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Developmental Exposure of Zebrafish (*Danio rerio*, Hamilton-Buchanan 1822) to Endocrine-Active Substances:

Impact on Vitellogenin, Aromatase and Sexual Differentiation

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Developmental Exposure of Zebrafish (*Danio rerio*, Hamilton-Buchanan 1822) to Endocrine-Active Substances:

Impact on Vitellogenin, Aromatase
and
Sexual Differentiation

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Contents

List of Abbreviations

ACTH	Adrenocorticotrophic hormone
ANOVA	Analysis of variance
βACT	Beta-actin
BPA	Bisphenol A
cAMP	Cyclic adenosine 5'-monophosphate
cDNA	Complementary deoxyribonucleic acid
CRE	cAMP responsive element
CYP19	Cytochrome P450 aromatase gene
DEPC	Diethyl pyrocarbonate
cNTP	Deoxyribonucleoside triphosphate
cpf	Days post-fertilization
EDC	Endocrine disrupting compound (chemical)
E_2	17β-Estradiol
EE_2	17α-Ethinylestradiol
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
ERE	Estrogen responsive element
FPLC	Fast protein liquid chromatography
GABA	Gamma aminobutyric acid
GnRH	Gonadotropin releasing hormone
GTH	Gonadotropic hormone
LH	Luteinizing hormone
LOEC	Lowest observed effect concentration
M-MLV	Moloney-murine leukemia virus
mRNA	Messenger ribonucleic acid
MT	17α-Methyltestosterone
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
NSB	Non-specific binding
OECD	Organization of Economic Cooperation and Development
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAH	Polycyclic aromatic hydrocarbon
PBR	Peripheral-type benzodiazepine receptor
pf	Post-fertilization
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl-sulphate
StAR	Steroidogenic acute regulatory protein
VTG	Vitellogenin
z-VTG	Zebrafish vitellogenin

I. Intention of this work

Although it is more than twenty years ago that there were first reports of incidences of sexual disturbances in wild fish populations in British rivers, endocrine disruption is still a matter of intensive scientific research activities and public concern. Albeit there has evolved an important scientific discipline, dealing in numerous studies all over the world with the topic endocrine disruption, particularly in fish, there are still numerous questions to be answered. The motivation of this thesis is, to answer at least some of the arising questions concerning endocrine and particularly reproductive disruption in fish.

Within the framework of research on the impact of endocrine-active substances on fish, the objectives of this thesis are a) to investigate the response of two potential molecular targets for the action of estrogenic compounds, vitellogenin and aromatase, b) to evaluate the relation between the molecular response and alterations of sexual differentiation and reproduction of the exposed organism and c) to consider the biomarker potential of both molecular targets.

The first two chapter of the present work deal with the egg-yolk precursor protein **vitellogenin (VTG)**, which is synthesized under estrogenic control in the liver, primarily of female fish. Induction of VTG especially in male fish that usually do not or only at very low amounts synthesize VTG (Copeland et al. 1986), has become a valuable and commonly applied biomarker for environmental exposure of fish to estrogenic (estrogen-like acting) chemicals (Sumpter and Jobling 1995; Kime et al. 1999). To show a causal relationship between estrogenic exposure and VTG induction in fish, it is necessary to apply a well validated and species-specific *in vivo* screening method. This is an indispensable prerequisite for a reliable and sensitive measurement and a critical assessment of VTG afterwards. Therefore, the first aim of this work was to develop and validate a homologous assay for the detection and quantification of VTG in the study organism, the zebrafish **(Chapter 1)**.

The following objective of this work is concerned with the applicability and suitability of the VTG assay to screen samples of different developmental stages of zebrafish for estrogenic exposure. Additionally, it was aimed at investigating the relationship between VTG induction and reproductive impairments as a basis for a critical evaluation of the

function of VTG as a biomarker of estrogenic disruption in the zebrafish. The link between VTG induction and reproductive disruption in the cause of estrogenic exposure of fish has been addressed by few studies only yet, thus the relationship between VTG and organizational reproductive effects is poorly understood. As an approach to this issue, the impact of developmental exposure of zebrafish to two model xenobiotics on VTG induction was investigated and correlated with the effects on sexual differentiation and reproduction **(Chapter 2)**.

The third chapter of this work deals with one of the key enzymes of steroidogenesis, the **P450 aromatase**, the enzyme that catalyzes the conversion of the androgens androstenedione and testosterone to estradiol. The synthesis of estradiol has been proposed to be the pivotal step in sexual differentiation, at least in gonochoristic fish (see Yamamoto et al. 1969; Nakamura et al. 1998; Baroiller et al. 1999 for review). To enlighten the physiological implication of aromatase and therefore the synthesis of estradiol in the sexual differentiation of a (juvenile) protogynic hermaphrodite like the zebrafish, the expression of the P450 aromatase genes **(CYP19A and CYP19B)** was investigated at different developmental stages. It was assumed that exogenous interference with the aromatase throughout sexual differentiation might disrupt or even change the differentiation process. A corroboration of this assumption would, in turn, provide evidence for a decisive function of aromatase gene expression in the process of sexual differentiation of the zebrafish.

This study belongs to the few recent approaches that also consider the direct interrelation between the molecular regulative pathways of the endocrine reproductive system (exemplified by the P450 aromatase gene expression) and the interference of endocrine disrupting chemicals with the reproductive function **(Chapter 3)**.

The following '**General Introduction**' to this work gives a survey of the basic subjects, the study organism and the chemicals concerned:

⇒ Section II.1 aims to introduce into the overall topic of the work that is 'endocrine disruption in fish'.

⇒ Section II.2 deals with the two model xenobiotics used in this study, which are able to act as estrogen mimics. These xenoestrogens are therefore suspected to modulate or even disrupt the reproductive endocrine system of fish.

⇒ Section II.3 conveys some information on the test organism used in this work, the teleost fish species zebrafish (*Danio rerio*).

⇒ Section II.4 describes the fundamental principles of vitellogenesis in teleost fish

⇒ Section II.5 gives a detailed review of what is basically known about the synthesis of steroid hormones (steroidogenesis) and the regulative pathways involved, in vertebrates and in fish in particular.

