Ex vivo and *in vivo* studies to assess chemical effects on steroidogenesis in fish: development and application of methods

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Table of contents

Table of contents

Table of contents

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Summary

The aquatic environment receives many chemical substances of natural or anthropogenic origin, which can influence the endocrine functions and health of wildlife. Various examples of endocrine disruption in wildlife were documented in aquatic organisms, for which associations between reproductive and developmental effects and exposure to endocrine-disrupting chemicals (EDCs) have been demonstrated. Since most of the endocrine-disrupting effects reported appear to be a consequence of feminization of males, most ecotoxicological research has been directed to identify estrogenic chemicals. However, the endocrine-disrupting effects exerted by EDCs can result from different mechanisms such as agonism or antagonism of endogenous steroid hormones via interaction with steroid hormone-receptors, or interference with the sex steroid hormone synthesis. Given the potential threat of these EDCs for wildlife, effective testing methods are required by regulatory agencies and industry to identify and assess the different mechanisms of action by which the EDCs exert their adverse effects. Testing strategies for endocrine disruption are being developed, in particular with fish test assays. These strategies are based on tiered approach, starting with fish *in vitro* and *in vivo* screening assays that identify and inform on potential endocrine mechanisms and effects. The results of the screening assays have then to be confirmed by higher tiered fish *in vivo* assays that characterize any apical adverse effect resulting from endocrine mode of action. Although the assays to screen for chemicals interacting with sex steroid receptor are widely available, tests to identify and inform on effects of chemicals that act via disrupting sex steroid biosynthesis still need to be developed. The aim of this thesis was therefore to develop and evaluate the potential of different fish test methods focused on chemicals that may interfere with the sex steroid biosynthesis.

In a first step, an *ex vivo* gonad assay from juvenile brown trout (*Salmo trutta fario*) was developed to specifically identify substances that disrupt the activity of enzymes involved in the sex steroid biosynthesis. The *ex vivo* gonad assay was applied to test model chemicals, known or suspected to inhibit sex steroid biosynthesis: 1,4,6-androstatriene-3,17-dione (ATD), an aromatase inhibitor pharmaceutical; prochloraz, an imidazole fungicide; tributyltin (TBT), an organotin compound and persistent organic pollutant. Their effects in the *ex vivo* gonad assay were assessed by measuring 17β-estradiol and testosterone

concentrations from the culture medium. The different profile of sex steroid concentrations obtained for each chemical exposure showed that the *ex vivo* gonad assay cannot only identify the chemicals disrupting the steroidogenesis, but has also the potential to inform on their specific mechanism of action.

To further evaluate the *ex vivo* gonad assay and its potential to inform on *in vivo* effects, the responses to prochloraz and TBT exposure were compared in the *ex vivo* and *in vivo* exposure assays of juvenile brown trout. The effects were again assessed by measuring 17β-estradiol and testosterone concentrations, and also by analyzing somatic indices and histopathology of gonads from fish exposed *in vivo* to the test chemicals. The results of this study demonstrated that the *ex vivo* gonad assay has the potential to inform on *in vivo* effects of chemicals disrupting the steroidogenesis and accordingly on their potential to affect sexual development of fish. This study highlights the potential of the *ex vivo* gonad assay to be a sensitive and informative tool for such EDCs.

The *ex vivo* gonad assay was then used to further analyze the potential of the steroidogenic inhibitors to impair the regulation of early sexual development of fish. This was investigated by comparing cellular and molecular effects of *ex vivo* and *in vivo* exposures to ATD, prochloraz and TBT. The *ex vivo* 17β-estradiol and testosterone concentrations were measured and *ex vivo/in vivo* gene expression of the aromatase and insulin-like growth factors (IGFs), involved in the regulation of sexual development, were compared. It was shown that the test chemicals could interfere with both the sex steroid and IGF systems and potentially lead to altered sexual development.

Finally, to confirm the potential of steroidogenic inhibitors to impair sex differentiation and development, a higher tier fish *in vivo* test, a Fish Sexual Development Test (FSDT), was applied. Two model fish species, zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*) were exposed, from embryo to sexual maturity, to prochloraz and the effects on their sexual differentiation were compared by assessing the sex ratios, the histology of gonads, and the vitellogenin concentration. The results of this last study demonstrated that, although the different strategies of sexual differentiation of zebrafish and fathead minnow influence the response of their gonad morphology and their sensitivity to prochloraz exposure, the exposure to steroidogenic inhibitors has the potential to alter their sexual development and subsequently the reproductive success and population structure of fish.

To conclude, we suggest that the evaluation of the *ex vivo* and in vivo methods in our different studies are sensitive and valuable tools for application in environmental risk assessment of chemicals interfering with the sex steroid biosynthesis. Although further characterization and validation studies of the *ex vivo* gonad and FSDT assays are still required, the combination of both *ex vivo* and *in vivo* assays represents a good testing approach.

1. Introduction

1.1. Endocrine disruption in the aquatic environment

The endocrine system of vertebrates regulates vital life processes, such as development, growth, metabolism and reproduction (Di Giulio and Hinton, 2008; Lintelmann et al., 2003; Pait and Nelson, 2002) and is very sensitive toward disturbing influences. Research over the last two decades has established at the global scale that certain environmental contaminants have the potential to interfere and modulate endocrine functions of humans and wildlife (Kavlock et al., 1996; Tyler et al., 1998). Compounds that interfere with the endocrine system are termed endocrine disrupting chemicals (EDCs) and include a diverse group of synthetic industrial and agricultural chemicals and even some naturally occurring compounds (Jobling et al., 2003; Pait and Nelson, 2002). EDCs have been defined as exogenous substances or mixtures that alter function(s) of the endocrine system and consequently cause adverse effects in an intact organism, or its progeny, or even subpopulations (Kavlock et al., 1996; Mills and Chichester, 2005; Vos et al., 2000). According to the growing evidence of the possible harmful effects of EDCs for both wildlife and humans (Colborn et al., 1993), research on endocrine disruption has become a major area of environmental research (Sumpter, 2005).

The aquatic environment is especially vulnerable to chemical pollution due to its tendency to receive and accumulate relatively high concentrations of chemicals, which enter via a variety of point and non-point sources, e.g. direct domestic and industrial discharges, soil and pavement runoff, or atmospheric deposition (Di Giulio and Hinton, 2008; Sumpter, 2002). The ability of EDCs to affect endocrine functions of aquatic organisms depends on a number of factors, including the endocrine-disrupting potency, the concentration and duration of exposure, the bioaccumulative potential, the life stage of the exposed organism, and/or the presence of other environmental stressors such as temperature, salinity, and other contaminants (Pait and Nelson, 2002). In the aquatic environment, fish are organisms susceptible to be exposed and harmed by EDCs. They can take up EDCs either by ingestion of contaminated food or by contact of their gills and skin with contaminated water (Di Giulio and Hinton, 2008).

Many field and laboratory studies support the hypothesis that EDCs can impair endocrine functions and reproductive health of various fish species. Biological effects in freshwater fish that have been attributed to EDC exposure include impaired gonadal

development, abnormal blood steroid concentrations, altered sexual behavior, impaired reproductive output, reduced hatching success and/or larval survival, or altered growth and development (Jobling and Tyler, 2003; Mills and Chichester, 2005; Pait and Nelson, 2002; Scholz and Klüver, 2009). Although it is not yet clear whether EDCs can impact reproductive success and structure of wild fish populations (Mills and Chichester, 2005; Jobling and Tyler, 2003), there is strong evidence that EDCs can disrupt reproductive health of individual fish.

1.2. Endocrine control of reproductive function and interference of environmental toxicants with the hypothalamic-pituitary-gonadal axis

Interference of EDCs with the endocrine system may disrupt the reproductive, thyroid or adrenocordical functions that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior of all vertebrate species. To date, most evidence for endocrine disruption has been obtained in the reproductive function of vertebrates, especially of teleost fish (Di Giulio and Hinton, 2008; Pait and Nelson, 2002). The development of reproductive tissue and the coordination of the complex processes occurring during the annual reproductive cycle of fish are controlled by hormones secreted along the hypothalamic-pituitary-gonadal (HPG) axis (Norris, 2007; Fig. 1). The hypothalamus integrates environmental stimuli (e.g. photoperiod and temperature), which results in secretion of various neurotransmitters and neuropeptides that influence the action of the gonadotropin-releasing hormone (GnRH). This neurohormone in turn regulates directly the synthesis and secretion of gonadotropins (follicle-stimulating hormone, FSH and luteinizing hormone, LH) from the pituitary gland. These gonadotropins, distributed via the bloodstream, cause alterations in the synthesis of sex steroid hormones, particularly estrogens and androgens (Bone et al., 1995). Estrogens are primarily secreted by follicle cells surrounding the oocytes of the ovaries (i.e. theca and granulosa cells), while androgens are primarily secreted by the interstitial Leydig cells of the testes. Sex steroid hormones may display their actions by binding to specific cell-surface receptors, but principally by binding to nuclear steroid receptors (i.e. estrogen (ER), androgen (AR) receptors), resulting in alteration of gene expression (Tyler et al., 1998).

Estrogens and androgens have a variety of functions, many of which are similar in various classes of vertebrates, including sex determination and differentiation, sexual development, control of the reproductive cycle and behavior, and development of

secondary sex characteristics (Bone et al., 1995). The response of target tissues (mainly gonads, brain and liver) to sex steroid hormones is largely dependent on the expression of steroid hormone receptors, which in turn is regulated by steroid hormones and therefore fluctuates in response to alterations of the sex steroid biosynthesis (Tyler et al., 1998). The concentration of steroids and their receptors change during the reproductive life of fishes; consequently, the degree of adverse effects caused by exposure to EDCs varies depending on the developmental and reproductive stage of fish (Di Giulio and Hinton, 2008).

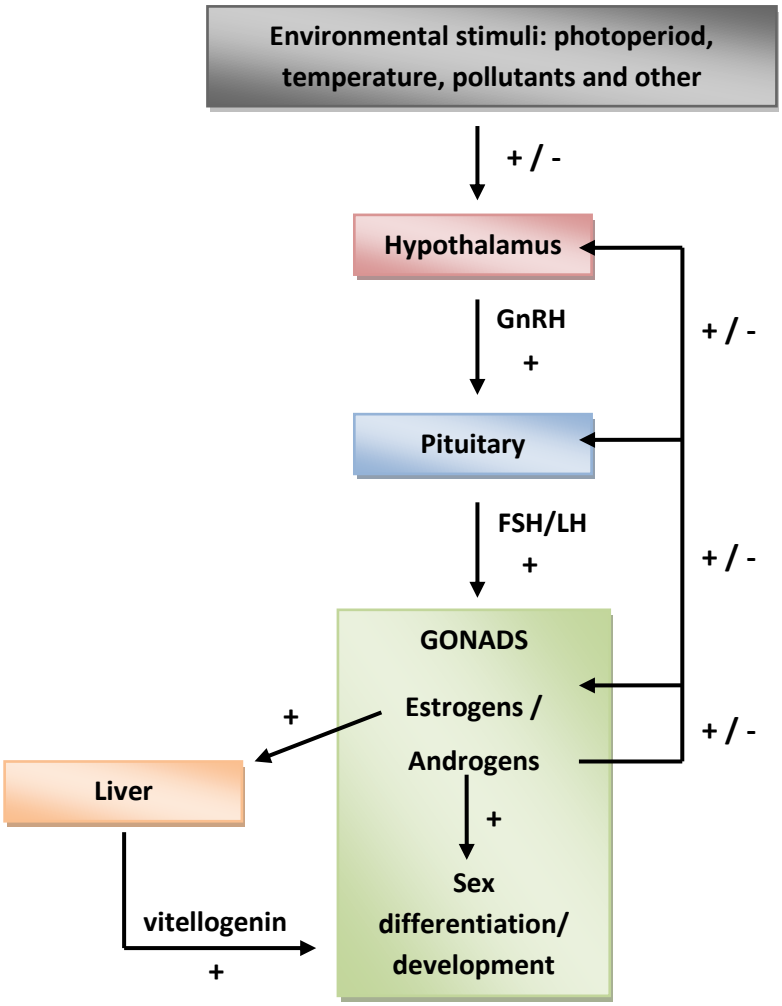


Figure 1. Schematic representation of the HPG axis controlling reproduction in fish. Feedback effects of steroids and other hormonal positive or negative effects (+ or -), illustrated by arrows, maintain a dynamic control of the reproductive endocrine system.

The HPG axis provides several possible points of interaction for EDCs that may result in interference with the reproductive endocrine function (Scholz and Klüver, 2009). Chemicals may act in the hypothalamus to influence GnRH secretion, which would secondarily affect pituitary gonadotropin secretion in response to GnRH (Khan et al., 2001). Other chemicals can act directly on the pituitary by influencing GnRH signaling pathways and gonadotropin secretion (Thomas, 1993). Exposure to these chemicals may result in alterations of gonadal development and function, for example in impaired steroid hormone or gamete production. In addition, some chemicals exert their effect directly on the gonads by influencing the activities of gonadotropin pathways and enzymes involved in the synthesis of sex steroid hormones (Ankley et al., 2005; Benninghoff et al., 2005). Alterations in the steroid hormone synthesis can dramatically influence steroid levels that target tissues such as gonads, and other organs important for reproduction, like the liver and the brain. Steroid levels may also be influenced by increases in metabolic clearance rate of steroids due to chemical induction of hepatic cytochrome P450 enzymes (Qian et al., 2007). Furthermore, steroid hormone action can also be affected in target tissues by direct interaction of chemicals with steroid receptors, resulting in agonism and antagonism of steroid hormone action. The agonist or antagonist mechanisms of EDCs may impair reproductive function for example by influencing the steroid feedback system (Harris et al., 2001), or the vitellogenin (precursor protein of the egg yolk) production in the liver (Jobling and Sumpter, 1993). Therefore, chemicals that can interact with the HPG axis could have serious effects not only on the development and well being of individual organisms, but also more importantly on the ability of these organisms to reproduce, and their offspring to survive and eventually reproduce (Lintelmann et al., 2003).

1.3. Morphology, endocrinology and environmental modulation of sex differentiation in teleost fish

1.3.1. Sexual differentiation and gonadal development

The sex differentiation refers to gonadal development once sex has been determined, i.e. when the undifferentiated gonad follows the ovarian or testicular differentiation pathway (Guerrero-Estevez and Moreno-Mendoza, 2010; Piferrer and Guigen, 2008). Among all different fish species, there is a large variety of patterns of sexual differentiation, from hermaphroditism to gonochorism. In most gonochoristic species, the

undifferentiated bipotential gonad develops directly into ovary or testis (an exception is juvenile hermaphroditism for which gonads are initially hermaphroditic, but later develop as ovaries or testes; Devlin and Nagahama 2002), while hermaphroditic species may be synchronic (ovarian and testicular tissue evolving simultaneously) or sequential (protandric, inversion from male to female; or protogynic, inversion from female to male; Sadovy and Shapiro, 1987). The different processes of sex differentiation are highly plastic and certain environmental factors, such as temperature or pH, or exposure to EDCs, may influence the sex differentiation towards male or female tissue development (Devlin and Nagahama, 2002; Nakamura, 1998).

Sex differentiation has been shown to begin first in the gonads of females than in males in the majority of the gonochoristic teleosts examined to date. The onset of ovarian differentiation and oogenesis starts with differentiation of somatic and germ cells and intensive proliferation of germ cells (mitosis), which is followed by their entry into meiosis to form follicles, composed of the oocyte and a layer of granulosa cells surrounded by an external layer of theca cells (Strüssman and Nakamura, 2002). The differentiation of the primary ovarian follicle into an egg is a process accompanied by its growth and massive structural and functional changes. The oogenesis starts with the transformation of primordial germ cells into oogonia, followed by transformation into primary oocytes; the meiosis starts at this moment and is followed by a massive growth of the oocyte during vitellogenesis, whereby the oocyte accumulates nutritional reserves needed for the development of the embryo (Patiño and Sullivan, 2002). During this time the oocyte remains in meiotic arrest till the maturation process, which is characterized by the resumption of meiosis. Finally ovulation takes place at the end of the maturation process (Lubzens et al., 2010). During oogenesis, the oocytes can be divided into different stages of maturity (Wolf et al., 2004): perinucleolar, cortical alveolar, early vitellogenic, late vitellogenic, and mature oocytes (Fig. 2).

In juvenile hermaphrodites, where gonads of genotypic males develop first female characteristics, the first sign of male differentiation is the regression of female tissue. In differentiated gonochorists, the onset of spermatogenesis starts with intensive germ cell mitosis and formation of germ cell cysts and tubules (Schulz et al., 2010). The testis is composed of the intertubular compartment and the tubular compartment. The intertubular compartment contains the steroidogenic Leydig cells. The tubular compartment contains the

spermatogenic tubules with Sertoli cell and the germ cells. Within the spermatogenic tubules, several cysts, deriving from a single spermatogonium, can develop independently. Different stages of maturity of the germ cells can be distinguished (Schulz et al., 2010): spermatogonia, spermatocytes, spermatids and spermatozoa (Fig. 2).

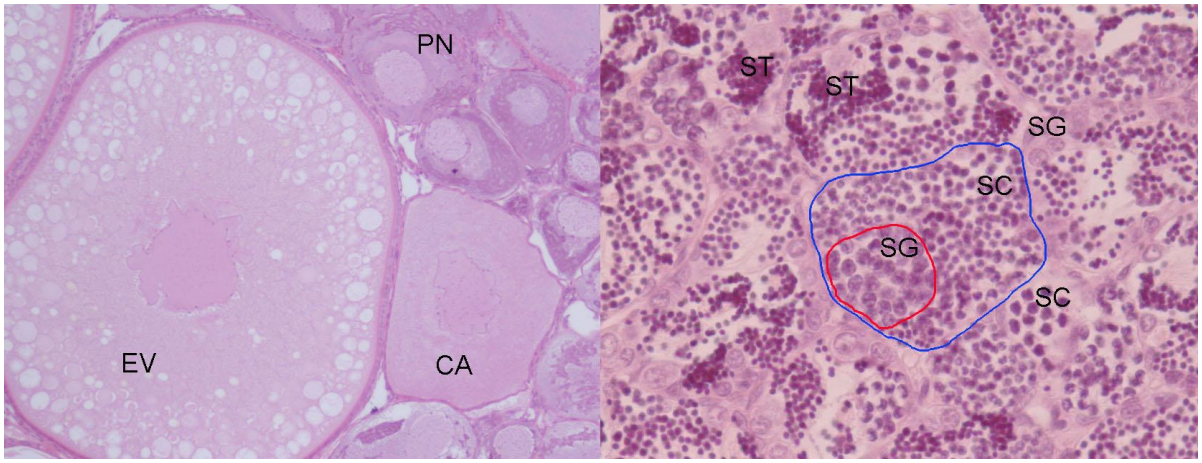


Figure 2. Images showing, on the left ovary, and on the right testis from juvenile brown trout (1+). Gonads present germ cells at different stages of maturity: perinucleolar (PN), cortical alveoli (CA) and early vitellogenic (EV) oocytes in ovary, and spermatogonia (SG), spermatocytes (SC) and spermatids (ST) in testis. The testis is composed of several tubules (delimited in blue) containing several cysts (delimited in red) of germ cells in a particular stage of maturity.

1.3.2. Endocrine control of sexual differentiation and development

In fish, as in other vertebrates, the main endocrine regulators of sexual differentiation and development are sex steroid hormones. Treatment of sexually undifferentiated fish with androgens or estrogens results in most cases in the production of males and females, respectively, regardless of genotypic sex (Piferrer, 2001). Several studies suggest that the balance between androgens and estrogens, rather than their absolute amount, is determinant for the direction of sexual differentiation (Piferrer and Guiguen, 2008). In all vertebrates, this balance is determined by the activity of the aromatase enzyme that converts androgens into estrogens. Accordingly, the aromatase plays an essential role in regulating fish sex differentiation (Guiguen et al., 2010). The main estrogen, 17β-estradiol, is found in much higher levels in females than in males, and is assumed to be the major sex steroid responsible for inducing and maintaining ovarian development (Norris, 2007). Both testosterone and 11-ketotestosterone are found in males, but also in females in a smaller

amount, and 11-ketotestosterone is the major androgen responsible for testicular development (Borg, 1994). In addition, other steroids like the 17α, 20β-dihydroxy-4-pregnen-3-one (the maturation-inducing steroid hormone) and other precursors of these hormones (from pregnenolone) play further roles during sexual development (Norris, 2007). Plasma levels of steroid hormones show important variations during male and female gonad differentiation and development and each steroid hormone has a distinct role in the regulation of sexual development (Lubzens et al., 2010; Schulz et al., 2010). For example, a major role of 17β-estradiol in female fish is to stimulate the liver to produce vitellogenin, the precursor of yolk, and the vitelline envelope proteins, which form the egg shell. Thus, in oviparous animals, estrogens are vital for oocyte growth, egg formation, and provision of yolk for the developing embryo (Tyler and Sumpter, 1996).

The sex steroids are not the only factors implicated in the endocrine control of sexual differentiation and development. Besides the complex interaction between the brain and gonads via the production of gonadotropins and sex steroids (Fig. 1), sexual development involves also cell proliferation and tissue growth, suggesting that growth hormones, particularly insulin-like growth factors, may also take part in fish gonadal development (Reinecke, 2010). Consequently, the disruption of different hormonal regulatory pathways of the sexual development may result in impaired sexual differentiation and development.

1.3.3. Fish early sexual development: a sensitive period of life

The early gonadal development, until the sexual maturity, appears to be especially sensitive to chemicals that affect the HPG axis and alter the sex hormone levels (Ankley et al., 2004; Tyler et al., 1998; Scholz and Klüver, 2009). Many laboratory studies indicate adverse effects or aberrations in sexual development associated with exposure to EDCs (effluent or single chemical) during the sexual differentiation and development of fish. For example, juvenile zebrafish (*Danio rerio*) exposed to pulp mill effluent exhibit high levels of vitellogenin in males (an estrogenic effect), a male-biased sex ratio and occurrence of intersex (Örn et al., 2006). Male juvenile roach (*Rutilus rutilus*) exposed to sewage treatment effluent presented increased vitellogenin concentration and feminization of the reproductive ducts in male (Liney et al., 2005). Further, in juvenile zebrafish exposed to either natural estrogens or trenbolone (synthetic androgenic steroid), the sex ratio was skewed either towards females or complete masculinization was observed, respectively (Holbech et al.,

2006). Other adverse effects such as reduction of gonads weight, delayed or increased gametogenesis, have been observed (Mills and Chichester, 2005; Scholz and Klüver, 2009). All these adverse outcomes have the potential to impact the reproductive health at the individual level and subsequently the maintenance of stable populations of fish (Hutchinson et al., 2006).

1.4. Chemicals interfering with the steroidogenesis

EDCs can influence the endocrine functions by affecting hormone levels (synthesis or metabolism of hormones), by interfering with hormone action in the targeted tissue (mimicking or antagonizing hormone action), or by modifying hormone receptor levels (Sonnenschein and Soto, 1998). So far, ecotoxicological research on chemical interference with endocrine functions in vertebrates has mainly focused on chemicals interacting with nuclear steroid receptors, especially the nuclear ER (Sonnenschein and Soto, 1998; Tyler et. al., 1998). However, a range of chemicals have been shown to interfere and exert direct effects in the steroid hormone biosynthesis (steroidogenesis) in teleost fish (Cheshenko, 2008; Sanderson, 2006). There is increasing evidence that estrogenic substances are not the only substances present in the aquatic environment that exert endocrine disrupting effects. The research on endocrine disruption needs, therefore, to orientate and develop also towards EDCs exhibiting other endocrine modes of action, like interference with steroidogenesis, to assess the potential risk of such chemicals for sexual development and reproductive health of aquatic organisms.

Several organs synthesize active steroids, including the adrenal glands, testis, ovary and brain, with sex steroids being mainly produced in gonads. Their synthesis (Fig. 3) is controlled by the activity of several cytochrome P450 enzymes (CYPs) and a number of hydroxysteroid dehydrogenases (HSDs; Sanderson, 2006). The synthesis of all steroid hormones starts with the conversion of cholesterol to pregnenolone by CYP11A (cholesterol side-chain cleavage). Pregnenolone is mainly converted to progesterone by 3β-HSD. Progesterone is then mainly converted into androstenedione by the CYP17 hydroxylase/lyase, which is first responsible for the 17α-hydroxylation of C17-20 steroid structures and its 17,20 lyase activity directs the biosynthesis of steroids toward sex steroids. Androstenedione, a weak androgen, is then mainly converted into testosterone by the 17β-HSD. Principally in the testes, 11-ketotestosterone, the prominent androgen, is formed from

testosterone by 11β-HSD. Finally, CYP19 or aromatase, which is expressed at a low level in testes and mainly in ovaries, is the key enzyme that converts androgens to estrogens (Cheshenko et al., 2008).

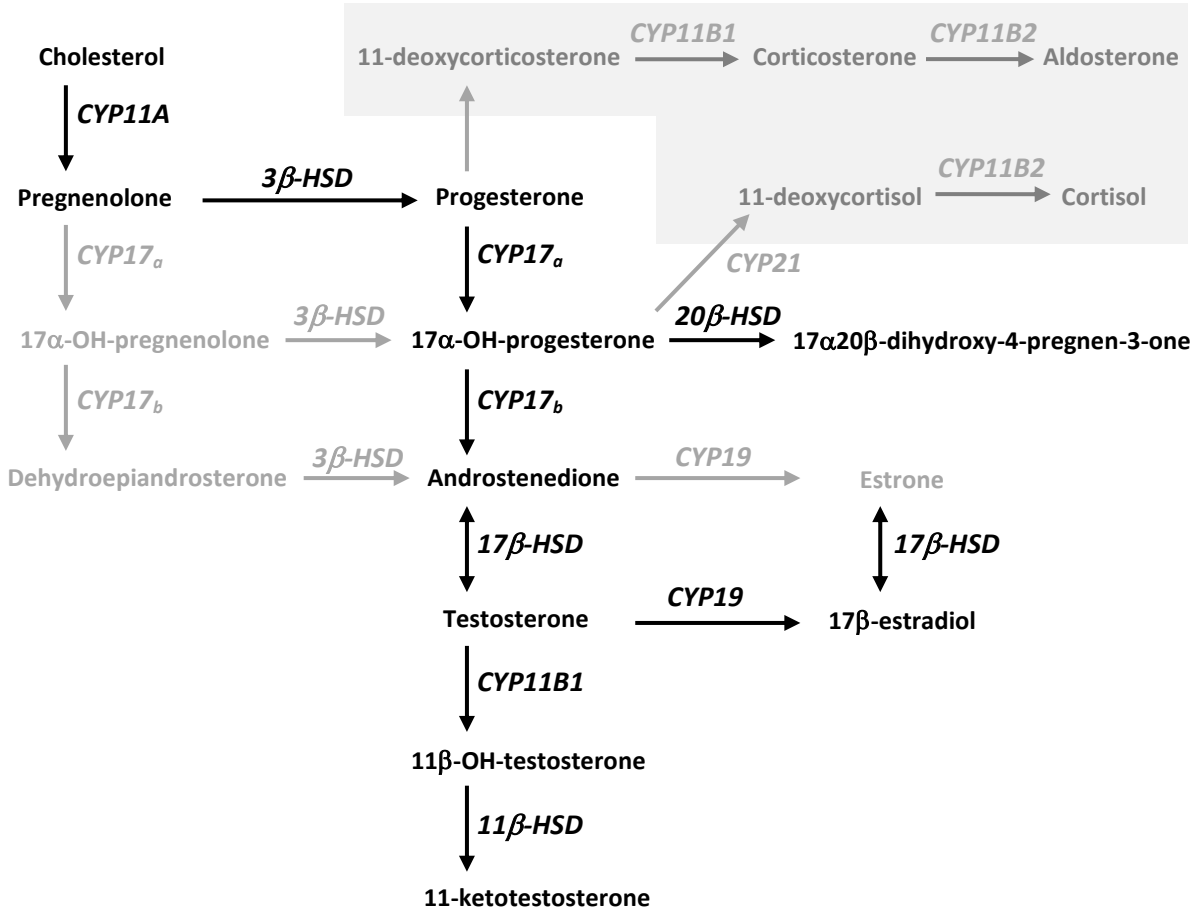


Figure 3. Enzymes involved in the biosynthesis of androgens and estrogens. Cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD) enzymes. CYP17 activity is indicated as (a) hydroxylase or (b) lyase activity. The major sex steroid biosynthesis pathway in fish gonad tissue is indicated in black. Gray background indicates steroidogenic pathways in extra gonadal tissues.

The steroidogenic pathway may be chemically disrupted by several mechanisms, including direct reversible or irreversible catalytic inhibition of steroidogenic enzymes, or indirect up- or down-regulation of steroidogenic enzyme expression. Indirect effects on the steroidogenic enzymes may occur via different interactions along the HPG axis (Sanderson and van der Berg, 2003). An analysis of the current literature indicates that still relatively little is known about the underlying mechanisms of interference of chemicals with

steroidogenesis and particularly about their potential toxicity in steroidogenic tissues, neither in humans nor in wildlife. Recent studies have reported modulations of sex steroid levels and impairment of gonadal development in wild fish collected from contaminated sites (Hecker et al. 2007; Hinfray et al., 2010; Lavado et al., 2004; Noaksson et al., 2001, 2003) suggesting that wild fish populations are indeed exposed to substances that can perturb steroidogenesis and potentially the sexual development of fish. However the nature and bioavailability of substances involved in these biological responses remain to be determined. Few laboratory studies have shown that chemicals interfering with the activity and/or the expression of one or several steroidogenic enzymes could lead to adverse effects that may potentially impair development and reproduction of fish (Table 1). These chemicals include natural products (i.e. phytosterols; MacLatchy et al., 1997), pesticides (i.e. prochloraz, fenarimol; Ankley et al., 2005), pharmaceuticals (i.e. ketoconazole; trilostane; Ankley et al., 2007; Villeneuve et al., 2008) and other industrial compounds (i.e. flame retardants; Deng et al., 2010). Most of them may potentially be found in the environment. Thus, there is a need to provide further knowledge on chemicals interfering with the steroidogenesis for a better understanding of the risk of this type of EDCs to humans and wildlife.

Among the studies on the mechanisms by which EDCs interfere with the sex steroid hormone homeostasis and function, particular attention has been given to CYP19. In several teleost fish such as zebrafish (*Danio rerio*), goldfish (*Carassius auratus*), tilapia (*Oreochromis niloticus*), fathead minnow (*Pimephales promelas*) or rainbow trout (*Oncorhynchus mykiss*), CYP19 has been found to be encoded by two different genes: *cyp19a* and *cyp19b* (Chang et al., 2005; Fenske and Segner, 2004; Tchoudakova and Callard, 1998; Toffolo et al., 2007; Villeneuve et al., 2006). These two distinct genes and encoded CYP19 proteins are differentially expressed, i.e. *cyp19b* mainly in the brain and *cyp19a* mainly in the gonads. Careful spatial and temporal balance of estrogens in the body is crucial in reproduction-related processes, including sexual differentiation and determination (Cheshenko et al., 2008), and the inhibition of the gonadal CYP19 activity and/or of gonadal *cyp19a* expression has been related to masculinization process of several fish species (Fenske et al., 2004; Guigen et al., 2010; Vizziano et al., 2008).

Table 1. Effects of chemicals disrupting the steroidogenesis on the development of the teleost gonads (reviewed in Schloz and Klüver, 2009).

