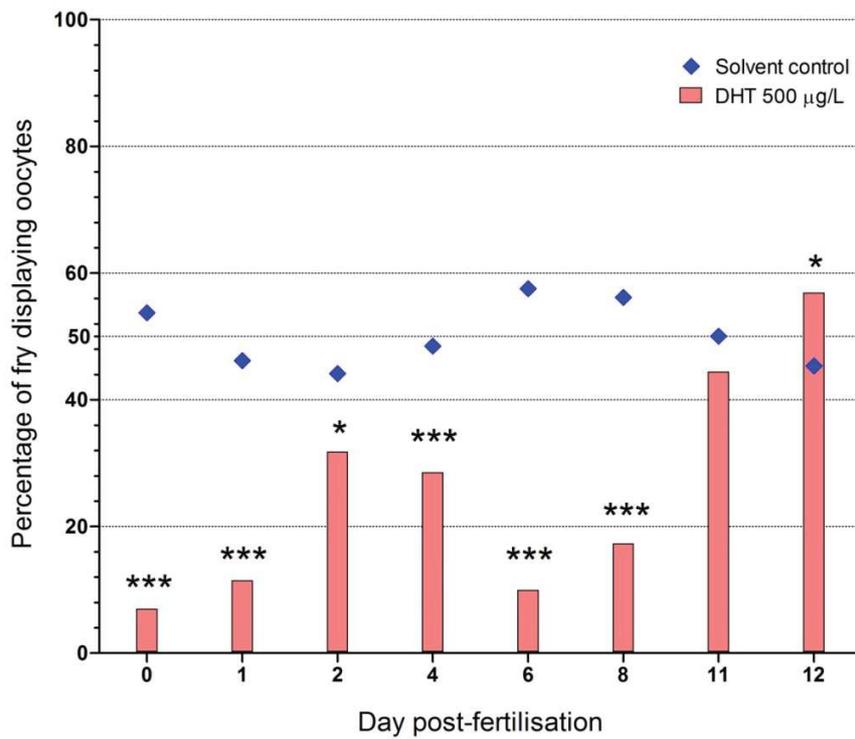


Figure 6: Percentage of fry exhibiting gonadal fluorescence at DPF16. The fry were treated with a 24 h pulse of DHT (500 µg/L) at different developmental time points (x-axis). Following this treatment they were returned to clean media. The percentage of fry displaying GFP+ cells in their gonads at DPF16 following 24 h treatment with a solvent control solution at different developmental time points are shown in blue.

Day post fertilisation	0		4		6		12	
	XY	XX	XY	XX	XY	XX	XY	XX
Male phenotype	15 100%	16 84%	11 85%	13 76%	19 95%	12 86%	17 100%	0 0%
Female phenotype	0 0%	3 16%	2 15%	4 24%	1 5%	2 14%	0 0%	14 100%
n	34		30		34		31	

Table 4: Genotyping results of a subset of fry from each exposure group. Phenotype is as determined by the presence or absence of fluorescence in the gonads. Percentages indicate relative numbers of fry developing normally or abnormally by genotype.

2.3.3 Flutamide treatment

A range finder experiment carried out at dpf 4 with the AR antagonist flutamide (data not shown) indicated that 500 µg/L was ineffective at altering the sex ratio and 50 mg/L was lethal, whereas the intermediate concentration of 5 mg/L was effective and was retained for all further exposures.

There was a slight increase in the number of fry exposed to flutamide exhibiting ovarian tissue at dpf 0 and dpf 4 (Figure 7). Interestingly, this roughly corresponds to the two developmental periods which were the most sensitive to high dose DHT and as would be expected for an AR antagonist, the opposite effect is observed as with DHT. The weak effect may suggest that blocking androgen signalling at these stages may not necessarily lead to female gonadal differentiation, perhaps due to the low endogenous levels of testosterone (Iwamatsu et al., 2006). It may also suggest that most of the flutamide in the exposure medium cannot pass through the chorion of the developing eggs. This theory is supported by the most pronounced effect of flutamide exposure which was mortality within hours when the 24 h exposure was carried out on hatched fry. This suggests that the selected concentration, based on a concentration range tested on unhatched embryos, is in fact lethal and the embryos are protected by their egg envelope prior to hatching.

Genotyping showed that the slightly increased numbers of phenotypic females at dpf 0 and dpf 4 compared to controls could be explained by increased numbers of XY females (17% at dpf 0 and 8% at dpf 4 cf. 5% and 0% for the control group respectively, Table 5). At dpf 6 the phenotypic and genotypic sex was in agreement for all fry genotyped.

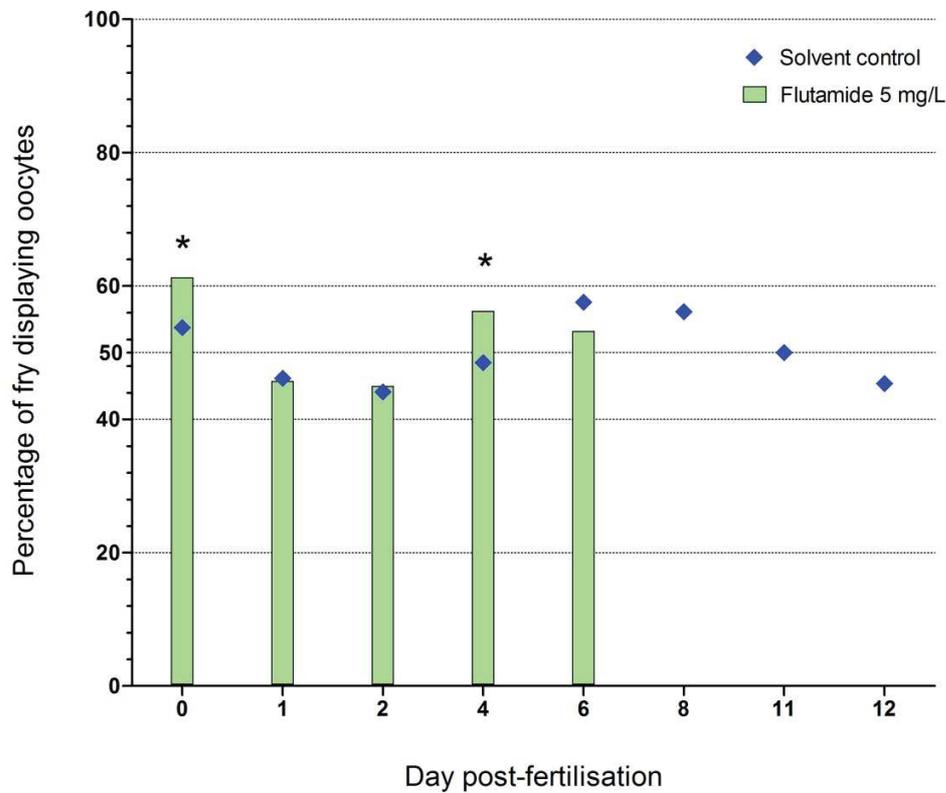


Figure 7: Percentage of fry exhibiting gonadal fluorescence at DPF16. The fry were treated with a 24 h pulse of flutamide (5 mg/L) at different developmental time points (x-axis). Following this treatment they were returned to clean media. The percentage of fry displaying GFP+ cells in their gonads at DPF16 following 24 h treatment with a solvent control solution at different developmental time points are shown in blue.

Day post fertilisation	0		4		6		12	
	XY	XX	XY	XX	XY	XX	XY	XX
Male phenotype	15 83%	1 8%	12 92%	0 0%	17 100%	0 0%	-	-
Female phenotype	3 17%	12 92%	1 8%	17 100%	0 0%	14 100%	-	-
n	31		30		31			

Table 5: Genotyping results of a subset of fry from each exposure group. Phenotype is as determined by the presence or absence of fluorescence in the gonads. Percentages indicate relative numbers of fry developing normally or abnormally by genotype.

3. Conclusions

The perfect concordance between genotypic sex and phenotypic sex for the unexposed control group demonstrates the ability of this *42sp50-GFP_ChgH-GFP* line to accurately identify the phenotypic sex of fry at dpf 16 (table 1).

A previous study in medaka tested a number of developmental time points for sensitivity to E2 and testosterone induced intersex (Koger et al., 2000). Koger et al. tested six day exposures commencing at dpf 0, dph 0, dph 7 or dph 21, with histological examination of the gonads at five months of age and found that the highest sensitivity was at dph 0. In our study, dph 0, defined as the day that greater than 50% of the fry had hatched, corresponded to dpf 9 for all three of the replicates experiments which were pooled to give the unexposed control group (table 1).

We therefore decided to carry out a systematic screening of developmental sensitivity to waterborne agents activating the ER and AR, as well as an AR antagonist from dpf 0 to dpf 12 (or dph 3). Our rationale was that a one day, rather than six-day, protocol and a greater number of developmental time points would allow the developmental dynamics of susceptibility to hormonally induced disruption of sex determination to be determined with greater precision.

Exposure studies with DHT and flutamide showed two periods of increased sensitivity between 0-1 dpf and 5-8 dpf. Sensitivity to E2 was greatest between dpf 1-5. No effect on phenotypic sex ratio was observed with DHT at dpf 11 or 12 or with E2 at dpf 12. It is of course possible that the chemicals used for the exposures were retained within the developing embryos following the rinsing of the exposure vessels and that their effects on sex determination were occurring several hours or days after exposure. Although it is difficult to determine the latency period between exposure and effect, what is sure is that by dpf 11 or 12 sex determination was stable to the tested exposure regimes. In addition, the fluctuating nature of the phenotypic effects observed with the higher concentration of DHT (Figure 6) suggests that either embryonic levels of DHT may drop considerably within 48 h, or that embryonic sensitivity may fluctuate with developmental age. A period of low expression of ARs during the switch from maternal to zygotic RNA expression cannot be excluded as a factor.

In our study, DHT treatment was ineffective when fry were exposed at dpf 11 or 12, whereas in the study carried out by Koger et al. the highest sensitivity was observed with a six day exposure from dph 0-6 (corresponding to dpf 9-15) (Koger et al., 2000). This intriguingly suggests that, in

addition to the two periods of heightened sensitivity to DHT observed between dpf 0-8, it is possible that a third period of sensitivity lies between dpf 12-15 (corresponding to dph 3-6).

Flutamide seemed unable to penetrate the chorion of the eggs as it was tolerated in unhatched embryos and lethal in hatched fry. Interestingly however, it did exert some weak effects on sex determination. This suggests that either: a small amount of flutamide passed the chorion; or that it was entering the eggs, is not toxic at pre-hatch stages of development and that antagonism of AR at these stages does not illicit high levels of sex reversal. Some light could be shed on this question by repeating the high concentration DHT exposure at one of the more sensitive time points (eg. dph 0 or dph 6) and co-treating the fry with flutamide. The degree of protection provided by flutamide to the DHT induced male-biased sex ration should give some indication as to the ability of flutamide to traverse the chorion.

It has yet to be shown whether this model is sensitive to disruption of sex determination when short-term exposures are carried out at developmental stages beyond dpf 12. This would allow molecules to be tested without consideration for their ability to pass the chorion and would allow the model to be used for screening of pure chemicals at high concentrations in a short-term assay.

Another interesting finding of this study was that DMSO appeared to be able to disrupt normal sexual differentiation at relatively low concentrations. Although a small percentage of XX medaka embryos develop as phenotypic males under normal rearing conditions (Nanda et al., 2003), some XY phenotypic females were observed in groups exposed to 0.05% DMSO at dpf 6 or 12. Further studies should be performed to confirm this result.

In this study we confirm that a 24 h treatment of medaka eggs with 500 μ g/L of E2 immediately after fertilisation results in a high incidence of XY females and a skewed phenotypic sex ratio towards female. Our choice to use the natural hormone DHT rather than its synthetic analogue methyl-DHT allows the effects of DHT at dpf 0 (this study) to be compared with those previously published for methyl-DHT (Kobayashi et al., 2011). We observed a roughly 50:50 sex ratio in fry exposed to 2.5 μ g/L DHT at dpf 0 but genotyping showed the presence XX males and XY females as described by Kobayashi et al. with higher concentrations of methyl-DHT, suggesting that DHT may be the more powerful molecule at this developmental time-point.

Further development of this model will involve a deeper analysis of the GFP expression patterns in order to identify any possible links between the number of fluorescent cells and the status of

the embryo in terms of agreement between genotypic and phenotypic sex. Crossing of this line with another line harbouring a fluorescent reporter cassette on the Y-chromosome is also envisaged with the aim of increasing throughput and reducing time and costs related to genotyping. We then plan to take advantage of the ChgH-GFP transgene carried by this line to investigate links between the level of ChgH transcription induced by environmentally relevant chemicals and disorders of sexual differentiation in fish. The investigation of these links is a timely and important subject in terms of the development of adverse outcome pathways for the identification of endocrine disruptors and the evaluation of their predictability.

4. Conflict of Interest

The authors declare the following competing financial interest(s). Two of the authors (G.L. and B.D.) have financial interests in WatchFrog, which plans to commercialise the test described here. A.T. and G.L. are currently employed by WatchFrog. P.S., P.T., C.B. and A.S. were employed by WatchFrog whilst they were involved in this study. A.K. declares no competing interests.

5. Acknowledgements

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Publication n°4

Presentation of publication n°4

Rapid Fluorescent Detection of (Anti)androgens with spiggin-gfp Medaka

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Anti-androgens are currently of interest to environmental scientists, as several case studies on wildlife have linked contamination of European rivers by anti-androgens (working in synergy with estrogenic compounds) to the feminization of male fish (Jobling et al., 2009; Urbatzka et al., 2007). Studies in mammals have shown that exposure to (anti-) androgens during sensitive developmental periods causes physiological and morphological abnormalities such as low sperm count and motility in male offspring after mother was exposed to testosterone during pregnancy (Recabarren et al., 2008).

Several bioassays have been developed to detect (anti-) androgenic activity, for example the AFSS assay described in the introduction of this thesis, but a specific, rapid and robust screening tool for androgen receptor modulation remained to be developed, partly because of the lack of specific target genes and the similarity of the consensus androgen response element with the response elements of glucocorticoids, progesterone and mineralocorticoids (Horie-Inoue et al., 2006).

The purpose of this study was to create a stable transgenic medaka line harboring the spiggin gene (originally an androgen-responsive gene specific to three-spined stickleback) driving the expression of GFP. We also aimed to develop a rapid protocol to detect (anti-) androgens using this line. Germinal transgenesis resulted in a stable line. No GFP was visible in non-treated fry, however *gfp* expression can be induced by exposure to exogenous androgens (DHT, 17MT). The specificity of the signal was tested by treatment with aldosterone, dexamethasone and progesterone which failed to induce fluorescence in the kidneys.

To demonstrate, that our assay is also a suitable tool for the detection of anti-androgens, we exposed the Spiggin-gfp fry to a dose range of four environmentally relevant anti-androgens: flutamide, fenitrothion, linuron and vinclozolin in co-treatment with 17MT. With the Spiggin-gfp model we achieved comparable sensitivity to anti-androgens as the AFSS assay. We successfully reduced the duration of the test from 21 days (AFSS assay) to 4 days. We developed a novel, unique, sensitive model providing a rapid and inexpensive tool to detect and quantify (anti-)androgenicity.

Rapid Fluorescent Detection of (Anti)androgens with *spiggin-gfp* Medaka

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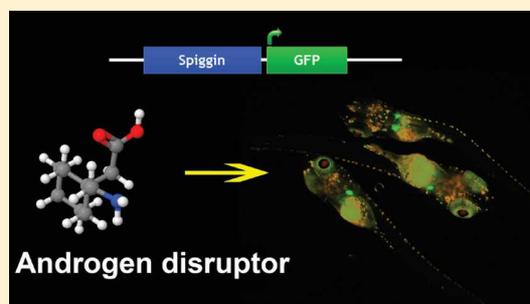
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Supporting Information

ABSTRACT: Widespread environmental antiandrogen contamination has been associated with negative impacts on biodiversity and human health. In particular, many pesticides are antiandrogenic, creating a need for robust and sensitive environmental monitoring. Our aim was to develop a sensitive and specific transgenic medaka (*Oryzias latipes*) model bearing an androgen responsive fluorescent reporter construct for whole organism-based environmental screening of pro- and antiandrogens. We analyzed the 5' regions of the androgen responsive three-spined stickleback (*Gasterosteus aculeatus*) *spiggin* genes in silico, revealing conserved blocks of sequence harboring androgen response elements. Identified putative promoters were cloned upstream of GFP. Germinal transgenesis with *spg1-gfp* led to stable medaka lines. GFP induction was exclusive to the kidney, the site of spiggin protein production in sticklebacks. Significant GFP expression was induced by three or four-day androgen treatment of newly hatched fry, but not by estrogens, mineralocorticoids, glucocorticoids or progestogens. The model responded dose-dependently to androgens, with highest sensitivity to 17MT (1.5 $\mu\text{g/L}$). In addition to flutamide, the biocides fenitrothion, vinclozolin and linuron significantly inhibited 17MT-induced GFP induction, validating the model for detection of antiandrogens. The *spg1-gfp* medaka model provides a sensitive, specific, and physiologically pertinent biosensor system for analyzing environmental androgen activity.



INTRODUCTION

Antiandrogenic activity in UK river water has been linked to the feminization of wild fish,¹ suggesting a risk to biodiversity. Specifically antiandrogens appear to be acting synergistically with estrogens, resulting in dose-dependent increases in ootestes, oviducts and vitellogenin in male fish. Previous identification of substantial antiandrogenic activity in the River Lambro in Italy suggests that the biological effects observed in the UK are not unique.² This is hardly surprising considering that an *in vitro* study showed that 66/200 pesticides inhibited 5 α -dihydrotestosterone (DHT) induced androgen receptor (AR) transcriptional activity.³ In an unrelated study 37/134 pesticides were found to be antiandrogenic *in vitro* with some mixtures of pesticides showing a concentration addition relationship,^{4,5} resulting in a higher overall antagonistic effect than the most powerful antiandrogen alone. In addition to pesticides, identified sources of androgen axis active chemicals include effluent from wastewater treatment works⁶ and kraft pulp mills.⁷

The observed predictability of fish models for mammalian responses to hypothalamic–pituitary–gonadal (HPG) axis active chemicals,⁸ suggests that the majority of AR agonists/antagonists active in fish species are also active in humans. Yeast and mammalian cell culture lines transfected with human AR have proved valuable for assessing transactivation potential in humans, but are limited to identifying substances that act directly on the receptor.^{9,10} Mouse models for reporter gene based measurement of AR activity have emerged,^{11,12} but no aquatic equivalents exist. Despite these tools, definitive proof is lacking that environmental androgen active chemicals are responsible for deleterious effects in humans. However, it is clear that disruption of the androgen axis in humans, particularly within critical developmental periods, can have physiological consequences. For example, modulation of AR

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signaling in humans by synthetic progestins has been linked to breast cancer and exposure of pregnant women to testosterone resulted in reduced sperm count and motility in male offspring.^{13,14} Interestingly, all symptoms of testicular dysgenesis syndrome, apart from germ cell cancer, can be reproduced in animal models by exposing them to endocrine disruptors during embryonic or perinatal development.¹⁵ Robust screening models are therefore needed to identify androgen axis disruptors among the thousands of currently untested chemicals in our environment.