II. General Introduction

II.1 Endocrine disruptive effects in the aquatic environment

First indications of pollutants in aquatic systems with hormone-like activity were found in the early 80's, when there were first reports of intersexuality or hermaphroditism in wild populations of roach (*Rutilus rutilus*) in British rivers (Sweeting 1981), predominantly downstream of sewage treatment works (STW). Intersexuality or hermaphroditism, even at low incidences, were believed to be rather uncommon in gonochoristic temperate fish species like the roach (Jafri and Ensor 1979). Almost at the same time British scientists were able to measure considerable amounts of the egg-yolk precursor protein vitellogenin (VTG), usually synthesized by only female fish, in male rainbow trout from a research station also situated in the discharge of a STW. As the vitellogenin gene expression is estrogen-dependent and is inducible also in male fish (Copeland et al 1986; Sumpter 1995; Sumpter and Jobling 1995 etc.), this was the first indication of the presence of estrogen-like compounds in the aquatic environment. Thereafter, similar observations in wild fish populations were reported from all parts of Europe. Very recently, studies have shown that disruptions in gonad development in wild fish result in reduced reproductive output (Jobling et al. 2001, 2002), which, in turn, may have population level consequences.

Furthermore, the effects seen in wildlife are not limited to 'hot spots' of chemical discharge but occur in the wider ambient environment across Europe. These findings raised some serious concerns about "artificial estrogens" and their potential impact on aquatic organisms. A growing awareness has been developed that these chemicals were more abundantly as expected and that there might be a reasonable hazard arising also for humans, especially when considering their role as a top predator. Therefore, investigations on effects of environmental compounds with estrogenic activity on the endocrine system in humans and in wildlife emerged as a major issue in science and public policy in recent years.

Interestingly, most reported examples of endocrine disrupting effects in wildlife have occurred in the aquatic environment or were associated with aquatic animals,e.g., as food source. This is no coincidence, and one simple reason for it is the fact that the aquatic environment serves as the ultimate sink for most waste and pollutants. Moreover, aquatic organisms like fish were exposed to higher concentration of contaminants than terrestrial organisms, due to direct exposure of the whole body surface to the contaminated environment. The direct interaction between environmental chemicals and highly permeable surfaces, like the gills, facilitates the uptake and permits the direct transport to the blood circulation. High respiratory water volumes increase the bioconcentration in aquatic organisms, and water as a very suitable solvent for (hydrophilic) chemicals, also increases their bioavailability. Terrestrial species, however, take up endocrine disrupting chemicals (EDCs) predominantly via food and digestive processes may therefore degrade, metabolize and detoxify chemicals.

Aquatic organisms and especially fish have been shown to function as sensitive sentinels for the detection of potential hazards arising from environmental pollutants that may act as EDCs.

II.2 "Endocrine disruptors"-what do we know about the chemicals applied in the studies?

17α-Ethinylestradiol (EE_2; 17alpha-Ethynyl-1,3,5(10)-estratriene-3,17 beta-diol)

Ethinylestradiol (EE_2) is a potent synthetic estrogen and the main compound of most oral contraceptives. It is therefore produced in large quantities by the pharmaceutical industry. Consequently, contamination of surface water with EE_2 is attributed to anthropogenic origin. EE_2 is largely excreted via the urine in inactive conjugates (mainly

as sulfates but predominantly as glucuronides and hydroxy-metabolite) or in the unconjugated form with other metabolites via the faeces (Ranny 1977; Guengerich 1990)., EE_2 and its conjugated metabolites thus reach the aquatic environment mainly through the discharges of STWs (sewage treatment works). Wegener et al. (1999) showed that the glucuronides of EE_2 are easily biotransformed back into their unconjugated active forms by microbial action. These processes of microbial cleavage of EE_2 conjugates can either take place already during the sewage treatment processing or in the receiving water, after discharging from the STW. High synthesis rates of β-glucuronidase, the steroid glucuronide-cleavage enzyme, and of sulfatases were demonstrated for common aquatic bacterial species like *Escherichia coli* (Tabak and Bunch 1970; Tabak et al. 1981). Due to high densities of microbial populations found in the sewage and sludge of the STW, a predominant release of unconjugated, bioactive EE_2 within the STW is implied. This is further supported by studies on glucuronidase enzyme activity in activated sludge that showed turn over rates approximately at steady state (Ternes et al. 1999b). Concentrations of unconjugated EE_2, measured in effluents of some STW, were shown to be higher than the concentrations of the natural estrogen. This is surprising given that the proportion of natural estrogens (estradiol, estrone and estriol, mainly as conjugates) excreted by women and female animals, should exceed the amount of excreted synthetic estrogens like EE_2. Blok and Woesten (2000) calculated the total daily amount of excreted natural estrogens of livestock and human population of the Netherlands. The anthropogenic supply was at least only one tenth of that of livestock, reaching 46 kg/day. Concentrations between 1.0 ng/L and 17 ng/L (median value), e.g., found in effluents of municipal German STPs (sewage treatment plants) could not be reached if EE_2 would not have been re-bioactivated during STW processing (Stumpf et al. 1996; Ternes et al. 1999a). Although synthetic estrogens like EE_2 were eliminated by approximately 80 to 90 % (Stumpf et al. 1996; Ternes et al. 1999a; Adler et al. 2001), the remaining levels in the lower ng/L range may reach or exceed threshold levels of adverse endocrine effects in aquatic organisms. An additional aspect to be considered results from the poor biological degradation of synthetic estrogens to that of the natural hormones. EE_2, e.g., was degraded in effluent water of a STW by only 15 % at aerobic conditions within a few days whereas the natural estradiol was degraded by almost 95 % (Wegener et al. 1999) during the same period.