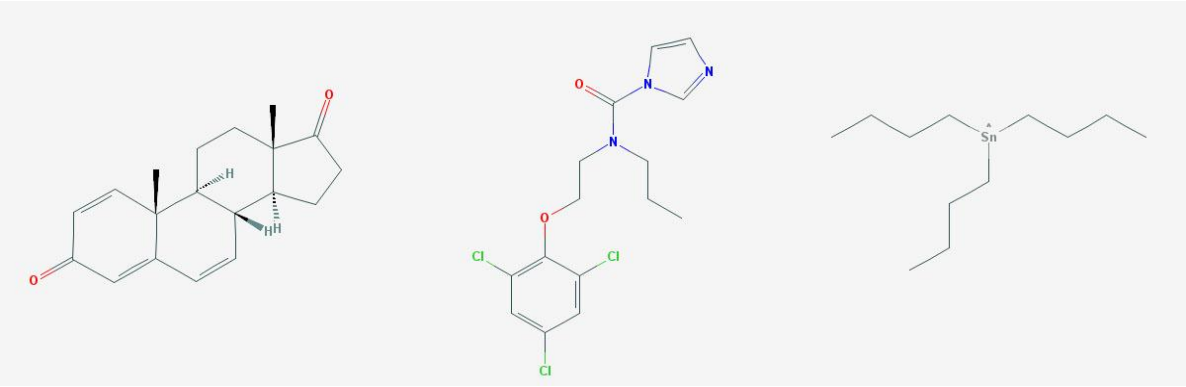
Chemical	Species	P	Effect level (µg/L)	Sex ratio	TSI	OSI	Sp	Oo	Other effects
Ketoconazole	*Pimephales promelas*	A	6		I	I			Increase proliferation of Leydig cells
Fadrozole	*Danio rerio*	J	10	m					
	Pimephales Promelas	A	50-100		I	R	S	D	Sertoli cells hypertrophy, increased of Leydig cells
Letrozole	*Oryzias* latipes	A	125		I	I	S	D	Enlarged lumen of seminferous tubules

A = exposure of adult fish ; D = delayed ; I = increased ; J = exposure of juvenile fish ; m = male-biased sex ratio ; Oo = oogenesis ; OSI = ovarian somatic index ; P = period of exposure; R = reduced; S = stimulated; Sp = spermatogenesis; TSI = testicular somatic index.
References: Ankley et al., 2007; Andersen et al., 2004; Panter et al., 2004 ; Sun et al., 2007 ; OECD, 2006

In this thesis, the following model chemicals, known or suspected to alter the steroidogenesis in addition to sexual differentiation and reproductive health of fish, have been studied:

1.4.1. 1,4,6-androstatriene-3,17-dione, an aromatase inhibitor

The chemical 1,4,6-androstatriene-3,17-dione (ATD; Fig. 4) is a potent irreversible CYP19 aromatase inhibitor that inhibits estrogen biosynthesis by permanently binding and inactivating CYP19 (Covey and Hood, 1981). It has also been used in few studies in a range of 0.5 to 5 mg/L to analyze the role of CYP19 expression in sex differentiation of several fish species, where it induces masculinization (Vizziano et al., 2008; Lee et al., 2003; Lee et al., 2001). So far, this chemical has not been reported in the environment. However, as ATD is used in some pharmaceutical products (e.g. body building supplements), surface waters may receive this chemical via domestic discharges.



ATD Prochloraz TBT

Figure 4. Chemical structure of ATD, prochloraz, and TBT (http://pubchem.ncbi.nlm.nih.gov/).

1.4.2. Prochloraz, an imidazole fungicide

Prochloraz (*N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)ethyl]imidazole-1-carboxamide; Fig. 4) is an imidazole fungicide widely used all around the world. The action of imidazoles are based on the inhibition of the cytochrome P450-dependent 14α-demethylase activity, which is required in the conversion of lanosterol to ergosterol (Henry & Sisler, 1984), an essential component of fungal cell membranes. Due to its unspecific interaction with cytochrome P450, it also inhibits a broad spectrum of other cytochrome P450-dependent enzymes, including key enzymes of the sex steroid biosynthesis, e.g. CYP19 aromatase (Vinggaard et al., 2006). *In vitro* studies have shown that prochloraz elicits multiple mechanisms of action: it antagonizes the AR and ER; it agonizes the aryl hydrocarbon receptor (AhR, which subsequently induces gene expression modulation) and, principally, inhibits aromatase activity (Andersen et al., 2002; Hecker et al., 2006; Hinfray et al., 2006; Laville et al., 2006; Villeneuve et al., 2007). *In vivo* studies with rats showed that prochloraz could also inhibit testosterone production possibly by altering CYP17 hydroxylase/lyase activity (Blystone et al., 2007; Vinggaard et al., 2006). In addition, few fish *in vivo* studies demonstrated the sublethal adverse effects of prochloraz exposure in a range of 3 to 300 μg/L, which is much lower as its acute toxicity value for rainbow trout (96-h LC_{50} = 1.43 mg/L). The studies of Ankley et al. (2005) and Zhang et al. (2008) showed a reduced fecundity of fathead minnow and Japanese medaka (*Oryzias latipes*), respectively, as response to prochloraz. In the study of Ankley et al. (2005), this effect was accompanied by decreased plasma sex steroids (17β-estradiol, testosterone and 11-ketotestosterone) and vitellogenin concentrations, and altered gonadal histology. Both studies further

demonstrated an indirect effect of prochloraz on the sex steroid synthesis via up-regulation of CYP17 and CYP19 gene expression. Furthermore, Kinnberg et al. (2007) reported, in an early life stage assay with zebrafish, the potential of prochloraz to induce a male-biased sex ratio, altered gonadal development and vitellogenin production.

Since prochloraz is a widely used fungicide, it can be found in surface water and transported by runoff from the terrestrial to the aquatic environment. In Switzerland, it is has been detected in a range of few ng/L in groundwater (NAQUA, OFEV, 2009). In streambed sediment analysis from several Danish rivers, it has also been demonstrated that it can accumulate in sediments, possibly resulting in locally elevated concentration of the substance in the aquatic environment (Kronvang et al., 2003). In addition, few metabolic studies in rainbow trout indicated that prochloraz may be widely distributed in the body and is extensively metabolized. The highest levels of prochloraz residues were mainly found in the liver, in which prochloraz has been reported to induce the gene expression of CYP1A biotransformation enzyme (Cravedi et al., 2001; Debrauwer et al., 2001; Sturm et al., 2001).

1.4.3. Tributyltin, a persistent industrial compound

Tributyltin (TBT) is a toxic and bioaccumulative chemical that is used for various industrial purposes. While its use as biocide in antifouling agent in boat paints has been internationally banned, it is likely that organotin compounds continue to be produced and used in biocides for other functions (reviewed in Antizar-Ladislao, 2008). Due to its persistent nature, it is a common contaminant of marine and freshwater ecosystems exceeding acute and chronic toxicity levels. Extended research undertaken since the early 1970s has shown that TBT is very toxic to a large number of aquatic organisms, with acute toxicity concentrations in the range of 5 to 1000 ng/L, depending on the species, life stage, and other chemical and physical parameters (e.g. salt- or freshwater, temperature; reviewed in Antizar-Ladislao, 2008). In the aquatic environment, TBT adheres strongly to bed sediments and high levels up to 1500 ng/L have been reported. In recent investigations, it has been reported that TBT concentrations in water, sediment and biota have generally declined, and maximum concentrations in marine water rarely exceed 100 ng/L (Diez et al., 2006; Bhosle et al., 2004). The oral route is the most obvious source for organotin uptake in aquatic biota and transfer via the food chain, although direct uptake via skin or gills is also important in fish (Lee et al., 2006; Strand and Jacobsen, 2005). In addition, is has been

demonstrated that TBT alters the activity and/or expression of hepatic CYP enzymes in fish, which decreases the xenobiotic detoxification and increases the potential risk of organotin compound exposure (Fent et al., 1998; Mortensen and Arukwe, 2007).

The TBT-induced masculinization (imposex) in female marine mollusks is a well-known example of endocrine disruption in invertebrates that is causally linked to an environmental pollutant (Matthiessen and Gibbs, 1998). Imposex occurs when male sex characteristics develop in female gastropods and has resulted in decline or extinction of mollusk populations worldwide. The bioaccumulation of TBT in gastropods and its endocrine disruptive effects result in elevated testosterone levels giving rise to imposex (Gibbs and Bryan, 1996). The mechanism of action of TBT is not clear yet, but considerable weight of evidence suggests an inhibition of CYP19 as possible mechanism (Bettin et al., 1996). However, TBT also inhibited conjugation of testosterone, which would result in reduced elimination and accumulation of testosterone from the body, and subsequently stimulate the development of imposex (Ronis and Mason, 1996).

In fish, TBT in a range of 1 to 1000 ng/L clearly induces endocrine disrupting effects (Antizar-Ladislao, 2008). In laboratory studies with zebrafish, TBT has been shown to impair reproductive function by causing high incidence of abnormal sperm and a male-biased sex ratio, which might be related to CYP19 inhibiting properties of TBT (McAllister and Kime, 2003). Similarly, fertilization success was decreased in medaka exposed to TBT (Nakayama et al., 2004). Further, TBT could modulate sex hormone levels in gonads of *Sebastiscus marmoratus* and impair gonadal development (Zhang et al., 2008, 2009). In addition, a long-term exposure of the mummichog (*Fundulus heteroclitus*) to TBT affected not only gonadal sex differentiation and spermatogenesis, but also spawning and egg quality (Mochida et al., 2010).

1.5. Fish test methods for endocrine disrupting chemicals

1.5.1. Fish in vivo testing

Given the occurrence of EDCs in the environment and their potential threat for reproductive health of fish populations, international research efforts on the development of testing strategies for EDCs have arisen [1] [2] (Fenner-Crisp et al. 2000; Huet, 2000;

[1] http://www.oecd.org/document/42/0%2C2340%2Cen_2649_34377_2348650_1_1_1_1%2C00.html
[2] http://www.epa.gov/endo/

Hutchinson et al., 2006). These strategies are based on tiered approach. The first step is the performance of a screening test. Screening assays have been defined as lower tier *in vitro* or *in vivo* investigations which allow the identification and classification of substances relative to their potential interaction with endocrine systems (Ankley et al., 1998). If a substance is suspected to have an endocrine mode of action, it has to be tested in an assay that can be used in risk assessment. These tests are defined to be higher tier *in vivo* methods (e.g. fish full or partial life cycle assays) to confirm the screening results and to characterize any adverse effects at the apical level of the biological organization (e.g. on survival, growth, morphological development, and reproduction) that may result from the endocrine mode of action of EDCs (Hutchinson et al., 2006; Matthiessen and Johnson, 2007).

Since the most evident impacts of EDCs in the environment are on aquatic organisms (Hutchinson and Pickford, 2002), it has stimulated research efforts to develop fish test methods. The fish species recommended by international regulatory agencies are: the fathead minnow (*Pimephales promelas*), the medaka (*Oryzias latipes*), the zebrafish (*Danio rerio*), and the three-spined stickleback (*Gasterosteus aculeatus*). Currently, two *in vivo* fish screening assays have been adopted by the OECD (Knacker et al., 2010): the fish short-term reproduction assay (OECD, 2009a) and the 21-day fish assay (OECD, 2009b), which are both conducted with reproductively active fish. The endpoints of these two screening assays include vitellogenin induction and evaluation of secondary sex characteristics in sexually dimorphic species (i.e. fathead minnow and medaka); further endpoints of the short-term reproduction assay include fecundity, fertility and histolopathology of gonads. In addition, higher tier fish *in vivo* methods providing data on adverse effects at the apical level have been proposed: the fish full life cycle test (FLCT; Seki et al., 2003), the fish 2-generation test (2-genT; Braunbeck et al., 2009; EPA, 2002) and the fish sexual development test (FSDT). The FSDT allows measuring sexual development endpoints (i.e. sex ratio and vitellogenin production; Holbech et al., 2006). The FLCT and the 2-genT allow assessing the effects on developmental and reproductive endpoints (i.e. hatching, sex ratio, survival, growth, time to first spawn, fecundity, fertility, and behavior) as well as biochemical, histological, and morphological markers. Additionally, the 2-genT allows determining trans-generational transfer of effects.

1.5.2. Fish in vitro testing

The justification for *in vivo* studies in ecotoxicology is derived from its objective to evaluate chemical effects at population and ecosystem levels (Castaño et al., 2003). Furthermore, due to the complexity of the endocrine system, extrapolation of *in vitro* effects to *in vivo* situation is not straightforward and requires further, often *in vivo*, investigations (Sanderson, 2006). In addition, *in vitro* assays do not provide biological responses and endocrine-mediated adverse effects at the apical level, which are needed for environmental risk assessment (Ankley et al., 2009). In contrast, a number of ethical, technical, scientific and economic reasons support the development of fish *in vitro* methods to be used as screening assays. They are rapid screening tools to evaluate the toxicity of a large number of individual compounds and environmental samples. Further, they reduce the number of animal used in toxicity testing (Castaño et al, 2003; Matthiessen and Johnson, 2007). *In vitro* cell-based methods provide the best experimental system for studying toxic mechanisms at the molecular and cellular levels in a controlled environment and isolation from the multiple physiological pathways interacting *in vivo* (Castaño et al., 2003). The identification of specific mechanism of action in *in vitro* assays can trigger more advanced and comprehensive *in vivo* testing, thereby optimizing time and resource use (Ankley et al., 2009; Hutchinson et al., 2006). In addition, i*n vitro* assays can lead to the development of biomarkers, which can be used to measure *in vivo* responses and can permit the evaluation of the effects of toxicants in animals (Castaño et al., 2003).

1.5.3. In vitro screening assays for endocrine disrupting chemicals interfering with steroidogenesis

To date, most of the developed *in vitro* assays have focused on EDCs binding to steroid hormone receptors (i.e. receptor binding assays, cell proliferation assays, reporter gene assays; Baker et al., 2000; Gray et al., 1997; Soto et al., 1995). To investigate effects of EDCs interfering with the steroidogenesis, various *in vitro* methods from different biological systems are possible. The catalytic activity of individual steroidogenic enzymes can be either measured in microsomal fraction of tissues that express the enzyme of interest, or in cell cultures by using selective radiolabeled substrate for the enzyme in combination with specific inhibitors of the enzyme (Hinfray et al., 2006; Vingaard et al., 2000; Sanderson et al., 2002). In cell lines, primary cell or tissue cultures, alterated enzyme expression can be

determined via northern blotting or RT-PCR (Hilscherova et al., 2004). An indirect way to measure chemical effects on steroidogenic enzyme function in cell cultures is to measure alterations in excretion of certain steroid hormones as an indicator of chemical effect on steroidogenesis (Hecker et al., 2006; Lee et al., 2006; Villeneuve et al., 2007). An advantage of this approach is that alterations in the profile of the secreted steroid hormones provide an indication of the identity of the enzymes affected by the xenobiotic treatment, without the need to examine each enzyme activity individually (Sanderson et al., 2003).

1.5.4. Ex vivo organ cultures

The *in vitro* fish cell systems which are currently used for toxicological studies are based either on established fish cell lines, primary fish cells or organ *ex vivo* cultures (Castaño et al., 2003). An organ culture has the advantage over cell lines that it possesses the morphological and cellular structure and function of its source tissue. The use of organ cultures is therefore physiologically relevant, and is particularly suitable to study specific mechanisms of action of toxicants in particular target organs. Furthermore, the preparation of *ex vivo* organ cultures is rather simple: organs are removed, dissected and maintained in a serum free culture medium. It is therefore uncomplicated compared to primary cell cultures that need an isolation procedure (Castaño et al., 2003). According to these advantages of organ cultures, and to analyse the effects of chemicals interfering with steroidogenesis, a fish *ex vivo* gonad culture was developed here (Fig. 5).

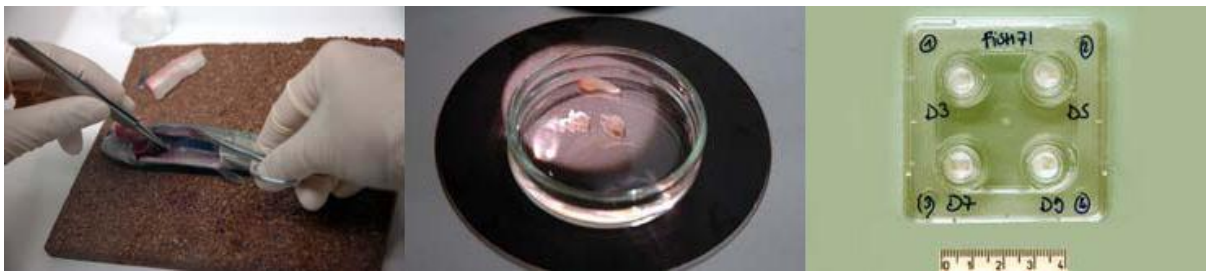


Figure 5. Images illustrating the developed gonad organ culture from juvenile brown trout. From left to right: removal of gonads from juvenile fish; dissection of gonadal fragments in Petri dish filled with fresh medium; gonadal fragments on insert filters transferred in a 4-well plate for incubation in fresh medium.

1.6. Test fish species

The bony fish represent the largest group of vertebrates both in number of species and in number of individuals. Their evolution and adaptation to different habitats has led to a high diversity of physiological, anatomical, behavioral and ecological strategies in fish (Bernanke and Köhler, 2008). Endocrine disruption has been studied in a variety of freshwater teleost fish species, but cyprinids (e.g. fathead minnow, zebrafish, carp) and salmonids (e.g. rainbow trout, brown trout, Atlantic salmon) are the best represented (Jobling and Tyler, 2003). Chemicals altering the sex hormone levels may induce quantitatively and qualitatively different biological responses and adverse apical effects in various fish species because of the different mechanisms for sexual development and different reproductive strategies (Hutchinson et al., 2006). Moreover, the sex, life stage and the endogenous hormone levels during the reproductive cycle of fish are other important physiological factors that may influence the effects of EDC exposure (Kawai et al., 2003). Therefore, it is important to analyze the differences in the sensitivities of different fish species to effects of EDCs for interspecies extrapolation from laboratory fish tests to wild fish populations (Hutchinson et al., 2006). For the experiments of this thesis, three different fish species with different sexual development and/or different reproductive strategies have been used.

1.6.1. Fathead minnow (Pimephales promelas) and zebrafish (Danio rerio)

Fathead minnow and zebrafish are small fish species currently used for EDC testing. Established methods for maintaining both species in continuous cultures in laboratory exist and the fish are easy to handle (Ankley et al., 2004). Both species have a short life cycle and start to spawn early in their life (Hutchinson et al., 2006). They are fractional spawners and produce a high number of eggs (Mills and Chichester, 2005). Their spawning activity can be precisely controlled by manipulating the temperature, photoperiod, and spawning substrate. Determination of fertility of the eggs can be achieved easily using a microscope. Thus, they are successfully used for partial and full life cycle tests to investigate reproductive effects of xenobiotics (Ankley et al., 2004).

The fathead minnow has a ubiquitous distribution across North America. Adults are approximately 50 to 75 mm long and weigh 2 to 5 g (Jensen et al., 2001). They have a generation time of 4 to 5 months. A reproductively active female fathead minnow typically

deposits clutches of 50 to 100 eggs on the bottom of the spawning substrate every 3 to 5 days (Jensen et al. 2001). Embryos hatch in approximately 4 to 5 days at 25°C (Ankley et al, 2004). Fathead minnows exhibit secondary sex characteristics and defined mating behavior, which makes them suitable to investigate phenotypic and behavioral changes associated with EDC exposure (Mills and Chichester, 2005). The gonadal differentiation of fathead minnow starts early in its development, and the gonads can be identified as either male or female from 10 to 25 days post-hatch (van Aerle et al, 2004; Uguz, 2008).

The zebrafish is a small sized fish species native to India and Burma. Adult zebrafish are approximately 40 to 50 mm long, and weigh about 1.5 g. The reproductive cycle takes 3 to 4 months. Reproductively active female zebrafish can spawn almost daily, with relatively large spawns (e.g. >150 eggs) occurring every 5 to 10 days. The eggs are released in the water column and settle to the bottom of the tank. The embryos hatch in about 3 days at 28°C (Ankley et al., 2004). In contrast to the fathead minnow, all juvenile individuals first develop ovary-like gonads. Only after approximately 35 to 45 days post-hatch, the ovarian tissue of male fish regresses and develop into testicular tissue (Maack and Segner, 2003).

1.6.2. Brown trout (Salmo trutta fario)

The brown trout is an important freshwater fish species in European rivers and has a high commercial and social value. Over the last 20 years, however, its catch has dramatically declined in Swiss rivers (Körner et al., 2005). To evaluate the causes for the observed decline, the project 'Fishnet' was established in 1998. Within the "fishnet" project, various hypotheses were put forward to explain the reduced catch (Burkhardt-Holm et al., 2002). Among others, a disturbed reproductive health as a consequence of endocrine disruption has been considered.

The brown trout is an annual spawner with a predominant spawning season in November and December. The brown trout is a gonochoristic species, in which gonadal development and differentiation happens during the first two to three years before attaining sexual maturity, with the males usually maturing before females (Billiard, 1987; Elliott, 1994). Signs of sex differentiation appear first in females at approximately 20-30 days post-hatch, and later on in males. According to their reproductive cycle, the morphology of their gonads follows seasonal changes. In males, gonads remain undifferentiated until the initiation of the first spermatogenic cycle (i.e. 1 to 2 years), and the complete

spermatogenesis and the first reproductive cycle usually takes place during the second year of life. In contrast, the completion of oogenesis till the ovulation and the first reproductive cycle happens for most females only in the third year of life (Billard, 1987). During the second year of life, some vitellogenic activity can, however, be observed in some females, although final ovulation does not take place. In salmonids, the capacity to synthesize and metabolize sex steroids is active from the time of egg fertilization but changes seasonally throughout the sexual differentiation and development of gonads (Billard et al., 1992; Feist and Schreck, 1996; Yeoh et al., 1996). Sex differentiation is labile during early life stages since sex inversion can easily be obtained by feeding the fry with food containing androgens or estrogens (Hunter and Donaldson, 1983).

1.7. Objectives of the thesis

The objective of the present thesis was to develop and/or test the potential of *ex vivo* and *in vivo* methods to identify and assess the effects of model chemicals disrupting the steroidogenesis in fish. To investigate this potential, the following key questions were posed:

- Does an *ex vivo* gonad assay have the potential to identify and discriminate between different effects of chemicals interfering with the steroidogenic pathyway?
- Does the *ex vivo* gonad assay inform on *in vivo* effects?
- Do chemicals interfering with steroidogenesis impact the regulatory processes of early sexual development and potentially the reproductive health of fish?
- Is the FSDT a suitable method to identify and assess the risk of chemicals interfering with steroidogenesis?

To answer to these questions, in a first step, an *ex vivo* gonad assay was developed to rapidly identify and quantify the effect of chemicals inhibiting the steroidogenic pathway. **Chapter 2** describes the development of this *ex vivo* gonad assay from juvenile brown trout, as well as the methodology and characterization of the assay. The advantages and limitations of this novel method are discussed. In **Chapter 3**, the potential of the *ex vivo* assay to predict *in vivo* physiological responses is presented. The *ex vivo* assay could thereby provide a suitable tool to bridge the gap to *in vivo* effects and a valuable screening and

informative tool for hazard assessment of EDCs. For this, *ex vivo* and *in vivo* biochemical responses to chemicals known to disrupt steroidogenesis were compared. This investigation was complemented by a 19-day *in vivo* study using the same chemicals to further analyze the potential effects of steroidogenesis disruption on sexual development of juvenile brown trout.

The endocrine regulatory processes of the early life period and all the endocrine factors that play a role during this period are not fully understood and identified. Hence, the developed *ex vivo* gonad assay could also be a suitable tool to study the regulatory processes during the early sexual development of fish and the potential impact of chemicals interfering with steroidogenesis during this sensitive life stage. Accordingly, the study in **chapter 4** analyses cellular and molecular effects of chemicals interfering with steroidogenesis on the regulation of early sexual development in brown trout by employing *ex vivo* and *in vivo* methods and comparing their biochemical and molecular responses.

Apart from the importance of extrapolation of the *ex vivo* results to *in vivo* effects in risk assessment, the difference of sensitivity to EDCs between fish species may also pose a problem for qualitative and quantitative extrapolation of data across fish species. Indeed, the difference in modes of sexual development and reproduction between fish species may influence their sensitivity to EDCs. To provide further knowledge on the influence of the gonadal differentiation mode on qualitative and quantitative effects caused by EDCs, the responses of two model fish species with different sexual differentiation strategies were characterized and compared in a FSDT **(chapter 5).**

Finally, the results of all the different studies of this thesis are summarized to evaluate the potential of the test methods to assess effects of chemicals interfering with steroidogenesis on sexual development and reproductive success of fish **(chapter 6)**.

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2. Paper 1

Development of an *ex vivo* brown trout (*Salmo trutta fario*) gonad culture for assessing chemical effects on steroidogenesis

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Abstract

A variety of natural and synthetic environmental substances have been shown to disrupt vertebrate reproduction through mimicking or modifying the regulation of the endocrine system. Tests to screen for any such chemicals that directly interact with the steroid hormone receptors are widely available; however, few tests have been developed to identify chemicals that affect endocrine function through non-receptor mediated mechanisms. The aim of this study was, therefore, to develop an assay for the identification of substances that disrupt the activity of enzymes involved in the sex steroid biosynthesis cascade, in particular the aromatase enzyme, CYP19, that catalyses the final conversion of androgens to estrogens. A gonad *ex vivo* assay was developed using gonad explants harvested from juvenile brown trout and cultured in a modified Leibovitz medium. Effects on sex steroid biosynthesis were quantified through measurement of 17β-estradiol (E2) and testosterone (T) concentrations in the medium after 2 days incubation. Exposure of ovary explants to 100 ng/ml 1,4,6-androstatriene-3,17-dione (ATD), a potent pharmaceutical aromatase inhibitor, reduced E2 concentrations and elevated T concentrations confirming that CYP19 activity could be inhibited in the assay. Exposure of ovary explants to 250 ng/ml prochloraz, an imidazole fungicide, also reduced E2 concentrations but did not affect T levels, consistent with reports that in addition to inhibiting CYP19 activity, prochloraz also inhibits enzymes in the steroidogenic pathway upstream of the CYP19 enzyme. Exposure to a third chemical, tributyltin (TBT), did not affect T or E2 concentrations, further supporting previous evidence that the CYP19 modulating effects of this chemical are not mediated through direct inhibition of CYP19 activity. These results demonstrate that the gonad *ex vivo* assay developed here can be successfully used to identify substances that disrupt sex steroid biosynthesis and further that it has the potential to inform on their specific mode of action.

1. Introduction

Estrogens and androgens play pivotal roles during sexual development in all vertebrates, and are responsible for the development of female and male characteristics, respectively, and for controlling reproductive cycles and fertility (Crisp et al., 1998; Danzo, 1998; Tyler et al., 1998). The amount of endogenous estrogens and androgens, and also their secretion to target tissues, are carefully controlled by the hypothalamic pituitary gonadal axis of the endocrine system (Norris, 2007). In the past few decades, it has been demonstrated that exposure to a range of exogenous natural and synthetic substances can alter the normal function of the endocrine system and lead to adverse effects. These effects include altered sex ratios and reduced reproductive success of vertebrates (Danzo, 1998; Tyler et al., 1998; Vos et al., 2000). In recognition of the potential ecological consequences of exposure to these endocrine disrupting chemicals, considerable research effort has been invested internationally to identify the causative agents and to determine their prevalence in the environment. To date, these efforts have largely focused on identifying and quantifying the effects of chemicals that mediate their effects via interaction with hormone receptors, predominantly the estrogen (Sonnenschein and Soto, 1998; Zacharewski, 1997) and androgen (Kelce and Wilson, 1997; Sohoni and Sumpter, 1998) receptors. However, there are many mechanisms via which chemicals may exert their effects on the endocrine system, including through disruption of steroid biosynthesis. For example, the conversion of androgens to estrogens through the catalysis of the aromatase cytochrome P450 enzyme (CYP19) is a critical step of the steroidogenic pathway (Cheshenko et al., 2008; Sanderson, 2006). *In vitro* studies have demonstrated that some substances, including pesticides (e.g. prochloraz, fenarimol; Hecker et al., 2006; Laville et al., 2006), pharmaceuticals (e.g. fadrozole, ketoconazole; Lee et al., 2006; Ankley et al., 2007) polychlorinated biphenyls (PCBs; Drenth et al., 1998), and flavonoids (Pelissero et al., 1996) can inhibit the activity of CYP19. *In vivo* experiments, to determine the consequences of such an inhibition for fish, have shown that inhibition of CYP19 activity in reproductively active females not only reduces plasma estradiol concentrations, but also reduces circulating concentrations of the egg yolk protein vitellogenin, and results in an associated decrease in egg production (Miller et al., 2007; Thorpe et al., 2007). Further, it has been shown that exposure of fish to aromatase inhibiting substances during early-life can result in male biased sex-ratios (Fenske and Segner, 2004; Guigen et al., 1999; Kinnberg et al., 2007; Lee et al., 2003).

At present, a causal link between exposure to chemicals that disrupt steroidogenesis and endocrine disruptive effects in wild fish populations has not been investigated. However, it is increasingly clear that estrogenic substances, although prevalent in the environment, do not account for all reproductive abnormalities reported in the aquatic wildlife. Indeed, it has already been suggested that chemicals that elicit effects via the androgen receptor, either as agonists (Miller and Ankley, 2004) or antagonists (Jobling et al., 2009) may be important contributors to reproductive disturbances in wild fish populations. Further, several studies have reported correlations between reduced androgen and estrogen plasma levels, decreased CYP19 activity and inhibition of gonadal growth in wild fish populations living in contaminated areas of European rivers and lakes (Hecker et al., 2002; Lavado et al., 2004; Noaksson et al., 2001), which may be consistent with exposure to pollutants targeting the steroidogenic pathway. In support of this, Hinfray et al. (2010) recently demonstrated the presence of aromatase inhibiting compounds in sediments of contaminated sites of French rivers. Although they did not determine the precise nature of the contaminants and their concentrations, their study does show that these aromatase inhibiting chemicals can persist in the environment and thus highlights the need to consider their potential effects on exposed wild populations. Thus, tests capable of screening for, and informing on the mode of actions of chemicals that disrupt endocrine function via non-estrogen receptor mediated mechanisms would be of considerable valued in helping us to understand the potential risk posed by such chemicals to the reproductive health of aquatic organisms.

To date several *in vitro* assays from different tissues and species have been developed to screen for chemicals that interfere with the steroidogenic pathway. This includes human placental microsome assays (Vingaard et al., 2000), trout ovarian and brain microsome assays (Hinfray et al., 2006a; Shilling et al., 1999) and cell-based assays using different human cell lines such as the adrenocortical H295R cells (Sanderson et al., 2002; Hecker et al., 2006) or the JEG-3 choriocarcinoma cells (Drenth et al., 1998). The steroidogenesis H295R and human recombinant microsomal aromatase assays are included as regulatory guidelines in both the US-EPA's Endocrine Disruptor Screening programme[3] and at level 2 of the OECD's conceptual framework[4]. All these assays have proven to be

[3] http://www.epa.gov/scipoly/oscpendo/index.htm
[4] http://www.oecd.org/document/58/0,3343,en_2649_34377_2348794_1_1_1_1,00.html

effective at identifying chemicals capable of modulating steroid hormone synthesis. However, the capacity of the microsomal assays to inform on potential *in vivo* effects, and the extrapolation of results from human cell-based assays to predict the mechanism of action in other biological systems, may not be adequate.

The most evident impacts of endocrine disrupting chemicals in the environment published to date are on aquatic organisms (Hutchinson and Pickford, 2002), which has stimulated research efforts to develop fish screening assays. A series of *in vivo* fish screening assays were validated to assess the endocrine potential of a chemical, such as the OECD's 21-days fish screening assay (OECD Test Guideline 230)[5] or the fish short term reproduction assay (OECD Test Guideline 229)[6]. Fish *in vitro* assays have the advantage over *in vivo* assays in that they are less time and resource consuming and minimise the use of animals. Moreover the conditions of *in vitro* assays are more controlled to screen and provide mechanistic data for potential endocrine disruptors. In that regard, Lee et al. (2006) developed ovarian and brain tissue cultures from Atlantic salmon to assess the effects of aromatase inhibitors. Villeneuve et al. (2007), who worked with ovary explant cultures from fathead minnow exposed to several chemicals interfering with the steroidogenesis, have previously demonstrated that such methods provide a sensitive tool to detect effects on steroidogenesis. However, these *in vitro* studies use tissues from adult and mature fish to analyse the effects of the test chemicals. The early life stage is a particularly sensitive period to exposure of chemicals interfering with the steroidogenesis (Jobling and Tyler, 2003; Scholz and Klüver, 2009). Hence, the development of *in vitro* assays focusing on the early sexual development is of high value.