In their recent extensive "State of the Science of Endocrine Disrupting Chemicals" document, World Health Organization (WHO) and United Nations Environment Programme (UNEP) highlight improved testing for endocrine disrupting chemicals (EDCs), in particular in high-throughput format, as an important future need.¹⁶ Development of rapid screening tools for AR modulation has been hampered by the lack of specific target genes. Furthermore, the consensus androgen response element (ARE) is identical to that of glucocorticoid, progesterone, and mineralocorticoid response elements,¹⁷ rendering development of specific assays even more arduous.

One of the best-documented physiological responses under tight androgen control is Spiggin production in the three-spined stickleback (*Gasterosteus aculeatus*). In the breeding season, male sticklebacks produce Spiggin glue protein in their kidneys in response to elevated circulating androgen to allow the building of nests. The most effective androgen for inducing Spiggin production is 11-ketotestosterone (11KT).¹⁸ Despite the almost undetectable levels of Spiggin protein in females under natural conditions, females synthesize Spiggin protein in response to exogenous androgens.¹⁹ Androgen-dependent Spiggin synthesis in the stickleback is already used as a biomarker in vivo and in vitro for androgen axis disruption.²⁰

The draft directive androgenized female stickleback screen (AFSS) was established by The Organization of Economic Cooperation and Development (OECD) in 2009.²¹ It describes the detection of androgens and also, by simultaneously treating female sticklebacks with a reference dose of an androgen (5 µg/L DHT or 0.5 µg/L 17MT), the detection of antiandrogens. The potency of antiandrogens is expressed as equivalents of the model pharmaceutical antiandrogen, flutamide (flutamide equivalents, FL eq). The exposure phase of this test runs for 21 days, followed by dissection of the kidneys of the adult fish to allow quantification of Spiggin protein by enzyme-linked immunosorbent assay (ELISA).²² During validation of the AFSS flutamide, fenitrothion, vinclozolin, and linuron were used as model antiandrogens.

Here, we describe an alternative, in vivo test based on a fluorescent reporter gene driven by the *spiggin* promoter. Our assay can detect pro- and antiandrogens in medaka larvae 4 days post hatch (dph4), and could serve as a rapid additional screening tool in the testing of chemicals and environmental samples.

MATERIALS AND METHODS

Chemicals and Solutions. Chemicals were purchased from Sigma-Aldrich (MO): 17MT (CAS: 58-18-4), DHT (CAS: 521-18-6), 11KT (CAS: 564-35-2), flutamide (CAS: 13311-84-7), linuron (CAS: 330-55-2), fenitrothion (CAS: 122-14-5), vinclozolin (CAS: 50471-44-8), anastrozole (CAS: 120511-73-1), 17β-estradiol (E₂, CAS: 50-28-2), aldosterone (CAS: 52-39-1), dexamethasone (CAS: 50-02-2), progesterone (CAS: 57-83-0). Medaka medium contained

per liter: 10 g NaCl, 0.4 g CaCl₂·2H₂O, 0.4 g MgSO₄·7H₂O, 0.3 g KCl, 0.02 g methylene blue adjusted to pH 7.2–8.0. Ethyl 3-aminobenzoate methanesulfate salt (MS222, CAS: 886-86-2) was prepared with an equal mass of sodium bicarbonate (CAS: 144-55-8) and adjusted to pH 7.5–8.0.

Characterization and Cloning of *spiggin* Promoters. The in silico analysis of the *spiggin* promoter regions is described in detail in the Supporting Information (SI). Briefly, the *spiggin* gene locus (linkage group IV: 21 000 000–21 220 000) in the three-spined stickleback genome (BROAD S1 assembly February 2006) was analyzed with Dotter software²³ and PipMaker.²⁴ mRNA-DNA alignments were carried out using Spidey, and nuclear receptor binding sites were predicted with NHR scan.²⁵

Sticklebacks were caught off the Taivassalo shore, Finland. Genomic DNA was extracted using PureLink Genomic DNA mini kit (Invitrogen, CA, USA) and used for PCR of *spiggin* promoters. Primers used in successful promoter amplification, with the added AgeI and XhoI restriction sites underlined, were *spg1_for* GTCTCGAGCATCTGGTTTTAAGACTGACTGAACCGACGAAG; *spg1_rev* GTACCGGTTGATGCTGCACGAGAGCAGTTGAAAGAAACAAC; *spg4_for* ACCTCGAGCAGGTGGGTTTCATGCAGTTC; *spg4_rev* ACACCGGTGAAACAAACAACCCACCACAT.

PCR products were resolved on agarose gel, purified and cloned into TOPO vector (Invitrogen, CA), sequenced prior to excision and ligated into pUC57 with AgeI and XhoI. The eGFP coding sequence from pEGFP-1 (Clontech, CA) was excised using AgeI and NotI and ligated into the pUC57-Spg plasmids.

Germline Transgenesis. Briefly, one-cell red-orange strain medaka embryos were injected with a 20 ng/µL nonlinearized solution of plasmid DNA in the absence of meganuclease as previously described by Kinoshita et al. 1996.²⁶

EDC Exposure Studies. Exposures were carried out in six-well plates. Stocks were made up in DMSO (final concentration 0.2%). Solutions were renewed every 24 h. Five newly hatched (dph0) F2–F5 *spg1.22-gfp* medaka fry were placed in each well and 12 wells were used for each exposure group giving a total of 60 fry per exposure group. For the estrogen responsive line (Figure 4B): five newly hatched (dph0) F12 *chgh-gfp* medaka fry were placed in each well and 8 wells were used for each exposure group giving a total of 40 fry per exposure group.

Image Capture. Fry were anesthetized with 200 mg/L MS222 and positioned ventrally for imaging of the dorsal region of the abdomen. Fluorescence was imaged using a 120W fluorescence source and ET-GFP long-pass filters (Excitation 480/40, Emission 510LP, Leica Microsystems GmbH, Germany). Images were captured with a 0.3 s exposure time (Figure 3A–C) or 0.75 s (Figure 3D–F and Figure 4) at 8× magnification with an Infinity 1-3C camera (Lumenera Corporation, ON, Canada) fitted to a Leica MZ10F stereomicroscope (Leica Microsystems GmbH, Germany). For the estrogen responsive line (Figure 4B) identical conditions were used except images were captured of the ventral region with a 0.3 s exposure time.

Data Analysis. ImageJ (Rasband, W.S., ImageJ, U.S. National Institutes of Health, MD) was used for image analysis. Color images of medaka fry were separated into red, green and blue layers. To remove background fluorescence resulting from endogenous pigments, intensity for each pixel in the red layer was doubled and subtracted from the green layer. This method efficiently removes the endogenous pigmentation appearing red

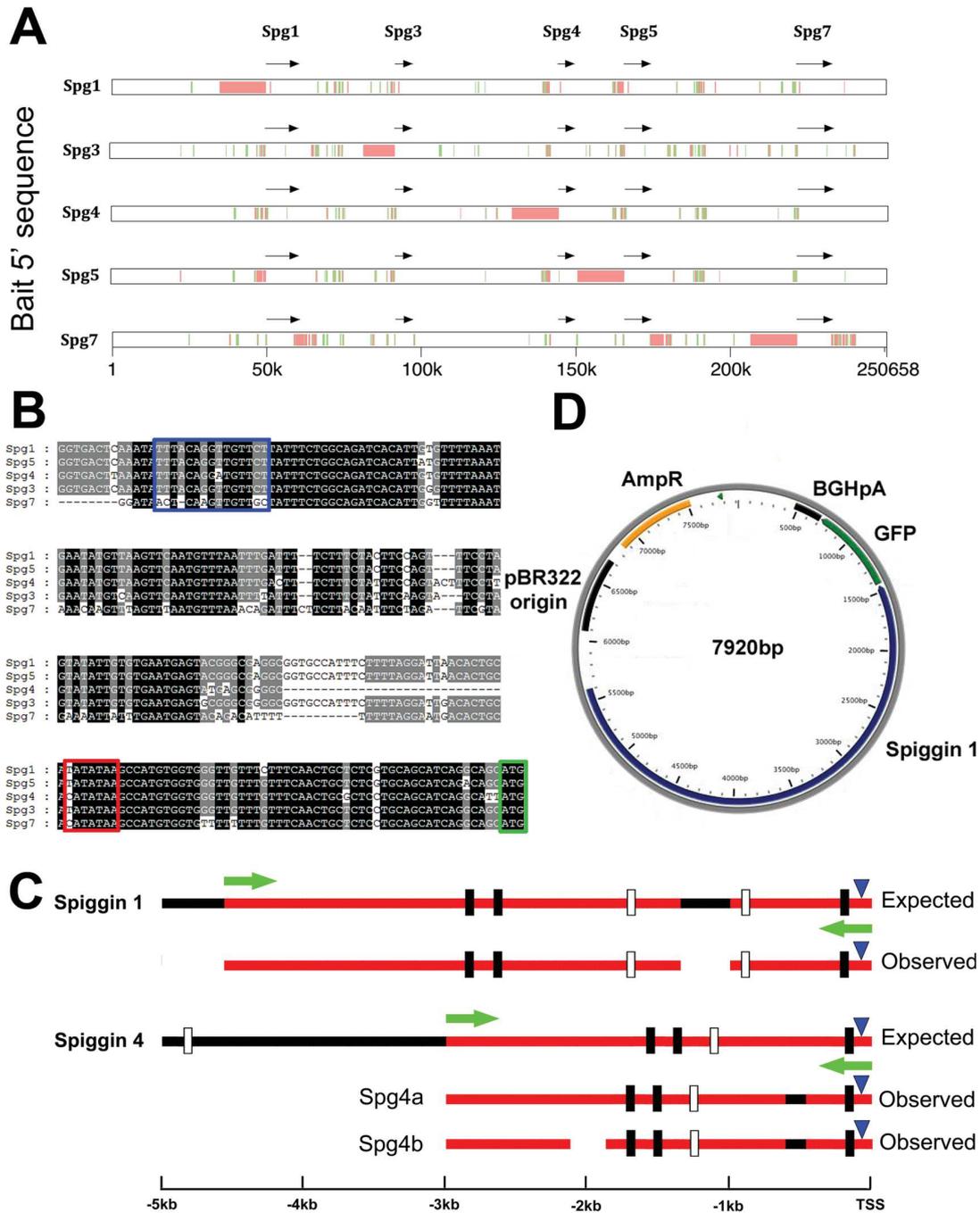


Figure 1. In silico *spiggin* promoter characterization. (A) Overview of the PIPs. Different *spiggin* 5' regions (baits) were compared to the whole *spiggin* gene locus (20 968 314–21 218 972) (for full PIPs see SI, Figure S3). Arrows denote the location of *spiggin* genes based on mRNA-DNA alignments, and red blocks 100% similarity in sequence to bait. (B) Alignment of *spiggin* 5' regions upstream of putative ATG (green box). The locations of possible TATA box (red box) and ARE (blue box) are depicted. (C) Schematic presentation of expected (database sequence) and observed (cloned sequence) *spg1* and *spg4* promoters. Predicted ARE (black bars), ERE (white bars), TATA boxes (blue triangles) and primers (green arrows) are shown. Regions predicted as conserved based on the PIPs are shown in red. (D) Plasmid map of the construct used for the *spg1.22-gfp* line.

or yellow in the images captured with GFP long pass filters, leaving the green fluorescence emitted by GFP (SI, Figure S1). The region containing the kidney was manually selected and the mean intensity of these pixels was calculated. In the absence of a means to separate *spg-gfp* transgenic and wild-type fry prior to exposure, all fry were analyzed. Accordingly, only the highest 50% of values were considered from each group.

Statistical Analysis. Statistical analyses were carried out with GraphPad Prism version 5.04 (GraphPad Software, CA). A D'Agostino and Pearson omnibus normality test was carried out on all exposure groups. If data in all groups compared showed normal distribution, then a Student's *t*-test was carried out. Otherwise data were compared using a Mann-Whitney test. Statistical significance is indicated by * $P < 0.05$, ** $P <$

0.01, *** $P < 0.001$ compared to androgen alone; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared to vehicle control; other comparisons are indicated with a bar. Mean \pm SEM are given throughout.

RESULTS

Characterization and Cloning of *spiggin* Promoters.

The *spiggin* gene in the stickleback genome has been duplicated creating a locus of directed repeats with an estimated copy number of seven (please see SI for more detail).²⁷ The promoter regions of these genes have not been characterized. We searched for conserved elements within the *spiggin* gene locus with particular focus on the 5' regions of the genes. DotMatrix plots (SI, Figure S2) and Percent Identity Plots (PIPs) (Figure 1A, SI, Figure S3) suggested that conserved elements are enriched within a region of 5 kb immediately 5' to the ATG of the *spiggin* genes, and these regions were found to contain conserved ARE and ERE sites (Figure 1A, C, SI, Figure S4). Alignment of the -250 to $+3$ bp regions to each other revealed a conserved, putative TATA box in addition to candidate ARE in all but *spg7* (Figure 1B). Interestingly, the promoters of *spg1*, *spg3*, and *spg5* all contain an ARE at -203 to -218 bp and at -177 to -191 bp in the *spg4* promoter (Figure 1B, C) which may suggest that this element is crucial to the AR-mediated activation of the promoters. The position (52 – 58 bp upstream from the translation start) and sequence (TATA-TAA) of the putative TATA box is conserved in *spg1*, *spg3*, *spg4*, *spg5*, and *spg7*, although in *spg4* and *spg7* the sequence is CATATAA. The number of transcripts on the Ensembl database ranks the *spiggin* genes according to weight of evidence for expression as $spg1 > spg4 > spg3 = spg7 > spg5 = spg6$. Based on these data, primers were designed to clone the promoters of *spg1* (4.5 kb), *spg3* (8.0 kb), and *spg4* (2.9 kb) (Figure 1C). A more in-depth description of the promoter analysis results is available as SI.

Promoters of *spg1* and *spg4* were successfully cloned but amplification of *spg3* failed despite various attempts with different primer designs. The cloned promoters showed the highest sequence similarity to expected regions ($>98\%$ for *spg1*, $>95\%$ for *spg4*). However, the cloned *spg1* promoter had a 399 bp deletion corresponding to a segment that was not predicted as conserved in our analysis (Figure 1C). Sequencing of *spg4* promoter clones revealed two variants, suggesting the presence of two different alleles. Both contained a 140 bp insert compared to database sequence, but *spg4b* displayed an additional 264 bp deletion (Figure 1C). The lengths of the obtained promoters were therefore *spg1* 4160 bp (expected 4513 bp), *spg4a* 3002 bp, and *spg4b* 2747 bp (expected 2869 bp); Genbank accession numbers are KF716169, KF716170, and KF716171, respectively. Despite the observed variation in sequence compared to the database genome sequence, all predicted binding sites were conserved (Figure 1C). All three promoters were cloned upstream of *gfp*. The plasmid map of the construct used in the *spg1-gfp* line is provided in Figure 1D.

Creation of *spiggin-gfp* medaka. Medaka germline transgenesis was carried out, 1322 eggs were injected with *spg1-gfp* and 1021 with *spg4-gfp*, resulting in 73 adult *spg1-gfp* and one adult *spg4-gfp* which had exhibited GFP in the developing embryo. Screening of the *spg4-gfp* adult and 36 *spg1-gfp* adults for germline transmission, by androgen treatment of the F1 progeny, led to identification of seven stable independent lines for *spg1-gfp* and none for *spg4-gfp*. The *spg1-gfp* transgene consisted of 4.159 kb of sequence

upstream from the translation start site of the *spiggin1* gene (Figure 1D). Following androgen treatment of the F1 progeny, the *spg1.22-gfp* line was selected as this line demonstrated the strongest GFP signal and the shortest response time (data not shown).

GFP Expression in *spg1.22-gfp* Medaka Adults and Fry. GFP was observed without androgenic treatment in the kidneys of 6-month-old F2 adult male medaka but never in females (Figure 2A, B). Nevertheless, after a seven-day 17MT treatment ($302 \mu\text{g/L}$) of F4 adult females, GFP expression was observed in the kidneys (SI, Figure S5).

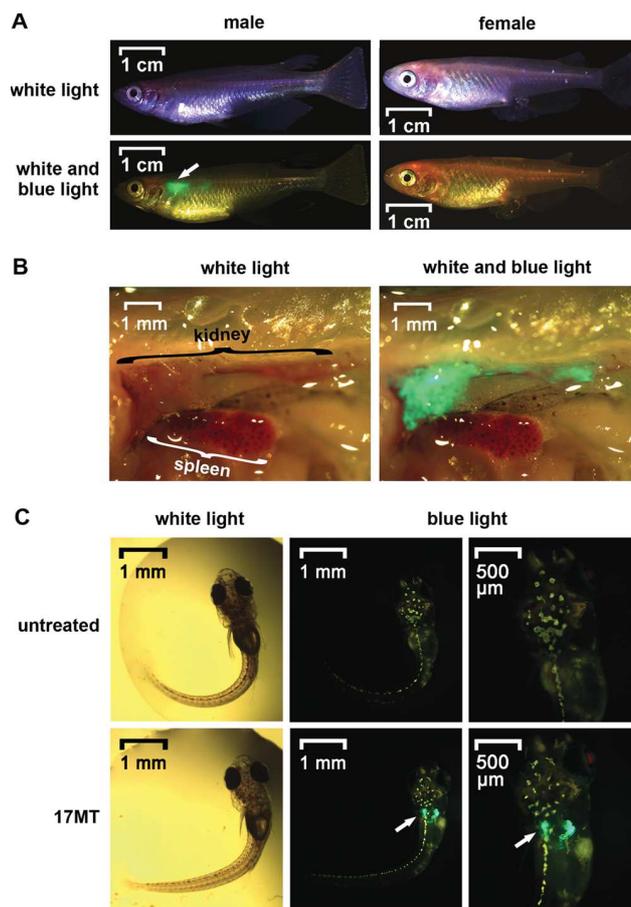


Figure 2. *Spg1.22-gfp* transgenic medaka. (A) Untreated, adult *spg1.22-gfp* fish under white light with or without additional blue light from a fluorescence source. Arrow denotes *gfp* expression in the kidneys. (B) Dissected untreated adult male medaka observed under white light with or without additional blue light from a fluorescence source. (C) Dph4 *spg1.22-gfp* fry treated for 4 days with vehicle or 17MT ($302 \mu\text{g/L}$) visualized under white and blue light. Arrows point to *gfp* expression in the developing mesonephros.