It has become obvious that the exogenous supply of natural and synthetic estrogens to surface waters is one principle cause of endocrine disruption in aquatic animals (Purdom et al. 1994; Desbrow et al. 1998; Routledge et al. 1998; Tyler and Routledge 1998). In fish,

EE_2 has been shown to act as a very potent inducer of vitellogenesis. Exposure of male rainbow trout by immersion over a period of ten days to EE_2 at concentrations of ≥ 0.1 ng/L caused a significant induction of vitellogenin synthesis already (Purdom et al.1994). Although environmental concentrations of EE_2 measured in most aquatic systems to date were mostly below the threshold levels known to induce endocrine effects in aquatic organisms, other factors, like the accumulation of EE_2, have to be considered. However, studies on bioaccumulation and -transformation of environmental estrogens, like EE_2, in fish are found rather scarce (Morrison et al. 1999; Olsen et al. 2000). Calculations of bioconcentration- or bioaccumulation factors are often derived from computer-based models or have been based on predictions using the octanol-water coefficient of the corresponding compound. Based on a specific food-web model, Lai et al. (2001), e.g., calculated bioaccumulation factors (BAF) for EE_2 in fish at different trophic levels. Predicted BAF values between 170 and 332 for EE_2 in fish were up to 57 times higher than those calculated for estradiol. Although the significance and impact of EE_2- (or other estrogens) bioaccumulation is strictly dependent on the physiological status of the organism during exposure, these data indicate that bioaccumulation of EE_2 is likely to occur (Larsson et al. 1999).

Natural endogenous steroid hormones are specifically bound mostly to high-affinity plasma steroid binding proteins to enable the transport of these lipophilic compounds through the blood circulating system. The rest of sex steroid hormones circulates in complex with low-affinity proteins like albumin. Steroid-specific binding protein systems, which bind the gonadal steroids testosterone and estradiol with high affinity (testosterone > estradiol), have been identified and described in plasma of fish species like in the rainbow trout, *Oncorhynchus mykiss* (Fostier and Breton 1975) or the spotted seatrout, *Cynoscion nebulosus* (Laidley and Thomas 1994). This SHBG (sex hormone binding globulin) is believed to be an important modulator of endogenous steroid hormone action (Hammond 1995). The presence of high-affinity steroid binding proteins in the plasma reduces the "free" hormone to very low levels (e.g., 1 % of the total), restricting their availability to target cells (Hammond 1995; Petra 1991). This regulates their biological activity. However, this could not be confirmed for exogenous steroids like EE_2 or xenoestrogens. As it was shown in studies with rainbow trout or carp, most environmental estrogenic agents are unlikely to produce biological effects by displacing endogenous steroids from plasma steroid binding proteins, unless they are present at very high concentrations (Kloas

et al. 2000; Milligan et al. 1998). In plasma of rainbow trout, EE_2 showed the highest binding affinity for the SHBG among most other estrogen mimics (Tollefsen 2002). In this study, EE_2 bound at about 130 times higher concentrations than the natural E_2, which corresponded to a relative binding affinity of 0.77 %. Industrial chemicals in contrast, only displayed very low binding affinities for SHBG. Bisphenol A (BPA), e.g., proved to be about 10^5 fold less potent than E_2 concerning binding. Although estrogen mimics have shown to be able to bind to SHBGs and to displace natural hormones (Milligan et al. 1998; Crain et al. 1998), they were only able to do so at very high concentrations. Therefore, EE_2 or BPA were presumably scarcely bound by SHBGs and may be expected to circulate unbound or weakly bound to low affinity proteins in plasma. This may consequently raise the bioavailability of these compounds relative to E_2 for certain target cells and therewith their biological effectiveness (Crain et al. 1998; Nagel et al. 1998).

Like other steroid hormones, EE_2 is thought to act primarily through the regulation of gene expression, inducing a severe increase in RNA and protein synthesis. Due to the nonpolar lipophilic nature of steroids, EE_2 is capable of penetrating through lipid membranes of all cells, presumably by means of passive or facilitated diffusion. In the cytoplasm EE_2 binds to estrogen receptors and generate a ligand-receptor complex. Once bound, this receptor complex undergoes allosteric conformational changes and converts from an inactivated into an activated form. This activated complex exerts estrogenic activity via specific interactions with differential nuclear EREs and thus regulates expression of the corresponding genes (Tsai and O'Malley 1994). Estrogen receptors are found in the reproductive tract, pituitary, hypothalamus, bone, liver and other tissues (Evans 1988).

Species-specific differences should always be considered when evaluating effect data for steroid hormones and their mimics in experimental animals. Administration of exogenous steroid hormones yields considerable interspecies variation in physiological responses due to potency differences between hormonal agents, differential pharmacokinetic patterns, and unique hormonal physiology (sensitivity) of each species. For EE_2, studies on the specific biovailability and pharmacokinetic of this compound in fish are rather scarce. This complicates the interpretation of dose-response relationships. In one of the few studies of that kind, the uptake and metabolism of EE_2 was characterized in male rainbow trout (Schultz et al. 2001). They showed that the concentration-time profile in plasma was particularly influenced by enterohepatic recirculation. After injection, fast glucuronidation and excretion to the bile predominantly eliminated EE_2. Only a minor

fraction of these EE_2 conjugates was actually eliminated. Most of the conjugates therefore appeared to be released from the bile into the gut, where they ware deconjugated and reabsorbed. The effect is that fish is 're-dosed' with EE_2 due to enterohepatic recirculation. Consequently, the concentration-time profile of EE_2 in fish plasma is probably not characterized by a fast linear decrease, but especially at higher doses indicates several peaks of re-increased plasma doses.

Bisphenol A (BPA; 2,2-bis[4-hydroxyphenyl]propane)

99.9 % of the industrially produced BPA is used as an intermediate in the production of polycarbonates, epoxy resins, flame-retardants and other specialty products from plastic industry (Staples et al. 1998). There are several potential routes how BPA enters the environment, direct as well as indirect through discharges and releases while processing, handling and manufacturing this chemical. BPA was characterized by the Society of Plastic Industry as "slightly to moderately toxic" to fish and by "having a low potential for bioaccumulation in aquatic organisms (Staples et al. 1998).