The aim of this study, therefore, was to develop a rapid and sensitive assay that can be applied to identify chemicals that disrupt steroidogenesis and further to inform on their potential *in vivo* effects in juvenile fish. For this reason, we decided to develop an *ex vivo* gonad culture assay using gonads explants from juvenile brown trout (*Salmo trutta fario*), an environmentally relevant native European fish species. Although current risk assessment strategies for endocrine disruption in freshwater fish are based on the responses of the standard laboratory fish species, they are unlikely to represent the full range of fish species that may be at risk in the wild (Jobling and Tyler, 2003). Indeed, the biology and reproductive

[5] http://www.oecd.org/dataoecd/62/36/38784404.pdf
[6] http://www.epa.gov/endo/pubs/fish_assay_isr.pdf

strategy of a relatively short-lived repetitive spawning fish species, such as the fathead minnow, differs to that of many European fish species which take longer to mature and typically spawn only once or twice per spawning season. Fluctuations in hormone levels during gonad development and maturation (Hutchinson et al., 2006a) are therefore likely to vary between such species, which may in turn affect their sensitivity to the exposure of endocrine disruptors. Our focus was thus to improve our understanding of the consequences of exposure to chemicals disrupting sex steroid biosynthesis for European fish species. Brown trout is a European fish species of significant economical and cultural value which is frequently used as bioindicator to detect adverse effects of potential pollutants. Although some biomarkers for exposure to endocrine disruptors are well established in brown trout (Körner et al., 2007; Vermeirrsen et al., 2005), as yet, indicators for inhibition of the sex steroid biosynthesis are missing.

2. Material and methods

2.1 General methods

2.1.1 Chemicals

Pituitary acetone powder from salmon (P3909), 1,4,6-androstatriene-3,17-dione (ATD, 95% purity, A7710), prochloraz (99.1% purity, 45631) and tributyltin chloride (TBT, 96% purity, T50202) were purchased from Sigma-Aldrich (Buchs, CH). Testosterone (99.5% purity) was purchased from VWR International AG, (Dietikon, CH). Salmon pituitary extract (SPE) stock solution was prepared with the pituitary acetone powder from salmon in distilled water at 2.5 mg/ml, filter sterilized, aliquoted, and stored at -20°C. The proportions and concentrations of the different gonadotropins of the SPE are unknown. Stock solutions of all other chemicals were prepared in absolute ethanol (Sigma; ACS reagent 99.5% purity) and stored at 4°C.

2.1.2 Test organisms

The experiments were conducted in accordance with Swiss guidelines for animal experimental use. At least one week prior to use, mixed sex immature (1+ year) brown trout (*Salmo trutta fario*) were collected from an organic fish farm (Nadler, Rohr, Switzerland) and transferred to 200 litre glass aquaria. The aquaria were held at a temperature of 12°C with a 16:8h light:dark photoperiod and received a continuous flow of water (4 litres/min) with

aeration. The fish were fed twice daily with pellets and/or insect larvae (~1% of the total body weight), but food was withheld for a least 12 hours prior to removal of the fish for an experiment. Mean wet body weight and length of fish used in the experiments were 24.44 ± 1.08 g (± SEM, n = 42) and 13.65 ± 0.24 cm (n = 42), respectively.

2.1.3 Ovary and testis explant preparation and incubation

The *ex vivo* gonad assay was adapted from the methods described by McMaster et al. (1995) and Miura et al. (1991). The fish were anesthetized in MS-222 (100 mg/L; ethyl 3-aminobenzoate methanesulfonate, Sigma, E10521), buffered to pH 7.4. Total length and wet body weight of the fish were recorded to the nearest 1 mm and 0.01 g, respectively. Gonads were removed and placed in a glass Petri dish containing ice-cold Lebovitz medium (Sigma, L4386) supplemented with 10 mM HEPES (Sigma, H3375) and 1% penicillin-streptomycin solution (Sigma, P0781), pH 7.4. The gonads were dissected into fragments of ~1 mm^3 and randomly transferred onto insert filters (Millicell CM culture plate inserts, Sigma, Z354988) in 24-well plates (4 fragments/well). Each well contained 600 μl L-15 medium (Lebovitz medium, 10 mM HEPES, 1% penicillin-streptomycin solution and 2% synthetic serum replacement [Sigma, S9388], pH 7.4). Immediately prior to incubation, the medium was replaced with L-15 only (absolute control), L-15 supplemented with solvent (solvent control; 0.1% ethanol) or L-15 supplemented with solvent containing graded concentrations of the test substance. To minimise inter-individual variation in steroidogenic enzyme activity amongst fish, gonads harvested from a single male or female fish were used for a single replicate of all treatment groups within an experiment. Gonads harvested from a total of three male and three female fishes were used for each test substance, to provide three independent replicates for each experiment. Gonad explants were incubated for between 2 and 8 days at 12°C (depending on the aim of the experiment) with medium renewal every 2 days. At the end of the incubation period, media was removed and frozen at -80°C, for subsequent determination of sex steroid concentrations, and the gonad explants fixed for either histological or immunohistochemical evaluation.

2.1.4 Histological analysis

Gonad explants were fixed for 1 hour in Bouin's solution (Sigma, HT10132), then rinsed twice with 70% ethanol. Explants were dehydrated in a series of ethanol dilutions,

cleared in Roti Histol (ROTH, 6640, Karlsruhe, DE) and embedded in paraffin (ROTH, 6642). Sections of each explant (4 μm thickness) were cut and stained with hematoxylin (ROTH, T865) and eosin (ROTH, 7089). Stained sections were examined using a light microscope (40x magnification) to compare the morphology of the non-exposed and exposed tissue fragments with the intact gonad tissue removed directly after dissection and to confirm the stage of gonad development.

2.1.5 Immunohistochemical analysis

Gonad explants were incubated with BrdU to assess cell proliferation. The BrdU is a thymidine analog which is incorporated into the newly synthesized DNA of replicating cells and can be detected using specific antibodies against BrdU by immunohistochemistry. After culture, gonad explants were fixed overnight in 10% formalin (Roti-Histofix, ROTH, A146) and then stored in formalin 4%. Explants were dehydrated by a series of ethanol dilutions, cleared in Roti-Histol, embedded in paraffin and 4 μm thick serial sections prepared. Antigen retrieval was performed by boiling the slides in citrate buffer (10 mM sodium citrate tribasic, 0.05% Tween-20, pH 6) for 5 min in the microwave. Sections were immunostained using the avidin-biotin-immunoperoxidase technique. A monoclonal anti-BrdU antibody (Abcam, ab8955) was used as primary antibody at a 1:200 dilution in PBS mixed with 1% skimmed milk and 0.05% Tween-20. The immunohistochemical staining was processed using the Histostain Bulk kit (Zymed Lab., Invitrogen, 959943B) according to the manufacturer's protocol. The antibody reaction was visualized using a fresh substrate solution containing 3,3'-diaminobenzidine tetrahydrochloride (DAB kit, Zymed Lab., Invitrogen, 002014). The sections were counterstained with hematoxylin.

For each treatment group, eight random digital images were analysed from testes fragments of each fish using the 40x microscope objective and four digital images analysed from ovary fragments of each fish using the 4x microscope objective. From each image, the BrdU index was assessed by counting the BrdU positive germ cells and expressed as a percentage of the total number of germ cells (BrdU index; immunolabeled germ cells/total germ cells x 100).

2.1.6 Sex steroid measurement

Media samples were thawed on ice and 400 μL aliquots removed and extracted twice with five volumes of diethyl ether (Sigma, 296082) in glass tubes. The two volumes of ether were pooled and evaporated under a gentle stream of nitrogen. Samples were resuspended in 400 μL of the immunoassay buffer and immediately analysed for 17β-estradiol (E2) and testosterone (T) using commercially available immunoassay kits (Cayman chemicals Europe, E2 EIA kit 582251, T EIA kit 582701, Tallinn, EE). Additional samples were extracted from L-15 medium samples supplemented with a high test concentration of ATD (10 μg/ml), prochloraz (25 μg/ml) and T (1000ng/ml) to determine the cross reactivity of each chemical with the E2 and T antibodies.

2.2 Experimental protocols

2.2.1 Experiment I: Ovary and testis morphology after 4 and 8 days of culture

To demonstrate that gonads maintain their structural integrity during the culturing procedures, gonad explant cultures were prepared, as described above using only a single gonad from each fish (3 replicate males and 3 replicate females were used). For each fish, the second gonad was removed and fixed whole in Bouin's solution for later histological comparison with fragments cultured from the same fish for 4 to 8 days in the L-15 medium.

2.2.2 Experiment II: Effect of exposure to SPE on cell proliferation and E2 and T concentrations

Gonad explants were incubated for 4 days at 12°C with L-15 culture medium only (absolute control) or supplemented with salmon pituitary extract (SPE) at nominal concentrations of 0.5, 5, 50 μg/ml. Gonad explants from all treatment groups were then co-incubated with 100 μM BrdU (5-bromo-2-deoxyuridine, Sigma, B5002) for the 8 last hours of culture. The effect of SPE on the cell proliferation of the gonad explants was examined by calculation of the BrdU index. After 2 and 4 days of culture and before to add BrdU, media was removed and frozen at -80°C for subsequent measurement of sex steroid concentrations. At the end of the incubation, the tissue fragments were removed and fixed in 10% formalin for subsequent immunohistochemical processing.

2.2.3 Experiment III: Effect of solvents on E2 and T concentrations

To select which solvent to use to deliver hydrophobic test chemicals in the *ex vivo* gonad assay, ovary and testis explant cultures were exposed for 2 days to the L-15 culture medium only (absolute control) and to L-15 supplemented with different concentrations of two organic solvents, which are currently used in cell culture assays: ethanol and dimethyl sulfoxide (DMSO; ovary explant only) at 0.01, 0.1 and 1% v/v. At the end of the exposure, the media was removed and frozen at -80°C for later measurement of sex steroid concentrations.

2.2.4 Experiment IV and V: Effects of testosterone, 1,4,6-androstatrien-3,17-dione (ATD), prochloraz and tributyltin (TBT) exposure on E2 and T concentrations

For experiment IV, the activity of the CYP19 enzyme was indirectly evaluated by analysing E2 production in the gonad explants exposed to T, which is the substrate of the aromatase enzyme. The optimized protocols were then used in experiment V to determine whether the gonad explant cultures could be used to identify chemicals that have the potential to disrupt steroid biosynthesis. For this, chemicals with known or suspected CYP19 inhibiting activity were tested in the assay. Explant cultures were exposed for 2 days to either L-15 medium, L-15 medium supplemented with 0.1% ethanol or L-15 medium supplemented with graded concentrations of each test chemical. The different stock solutions of each chemical, prepared in 100% ethanol, were diluted 1:1000 in the L-15 culture medium to give nominal exposure concentrations of 10, 100, 1000 ng/ml for T, 100 and 1000 ng/ml for ATD, 250 and 2500 ng/ml for prochloraz and 1, 10, 100 pg/ml for TBT. The test concentrations of T, ATD and prochloraz were based on sublethal data available in the published literature (Ankley et al. 2005; Lee et al., 2006). The TBT concentrations were selected according to environmental relevant concentrations and sublethal data from fish *in vivo* experiments in the published literature (Lyssimachou et al. 2006; McAllister and Kime, 2003; Zhang et al., 2007). At the end of each incubation period, medium was removed from the culture and frozen at -80°C. The media samples were extracted and concentrations of E2 and T measured using commercially available EIA kits to assess effects on steroid biosynthesis. The gonad fragments were fixed in Bouin's solution to assess effects on the morphology of the gonad tissue.

2.3 Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). All statistical analysis was performed using SigmaStat 2004 for Windows Version 9.01 (Systat Software GmbH, Erkrath, Germany). Significant differences, relative to the absolute control (Experiment II and III) or solvent control (Experiment IV and V) were analysed using one-way analysis of variance (ANOVA) where data met the assumptions of normality and homogeneity of variance, followed by a pair-wise multiple comparison procedure (Holm Sidak test). Data which failed to pass the normality test were analysed using an ANOVA of Ranks, followed by a pair-wise multiple comparison procedure (Tukey test). Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1 General characteristics of the gonad explant culture

3.1.1 Experiment I: Ovary and testis morphology after 4 and 8 hours of culture

Comparison of the intact ovary (Fig. 1A), removed from each fish, with the ovarian tissue fragments incubated for 4 and 8 days (Fig. 1B-1C) indicated that the structure of the ovary was maintained within the explant culture. Histological analysis of the ovarian fragments did not reveal evidence of necrosis; somatic cells were found in close association with oogonia and the oocytes were well enclosed by follicular cells. Further, the stage of development was consistent with that observed for the intact ovary; ovaries were at the previtellogenic stage. In 86% of the females, oogonia at different meiotic stages and perinucleolar oocytes with the Balbiani vitelline bodies could be observed. In the remaining 14% of females a few early vitellogenic oocytes were present.

The structure of the testis explants also appeared intact after 4 days of culture (Fig. 1E) when compared with the structure of the intact gonad (Fig. 1D). However, the tissue of 25% of the testis fragments cultured for 8 days showed some necrosis with degeneration of germ cells and vacuolation of Sertoli cells in the centre of the tissue fragments (Fig. 1F). All males used to prepare the testis explant cultures were in the pre-spermatogenic stage I containing only spermatogonia surrounded by somatic cells.

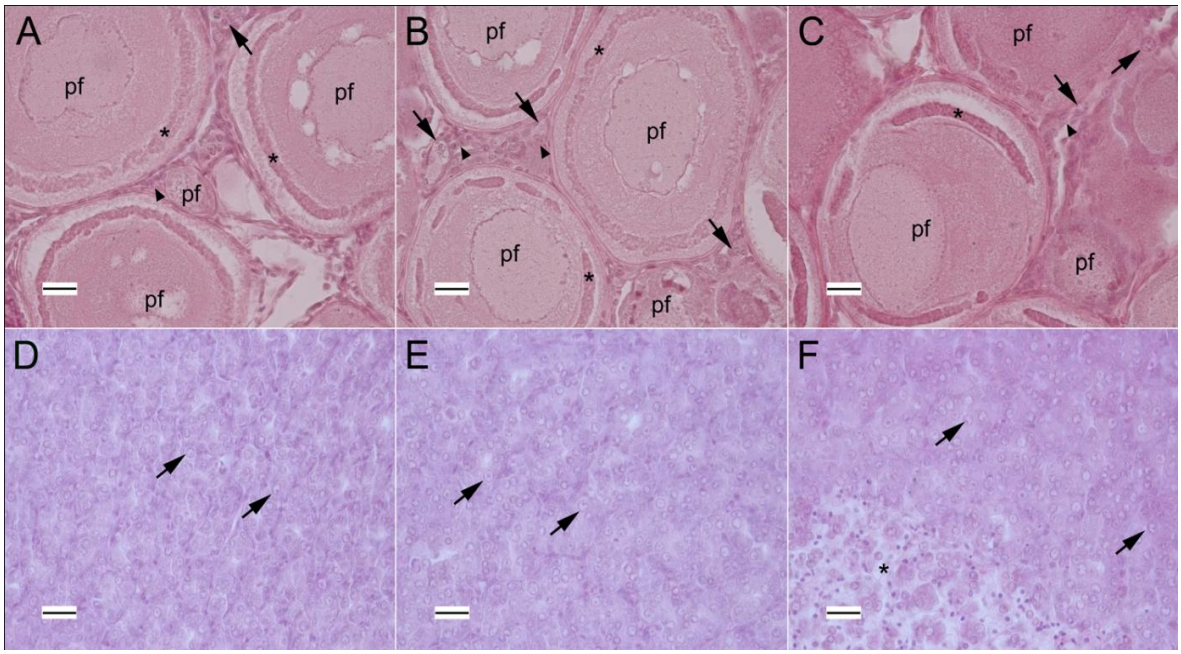

Fig. 1. Comparison of the morphology of intact gonads with ovary and testis cultured explants. A: Intact ovary fixed in Bouin's directly after dissection. B-C: Ovary explants cultured for 4 (B) and 8 days (C). Ovary explants in previtellogenic stage with oogonia (arrows), perinucleolar follicles (pf) with Balbiani bodies (asterisks). Bar = 50 μm. D: Intact testis fixed in Bouin's directly after dissection. E-F: Testis explants cultured for 4 days (E) and 8 days (F). Testis explants in pre-spermatogenic stage I with spermatogonia (arrows). F: Testis explants with degeneration of germ cells and vacuolation of Sertoli cells (asterisk). Bar = 25 μm.

These initial investigations confirmed that the methods described for the gonad cultures would enable successful culture of both ovarian and testicular tissue for a period of up to 4 days. At this stage of the study, a culture of 4-days was therefore used for the subsequent experiments to analyse if test chemicals could influence the morphology and the activity of the steroidogenic enzymes of the cultured explants.

3.1.2 Experiment II: Effect of exposure to SPE on cell proliferation and on E2 and T concentrations

BrdU-labelling of the germ cells was used firstly to determine whether the gonad explants would maintain their ability to proliferate *ex vivo*. Evaluation of both ovary and testis explants, after 4 days of culture, confirmed that BrdU was incorporated in both somatic and germ cells (Fig. 2A-3A) indicating that the cells continued to proliferate. In teleosts, as in other vertebrates, gonadotropins mediate their effects through the

production of sex hormones by the somatic cells in interaction with the germ cells (Nagahama, 1994). The BrdU staining of somatic cells, therefore, indicated that the gonad explants were physiologically functional, supporting the histological analysis that the tissue is healthy, and thus further demonstrating the suitability of the culture conditions described.

BrdU-labelling of the germ cells was also used to evaluate the capacity of the explant cultures to respond to gonadotropins at a cellular and physiological level after exposure for 2 and 4 days with graded concentrations of SPE. Gametogenesis is regulated by the pituitary-gonadal axis in vertebrates and gonadotropins have previously been shown to induce *in vitro* mitosis and germ cell proliferation in immature gonads of fish (Amer et al., 2001; Miura et al., 1991; Miura et al., 2007). As SPE contains gonadotropins, and both the luteinizing hormone and the follicle stimulating hormone have been shown to promote gametogenesis by stimulating the production of sex steroid hormones (Taranger et al., 2010), it was expected to increase germ cell proliferation in the *ex vivo* gonad assay. In our cultures, there was no evidence that exposure to graded concentrations of SPE for a period of up to 4 days affected cell proliferation (BrdU index) in either the ovary (Fig. 2B, $p = 0.381$) or testis (Fig. 3B, $p = 0.466$) explants, when compared to the control group. It should be noted, however, that although both Amer et al. (2001) and Miura et al. (2007) used a similar *ex vivo* gonad culture method to ours, albeit with different fish species, they exposed testis and ovarian fragments to various steroids and hormones for a longer period of 15 days before inducing gametogenesis and detecting an increased cell proliferation in the gonad explants. The lack of an effect of SPE in our cultures may, therefore, be a consequence of the relative short period of exposure used. It is possible that prolonged periods of culture are required to enable the gonadotropins to induce biosynthesis of the gonadal steroid hormones that mediate various stage of gametogenesis. If this is the case, the ability of our *ex vivo* gonad assay to detect rapid responses at the morphological level may be limited.

Biochemical analysis of E2 and T production in the media, after 2 and 4 days, indicated that E2 production in the ovary explants tended to increase with increasing concentrations of SPE at both day 2 (Fig. 2C, $p = 0.188$) and day 4 of culture (Fig. 2D, $p = 0.151$), although these trends were not significant. T production in the ovary explants exposed to 5 and 50 μg/ml SPE, however, was significantly increased after both 2 (Fig. 2E) and 4 (Fig. 2F) days of culture ($p < 0.05$). The higher levels of T production, compared to E2, in the ovary explants exposed to SPE most likely reflects that aromatase activity is quite low,

as it would be expected in previtellogenic ovary. Indeed, the CYP19 gene expression and activity increase during oogenesis till the oocyte final maturation (Cheshenko et al., 2008). Further, a number of studies report on the role of androgens in the regulation of oocyte growth in previtellogenic ovary (Kortner et al., 2008; Tosaka et al., 2010). Therefore, our results suggest that the induction of T production may dominate over the E2 production after exposure to SPE. It may also be possible that E2, once produced, has only a short-half life in the medium; Haddy and Pankhurst (1998) demonstrated that an incubation of isolated ovarian follicles of rainbow trout with human chorionic gonadotropin (hCG) resulted, after 12 to 18 hours, in metabolic losses of E2 from the incubation media.

Testis fragments exposed to SPE for 2 days exhibited an increase of T production at 50 μg/ml (Fig. 3E, $p < 0.05$), but in contrast to the ovarian cultures, E2 production showed a decrease at 50 μg/ml (Fig. 3C, $p < 0.05$). After 4 days of culture, the lower levels of E2 in all treatment groups meant that differences in E2 production between the SPE treated groups and the control could no longer be detected (Fig. 3D, $p = 0.109$), although T production was still increased at 5 and 50 μg/ml when compared to the control (Fig. 3F, $p < 0.05$). An increase in T production, in response to SPE exposure, is consistent with expectation as the regulation of spermatogenesis by gonadotropins is mediated by activation of the Leydig cells that produce sex steroid hormones (Nagahama, 1994; Schulz et al., 2010). The observed decreases in E2 after 2 days of exposure to SPE, however, were not expected. It should be noted that although estrogens are only present at low concentrations in the blood serum of male teleosts, they are involved in the regulation of spermatogenesis (Miura et al., 1999; Amer et al., 2001) and may, therefore, be influenced by exposure to gonadotropins. However, Miura et al. (1999) did not observe effects on serum levels of E2 in eel injected with hCG.

Collectively, the observed effects of SPE on the measured levels of E2 and T in the media, indicate that the gonad explants maintain their steroidogenic capacity and can respond to gonadotropin signalling. Although the patterns of E2 and T production for both ovary and testis explants were similar after 2 and 4 days of culture, the levels of both sex steroids were generally lower after 4 days of culture, indicating either a reduction in enzyme activities or that the exo- endogenous precursors are becoming limited. For the subsequent chemical exposures, a 2 day incubation time was therefore used.

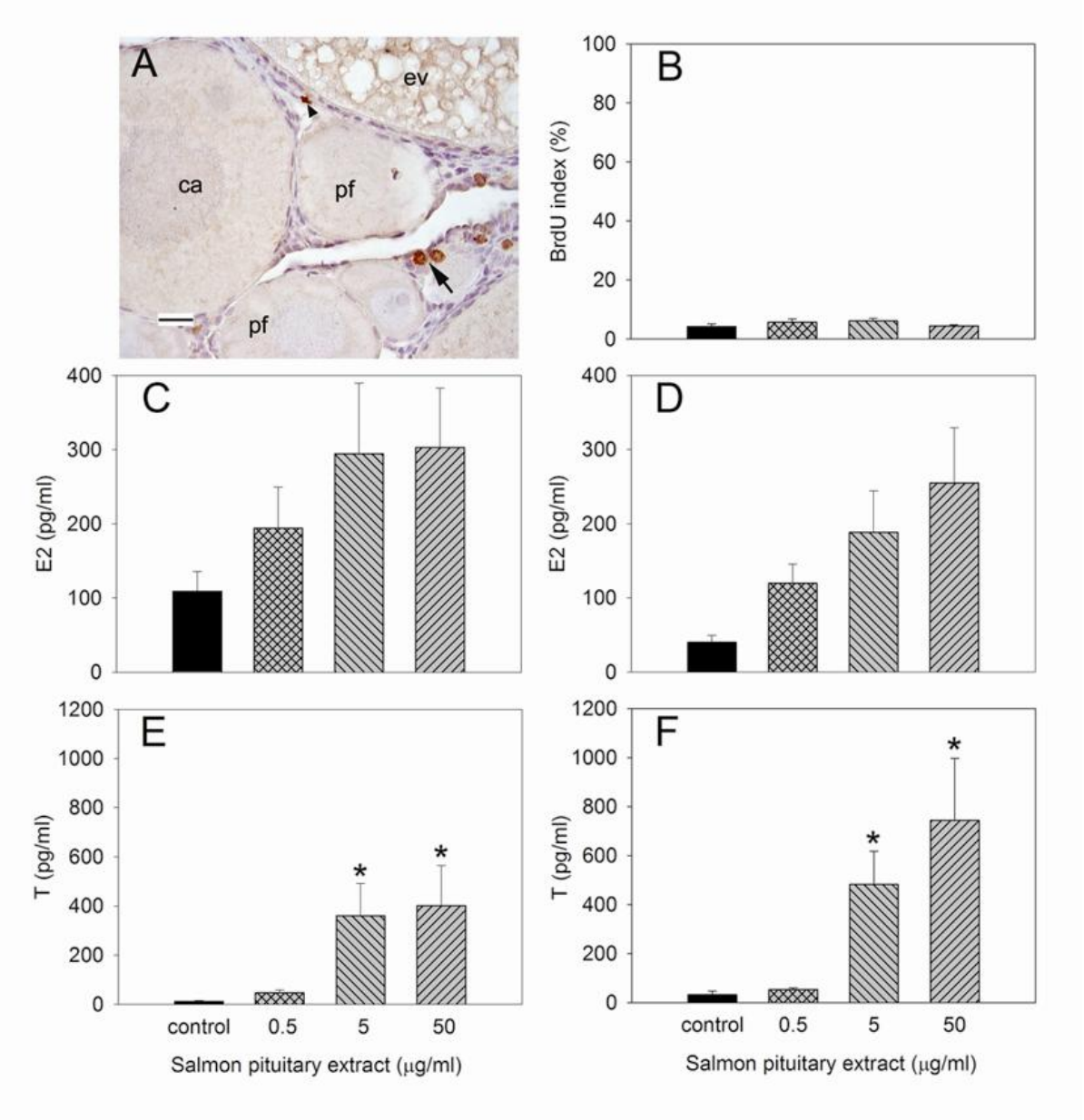

Fig. 2. Ovary explants exposed to SPE. A: Immunoreaction of BrdU in ovary explant with immunostained oogonia (arrow) and follicular cells (arrowhead). Pf, perinucleolar oocyte; ca, cortical alveolar oocyte; ev, early vitellogenic oocyte. Bar = 50 μm. B: BrdU index after 4 days of culture. The number of proliferated germ cells is expressed as a percentage of the total number of germ cells; n = 4. C-D: E2 levels of the ovary explant cultures exposed to SPE for 2 days (C) and 4 days (D); n = 3. E-F: T levels of the ovary explant cultures exposed to SPE for 2 days (E) and 4 days (F); n = 3. Results are given as means ± SEM. Bars marked with asterisk are significantly different from the control ($p < 0.05$).

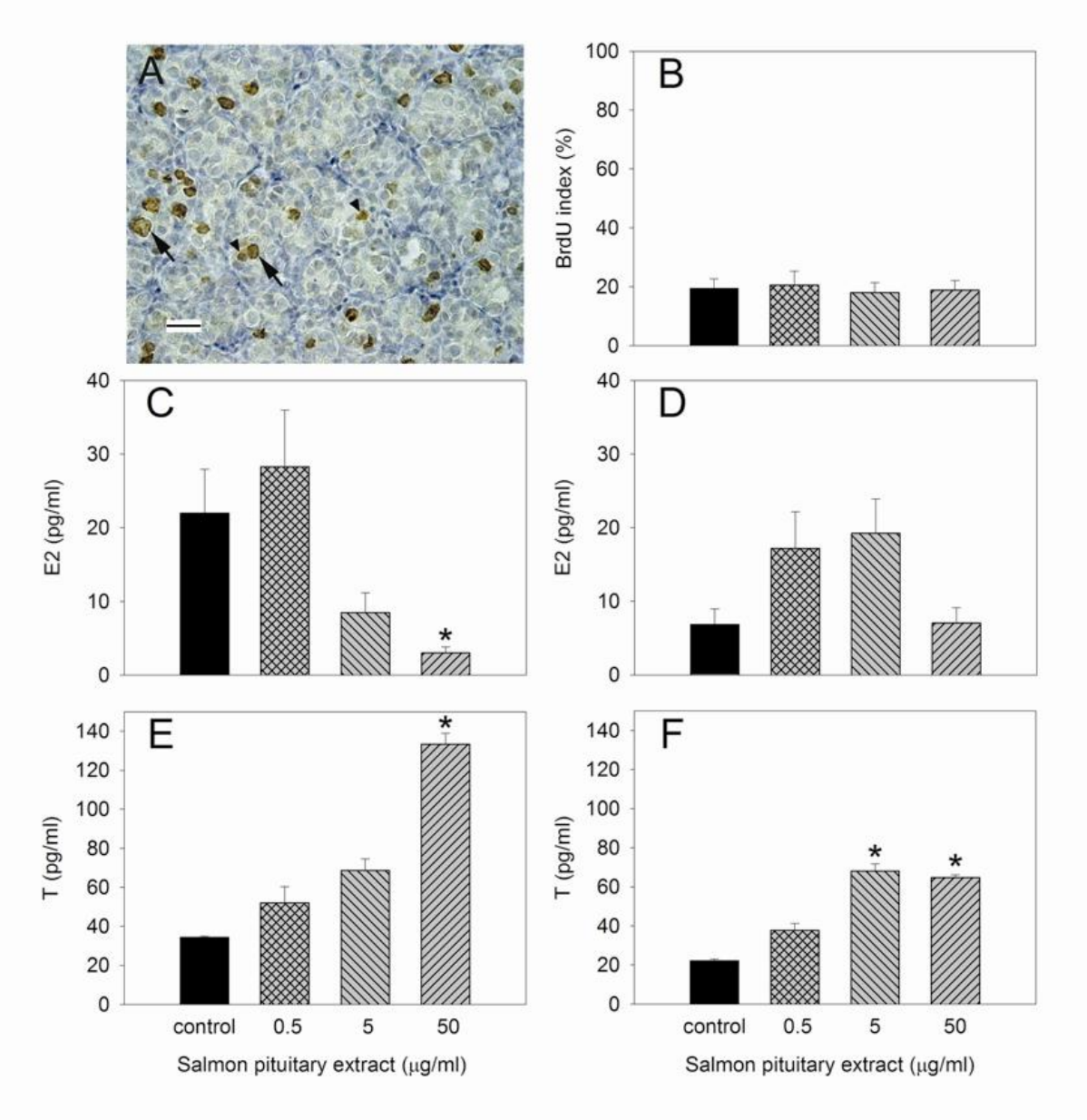

Fig. 3. Testis explants exposed to SPE. A: Immunoreaction of BrdU in testis explant with immunostained spermatogonia (arrow) and Sertoli cells (arrowhead). Bar = 25 µm. B: BrdU index after 4 days of culture. The number of proliferated germ cells is expressed as a percentage of the total number of germ cells; n = 8. C-D: E2 levels of testis explant cultures exposed to SPE for 2 days (C) and 4 days (D); n = 3. E-F: T levels of the testis explant cultures exposed to SPE for 2 day (E) and 4 days (F); n = 3. Results are given as means ± SEM. Bars marked with asterisk are significantly different from the control ($p < 0.05$).

3.1.3 Experiment III: Effects of solvents on E2 and T concentrations

Several *in vitro* and *in vivo* studies have reported that some organic solvents used as carriers to deliver hydrophobic substances in aquatic ecotoxicological studies may have the potential to modulate endocrine responses (see Hutchinson et al., 2006b). Therefore, exposure to different concentrations of ethanol (EtOH) and dimethyl sulfoxide (DMSO) were conducted here, to evaluate the potential effects of solvents on E2 and T biosynthesis in the gonad explant cultures. No significant difference of E2 ($p = 0.932$ and $p = 0.165$ for ovary and testis respectively) or T production ($p = 0.740$ and $p = 0.752$ for ovary and testis respectively) were observed between the different EtOH treatment groups in a concentration range of 0.01 to 1% v/v in both ovary and testis cultures (Fig. 4 A to D). Similarly exposure to DMSO in a concentration range of 0.01 to 1% v/v did not affect E2 ($p = 0.942$) or T production ($p = 0.904$) in the ovary explants (data not shown). Based on the clear lack of an effect of EtOH on either E2 or T production within both ovary and testis cultures, a concentration of 0.1% EtOH was selected for use in the subsequent experiments to evaluate the effects of exposure to different chemicals on sex steroid production.