No GFP was visible in untreated F1 fry of the *spg1.22-gfp* line until dph60, around the time when medaka become reproductively active, suggesting that the expression of *gfp* is induced by increasing levels of androgens. Consequently, we screened F1 fry with a high concentration of androgen (17MT, $302 \mu\text{g/L}$) to confirm germ line transmission. This treatment resulted in *gfp* expression in the developing mesonephros (Figure 2C). All further experiments were carried out using unsorted F2–F5 *spg1.22-gfp* fry resulting from a nontransgenic (red-orange strain)/ transgenic cross, exposed from dph0

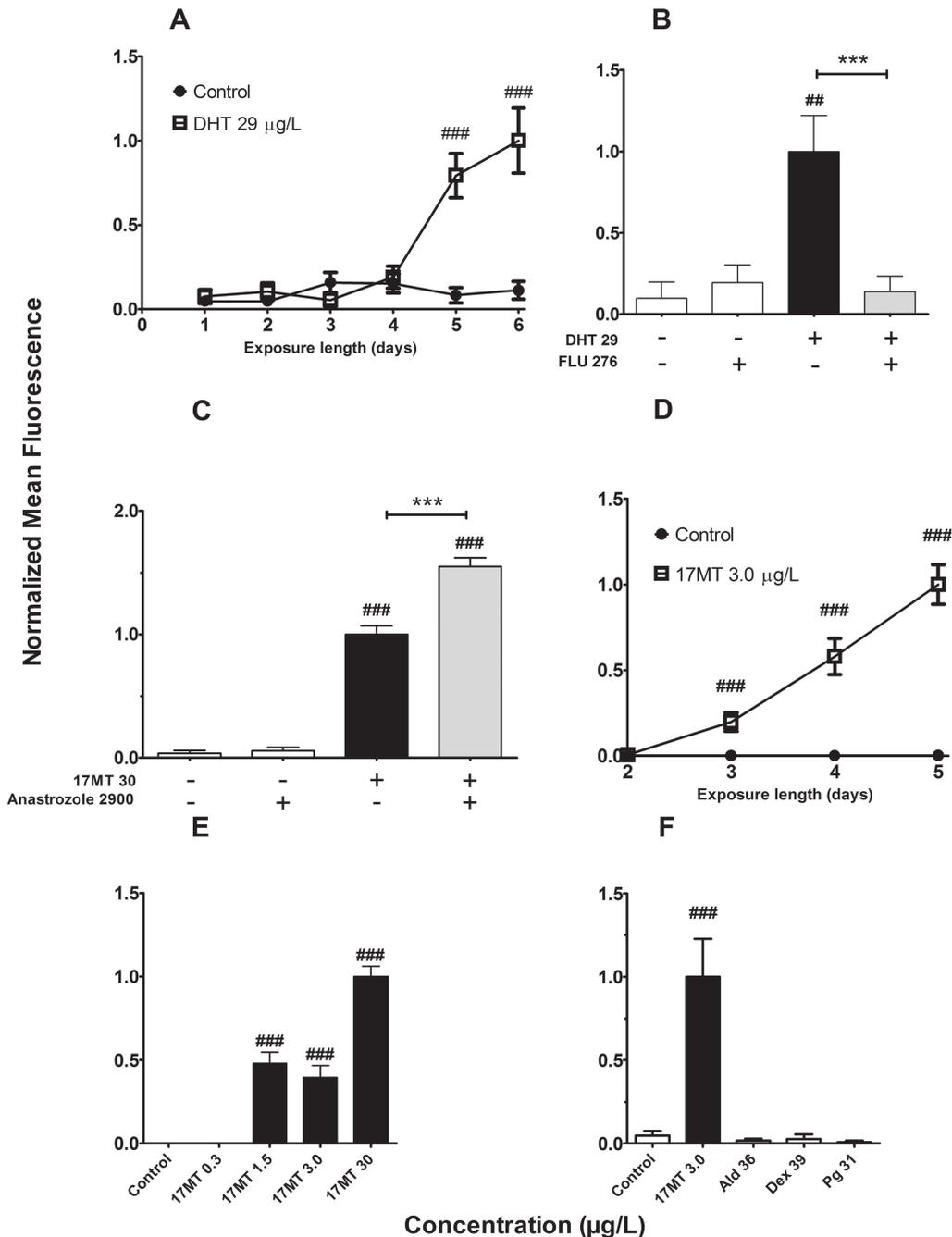


Figure 3. Androgenic specificity of the *spg1.22-gfp* line. (A) Evolution of fluorescent signal during exposure to DHT. (B) Inhibition of DHT induced fluorescent signal after 6 days of cotreatment with flutamide. (C) The effect of anastrozole on GFP signal induced following 3 days exposure to 17MT. (D) Evolution of fluorescent signal during exposure to 17MT 3.0 $\mu\text{g/L}$. (E) Dose-response relationship after 4 days of exposure to 17MT. (F) Quantification of GFP signal following a 4 day treatment with aldosterone (Ald), dexamethasone (Dex) or Progesterone (Pg).

onward. All groups contained approximately 50% transgenic and 50% wild-type fry.

GFP Induction with Androgens. GFP was significantly induced in fry exposed to 29 $\mu\text{g/L}$ of the nonaromatizable androgen DHT from hatching to dph5, showing that *spg1-gfp* can be induced by androgens (Figure 3A).

To determine specificity of the fluorescent response, fry (dph0) were cotreated for 6 days with DHT (29 $\mu\text{g/L}$) and/or the pharmacological antiandrogen flutamide (276 $\mu\text{g/L}$). DHT induced GFP production was entirely abolished by cotreatment

with flutamide, showing that the DHT effect is mediated via AR (Figure 3B).

A 72 h exposure to the pharmacological aromatase inhibitor anastrozole (2.9 mg/L) did not induce fluorescence (Figure 3C). However, the aromatizable androgen 17MT (30 $\mu\text{g/L}$) did induce significant fluorescence and cotreatment with 17MT and anastrozole induced a 1.5-fold higher signal than 17MT alone. This suggests that blocking the conversion of 17MT to methylestradiol by inhibition of aromatase maintains a greater degree of androgen signaling.

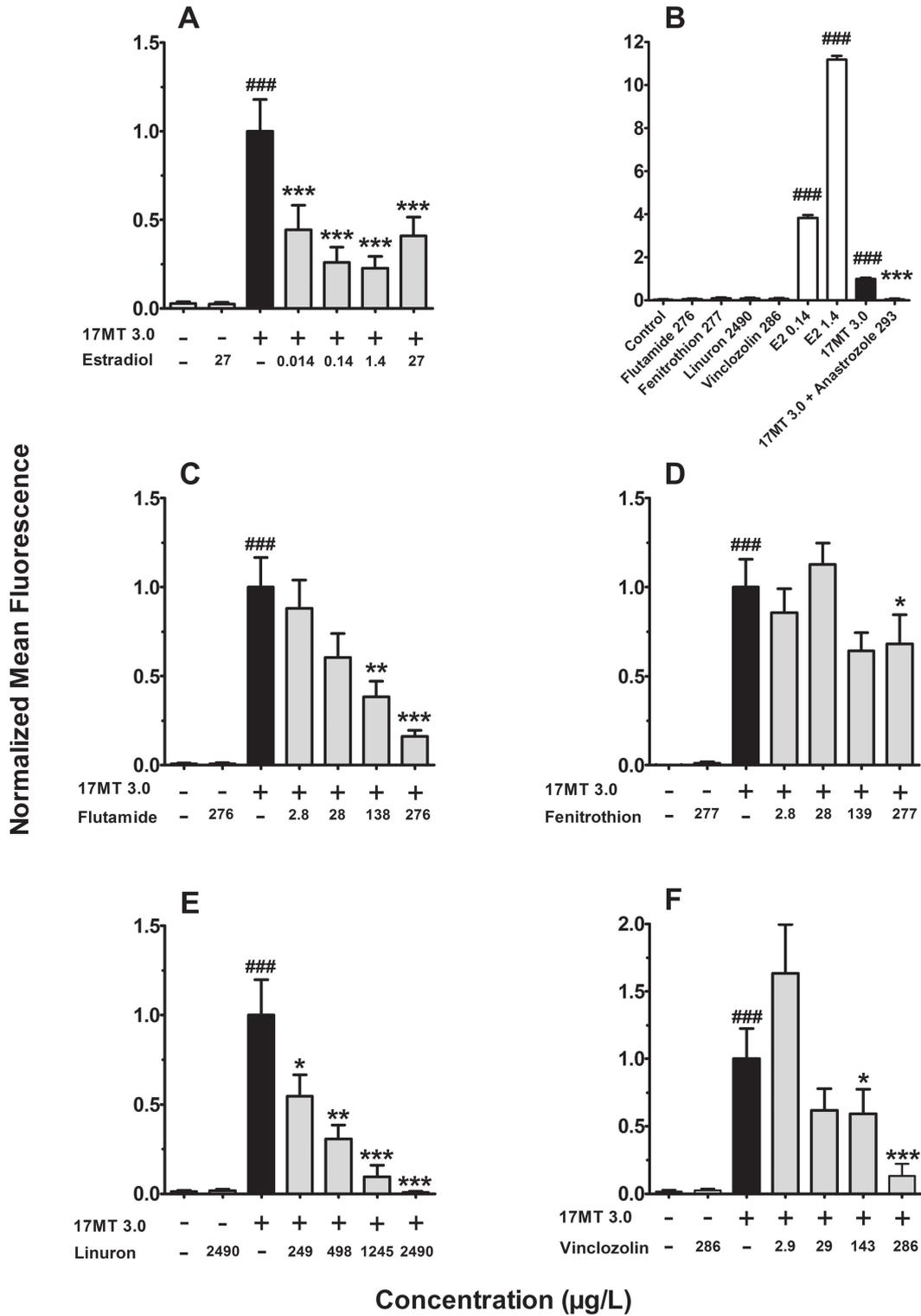


Figure 4. Antiandrogen detection assays with 17MT. (A) Quantification of GFP signal induced by E₂ alone, or in cotreatment with 17MT. (B) Lack of estrogen axis activation following 4 day treatment of *chgh-gfp* medaka with high concentrations of flutamide, fenitrothion, linuron and vinclozolin. (C–F) The effects of a 4 day cotreatment of *spg1.22-gfp* fry with 17MT and a range of concentrations of flutamide, fenitrothion, linuron, or vinclozolin.

Optimized Protocol. Due to the more rapid response observed with 17MT compared to DHT (compare Figure 3A and C), 17MT was selected as the cotreatment for the detection of antiandrogens. We conducted a time course experiment with 3.0 µg/L 17MT from dph0–5 (Figure 3D). A

statistically significant increase in GFP signal was induced after 72 h exposure, however, a more robust GFP signal was observed after 96 h, therefore, further exposures were carried out for 96 h.

Using this protocol, 17MT was tested at four concentrations (Figure 3E). The lowest observable effect concentration (LOEC) for 17MT was 1.5 $\mu\text{g/L}$.

Specificity of the Response. In addition to androgens, mineralocorticoids, glucocorticoids, and progestogens have been shown to induce some AREs. Therefore, the specificity of the response for AR agonists was tested using aldosterone, dexamethasone, and progesterone, respectively. A lack of GFP production following treatment with 10^{-7} M of each substance from dph0 to dph4 demonstrated the specificity of the response. Significant fluorescence was induced with a 10-fold lower concentration of 17MT (Figure 3F).

Estrogens have been reported to reduce androgen-stimulated *spiggin* response in adult stickleback fish in vivo and in stickleback kidney cells in vitro.^{20,28} The *spg1* promoter also contains putative EREs. We therefore tested the effect of E_2 on 17MT-induced GFP production with our medaka model. GFP signal was observable in fry exposed to 17MT (3 $\mu\text{g/L}$) for 4 days (Figure 4A). Co-treatment with E_2 (14 ng/L-27 $\mu\text{g/L}$) repressed androgen-induced *gfp* expression, whereas E_2 alone had no observable effect.

Detection of AR Antagonists Using *spg1.22-gfp*. Due to the observed repression of 17MT-induced *gfp* expression by E_2 , prior to determining the ability of the *spg1.22-gfp* line to identify antiandrogenic activity, we verified that the test substances were not estrogenic. Using an estrogen responsive transgenic medaka line harboring the promoter of the *choriogenin H* gene driving expression of *gfp* (*chgh-gfp* line), we carried out exposures within the same developmental window (96 h, dph0-dph4) and under the same exposure conditions as for the *spg1.22-gfp* line. This line was created using the same genetic construction described by Karauchi et al. for the creation of their line.^{29,30} All four antiandrogens (flutamide, fenitrothion, linuron, and vinclozolin) failed to induce *gfp* expression compared to the solvent control group, when tested at the maximum concentration to be utilized in the proceeding experiments (Figure 4B). In contrast E_2 strongly induced a fluorescent response as did 17MT to a lesser extent. The 17MT-induced fluorescent response was completely abolished when cotreated with anastrozole.

To determine the sensitivity of a four-day assay for the detection of antiandrogens, we first tested flutamide. A concentration dependent inhibition of GFP signal was observed at all concentrations tested. Flutamide significantly inhibited GFP signal at ≥ 138 $\mu\text{g/L}$ (Figure 4C).

We then tested the assay sensitivity for three well-characterized environmentally relevant antiandrogens. Fenitrothion significantly reduced GFP signal at 277 $\mu\text{g/L}$ and almost at 139 $\mu\text{g/L}$ ($P = 0.0657$ cf. 17MT alone) (Figure 4D). Linuron and vinclozolin significantly reduced the GFP signal at ≥ 249 $\mu\text{g/L}$ (Figure 4E) and ≥ 143 $\mu\text{g/L}$ (Figure 4F) respectively.

Therefore, the determined LOECs were 138 $\mu\text{g/L}$ (flutamide), 277 $\mu\text{g/L}$ (fenitrothion), 249 $\mu\text{g/L}$ (linuron) and 143 $\mu\text{g/L}$ (vinclozolin). This indicates the following order of antiandrogenic potency in terms of molarity flutamide = vinclozolin > fenitrothion = linuron.

DISCUSSION

Here, we describe an in vivo test for the detection of androgenic/antiandrogenic chemicals. Our aim was to create a medaka transgenic line suitable for detection of pro- and antiandrogenic compounds among industrial chemicals or present in environmental samples. The *spiggin* response from

the three-spined stickleback, which is under validation as an OECD guideline for testing of androgen axis disruptors (OECD 2009a), was modeled here in medaka using the *spiggin1* promoter driving expression of *gfp*. We believe that this tool will be useful for screening and prioritizing purposes, before carrying out more expensive and labor-intensive guideline tests.

The three-spined stickleback *spiggin* gene response is well-tested and androgen-specific.^{21,31} Interestingly, we identified a putative ARE with a conserved sequence (TTTACAggtTGTCT) and position (-203 to -218bp) in *spg1*, 3, and 5. This element resembles the classical inverted ARE (AGAACAAnnTGTCT) but displays changes in the first half site, of which the G \rightarrow T change in position -6 in particular may have consequences for functionality.³² It remains to be studied whether this element mediates the androgen-specific induction of the *spiggin* promoter. It is also important to note that the conserved regions between *spiggin* gene promoters extend up to -5kb from the ATG, suggesting the presence of other sequences of regulatory importance.

We confirmed that the transgene drives kidney-specific expression of *gfp* mimicking the physiological expression of *spiggin* observed in stickleback, possibly due to a kidney-specific transcription factor binding site being present within the promoter. This transcription factor must be sufficiently conserved between species to confer the same tissue specificity. In addition, the male specific expression of *spiggin*¹⁸ is faithfully mimicked in our transgenic medaka model. We show that in response to AR agonists, GFP is produced in the kidneys and is quantifiable in fry immediately after hatch. This method is noninvasive as fluorescence quantification can be carried out in intact, live fry. Interestingly, *spg1.22-gfp* fry appear to be less sensitive to aquatic exposures with DHT than 17MT. This is in accordance with the AFSS assay²¹ which is conducted with androgen cotreatments of either 5 $\mu\text{g/L}$ DHT or 0.5 $\mu\text{g/L}$ 17MT. The need for a higher concentration of DHT is hypothesized to be due to low bioavailability or stability.

With this in mind, we developed a four day protocol for the detection of antiandrogens by cotreating with 17MT. We determined that the sensitivity of our assay is 138 $\mu\text{g FL eq/L}$, less than half the LOEC that induces testis-ova in medaka³³ and reduces secondary sexual characteristics in stickleback.³⁴ This is sufficiently sensitive to detect environmentally relevant antiandrogenic activity, given levels found in river samples, for example, Lambro River, Italy (370–4723 $\mu\text{g FL eq/L}$)² as well as effluent from wastewater treatment plants (21.3–1231 $\mu\text{g FL eq/L}$ in the UK)⁶ and even some control sites (>150 $\mu\text{g FL eq/L}$ in the River Ock, UK).³⁵

We achieved comparable flutamide sensitivity to the AFSS (138 $\mu\text{g/L}$ and 10–250 $\mu\text{g/L}$ respectively).²¹ We expect a higher sensitivity to be achieved with a homozygous line, which have previously decreased interindividual differences in response caused by different copy numbers and genetic locations of the transgene in other transgenic models (data not shown). The “near miss” with 28 $\mu\text{g/L}$ flutamide ($P = 0.0588$ cf. 17MT alone) suggests that, with a tighter interindividual response, a greater sensitivity to flutamide can be achieved. Interestingly the LOECs determined here for environmental antiandrogens were also similar to those obtained during interlaboratory testing of the AFSS (fenitrothion: 277 $\mu\text{g/L}$ cf. 60 $\mu\text{g/L}$, linuron: 249 $\mu\text{g/L}$ cf. 250 $\mu\text{g/L}$ and vinclozolin: 143 $\mu\text{g/L}$ cf. 100 $\mu\text{g/L}$, respectively). We observed the following order of potency: flutamide =

vinclozolin > fenitrothion = linuron, which compares favorably with results obtained for the AFSS interlaboratory testing (fenitrothion > flutamide = vinclozolin > linuron)²¹ with the exception of fenitrothion. Interestingly, E₂ inhibited 17MT-induced activation of the *spg1* promoter in a concentration-dependent manner. This fits with observations of inhibition of spiggin production in vitro in stickleback kidney cells with 270 µg/L E₂²⁰ and in vivo in male stickleback with 20 ng/L ethinylestradiol,²⁸ although the in vivo study failed to reach statistical significance. The mechanism of the E₂ mediated inhibition of Spiggin production remains unclear. Negative feedback through the HPG axis is unlikely to be the only mechanism as the effect has been observed in vitro, although at much higher concentrations.²⁰ As proposed previously^{20,28} the effect may be local, acting e.g. at the level of AR expression regulation, through membrane bound ER or through the putative EREs in the spiggin promoters. Due to the well-described induction of aromatase expression by estrogens³⁶ it is entirely possible that all estrogens are also antiandrogenic in vivo, inducing aromatase expression, which in turn converts androgens to estrogens—reducing androgen axis signaling. In light of this finding, it is possible that the increase in GFP production observed with 17MT plus anastrozole compared to 17MT alone (Figure 3C) is due to a combinatorial effect. This may be due to an increase in available androgen leading to an increase in GFP production and/or a decrease in inhibition of *spiggin* transactivation by methylestradiol, if indeed it inhibits GFP production as we have observed with E₂.

As a screening tool prior to higher tier tests, our assay will reduce exposure length from 21 to 4 days, translating into a vast reduction in cost and turnaround time, making it ideal for adaptation to medium throughput screening. The ability to perform this assay prior to protected life stages considered as laboratory animals in Europe is an additional ethical advantage.³⁷ Finally, the use of the *spg1.22-gfp* medaka line is expected to facilitate interlaboratory comparisons as the AFSS assay suffers from variable sensitivity due to differences in the ages and maturities of the adult stickleback used.²¹

Medaka offer certain advantages over other fish species such as their adaptability to salt or fresh water allowing this model to be used to test unextracted, whole water sample from the entire length of a river, from its source to its estuary and out to sea. This will allow comparisons of androgen axis activity along rivers in an in vivo model and may lead to the identification of point sources of pollution. Additionally, medaka possess a well-characterized XX/XY sex determination system with the master sex determining gene, *dmy*, present on the Y chromosome^{38,39} allowing disparities between genetic and phenotypic sex to be linked to levels of androgen axis activity.

The experiments presented here were all carried out with transgenic medaka fry resulting from a cross between wild-type and heterozygous parents. This resulted in the production of an approximately equal mix of wild-type and heterozygous fry. As no basal fluorescence is observable in the fry of this line, it was impossible to exclude wild-type fry prior to exposure and all statistical analyses were carried out on the mixed population. A major goal in the near future is, therefore, the creation of a homozygous line. This feature was not achieved prior to this series of experiments as the procedure can take several years, but is ongoing. The transmission rate observed in all generations of *spg1.22-gfp* medaka used for this study was approximately 50%, suggesting a single transgene insertion site. However, it would require two additional generations before

obtaining homozygous fish, then a number of generations to reamplify the population to a level allowing sufficient egg production for large-scale experiments.