Pharmacokinetic characteristics of BPA were intensively studied in mammalian species, like especially rats and mice. Uptake, metabolism and excretion have been examined and characterized in the course of biotransformation processes. In those processes, lipophilic and unpolar compounds (with, e.g., aromatic of phenolic groups) are conjugated in the phase II reactions with polar molecules like glucoronyl or sulfonyl groups to increase their hydophilic character, facilitating metabolism and/or secretion. Glucuronidation has proven to be the main metabolizing reaction to BPA in mammals (Knaak and Sullivan 1966; Yokota et al. 1999).

In fish, toxicokinetic studies on metabolism and excretion of BPA or other bisphenols are rather scarce. Recently, the uptake, metabolism and excretion of BPA were characterized in rainbow trout (*Oncorhynchus mykiss*) after water-borne exposure or injection of the substance (Lindholst et al. 2001). When fish were exposed through the water, a steady-state level of absorbed in plasma, liver and muscle is reached after 6-12 h. Bioconcentration factors (BCF) were between 3.5 (plasma) and 5.5 (muscle). The uptake of BPA into the blood coincides with the appearance of the main degradation product of BPA, BPA-glucuronic acid (BPAGA). The plasma level of BPAGA exceeded the BPA very fast within 2-6 h. By this process of glucuronidation, the log K_{ow} (partition coefficient) of BPA was significantly reduced from 3.4 – 3.8 to 1.4 – 1.8 (for BPAGA) (Staples et al. 1998). The results from the BPA injection experiments gave a good

indication to the excretion behavior of BPA from plasma, liver and muscle. Maximum concentrations in all three compartments were reached within 2 h after intraperitoneal injection. Based on a first-order exponential decay regression model the plasma half-life of BPA was determined as 3.75 h. The excretion rates of BPA from the three compartments plasma, liver and muscle were compared and could be ranked in the order liver > plasma > muscle. The highest excretion rate of 92.3 % from the liver was reached within the first 12 h after injection. Concerning the induction of vitellogenin by BPA exposure, it is most interesting that the lag periods in male and female trout between injection of the substance and the significant increase of plasma VTG were different. The lag time in male was with 5-7 days approximately twice as high as in female trout. Moreover, it is intriguing that the highest VTG plasma levels were observed at a time when the BPA has already been cleared from the liver tissue. This means that the vitellogenin response persists for a longer time than the actual exposure. These findings are of considerably significance for the use of VTG as a biomarker for acute exposure to EDCs.

II.3 The zebrafish (*Danio rerio*) as test species for the assessment of endocrine disruption

The zebrafish, *Danio rerio,* has been a popular fish species in freshwater aquaria for a long time. This species belongs to the ubiquitous and ecologically and economically important Osteichthyes family of Cyprinidae, and as a tropic warm water species, it is native to India and Pakistan (Axelrod and Schultz 1955). As a typical r-strategist, the zebrafish reproduce the whole year through and the generation time of approximately three to four months is rather short. The zebrafish is easy to handle and to breed, and as a very special feature of this species, its eggs are not only numerous but transparent. This facilitates the observation and investigation of embryonic development. Due to these opportunities, the zebrafish came into focus of studies of developmental biologists and embryologists already in the thirties (Creaser 1934; Roosen-Runge 1936 – 1939). In 1958 the embryonic development of the zebrafish was first described in detail by Hisaoka and Battle (1958). In the seventies, the zebrafish was considered as a favored candidate for a 'standard' test organism for toxicological risk assessment studies on water pollutants (Laale 1977). In the early eighties, the zebrafish was then discovered as a genetic model of vertebrate development. In 1994 the first genetic linkage map for the zebrafish was presented (Postlethwait et al. 1994) and in the meantime the zebrafish is on track to

become the next vertebrate species of which the whole genome is going to be completely sequenced. The beginning of this successful story originates from the intensive studies on zebrafish development and genetics in the late seventies and in the eighties by Streisinger (1981) and colleagues from the University of Oregon, Eugene. Over the last decades, the zebrafish has also evolved as a well-established test organism in ecotoxicology (Roales and Perlmutter 1974; Thomas 1975; Nagel 1988; Braunbeck et al. 1989; Ensenbach 1991, etc). It is widely used as a study species, especially in Europe, and OECD and USEPA test guidelines for its use in chronic life-cycle and early life-stage survival and development tests have been developed. Due to the to the several advantage of the zebrafish, it has become a favorable model organism and laboratory test species. In recent times, the zebrafish has also evolved as one of the most commonly used fish species for work on EDCs, besides the fathead minnow (*Pimephales promelas*) or the medaka (*Oryzias latipes*). Importantly for the studies presented in this work, the ontogeny of gonadogenesis has recently been documented (Maack 2002). A significant uncertainty in using the zebrafish as a test organism for the assessment of adverse effects of endocrine-active compounds is the lack of knowledge concerning basic aspects of sexual determination and maintenance, location and expression of sex-determining genes. In this respect, indistinct secondary sex characteristics in zebrafish make it difficult to distinguish between the sexes, especially in immature fish. In adult reproducing zebrafish, females generally differ from males in the rounder body contour, whereas the males are slender in appearance, and during or shortly after courtship the male coloration is darker and more intensive. The identification and characterization of sexual dimorphically expressed genes would tremendously improve and facilitate zebrafish use. Generation of phenotypically distinct mutants may be the most innovative approach in the work with zebrafish.

II.4 Vitellogenesis in teleost fish

In all oviparous vertebrates, the development of embryos takes place outside the maternal body and thus the survival and development of the offspring ultimately depends on the nutrient supply in the eggs. The main nutrient source for the embryo and the most substantial components of the egg have been characterized as yolk. In most cases, the definition of egg-yolk is restricted to its protein components. Besides lipids, carbohydrates and other components like lectins etc., the yolk proteins contribute > 80 % of the dry weight of eggs. The two major protein groups, composing the egg-yolk are the predominantly lipoprotein (lipovitellins) and the phosphoproteins, like phosvitins of

phosphettes (Wallace 1985; Tyler 1991). It is well established that the egg yolk proteins are not synthesized in the oocytes themselves but derive from precursor proteins that are selectively incorporated during oocyte development. Most of the yolk proteins are derived from the phospholipoglycoprotein VTG. Therefore, vitellogenesis represents a major aspect of oocyte development in fish (Wallace 1985). Many authors have already reviewed the stages of oocyte development in teleost fish (Wallace and Selman 1981; Wallace 1985; Mommsen and Walsh 1988; Selman and Wallace 1989; Tyler 1991 etc.). Deviating from the observation that the yolk proteins in teleost fish species are mainly stored as fluid-filled spheres (Wallace and Selman 1981), the zebrafish stores the yolk in the oocytes in the form of crystalline platelets (Länge et al. 1983; Länge 1985; Selman and Wallace 1989). This has previously been characterized as a common feature of several actinopterygian species (Länge 1985) as well as of anamniots.