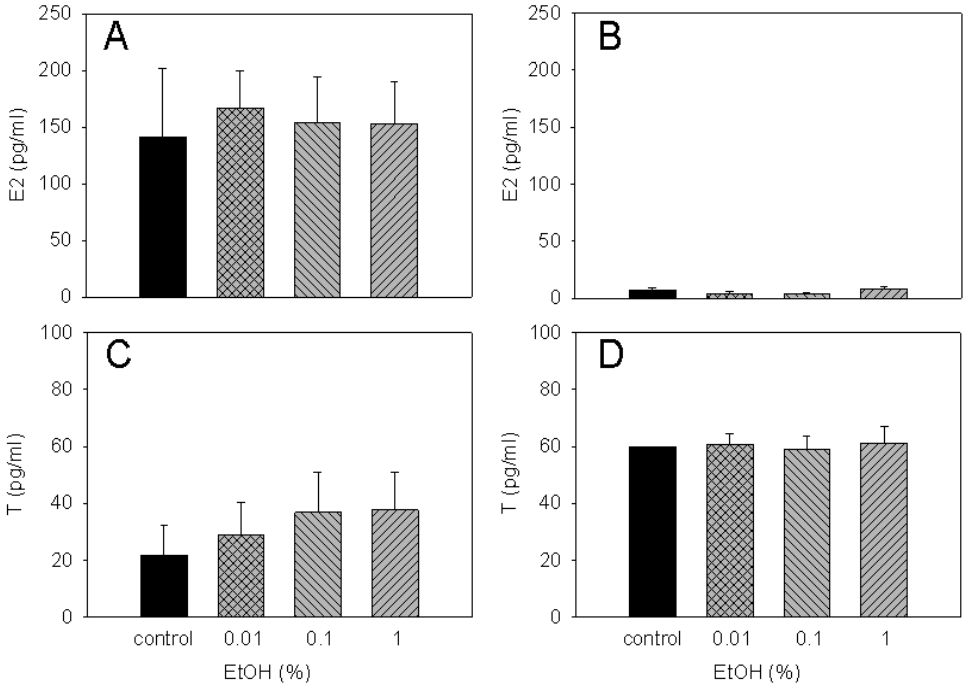

Fig. 4. E2 and T production of ovary and testis explants exposed to EtOH. A-B: E2 levels of ovary (A) and testis (B) explant cultures. C-D: T levels of ovary (C) and testis (D) explant cultures. Results are given as means ± SEM; n = 3 for control; n = 6 for EtOH treatment groups. Bars marked with asterisk are significantly different from the control ($p < 0.05$).

3.1.4 Experiment IV: Effects of T exposure on E2 and T concentrations

The ovary and testis explants exposed to a T concentration of 1000 ng/ml showed increased E2 levels ($p < 0.05$) compared with the solvent control (Fig. 5A). The relatively high concentration of T supplied to induce the E2 production suggested again that the CYP19 activity might be low in the ovarian tissue of our cultures. Moreover, cross-reactivity examination indicated that T at 1000 ng/ml could react with E2 antibodies of the immunoassay kit (245 ± 3 pg/ml E2 equivalence). Further, between 50.1 and 63% (Fig. 5C) and between 55.6 and 65.4% (Fig. 5D) of the T added to the medium was metabolized by the ovary and testis explants respectively. Therefore, the increase of E2 levels might be associated to the excess of T in the media and the cross-reactivity of T might account for the apparent E2 production observed. Earlier studies with vitellogenic follicles from mature fish (Afonso et al, 1997; Haddy and Pankhurst, 1998; Lee et al, 2006), indicated that T exposure could induce E2 synthesis and was a limiting factor for the production of E2. Our results do not suggest the same conclusion. Potentially this is due to differences in the amount of tissue and/or the maturity of fish. Indeed, the vitellogenic follicles used in these studies, which possess higher levels of aromatase activity, are stimulated to produce E2 after exposure of a low T concentration of 10 ng/ml.

Levels of E2 might also be lower because of losses of E2 from the incubation media. Haddy and Pankhurst (1998) showed that decreased levels of E2 after 18h incubation of ovarian vitellogenic follicles from rainbow trout were correlated with increased metabolism of E2 to E2-glucuronide over the culture time. These results suggest that incubation time is an important parameter to assess sex steroid levels in the media. Since the incubation time is a critical parameter and the goal was to develop a rapid assay, a time course experiment in a range of 4 to 48 hours culture would in the future work be highly relevant to optimize the assay. However, T and SPE exposure results indicated that the CYP19 activity was quite low. Hence, a shorter time of culture might not be sufficient to analyse effects of chemicals interfering with the whole steroidogenic pathway, including the CYP19. According to this analysis, for all subsequent chemical exposures, a 2 day incubation time was therefore used.

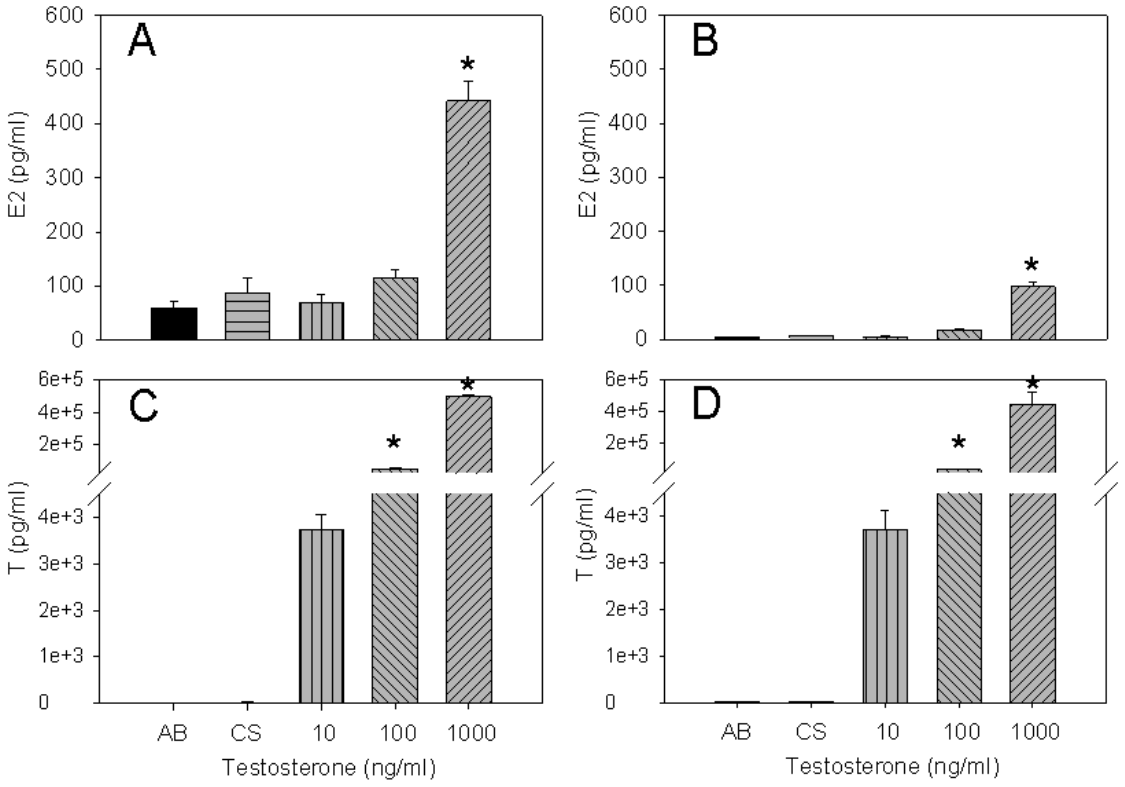

Fig. 5. E2 and T production of ovary and testis explants exposed to T. A-B: E2 levels of ovary (A) and testis (B) explant cultures. Part of the effect might be due to cross-reactivity of T with the E2 antibodies of the immunoassay kit (245 ± 3 pg/ml E2 equivalents of T at 1000 ng/ml). C-D: T levels of ovary (C) and testis (D) explant cultures. AB, absolute control; CS, control solvent. Results are given as means ± SEM; n = 3. Bars marked with asterisk are significantly different from the control solvent ($p < 0.05$).

3.2 Experiment V: Effects of 1,4,6-androstatrien-3,17-dione (ATD), prochloraz and tributyltin (TBT) exposure on E2 and T concentrations

There was no evidence that exposure of the gonad explants to each of the test chemicals affected the morphology of either the testis or ovary fragments when compared with either the control explants or the intact gonad removed directly after dissection (data not shown).

3.2.1 ATD and prochloraz exposures

Incubation of the ovary explants with ATD at concentrations of 100 and 1000 ng/ml reduced E2 production ($p < 0.05$) when compared to the control solvent (Fig. 6A). Together

with the E2 decreased levels, a proportional accumulation of T was expected and observed in the ovary cultures (Fig. 6C). An accumulation of T was also observed in the testis cultures (Fig. 6D), but contrary to expectation ATD did not inhibit E2 production ($p = 0.136$). Further analysis revealed that ATD was cross reacting with both the T and E2 antibodies in the immunoassay kits (211 ± 27 pg/ml E2 equivalence and 7750 ± 278 pg/ml T equivalence due to cross-reactivity of ATD added at 10 μg/ml in the medium). This would appear to explain the apparent E2 production at 1000 ng ATD/ml in the testis cultures. Lee et al. (2006) also observed that ATD cross-reacted with the E2 antibodies of their radioimmunoassay which highlights the need for caution when interpreting the data from immunoassays. However, the marked reduction in E2 production of the ovary explants exposed to the lower concentration of ATD (100 ng/ml) confirmed that ATD was acting as an aromatase inhibitor within our assay. This is consistent with the findings of Lee et al. (2006) who also observed a decrease of E2 production from salmon ovarian follicles exposed to 100 ng/ml ATD. Further, Hinfray et al. (2006b) demonstrated that aromatase activity was greatly decreased in the ovaries of zebrafish exposed for 7 days to ATD at 1 μM (~300ng/ml), indicating that the effect of ATD in our *ex vivo* gonad assay is highly comparable with the *in vivo* effects of this chemical.

Exposure to prochloraz also inhibited E2 production ($p < 0.05$) in the ovary culture, when compared to the solvent control, at concentrations of 250 and 2500 ng/ml (Fig. 6E). However, in contrast to ATD, exposure of the ovary explants to prochloraz did not induce an accumulation of T, rather T showed a tendency to decrease with increased prochloraz concentrations, although this was not significant when compared to the solvent control (Fig. 6G, $p = 0.356$). Exposure of testis cultures to prochloraz did not affect E2 production, when compared to the solvent control (Fig. 6F, $p = 0.131$), but T concentrations were decreased at a concentration of 250 ng/ml (Fig. 6H, $p < 0.05$). Cross-reactivity analysis of prochloraz at 25 μg/ml in the medium revealed a cross-reaction with E2 antibodies of 57 ± 22 pg/ml E2 equivalence and with T antibodies of 120 ± 28 pg/ml T equivalence. A cross- reactivity would explain that the inhibition of T production in testis cultures exposed to 2500 ng/ml of prochloraz was not significant. Nevertheless, the potential cross-reactivity does not modify the interpretation of the results. This profile of effects is highly consistent with the findings of Ankley et al. (2005) who similarly demonstrated that plasma E2 concentrations in male fathead minnow exposed *in vivo* to prochloraz (300 ng/ml) were not altered, while the

concentrations of T were significantly decreased. The inhibition of E2 and reduction of T production suggests that prochloraz may be inhibiting other CYP enzymes in the steroidogenic pathway upstream of CYP19. In support of this, Blystone et al. (2007) had previously hypothesised that the increased levels of progesterone and reduced production of T observed in *ex vivo* fetal testes taken from pregnant rats fed with prochloraz resulted from an inhibition of the lyase/hydroxylase enzyme (CYP17). Yet our data show that prochloraz is a more potent suppressor of E2 production than of T production. Hecker et al. (2006) likewise observed in the H295 R cell line exposed to prochloraz in a concentration range of 0.003 to 1 μM that the E2 production was more sensitively inhibited than the T production. Similarly the inhibitory effect of prochloraz in ovary explants from fathead minnows in a concentration range of 0.8 to 15 μM was more pronounced on E2 production than on T production (Villeneuve et al., 2007).

The results of this experiment imply that the *ex vivo* brown trout gonad assay has the potential to identify chemicals that disrupt the activity of enzymes involved in the synthesis of E2 and T, including the different CYP enzymes and hydroxysteroid dehydrogenases of the sex steroid biosynthesis. Further, the high consistency between our findings and those of the *in vivo* assay of Ankley et al. (2005) would suggest that the *ex vivo* gonad assay shows potential for use in predicting *in vivo* effects.

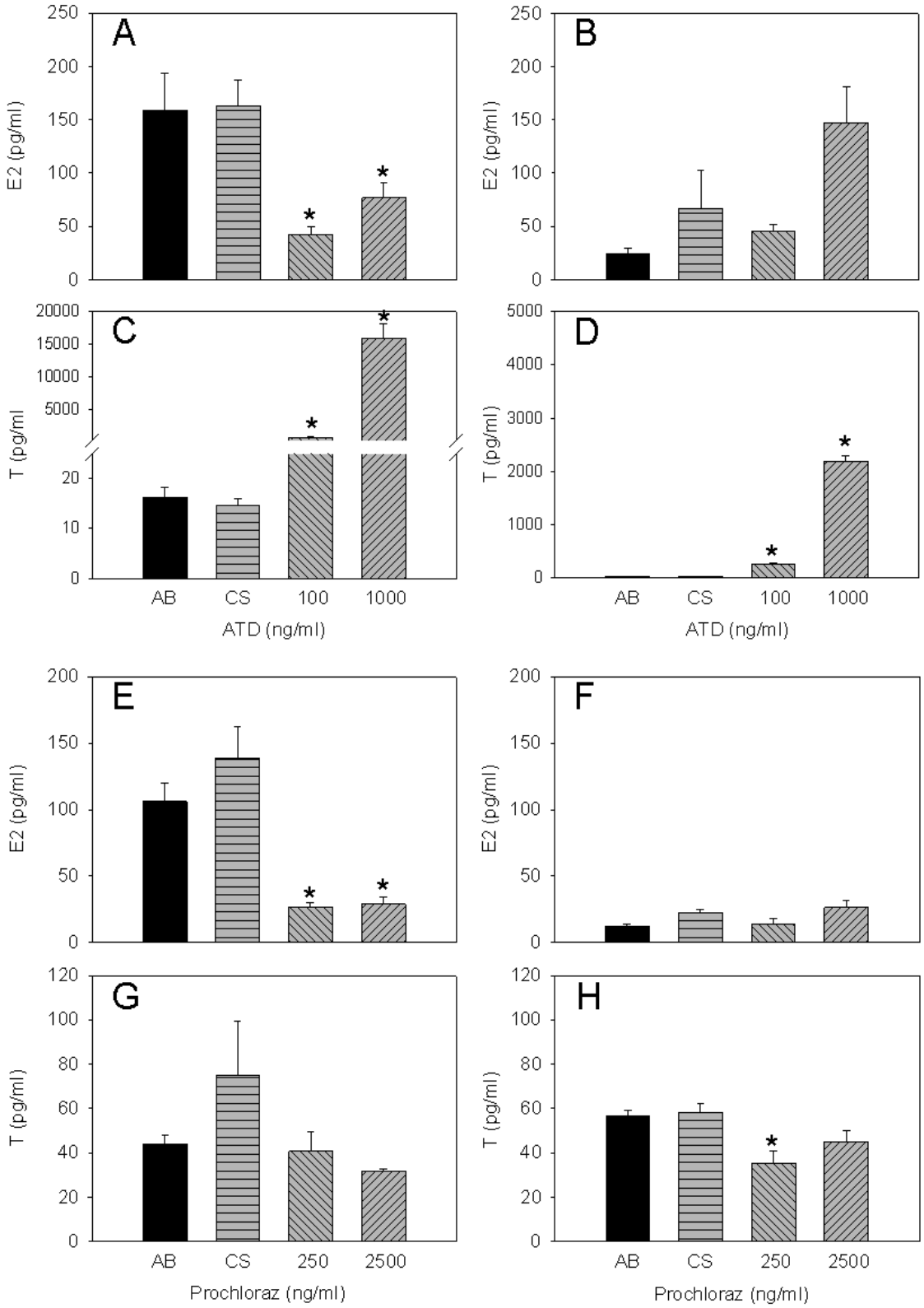

Fig. 6. E2 and T production of ovary and testis explants exposed to ATD and prochloraz. A-B: E2 levels of ovary (A) and testis (B) explant cultures exposed to ATD. C-D: T levels of ovary (C) and testis (D) explant cultures exposed to ATD. E-F: E2 levels of ovary (E) and testis (F) explant cultures exposed to

prochloraz. G-H: T levels of ovary (G) and testis (H) explant cultures exposed to prochloraz. AB, absolute control; CS, control solvent. Results are given as means ± SEM (n = 3). Part of the effect might be due to cross-reactivity of ATD and prochloraz with E2 and T antibodies of the immunoassay kits (211 ± 27 pg/ml E2 equivalents and 7750 ± 278 pg/ml T equivalents of ATD at 10 μg/ml; 57 ± 22 pg/ml E2 equivalents and 120 ± 28 pg/ml T equivalents of prochloraz at 25 μg/ml). Bars marked with asterisk are significantly different from the control solvent ($p < 0.05$).

3.2.2 TBT exposure

E2 production in the ovary and testis explants exposed to 1, 10 and 100 pg/ml of TBT was not modified when compared to the solvent control (E2 production, $p = 0.920$ and $p = 0.525$ for ovary and testis explant cultures respectively). The T production from ovary explant cultures remained unchanged ($p = 0.305$), but was reduced in the testis explant cultures when compared to the solvent control ($p < 0.05$). However, the T production was not modified when compared to the absolute control. This difference might be due to a possible false laboratory manipulation of the solvent used in the control solvent treatment group, which consequently might lead to a contamination of the solvent with a chemical that can cross-react in the T immunoassay. Another possibility might be a bad manipulation during the sex steroid extraction of the media. Therefore, the reduction of T compared with the control solvent in the testis culture might be a spurious result.

Exposure to TBT had previously been associated with imposex (the development of male sexual characteristics in females) in marine molluscs (Spooner et al., 1991), and it had been hypothesised that TBT and other organotin compounds might induce these effects through increasing androgen levels via inhibition of CYP19 activity (Bettin et al., 1996). In support of this, expression of the aromatase gene (*cyp19)* was previously shown to be suppressed in genetically female Japanese flounder that had been masculinised through TBT exposure (Shimasaki et al., 2003). TBT has also been shown to induce masculinisation of zebrafish at extremely low levels (0.1-100 pg/ml) and a high incidence of sperm lacking flagella (McAllister and Kime, 2003). Further, it was shown that this masculinising effect could be blocked and neutralised by co-exposing fish with TBT and ethinylestradiol (Santos et al., 2006). Another study with female cuvier exposed to environmental levels of TBT (1-100 pg/ml) also showed elevated testosterone levels and inhibition of ovarian development (Zhang et al. 2007).

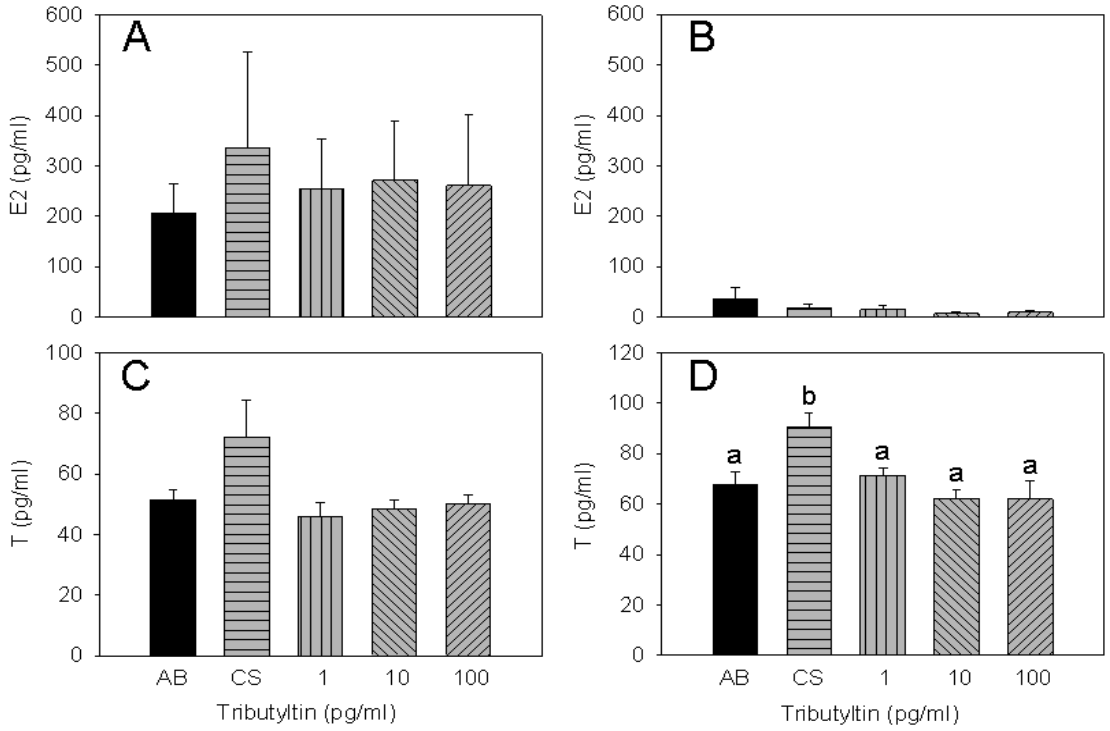

Fig. 7. E2 and T production of ovary and testis explants exposed to TBT. A-B: E2 levels of ovary (A) and testis (B) explant cultures. C-D: T levels of ovary (C) and testis (D) explant cultures. For the T levels of testis cultures (D), the results of the treated groups were compared to the absolute control because of a possible false positive reaction in the control solvent group. AB, absolute control; CS, control solvent. Results are given as means ± SEM (n = 3). Bars marked with different letters are significantly different ($p < 0.05$).

The apparent lack of effect of TBT in our assay would appear to imply that TBT is not acting as a direct inhibitor of aromatase activity. In support of this, several recent studies also report conflicting results for TBT exposure. For example, Hano et al. 2007 demonstrated that exposure to TBT caused concentration-dependent mortality and impaired embryonic development of injected embryos of medaka, but no masculinisation was observed. Further, Lyssimachou et al. (2006) demonstrated that brain CYP19 activity and mRNA expression could be both elevated or inhibited in juvenile Atlantic salmon depending on the concentration and on the time of exposure of TBT. In *in vitro* studies, TBT exhibits different effects depending on the cellular model used. In both H295R (Sanderson et al., 2002) and KGN cells (Ohno et al., 2004), TBT exhibited inhibitory effects on CYP19 activity, but only at very high concentrations, which was attributed to cell toxicity. On the other hand, TBT at

concentrations ranging from 10 to 100 nM, caused a dose-dependent increase of both CYP19 activity and mRNA expression in human choriocarcinoma cell lines (Laville et al, 2006; Nakanishi et al., 2002). Further, hepatocytes isolated from salmon exposed to 0.01-5 μM TBT showed differential expression patterns dependent on the exposure time and concentration of TBT (Mortensen and Arukwe, 2009). These studies indicate that the alteration of CYP19 activity by exposure to TBT might be regulated at the transcriptional level.

4. Conclusion

The establishment of our *ex vivo* gonad assay was important to identify endocrine disrupting chemicals that might interfere in the steroidogenesis of the juvenile brown trout, and therefore potentially impair its sexual development and reproductive capacity. The results of this study show that the *ex vivo* brown trout gonad assay has the potential to provide more understanding of fish reproductive toxicology and to inform on possible adverse effects of environmental hazardous substances.

The first steps of this study confirmed that, once established, preparation of the trout gonad culture was simple and rapid to achieve. The SPE and T experiments showed however some limitations of the assay in that the aromatase activity is quite low in the previtellogenic ovary explants and the responses of the assay vary according to the time of exposure. It would therefore be interesting to further characterize the assay by doing time course experiments with the focus to optimize the exposure time of the assay. Nevertheless, exposure of the juvenile brown trout gonad explants to different chemicals resulted in different hormone concentration patterns consistent with their mode of action. This indicated that hormone production could be used, within the *ex vivo* trout gonad assay, as a suitable and sensitive endpoint for identifying potential endocrine disruptor interactions in the steroidogenic pathway. In addition to screen for chemicals disrupting the steroidogenic pathway, such an assay gives the potential to study the mode of action of these chemicals. This assay has also an advantage over other *in vitro* assays using cancer cell lines, as it is more species/tissue relevant and should be more predictive of *in vivo* effects, although further work is required to confirm this. If this can be demonstrated, then the *ex vivo* gonad assay will offer the benefit, when compared to *in vivo* studies, in that it is less time and material consuming and uses considerably less fish.

Acknowledgements

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3. Paper 2

Comparative *ex vivo* and *in vivo* effects of prochloraz and tributyltin on sex hormone biosynthesis in juvenile brown trout (*Salmo trutta fario*)

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Abstract

Screening assays for chemicals that have the potential to disrupt reproduction in vertebrates through binding the sex hormone receptors are widely available. However, development of methods for identifying chemicals that disrupt steroidogenesis has received less attention. In this study, disruption of gonadal steroidogenesis in juvenile brown trout exposed to prochloraz and tributyltin was compared in a 2-day *ex vivo* gonad assay and a 2-day *in vivo* exposure, to assess the applicability of the *ex vivo* assay for informing on the effects of these chemicals. In both the *ex vivo* and *in vivo* assays, exposure to prochloraz (32 to 320 μg/L) induced concentration-related decreases in ovarian E2 concentrations ($p < 0.05$), while ovarian T concentrations remained unchanged. While concentrations of T were suppressed in the *ex vivo* testis cultures (320 μg/L; $p < 0.05$), there was no evidence for a suppressive effect on T concentrations in the 2-day *in vivo* exposure. Exposure to TBT did not affect ovarian concentrations of E2 and T in either the *ex vivo* or *in vivo* assays, but testicular concentrations of T were suppressed in the *ex vivo* assay (1 μg/L; $p < 0.05$) and in the 19-day *in vivo* exposure (1 μg/L; $p < 0.05$). The difference in sensitivity of response between the *ex vivo* and *in vivo* assays for both chemicals most likely reflects differences in the exposure route and uptake of the chemicals, in bioaccumulative and metabolic steps, and in different interactions of the test chemicals that take place in the *ex vivo* or *in vivo* exposures. Further, exposure of juvenile brown trout to TBT for 19 days (1 μg/L; $p < 0.05$) modified the gonad development stage and led to decrease of perinucleolar oocytes and increase of early vitellogenic oocytes in females, while in males, the area of spermatocyte and spermatid cysts increased. These histopathological changes in the gonads of juvenile brown trout are indicative of potential reproductive dysfunction of TBT long term exposure. These results suggest that the *ex vivo* assay has the potential to be used as a screening tool for identifying chemicals that have the potential to disrupt steroidogenesis in fish and potentially induce reproductive adverse effects.

1. Introduction

The regulation and balance between androgen and estrogen levels is essential during sexual development, differentiation and reproduction in all vertebrates, including fish (Norris, 2007). However, many chemicals found in the environment have the potential to disrupt the balance of these sex hormones leading to impaired sexual development and reproduction in aquatic organisms (Scholz and Klüver, 2009; Sumpter, 2005). These chemicals, termed endocrine active chemicals (EACs), have been shown not only to exert their effects through direct interaction with the sex hormone receptors, as agonists or antagonists (Sonnenschein and Soto, 1998), but also through disruption of sex steroid hormone biosynthesis (Sanderson, 2006). In vertebrates, the sex steroid hormones are synthesized in several organs and predominantly in the gonads (Norris, 2007). The key enzymes involved in their biosynthesis consist of several specific cytochrome P450 enzymes (CYPs), hydroxysteroid dehydrogenases (HSDs), and steroid reductases (Sanderson, 2006). Of particular importance are the key enzymes for the production of androgens and estrogens, 17a-hydroxylase/C_{17-20} lyase (CYP17) and aromatase (CYP19), respectively.

A number of EACs have been shown to alter the activity and/or the expression of one or more enzymes involved in sex hormone biosynthesis in both *in vitro* and *in vivo* studies. These chemicals include natural products, pesticides, pharmaceuticals and other environmental anthropogenic contaminants (Cheshenko et al., 2008; Sanderson, 2006). Given the potential occurrence of these steroidogenic inhibitors in the aquatic environment (Gracia et al., 2008; Grund et al., 2011) regulatory agencies and industry require bioassays that can be used to screen for such EACs. Ideally these bioassays should also further our understanding of the potential risks that these EACs pose to the health of wildlife and humans (Ankley et al., 2010). So far ecological risk assessment of chemicals has focused on whole animal testing, notably with fish, to provide meaningful apical and integrated endpoints suitable to assess the effects of potential EACs, including effects on survival, development and reproduction (Castaño et al., 2003). However, as an alternative to *in vivo* assays for potential use in risk assessment, *in vitro* (or *ex vivo*) testing may offer advantages in allowing rapid screening of large numbers of chemicals and, in some cases, for informing on their specific modes of action (Castaño et al., 2003).

The aim of this study was to determine whether an *ex vivo* brown trout (*Salmo trutta fario*) gonad assay, previously shown to be sensitive to the effects of EACs that interfere with

steroidogenesis (a Marca Pereira et al., 2011), could be used to inform on their effects *in vivo*. Juvenile brown trout were exposed for 2-days, in a continuous flow-through exposure system to graded concentrations of an imidazole fungicide, prochloraz, and an organotin compound, tributyltin (TBT). At the end of the exposure, gonadal levels of 17β-estradiol (E2) and testosterone (T) were determined to assess effects of exposure on steroidogenesis. The profile of effects on the concentration of the sex hormones were then compared with the results of the 2-day *ex vivo* gonad assay, in which extracts of water samples collected from the *in vivo* exposure tanks were tested, to determine whether effects on steroidogenesis were consistent between the *in vivo* and *ex vivo* assays. As there was no evidence for an effect of TBT in the 2-day *in vivo* exposure, a longer 19-day exposure duration was also included to confirm the results of the *ex vivo* assay and to further investigate whether an altered sex hormone profile would result in a altered gonadal development of the gonads.

2. Material and methods

2.1 Test organisms

All animal exposures were conducted in accordance with Swiss guidelines for animal experimental use. At least 10 days prior to use, mixed sex immature (1+ year) brown trout were collected from a local organic fish farm (Nadler, Rohr, Switzerland) and acclimated in 160 litre glass tanks in which conditioned water was continuously aerated (1:1 charcoal filtered tap water:reverse osmosis water, UV treated) at a temperature of 12°C with a 16:8h light:dark photoperiod. Stock fish were fed twice daily with pellets (~1% of the total body weight per day), with food withheld for at least 24 hours prior to exposure. Mean wet body weights and fork lengths of the fish used in the *in vivo* exposures were 34.88 ± 10.2 g and 13.26 ± 1.38 cm (mean ± standard deviation, N = 300), respectively.

2.2 In vivo exposure of brown trout to prochloraz and tributyltin (TBT)

2.2.1 Test apparatus and test chemicals

Exposure studies were conducted in 60 litre glass tanks. During the *in vivo* exposures, pH, dissolved oxygen and water temperature were monitored on days 0, 1 and 2 of both the 2- and 19-day exposures and twice weekly for the 19-day exposure study. Throughout the exposure, dissolved oxygen concentrations remained >80% of the saturation value. Water temperatures ranged between 11.2 and 14.3°C and pH values ranged between 7.35 and 7.90

and did not differ between the treatment groups (p = 0.816 and 0.820, respectively; n = 14). Dilution water and test chemical flow rates were checked every 2 days during the 19-day exposure study.

Prochloraz (99.1% purity) and TBT (96% purity) were purchased from Sigma-Aldrich, Buchs, CH. Stock solutions of the test chemicals were prepared in methanol (99.9% purity, Sigma-Aldrich, Buchs, CH) in 500 ml solvent rinsed glass bottles. Each stock solution was dosed to glass mixing vessels by means of a peristaltic pump at a rate of 0.05 ml/min to mix with the dilution water flowing to the flow splitting vessels at a rate of 500 ml/min. From the flow splitting vessels, the test solution flow was split to give a flow rate of 250 ml/min to each of the 2 replicate tanks per treatment group, providing a 99% replacement time of approximately 20 hours. The solvent control vessels received the same rate of addition of methanol such that the water in all test vessels, except the dilution water control, contained 0.1 ml methanol per litre.

2.2.2 Experimental design

The experimental design was adapted from the methods described by Thorpe et al. (2000). To initiate the exposure studies, stock fish were transferred randomly to 60 litre test vessels. Groups of 24 fish (2 replicate tanks of 12 mixed sex fish) were exposed to prochloraz at nominal concentrations of 32, 100, 320 μg/L for 2 days, and to TBT at nominal concentrations of 0.1, 0.32 and 1 μg/L, for 2 days and for 19 days. The exposure study included an absolute control (receiving dilution water only) and a methanol solvent control.

2.2.3 Fish sampling

At the end of the 2-day and 19-day exposures, fish were sacrificed in a lethal dose of MS-222 (500 mg/L; ethyl 3-aminobenzoate methanesulfonate, Sigma-Aldrich, Buchs, CH), buffered to pH 7.4. Total body weight was recorded to the nearest 0.01 g and fork length measured to the nearest 0.1 cm. Condition factor was derived by expressing the cube of the fork length (distance between the tip of the head and the fork of the caudal fin) as a percentage of total body weight. Gonads were removed and their wet weight recorded to the nearest 0.01 mg. One gonad from each fish was fixed in Bouin's solution (Sigma-Aldrich, Buchs, CH) for 4 hours, rinsed twice and stored in 70% ethanol (ROTH, Germany) for subsequent confirmation of gender. The second gonad from each fish was snap frozen and

stored at -80°C for subsequent sex steroid extraction. The gonadosomatic index (GSI) was derived by expressing the gonad weight, respectively, as percentages of total body weight.