It can realistically be envisaged that availability of a simple, rapid and relatively inexpensive in vivo test for androgen axis activity will lead to an increase in the number of raw chemicals and environmental samples tested. This should result in an increased identification of pro- and antiandrogenic EDCs and provide opportunities for regulatory authorities to reduce or eliminate human and wild-life exposure to these substances.

Spg1.22-gfp medaka fry provide a unique model that allows rapid detection and quantification of androgenic and antiandrogenic substances in the environment. Compared to the current OECD AFSS guideline, our model reduces the detection time from over 21 days to 4 days while maintaining sensitivity and eliminating labor-intensive steps (dissection, sample preparation, and ELISA). We consider that this model is adapted to environmental monitoring as well as medium-throughput screening of chemicals prior to higher tier tests such as the AFSS.

■ ASSOCIATED CONTENT

📄 Supporting Information

A more in-depth description of the promoter analysis results is available as supporting text, as well as five figures as described in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare the following competing financial interest(s): Two of the authors (G.L. and B.D.) have financial interests in WatchFrog, which is planning to commercialize the test described here. A.S., P.S., A.T., D.D.P. and G.L. are currently employed by WatchFrog, N.M. and P.D. were employees of WatchFrog when they were involved in this study. The other authors declare no competing interests.

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Manuscript n°5

Presentation of manuscript °5

Evaluation of the endocrine disrupting capacity of the pesticides linuron and fenoxycarb: effects on the thyroidal, estrogenic and androgenic systems

Having validated assays for the detection of estrogen and androgen axis active substances we decided to use these models, together with the THbZIP – gfp *Xenopus laevis* tadpoles to investigate the effects of two widely used pesticides- the herbicide linuron and the insecticide fenoxycarb.

Huge amounts of both compounds are used worldwide and have been detected in the environment in concentrations relevant to endocrine disruption. A variety of studies on vertebrates and invertebrates demonstrated the anti-androgenic effect of linuron, but it's interactions with other hormonal systems is much less documented. Nothing is known about the endocrine disrupting properties of fenoxycarb, however the molecular resemblance between fenoxycarb and thyroid hormone is striking.

In this study, we contribute to the general knowledge about the endocrine disrupting activity of linuron and fenoxycarb by assessing their (anti-)thyroidogenic, (anti-)androgenic, (anti-)estrogenic and aromatase disruption potential by using *in silico* and *in vivo* approaches. The (anti-)estrogenic and aromatase disruption potential were assessed using the ChgH-GFP transgenic medaka fry (Spirhanzlova et al., 2015). In the case of linuron, we complemented the protocol with an adapted mode which allows us to carry out more mechanistic studies. We exposed the CHgH-gfp fry to linuron in co-treatment with estradiol instead of testosterone in order to investigate whether the anti-estrogenic activity of linuron is due to aromatase inhibition. Potential (anti-) androgenicity was investigated using the Spiggin-gfp medaka (Sébillot et al., 2014) and the (anti-) thyroidogenicity was assessed by exposing the ThbZIP-gfp *Xenopus laevis* tadpoles (Fini et al., 2007).

Despite the fact that in manuscript n°2 of this thesis we describe the newly developed protocol of fluorescence quantification using automatic plate reader, in this study we used standard imagery. The protocol using fluorescence plate reader is more suitable in cases where a technology has to be transferred between laboratories as the equipment is less costly and takes less training to use.

In the case of this study it was more convenient to acquire images of the larvae in order to evaluate other features than fluorescence intensity for example development impairments.

Evaluation of the endocrine disrupting capacity of the pesticides linuron and fenoxycarb: effects on the thyroidal, estrogenic and androgenic systems

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Abstract

The herbicide linuron and the insecticide fenoxycarb are two chemicals commonly used in agricultural practices. Owing to agricultural runoff and other means of distribution, these compounds have been detected in the environment and food, where they could affect human and wildlife health due to their potential endocrine disrupting properties. While there are a number of studies that indicate that linuron is a likely endocrine disruptor, there is no information available on potential effects of fenoxycarb on vertebrate endocrine systems. Using sensitive *in vivo* exposure assays in transgenic *Xenopus laevis* tadpoles and medaka, complemented with *in silico* docking simulations of linuron and fenoxycarb to human hormone receptors, we assessed the potential of these pesticides to disrupt the thyroidal, androgenic and estrogenic systems. Linuron, at all doses tested, elicited thyroid hormone-like activity in tadpoles and, at concentrations of 2.5 mg/L or higher suppressed the estrogenic action of testosterone in medaka. Our experiments suggest that, in addition to its previously established anti-androgenic action, linuron produces weak thyroid hormone-like responses, as well as acting at the estrogen receptor level to inhibit estrogen signalling. Fenoxycarb on the other hand, did not cause any changes in the thyroidal, androgenic or estrogenic systems at the concentrations tested.

Keywords: linuron; fenoxycarb; endocrine disruptors; androgen; estrogen; thyroid

Abbreviations: 17MT: 17-methyltestosterone; E2: 17 β -estradiol; GFP: green fluorescent protein; JH: juvenile hormone; JHA: juvenile hormone analog; MS222: ethyl 3-aminobenzoate methanesulfate salt; TH: thyroid hormone; TR: thyroid hormone receptor; TSH: thyroid-stimulating hormone

1. Introduction

An increasing number of pesticides are proven or suspected endocrine disruptors in vertebrates. Many pesticides have been linked to the increased incidence of endocrine-related effects and diseases in both humans and wildlife (Bergman et al., 2013; McKinlay et al., 2008) and are believed to contribute significantly to the current decline in biodiversity (Zhou et al., 2010). It is well established that endocrine disruptors can act at extremely low levels, in the nanomolar to micromolar range, and some even at picomolar levels (Vandenberg et al., 2012). Here we focus on two chemically very different compounds, the herbicide linuron and the insecticide fenoxycarb, both of which are used extensively worldwide and have been shown to be present in the environment at levels potentially relevant to endocrine disruption.

Linuron, or 3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea, is a phenyl-urea herbicide that acts on target plants by targeting the photosystem II reaction center and inhibiting the electron transport responsible for photosynthesis (Jurado et al., 2011). This compound is used to control a number of broadleaf and grass weeds in the cultivation of a variety of vegetables and cereals. Linuron is known to enter surface waters in agricultural runoff, where it is moderately persistent (Patterson, 2004). Surface water concentrations have been reported to fall in the nanogram to microgram per liter range. Concentrations detected include 1.05 µg/L in a Canadian river within an agricultural catchment (Woudneh et al., 2009) and 4.42 µg/L in a Florida stream receiving agricultural runoff (Schuler and Rand, 2008). Linuron has also been detected in drinking water and food residues (Environmental Protection Agency, 2009; Pest Management Regulatory Agency, 2014). More than a decade ago, linuron was shown to exert anti-androgenic effects (Lambright et al., 2000), which was later confirmed by a number of other studies, in a variety of vertebrate species. While the anti-androgenic activity of linuron has been studied extensively, its interactions with other hormonal systems are less well documented. There is some evidence of *in vivo* disrupting effects on the thyroidal and estrogenic systems (George et al., 2003; Marlatt et al., 2012; O'Connor et al., 2002).

Fenoxycarb, or ethyl [2-(4-phenoxyphenoxy)ethyl] carbamate, on the other hand, is one of the most widely used juvenile hormone analogs (JHAs) and nothing is known about its endocrine disrupting properties. Juvenile hormone analogs are a class of insecticides that disrupt insect development and growth by mimicking the action of insect juvenile hormones (JHs) (Dhadialla et al., 1998; Grenier and Grenier, 1993). Fenoxycarb is marketed worldwide for use as an insecticide on orchards and crops, in nurseries and greenhouses, on ornamentals, in flea-control carpet sprays and as fire ant bait (Sullivan, 2010). JHAs are generally considered non-toxic to vertebrates; for example, toxicity studies for fenoxycarb have found low acute toxicity to mammals upon oral, dermal or inhalation exposure (European Food Safety Authority, 2010; Marrs, 2012a; Sullivan, 2010). However, fenoxycarb shows a remarkable structural resemblance to thyroid hormones (THs) (Davey and Gordon, 1996) (Fig. 1), a property shared with several groups of TH disruptors (Bergman et al., 2013). Furthermore, both fenoxycarb and THs cause locust ovarian follicle cells to shrink, mimicking JH effects (Davey and Gordon, 1996). Na^+/K^+ ATPase inhibitors were able to abolish this TH effect, indicating that THs have the same molecular mode of action as JH and fenoxycarb in locust ovaries (Davey, 2000; Davey and Gordon, 1996; Kim et al., 1999). Interestingly, the thyroid gland was one of the target organs of fenoxycarb identified in short-term toxicity studies, in which rats receiving high doses of fenoxycarb showed follicular hyperplasia, increased thyroid weight, and small, active thyroid follicles in females (European Food Safety Authority, 2010; Marrs, 2012b). It is therefore of concern that potentially significant doses of fenoxycarb are present in the environment. Despite its short half-life and strong sediment affinity (Sullivan, 2010), fenoxycarb has been detected in Australian streams at concentrations of up to 300 ng/L (Schäfer et al., 2011). For comparison, the no-observed-effect concentration for reproductive effects in *Daphnia magna* is 1.6 ng/L (European Food Safety Authority, 2010). In addition, fenoxycarb residues in wheat are thought to remain pharmacologically relevant for at least three years after spraying (Liu et al., 2008).

The aim of this study was to assess the endocrine disrupting properties of fenoxycarb and linuron by investigating their effects on the thyroidal, estrogenic and androgenic systems, using sensitive transgenic vertebrate models (Fini et al., 2007; Kurauchi et al., 2005; Sébillot et al., 2014) and *in silico* prediction of binding affinities to human hormone receptors (Vedani et al., 2012).

2. Materials and methods

2.1. Chemicals and solutions

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), including 17 α -methyltestosterone (17MT, CAS: 58-18-4), testosterone (CAS: 58-22-0), 17 β -estradiol (E2; CAS: 50-28-2); 3,3',5-triiodo-L-thyronine (T₃; CAS: 6893-02-3), fenoxycarb (CAS: 72490-01-8), and linuron (CAS: 330-55-2). All stock solutions of hormones, fenoxycarb and linuron were made in dimethyl sulfoxide, its final concentration being 0.2% in all treatments (including vehicle controls). Ethyl 3-aminobenzoate methanesulfate salt (MS222; CAS: 886-86-2) solution was prepared with the same mass of sodium bicarbonate and adjusted to pH 7.5–8.0. Medaka medium contained the following quantities of salts per liter: 10 g NaCl, 0.4 g CaCl₂·2H₂O, 0.4 g MgSO₄·7H₂O, 0.3 g KCl, 0.02 g methylene blue, and its pH was adjusted to 7.2–8.0.

2.2. In vivo exposure studies

2.2.1. Transgenic medaka models

An exposure study using transgenic *ChgH-gfp* medaka (*Oryzias latipes*) was carried out in order to investigate the potential impact of fenoxycarb and linuron on the estrogenic system. The *ChgH-gfp* medaka line harbors a genetic construct composed of 2047 bp of the upstream region of the medaka *choriogenin H* gene and the coding sequence of *gfp* (Kurauchi et al., 2005; Spirhanzlova et al., 2015). In response to estrogenic compounds, *gfp* is expressed in the liver of the fish, while no GFP signal is visible in the absence of estrogen treatment. Ectopic fluorescence is observable in the head and the heart of the fish. To identify any potential (anti-)androgenic activity of fenoxycarb, transgenic *Spig1.22-gfp* medaka were used. This line contains the stickleback (*Gasterosteus aculeatus*) *spiggin-1* gene promoter driving the expression of *gfp* and has been previously used to demonstrate the anti-androgenic action of linuron (Sébillot et al.,

2014). In response to androgen treatment, this line expresses *gfp* in the kidneys, whereas no GFP signal is visible in the kidneys without androgen treatment.

Fry were exposed to the test chemicals in six-well plates. Five newly hatched (dph-0) transgenic medaka were placed in 8 ml of medaka medium in each well. Four wells (n = 20 fish) constituted an exposure group. The fish were incubated at 26°C for 24 and 96 h for *ChgH-gfp* and *Spg1.22-gfp*, respectively. The test solutions were renewed every 24 h. *ChgH-gfp* fry were exposed to a range of fenoxycarb concentrations (1 ng/L to 0.1 mg/L) in the presence or absence of testosterone (28.57 µg/L). Due to toxicity of linuron to medaka fry which was observed above 5 mg/L (results not shown), concentrations of 1–5 mg/L were chosen to investigate the activity of linuron on the estrogenic system. The effect of these linuron doses was also tested in the presence of E2 (180 ng/L). *Spg1.22-gfp* fry were exposed to the highest concentration of fenoxycarb (0.1 mg/L) only, with or without 17MT (5 µg/L). All exposure studies were carried out in duplicate.

Prior to quantification of fluorescence, fry were rinsed in medaka medium, anesthetized in 200 mg/L MS222 in Evian water, and positioned manually to expose the ventral part of the body of the fry in case of *ChgH-gfp* model, or positioned ventrally to allow access to the dorsal region in the case of *Spg1.22-gfp* fish. A Leica MZ10F stereomicroscope (Leica Microsystems, Wetzlar, Germany) in combination with an Infinity 1-3C camera (Lumenera, Ottawa, ON, Canada) was used to image the fluorescence of the fry. A 120W fluorescence source and ET-GFP long-pass filters (excitation 480/40, emission 510LP) (Leica) were used for fluorescence illumination. Images were captured at 8x magnification with 0.3 s and 0.75 s exposure time for *ChgH-gfp* and *Spg1.22-gfp* fish, respectively. Images were saved as 8-bit colour jpg files. An ImageJ macro (W.S. Rasband, U.S. National Institutes of Health, Bethesda, MD, USA) was used for image analysis as previously described (Sébillot et al., 2014). To eliminate residual autofluorescence from endogenous pigments, a 10-255 threshold was applied for the *ChgH-gfp* model. Depending on the model, the liver or kidney region of the fry was manually selected, and the total (*ChgH-gfp* fry) or mean (*Spg1.22-gfp* fry) intensity of the pixels in the selected area was calculated.

2.2.2. Transgenic *Xenopus laevis* model

To detect any potential effects of fenoxycarb and linuron on the thyroidal system, we carried out an exposure study using transgenic *Xenopus laevis* tadpoles of the *TH/bZIP-gfp* line. In this line, the promoter of the leucine zipper transcription factor TH/bZIP drives expression of the *gfp* coding sequence, resulting in GFP signal in the head area, primarily the brain, in response to thyroid hormone receptor (TR) activation (Fini et al., 2007).

Per treatment group, 20 Nieuwkoop-Faber stage-45 tadpoles were incubated in six-well plates containing 8 ml of exposure solution, with ten tadpoles per well. The exposure solutions were prepared in Evian water on the first day of the experiment, stored at 4°C for the entire exposure period, and removed to room temperature two hours before renewal, to equilibrate the temperature. The tadpoles were incubated for 72 h in the dark at a temperature of 21°C and the exposure solutions were renewed every 24 h. To ascertain whether or not the test chemicals affect the thyroidal system, tadpoles were exposed to fenoxycarb (1 ng/L to 0.1 mg/L) or linuron (1 mg/L to 20 mg/L) with or without T₃ (3.25µg/L). All exposure studies were carried out in duplicate.

At the end of the experiment and before the image capture, tadpoles were rinsed in Evian water, anesthetized in 100 mg/L MS222, and placed one per well into black 96-well plates with conical wells (Greiner Bio-One, Courtaboeuf, France). Two empty wells per line were used for the quantification of background fluorescence. Tadpoles were manually positioned to expose the ventral side of the body, with the dorsal surface in contact with the bottom of the well and the head in the center of the well. An automatic imaging system was used to photograph each well, composed of a macrofluostereomicroscope with band-pass GFP filters (excitation 475-495 nm, emission 495-545 nm) (Leica), a robotized platform (Märzhäuser Wetzlar, Wetzlar, Germany) and a high-resolution ORCA-AG camera (Hamamatsu Photonics, Hamamatsu City, Japan). Using SimplePCI software (Hamamatsu Photonics), mean pixel intensity of each well was quantified. Mean pixel intensity of empty wells was subtracted from the wells on a plate-by-plate

basis for normalization of background fluorescence. Values outside of +/- 2 SD from the mean were eliminated prior to calculation of the mean fluorescence intensity of each exposure group.

2.2.3. Statistical analysis

GraphPad Prism version 5.04 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. A D'Agostino and Pearson omnibus normality test was used to determine the distribution of values in each of the exposure groups. A one-way ANOVA and Dunnett post test was carried out if all groups showed a normal distribution. A Kruskal-Wallis test with Dunns post test was used if at least one of the compared groups did not pass the normality test. All groups were compared to the appropriate control group. Differences were only considered significant if they were greater than +/- 12% compared to the relevant control and $P < 0.05$.

2.3. In silico prediction of binding affinities

The chemical structures of fenoxycarb and linuron, in mol or pdb file format, were downloaded from the ChemSpider database (www.chemspider.com). Potential receptor binding was tested using Open VirtualToxLab software version 5.0 (Vedani et al., 2012). The fenoxycarb and linuron structures were docked against the following human nuclear receptors: TH receptors α and β (TR α and TR β), androgen receptor (AR), aryl hydrocarbon receptor (AhR), estrogen receptors α and β (ER α and ER β), glucocorticoid receptor (GR), liver X receptor (LXR), mineralocorticoid receptor (MR), peroxisome proliferator-activated receptor γ (PPAR γ) and progesterone receptor (PR). Predicted inhibition constants (K_i) were generated by the software by simulating and quantifying the interactions of the test compounds with the potential target proteins using automated flexible docking combined with 4D Boltzmann scoring (Vedani et al., 2012).

3. Results

3.1. Fenoxycarb

When the thyroidal activity of fenoxycarb was assessed in *TH/bZIP-gfp* tadpoles, no observable increase in fluorescence was detected compared to the relevant control in either the T₃-spiked or unspiked mode (Fig. 2A). None of the tested concentrations of fenoxycarb induced fluorescence in *ChgH-gfp* fish in the absence of testosterone, and no significant changes in the fluorescence intensity compared to the positive control were detected in the presence of testosterone (Fig. 2B). No fluorescence increase was observed in *Spg1.22-gfp* fry exposed to 0.1 mg/L fenoxycarb in the absence of 17MT compared to the negative control; likewise no difference in fluorescence intensity was detected in the presence of 17MT (Fig. 2C). Predicted K_i values generated by docking fenoxycarb to potential human target proteins were in the low μ M range for most receptors (including AR, ERs and TRs), but slightly higher predicted binding affinities (nM range) were found for GR and PR (Table 1).

3.2. Linuron

Compared to the negative control, all tested concentrations of linuron caused a significant increase of fluorescence in the head of the *TH/bZIP-gfp* tadpoles; however, when tadpoles were co-treated with T₃, this effect was masked (Fig. 3A). No significant increase of fluorescence was detected when linuron doses were tested in *ChgH-gfp* fish in the absence of testosterone in comparison to the negative control. However, in the presence of testosterone or E2, a significant decrease in fluorescence was observed for fry exposed to 2.5 mg/L of linuron or greater (Fig. 3B, Fig. 4). All predicted K_i values for linuron were found to be in the low μ M range, with no affinity to the AhR (Table 1).