Regulation of vitellogenesis in hepatocytes of teleosts, like in oviparous vertebrates in general, is mediated by the availability of estradiol (E_2) and the subsequent interaction with the estrogen receptors (ERs). Therefore, the higher level of regulative processes of vitellogenesis mainly bases on the hormonal control of steroidogenesis via the hypothalamus-pituitary axis. Main principles of steroidogenesis control will be described in the following section II.5. The induction of vitellogenin synthesis in oviparous vertebrates represents a direct response to ER-mediated stimuli in target cells that is in liver cells. VTG gene transcription is activated by the direct interaction of estradiol-estrogen receptor-complexes with EREs (estrogen responsive elements) of the VTG gene(s) promoters. The precursor VTG protein passes through intensive post-translational modifications in the endoplasmic reticulum and the Golgi apparatus (Wallace 1985; Chen et al. 1994) before it is released into the blood stream. VTG gene expression does not affect only one single VTG gene. As it has been proven for many other oviparous vertebrate species, vitellogenin genes belong to a multigene family and are often found in several copies in the genome. From the zebrafish genome, at least seven distinct VTG genes have already been cloned (Wang et al. 2000). Mommsen and Welsh (1988) or Silversand (1996), for instance, have reviewed vitellogenesis in teleost fish and the characterization of the VTG protein in detail.

In the last ten to twenty years, an increasing awareness on the hazards arising from hormonal acting, and especially estrogen-like acting contaminants in the aquatic

environment has developed. Much of the evidence from effects of EDCs has come from the observations of reproductive impairments in wildlife fish. These effects have been ascribed predominantly to the exposure to environmental estrogens (e.g., Purdom et al. 1994; McMaster et al. 1995; Jobling et al. 1998). The induction of VTG in male fish has become one of the most valuable indicator of estrogenic contaminants in the aquatic environment (Heppell et al. 1995; Sumpter and Jobling 1995; Tyler et al. 1996; Denslow et al. 1999). This yolk precursor protein is usually only expressed in female fish, but the estrogen-responsive vitellogenin gene(s) are also present in the genome of male fish. Due to the deficiency of plasma E_2, these VTG genes generally remain silent or predominantly silent in male fish. Since, however, hepatic estrogen receptors are also present in male fish, the VTG gene expression is triggered by the exposure to exogenous estrogens. The induction of VTG expression by the exposure to exogenous estrogens, thus beings a highly sensitive process. Therefore, the detection of VTG in the plasma of male fish is employed as a useful feature for detecting estrogenic contamination and even for the quantification of the estrogenic activity and strength of the contaminants.

II.5 Review of steroidogenesis

Steroidogenesis is accomplished by the action of two major enzyme families, the hydroxylases and the steroid dehydrogenases. The former belongs to the cytochrome P450 enzymes, which are encoded by genes of the cytochrome P450 superfamily. These cytochromes are located in the mitochondria (using ferredoxin and ferredoxin reductase as electron donors) or in the endoplasmic reticulum (using the ubiquitous electron donor NADPH reductase). The second enzyme family, the steroid dehydrogenases, consists of members of short-chain alcohol dehydrogenases/reductases or aldo-keto reductases. They all use either NAD^+/NADH or $NADP^+$/NADPH as cofactors. All steroid hormones are derived from the substrate cholesterol, which is either synthesized *de novo* from acetyl-CoA or is obtained from exogenous sources by receptor-mediated uptake of lipoproteins. It may also be released by hydrolyses of stored cholesterol esters in lipid droplets.

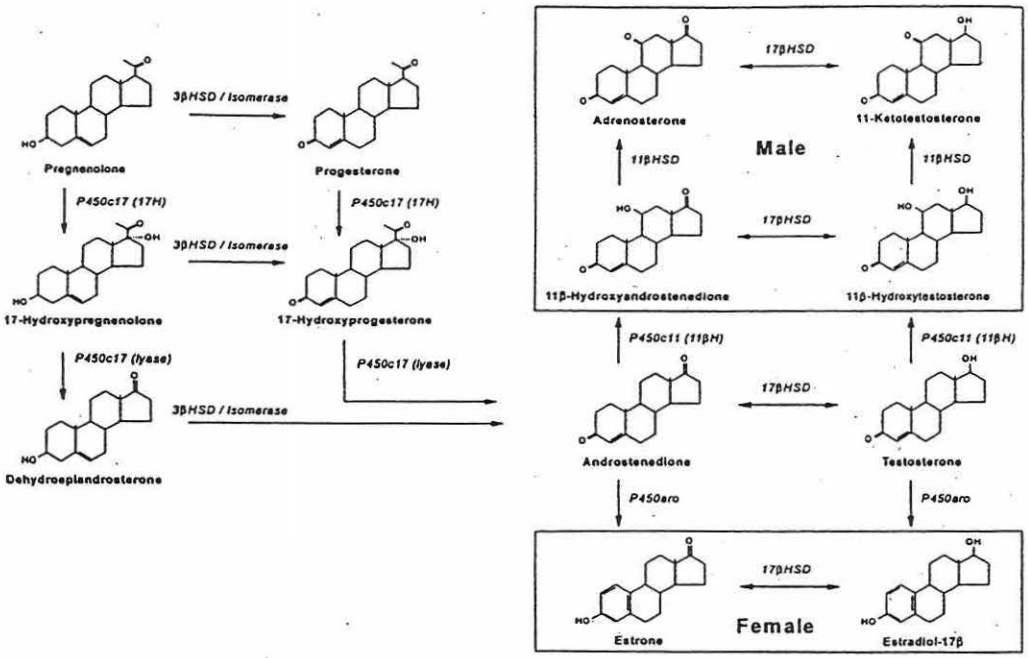

Fig. 1. Schematic illustration of some of the gonadal steroidogenic pathways in fish and participating enzymes. Enzyme activities are: aromatase (P450aro), 3β-hydroxysteroid dehydrogenase/Isomerase (3βHSD/Isomerase), 11β-hydroxysteroid dehydrogenate (11βHSD), 17β-hydroxysteroid dehydrogenate, 11β-hydroxylase P450c11 (11βH), 17α-hydroxylase/17,20 lyase P450c17 (17H/lyase).
(From: Baroiller et al 1999)