2.2.4 Extraction of test water samples

A direct aim of this investigation was to compare the effects of the test chemicals in the *ex vivo* and *in vivo* assays. Therefore, extracted water samples collected directly from the 2-day exposure vessels were tested in the *ex vivo* assay (see section 2.3). At the end of the 2-day exposure, water samples (1 litre volume) were collected from one replicate tank per treatment group into solvent-cleaned flasks. Immediately after collection, the samples were spiked with 0.5% v/v methanol and extracted onto preconditioned Sep-Pak Classic C18 cartridges (Waters Ltd, Hertfordshire, UK). The samples were eluted with 5 ml methanol, evaporated under a gentle stream of nitrogen, redissolved in 1 ml ethanol (ACS reagent 99.5% purity; Sigma-Aldrich, Buchs, CH) and stored overnight, at 4°C, before use in the *ex vivo* gonad assay.

2.2.5 Gonad sex steroid extraction and sex steroid concentrations measurement

To assess the effects of prochloraz and TBT on sex steroid synthesis, the frozen gonads from the 2-day and 19-day *in vivo* exposures were thawed on ice and ground manually in 2 ml autoclaved tubes with ice cold 10 mM PBS, pH 7.4 (0.1ml / 10 mg tissue) using tissue grinder pestles. Tubes were then centrifuged at 9300 *g* for 15 min at 4°C and the supernatant transferred to glass tubes. The pellet was re-suspended with 400 ml 10 mM PBS and transferred to a new glass tube. The supernatant and the pellet suspension were extracted twice with 2 ml of diethyl ether (Sigma-Aldrich, Buchs, CH). For each extraction phase, the samples were vortexed for 10 sec and centrifuged at 2000 *g* for 5 min at 4°C. The ether layers from the supernatant and pellet suspensions for each sample were pooled into new glass vials and evaporated under a gentle stream of nitrogen. Samples were re-suspended in 400 μL of the immunoassay buffer (EIA buffer, Cayman Chemicals Europe, Tallinn, Estonia) and stored overnight at 4°C. The following day, E2 and T concentrations were assessed using commercially available E2 and T immunoassay kits (Cayman Chemicals Europe, Tallinn, Estonia); limits of detection for the E2 and T immunoassay kits are 19 pg/ml and 6 pg/ml, respectively. The cross reactivity of the immunoassay antibodies with other steroid hormones ranges between <0.01 and 14.5% for E2 antibodies and between <0.01

and 27.4% for T antibodies. The cross reactivity for the test chemicals was tested in our previous study (a Marca Pereira et al., 2011) and is very low for the test concentrations used in this study (0.0001-0.0003% for E2 antibodies and 0.0004 to 0.0006% for T antibodies). The gonadal sex steroid concentrations were evaluated in pg per mg of gonadal tissue

2.2.6 Histology

Gonads collected from male and female brown trout exposed for 19 days to the solvent control and the 1 μg/L TBT treatment were examined histologically to determine effects on gonad morphology. Six males and six females from each treatment group were analysed. The gonads fixed in Bouin's solution were dehydrated in a series of ethanol dilutions, cleared in Roti Histol (ROTH, Germany) and embedded manually in paraffin (ROTH, Germany). Sections of each gonad (4 mm thick) were cut longitudinally, and stained with hematoxylin and eosin (ROTH, Germany). The histomorphometrical analysis was adapted from the methods described by Van der Ven et al. (2003) and involved identification of the proportion of gonad cell types on digital microphotographs. For the testes, three random digital images from each fish were examined using the 40x microscope objective and the relative spermatogenic cyst area assessed and categorized into three major classes: spermatogonia (SG), spermatocytes (SC), or spermatids (ST). The development stage of the testes was characterized into four stages: stage I – testis with spermatogonia I and II; stage II – testis tubules with lumen and filled with spermatogonia and spermatocytes; stage III – testis tubules filled with spermatogonia, spermatocytes and spermatids; stage IV – testis tubules with spermatids which are transformed into spermatozoa. For the ovaries, three to four digital images from each fish were examined using the 10x microscope objective. On each ovary microphotograph, all oocytes present were counted and categorized into three major groups: perinucleolar oocytes (PN), cortical alveolus oocytes (CA) and early vitellogenic oocytes (EV). The development stage of ovaries was characterized into three different stages: stage I – presence of PN with Balbiani corpse; stage II – occurence of CA with small vacuoles in the cytoplasm; stage III – occurrence of EV filled with vacuoles.

2.3 Ex vivo gonad assay

A full description of the *ex vivo* gonad assay is provided elsewhere (a Marca Pereira et al., 2011). In brief, fish were anesthetized in MS-222 (100 mg/L; ethyl 3-aminobenzoate

methanesulfonate; Sigma-Aldrich, Buchs, CH), buffered to pH 7.4. Gonads were removed and placed in a glass Petri dish containing ice-cold Lebovitz medium (Sigma-Aldrich, Buchs, CH) supplemented with 10 mM HEPES (Sigma-Aldrich, Buchs, CH) and 1% penicillin-streptomycin solution (Sigma-Aldrich, Buchs, CH), pH 7.4. The gonads were dissected into fragments of ~1 mm^3 and randomly transferred onto insert filters (Millicell CM culture plate inserts; Sigma-Aldrich, Buchs, CH) in 24-well plates (4 fragments/well). Each well contained 600 µl L-15 medium (Lebovitz medium, 10 mM HEPES, 1% penicillin-streptomycin solution and 2% synthetic serum replacement [Sigma-Aldrich, Buchs, CH], pH 7.4). To minimise inter-individual variation in steroidogenic enzyme activity amongst fish, gonads harvested from a single male or female fish were used for a single replicate of all treatment groups within an experiment. For each test substance, gonads harvested from a total of three male and three female fishes were used to provide three independent replicates. Immediately prior to incubation, the medium was replaced with L-15 only (absolute control), or L-15 supplemented with the extracted water samples (diluted 1:1000 in L-15 culture media) from the SC, prochloraz and TBT treatment groups of the 2-day *in vivo* exposure study. Gonad explants were incubated for 2 days at 12°C. At the end of the incubation period, 400 mL aliquots of the media were removed and extracted twice with five volumes of diethyl ether in glass tubes. The two volumes of ether were pooled and evaporated with a gentle stream of nitrogen. Samples were re-suspended in 400 µL of the immunoassay buffer and immediately analysed for E2 and T concentrations using the Cayman immunoassay kits. The sex steroid concentrations were evaluated in pg per ml of culture medium.

2.4 Statistical analysis

Statistical analysis was performed using SigmaStat 2004 for Windows Version 9.01 (Systat Software GmbH, Erkrath, Germany). All results are expressed as mean ± standard error of the mean (SEM). To normalize the data of the *in vivo* and *ex vivo* assays, all values have been transformed using $\log_{10}$. To investigate effects of prochloraz and TBT 2-day *in vivo* exposures, data from the absolute and solvent controls were pooled after confirming that both controls did not differ significantly (t-test). Based on these results all further analyses were conducted using pooled data for each of the treatments. Due to technical difficulties, the absolute control line from the TBT 19-day *in vivo* exposure was terminated, and data were compared to the solvent control. Significant differences relative to the control were

assessed using one-way analysis of variance (ANOVA) where data met the assumptions of normality and homogeneity of variance, followed by a multiple comparison procedure versus the control (Dunnett's test). For data that did not conform to parametric assumptions, significant differences were assessed using a non-parametric test (Kruskall-Wallis ANOVA on ranks), followed by non-parametric multiple comparisons versus the control (Dunn's test). Differences were considered significant at $p < 0.05$. Correlation analysis was based on Spearman rank correlation coefficient *r* which was considered significant at $p < 0.05$.

3. Results

3.1 Test organisms

No mortalities were observed during the 2-day *in vivo* exposure study. Condition indices (weight, length, condition factor and GSI) for both the male and female brown trout did not differ between treatment groups (data not shown) indicating that fish size and maturity was comparable between the groups. However, mortalities occurred in control and treated groups during the 19-day exposure study (total of dead female and male fish per treatment group: 2 in the solvent control group; 2, 1 and 0 in the 0.1, 0.32 and 1 □g/L TBT treated groups, respectively). There was no evidence, however, that these mortalities were linked to chemical treatment ($r = -0.697$, $p > 0.05$). At the time of sampling, the number of female and male individuals per treatment group was high enough ($n \geq 8$) to obtain an adequate statistical power. The condition indices (condition factor and GSI) of male and female brown trout of the 19-day *in vivo* exposure to TBT did not differ between the control and treated fish (data not shown).

3.2 In vivo and ex vivo effects of prochloraz on gonadal E2 and T production

After 2 days *in vivo* exposure of female brown trout to prochloraz, ovarian E2 production was inhibited in a concentration-dependent manner relative to the control ($p < 0.05$, Fig. 1A), with a significant decrease significantly at 32 □g/L prochloraz. Incubation of the ovary cultures with extracts from the prochloraz exposure tanks decreased the E2 production in a concentration-dependent manner ($p < 0.05$, Fig. 1B). The 2 days *in vivo* and *ex vivo* exposures to prochloraz did not alter significantly ovarian production of T ($p > 0.05$, Fig. 1C-1D).

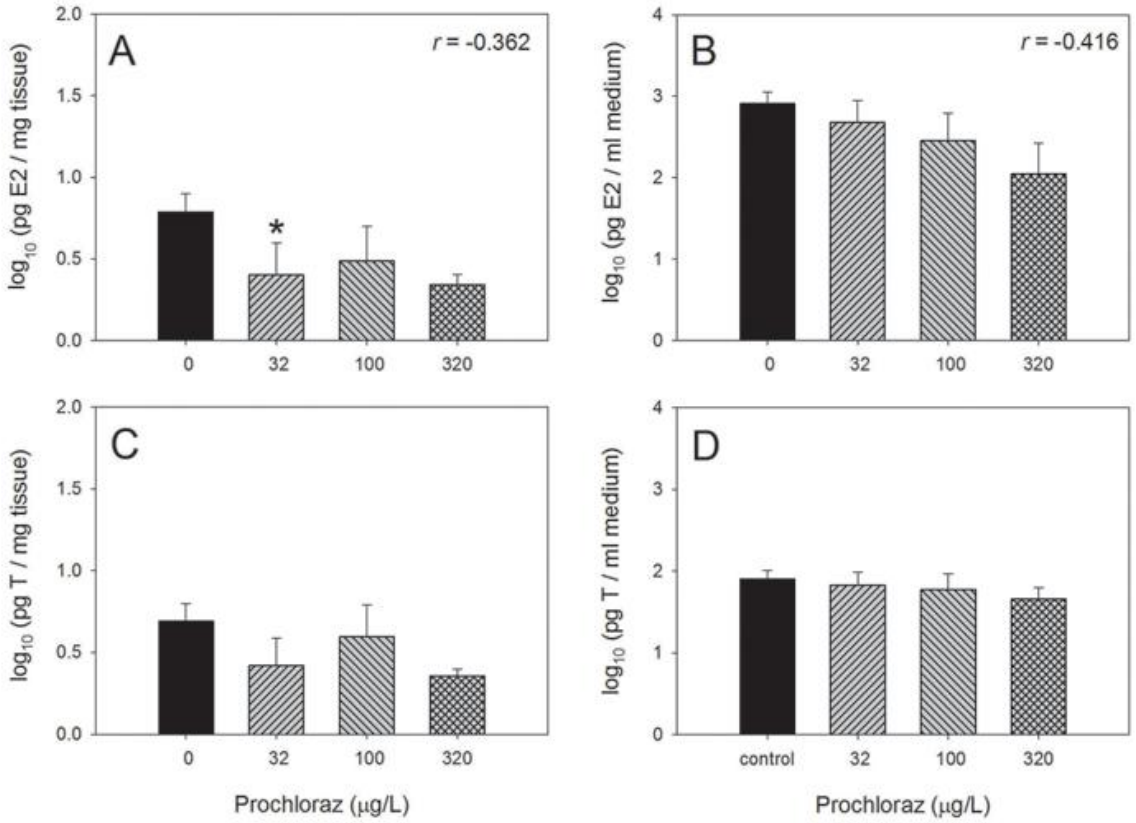

Fig. 1. Ovarian E2 and T concentrations of female juvenile brown trout (A and C) and of ovary cultures (B and D) exposed for 2 days to graded concentrations of prochloraz. Results are given as means ± SEM; n ≥ 9 for *in vivo* exposure and n = 3 for *ex vivo* exposures. Bars marked with asterisk are significantly different from the control group ($p < 0.05$). The Spearman rank correlation coefficient *r* is indicated for a significant concentration-dependent effect ($p < 0.05$).

The 2 days *in vivo* exposure of males to prochloraz did not alter the testicular T production ($p > 0.05$, Fig. 2A). However, a concentration-dependent decrease of T concentrations was observed in testis cultures incubated with extracts from the prochloraz exposure tanks with a significant reduction at 320 μg/L prochloraz when compared to the control ($p < 0.05$, Fig. 2B).

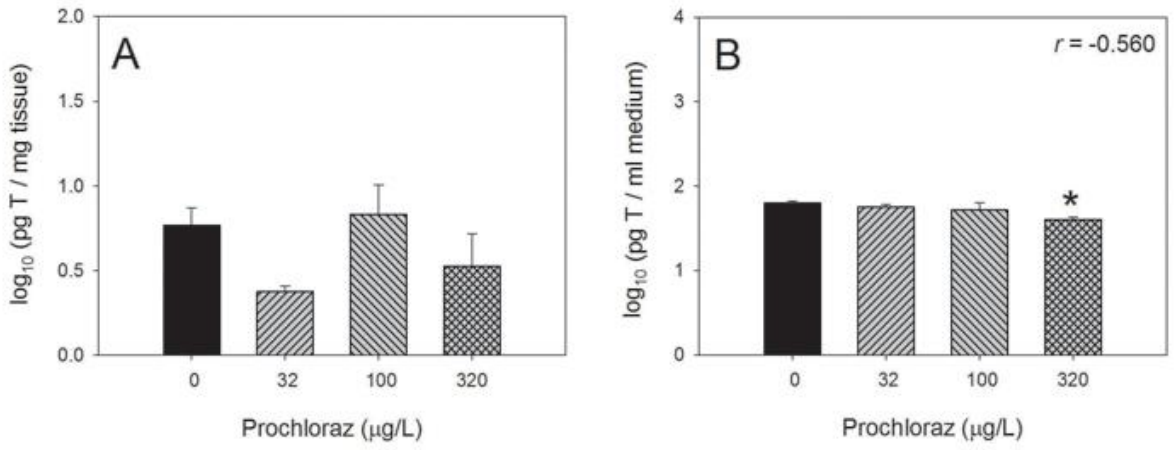

Fig. 2. Testicular T concentrations of male juvenile brown trout (A) and of testis cultures (B) exposed for 2 days to graded concentrations of prochloraz. Results are given as means ± SEM; n ≥ 8 for *in vivo* exposure and n = 3 for *ex vivo* exposures. Bars marked with asterisk are significantly different from the control ($p < 0.05$). The Spearman rank correlation coefficient *r* is indicated for a significant concentration-dependent effect ($p < 0.05$).

3.3 In vivo and ex vivo effects of TBT exposure on the gonadal E2 and T production

There was no evidence that *ex vivo* exposure of ovary cultures and *in vivo* exposure of female brown trout to TBT for 2 days and 19 days affected concentrations of E2 and T relative to the control ($p > 0.05$, data not shown). Similarly, the T levels of male brown trout exposed *in vivo* for 2 days to TBT were not modified ($p > 0.05$, Fig. 3A). However, the T concentrations of the testis cultures exposed to the extracts of the TBT exposure tanks were decreased in a concentration-dependent manner with a significant reduction at 1 µg/L TBT relative to the control ($p < 0.05$, Fig. 3B). Furthermore, a concentration-dependent reduction in T concentrations was observed in testis of juvenile fish exposed to TBT for 19 days, with a significant decrease at 0.32 and 1 µg/L TBT ($p < 0.05$, Fig. 3C).

3.4 Influence of TBT exposures on gonadal histomorphology

In the 19-day exposure study, the proportion in the ovarian development stages of females was modified between the solvent control and the 1 µg/L TBT treated group. In the solvent control group, 16.7% females were categorized as stage I, 58.3% as stage II and 25% as stage III (Fig. 4A). In the 1 µg/L TBT treatment group, 4.5% of the female gonads were in stage I, 4.5% in stage II, and 91% in stage III. Furthermore, compared to the solvent control,

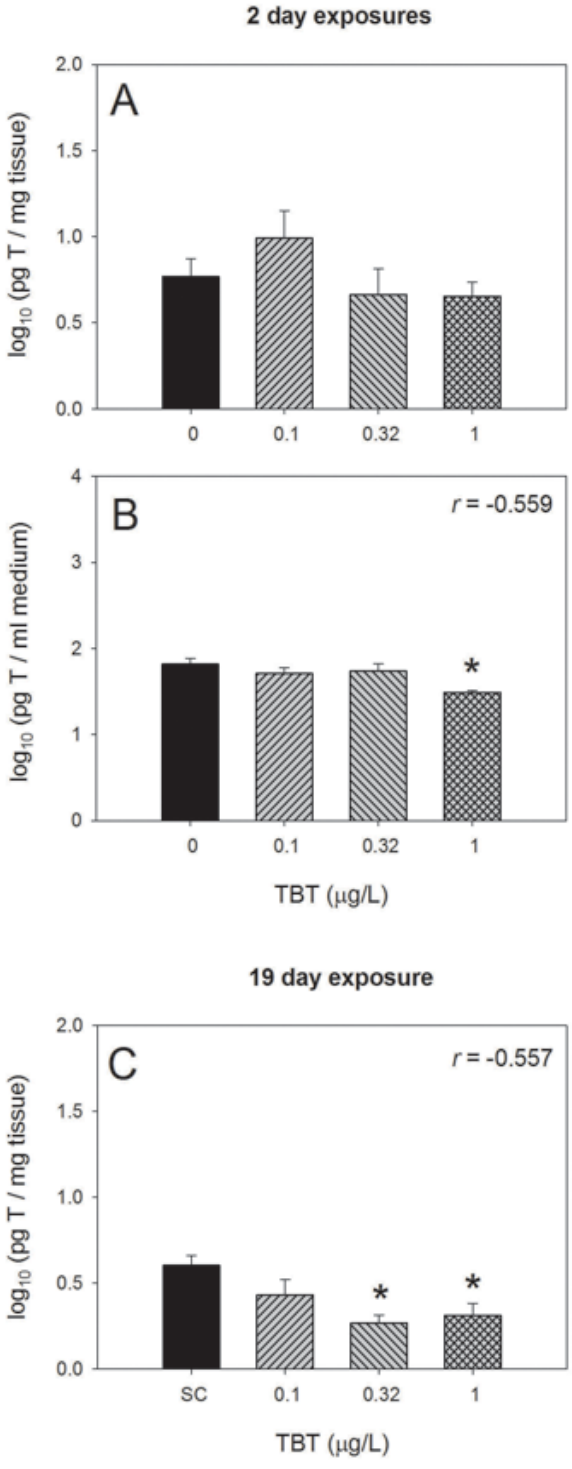

Fig. 3. Testicular T concentrations of male juvenile brown trout (A and C) and of testis cultures (B) exposed for 2 and 19 days to graded concentrations of TBT. Results are given as means ± SEM; n ≥ 8 for *in vivo* exposure and n = 3 for *ex vivo* exposures. Bars marked with asterisk are significantly different from the control group ($p < 0.05$). The Spearman rank correlation coefficient *r* is indicated for a significant concentration-dependent effect ($p < 0.05$).

the PN oocyte proportions were decreased 1.2-fold ($p < 0.05$), while the EV oocyte proportions were increased 11-fold ($p < 0.05$) in the TBT treatment group (Fig. 4B). No incidence of oocyte atresia was observed in the solvent or TBT treated group.

The male gonad morphology presented a change in the testicular development stages of the TBT treatment group, with gonads in a more advanced development stage. In the solvent control group, 66.6% of the male gonads were categorized as stage I and 33.3% as stage II. In the 1 µg/L TBT treatment group, 33.3% of the male gonads were categorized as stage I, 33.3% as stage II, 16.6 as stage III (Fig. 4C), and 16.6% as stage IV. In the TBT treated group, the spermatogonia cyst area tended to decrease compared to the

solvent control group, without being significant (p = 0.214), while the spermatocyte and spermatid cyst area were significantly increased (p < 0.05, Fig. 4D).

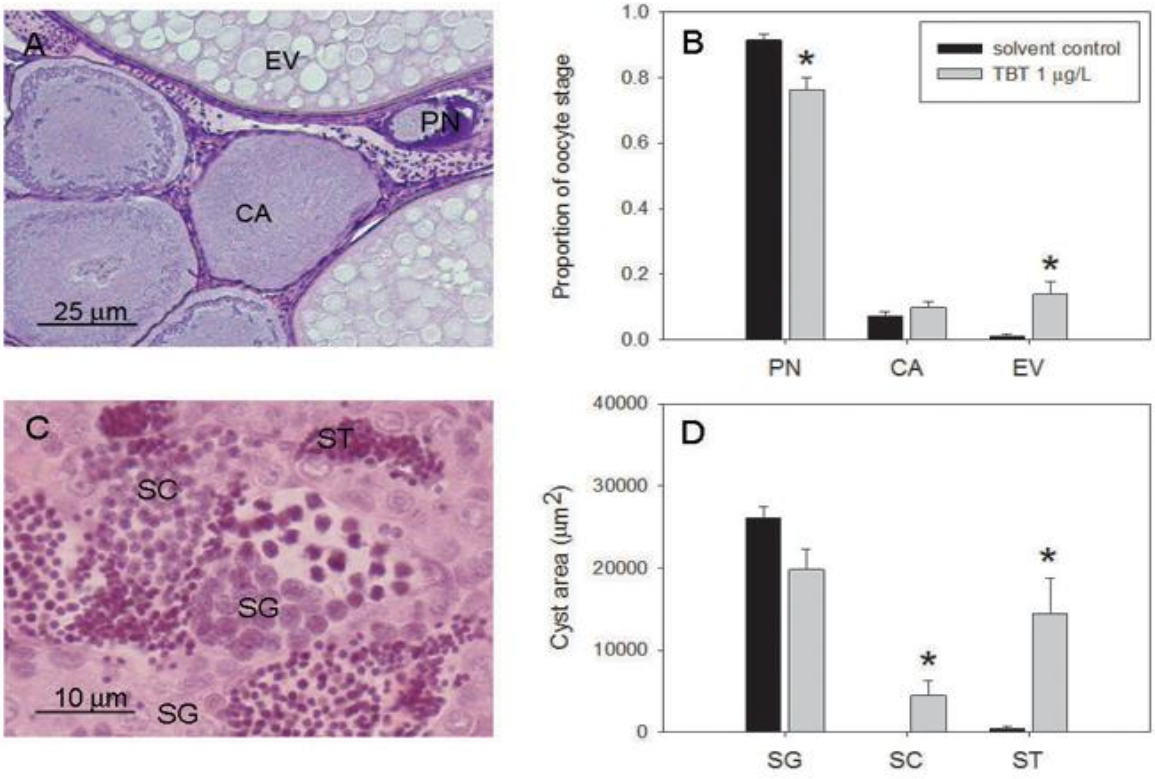

Fig. 4. Histomorphometrical effects of TBT at 1 µg/L on ovaries and testes of brown trout exposed for 19 days. Images of ovaries in stage III (A) with PN, perinucleolar oocyte, CA, cortical alveolar oocyte, and EV, early vitellogenic oocyte, and testes in stage III (C) with SG, spermatogonia, SC, spermatocyte, and ST, spermatid. Effects of TBT on the oocyte stage proportions of PN, CA and EV in ovaries (B). Effects of TBT on the relative spermatogenic cyst areas of SG, SC and ST in testes (D). Results are given as means ± SEM; n = 6. Bars marked with asterisk are significantly different from the solvent control (p < 0.05).

4. Discussion

4.1 Effects of prochloraz on in vivo and ex vivo gonadal E2 and T production

Comparison of the results from the 2-day *in vivo* and *ex vivo* exposures to prochloraz demonstrates that the effects of prochloraz on sex hormone biosynthesis in the gonads of juvenile brown trout are comparable in both assays. Prochloraz was expected, as a CYP inhibitor fungicide, to inhibit CYP19 aromatase activity *in vivo* and *ex vivo* (Andersen et al., 2002; Ankley et al., 2005). The observed decrease in E2 concentrations relative to the T synthesis in both the 2-day *in vivo* and *ex vivo* assays supports this mechanism of action.

However, the response sensitivity to prochloraz exposure is higher in the *in vivo* assay than in the *ex vivo* assay. These differences in sensitivity may result, at least in part, to differences in the target exposure concentrations of prochloraz in the *ex vivo* and *in vivo* assays. Through conducting the *ex vivo* exposures using water samples collected and extracted from the *in vivo* exposure vessels, we had hoped to standardize the exposure concentrations between the two assays. However, although previous studies have shown prochloraz to be stable in fish flow-through systems (Thorpe et al., 2011), if prochloraz had undergone any degradation within the exposure system, the real concentration of prochloraz would be lower as the nominal concentration. The lower concentration within the exposure system could potentially explain the lower *ex vivo* response sensitivity. Another possibility could relate to different toxicokinetics in the *ex vivo* versus the *in vivo* assay. Cravedi et al., 2001 showed that prochloraz was widely distributed in the whole body of rainbow trout exposed by gavage, including the gonads, and that it was rapidly and extensively biotransformed to more polar metabolites. Although the biological activity of these metabolites is not known, it is possible that they could have modified the *in vivo* potency of prochloraz.

According to previous *in vitro* and *in vivo* studies, in addition to inhibiting E2 synthesis, prochloraz alters CYP17 enzyme activity earlier in the sex hormone biosynthesis pathway, which might subsequently affect T production (Blystone et al., 2007; Villeneuve et al., 2007; Vinggaard et al., 2002). A reduction of T production is indeed detected in the *ex vivo* testis culture exposed to prochloraz, although this decrease in T levels is not observed in testis of males exposed *in vivo* to prochloraz. Yet, the *in vivo* profile of testicular T levels might indicate an effect of prochloraz on T production. Indeed, if prochloraz acted on the testicular steroidogenesis only through its aromatase inhibiting mode of action, increased T levels would be expected. Consistent with this hypothesis, the exposure of coho salmon (Afonso et al., 1999) and fathead minnow (Ankley et al., 2002) to a specific aromatase inhibitor, fadrozole, increased the plasma T concentrations while E2 production was inhibited. Therefore, prochloraz exposure might also alter *in vivo* T production in our *in vivo* assay. However, prochloraz has a greater effect on T synthesis in the *ex vivo* assay and thus the response sensitivity between the *ex vivo* and *in vivo* assays is again different. The absence of a clear inhibition of the *in vivo* testicular T synthesis to prochloraz in our study may indicate a possible feedback control mechanism of the hypothalamic-pituitary-gonadal (HPG) axis that could occur to compensate the lack of plasma T. In support of this, it was

demonstrated that adult fathead minnow exposed to prochloraz for 8 days at 30 to 300 μg/L could compensate and rapidly recover steroid production by up-regulating the transcripts of important steroidogenic enzymes in gonads (i.e. CYP17 and CYP19; Ankley et al., 2009). As the full HPG axis is not represented in the *ex vivo* assay, a positive feedback on T production would not occur, which may account for the higher sensitivity of the testis to the suppressive effects of prochloraz on T levels when exposed *ex vivo*.

Further analyses would be required to determine the underlying cause of the different effects of prochloraz on the steroidogenesis of the gonads when exposed *in vivo* rather than *ex vivo*. However, despite the differences of sensitivity in the testis exposures, it is clear that the profiles of effect observed on the E2 and T concentrations in the 2-day *in vivo* and *ex vivo* exposures were comparable, and in both assays prochloraz was shown to have multiple modes of action and to suppress T production, in addition to inhibiting the conversion of T to E2.

4.2 Effects of TBT on in vivo and ex vivo gonadal E2 and T production

The observed decrease of T concentrations in the *ex vivo* testis cultures exposed to 1 μg/L TBT was contrary to expectation, since previous studies have associated TBT masculinising effects with decreased E2 levels and increased T titers in marine mollusks (Bettin et al., 1996) and fish (Zhang et al., 2007 and 2009). However, our results were consistent with those of Ohno et al. (2005) and Nakajima et al. (2005), who investigated the effects of organotin compounds on testosterone biosynthesis in pig testis. They reported that TBT at 0.1 μM (approximately 30 μg/L) suppressed *in vitro* T production in pig Leydig cells by inhibiting the activity and/or gene transcription of several steroidogenic enzymes responsible for the T synthesis. Kim et al. (2008) also demonstrated reduced serum T concentrations due to reduction in the expression of steroidogenic enzymes responsible for T production in immature male mice fed with TBT. Similarly, McVey and Cooke (2003) showed that organotin compounds including TBT affected the activity of steroidogenic enzymes responsible for T synthesis in rat testis microsomes.

An inhibition of T production was not detected in the 2-day *in vivo* study. However, when male juvenile brown trout were exposed to TBT for 19 days, the testicular T levels were decreased. The different measures of the *in vivo* study compared to the *ex vivo* study are most likely due to the different toxicokinetic processes of TBT in the *in vivo* situation. It is

known that TBT is widely distributed and accumulates in the whole body of fish (mainly in the liver, but also in the gonads), and further is slowly eliminated (reviewed by Antizar-Ladislao, 2008). Hence, a longer period of exposure might be required *in vivo* for TBT to accumulate to an effective concentration and exert its effect in the gonads. In the *ex vivo* gonad assay, the route of exposure allows a direct effect of TBT on the steroidogenic activity. Nevertheless, both *ex vivo* and *in vivo* assays demonstrate that TBT might affect steroidogenesis. Indeed, the same profile of testicular T concentrations was observed from gonads exposed either *in vivo* for 19 days or *ex vivo* for 2 days to TBT, indicating that the *ex vivo* gonad assay has the potential to inform on the *in vivo* effects of a chemical that disrupts steroidogenesis.

4.3 Effects of TBT on the histomorphology of gonads of female and male brown trout

Earlier studies have reported damage of fish reproductive organs after TBT exposure. For example, Mochida et al. (2007) investigated the toxicity of TBT exposure, in a range of 0 to 10 μg/L, on spermatogenesis of the marine fish *Fundulus heteroclitus* exposed for 2 weeks and observed serious testicular damage. Zhang et al. (2007; 2009) observed that the exposure of male and female cuvier (*Sebasticus marmoratus*) to TBT in a range of 1 to 100 ng/L could inhibit gonadal development. In contrast our results indicate a direct or indirect developmental effect of TBT in gonads. Consistent with that, Thibaut and Porte (2004) showed that organotin compounds could enhance the activity of the 17α-20β-hydroxysteroid dehydrogenase responsible for the production of 17,20βP, which might subsequently enhance gamete growth and maturation. However, further research would be necessary to determine whether TBT enhanced activity of 17α-20β-hydroxysteroid dehydrogenase in our investigation. Nevertheless, these results indicate that an altered sex hormone profile might be linked to an altered gonadal development of the gonads.

5. Conclusion

The results of this study demonstrate that both *ex vivo* and *in vivo* assays exhibit a qualitatively similar profile in sex hormone level changes after exposure to prochloraz or TBT and also imply comparable interpretation of the modes of action for each test substance. Indeed, both *ex vivo* and *in vivo* assays suggest that prochloraz inhibits CYP19 activity and can also interfere with the activity of other enzymes involved in the steroidogenic pathway.

Similarly, the inhibition of T production in testis by TBT, which has not been previously reported in fish, was consistently observed in both the *ex vivo* and the *in vivo* assay. This highlights the potential of the *ex vivo* gonad assay as an informative bioassay for both identifying and informing on the mechanism of action of substances interfering with steroidogenesis in fish.