4. Discussion

The potential effects of the herbicide linuron and the insecticide fenoxycarb on the thyroidal and gonadal axes were assessed using established transgenic vertebrate models. Our results show a stimulating effect of linuron on the thyroidal pathway, detectable only in the absence of T₃. In the male juvenile rat test, this compound has been shown to induce a decrease in thyroxine (T₄) and thyroid-stimulating hormone (TSH) concentrations after 30 days of gavage at 50 or 100 mg/kg/day (George et al., 2003), and dose-dependent decreases in T₃ and T₄ levels were observed in a 15-day adult rat screening study (O'Connor et al., 2002). Together with the results presented here, these observations suggest that linuron produces a T₃ like response. Thyroid receptor agonists, such as T₃ and T₄, induce the expression of TH target genes, increasing the GFP level in our xenopus assay, but they also exert a negative feedback within the thyroidal axis, explaining the reduction in THs and TSH concentrations in the rat tests. The lack of a dose response over a 20-fold increase in concentration and the absence of significant effects in the T₃-spiked condition suggest that linuron has weak T₃-like effects. This observation is in accordance with the result of an *in vitro* transcriptional activation assay using HepG2 cells transfected with a luciferase-based reporter gene and a T₃-responsive element, reporting linuron to have weak T₃-like activity (Hofmann et al., 2009), though even in this *in vitro* system effects could be played out by interference with inactivating deiodinases or comodulator proteins.

In our estrogenic test, no estrogenic effects of linuron were detected, supporting earlier cell culture studies demonstrating that linuron is not an estrogenic molecule (Gordon et al., 2015; Vinggaard et al., 1999). Instead, we found linuron to be an anti-estrogenic compound, reducing the fluorescence in the liver of *ChgH-gfp* medaka in the presence of testosterone. This decrease of fluorescence, however, can be caused by effects at different levels of the estrogenic system, including enzymatic aromatization of testosterone into E2, metabolism of testosterone or E2, and transcription of target genes controlled by ERs. We therefore tested linuron in co-treatment with E2 to determine whether this decrease in fluorescence could be due to inhibition of aromatase activity. As the result was similar to the one obtained with testosterone, we can conclude that aromatase inhibition is not the major cause of decrease in fluorescence. The effect

of linuron on aromatase activity is still equivocal (Environmental Protection Agency, 2015; Vinggaard et al., 1999). In a yeast assay evaluating ER activation (Orton et al., 2009), linuron also demonstrated anti-estrogenic activity at concentrations of 1.9–31.3 μM (0.47–7.8 mg/L), making it likely that our results are linked to ER antagonism. Our data obtained with *ChgH-gfp* medaka are in accordance with observations made in fathead minnow (*Pimephales promelas*), where linuron has been demonstrated to reduce plasma vitellogenin concentrations in females (Marlatt et al., 2012).

The T3-like effects and anti-estrogenic activity of linuron is in addition to its earlier described anti-androgenic action. *In vitro* studies have shown that linuron competitively inhibits androgen binding to AR in mammals, fish, and amphibians (Kojima et al., 2004; Orton et al., 2009; Wilson et al., 2007). In rats, linuron reduced the weight and development of androgen-sensitive reproductive tissues (Lambright et al., 2000) and was shown to have adverse impacts on male reproductive health, including abnormal reproductive development following *in utero* exposure, and reduced testosterone production *in vitro* and *in vivo* (Hotchkiss et al., 2004; Wilson et al., 2009). We have also reported previously that linuron is an anti-androgenic molecule using the *Spg1.22-gfp* medaka transgenic line (Sébillot et al., 2014). Similarly, in stickleback, linuron was reported to suppress dihydrotestosterone-induced spiggin production *in vivo* at concentrations of 100 and 250 $\mu\text{g/L}$ at both the transcript and protein level. This is assumed to occur specifically through AR antagonism (Hogan et al., 2012; Jolly et al., 2009). Strangely, the *in silico* predicted K_i values for linuron do not indicate significant binding affinity of the chemical to human AR, ERs or TRs. Whether this is because of false-negative docking data or non-competitive antagonism where linuron binds to an allosteric site of the receptor, remains to be established, but our linuron exposure studies underscore the importance of confirming negative predictions *in vivo* or *in vitro* when assessing a chemical's endocrine disrupting potential.

Our experimental data for fenoxycarb show the absence of disrupting effects on the thyroidal, estrogenic and androgenic systems. While THs may activate the same insect receptors as JH and fenoxycarb in locust ovaries (Davey, 2000; Davey and Gordon, 1996; Kim et al., 1999), the

opposite does not seem to be the case, *i.e.* despite its structural resemblance to THs, fenoxycarb does not bind vertebrate TRs with significant affinity. Our *in silico* data predicted an interaction with human TR α and β with a K_i of ~ 5 μ M (1.5 mg/L), but our *in vivo* data indicate an absence of effects on the thyroidal system. In our experiments, xenopus tadpoles were exposed to the maximum tolerated concentration of 0.1 mg/L, which is 15x lower than the predicted K_i . In rodent toxicity studies, rats needed to be exposed for 30 days to high amounts of the chemical (50 and 100 mg/kg/day) to detect an effect on the thyroid gland. Taken together, these observations suggest that fenoxycarb could probably impact the thyroidal axis at high doses far above the environmental concentrations. We speculate that in xenopus tadpoles, fenoxycarb exhibits an acute toxic effect at concentrations lower than those able to impact the thyroidal system.

To our knowledge, there are no published studies investigating the activity of fenoxycarb on vertebrate androgenic and estrogenic pathways. To assess the potential effect of fenoxycarb on these systems, medaka fry were exposed to the maximum tolerated concentration of fenoxycarb. This concentration was found to be 0.1 mg/L (results not shown), *i.e.* 300x higher than the maximal environmental concentration reported for this pesticide (Schäfer et al., 2011). However, we could not detect any effect of fenoxycarb at this dose on the gonadal endocrine systems, which is further supported by the high predicted K_i values of fenoxycarb for the human AR and ERs. Our *in silico* docking analysis, however, suggests that possible effects of fenoxycarb on the adrenal system could be an interesting avenue for further research.

In summary, this study reinforces the evidence for interactions of linuron with the thyroidal and estrogenic pathways, in addition to its established anti-androgenic action. The experiments carried out in xenopus tadpoles and medaka also show that fenoxycarb, at the levels found in the environment, does not affect the thyroidal, estrogenic and androgenic systems.

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Table 1. Predicted inhibition constants (K_i) of the interactions of fenoxycarb and linuron with several human nuclear receptors.

Receptor	K_i (fenoxycarb)	K_i (linuron)
AR	65.3 μ M	16.0 μ M
AhR	59.2 μ M	-
ER α	54.4 μ M	54.5 μ M
ER β	2.81 μ M	13.1 μ M
GR	42 nM	16.9 μ M
LXR	22.3 μ M	4.28 μ M
MR	2.11 μ M	27.1 μ M
PPAR γ	44.2 μ M	11.2 μ M
PR	365 nM	5.03 μ M
TR α	5.77 μ M	56.5 μ M
TR β	3.95 μ M	7.05 μ M

Figures

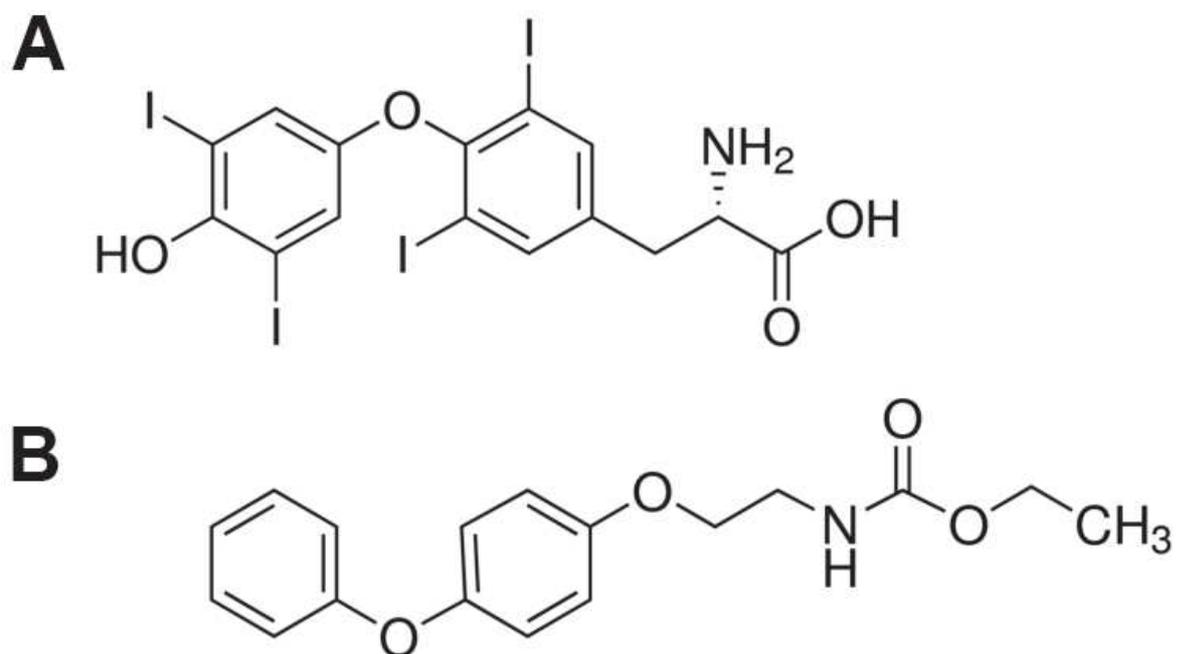


Figure 1 Chemical structures of **(A)** thyroid hormone (L-thyroxine) and **(B)** fenoxycarb. Both chemicals are phenoxyphenol derivatives.

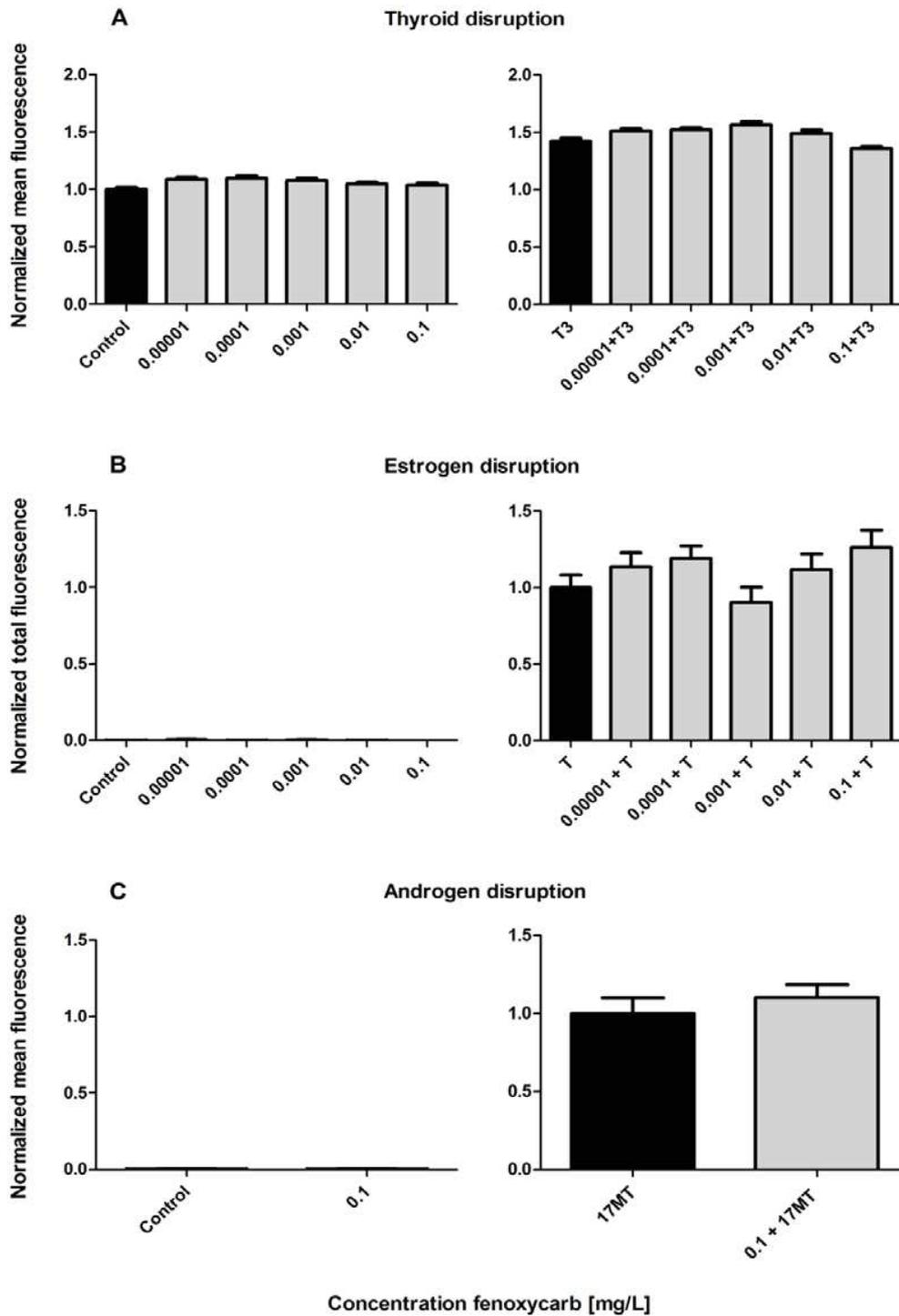


Figure 2. *In vivo* exposure study of fenoxycarb. (A) Exposure of *TH/bZIP-gfp* transgenic *Xenopus laevis* tadpoles to fenoxycarb showed no thyroidal activity with or without T₃. (B) Fenoxycarb exhibits no activity on the estrogenic system with or without testosterone co-

treatment in *ChgH-gfp* transgenic medaka. (C) No androgenic activity was detected after exposure of *Spg1.22-gfp* transgenic medaka with or without 17MT. Results are shown as means \pm SEM (n = 40 per treatment).

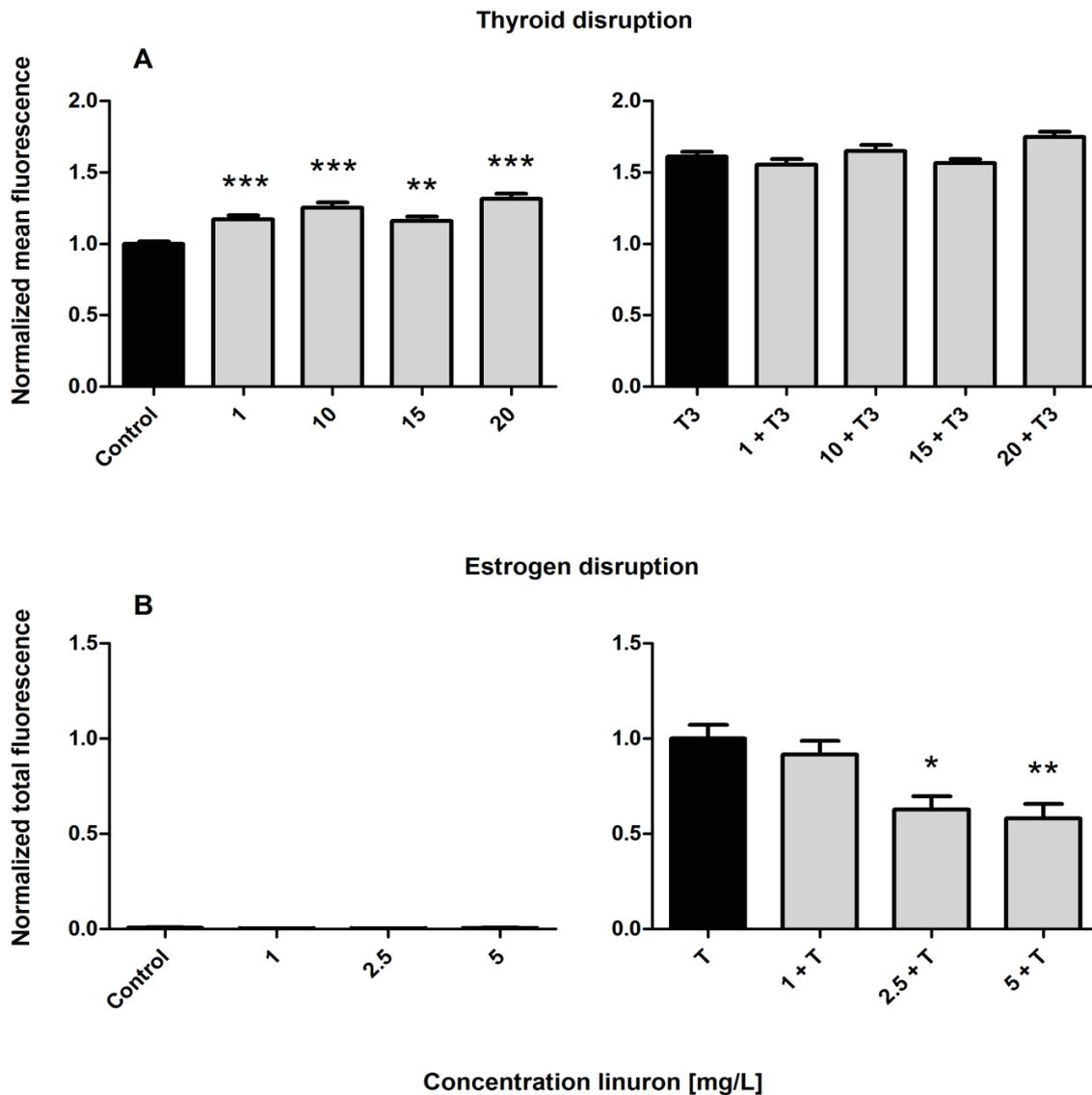


Figure 3. *In vivo* exposure study of linuron. (A) Linuron significantly induced fluorescence in the head of *TH/bZIP-gfp* transgenic *Xenopus laevis* tadpoles without T₃ co-treatment, but no significant additional induction was detected in the presence of T₃. (B) No significant fluorescence was induced by linuron in the liver of *ChgH-gfp* transgenic medaka without

testosterone co-treatment. Fluorescence induced by testosterone was significantly reduced by concentrations of linuron of 2.5 mg/L or above. Results are shown as means \pm SEM (n = 40 per treatment). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

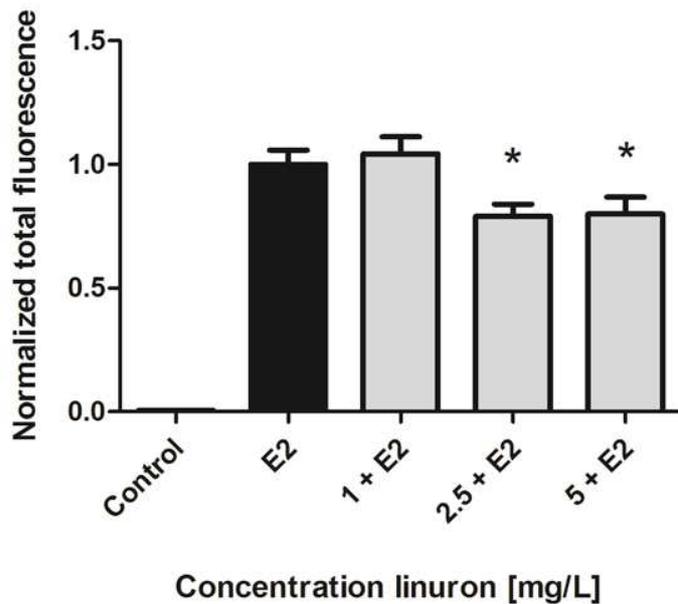


Figure 4. *In vivo* exposure of *ChgH-gfp* transgenic medaka to linuron in the presence of E2. Fluorescence induced by E2 was significantly reduced by concentrations of linuron of 2.5 mg/L or above. Results are shown as means \pm SEM (n = 40 per treatment). * $P < 0.05$

Discussion

1. What is an endocrine disruptor?

The definition of endocrine disruptors and other terms related to endocrine disruption such as “adverse effects”, “potency” and “endpoints” have been a topic of intense debate for some time. The necessity for clarification of these definitions has led to a number of publications documenting a discussion between the scientists (Kortenkamp et al., 2011, 2012; Rhomberg et al., 2012). The major question addressed is “What is an endocrine disruptor?”. A number of definitions have been suggested over the last two decades (Table 4. – adapted from (Zoeller et al., 2014)).