The first rate-limiting step in steroidogenesis is the conversion of cholesterol to pregnenolone by cholesterol side-chain cleavage enzyme ($P450_{scc}$; gene product of CYP11A) at the inner mitochondrial membrane. The most critical step in this reaction is the trans-membrane transport of cholesterol to the inner mitochondrial membrane by the mitochondrial peripheral-type benzodiazepine receptor (PBR) and the steroidogenic acute regulatory protein (StAR). This first reaction step can be influenced on different levels by acute or long-term actions, e.g., by LH (luteinizing hormone) or ACTH (adrenocorticotrophic hormone) or through translational effects on StAR.

The following reaction steps are catalyzed by the same single enzyme, the 17α-hydroxylase/17,20 lyase ($P450_{17\alpha}$; CYP17 gene product(s)), which is localized in the endoplasmic reticulum. Expression/activity of this enzyme is also regulated through multiple regulative and tissue-specific mechanisms.

The subsequent aromatization of C_{19} steroids (androstenedione or testosterone) to estrogens is catalyzed by the aromatase ($P450_{arom}$; CYP19 gene product). The underlying reaction involves the conversion of Δ^4-3-one A-ring of androgens to the corresponding

phenolic A-ring characteristics of estrogens (C_{18}). This enzyme is localized in the endoplasmic reticulum and predominantly expressed in brain and gonad tissue. Transcription of CYP19 is tissue-specifically regulated by the use of different specific promoters, resulting in differential splicing.

This last committed step in estrogen formation maintains the critical balance of androgen and estrogen synthesis. These sex steroid hormones are essential for the endocrine control of reproduction and reproductive development in mammals and play a crucial role in other vertebrates as well.

Cytochrome P450 aromatase reaction

The aromatase complex consists of two components: a) the aromatase cytochrome P450 and b) the NADPH-cytochrome P450 reductase (reductase or oxidoreductase). The former part is responsible for the substrate binding, the reductase facilitates the flow of electrons necessary for the oxidation of the substrate. As a member of the cytochrome P450 enzyme superfamily, the aromatase complex is membrane bound like all other eukaryotic P450's. In general, both components are believed to be associated only with microsomal compartments, mainly in the endoplasmic reticulum. The chemical mechanisms of androgen conversion, catalyzed by the aromatase complex, seem to be unique in all vertebrates as it comprises three consecutive oxidation reactions. It necessitates the sequential transfer of three pairs of electrons, consuming 3 mol of oxygen and 3 mol of NADPH for 1 mol of estrogen.

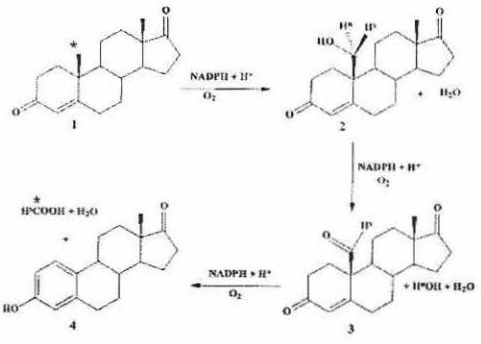

Fig. 2. Aromatization of androgens to estrogens. (1) Androstenedione or testosterone substrate; (2) and (3) intermediates of reaction; (4) Estrone and estradiol products.
(From: Brodie et al. 2000)

The aromatase cytochrome P450 owns a heme prosthetic group with a Fe^{3+}-ferric ion center. The NADPH-cytochrome P450 reductase contains two flavin prostethic groups, FMN and FAD, as cofactors within its catalytic side. Only upon association of these two catalytic components, the aromatase complex is able to express its catalytic activity. After the substrate has been bound to the binding cavity of the P450, the reductase associates

with this complex. A pair of electrons is transferred from NADPH (e^--donor) via the two flavin prosthetic groups to the ferric ion of the P450. Interaction with this heme group and electron transfer activates oxygen and enables its attack on the C19 of the methyl group of the substrate, leading to a hydroxylation of this group. Repetition of this process of electron transfer and oxygen reduction results in the second reaction in a conversion of this hydroxyl-group at C19 to an aldehyd group. The concrete mechanism of the following third oxidation, in which the aromatic ring is formed, is still debated lively. Akhtar et al. (1997) propose a mechanism in which the aldehyd carbon is expelled as formic acid (this expulsion was proven in using isotopic experiments by Skinner and Akhtar, 1969), and the 3-keto function should be converted to an enol-intermediate before the cleavage of the C10-C19 bound (Oh and Robinson 1993).

Reported affinity constants K_M for the two main $P450_{arom}$ substrates androstenedione and testosterone show generally high values. Although, due to the lack of expression of 17β-hydroxy-steroid dehydrogenase ($P450_{c17}$, the enzyme converting androstenedione to testosterone, androstenedione often seems to have a higher metabolizing rate V_{max}. The metabolism of androstenedione to estrogen is generally favored over testosterone, at least in human ovarian cells. As a consequence of the high catalytic activity and substrate affinity of the $P450_{arom}$, and presumably in need of a coordination of $P450_{c17}$ and $P450_{arom}$-expression regulation, a compartmental separation of estrogen and androgen synthesis is shown by many studies, especially in humans. In the ovary, this phenomenon is described as the "two-cell theory" of follicular estrogen synthesis (Hillier et al. 1994). A two-cell type model of estrogen synthesis in follicle cells of fish has at least been described for salmonid species (Kagawa et al. 1985).