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4. Paper 3

Molecular and cellular effects of chemicals disrupting steroidogenesis during early ovarian development of brown trout (*Salmo trutta fario*)

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Abstract

A range of chemicals found in the aquatic environment have the potential to influence endocrine function and affect sexual development by mimicking or antagonizing the effects of hormones, or by altering the synthesis and metabolism of hormones. The aim of this study was to evaluate whether the effects of chemicals interfering with sex hormone synthesis may affect the regulation of early ovarian development via the modulation of sex steroid and insulin-like growth factor (IGF) systems. To this end, *ex vivo* ovary cultures of juvenile brown trout (*Salmo trutta fario*) were exposed for 2 days to either 1,4,6-androstatriene-3,17-dione (ATD, a specific aromatase inhibitor), prochloraz (an imidazole fungicide) or tributyltin (TBT, a persistent organic pollutant). Further, juvenile female brown trout were exposed *in vivo* for 2 days to prochloraz or TBT. The *ex vivo* and *in vivo* ovarian gene expression of the aromatase (CYP19), responsible for estrogen production, and of IGF1 and 2 were compared. Moreover, 17β-estradiol (E2) and testosterone (T) production from *ex vivo* ovary cultures was assessed. *Ex vivo* exposure to ATD inhibited ovarian E2 synthesis, while T levels accumulated. However, ATD did not affect *ex vivo* expression of *cyp19*, *igf1* or *igf2*. *Ex vivo* exposure to prochloraz inhibited ovarian E2 production, but did not affect T levels. Further prochloraz up-regulated *igf1* expression in both *ex vivo* and *in vivo* exposures. TBT exposure did not modify *ex vivo* synthesis of either E2 or T. However, *in vivo* exposure to TBT down-regulated *igf2* expression. The results indicate that ovarian inhibition of E2 production in juvenile brown trout might not directly affect *cyp19* and *igf* gene expression. Thus, we suggest that the test chemicals may interfere with both sex steroid and IGF systems in an independent manner, and based on published literature, potentially lead to endocrine dysfunction and altered sexual development.

1. Introduction

The sexual development of fish is known to be regulated by gonadotropins and sex steroid hormones (Nagahama, 1994), but other factors such as growth hormones might also be involved in fish gonadal development (Reinecke, 2010). The presence of the insulin-like growth factor (IGF) peptide and/or mRNA and/or receptors has been demonstrated in ovaries of fish at the onset of their development (Berishivili et al., 2006; Schmid et al., 1999). Further, recent studies have reported the involvement of IGF1 in fish oocyte growth (Campbell et al., 2006) and maturation (Weber et al., 2007). Although the physiological role of IGF2 in ovarian development is unclear, some studies imply involvement of IGF2 in the maturational competence of oocytes (Bobe et al., 2003; Nelson and Van der Kraak, 2010). In addition, recent studies suggest that exposure to chemicals that target the endocrine system could compromise IGF signalling during ovarian development, and thereby potentially affect reproductive success of fish (Filby et al., 2007; Shved et al., 2008). Nonetheless, to better understand the impact of endocrine active chemicals on fish reproduction, further studies about the underlying processes triggering gonadal development are needed.

Cross talks between sex steroid and growth hormone signalling pathways have been reported during the regulation of gonadal development, but are not fully understood yet. Indeed, several *in vitro* studies show that IGF1 stimulates ovarian steroidogenesis, though the underlying mechanisms are unclear (Kagawa et al., 2003; Nakamura et al., 2003; Paul et al., 2010). In addition, exposure of fish to estrogens affect *igf*1 hepatic and gonadal expression and/or IGF1 serum levels (Davis et al., 2007; Filby et al., 2007; Shved et al., 2007; Shved et al., 2008). Only a few studies have addressed whether exposure to chemicals targeting sex hormone biosynthesis, in particular inhibiting 17β-estradiol ovarian production, can alter the IGF signalling pathway during ovarian development. For example, Rime et al. (2010) reported that prochloraz (a fungicide known to disrupt the steroidogenesis) was able to induce oocyte maturation and stimulated gene expression of *igf1* and *igf2* in *in vitro* post-vitellogenic ovarian follicles from rainbow trout. However, to date the effects of chemicals disrupting steroidogenesis on both the sex steroid and the IGF systems during the early period of gonadal development has not been studied.

The aim of this study was to analyse the impact of chemicals that are known to interfere with steroidogenesis on the IGF and sex steroid signalling pathways during the early period of ovarian development of fish. To this end, we investigated the *in vitro* and *in*

vivo effects of the test chemicals on the mRNA expression of the aromatase (*cyp19*) gene, the key steroidogenic enzyme for the conversion of androgens to estrogens, as well as on the expression of *igf1* and *igf2* genes. In a first part, *ex vivo* ovary cultures of juvenile brown trout (*Salmo trutta fario*) were exposed for two days to three different substances: 1,4,6-androstatriene-3,17-dione (ATD), a potent pharmaceutical aromatase inhibitor; prochloraz, a sterol biosynthesis inhibiting fungicide; and tributyltin (TBT), a well known persistent organic pollutant. The sex steroid production of the ovary explants cultures was assessed. In a second part, ovarian tissue samples of juvenile brown trout from a previous *in vivo* experiment (a Marca Pereira et al., *submitted*) were used. In this experiment, juvenile brown trout were exposed for two days to prochloraz and TBT. Both *ex vivo* and *in vivo* exposed ovarian tissues were analysed for *cyp19*, *igf1* and *igf2* expression and *ex vivo* and *in vivo* effects were compared.

2. Material and methods

2.1 Test organisms

The experiments were conducted in accordance with Swiss guidelines for animal experimentation. Mixed sex immature (1+ year) brown trout (*Salmo trutta fario*) were collected from a local organic fish farm (Nadler, Rohr, Switzerland). All fish were kept in conditions described in our previous studies (a Marca Pereira et al., 2011). Mean wet body weights and fork lengths of the fish used for the *ex vivo* gonad assay and the *in vivo* short term exposure assay (see below) were 26.35 ± 11.98 g and 12.36 ± 1.7 cm (± standard deviation, N = 48), respectively.

2.2 Ex vivo gonad assay and sex steroid measurement

The experimental design of the *ex vivo* gonad assay was previously described by a Marca Pereira et al. (2011). In brief, fish ovaries in previtellogenic stage were dissected into fragments of approximately 1 mm^3 and randomly transferred onto insert filters (Millicell CM culture plate inserts; Sigma-Aldrich, Buchs, CH) in 24-well plates (4 fragments/well). Each well contained 600 µl L-15 medium (Lebovitz medium, 10 mM HEPES, 1% penicillin-streptomycin solution and 2% synthetic serum replacement, pH 7.4). Prior to incubation, the medium was replaced with L-15 only (absolute control), L-15 supplemented with solvent (solvent control; 0.1% ethanol; concentration based on our previous study) or L-15

supplemented with ATD (>95% purity), prochloraz (99.1% purity) or TBT (96% purity). All test substances, purchased from Sigma-Aldrich, Buchs, CH, and prepared in pure ethanol, were diluted 1:1000 in the L-15 culture medium to provide nominal concentrations of 1, 10, 100 μg/L ATD, 2.5, 25, 250 μg/L prochloraz and 0.01, 0.1 and 1 μg/L TBT. A total of six replicates per test concentration were provided. Gonad explants were incubated for 2 days at 12°C. At the end of the incubation period, media was removed and frozen at -80°C for subsequent determination of sex steroid concentrations, and the cultured gonad explants were fixed in 1ml RNA*later* (Sigma-Aldrich, Buchs, CH), kept at 4°C overnight and stored at -20°C for later extraction of total RNA. The gonad explants were then analysed for the mRNA expression of *cyp19*, *igf1* and *igf2*. The sex steroid measurement from the media samples of the *ex vivo* ovary cultures were done as described by a Marca Pereira et al. (2011). 17β-estradiol (E2) and testosterone (T) concentrations were analysed using commercially available immunoassay kits (Cayman chemicals Europe, Tallinn, Estonia).

2.3 In vivo short term exposure assay

To directly compare the *ex vivo* and *in vivo* molecular response to the test chemical exposures, female juvenile brown trout were exposed for 2 days to a solvent control (0.01% methanol), prochloraz at 320 μg/L and to TBT at 1 μg/L for 2 days. The detailed experimental procedures are described in a Marca Pereira et al. (*submitted*). At the end of the exposure study, the fish were sacrificed in a lethal dose of MS-222 (500 mg/L; ethyl 3-aminobenzoate methanesulfonate, Sigma-Aldrich, Buchs, CH), buffered to pH 7.4. One gonad from each fish was fixed in RNA*later*, kept at 4°C overnight and stored at -20°C until the total RNA extraction. Gonads from a total of eight female fish from each treatment group were analysed for the mRNA expression of *cyp19*, *igf1* and *igf2*.

2.4 Gene expression analysis

2.4.1 Design of primers and probes for real-time PCR

Based on the mRNA sequences of *Salmo trutta* aromatase (GenBank Accession number AY427786.1), *Salmo salar* IGF-I (Accession number EF432852.2), and *Salmo salar* IGF-II (Accession number EF432854.1), sense and antisense primers and a probe were created as described by Shved et al. (2007) and Caelers et al. (2004) for each target gene. The real-time PCR primers with annealing temperature of 60°C were designed using the

Primer3 software, version 0.4.0 (http://frodo.wi.mit.edu/primer3/). The primer sequences and the amplicon size are given in Table 1. The R18S gene was used as housekeeping gene and its primers and probe were based on the mRNA sequence of *Oreochromis mossambicus* (Accession number AF497908.1). For each primer set, the efficiency of the PCR reaction was measured in triplicate on 5-fold serial dilutions of the control templates. Real-time PCR efficiencies for each reaction were then calculated using the following equation: E = (10-1/slope – 1) x 100, and were in a range of 97 to 99%. Melting curve analysis was performed for each product to check the specificity and identity of the RT-PCR products.

Table 1

Primer and probe sequences and amplicon size of the studied genes

Primer/probe	Sequence (5' → 3')	Amplicon size (nucleotides)
cyp19 sense	CTGAGACAGGGGCGTTACAC	74
cyp19 antisense	CCTCTCTCATCCATGCCAATAC	
cyp19 probe	[a] CGGTTCGGCAGTAAACAGGGTCTGA [b]	
igf1 sense	CGTGGTATTGTGGACGAGTG	79
igf1 antisense	CAGACTTGACAGGGGCACAG	
igf1 probe	[a] CTTCCAGAGTTGCGAGCTGCGG [b]	
igf2 sense	CACCACTCAGTTTGCCACAC	84
igf2 antisense	CAGCACTCGATTTGACGAAG	
igf2 probe	[a] CGGAGAACGGAAAACACAAGAATGAAGG [b]	
R18S sense	GGTTGCAAAGCTGAAACTTAAAGG	85
R18S antisense	TTCCCGTGTTGAGTCAAATTAAGC	
R18S probe	[a] ACTCCTGGTGGTGCCCTTCCGTCA [b]	

[a] Reporter dye (FAM)-labelled 5' nucleotide.
[b] Quencher dye (TAMRA)-labelled 3' nucleotide.

2.4.2 Gonad RNA extraction and real-time PCR quantification of cyp19, igf1 and igf2 gene expression

The total RNA from the *in vitro* and *in vivo* gonad samples was extracted using TRIzol reagent (Invitrogen), treated with RQ1 – RNase – Free DNase (Promega, Dübendorf, CH), and resuspended in diethylpyrocarbonate-treated water (Sigma-Aldrich, Buchs, CH). The total RNA was photospectrometrically quantified and its purity was assessed by the $A_{260/280nm}$ ratio. The cDNA was synthesized from 800 ng total RNA using 1x TaqMan RT buffer, $MgCl_2$

(5.5mM), 1.25 U/μl murine leukemia virus (MuLV) reverse transcriptase, 2.5 μM random hexamers primers, 0.4 U/μl ribonuclease inhibitor, and 500 μM each dNTP (Applied Biosystems, Rotkreuz, CH) for 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C. From 10 ng/μl total RNA, 2 μl cDNA were obtained and were subjected, in duplicates, to real-time PCR using the FastStart Universal Probe Master (ROX) (Roche, Rotkreuz, CH), including 300 nM of each primer and 150 nM of the fluorogenic probe. Amplification was performed with 10 μl in optical 96-well plate using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) with the conditions: 15 min at 95°C, followed by 45 cycles for 15 s at 95°C and for 1 min at 60°C.

2.4.3 Relative quantification of treatment effects using the $\Delta\Delta C_T$ method

The comparative threshold cycle ($\Delta\Delta C_T$) method of Livak and Schmittgen (2001) was used to calculate relative gene expression ratios between ATD, prochloraz and TBT treated and control groups. Data were normalized to R18S as the reference gene. Efficiency tests for R18S, IGF1, IGF2 and CYP19 assays permitted the accurate use of the $\Delta\Delta C_T$ method (data not shown). Relative changes induced by ATD, prochloraz or TBT exposures were calculated using the formula $2^{-\Delta\Delta C}{}_T$, with $\Delta\Delta C_T = \Delta C_T$ (treated group) – ΔC_T (control group), and $\Delta C_T = C_T$ (target gene) – ΔC_T (reference gene). All data are expressed as *n*-fold changes of gene expression in the exposure group relative to the control group, displayed in the graphs as $\log_2$ scale.

2.5 Statistical analysis

To investigate effects of prochloraz exposure in the *ex vivo* gonad assay, data from the absolute and solvent controls were pooled after confirming that both controls did not differ significantly. All results are expressed as mean ± standard error of the mean (SEM) and were analysed relative to the control. The statistical analysis was performed using SigmaStat 2004 for Windows Version 9.01 (Systat Software GmbH, Erkrath, Germany). Significant differences were analysed using a Kruskal-Wallis ANOVA on Ranks, followed by a pair-wise multiple comparison procedure (Dunn's test). For the gene expression results of the *in vivo* exposure experiment, significant difference from the solvent control group was calculated using a t-test. Differences from the control group were considered significant at $p < 0.05$.

3. Results

3.1 Effects of ATD, PZ and TBT on the E2 and T production of ovary cultures

No significant difference of E2 and T levels was observed between the absolute and the solvent controls (data not shown). The exposure of ovary cultures to ATD at 100 μg/L inhibited E2 production ($p < 0.05$, Fig. 1A) whereas T levels accumulated at 10 μg/L ATD ($p < 0.05$, Fig. 1B) compared to the control. Prochloraz also reduced E2 production at 250 μg/L ($p < 0.05$, Fig. 1C), but did not alter T production (data not shown). TBT had no effect on either E2 or T synthesis in the ovary cultures (data not shown).

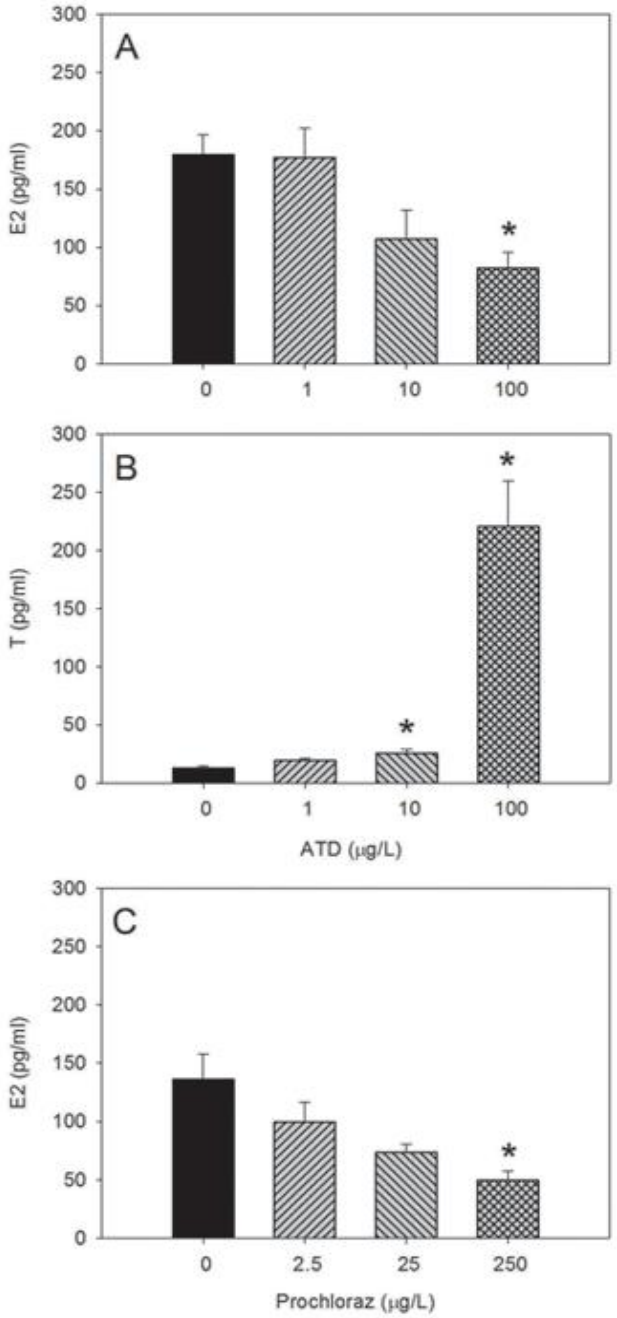

Fig. 1. E2 and T production of *ex vivo* ovary cultures exposed to ATD and prochloraz. A: E2 concentrations of ovary explants exposed to ATD. B: T concentrations of ovary explants exposed to ATD. C: E2 concentrations of ovary explants exposed to prochloraz. Results are given as mean ± SEM ($n \geq 3$). Significant differences between the control and exposure groups are marked as * $p < 0.05$.

3.2 Effects of ATD, PZ and TBT on the ex vivo and in vivo expression of the cyp19, igf1 and igf2 genes

No significant difference in gene expression was observed between the absolute and solvent controls in the *ex vivo* assay (data not shown). The exposure of ovary cultures to ATD and TBT did not cause any change of *cyp19*, *igf1* or *igf2* transcript levels (data not shown). Similarly, prochloraz did not influence the *ex vivo* expression of *cyp19* and *igf2*. However, *igf1* was up-regulated in ovary explants exposed to prochloraz at 250 μg/L ($p < 0.05$, Fig. 2A). In ovaries of female brown trout exposed to prochloraz at 320 μg/L, there was a trend of reduction of *cyp19* mRNA levels compared to the solvent control, though the difference was not significant ($p = 0.117$). The *igf1* transcripts were up-regulated ($p < 0.05$, Fig. 2B), while the *igf2* expression was not modified by prochloraz *in vivo* exposure. The TBT *in vivo* exposure of 1 μg/L showed a trend of reduction of *cyp19* mRNA levels, though not significant ($p = 0.149$). The *igf1* expression was not modified. However, *igf2* expression was down-regulated in ovaries of fish exposed to 1 μg/L of TBT ($p < 0.05$, Fig. 2C).

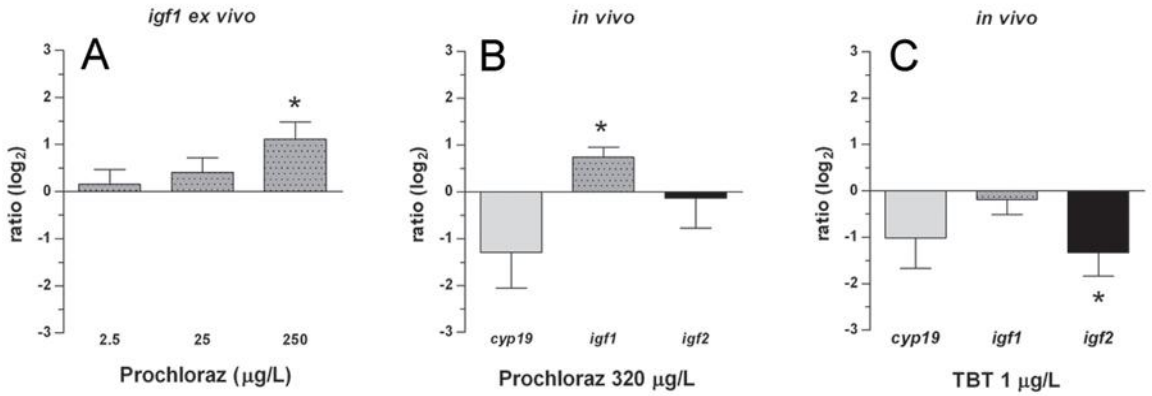

Fig. 2. *Ex vivo* and *in vivo* influence of prochloraz and TBT exposure on *cyp19*, *igf1* and *igf2* mRNA expression. A: Relative changes (log_2) to the control group of *igf1* expression in ovary explants exposed to prochloraz. B: Relative changes (log_2) to the solvent control group of *cyp19*, *igf1* and *igf2* expression in ovaries of brown trout exposed to 320 μg/L prochloraz. C: Relative changes (log_2) to the solvent control group of *cyp19*, *igf1* and *igf2* expression in ovaries of brown trout exposed to 1 μg/L TBT. Normalization was performed with the R18S as housekeeping gene. Results are given as mean ± SEM (n = 6 for *ex vivo* data; n = 8 for *in vivo* data). Significant differences between the control and exposure groups are marked as * $p < 0.05$.

4. Discussion

4.1 Effects of ATD exposure

The levels of the sex steroids obtained from the *ex vivo* gonad assay exposed to ATD were consistent with the results of our previous study (a Marca Pereira et al., 2011). Given that ATD is a specific CYP19 aromatase inhibitor, the observed T accumulation was expected. However, our results showed that inhibition of E2 production did not affect expression of *cyp19* in our explant cultures. This contrasted with the results of an earlier study, in which fathead minnow exposed to fadrozole (another specific CYP19 inhibitor) for seven days showed concentration-dependent up-regulation of the *cyp19* expression (Villeneuve et al., 2006). The results of that study indicated that a compensatory response normally takes place via a feedback regulation of the hypothalamic-pituitary-gonadal (HPG) axis, which is missing in our *ex vivo* cultures. Villeneuve et al. (2009) found that female and male fathead minnow exposed to fadrozole for 8 days could rapidly compensate and recover from E2 inhibition and up-regulate several HPG axis genes that would contribute to increase sex steroid production. Given the absence of the HPG axis in the *ex vivo* assay, these results suggest that ATD might not act directly on the transcription of *cyp19*. Instead, *cyp19* expression might be altered by a feedback regulation of the HPG axis after the E2 synthesis inhibition.

In addition, our results indicated that neither *igf1* nor *igf2* expression was affected by a decrease of E2 and/or subsequent accumulation of T. The IGF signalling pathway is endocrine and/or paracrine/autocrine. In the first case, the release of IGFs from the liver into the circulation is regulated by the growth hormone (GH) from the pituitary (Reinecke, 2010). In the gonads, the IGF system is also thought to act in a paracrine or autocrine manner (Reinecke, 2010). Several *in vitro* studies reported the increase of both the CYP19 activity and gene expression in ovarian follicles exposed to IGF1 (Weber et al., 2007; Paul et al., 2010; Kagawa et al., 2003). Therefore, we assume that an inhibition of E2 synthesis would up-regulate *igf* gene expression as a potential compensatory response. Our results showed, however, that an *ex vivo* inhibition of the CYP19 activity might not directly induce an up-regulation of the IGF system. Hence, we hypothesize that a feedback mechanism of the HPG might be necessary for a regulation of the IGF system. The absence of this mechanism in our *ex vivo* cultures could explain the lack of response in gene expression after ATD exposure.

4.2 Effects of prochloraz exposure

Prochloraz is an imidazole fungicide that displays several modes of action, including inhibition of the CYP19 activity, inhibition of androgen synthesis and antagonism of the androgen receptor (Vinggaard et al., 2006). This androgen synthesis inhibiting effect of prochloraz could explain why no T accumulation was observed in the *ex vivo* ovary explant cultures. These results were consistent with our previous study (a Marca Pereira et al., 2011). In addition, the *ex vivo* inhibition of E2 production was not paralleled by a change in the transcript levels of *cyp19*, and prochloraz exposed fish presented a trend of reduced *cyp19* mRNA levels, albeit not significant. These findings contrast with the results of Ankley et al. (2009) and Liu et al. (2011) who observed an association of prochloraz induced reduction of steroid production with an up-regulation of *cyp19* and adaptation of the HPG axis in adult fathead minnow and zebrafish exposed to prochloraz. It is important to observe that the adult fish life stage of these two studies differ from the juvenile stage studied in our study. This indicates that an indirect effect of prochloraz and a compensatory mechanism through the HPG axis is possible during the adult life stage. However, the exposure of zebrafish and fathead minnow during early life stages leads to male-biased sex-ratios and decrease of vitellogenin concentrations in both female and male fish (Kinnberg et al., 2007; Thorpe et al., 2011), which indicates that a potential up-regulation of the HPG axis genes do not compensate for the adverse effects in fish exposed to prochloraz during the early life stage.

Interestingly, an up-regulation of *igf1* was observed *ex vivo* and *in vivo*. In support of these results, we demonstrated in a previous *in vivo* study that prochloraz could enhance maturation of ovaries of juvenile brown trout exposed *in vivo* for 19 days (data not published). Similarly, Rime et al. (2010) observed *in vitro* maturation of post-vitellogenic ovarian follicles of rainbow trout exposed to prochloraz and up-regulation of *igf1* and *igf2* transcript levels. Further, an *in vitro* effect of IGFs on oocyte maturation was previously reported in ovarian follicles of several fish species (Nelson et al., 2010; Weber et al., 2007). These studies have focused on post-vitellogenic or vitellogenic follicles and on the role of the IGF system in ovarian maturation. Therefore, in our study, the absence of effect of prochloraz on *igf2* expression in previtellogenic ovaries of brown trout might suggest that IGF2 exerts its role predominantly in mature ovaries and that the maturational effect of prochloraz might be exerted via the IGF signalling pathway. In addition, since the up-

regulation of *igf1* was not observed in the *ex vivo* ATD exposure, we suggest that prochloraz may not exert its effect on *igf* expression via its aromatase inhibiting mechanism of action, but rather through antagonism of the androgen receptor (Vinggaard et al., 2002).

4.3 Effects of TBT exposure

Our previous study similarly indicated that the sex steroid levels in the *ex vivo* ovary cultures were not affected by TBT exposure. This is consistent with the absence of an *ex vivo* effect of TBT on *cyp19* or *igf* expression. Few *in vivo* studies reported a masculinisation effect of TBT on fish (McAllister et al., 2003). They attributed this effect to a potential inhibition of the CYP19 activity and/or expression. However, this hypothesis was to date never investigated in fish gonads. In our *in vivo* experiment, the TBT exposure showed a trend of reduction of *cyp19* ovarian expression. Although it is not a potent effect, this reduced *cyp19* transcript level might indicate that TBT interferes with the steroidogenic pathway at a transcriptional level.

The observed *in vivo* down-regulation of *igf2* in the previtellogenic ovaries might indicate that TBT interferes with factors that are absent in the *ex vivo* gonad cultures. Given that IGF2 is mainly expressed in the preovulatory ovary of rainbow trout, potentially promoting follicular maturational competence (Bobe et al., 2003), our results might indicate a potential impact of TBT on the late maturational stages of oocytes. Unfortunately, we cannot test this hypothesis directly since the gonads used in our study were in the previtellogenic stage. Accordingly, we cannot conclude here why *igf2* was down-regulated by TBT exposure. Nonetheless, in a previous study TBT enhanced maturation of female brown trout exposed *in vivo* for 19 days (data not published). Hence, these observations might suggest that TBT influences the early gonadal development, possibly via the modulation of *igf2* expression.

5. Conclusion

Here we demonstrate that *ex vivo* exposure of ovary explants to ATD, a specific aromatase inhibitor, did not influence the expression of either the *cyp19*, *igf1* or *igf2*. We also demonstrated that both *ex vivo* and *in vivo* exposure of the ovaries to prochloraz, a fungicide with multiple modes of action, induced an up-regulation of *igf1* possibly via its anti-androgenic mechanism of action. Furthermore, the *in vivo* exposure of TBT had a

negative effect on *igf2*, without influencing E2 levels. Therefore, from the comparison of ATD, prochloraz and TBT exposures, we suggest that alteration of CYP19 activity and/or expression might not have a direct influence on *igf* expression in brown trout ovaries. On the other end, these chemicals might impact, directly or indirectly, the sex steroid and the IGF pathways in an independent manner, which might lead to altered gonadal development. To make the link between the observed effects of the test chemicals and impaired ovarian development, additional *in vitro* and *in vivo* studies on gonadal development and reproduction of brown trout are required.

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5. Paper

Mode of sexual differentiation and its influence on the relative sensitivity of the fathead minnow and zebrafish in the fish sexual development test

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Abstract

Exogenous treatment of fish with natural sex hormones and their mimics has been shown to influence gonadal differentiation resulting in biased phenotypic sex-ratios. This has lead to the development of the Fish Sexual Development Test (FSDT) as a method for the detection of endocrine active chemicals. Proposed test organisms include the medaka, zebrafish (ZF) and stickleback, although the guideline also allows for inclusion of species such as the fathead minnow (FHM), provided the test duration allows for sufficient sexual differentiation. However, although the processes underlying sexual differentiation are known to differ for each of these species, it is not known how, or if, these differences would influence the results of the FSDT. In the experiments reported here, responses of the ZF and FHM to prochloraz, a sterol biosynthesis inhibitor and androgen antagonist, were characterized and compared. Exposure to 320 μg/L of prochloraz, from embryo until 60 (ZF) or 95–125 (FHM) days post hatch inhibited somatic growth of both species, but while a negative impact on ZF larval survival was observed (LOEC 32 μg/L) there was no evidence for an effect on FHM larval survival. Prochloraz influenced sexual differentiation in both species by decreasing the proportion of females (LOEC 100 μg/L (ZF), 320 μg/L (FHM)) and delaying completion of sexual differentiation; manifest as an increased incidence of ovotestis in the ZF (LOEC 100 μg/L) and as an increased number of fish with undifferentiated gonads in the FHM (LOEC 320 μg/L). However, while exposure to 320 μg/L prochloraz delayed maturation of the differentiated FHM testis, there was no such effect in the ZF. These results demonstrate that the different strategy of sexual differentiation in the ZF and FHM influences the profile of responses of their gonads to the masculinising effects of prochloraz, but does not affect their overall sensitivity.

1. Introduction

Sexual determination and differentiation are highly variable and plastic in fish (Francis, 1992) with the steroidogenic sex hormones playing an important role in influencing differentiation of the gonads. As a consequence exogenous treatment of fish with natural hormones and their mimics can functionally override genetic sex in fish leading to biased phenotypic sex ratios (reviewed in Scholz and Klüver, 2009). This knowledge has led to the development of the Fish Sexual Development Test (FSDT) which has been proposed to OECD as a chronic test for the detection and characterization of potential endocrine active chemicals (EACs[7]). The FSDT is at level 4 of the OECD's Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals as an in vivo assay providing data about multiple endocrine mechanisms and adverse effects. The test is based on the existing Fish Early Life Stage Toxi-city Test (OECD Guideline No.210[8]), in which exposure is initiated with fertilized eggs and effects on apical endpoints (hatching suc-cess, larval/juvenile survival and growth) determined. In the FSDT the exposure is extended until the period of sexual differentiation has completed, allowing measurement of endocrine-associated endpoints to be included (vitellogenin levels and gonadal differ-entiation and development).

The FSDT was initially developed for the zebrafish (ZF; Danio rerio) whose gonads have been shown to be especially sensitive to EACs between 20 and 60 days post hatch (dph) (Andersen et al., 2003). The ZF is an undifferentiated gonochoristic species in which ovary-like gonads initially develop in all juveniles regardless of future sex. In those fish destined to be female, development of the ovary continues, but future males must pass through a stage of juvenile hermaphroditism in which the ovary-like tissue undergoes apoptosis and the development of testicular tissue begins (Takahashi, 1977; Uchida et al., 2002; Maack and Segner, 2003). However, it is known that phenotypic sex ratios may shift in species of fish with other strategies of sexual differentiation (Piferrer et al., 1994; Kitano et al., 2000; Kwon et al., 2000), which has in turn led to a desire to adapt the FSDT to additional OECD test species, such as the fathead minnow (FHM; Pimephales promelas) (Panter et al., 2006; Bogers et al., 2006). The FHM is a differentiated gonochorist, in which formation of the indifferent gonad proceeds directly to testis or ovary; primordial germ cells differentiate into

[7] http://www.oecd.org/document/62/0,2340,en 2649 34377 2348606 1 1 1 1,00.html

[8] http://www.oecd.org/dataoecd/17/62/1948269.pdf

either oogonia, from 10 dph, or into spermatogonia from approximately 25 dph (van Aerle et al., 2004).