Table 4.

1996 US EPA	An exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes.
1996 EU	An exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function. A potential ED is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism.
1998 The Environment Agency	An endocrine disruptor is an exogenous substance that causes adverse health effects in an organism, or its progeny, consequent to endocrine function
1999 National Academy of Science	The term hormonally active agents (HAAs) is used to describe substances that possess hormone-like activity regardless of mechanism. Convincing evidence that an HAA can affect the endocrine system would be its ability to bind to classic hormone receptors and promote measureable responses such as the induction of hormone-responsive genes or gene products. However, chemicals can disrupt hormonal processes by a variety of other mechanisms.
2000 The Royal Society	EDCs are substances which may interfere with normal function of the endocrine (hormone) system of human and animals, since many of them mimic the structure of natural hormones produced in the body.
2000 German Consultative Study	Substances able to disrupt endocrine processes with the potential for impairing development and reproduction or increasing the risk of cancer
2002 WHO/IPCS	An exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse effects in an intact organism, or its progeny, or (sub)populations. A potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations.
2012 Endocrine Society	An exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action

Several opinions which were accepted in past are being reviewed and clarified, for example the term “functions of the endocrine system” previously stood for “change in hormone concentrations in the blood”, but nowadays it is promoted to rather focus on hormone action instead of hormone concentrations in blood when “hormone action” is understood as “hormone receptor activation” that leads to developmental or physiological effect. With this in mind the EDCs would be nowadays defined as compounds which have the capacity to provoke an adverse physiological effect secondary to a modification of endocrine signaling. It is also necessary to emphasize the difference between endocrine disrupting chemicals and endocrine active chemicals. These two terms are often exchanged by the scientists, which identify results obtained from *in vitro* bioassays as endocrine disruption while this term should be used only in case a physiological effect is observed, which is not possible in the case of *in vitro* bioassays.

Because the confirmation of adverse physiological effects often requires raising the animals to adult life stages and even breeding them to obtain the next generations, new strategies such as the Adverse Outcome Pathways (AOPs) framework are emerging as a solution for the risk assessment of potential endocrine disruptors. According to the OECD, AOPs are analytical constructs that describe a sequential chain of causally linked events at different levels of biological organization that lead to an adverse health or ecotoxicological effect. The AOP is relevant to risk assessment and it is linearly linking existing knowledge along series of connected key events between the two points - molecular initiating event and an adverse outcome. Since its launch by the OECD in 2012, the AOP program has been adopted by many laboratories and contributes greatly to the risk assessment of chemicals.

2. Screening tools for endocrine disruptors – *in vivo* vs *in vitro*

Concern and awareness regarding the presence of endocrine disruptors in the environment has increased during the last decades. A high number of chemicals have been demonstrated to cause a hormonal axis disruption in wildlife and many laboratories have attempted to develop screening assays to detect endocrine disruptors in the environment, or to test the endocrine activity of pure chemicals. Bioassays have been used to detect endocrine disruptors for many years. As reviewed in the introduction of this thesis, transgenic *in vivo* and *in vitro* bioassays have been developed

for investigating the disruption of different hormonal axis. Both *in vivo* and *in vitro* bioassays have their advantages. The advantages of *in vitro* studies are numerous:

- Reduction of the use of laboratory animals
- High throughput for large scale production
- Lower cost
- Higher sensitivity
- Numerous cell lines are human derived, therefore can offer an insight into the effect of the chemical in human species

The disadvantages are mostly due to the fact, that cells do not possess the metabolism and physiology of a complex organism, which can lead to differences in results between *in vitro* and *in vivo* bioassays (Charles et al., 2005; Folmar et al., 2002). False positive results can be obtained when a compound isn't metabolically inactivated, as demonstrated in the case of phthalate esters which induced weak estrogenic effects *in vitro*, but were inactive in an *in vivo* study using rodents (Zacharewski et al., 1998). The study however doesn't include data from other *in vivo* bioassays using other animal species so the possibility of the compound being active in another species should not be excluded. More problematic might be the case of false negative results, when a compound exhibits activity *in vivo*, but lacks activity *in vitro*, which can be due to an absence of metabolic activation or an action on hormone synthesis in a cell type other than the one used for the *in vitro* assay. An example of compounds which require bio-activation is PCBs (Fielden et al., 1997). As endocrine hormones, by definition, are produced in one part of the body and act on another, metabolism and physiology are greatly involved in the production, transport and action of the hormone in the target tissue. This is a reason why *in vivo* studies allow us to predict the potential endocrine disrupting effect of a compound more clearly than *in vitro* assays. However *in vivo* bioassays also have a number of disadvantages:

- As with *in vitro* bioassays difficulties exist in extrapolating from one species to another, however an additional complication with *in vivo* bioassays is that in most cases human data is not available.
- The cost of *in vivo* bioassays is greater, than the cost of *in vitro* bioassays
- Laboratories using animals for their experiments have to have a license

- *In vivo* bioassays are often time consuming and can last up to several weeks or even generations
- Society in general calls for reduction of animal use in science

The panel of *in vitro* bioassays used to assess endocrine disrupting potential is wide and contains bioassays for almost every hormonal axis, but the question remains if the information received from the *in vitro* bioassays is sufficient to label a compound safe for use, without confirmation *in vivo*. An ideal solution for thorough investigation of the full endocrine disrupting potential of a chemical would be the combination of *in silico*, *in vivo* and *in vitro* assays, however it has been demonstrated in manuscript n°5 of this thesis, that even if *in silico* docking predicts high affinity for a receptor, the *in vivo* bioassays do not necessarily confirm this result. Currently, recommendations by the OECD are published in the guidance document “Conceptual framework for testing and assessment of endocrine disrupters” adopted in 2002 and updated in 2012 including different guidelines, some of them mentioned in the introduction of this thesis (Fig. 16). This guideline which is also referred to as a “toolbox” is composed of five levels each corresponding to a different level of biological complexity, starting from data mining to multigenerational *in vivo* bioassays (Hecker and Hollert, 2011).

<p>Level 1 Sorting & prioritization based upon existing information</p>	<ul style="list-style-type: none"> •Physical & chemical properties, e.g., MW, reactivity, volatility, biodegradability •Human & environmental exposure, e.g., production volume, release, use patterns •Hazard, e.g., available toxicological data
<p>Level 2 <i>In vitro</i> assays providing mechanistic data</p>	<ul style="list-style-type: none"> •ER, AR, TR receptor binding affinity •Transcriptional activation •Aromatase & Steroidogenesis <i>in vitro</i> •Aryl hydrocarbon receptor recognition/binding •High Through Put Prescreens •Thyroid function •Fish hepatocyte VTG assay •QSARs; Others (as appropriate)
<p>Level 3 <i>In vivo</i> assays providing data about single endocrine Mechanisms and effects</p>	<ul style="list-style-type: none"> •Uterotrophic Assay (estrogenic related) •Hershberger Assay (androgenic related) •Non-receptor mediated hormone function •Fish VTG assay (estrogenic related) •Others (e.g. thyroid)
<p>Level 4 <i>In vivo</i> assays providing data about multiple endocrine mechanisms and effects</p>	<ul style="list-style-type: none"> •Enhanced OECD 407 (endpoints based on endocrine mechanisms) •Male and female pubertal assays •Adult intact male assay •Fish gonadal histopathology assay •Frog metamorphosis assay
<p>Level 5 <i>In vivo</i> assays providing data on effects from endocrine & other mechanisms</p>	<ul style="list-style-type: none"> •1-generation assay (TG415 enhanced) •2-generation assay (TG416 enhanced) •Reproductive screening (TG421 enhanced) •Combined 28 day/reproduction screening test (TG 422 enhanced) •Partial and full life cycle assays in fish, birds, amphibians & invertebrates (development & reproduction)

Figure 16. Different levels of “Conceptual framework for testing and assessment of endocrine disruptors” adapted from (Hecker and Hollert, 2011).

Multigenerational studies offer insight into the long term effect of endocrine disruptors. The majority of the *in vivo* bioassays focus on short term effects, which allow rapid data acquisition. However, they do not allow the study of long term effects such as fertility or cancer development or transgenerational effects. This is an important point as there have been cases, when an endocrine disruptor did not affect the mother, but affected the children, for example in the case of DES, described in the introduction of this thesis. Multigenerational studies are nevertheless time consuming and thus costly and with the amount of potential endocrine disruptors to be tested, it is not feasible to assess all with these kind of studies.

A question remains, if rapid tests using small model organisms like medaka, zebrafish or xenopus described in this thesis are suitable solution for EDC testing. They surely fill the gap between *in vitro* and *in vivo* bioassays, however while they exhibit many advantages in terms of speed or price, they also lack the availability to prove adverse physiological outcome like *in vitro* tests. They can however predict it to some extent, so they make suitable models for preliminary testing before full term *in vivo* bioassays.

3. Establishment of a panel of endocrine disruption bioassays

In this thesis, three novel transgenic medaka based models were introduced which allow rapid detection of disruption in sex hormone signaling and gonadal development and therefore prediction of OECD physiological endpoints. This bioassay battery completes a previously developed assay using *ThbZIP-gfp* tadpoles to detect disruption of thyroid axis. We are therefore capable of assessing the endocrine disruption of three major hormonal pathways – thyroid, estrogen and androgen, in addition to the possibility to detect the presence of sex reversal in the gonads.

Trends in bioassays nowadays ask for higher throughput, lowering the costs and decrease of the time. Already the creation of transgenic aquatic *in vivo* bioassays greatly improves the time needed to carry out bioassays, for example as demonstrated in publication n°4 in this thesis, using

the *spiggin-gfp* medaka allows us to detect (anti-)androgens in just 72-96 hours. It is a great time saving improvement compared to AFSS assay (described in the introduction of this thesis) in which non-transgenic stickleback females are exposed to (anti-)androgens and raised to maturity before the quantification of spiggin protein by ELISA, especially as the sensitivity of both assays is comparable (Katsiadaki et al., 2006; Sébillot et al., 2014).

Improvement in the time required to carry out an *in vivo* bioassay was as well demonstrated in publication n°1 of this thesis. The *ChgH-gfp* transgenic medaka used in this publication allow the detection of (anti-)estrogenic compounds and aromatase inhibitors in 24-48h, which is significantly less than any non-transgenic *in vivo* bioassay described previously in the introduction (Spirhanzlova et al., 2015). The exposure time of *ChgH-gfp* transgenic medaka assay is also shorter than the exposure time in similar assays using transgenic fish embryos, for example *cyp19a1b-gfp* zebrafish (5d), *ere-zvtg1: gfp* zebrafish (13d), *ChgL-gfp* medaka (6d) or *5xERE:GFP* zebrafish (3d) (Brion et al., 2012; Chen et al., 2010; Gorelick and Halpern, 2011; Salam et al., 2008). Moreover in both of our newly developed assays (*spiggin-gfp* and *chgh-gfp*) exposure times do not exceed the time when the larvae takes nutrition from their own yolk sacks and therefore they are not considered as laboratory animals under European legislation, which is a great advantage.

An important point is the sensitivity of the assays. As mentioned above, the sensitivity of the *spiggin-gfp* assay is comparable to the one of original AFSS assay and to this date, no similar transgenic model using aquatic larvae to detect (anti-)androgens has been developed. In the case of *ChgH-gfp* assay, among transgenic models responding to estrogens there are less sensitive models (*5xERE:GFP* - 0.5µg/L), those with a comparable detection limit (*ChgL-gfp* - 25ng/L) and more sensitive models (*ere-zvtg1* - 0.1ng/L or *cyp19a1b-gfp* - 2.9ng/L). A lower detection limit is surely a great advantage, because it allows the detection of environmentally relevant concentrations, however the question remains if such low concentrations have the potential to cause harmful effects in wildlife, for example the development of intersex.

One way to ensure that the screening methods used cover several aspects of possible endocrine disruption would be the combination of quantifiable markers for endocrine activity and

physiological markers capable of indicating adverse effects, as we demonstrate in the case of *ChgH-gfp_42sp50-gfp* transgenic medaka model described in manuscript n°3. After the assay is fully established, this model will allow sensitive screening of (anti-)estrogenic molecules and aromatase inhibitors and at the same time detect any potential sex reversal in the gonads. Combination of several models in one as we demonstrated here also follows the general need of time and cost saving. Moreover at the moment a new *ChgH-gfp_42sp50-gfp* generation with crystalline-RFP linked to the Y chromosome is being developed, which will greatly reduce the time of the assay allowing us to skip the genotyping step as genetic XY males will be easily identified by RFP signal in the eyes. The development of the protocol using *ChgH-gfp_42sp50-gfp* to detect sex reversal is currently at its starting point. In manuscript n°3 we determined the sensitive exposure window for sex reversal using 24 hours lasting pulses of high doses of E2 and DHT, however several other points will have to be determined, before we will be able to include this assay to the bioassay battery in our laboratory. In the future we will investigate the low detection limit of this model both for the *ChgH-gfp* and *42sp50-gfp* transgenes. A protocol for the study of anti-androgens will also have to be improved for this model. An interesting study could be carried out investigating whether the sex reversal caused by short administration of androgens and estrogens can remain until the maturity and affect the fertility of adults. Another step would be to adapt the *ChgH-gfp_42sp50-gfp* assay for the testing of environmental water samples.

Another possibility to decrease the time of assays and in certain cases increase their sensitivity limit is the creation of homozygous lines, which eliminates the step of sorting of transgenic and non-transgenic embryos. A stable homozygous line of *ChgH-gfp* is already in use and the creation of homozygous *spiggin-gfp* has recently been completed.

The fluorescence readout and quantification of the transgenic larvae expressing GFP at the end of the exposure also takes a great amount of time. For *in vitro* studies, automatic platforms and fluorescence plate readers are widely used, but the quantification of fluorescence in transgenic larvae during *in vivo* studies is mainly carried out by imagery followed by fluorescence quantification using programs like ImageJ. Those laboratories which use automatic platforms to read *in vivo* experiments often still need to anaesthetize and manually position the transgenic larvae prior to image acquisition. This step is not only time consuming and labour intensive, but

the anesthesia may also interfere with the physiology of the larvae in the case of experiments with repeated measurements. A simple solution to this issue is demonstrated in manuscript n°2 of this thesis, where we describe a protocol of fluorescence detection in free swimming unanaesthetized larvae. So far we established protocols to measure fluorescence of *ChgH-gfp* medaka fry and *ThbZIP-gfp Xenopus laevis* tadpoles which result in comparable detection limit to fluorescence quantification by imagery and greatly facilitate the procedure by reducing its complicity and operating time. In addition, in the future we plan to develop a similar protocol for the *spiggin-gfp* model. The newly developed protocols can be easily used during inter-laboratory screenings, because they are simple and every laboratory can easily perform the procedure without any special training once they acquire the fluorescence plate reader. Moreover they are also economically advantageous, as fluorescence plate readers are cheaper than fluorescence microscope. The imagery method of capturing images of the larvae has nevertheless its advantages. An actual image can be useful in further observations of the activity of a compound connected for example with pigmentation, size or developmental aberrations of the larvae, which is not the case of the results obtained from fluorescence plate reader.

The applicability of the newly developed battery, with the exception of the *ChgH-gfp_42sp50-gfp* assay, was demonstrated in manuscript n°5 in addition to *in silico* docking prediction.

The long term goal in our laboratory is standardization of the protocols of our models so they can be used within the REACH framework to assess endocrine disrupting potential of individual chemicals before they are allowed on the market.

4. The use of the newly developed models in EDA – environmental water sample testing

This thesis was part of the EDA-EMERGE program financed by Marie Curie ITN. The EDA-EMERGE project included private and public laboratories from eight European countries and its overall goal was to “train a new generation of young scientists in the interdisciplinary techniques required to meet the major challenges in the monitoring, assessment, and management of toxic pollution in European river basins” (Brack et al., 2013). Effect directed analysis (EDA) is a new approach to identification and monitoring of endocrine disruptors and other emerging pollutants

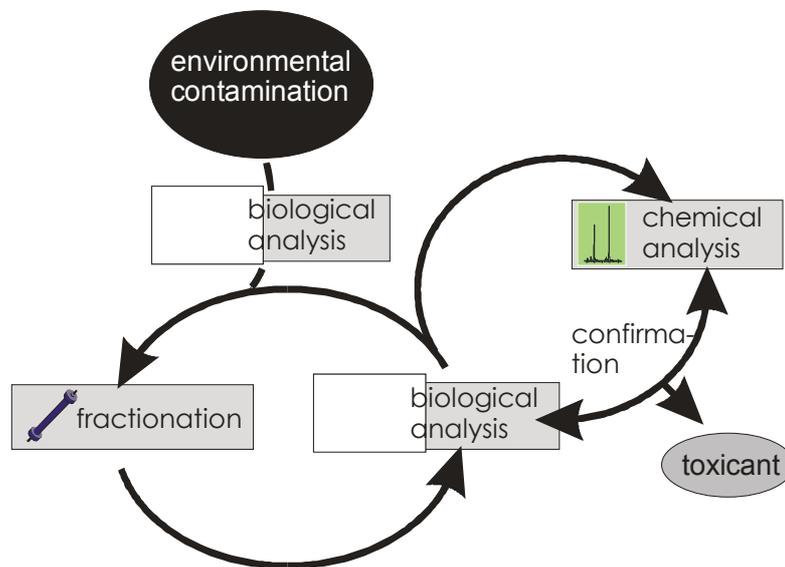
by reducing the complexity of the mixtures by combining fractionations, bioassays and chemical analysis (Fig. 17).

The work on this thesis regarding the EDA-EMERGE program included the development of new bioassays to detect endocrine disruptors (publication n°1 and n°4 of this thesis) and demonstrating their applicability by using them to test whole water samples collected by EDA-EMERGE partners on different sampling regions in Germany, Croatia, Czech republic and Switzerland in a subprogram called the European Demonstration Program (EDP).

While testing environmental whole water samples or their fractions, the volume of the sample available for *in vivo* bioassays is often limited and it is desirable to decrease the volume needed for the exposure study. There are several ways we try to achieve this goal in our laboratory. For example in the case of *ChgH-gfp* assay, we developed a protocol which requires reduced number of test fry per exposure group (eight instead of twenty) and decreasing the exposure time from 48 hours to 24 hours. This doesn't only contribute to the overall need to reduce the number of test animals but also reduces 8× the volume needed to carry out the bioassay.