Besides all differences in the regulation and localization of expression among species, each component of the aromatase complex is highly conserved. Among all vertebrates, peptides demonstrate 50 – 90 % sequence identities between fish and mammals. The degree of conservation is even more pronounced on the genomic level. The general organization of the gene is quite similar, despite considerable variances in size between species of diverse taxonomic levels. This high level of conservation of the aromatase complex is consistent with its relatively recent evolution that was accompanied by a high degree of selection pressure.

A high diversity of expression sites of $P450_{arom}$ among vertebrate species is most typically found and the reasons for species-specific tissue distribution patterns of estrogen

synthesis are often still unclear. Principally, aromatase activity can be found in gonad and brain tissue of almost all vertebrates. Expression of $P450_{arom}$ in the central nervous system emphasizes a probable key function of this enzyme in neuroendocrine regulative processes (Naftolin et al. 1975). Several studies on humans gave strong indications that tissue-specific promoters and alternative splicing mechanisms are essentially responsible for a high variability in the expression regulation (Simpson et al. 1997; Kamat et al. 1999; Means et al 1991; etc). In mammals the P450arom is generally encoded by one single CYP19 gene, besides the discovery of duplicated entirely functional copies of P450arom in the genome of the pig (Corbin et al. 1995; Graddy et al. 2000).

Regulation of brain aromatase:

Expression of $P450_{arom}$ in the central nervous system, its function in neuroendocrine processes and mechanisms of regulation in different vertebrate species have already been in focus of many recent studies. Sex steroid hormones, such as testosterone and estradiol, exhibit key role functions in behavioral and physiological, sex-specific processes. The conversion reaction of androgens into estrogens plays a crucial role in the regulation of reproductive behavior, sexual differentiation and feedback control of gonadotropin secretion (Balthazart 1989; Balthazart and Foidart 1993). Immunocytochemical studies on the cellular localization of aromatase in the brain of birds gave strong indications that aromatase is in certain brain areas often co-localized with estrogen receptors (Balthazart et al. 1991; Balthazart and Ball 1998). Similar findings in specific parts of the brain in mice and rats (Tsuruo et al. 1995, 1996) suggest that this might be a common feature among vertebrates. Nevertheless, a direct intracrine control of aromatase synthesis by locally formed estrogens could not be observed. In most aromatase-active brain areas of rodents and birds, an intracellular co-localization of ER was not found. Moreover, as yet, no estrogen-responsive element have been found upstream of or in the aromatase exon 1 in rat (Kato et al. 1997). Accordingly, a trans-synaptic, catecholaminergic control of aromatase by estrogens was postulated. At least a close anatomical relationship between aromatase active cells and fibers with tyrosine hydroxylase (TH), the rate-limiting enzyme of catecholamin synthesis, expression in almost all brain areas would confirm this assumption (Balthazart and Ball 1998). Further support of this hypothesis of an afferent catecholaminergic regulation of aromatase activity is given by the detection of a regulative capacity of cyclic AMP (cAMP) on aromatase action in a variety of rodent tissues including brain (Mendelson 1984; Abe-Dohmae et al. 1997). The concentration of cAMP is modulated via dopamine (DA) and noradrenaline (NA) release and their postsynaptic

binding to dopaminergic receptors which, in turn, increases adenylyl cyclase activity (Etgen et al. 1986; Hickey et al. 1990; Simpson et al. 1997; Furbass et al. 1997). CAMP responsive elements (CRE) moreover could be confirmed for exon 1 of the aromatase gene, expressed in lower amounts in rodent brain (Hickey et al. 1990; Simpson et al. 1997). *In vitro* studies even gave some indication for a direct inhibitory capacity of DA which presupposes that aromatase metabolizes DA (Osawa et al. 1994; Baillien and Balthazart 1997). Besides all these complex regulative effects on aromatase function, estrogens, the enzyme products, also directly influences aromatase activity by positive feedback control. This explains the positive (indirect) effect of testosterone on aromatase activity and mRNA expression, demonstrated by different studies in mammals and birds (Balthazart et al. 1990, 1992; Roselli and Resko 1984, etc.). A direct action of androgens on the aromatase expression on DNA-level is assumed but still has to be verified.

As useful tools to enlighten still undefined or putative controls, aromatase inhibitors were successfully applied in several studies. These aromatase inhibitors are structurally divided into steroidal and non-steroidal compounds. Independently of their structure, these inhibitors interact directly with the enzyme and not with the mRNA (Balthazart 1997), competing with the androgen substrate for the enzyme binding site. In general, steroidal inhibitors act as analogues of androgen substrates whereas the non-steroidal inhibitors perturb the catalytic properties of the heme prosthetic group of the aromatase complex. Especially the non-steroid aromatase inhibitors have provided evidence for their high inhibitory potency in numerous breast cancer studies in humans, an application they have originally been developed for (Brodie et al. 1977, 1999). Studies in birds with non-steroidal aromatase inhibitors revealed unexpected effects of these compounds (Balthazart et al.1997, Schlinger and Callard 1990). Non-steroidal aromatase inhibitors markedly decreased aromatase activity in brain cells of birds, whereas in certain areas of the brain they were able to increase the concentration of the aromatase protein and after longer periods of treatment, even of the mRNA. This increase in protein content could not directly be explained by a stimulation of gene transcription, as RT-PCR assays could not confirm an acute increase in mRNA expression in brain areas of increased aromatase-active cells. A translational regulation was postulated, but post-transcriptional mechanisms are, in general, experimentally difficult to verify (Balthazart et al. 1997). Harada et al. (1999) observed a time-dependent increase in the aromatase protein as well as in the mRNA level in the ovary of mice after eight-day daily administration of fadrozole (10 mg/kg body weight per

injection). From this result it was assumed, that the increase of the aromatase protein was caused by a decrease in the enzyme turnover. At least in mammals, aromatase inhibitors like fadrozole have proven to bind competitively to the enzyme binding site (Steel et al. 1987).