Exposures conducted using the FSDT, have confirmed that estrogens (Holbech et al., 2006; Panter et al., 2006), androgens (Holbech et al., 2006), anti-estrogens (Andersen et al., 2004) and inhibitors of steroidogenesis (Andersen et al., 2004; Kinnberg et al., 2007) produce similar profiles of effect on phenotypic sex ratios in both the ZF and the FHM. However, due to a lack of comparable studies in which the same chemical and concentrations have been tested in different species, it has yet to be determined how, or if, the threshold effective concentrations derived from a FSDT would be influenced by differences in the timing and strategy of sexual differentiation for each species. The aim of this investigation was, therefore, to compare the responses of the FHM and ZF exposed to prochloraz, a sterol biosynthesis inhibitor and androgen antagonist (Ankley et al., 2005; Kinnberg et al., 2007; a Marca Pereira et al., 2011), in the FSDT. Effects on apical endpoints (hatching rate, survival, growth) and endocrine-associated developmental endpoints (sex-ratio, gonadal maturity and peritoneal attachments and vitellogenin levels) were evaluated and compared to determine the influence of species on the results of the FSDT.

2. Material and methods

2.1 Test Organisms

FHM embryos were supplied by Smithers Visient (Horn, Switzerland). ZF embryos were obtained from broodstock fish held at the University of Basel (Switzerland). For both species embryos (<24 h post-fertilization) were checked for successful fertilization, under a light microscope, before random allocation to incubation cups (glass cylinders with a base of 0.4 μm nylon mesh) for the subsequent control and chemical exposures. Throughout the experiments a 16 h light:8 h dark photoperiod was used. Newly hatched larvae were fed twice daily with rotifers (Brachionus plicatilis) until 7 dph, and twice daily with Artemia nauplii (<24 h after hatching) from 5 to 30 dph, supplemented once daily with ground pellet food (Silver Cup Trout Pellets, Hofmann AG, Switzerland). From 30 dph all fish were fed twice daily with adult brine shrimp (Swiss Tropical Fish, Switzerland) supplemented once daily with pellet food.

2.2 Test Substance Preparation and Dosing

Prochloraz (99.1% purity) was purchased from Sigma–Aldrich Laborchemikalien GmbH (Germany; lot no. 6220X). To avoid the use of solvents within the exposure system, solvent-free stocks of prochloraz (nominally 32 mg/L) were prepared daily by coating 10 L glass vessels with 8 mL of a concentrated acetone-based stock (prepared every 10 days by dissolving 4.0 g prochloraz in 100 ml of acetone; stored at 4 ◦C). After evaporation of the acetone, under a gentle stream of nitrogen, 10 L of dilution water was added and the vessel left to stir at room temperature (25 ◦C) for 24 h to ensure solubilisation of the test chemical. To produce the desired nominal exposure concentrations of 32, 100 and 320 μg/L, the water-based stock solution was dosed, via a peristaltic pump, to glass mixing vessels at nominal flow rates of 0.40, 1.25 and 4.00 ml/min, respectively, to mix with the dilution water flow of 400 ml/min. The diluted test solution then flowed from each mixing vessel, via gravity, to flow-splitting devices where it was split to flow to each of 10 replicate test vessels (10 L glass aquaria) per concentration, at nominal flow rates of 40 ± 4 ml/min. Dilution water and test chemical flow rates were checked twice weekly, and flow rates to the individual test vessels once weekly.

2.3 Water Supply and Test Apparatus

Dilution water from the municipal supply to Basel (Switzerland) was filtered (activated carbon), mixed 50:50 with reverse-osmosis treated water, sterilized (UV sterilizer) and then fed into a temperature-controlled aerated tank (nominal temperature of 25 ◦C). Conductivity and pH of the mixed water were monitored daily and remained between 200 and 250 μS/cm, and between 7.5 and 8.5, respectively. All test materials were constructed of glass with a minimum of other materials (silicone rubber tubing and adhesive) used. Aquaria heaters were used in each ZF test vessel to raise the nominal temperature to 27 ◦C. Dissolved oxygen concentration and pH levels were determined in the individual test vessels on days 0 and 1 and then once weekly throughout the expo-sure period. The dissolved oxygen concentration remained >70% of the air saturation value throughout and pH values ranged from 6.74 to 7.99. Water temperatures were monitored twice weekly and ranged from 24.8 to 26.8 ◦C in the FHM tanks and from 26.0 to 27.6 ◦C in the ZF tanks over the course of the experiment. On each monitoring occasion, however, temperatures between the replicates and treatments did not differ by more than 0.5 ◦C for each species.

2.4 Experimental Design

For both the FHM and the ZF exposures, the test was initiated by suspending incubation cups (2 cups/aquaria) containing the fertil-ized embryos (25 embryos/cup for the FHM and 30 embryos/cup for the ZF) into replicate exposure tanks for each species (6 replicates for the FHM and 4 replicates for the ZF). The incubation cups were removed daily to check for the number of live and dead embryos and any dead embryos discarded. When hatching was completed in each pair of cups, the larvae were released directly into the exposure tank. Numbers of dead larvae were recorded daily and any dead removed. To minimize for any conflicting density dependence effects that may result from differential survival during early development between each tank, the numbers of surviving fish in each tank were confirmed at 30 dph and adjusted to give final numbers of 30 fish/tank, for both species; surplus fish in each tank were recorded, randomly removed and sacrificed in a lethal dose of MS222. Any further mortalities observed after this time were noted and removed but no further adjustments to the numbers of fish in each tank were made. At 60 dph, each FHM aquaria was replaced with 20 L aquaria to minimize for density dependence effects as the fish continued to grow.

To determine the effect of prochloraz exposure on somatic growth and gonadal sexual differentiation and determination in each species, the surviving ZF in each of the four replicate tanks in each treatment group were sampled when the fish reached 60 dph, using the procedures outlined below. As the period of time required for the gonad of the FHM to complete sexual differentiation has not been clearly defined an extended exposure period was used for this species and the fish sampled on two different sampling occasions; three of the replicate FHM tanks in each treatment group were sampled when the fish reached 95 dph while the remaining three replicate tanks in each treatment were sampled when the fish reached 125 dph.

2.5 Analytical chemistry

Water samples were collected from the solvent-free stock solutions and from a single replicate FHM aquaria and ZF aquaria within each treatment group at weekly intervals throughout the experiment to determine prochloraz exposure concentrations. The samples were diluted 1:1000 with water and analysed by HPLC with UV/VIS detection by. A Merck-Hitachi L-2200 autosampler was used to inject a 250 μL volume onto a Luna C18(2) 5 μm

(150 mm × 4.6 mm) column. Prochloraz was eluted isocratically using 75:25 methanol:water at a flow rate of 0.8 ml/min, delivered with a Merck-Hitachi L-2130 pump, and detected used a Merck-Hitachi L-2400 detector, set at a detection wavelength of 220 nm. The limit of quantification was 10 μg/L.

2.6 Fish sampling

All fish were starved for a period of 24 h prior to sampling. Fish were sacrificed in a lethal dose of MS222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt; Sigma), buffered to pH 7.4. Total lengths and wet body weight of the fish were recorded to the nearest 1 mm and 0.01 g, respectively. For the FHM, blood was collected by cardiac puncture, using a heparinised syringe (1000 Units heparin/mL), centrifuged (10,000 × g; 5 min, 4 ◦C) and the plasma removed and stored at −80 ◦C for later analysis of plasma vitellogenin concentrations using a commercially available FHM vitellogenin ELISA kit (Biosense Laboratories AS, Norway). The smaller size of the ZF made it difficult to reliably obtain blood; therefore, livers were removed, weighed to the nearest 0.01 mg, snap frozen using liquid nitrogen and stored at −80 ◦C for later analysis of hepatic vitellogenin concentrations using a commercially available ZF vitellogenin ELISA kit (Biosense Laboratories AS, Norway). The head (cut immediately behind the operculum) and tail (behind the dorsal fin) were then removed from each fish and the remaining body part fixed for 18 h in Bouins fixative, then rinsed and stored in 70% ethanol for subsequent histological evaluation.

2.7 Histological Evaluations

Each sample was processed, embedded in paraffin wax and transverse sections cut through the gonads, gonadal ducts and their mesenteric attachments at a thickness of approximately 2–4 μm. The sections were mounted on glass slides, stained with haematoxylin and eosin and examined microscopically to determine, where possible, fish sex and stage of testicular or ovarian development (based on Leino et al., 2005). Based on these evaluations, at each sampling point, each fish was categorized as male, female, sexually indifferent (germ cells not yet differentiated), or ovotestis (both male and female germ cells present). Morphology of the reproductive tract was also assessed, where possible, and defined as being a double or single attachment or not assessed.

2.7 Statistical analyses

To investigate effects of the prochloraz exposure, data were pooled within each treatment group (after confirming that the replicates did not differ significantly) and compared to the DWC using GraphPad Prism Version 5.03 for Windows. Data meeting the assumptions of normality and homogeneity of variance were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test. The VTG data, which was non-normally distributed, was analysed using a Kruskal–Wallis test, following by Dunn's Multiple Comparison Test. Effects of prochloraz treatment on the proportion of male, female and indifferent/ ovotestis fish were analysed using the exact Fishers test.

3. Results

3.1 Analytical Chemistry

The method of preparation for the solvent-free stock solutions of prochloraz produced consistent stock concentrations that were close (96% of nominal) to the target concentration of 32 mg/L; mean measured concentrations were 30.8 ± 2.2 mg/L (n = 33). The measured concentrations of prochloraz in the exposure vessels were also stable and close to the target concentrations of 32, 100 and 320 μg/L; mean measured concentrations over the duration of the test were 31.7 ± 1.8, 81.9 ± 5.4 and 297.4 ± 39.4 μg/L (n = 10 sampling occasions), respectively, in the zebrafish exposure vessels and 34.4 ± 4.9, 87.8 ± 12.7 and 293.9 ± 33.1 μg/L (n = 18 sampling occasions), respectively, in the FHM exposure vessels. Prochloraz was not detected in the DWC.

3.2 Hatching Success, post-hatch survival and growth

The mean (±SD) hatching success of the ZF and FHM embryos in the control vessels was 91.7 ± 0.01% (n = 4) and 99.3 ± 0.01% (n = 6), respectively. There was no evidence that exposure to prochlo-raz affected embryo hatching success for either species ($p > 0.05$; data not shown). Mean (±SD) survival until 30 dph in the con-trol vessels was 66.9 ± 7.3% (n = 4) and 67.9 ± 3.4% (n = 6) for the ZF and FHM, respectively. Survival of the juvenile ZF to 30 dph was lower, when compared to the controls, for each of the prochloraz treatment groups (52.1, 55.3, 27.4% survival in the 32, 100 and 320 μg/L prochloraz treatments, respectively;

p < 0.001; n = 4). In contrast, survival of the juvenile FHM until 30 dph was observed to be comparable to the controls in the 32 μg/L prochloraz treatment (70% survival), but higher in both the 100 μg/L (79% survival) and 320 μg/L (78% survival) prochloraz treatments (p = 0.02; n = 6). Mortality post 30 dph was less than 5% for both species, and was not treatment related.

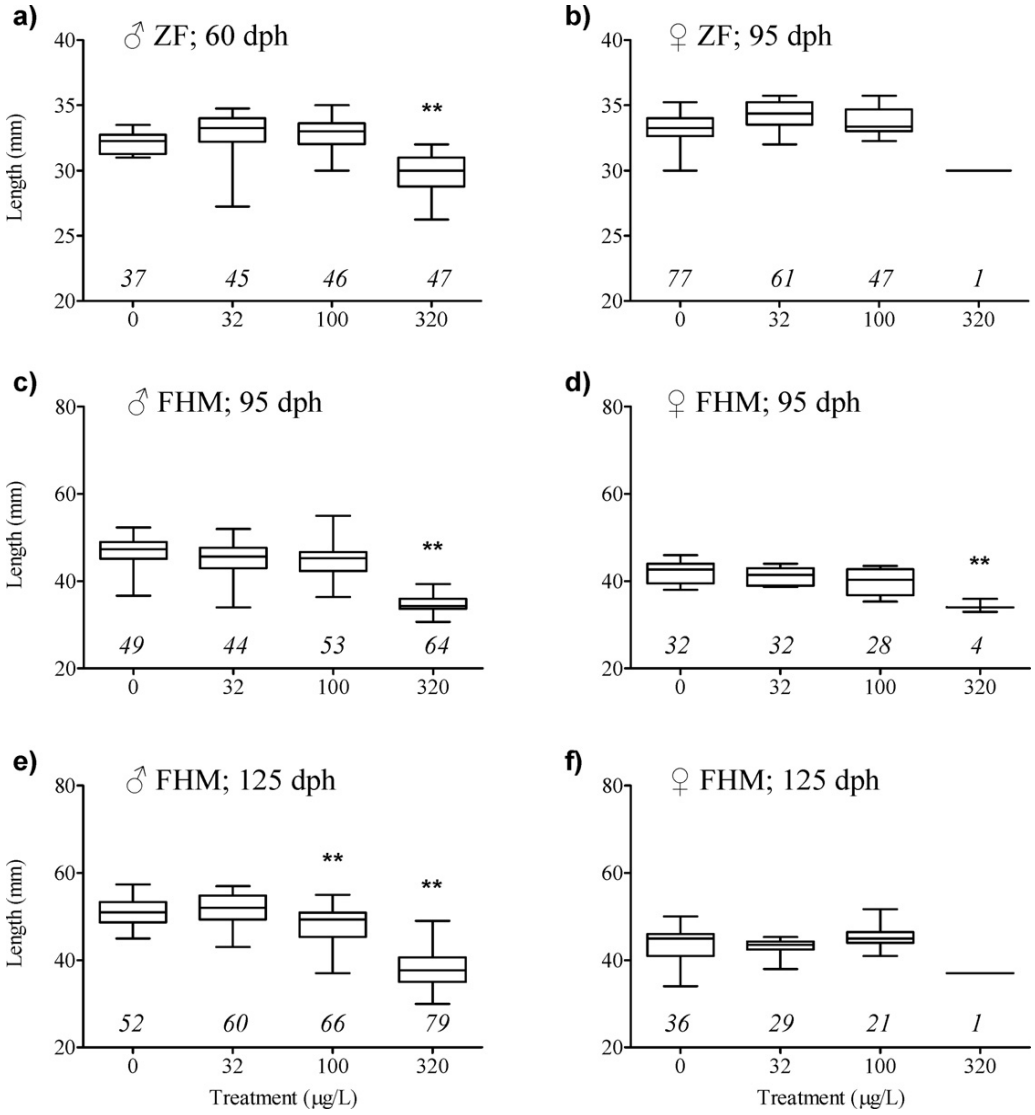

Fig.1. Total lengths in zebrafish (ZF) exposed to graded concentrations of prochloraz from embryo to 60 dph (a and b) and in fathead minnow (FHM) exposed from embryo to 95 dph (c and d) and 125 dph (e and f). Each box shows the lower quartile, median and upper quartile values for all replicates within each treatment group and the whiskers show the minimum and maximum range of the data. The italic numbers above the x-axis show the sample size for each treatment group. Significant differences between the control and exposure groups are denoted as **p < 0.05. Due to the low numbers of females in the 320 μg/L exposure group, effects of this treatment on female length were not determined for the ZF, at 60 dph, and the fathead minnow at 125 dph.

Mean (±SEM) total lengths for the control male and female ZF sampled at 60 dph were 32 ± 3 mm (n = 37) and 33 ± 0.3 mm (n = 77), respectively. For the control FHM sampled at 95 dph and 125 dph mean values were 47 ± 0.9 mm (n = 49) and 52 ± 0.8 mm (n = 52), respectively for the males, and 42 ± 0.7 mm (n = 32) and 43 ± 1.0 mm (n = 36), respectively, for the females. Exposure to prochloraz negatively impacted the growth of both species, when compared with the respective controls (ZF LOEC 320 μg/L; FHM LOEC 100 μg/L; $p < 0.05$; Fig. 1).

3.3 Histological evaluations: gonadal differentiation

In the surviving ZF controls at 60 dph (n = 4 replicate tanks), 32 ± 2.7% of the fish were sexed as male, compared with 65 ± 3.3% sexed as female. The remaining 3 ± 1.4% of control ZF were observed to have ovotestis. A comparable sex ratio and incidence of ovotestis was also observed in the lowest prochloraz exposure group (32 μg/L; Fig. 2a), but exposure to higher concentrations influenced the sex ratio toward male and increased the proportion of fish with ovotestis (LOEC 100 μg/L; $p < 0.05$).

In the surviving FHM controls, 56 ± 2.6% of the fish were sexed as male and 37 ± 5.4% as female at 95 dph (n = 3 replicate tanks) and 58 ± 5.1% as male and 40 ± 5.9% as female at 125 dph (n = 3 replicate tanks). At both sampling time-points, a small proportion of the control fish (7 ± 5.1% at 95 dph and 2 ± 2.2% at 125 dph) had gonads that had not yet completed sexual differentiation. Exposure to prochloraz (320 μg/L) influenced sexual differentiation of the FHM gonads decreasing the proportion of females at both time points ($p < 0.05$; Fig. 2b and c) and increasing the number of fish with gonads that had not yet differentiated into either male or female germ cells at 95 dph ($p < 0.05$; Fig. 2b). However, although 9 ± 1.2% of FHM in the 320 μg/L prochloraz treatment were still sexually undifferentiated at 125 dph, this was no-longer significantly higher than the incidence observed in the controls ($p > 0.05$; Fig. 2c). There was no evidence of ovotestis occurring in the control FHM tanks, and although one case was observed in both the 32 μg/L and 100 μg/L treatments at 95 dph and one case in the 100 μg/L treatment at 125 dph, there was no evidence that this was related to exposure to prochloraz.

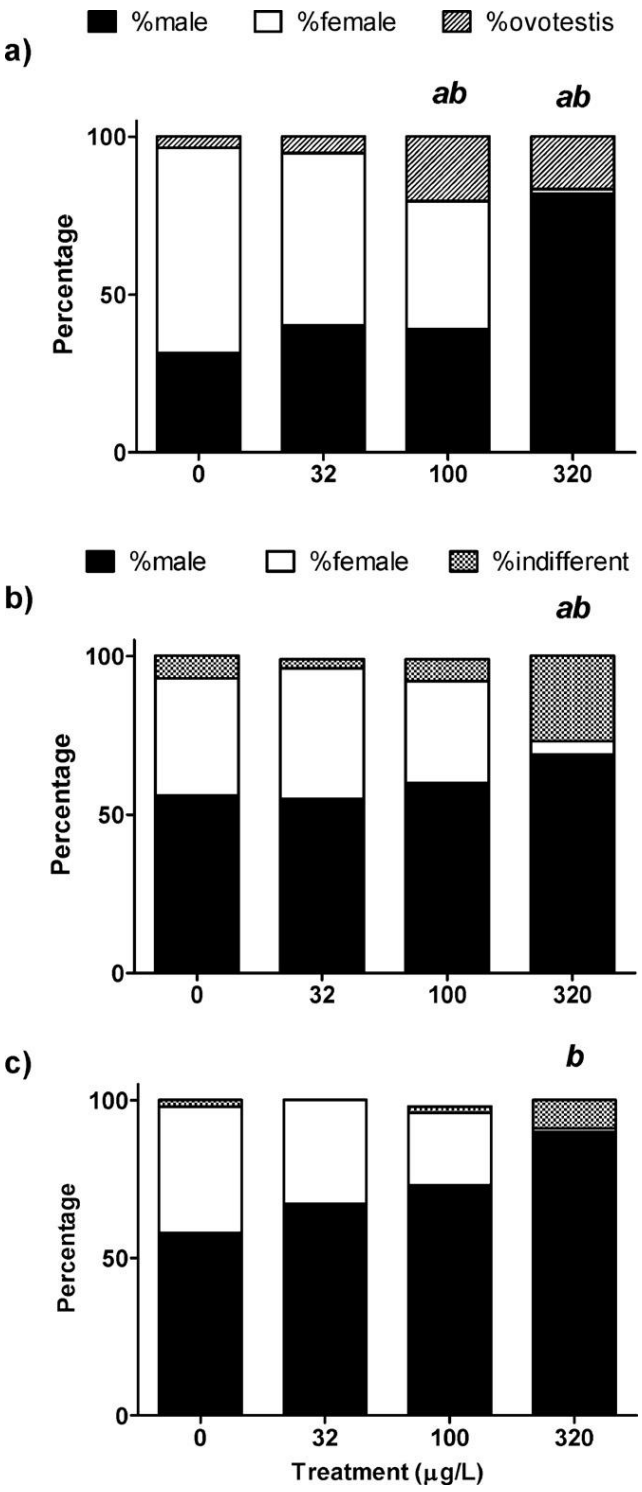

Fig.2. Sex ratios in zebrafish exposed to graded concentrations of prochloraz from embryo to 60 dph (a) and in fathead minnow exposed from embryo to 95 dph (b) and 125 dph (c). Each bar shows the percentage of males (black) and females (white), while those possessing ovotestis (zebrafish) or indifferent (fathead minnow) are represented by the hashed regions. Significant differences in the percentage of fish with indifferent/ovotestis gonads, between the control and exposure groups, are denoted with the letter 'a' ($p < 0.05$). A significant difference in the male:female sex ratio (proportion of females) between the control and exposure groups is denoted with the letter 'b' ($p < 0.05$).

3.4 Histological evaluations: gonadal development

In the control ZF with differentiated gonads, the testes were in stage 4 (70% of males; spermatids/some spermatozoa in the lumen of the seminiferous tube) or stage 5 (30% of males; abundant sperm present in the lumen) of development, and the ovaries were in stage 3 (14% of females; early vitellogenic), stage 4 (34% of females; late vitellogenic) or stage 5 (52% of females; mature oocytes) of development. Consistent with expectation the testis of

the control ZF had single attachments to the coelomic wall while double attachments were observed for the ovaries. For the control ZF with ovotestis, double attachments to the coelomic wall were observed. Exposure to prochloraz did not affect either gonadal stage ($p > 0.05$, data not shown) or the number of attachments of the gonads to the coelomic wall in the differentiated male or female ZF. However, in the lowest and highest treatment groups all fish with ovotestis had single attachments to the coelomic wall, where assessments of ducts were possible (i.e. 4 fish out of 6 with ovotestis assessed in the 32 μg/L treatment group and 5 out of 8 in the 320 μg/L group). In the 100 μg/L treatment group 50% of the fish with ovotestis had only a single attachment to the coelomic wall, while 50% had double attachments (attachment of the gonad to the coelomic wall was not assessable for one fish within this group).

At 95 dph, the gonads of a large proportion of the control FHM males were at an early stage of development with only resting germ cells (stage 1; 31% of males) or spermatogonia (stage 2; 31% of males) evident, although more advanced stages of testicular devel-opment were observed in some males (10% of males at stage 3, 14% at stage 4 and 14% at stage 5). Ovarian development was more advanced at this time, with 12% of females at stage 2, 19% at stage 3 and 28% at stage 4, while mature/spawning oocytes (stage 5) were present in the ovaries of 41% of the control females. By 125 dph, both testicular and ovarian development had advanced in the control fish; 9% of the control males were at developmental stage 1, 25% at stage 2, 21% at stage 3, 23% at stage 4 and 21% at stage 5; 17% of the control females were at developmental stage 2, 8% at stage 3, 14% at stage 4 and 61% at stage 5. Exposure to the highest concentration of prochloraz (320 μg/L) appeared to inhibit testicular maturation, with only 6% of males having testis that had advanced beyond developmental stage 2 at 95 dph, and only 41% at 125 dph, compared with 38% and 65% in the respective controls ($p < 0.05$; Fig. 3a and c). There was, however, no evidence for an effect of prochloraz exposure on ovarian maturation in those fish that had differentiated into females ($p > 0.05$; Fig. 3b and d).

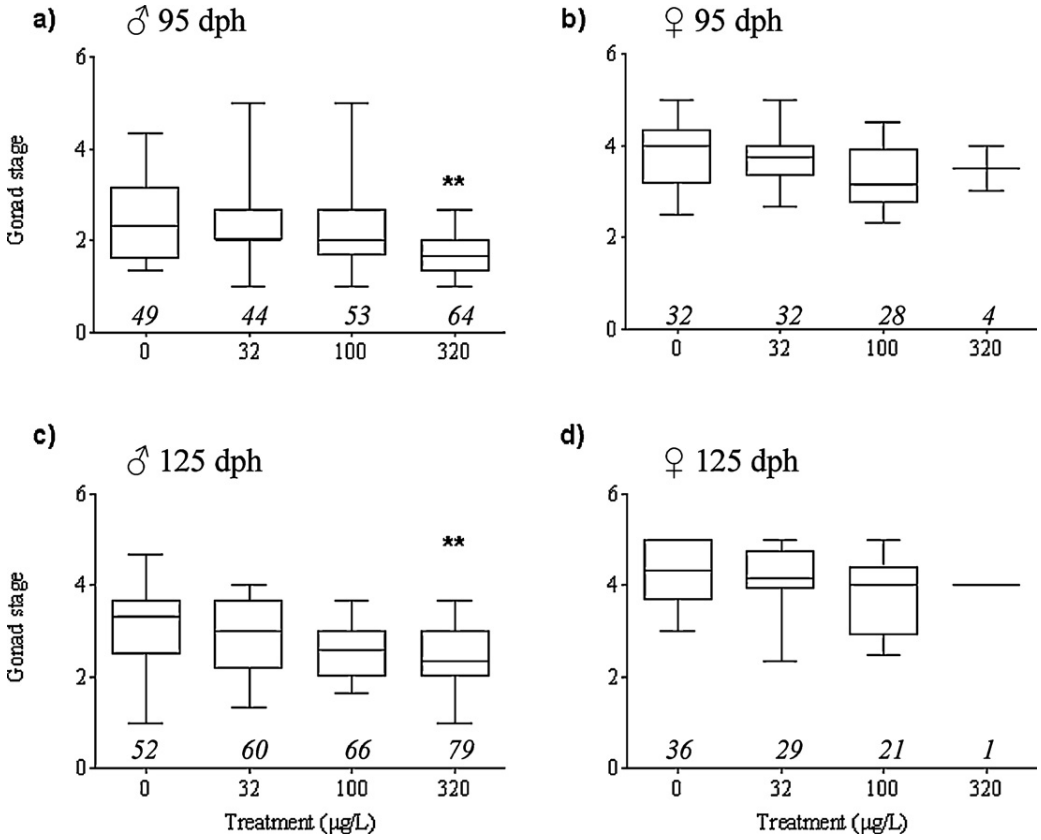

Fig. 3. Gonadal maturity stage in male (a and c) and female (b and d) fathead minnow exposed to graded concentrations of prochloraz from embryo to 95 dph (a and b) and 125 dph (c and d). Each box shows the lower quartile, median and upper quartile values for all replicates within each treatment group and the whiskers show the minimum and maximum range of the data. The italic numbers above the x-axis show the sample size for each treatment group. Significant differences between the control and exposure groups are denoted as **$p < 0.05$.

Attachment of the differentiated gonads to the coelomic wall was consistent with expectation at both time points in the control and prochloraz exposed FHM with single attachments observed for the testis and double attachments for the ovaries. Among those fish that had not completed sexual differentiation at 95 dph, single attachments of the gonad to the coelomic wall were observed in the controls and lower prochloraz treatments, but in the 320 µg/L prochloraz exposure group double attachments were observed for 20% of the fish, although this was not significantly different to the control ($p > 0.05$). At 125 dph, however, of the two fish identified in the controls as having not yet completed sexual differentiation, one had gonads with a single attachment to the coelomic wall, while the other fish had gonads with double attachments. Similarly, no clear patterns of duct

formation were observed in the prochloraz treated fish; both single and double attachments (1:1) were observed in the undifferentiated fish exposed to 100 μg/L prochloraz while only single attachments were observed for the undifferentiated fish exposed to 320 μg/L.

3.5 Vitellogenin concentrations

Mean (± SEM) hepatic vitellogenin concentrations in the male and female control ZF sampled at 60 dph were 17,400 ± 4900 ng/mg (n = 37) and 2,835,200 ± 889,300 ng/mg (n = 77), respectively. Hepatic concentrations of vitellogenin were reduced in the male ZF exposed to the highest concentration of prochloraz (320 μg/L; $p < 0.05$; n = 47; Fig. 4a), when compared to the control, and were also low in the one female found at this concentration of prochloraz, although the significance of this could not be tested (Fig. 4b). Similarly the numbers of ZF with ovotestis were too low to determine whether vitellogenin concentrations were influenced by exposure to prochloraz although comparison of the mean values would imply that this was the case; hepatic concentrations of vitellogenin were 7400 ± 3700 ng/mg (n = 4) in the control fish with ovotestis, com-pared with 400 ± 60 ng/mg (n = 7) in the fish exposed to 320 μg/L of prochloraz (data not shown). Mean (±SEM) plasma vitellogenin concentrations in the control male and female FHM sampled at 95 dph were 21.8 ± 1.0 ng/ml (n = 49) and 770,420 ± 125,373 ng/ml (n = 32), respectively, and 92.3 ± 48.7 ng/ml (n = 52) and 1,687,170 ± 180,387 ng/ml (n = 34), respectively, at 125 dph. Although circulating plasma concentra-tions of vitellogenin in the control male FHM were low, they were found to be significantly reduced by exposure to 100 μg/L of prochloraz at both time points ($p < 0.05$), and by exposure to 320 μg/L of prochloraz at 125 dph ($p < 0.05$), although not at 95 dph ($p > 0.05$), when compared to the respective controls (Fig. 4c and e). Significantly lower concentrations of plasma vitellogenin were also observed in the female FHM exposed to 100 μg/L at both time-points ($p < 0.05$) and to 320 μg/L at 95 dph ($p < 0.05$; Fig. 4d and f). Numbers of FHM with undifferentiated gonads were too low to determine whether vitellogenin concentrations were influenced by exposure to prochloraz.

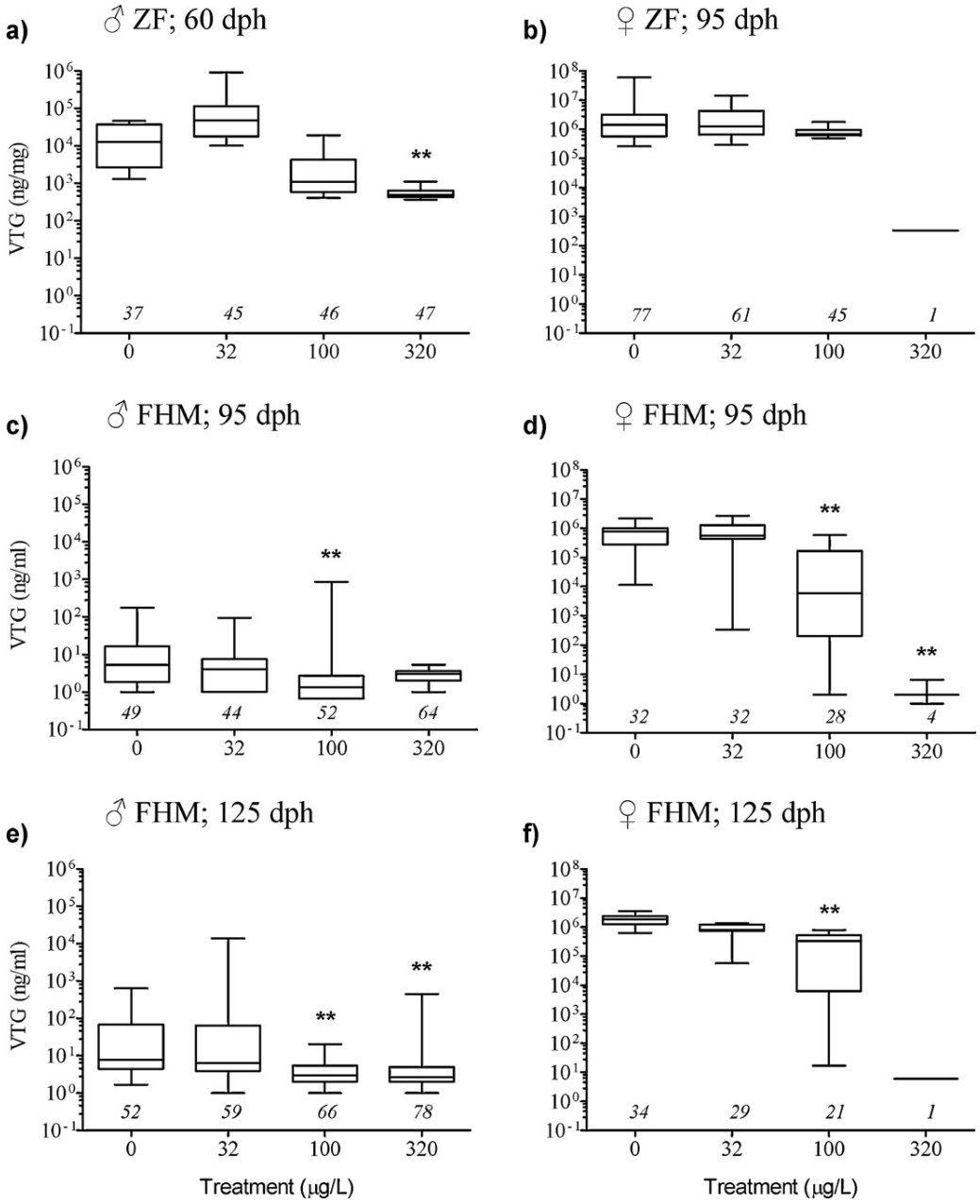

Fig. 4. Vitellogenin concentrations in zebrafish (ZF) exposed to graded concentrations of prochloraz from embryo to 60 dph (a and b) and in fathead minnow (FHM) exposed from embryo to 95 dph (c and d) and 125 dph (e and f). Each box shows the lower quartile, median and upper quartile values for all replicates within each treatment group and the whiskers show the minimum and maximum range of the data. The italic numbers above the x-axis show the sample size for each treatment group. Significant differences between the control and exposure groups are denoted as **$p < 0.05$.