To better interpret the results of the *ChgH-gfp* assay while testing environmental water samples with unknown chemical composition present, we developed a protocol using a range of known concentrations of EE2 followed by curve fitting of a sample which exhibited an activity in our assay, which allows us to determine the EE2 equivalent of a sample. In the future we would like to implement a similar strategy of curve fitting for the *spiggin-gfp* medaka and *ThbZIP-gfp Xenopus laevis* assay. We have also increased the sensitivity of the assay by working with dry extract samples sent to us by EDA-EMERGE partners which can be diluted to higher concentrations than the initial environment concentration, therefore increasing sensitivity.

Dozens of whole water environmental samples were tested using the bioassay battery available in our laboratory demonstrating their applicability in EDA. Two endocrine active hotspots were identified amongst the different sampling site within the EDP project, one in Germany with pro-thyroidogenic and anti-androgenic activity and one in Croatia exhibiting estrogenic activity. The results of the EDP obtained in our laboratory are preliminary and they are included in the annex of this thesis together with the publication n°6, which is an EDA-EMERGE introductory paper. The manuscript including the EDP results of all the partners is currently in preparation.



*Figure 17. **Effect-directed analysis.** To simplify the process of identification and monitoring of endocrine disruptors and other emerging pollutants a new approach was developed called Effect directed analysis. This method combines bioassays (in vivo and in vitro), fractionation and chemical analysis to reduce the complexity of the mixtures. First a whole water sample is tested on a set of bioassays, if it is active, the sample is fractionated and each fraction is tested again in bioassays to determine, which fraction is responsible for the endocrine activity. Chosen fractions undergo chemical analysis. The identified toxicant is tested again on bioassays to confirm its endocrine activity (Brack et al., 2013).*

RESULTS
AND
PERSPECTIVES

Results and perspectives

The experiments included in this thesis were carried out during three years in the laboratory of WatchFrog situated in Evry under the supervision of Dr. Andrew Tindall. The thesis was financed by Marie Curie ITN program EDA-EMERGE. The main outcomes of this thesis are:

- 1) The development and characterization of the *ChgH-gfp* transgenic medaka model along with a protocol for its application to detect (anti-)estrogens and aromatase inhibitors.
- 2) The conception and optimization of a functional protocol to facilitate readout of fluorescence of unanaesthetized *ChgH-gfp* transgenic medaka and *ThbZIP-gfp* transgenic *Xenopus laevis* using a fluorescence plate reader.
- 3) The development of a double transgenic medaka model *ChgH-gfp_42sp50-gfp* with GFP expressed in the developing ovaries. This model links sex reversal to estrogen axis activity that is quantified using the ChgH-GFP biomarker.
- 4) The conception, production and characterization of *spiggin-gfp* transgenic medaka model and optimization of a protocol to detect (anti-)androgens.
- 5) Demonstration of the applicability of the newly developed *in vivo* bioassay battery by assessing the potential endocrine disrupting potential of the herbicide linuron and insecticide fenoxycarb.
- 6) Demonstration of the applicability of the newly developed *in vivo* bioassay battery to detect endocrine disruption in environmental whole water samples within the EDA-EMERGE program.

Regarding the *ChgH-gfp* model, our next steps will be to improve the current protocol to reduce fry numbers per exposure group. The assessment of endocrine disruption in environmental water samples could also be further optimized by using dried whole water samples, as already mentioned in the discussion of this thesis. We are also currently trying to lower the detection limit by selective breeding of animals displaying high estrogen-GFP responses.

To improve the *spiggin-gfp* protocol we have recently established a homozygous line. This will result in significant assay preparation time reduction, as it eliminates the need to sorting transgenic and non-transgenic larvae. It is hoped that use of the homozygous line increases sensitivity, but this remains to be verified. The protocol for fluorescence quantification by plate

reading in unanaesthetized *spiggin-gfp* could also be further optimized and the sensitivity of the model to estrogen inhibition will also be investigated.

A *ChgH-gfp_42sp50-gfp* medaka line harboring *crystalline-rfp* linked to the Y chromosome is currently under development. This will accelerate the process of sex reversal detection, by omitting the need for genotyping. More work is still required to fully characterize the *ChgH-gfp_42sp50-gfp* transgenic medaka, notably by increasing the sensitivity of *ChgH-gfp* and investigating in greater depth the detection limit for sex reversal using lower doses of estrogens and androgens. Also the protocol still has to be optimized for the use in environmental water sample testing and eventually a homozygous line will be developed.

We believe, that this thesis will greatly contribute to the overall knowledge about endocrine disruptors by introducing a toolbox of three new medaka based transgenic models suitable for detecting the endocrine disrupting potential of chemicals and environmental water samples.

Acknowledgements

What a journey it has been!

During my undergrad studies I never thought that I might receive a PhD, even less doing so in foreign country. Yet here I am, holding a freshly printed copy of my thesis, which is written in English, 1000 km from my hometown. The transition from a student, who had problems loading an electrophoresis gel without shaking hands (I am sorry Tereza) to a (relatively) confident (almost) doctor wouldn't have been possible without a whole regiment of people. Now is the time to say, that when I read examples of theses written by French students, I was surprised by the excessive acknowledgments they wrote. My thoughts were "Are they thanking absolutely everybody in their lives, possibly with personal message included? There is no way, I will do that". But now I am sitting here, with a list of people I can't forget beginning with my elementary school biology teacher and ending with the nice lady from the canteen called Therese. After some thinking, I reduced the list to people who were crucial to the success of my thesis, but of course, if you are reading this and you can't find yourself here, give me a call, and I will acknowledge you personally.

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ANNEXE I

DISCUSSION

Open Access

EDA-EMERGE: an FP7 initial training network to equip the next generation of young scientists with the skills to address the complexity of environmental contamination with emerging pollutants

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Abstract

The initial training network consortium novel tools in effect-directed analysis to support the identification and monitoring of emerging toxicants on a European scale (EDA-EMERGE) was formed in response to the seventh EU framework program call to train a new generation of young scientists (13 PhD fellows and 1 postdoctoral fellow) in the interdisciplinary techniques required to meet the major challenges in the monitoring, assessment, and management of toxic pollution in European river basins. This 4-year project is of particular relevance considering the multidisciplinary analytical chemistry and biology skills required to investigate the enormous complexity of contamination, effects, and cause-effect relationships. By integrating innovative mode-of-action-based biodiagnostic tools including *in vitro* and *in vivo* tests, transgenic organisms, and 'omics' techniques with powerful fractionation and cutting edge, analytical, and computational structure elucidation tools, powerful new EDA approaches are being developed for the identification of toxicants in European surface and drinking waters. Innovative method development by young researchers at major European universities, research centers, and private companies has been closely interlinked with a joint European demonstration program, higher-tier EDA, and specialized training courses and secondments. Using a simplified protocol based on existing EDA tools, EDA-EMERGE fellows are also being trained to organize and run international and interdisciplinary sampling and monitoring campaigns within selected European river basin sites. Strong networking between academia, the private sector, and leading regulators in the field of river basin management and pollution management ensures the relevance of the research for practice and excellent employment opportunities for the fellows. Additionally, an internationally composed advisory board has been tasked to introduce new perspectives on monitoring, assessment, and management of emerging pollutants within and outside of Europe. The combination of cutting edge science with specialized training in

(Continued on next page)

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complimentary soft skills is being offered with a strong emphasis on commercial exploitation and media competence which further enhances the employability of the fellows in research, academia, and beyond.

Keywords: Effect directed analysis; Emerging chemical pollutants; EU river basins; Bio-assays; Analytical chemistry; EDA-EMERGE

Background

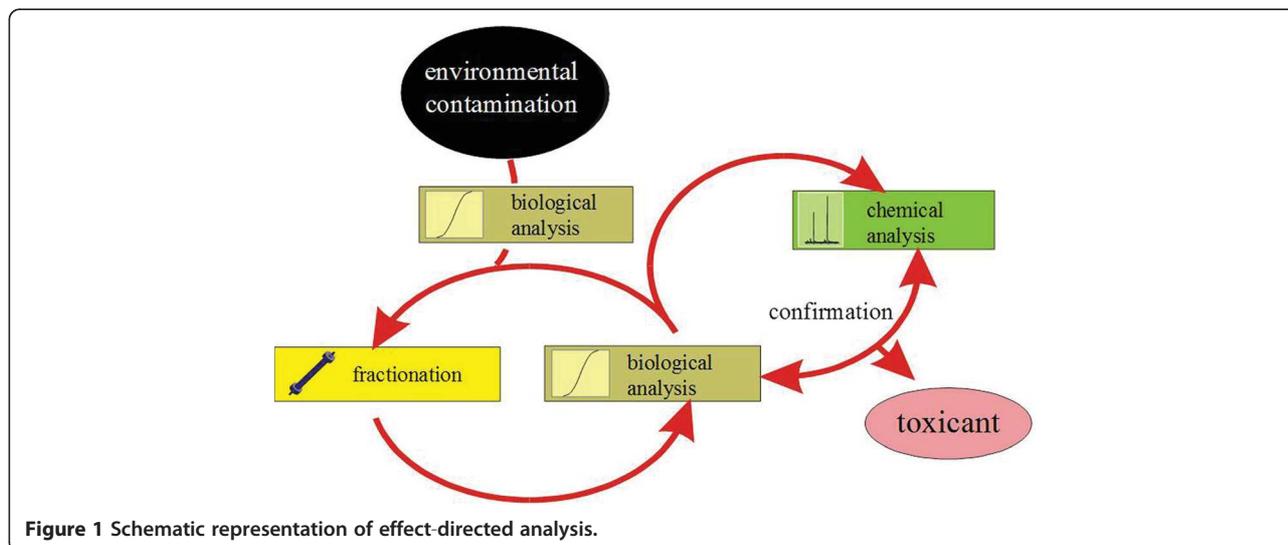
Water quality in many European rivers improved significantly over the last 30 years, and river waters exhibiting acute lethality to fish or invertebrates are an exception today. However, toxic pressure by pesticides [1], biocides [2], endocrine disruptors [3,4] and many other chemicals [5] still presents a large-scale risk and causes community shifts and the disappearance of sensitive species in many river basins. The European Water Framework Directive (WFD) [6] provides a new holistic approach for the assessment and management of the ecological status of water bodies. However, the WFD chemical monitoring and classification is still centered on the traditional approach of regulating individual chemicals, the priority substances (PS) and the river basin specific pollutants, via compliance with environmental quality standards. This approach, based on chemical analysis of single substances, cannot cover the enormous variety of potentially hazardous chemicals present in the environment, which occur in complex and time variable mixtures. Subsequently, the observation of adverse effects in biota is often not reflected by the occurrence and concentrations of PS but caused by other non-regulated contaminants of emerging concern. In a discussion paper by Christian Daughton, US Environmental Protection Agency (USEPA) stated that 'an ever-expanding universe of unique chemicals, untold numbers of which have yet to be recognized or revealed, continually perfuse our environment and contact living systems' [7]. In 2005, 26 million individual chemical substances were identified and registered in the Chemical Abstract Service (<http://www.cas.org/>), with one-third being commercially available [7]. By June 2013, this number had increased to 72 million. The continuous increase in the diversity of chemicals being used makes identifying those that occur in our environment an increasingly difficult task. The screening of environmental samples, such as extracts of water, sediments, or biota, with liquid or gas chromatography coupled with high-resolution mass spectrometry (LC-HRMS) typically reveals tens of thousands of peaks, the majority of which are unknown.

Nationally and across the member states, there is an increasing awareness that the analysis of PS alone is insufficient to characterize the toxic pressure to aquatic ecosystems and water resources [8,9]. In response, powerful new screening methods are being developed.

These include multi-target analyte approaches together with suspect screening, e.g., the use of transformation prediction models to propose transformation products without available standards and the screening of unknowns including all detected masses or peaks [10]. However, the identification of unknowns, particularly with LC-MS techniques, is very challenging and time-consuming and therefore unlikely to be available as a routine technology for all chemicals in the complex mixtures that are found in the environment. Approaches are required to focus analytical efforts on those chemicals that actually pose a risk or cause an effect. Two of the most prominent methodologies to approach this challenge, effect-directed analysis (Figure 1) and toxicity identification evaluation, apply biological tools to select fractions of a mixture and individual components for identification and structure elucidation efforts according to measurable effects [11-15].

Significant methodological development in EDA has been achieved within the last years as reflected by a recent book on this topic [16]. However, significant bottlenecks remain which are now being addressed by the Marie Curie initial training network (ITN) project, EDA-EMERGE. Research within EDA-EMERGE is directed towards developing powerful new bio-analytical detection tools to significantly improve the analytical power of LC-HRMS-based structure elucidation by interlinking with novel, powerful computer tools, to enhance EDA throughput, and to reduce sample requirements and the risk of artifacts by means of miniaturization and automated online combination of fractionation, biotesting, and chemical analysis. The most important aspect of EDA-EMERGE, however, is its people. This ITN has been designed to produce a new generation of experts that are trained during their PhD studies in biological and chemical tools and the attendant integration in order to unravel and assess complex contamination of the aquatic environment for the sustainable management of European water resources in close collaboration with key players in WFD implementation such as the European Commission Joint Research Centre (JRC), the European Environmental Agency, and the German Federal Environmental Agency.

The EDA-EMERGE consortium includes two universities (Free University of Amsterdam and the RWTH Aachen University), six research centers (Helmholtz



Centre for Environmental Research (UFZ), Institut National de l'Environnement Industriel et des Risques (INERIS), Swiss Federal Institute of Aquatic Science and Technology (Eawag), Rudjer Boskovic Institute, Norwegian Institute for Water research, the Italian Institute of Health, the JRC), and five private companies (Environmental Institute, KWR Watercycle Research Institute, WatchFrog, HighChem, and gaia) (Figure 2). EDA-EMERGE is an outcome of the European Network of Reference Laboratories for Monitoring of Emerging Environmental Pollutants (NORMAN) and strongly benefits from this platform, bringing together 55 partners from science, monitoring, and regulation in 19 countries [17]. Since complex contamination is not exclusively a European problem, EDA-EMERGE is also networking with renowned overseas partners such as advisors from the US-EPA and Environment Canada.

Novel scientific tools

On the basis of the outstanding expertise of their host institutes in EDA, the 13 PhD fellows are developing novel methods for advanced EDA in three work packages, *viz.* bio-analytical tools, chemical tools, and hyphenated tools. A specific focus of bio-analytical tool development will be on vertebrate *in vivo* and *in vitro* models for genotoxicity, mutagenicity [18] and AhR-mediated toxicity, and for endocrine disruption [19]. This will include innovative transgenic models using multi-well-based fluorescent screens for androgenic and corticosteroid disruptors on the basis of extensive experience with tadpole fluorescent screens for thyroid hormone disruptors (Figure 3) and fluorescent zebrafish transgenic embryos for estrogens [20].

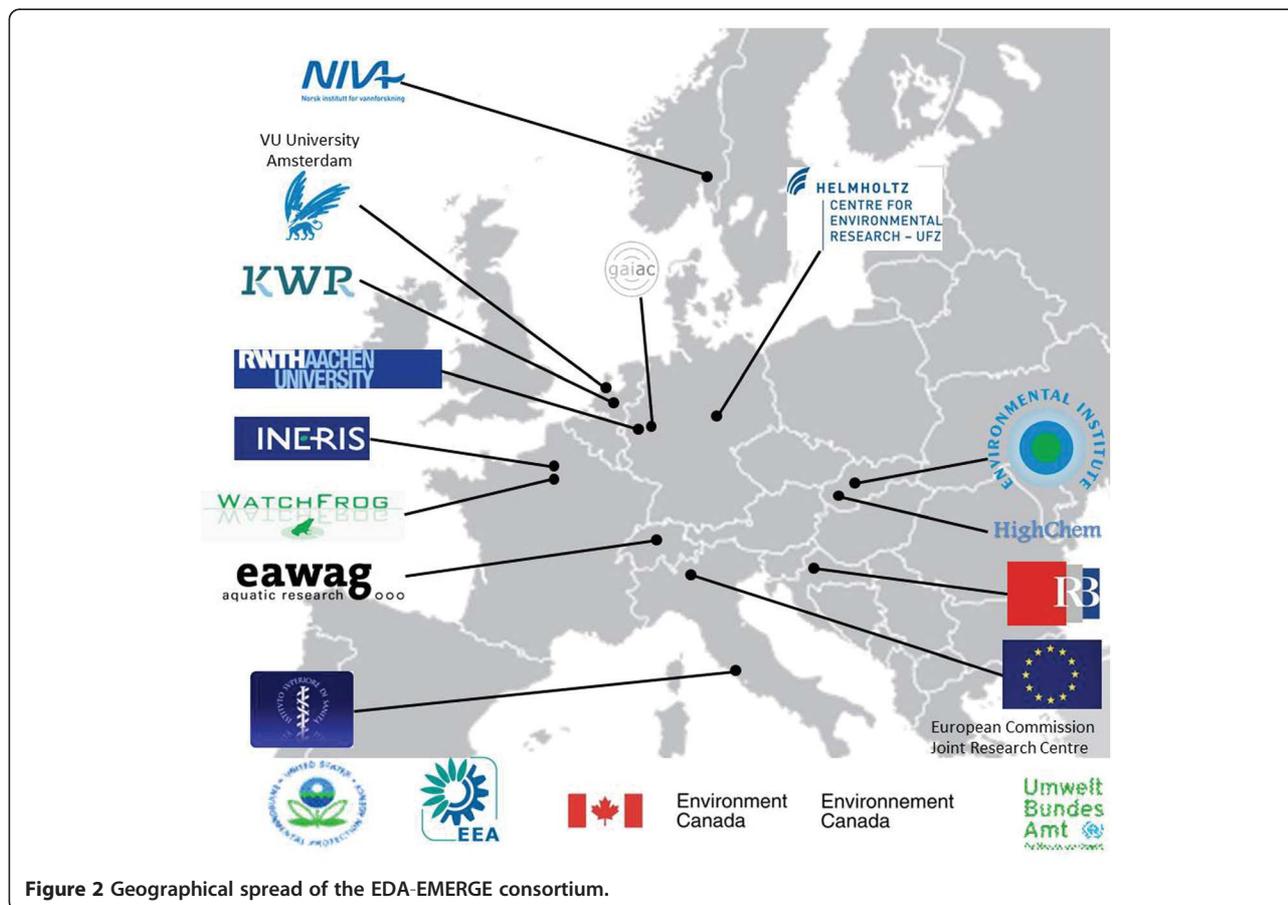
Existing EDA approaches are limited by the lack of knowledge and availability of toxicological endpoints to

direct toxicity isolation and identification. This limitation is addressed by EDA-EMERGE with the investigation of toxicogenomic tools to detect and unravel toxicants with unknown receptors and modes of action. The new approaches include DNA microarray fingerprints in algae [21,22], a proteomic approach in zebrafish embryos previously established in adult fish tissue [23] to direct EDA, and a metabolomics EDA approach using invertebrate and fish cell lines *in vitro*.

Together with the detection of adverse effects, the identification and structure elucidation of components of toxic fractions are a bottleneck in EDA, particularly of polar compounds requiring LC-MS analysis. Tool development focuses particularly on LC-HRMS methods that have successfully been applied by EDA-EMERGE partners for the identification and quantification of glucocorticoid compounds [24], drugs [25], and other polar micropollutants [26]. EDA-EMERGE combines the analytical power of HRMS with computer tools to predict fragmentation, chromatographic retention, and stability [27,28]. Mass spectra of newly identified compounds will feed the community-driven, open-access, accurate mass spectral database MassBank [29] maintained by NORMAN. Significant enhancement of the throughput of EDA towards more routine application will be achieved by automated approaches to directly link fractionation, biotesting and identification [30].