4. Discussion

In these experiments, responses of the ZF and FHM to prochloraz were characterized and compared to assess the influence of mode of gonadal differentiation on endpoints from the proposed OECD FSDT. The results demonstrate that although the two species do show some differences in their profile of responses, in both species differentiation and development of the gonads was sensitive to the effects of prochloraz.

Somatic growth of both species was inhibited following expo-sure to prochloraz (ZF LOEC 320 μg/L; FHM LOEC 100 μg/L), but while a negative impact on larval survival was also observed for the ZF, FHM survival was not affected. Although Kinnberg et al. (2007) had not previously reported an effect of prochloraz (up to 202 μg/L) on ZF growth (or survival), our observations are consis-tent with reports that prochloraz suppresses concentrations of the sex hormones, testosterone and estradiol (Ankley et al., 2005; a Marca Pereira et al., 2011) which are involved in the promotion and maintenance of somatic growth in fish (Matty, 1986). Further, they support an earlier investigation in which exposure to prochloraz was shown to reduce muscle growth in juvenile rainbow trout, through reducing food intake (Fauconneau and Paboeuf, 2001). The negative effect of prochloraz on ZF survival, however, was unex-pected given that the reported LC50 for prochloraz (96 h LC50 in rainbow trout of 1.43 mg/L; FAO, 2009) exceeds the concentrations utilized here and further that there was no evidence of toxicity in the FHM. It should be noted, however, that control post-hatch ZF (larval) survival (68% survival) was suboptimal, which may indicate poor feeding of the larvae and this may in turn have increased the susceptibility of the ZF to treatment-induced mortality (Goolish et al., 1999; Diekmann and Nagel, 2005). A low post-hatch survival (67%) was also observed for the FHM, however, there was no evidence that exposure to prochloraz increased mortality of the FHM larvae; levels of mortality were comparable in all FHM tanks and appeared to relate to the presence of a deformity (curved spine) in the supplied stock of fish. Although, for both species, survival in the controls was below the 70% post-hatch requirement for a valid FSDT, there was no evidence that this influenced the response of other endpoints included within the FSDT (as discussed in subsequent sections), when compared with the investigation of Kinnberg et al. (2007).

Consistent with previous reports that early-life exposure to the aromatase inhibitors fadrozole (Piferrer et al., 1994; Kitano et al., 2000; Kwon et al., 2000; Andersen et al., 2004) and prochloraz (Kinnberg et al., 2007) leads to male-biased sex-ratios in fish, prochloraz

influenced sex-ratios of both the ZF and FHM in this study. Significantly lower proportions of phenotypic females were observed at 60 dph for the ZF (LOEC 100 μg/L) and at 95 and 125 dph for the FHM (LOEC 320 μg/L), following exposure to prochloraz, suggesting that ZF gonadal differentiation may be more sensitive to the effects of prochloraz than the process of gonadal differenti-ation in the FHM. This would appear to support an earlier report that the process of sexual differentiation in the ZF is more sensi-tive to the disruptive effects of E2 when compared to the medaka (Holbech et al., 2006). It is possible that the late timing for the period of enhanced sensitivity to EACs in the ZF (between 20 and 40 dph; Andersen et al., 2003), when compared to the FHM (between 10 and 15 dph; van Aerle et al., 2002) and the longer duration of the sexually indifferent period in the ZF, could be contributing to an increased sensitivity of their gonads to the effects of EACs.

Consistent with the study of Kinnberg et al. (2007) exposure to prochloraz increased the incidence of ovotestis in ZF (LOEC 100 μg/L), suggesting that the process of sexual differentiation had been arrested at the juvenile hermaphrodite stage. There was no evidence for an increased incidence of ovotestis in the prochloraz-exposed FHM. However, an increase in the number of fish with undifferentiated gonads was observed at 95 dph (LOEC 320 μg/L), suggesting that exposure of FHM to prochloraz impedes differen-tiation of the primordial germs into oogonia/spermatogonia. This indicates that, although manifest differently as a consequence of their different modes of sexual differentiation, exposure to prochloraz delays the timing of differentiation in both the ZF and the FHM. Such an effect would be consistent with prochloraz influencing dif-ferentiation toward male; differentiation of spermatogonia occurs later than differentiation of the oogonia in both the ZF and the FHM (Maack and Segner, 2003; van Aerle et al., 2004). Further the lack of a significant difference in the number of FHM with undifferentiated gonads at 125 dph suggests that prochloraz delays, rather than permanently inhibits, the process of gonadal differentiation. This supports an earlier report that exposure of rainbow trout to prochloraz induced a reversible delay in spermatogenesis, rather than a permanent defect of the testes (Le Gac et al., 2001).

In agreement with this effect on the timing of gonadal differ-entiation, exposure to prochloraz also delayed maturation of the differentiated testis in the FHM (LOEC 320 μg/L). There was, however, no evidence for such an effect on the ZF testis and there was no evidence for an effect on ovarian maturation in either species. These observations

contrasted with the earlier investigation of Kinnberg et al. (2007) who reported an advanced maturation and increased accumulation of spermatozoa in the testis of ZF males, exposed to prochloraz from fertilization until 60 dph, and a delayed ovarian maturation. It is not known why our observations differ, however, quantification of gonadal stage is subjective, and the protocol used in our study (5-stage classification scheme; Leino et al., 2005) differs from the 3-stage classification scheme used by Kinnberg et al. (2007). Given that the magnitude of differences in gonadal stage between the control and prochloraz exposed fish was small in both studies, it is possible that these differences in classification resulted in the different interpretation of effects.

Disruption of the peritoneal attachment has previously been shown to be a sensitive endpoint for assessing the effects of EACs on gonadal duct development in fish exposed during early-life to potent and weak estrogens (Gimeno et al., 1998; van Aerle et al., 2002; Panter et al., 2006) and androgens (Bogers et al., 2006). In our investigation, assessment of duct formation complemented the histological assessments of gonadal differentiation in the differentiated control and prochloraz-exposed fish, indicating that for those fish where differentiation of the gonads had been influenced toward male, duct formation was also masculinised. However, there was no evidence for a clear effect of prochloraz exposure on gonadal duct formation in those fish possessing undifferentiated gonads in either species. Thus, at least for exposure to prochloraz, conducting assessments of the effects of exposure on formation of the gonadal ducts does not appear to increase the sensitivity of the FSDT. Given the increased efforts required to conduct a histological assessment of the gonadal attachments, this suggests the need for further investigation in to the potential additional value that may be gained through conducting such assessments before advocating inclusion of gonadal attachments as an endpoint within the FSDT.

In both species, exposure to prochloraz suppressed concentrations of vitellogenin in males and females, with the FHM appearing to be the more sensitive of the two species (LOEC 100 μg/L in the FHM, compared with 320 μg/L in the ZF). Likewise, previous investigators have shown that exposure to prochloraz suppresses VTG concentrations at concentrations of 202 μg/L in the ZF (Kinnberg et al., 2007) and at concentrations of 116 or 311 μg/L in mature FHM (Ankley et al., 2005). Such a decrease in vitellogenin levels is consistent with the sterol biosynthesis (including aromatase) inhibiting mode of action of prochloraz causing reduced endogenous 17β-estradiol levels and thereby inhibiting

vitellogenin synthesis (Ankley et al., 2005). The apparent higher sensitivity of the FHM when compared to the ZF, however, was unexpected but was most likely a consequence of the differ-ent tissues used to assess vitellogenin concentrations; in the FHM, circulating plasma concentrations were measured, whereas in the ZF stored liver concentrations of vitellogenin were measured. The lower concentrations of vitellogenin in the plasma versus the liver, may be influencing the ability to detect deviations from baseline, however, it could also be possible that prochloraz has a greater suppressive effect on secretion of vitellogenin from the liver than on synthesis in the liver, although further work would be necessary to confirm this.

The results from this investigation demonstrate that the FSDT can be successfully applied to assess EAC effects on sexual differ-entiation and development in both the ZF and the FHM. However, comparison of the effects of prochloraz on each of the measured endpoints suggests that the different processes by which their gonads differentiate does have some influence on the response of their gonads to the effects of prochloraz. Sexual differentiation was delayed and influenced toward male in both species, but these effects were detected at a lower concentration of prochloraz in the ZF (LOEC 100 μg/L) than in the FHM (LOEC 320 μg/L). However, while maturation of the differentiated testis was inhibited in the FHM (LOEC 320 μg/L), there was no evidence for such an effect in the ZF. Similarly, the suppressive effects of prochloraz on concen-trations of vitellogenin were observed at a lower concentration in the FHM (LOEC 100 μg/L) than in the ZF (LOEC 320 μg/L). However, when all endpoints are considered together, a LOEC of 32 μg/L would be derived for the ZF, due to the effect on larval survival, and a LOEC of 100 μg/L would be derived for the FHM.

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6. Final discussion

6.1. The *ex vivo* gonad assay as tool to identify steroidogenesis disruptors

This thesis has focused on the effects of chemicals interfering with steroidogenesis. Given the occurrence of this type of EDCs in the environment and their potential threat for sexual development and reproductive success of fish, it is important to provide new tools to identify them (Sanderson and van den Berg, 2003). For this, the *ex vivo* gonad assay has been developed and characterized.

The first study of this thesis (chapter 2) indicated that the *ex vivo* gonad assay was a promising tool to address effects on the steroidogenic production of E2 and T. The exposure of the *ex vivo* assay to a salmon pituitary extract has shown that the E2 and T production could be induced, suggesting that the gonad culture could positively respond to pituitary hormones and was physiologically functional; in addition, exposure to different steroidogenesis inhibiting chemicals could clearly decrease E2 and/or T synthesis in ovary and testis explant cultures. Thus, these experiments demonstrated that the *ex vivo* gonad assay was able to detect the effects of exogenous compounds that induce or inhibit the activity or expression of the steroidogenic enzymes. Furthermore, the *ex vivo* gonad assay responded differently to exposure of ATD, prochloraz and TBT through different profiles of E2 and T levels consistent with their mechanism(s) of action, and the results were reproducible between our different studies (chapters 2, 3 and 4). Hence, it was shown that the *ex vivo* gonad assay could distinguish between different effects of chemicals interfering with steroidogenesis and might be a suitable tool to identify and study the specific mechanisms of action of the test chemicals.

The test chemicals were chosen according to their known or suspected different mechanism of action on the steroidogenic pathway. The biochemical results of the *ex vivo* gonad assay in our different studies (chapter 2, 3 and 4), consistent with other research studies (see literature references from chapers 2 and 3), demonstrated that: 1) ATD is a specific aromatase inhibitor that induces inhibition of E2 production with subsequent accumulation of T (Lee et al., 2006); 2) prochloraz possesses different mechanisms of action, e.g. it inhibits the activity of several steroidogenic enzymes responsible for E2 and T synthesis (Ankley et al. 2005); and 3) TBT may act directly and/or indirectly on the activity and/or expression of the steroidogenic enzymes depending on its concentration and time of exposure. The precise target molecules of both prochloraz and TBT were, however, not

identified in our experiments. Based on published literature, it is suspected that prochloraz inhibits CYP17 and reduces T production by this way (Blystone et al., 2007). Further, the inhibition of T by TBT might be due to reduced activity or gene expression of 17β-HSD or 3β-HSD, although this was not shown in fish (McVey et al., 2005; Ohno et al., 2005). To analyze thoroughly the mechanism of action of diverse compounds and given that EDCs may influence the activity and/or the expression of several enzymes of the steroidogenic pathway, other endpoints in addition to the E2 and T concentrations should be measured, e.g. progesterone production, or gene/protein expression of key steroidogenic enzymes, such as 3β-HSD, CYP17 and CYP19, or of the sex steroid receptors (i.e. ERs or ARs). These endpoints simultaneously measured would allow gaining more detailed information on the mechanism of action of xenobiotics.

Although the *ex vivo* assay can be used to identify and assess EDCs interfering with steroidogenesis, it presents some limitations. Indeed, the incubation of the *ex vivo* gonad explants with testosterone (chapter 2) suggested that the aromatase activity might be low in our cultures. It was also observed that the responses of the *ex vivo* assay to exogenous compounds could vary according to the exposure time. Since the gonad organ culture may metabolize the produced steroids to unknown degree during the incubation, it would be appropriate to further characterize the *ex vivo* gonad assay by doing time course experiments to optimize the exposure time and efficiency of the assay. Moreover, to further develop and determine the utility of the *ex vivo* gonad assay to identify EDCs, other xenobiotics with different mechanisms of action, e.g. chemicals stimulating the activity of steroidogenic enzymes, or chemicals mimicking or antagonizing estrogen/androgen actions, should be tested in the *ex vivo* assay. In addition, it would be of interest to investigate effects of mixtures of substances with known and different mechanisms of action.

After adequate characterization of the *ex vivo* gonad assay, a possible future utility of the test system could be the ecotoxicological evaluation of environmental samples, such as industrial or wastewater effluents. At present, effluent and environmental monitoring is mainly focused on chemical detection of some known pollutants, which means that newly produced chemicals that might present a risk for the environment are not detected until they have done significant environmental damage (Matthiessen and Johnson, 2007). Therefore, instead of waiting for adverse effects on the population level, a more rational approach for EDCs detection and for warning of pollution problems would be the use of

biological monitoring methods which integrate effects of given classes of EDCs. Thus, by applying effluents samples to the *ex vivo* gonad assay, the presence of active substances could be detected and used to trigger detailed investigations to identify causative chemicals and pollution sources.

6.2. The *ex vivo* gonad assay as potential tool to inform on *in vivo* effects

So far, the ecotoxicological research on fish is largely based on *in vivo* studies, in which endpoints that measure effects of toxicants on survival, development and reproduction of fish, allow to identify the adverse effects at the individual level and extrapolate the responses to population level. Yet to provide hazard assessment and predictions of sublethal effects of EDCs, a good understanding of the chemical mechanisms of action is indispensable, and cell cultures are precisely recognized as valuable methods to study them (Castaño et al., 2003; Segner et al., 2001).

The *ex vivo* gonad assay has several advantages over *in vivo* screening assays: in principle, it requires smaller volumes of test chemicals for the exposure, less exposure material and time of preparation, and is therefore less expensive. Further, the number of fish required for *in vivo* assays is considerably higher than for the *ex vivo* assay, which is why this latter is ethically preferred in a society who desires to reduce the animal use in toxicological research. In addition, the mechanism of action of xenobiotics can appropriately be studied because the experimental conditions of the *ex vivo* assay are well controlled, and the *ex vivo* assay is isolated from the superimposed physiological processes taking place in an intact organism, which makes the responses of *in vivo* assays difficult to interpret. Furthermore, the gonad organ culture maintains the same structure and functions as gonads *in vivo* and is more species/tissue relevant as other *in vitro* screening assays using cancer cell lines. Hence, this last characteristic renders the *ex vivo* assay more appropriate to predict *in vivo* effects. The challenge is, however, to allow extrapolation from *ex vivo* results to *in vivo* effects and to provide information on the biological responses at the individual level.

In chapter 3, it was shown that the *ex vivo* gonad assay had the potential to predict *in vivo* effects. The exposures to prochloraz and TBT induced comparable E2 and T level patterns and suggested similar modes of action of the test chemicals between *ex vivo* and *in vivo* assays. Indeed, in both *ex vivo* and *in vivo* assays, the inhibition of both estrogen and androgen production by prochloraz and the inhibition of T by TBT were observed. These

observations indicated that the *ex vivo* gonad assay was able to qualitatively predict the effects of chemicals interfering with the steroidogenesis by using the gonadal sex steroid production as endpoint. However, by comparing the LOEC (Lowest Observed Effect Concentration) between *in vivo* and *ex vivo* assays, the *in vivo* assay proved to be more sensitive than the *ex vivo* assay to chemical effects on steroidogenesis. The ovarian E2/T ratio was inhibited by prochloraz at lower concentrations in the *in vivo* assay (LOEC 32 μg/L *in vivo*, whereas the inhibition was concentration-dependant *ex vivo*, but not significantly different from the control group). The testicular T/E2 ratio was reduced by prochloraz *in vivo* at a LOEC of 320 μg/L, whereas this ratio was not modified *ex vivo*. Further, the testicular T production and the T/E2 ratio were inhibited by TBT at a LOEC of 0.32 μg/L, whereas in the *ex vivo* assay, the testicular T production was reduced at a LOEC of 1 μg/L. The difference of sensitivity between the *ex vivo* and *in vivo* assays might be due to different reasons: first, the route of exposure is different and the *ex vivo* assay cannot mimic the uptake, metabolism, distribution and targeting of xenobiotics; second, in the *in vivo* situation, the chemical endocrine effects of xenobiotics result from a combination of complex interaction between different hormone systems, interaction in different target tissues and feedback control reactions between the target tissue and the central nervous system, which are partly absent in the *ex vivo* situation. Therefore, according to the consistency of the effect patterns of the test chemicals among the *ex vivo* and *in vivo* assays, we can conclude that the *ex vivo* gonad assay may represent a valuable screening and informative test method for chemicals disrupting the steroidogenesis, although it might not be suitable to identify threshold effective concentrations of the tested substances for hazard assessment.

In chapter 4, the combination of *ex vivo* and *in vivo* results provided additional and valuable information on molecular effects of prochloraz and TBT exposures and on the regulation of early sexual development of fish. The lack of the complete HPG axis in *ex vivo* assay is a limitation to the assessment of indirect effects of toxicants on the gonadal steroidogenesis. However, the comparison of the *ex vivo* and *in vivo* results permitted to suggest potential direct and indirect target molecules of the test chemicals involved in the regulatory pathways of the sexual development. Consistent with other *in vivo* fish studies (Ankley et al., 2009; Zhang et al., 2008), our results indicated that the interference of the steroidogenic pathway by the test chemicals might be related to feedback control responses of the HPG axis and up- or down-regulation of several hormone systems involved in the

regulation of sexual development. Although further studies are necessary to confirm this, it would have been impossible to formulate this hypothesis with only the results of the *in vivo* assay. In conclusion, the *ex vivo* assay might not be suitable as predictive tool for quantitative effects in EDC testing. Nevertheless, the combination of *ex vivo* and *in vivo* assays has proven to be valuable to provide more information on the mechanism of action of the test chemicals, which can further facilitate the organization and optimization of subsequent directed *in vivo* testing. This is in agreement with strategies on EDC testing of international regulatory agencies that apply a tiered approach involving a complementary battery of short- and long-term *in vivo* and *in vitro* assays to identify and assess potential EDCs (Zacharewski, 1998).

6.3. Impaired sexual development potentially caused by steroidogenesis disruption

The 19-day exposure of juvenile brown trout to prochloraz and TBT (chapter 3) indicated the potential of both chemicals to modulate the early sexual development of male and female gonads. It was demonstrated that both prochloraz and TBT interfere with the steroidogenic pathway and both enhanced maturation status of juvenile brown trout. Whether this effect might impact sexual development of brown trout is not known, and further long-term *in vivo* studies with apical endpoints are needed to elucidate this point. Both prochloraz and TBT have been shown to enhance the production of 17α-20β-dihydroxy-4-pregnen-3-one steroid responsible for the maturation of both male and female gametes (Rime et al., 2010; Thibaut and Porte, 2004). However, a disruption of E2 production by prochloraz or TBT might block the male and female germ cell proliferation, which has been shown to be dependent on E2 in several fish species (Miura et al., 1999; Miura et al., 2007). It is, therefore, possible that both chemicals enhance the maturation of differentiated germ cells that are already in a stage of advanced gametogenesis, whereas the proliferation of oogonia and spermatogonia might be blocked, thereby impeding a normal sexual development. Furthermore, the alterations of CYP19, IGF1 and IGF2 gene expression by ATD, prochloraz and TBT exposures in both *ex vivo* and *in vivo* conditions (chapter 4) indicated that these EDCs have the potential to affect directly (enzyme activity) and indirectly (gene expression) two important hormone systems essential in the regulation of early sexual development in fish, namely the sex steroid and IGF regulatory pathways. The modulation of IGF gene expression could, however, not be directly linked to the

steroidogenesis inhibiting mechanism of action of the test chemicals. It is possible that the maturational effect and the up-regulation of *igf1* stimulated by prochloraz exposure are due to its anti-androgenic mechanism of action (Vinggaard et al., 2006). Further, the maturational effect and negative effect of TBT on *igf2* expression might be due to indirect interaction of TBT anywhere along the HPG axis. Nevertheless, our studies suggested that these test chemicals have the potential to disrupt the regulation of sexual development, which might subsequently impair the reproductive success of fish.

The exposure of both zebrafish and fathead minnow to prochloraz during their sexual differentiation and development (chapter 5), indicated that the masculinising effect (leading to male-biased sex-ratios) of prochloraz might be linked to inhibition of the steroidogenesis. A skewed sex ratio is a clear indicator of adverse apical effect that has the potential to impact fish population structure. Therefore, this study clearly shows the potential threat of prochloraz exposure for aquatic species. Yet this study does not directly associate the impaired sexual differentiation with inhibition of the steroidogenesis. Further, the sex steroid profiles were not assessed in this study. However, the decrease in vitellogenin levels is consistent with the aromatase inhibiting mechanism of action of prochloraz, which reduced E2 levels and consequently vitellogenin production (Kime et al., 1999).

Additional laboratory studies have demonstrated that EDCs inhibiting the steroidogenesis might impact sexual development leading to adverse effects that might further impair reproductive success of fish (reviewed in Scholz and Klüver, 2009). For example, in juvenile zebrafish exposed for 40 days to a CYP19 inhibitor, fadrozole, differentiation and development of female gonads were inhibited (Andersen et al., 2004). Similarly, undifferentiated sea bass (*Dicentrarchus labrax*) that were administered fadrozole resulted in inhibition of ovarian differentiation and a skewed sex ratio towards male (Navarro-Martin et al., 2009). These studies demonstrate the importance and pivotal position of the CYP19 enzyme in the female and male sex differentiation of fish (Guiguen et al., 2010) that maintain a specific androgen/estrogen balance. Therefore, the exposure to steroidogenesis disruptors interfering with steroidogenic enzymes might be a cause for impaired gonadal differentiation and development in fish. To demonstrate this, further *in vivo* studies with model chemicals inhibiting steroidogenesis should be done to link the steroidogenesis inhibiting endpoint to adverse effects on the sexual development (e.g. sex ratio and histopathology). In conclusion, the analysis of the sex steroid profile might be a

good indicator of impaired sexual development and should always be linked to the analysis of apical endpoints (Mills and Chichester, 2005; Ankley et al., 2008).

6.4. Evaluation of the Fish Sexual Development Test

The Fish Sexual Development Test (FSDT) has several advantages. The sexual development phase has been shown to be the most critical life stage with respect to sensitivity and maintenance of effects of EDCs (Knacker et al., 2010). Further, the FSDT integrates endpoints that might both indicate a mode of action and that are ecologically relevant to conduct quantitative risk assessment of the test chemicals. Therefore, the FSDT might be appropriate to replace other higher tiered *in vivo* fish tests that are more time and resource consuming, i.e the fish full life cycle test and the fish 2-generation test, as long as the most sensitive time window of exposure and the most sensitive endpoints related to the specific endocrine mechanism of action of the tested substance are covered (Knacker et al., 2010).

Studies on the development and application of the FSDT have shown that several classes of EDCs, i.e. (anti-)estrogens, (anti-)androgens and steroidogenic inhibitors have the potential to influence sex ratios and vitellogenin concentrations in model fish species (Andersen et al., 2004; Bogers et al., 2006; Holbech et al., 2006; Kinneberg et al., 2007; Panter et al., 2006) and that the assay is suitable to screen EDCs. The vitellogenin concentration is widely used as endpoint for estrogenic exposure in fish (Jobling and Sumpter, 1993), and the sex ratio endpoint has been shown to be an effective biomarker for estrogenic and androgenic chemicals that influence a skewed sex ratio towards females or males, respectively (Andersen et al., 2003; Örn et al., 2003). However, by comparing all these studies, the precise mechanism of action of the test chemical cannot be identified by only the sex ratio and vitellogenin endpoints. Indeed, it has been shown that exposure of fish to an anti-estrogen or to an aromatase inhibitor induced a male-biased sex ratio, which could also be interpreted as an androgenic mechanism of action. Furthermore, inconsistencies in the vitellogenin endpoint are found between these studies. For example, the exposure of juvenile zebrafish to an anti-estrogen or an aromatase inhibitor caused an increase in vitellogenin concentrations, whereas a reduction would have been expected (Andersen et al., 2004). In addition, consistent with the report of Kinnberg et al. (2007), our FSDT study (chapter 5) showed that fish exposed to prochloraz presented a male-biased sex ratio. Yet

prochloraz possesses several known mechanism of action that cannot be clearly identified in this study. These observations suggest that *in vivo* screening assays shoud be accompanied by further *in vitro* studies to reveal additional information on the mechanism of action of EDCs.

The results of our FSDT study show that the choice of fish species is essential to identify a threshold effective concentration for hazard assessment. Indeed, the exposure to prochloraz influenced the sexual differentiation of zebrafish and fathead minnow in different patterns of response. Further, according to the chosen endpoint, the threshold concentration could vary between the two fish species. With regard to the most relevant endpoint of this study, the sex ratio, the zebrafish was more sensitive to the effects of prochloraz as the fathead minnow. One explanation for this difference could be the different timing of sexual differentiation between the two fish species. The gonads of the zebrafish stay longer in a "juvenile hermaphrodite" or undifferentiated state as the gonads of the fathead minnow (Maack and Segner, 2003; van Aerle et al., 2002), thereby leading to a larger bioaccumulation of prochloraz till this critical period of life. If we follow this hypothesis, then the juvenile brown trout, whose period of undifferentiated gonads is much longer (Billard, 1987), would be much more sensitive to effects of prochloraz on sexual development as the fathead minnow and the zebrafish. However, a similar FSDT assay with juvenile brown trout exposed to prochloraz would be required to confirm this. Nevertheless, so far, the zebrafish would appear to be the most suitable fish species to evaluate EDCs in the FSDT, since it was more sensitively influenced in the sex ratio endpoint as was the fathead minnow.

In conclusion, in this thesis we have developed, applied and evaluated *ex vivo* and *in vivo* testing methods to identify and assess potential endocrine active substances that specifically interfere with the sex steroid biosynthesis pathway. Although the *ex vivo* gonad assay need further characterization, we suggest that it is a suitable and powerful tool to identify steroidogenic inhibitors and to be applied for investigating their mechanisms of action. Furthermore, it could be used in the future for environmental monitoring. The *ex vivo* assay represents a first step in the tiered approach of EDCs hazard assessment that allows to decide if further testing is required, and if this is the case, to properly chose and organize the

type of *in vivo* screening assay and the most relevant endpoints to confirm the endocrine-disruptive effects of the test chemicals. Further, to bridge the gap between endocrine mechanistic data of the *ex vivo* gonad assay and the characterization of apical endocrine adverse effects in the FSDT, additional common endpoints between the two assays (i.e. sex steroid profiles) should be integrated. Finally, although further studies would be indicated to confirm the sensitivity of the FSDT assay and to allow proper extrapolation of the results between different fish species, this *in vivo* assay is suitable for both characterizing endocrine disruptive effects and determining regulatory useful threshold concentrations of the tested substances.

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2008 – 2009	Organization of a seminar for the Master of Sustainable Development, Title: «Integrated Urban Water Management» Department of Environmental Sciences, University of Basel
Jan 2005 – March 2006	Work experience at the Federal Office for the Environment FOEN: Notification procedures for substances submitted to the Rotterdam Convention (PIC Convention); Reports on substances submitted to the OSPAR Commission (Commission for the protection of the marine environment of the North-East Atlantic); Annual activity report for the MONARPOP project (Monitoring network in the Alpine region for persistant and other organic pollutants) Industrial Chemicals Section, Waste management, Chemicals and Biotechnology Division, FOEN, Ittigen, Switzerland
July 2004 – Dec 2004	Work experience in environmental education: conception of the annual exhibition «Le karst et le monde magique des grottes»; pedagogic activities and guided tours Centre Nature Les Cerlatez, Saignelégier, JU – Switzerland
July 2003 – Dec 2003	Work experience in project management in biotechnology: coordination of scientific Swiss and European teams for the development of a new technology for the treatment of industrial pollutants Start-up BELAIR Biotechnologie, EPFL, Lausanne, Switzerland
April 2003 – June 2003	Technician in biological control of arthropods CABI Biosciences Switzerland Centre, Delémont, Jura
2002 – 2003	Teaching in secondary and high schools in the Canton of Jura
2001	Research work in biochemistry in the group of Dr. R. Chiquet-Ehrismann Friedrich Miescher Institute, Basel, Switzerland

Platform presentations and posters

2009	1st Young Environmental Scientists (YES) Meeting, Landau, Germany Presentation: Development of an *in vitro* gonad culture to quantify the effects of aromatase inhibitors in brown trout (*Salmo trutta fario*)
2009	Syngenta Product Safety External Collaborations Day, Berkshire, UK Poster: Description of an *ex vivo* brown trout (*Salmo trutta fario*) gonad culture suitable for assessing chemical effects on the steroidogenic pathway of a native European fish species
2007	8th International Symposium on Reproductive Physiology of Fish, Saint Malo, France Poster: Development of an *in vitro* gonad culture to quantify the effects of aromatase inhibitors in brown trout (*Salmo trutta fario*)
2007	Conference NRP50 «Endocrine Disruptors; Relevance to Humans, Animals and Ecosystems», Biel, Switzerland Poster: Development of an *in vitro* gonad culture to quantify the effects of aromatase inhibitors in brown trout (*Salmo trutta fario*)

Publications

2011	a Marca Pereira M.L., Thorpe K.L., Wheeler J.R., Burkhardt-Holm P. Development of an *ex vivo* brown trout (*Salmo trutta fario*) gonad culture for assessing chemical effects on steroidogenesis. *Aquatic Toxicology*, 101, 500-511.
2011	Thorpe K.L., a Marca Pereira M.L., Schiffer H., Burkhardt-Holm P.,Wheeler J.R. Mode of sexual differentiation and its influence on the relative sensitivity of the fathead minnow and zebrafish in the fish sexual development test. *Aquatic Toxicology*, 105, 412-420.
Accepted	a Marca Pereira M.L., Eppler E., Thorpe K.L., Wheeler J.R., Burkhardt-Holm P. Molecular and cellular effects of chemicals disrupting steroidogenesis during early ovarian development of brown trout (*Salmo trutta fario*). *Environmental Toxicology*.
Submitted	a Marca Pereira M.L., Thorpe K.L., Wheeler J.R., Burkhardt-Holm P. Comparative *ex vivo* and *in vivo* effects of prochloraz and tributyltin on sex hormone biosynthesis in juvenile brown trout (*Salmo trutta fario*). For *Comp Physiol Biochem*.

Subventions

2009	Grant of 15‘000 CHF from the Foundation Emilia Guggenheim-Schnurr der Naturforschenden Gesellschaft, Basel, Switzerland, awarded to Maria L. a Marca Pereira and Dr. Karen L. Thorpe. Project: Application of a brown trout *ex vivo* gonad culture for investigating the effects of chemical exposure on steroidogenesis
2004	Grant of 33’000 CHF from Syngenta, UK Collaborative project with Prof. P. Burkhardt-Holm, University of Basel Project: Solvent mode of action on fish endocrinology: interactions with endocrine disrupter modes of action

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