European demonstration program

Applying a simplified EDA protocol featuring a novel on-site large-volume solid phase extraction apparatus with limited fractionation, a selected set of bioassays and chemical screening analysis will be used to characterize about 20 sites within European river basins. This approach combines sites of interest according to sources of

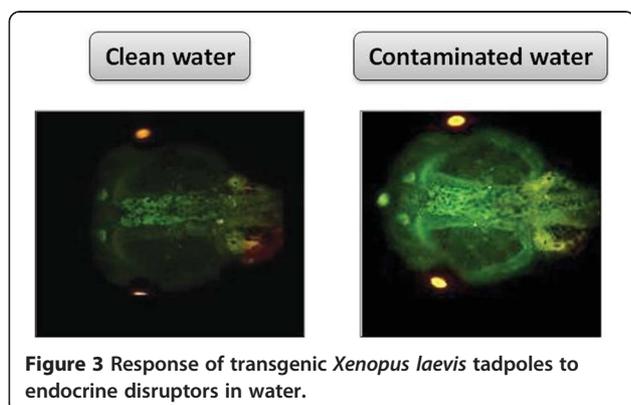


contamination such as municipal wastewater, treated hospital effluents, industrial sites affecting water quality, untreated wastewater entering the river, and reference sites with European sampling stations downstream of impacted headwaters. The European Demonstration Program will allow a comparison of classical monitoring approaches with the new tools and will train the PhD fellows in organizing and running common sampling campaigns on a European scale.

Training program

In line with the major objective of equipping young interdisciplinary researchers with the skills to address the challenges of complex contamination and its effects on aquatic ecosystems and to support WFD implementation, an extensive training program was set up and was enthusiastically followed by the PhD fellows. Although having a primary focus on EDA-EMERGE PhD students, the courses are open to external participants within the attendant maximum capacity, and this opportunity has been intensively utilized by PhD fellows from across Europe.

The formal training program kicked off with a week-long summer school (25 to 29 June 2012). The PhD fellows were lectured by leading scientists within the EDA-EMERGE consortium and beyond on the European regulatory framework, EDA, sampling and sample preparation, separation and fractionation, biodiagnostic tools, and chemical analysis. Additionally, there was also a PC-based workshop on structure elucidation along with virtual case studies. The scientific program was supplemented with a half day excursion to Wechselburg in Saxony where fellows and lecturers learnt about the river Mulde, a previously heavily



contaminated river in a now scenic, tourist-friendly setting, which is slowly recovering (Figure 4). This also provided an informal and relaxed atmosphere for intensive and fruitful networking that significantly promoted EDA-EMERGE progress.

The summer school was followed by several specialized training courses including a course on advanced LC-MS and multivariate statistics at the UFZ, Leipzig, Germany, a course on *in vitro* and *in vivo* assays of endocrine disrupting chemicals using fish models at INERIS, Verneuil-en-Halatte, France, and a course focusing on chemical screening, prioritization of environmental pollutants and data storage in European databases in the Jasna Valley, Slovakia. In total, ten specialized courses complemented by local scientific and soft skill training courses provide a comprehensive supplement to the scientific work in a traditional PhD thesis. An additional component of the training program involves 1- to 3-month secondments (at least one secondment per fellow) hosted by EDA-EMERGE partner institutes to work on aspects of the PhD research where the host institute possesses specialized expertise in analytical techniques or data analysis not typically associated with the home institute. The advantages of the secondment initiative is that, in addition to the specialized skills and expert training acquired, the fellows also broaden their professional network and obtain a different research or working perspective.

Discussion

Within the context of EDA-EMERGE research goals, a number of scientific work packages are being implemented such as developing novel bio-analytical tools, novel chemical tools, hyphenated tools, a higher-tier EDA study, establishing a simplified protocol, and conducting,

monitoring, and sampling within the European Demonstration Programme (EDP). Bio-analytical methods being developed include mechanism-specific screening tools in early life stages of zebrafish, novel fish-based biological tools, and fluorescing vertebrate models for EDA of endocrine disrupting chemicals. DNA microarray fingerprints, a proteomics and a metabolomics approach to direct EDA, are also in progress, linking multiple contaminations with multiple toxicological end points. A particular emphasis is given to toxicogenomic tools including proteomics and metabolomics to achieve unbiased effect detection. These innovative biological tools are supplemented with sophisticated chemical tools for fractionation and structure elucidation. Extensive separation and characterization of contaminants are achieved with novel fractionation tools. Comprehensive multidimensional GC/MS is used for both identification and quantitative screening. Since the identification of polar toxicants is still one of the big bottlenecks of EDA; a specific focus is given to structure elucidation techniques using LC-MS/MS. This will be achieved by developing integrated analytical and computer tools for structure elucidation of polar compounds, including the development of new approaches for retention and fragmentation prediction, diagnostic derivatization techniques, as well as metabolite prediction and identification in treatment processes in the water cycle. Innovative automated high-throughput EDA approaches are in development that directly link fractionation, biotesting, and identification. This includes two-dimensional microfractionation approaches prior to effect detection and chemical analysis.

Beyond these method developments within the individual PhD projects, there are also integrated activities in the scientific work packages focused on a simplified protocol that will be applied in the EDP. This will provide the PhD fellows with experience in planning and implementing large, collaborative, EU-wide sampling campaigns. Standard operational procedures are in development for all steps of the simplified protocol including an integrated large-volume fractionated *in situ* solid phase extraction, a large set of bioassays, and chemical screening analysis for about 300 potentially relevant compounds. The EDP is being performed by PhD pairs (biology and analytical chemistry) from different partner institutions with complementary expertise. Each pair selected a river basin as well as relevant sampling sites representing specific pollution situations and sources and typically including a reference site as well as one integrative sampling site related to monitoring (e.g., the Watch List Study sampling sites).

The fellows have been trained in all relevant techniques such as chemical structure elucidation, chemometrics, sampling/extraction, biotesting, and chemical analysis. The training courses and personalized career development



Figure 4 EDA-EMERGE fellows and lecturers. They were in front of the viaduct in Göhren, Germany bridging the valley of the Zwickauer Mulde River. Completed in 1871 and still in operation, it is one of the most impressive bridges in Germany demonstrating early railway architecture.

plans were designed after a baseline study was performed using an initial training needs analysis survey sent to all EDA-EMERGE fellows at the outset of the project. These specialized technical and scientific training courses together with secondments at partner institutes were supplemented by soft-skill courses such as presentation skills and a film/media course in conjunction with standard local courses such as scientific writing, language skills, and time management offered at most host institutes. According to a basic EDA-EMERGE dissemination plan, ESR fellows have been encouraged from the outset to disseminate their research output via online open-source forums, conferences, and journal publications.

Conclusions

In response to the call to train a new generation of young scientists in the interdisciplinary skills related to EDA and the management of European water sources, EDA-EMERGE provides a highly systematic and comprehensive research and training program within a consortium that brings together key competences from universities, research centers, private companies, and regulatory authorities. A balanced mix of 14 fellows from biology and analytical chemistry backgrounds were linked by research work packages focused on novel analytical tool development. Applications in a simplified protocol and an EDP provided an ideal basis for specialized training and structured public dissemination of the results. Networking is achieved via a range of activities from opening training course participation to external fellows and external experts, a summer school, conference presentations, dissemination and outreach activities as Marie Curie ambassadors, and interaction with other networks to secondments with EDA-EMERGE partners and associated partners.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

WB was responsible for the general design of the project and wrote the first draft of the manuscript. The other authors - SG, TS, MK, MH, MM, JH, KS, JS, AH, JS, ZR, SAA, MS, MC, ML, PL, ST, XO, MS, KT, ACdA, JF, MHW, MA, SK, HH, TBS, CDP, AT, and PS - contributed with specific information concerning their respective methods. All authors helped revise the draft of the manuscript. All authors read and approved the final manuscript.

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ANNEXE II

European demonstration program

Within the European demonstration program previously described in Annex I. (Brack et al., 2013) we assessed the potential endocrine activity of environmental whole water samples provided by EDA-EMERGE partners. The samples were acquired in ~20 different sampling sites in Europe (Croatia, Switzerland, Czech Republic and Germany). The sampling sites were chosen because of their proximity to municipal wastewater treatment plants, hospitals and industrial sites. To assess the (anti-)thyroidogenic potential of the samples, ThbZIP-gfp transgenic *Xenopus laevis* tadpoles were exposed to non-diluted whole water samples with or without the co-exposure to 5nM T3. Out of all the samples, only one sample exhibited pro-thyroidogenic activity (Fig. 18, 19A). Three samples from Germany were also tested using the Spiggin-gfp transgenic medaka fry to detect disruption of androgen axis with and without the 5µg/L 17MT co-treatment. The same sample which exhibited pro-thyroidogenic activity in the ThbZIP-gfp assay also exhibited slight anti-androgenic activity (Fig. 19B).

ChgH-gfp transgenic medaka fry were used to assess the potential of samples from Croatia to disrupt estrogen signaling (Fig. 20). In this case we received dried whole water samples which were resuspended in DMSO prior to dilution in Evian water. This method allows the samples to be tested in more concentrated forms than the original water samples, allowing the detection of EDCs existing below the assay detection limit in the original sample. A standard curve of EE2 was included to allow determination of the EE2 equivalence of the samples. One sample exhibited estrogen activity equivalent to 21.6 ng/L EE2 (Fig. 21A). Three samples including the blank exhibited anti-estrogenic activity in the presence of testosterone and one sample significantly increased the fluorescence induced by the testosterone co-treatment (Fig. 21B).

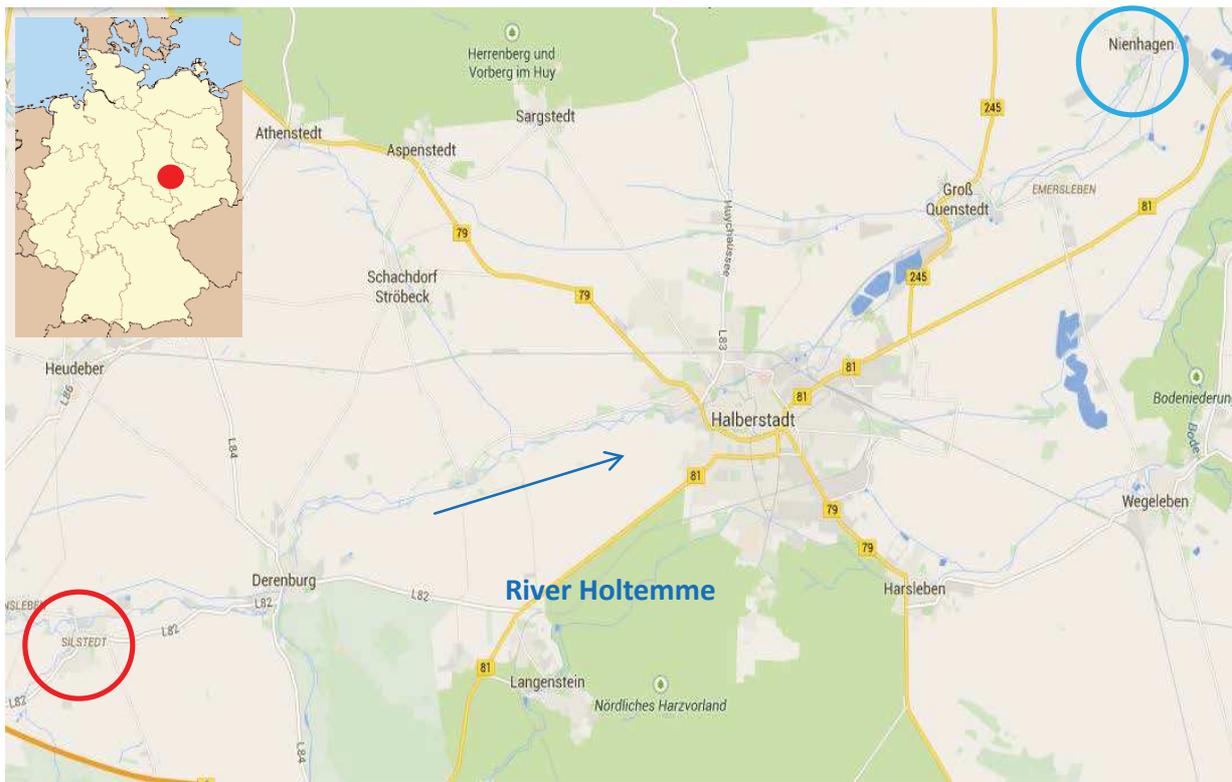


Figure 18. **European demonstration program –sampling sites in Germany.** *Three sampling sites at the river Holtemme around the city Halberstadt were selected. The first sampling site – Steinerne Renne is located near the source of the river Holtemme and is considered as a clean control site. This site is not visible in the figure due to its distance from the other sampling sites. The second site is located near Silstedt (red circle), in the middle of an agriculture area, possibly heavily polluted by pesticides and upstream from Halberstadt city. The third sampling site is located near Nienhagen (blue circle), downstream from Halberstadt. Whole water samples from these three sampling sites were tested for their possible thyroid and anti-androgenic activity.*

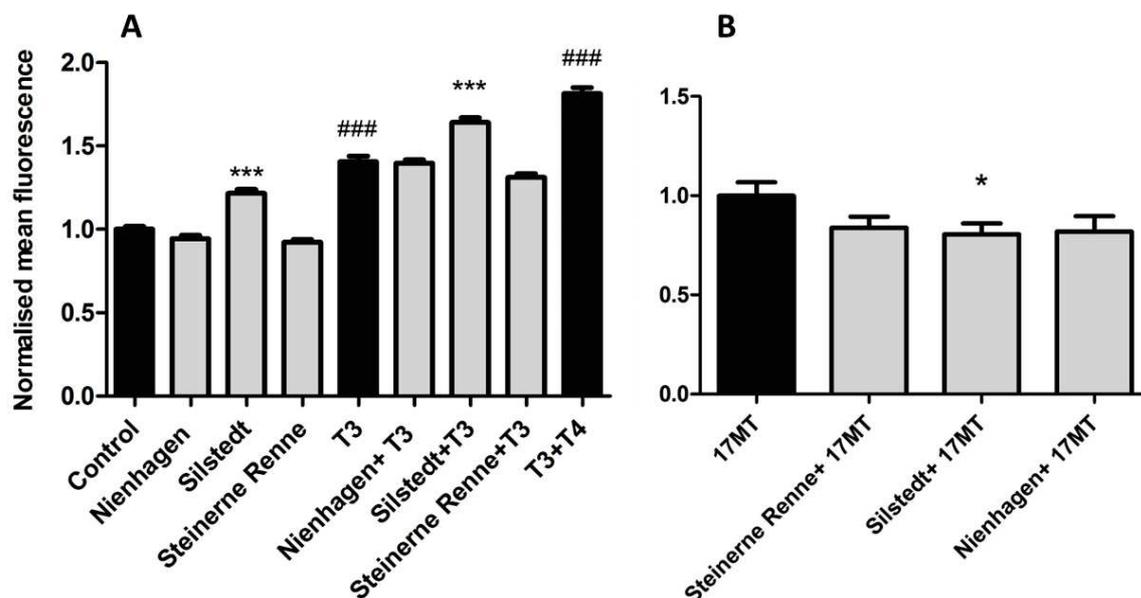


Figure 19. European demonstration program- results from *in vivo* bioassays using transgenic aquatic larvae. A. The whole water sample from Silstedt was active in the XETA *in vivo* bioassay using *ThbZIP-gfp* transgenic *Xenopus laevis* tadpoles to detect disruption of the thyroid axis with and without the co-treatment with 5nM T3. B. The whole water sample from Silstedt exhibited significant anti-androgenic activity in the spiggin-gfp *in vivo* assay in the presence of 5µg/L 17MT.



Figure 20. **European demonstration program –sampling sites in Croatia.** Six sampling sites at the river Sava around the city of Zagreb were selected. All selected sites were located in the proximity of water treatment plants or other possible sources of pollution. Dried whole water samples from these sampling sites were resolubilised in the minimum volume possible which resulted in non-lethal test solutions and then tested for their possible (anti-)estrogenic activity using transgenic ChgH-gfp medaka.

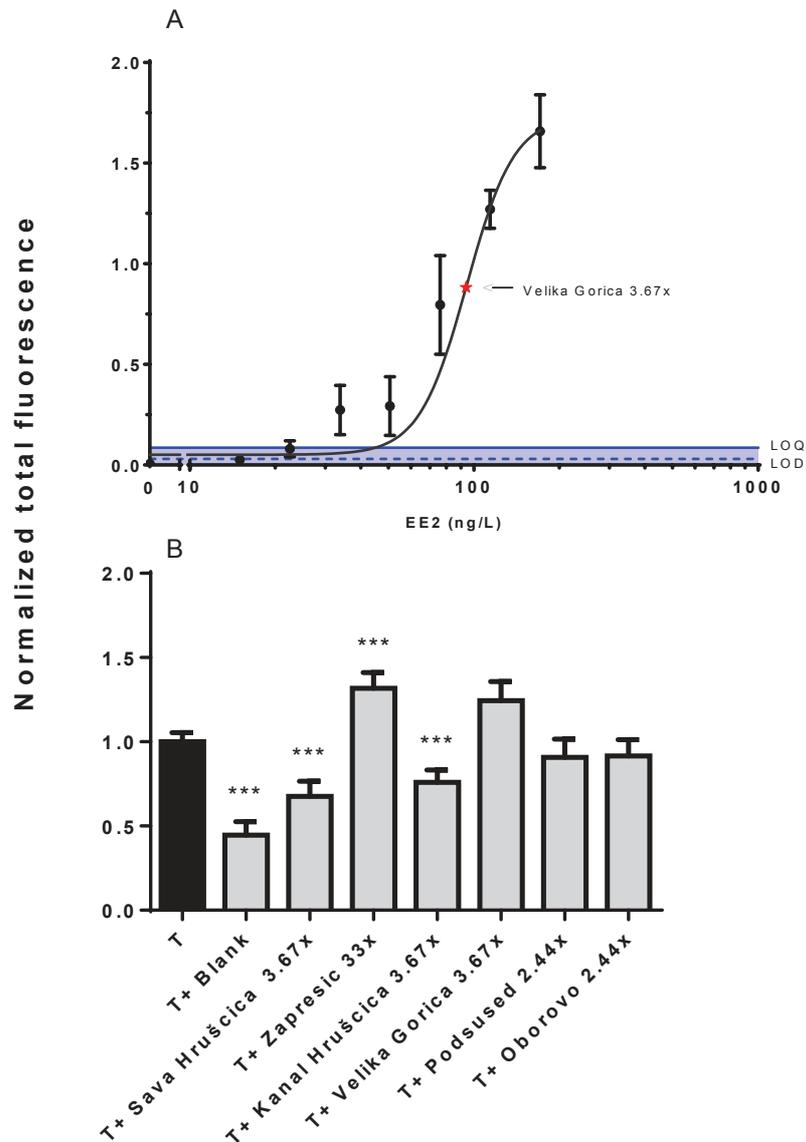


Figure 21. European demonstration program - results from *in vivo* bioassays using transgenic *Chgh-gfp* medaka. A. The whole water sample from Velika Gorica exhibited estrogenic activity of approximately 21.6 ng/L EE2 equivalents. B. The whole water samples from Sava, Kanal Hrušćica and the blank exhibited significant anti-estrogenic activity in the presence of testosterone. The sample from Zapresic significantly increased the fluorescence induced by testosterone. The Velika Gorica sample which previously exhibiting estrogenic activity in absence of testosterone was not significantly different to the control in the presence of testosterone.