Further assumptions postulate a direct stimulation of the aromatase transcription and/or translation by steroid-independent mechanisms. A fast, non-genomic regulative pathways, e.g., via phosphorylation / dephosphorylation of the aromatase enzyme, has been identified in the avian brain (Balthazart et al. 2001). Thereby a kinase catalyzed phosphorylation with ATP and divalent cations (Mg^{2+} / Ca^{2+}) inhibited the aromatase activity in preoptic area-hypothalamus homogenates of quail. These phosphorylation reactions are reversible and the aromatase activity could be restored by the activity of phophatases. Electrochemical and/or neurochemical events as novel mechanisms of a very rapid aromatase control and accordingly estrogen availability in the brain were also discussed (Abe-Dohmae et al. 1996; McEwen and Alves 1999). Moreover, the detection of several other genomic factors that affect aromatase gene expression and the process of sexual differentiation of lower vertebrates may be expected, especially as they have already been confirmed in mammals.

In conclusion, such studies demonstrate the complexity of regulative mechanisms affecting aromatase enzyme activity and expression, and furthermore, the divergence of effects of non-steroidal inhibitors on aromatase, especially in the brain.

The role of sex steroids in neuroendocrine regulative processes

Steroidogenesis, spermatogenesis and oogenesis are regulated extrinsically by pituitary hormones, the gonadotropins. Gonadotropins can be divided into two main groups, the GTH (gonadotropic hormone) I-like hormones and the GTH II-like hormones. Structurally, the gonadatropins are glycoproteins with heterodimeric structure. They are composed of two subunits, a common α-subunit and a hormone-specific β-subunit. The synthesis and release of gonadotropins from the pituitary is predominantly controlled by hypothalamic releasing hormones, the GnRHs (gonadotropin-releasing hormone). Vice versa, gonadal steroid hormones exert regulatory control action on GTH levels by feedback mechanisms, directly or indirectly, by influencing the activity of GnRH neurons. The physiological effects of gonadotropin release generally depend on their binding to and activation of specific GTH receptors in target cells. Another modulating factor in gonadotropin release is the catecholamin DA. Even in fish, DA seems to act as the principal hypothalamic suppressor of GTH II release as, e.g., shown in tilapia (Levavi-Sivan et al. 1995) and goldfish (Chang et al. 1990, 1993). Both piscine gonadotropin forms exert important

functional roles in gonadal steroidogenesis and gametogenesis but, however, there is still little information on their neuroendocrine function, especially for GTH I. In difference to mammals, teleost fish lack a functional hypothalamo-pituitary portal blood system (Gorbman 1995, Kah et al. 1993), alternatively the regulation of pituitary hormone release, like GTH II, in fish is regulated by direct neuronal innervation. GTH I and GTH II in fish are structurally similar to the mammalian follicle stimulating hormone (FSH) and the luteinizing hormone (LH) (Quérat 1994). For the gonadal steroidogenesis in teleost species, a two-receptor model for GTH I and II, comparable to other vertebrates, has been proposed, based on studies in salmon (Yan et al. 1992). In conjunction with DA, GnRH work as the main modulating factor of gonadotropin release, even in fish. At least two genomic isoforms of GnRH have already been found in fish, in which the evolutionary oldest and most widely distributed form of GnRH in vertebrates, the chicken GnRH II, is one of the forms expressed in all teleost species examined thus far. In brain extracts of medaka and zebrafish, e.g., the salmon GnRH was detected as the second molecular form (Powell et al. 1996). For some fish species, a third form of GnRH has already been characterized in the brain (Powell et al. 1994, 1996; White et al. 1995). The existence of several GnRH forms in one species generally correlates with more complex GnRH receptor-signal transduction pathways. Besides the control of gonadotropin release, several GnRH-forms have been proved their ability to stimulate growth hormone (GH) release in some fish species like the common carp (Lin et al. 1993). Here again a conjunctive action of GnRH and DA could be proposed, as also for DA a stimulating effect on GH release was detected in goldfish and carp (Peter and Marchant 1995).

Apart from these poorly understood neuroendocrine control mechanisms by neuropeptides or neurotransmitter, sex steroids significantly influence many neuroendocrine processes. Modes of steroid action in reproductive neuroendocrinology can hardly be generalized in fish as they show an incredible diversity in their reproductive strategies. Most information available bases on studies in goldfish (*Carassius auratus*). Many of these studies focus on feedback control mechanisms of gonadal sex steroids on pituitary GTH II release, the process which in turn triggers gonadal growth, steroidogenesis, ovulation and sperm release. *In vivo* and *in vitro* studies in goldfish, for example, indicate that testosterone, though indirectly through aromatization to estradiol, exert a positive feedback effect on GnRH-stimulated GTH II release (Trudeau et al. 1991, 1993a). The detailed mechanism of this testosterone-mediated stimulation of GTH II release is still hardly understood. Testosterone seems to enhance GTH II release directly on

the pituitary level by a protein-synthesis mediated process (Trudeau 1993a), obviously independent of modulating effects on GnRH receptor activity or pituitary GTH II content. Furthermore, treatment with testosterone was also found to stimulate GTH II subunit-mRNA production in pituitary glands obtained from both sexually immature and sexually mature goldfish (Huggard et al. 1996). Similar effects could be observed in trout (Crim et al. 1979; Trinh et al. 1986) or other species, but the extent of the effect strongly depends on the sexual developmental stage. Nevertheless, the enhancing effect of steroids on GTH IIβ-subunits transcription could be supported by the discovery of EREs in the promoter region of GTH IIβ gene of chinook salmon (Liu et al. 1995; Xiong et al. 1994). Otherwise, negative feedback effects of steroid hormones are predominantly exerted by interactions with the GTH II-release inhibitory function of DA. Inhibitory effects of estradiol and testosterone on GTH II release could be observed in several teleost species like the goldfish or African catfish (Trudeau et al. 1993a; Vermeulen 1994). Indications for a direct estrogenic responsiveness of dopaminergic, inhibitory neurons in the brain are given in goldfish studies (Trudeau 1997) and probably explain the mechanism of steroid negative feedback on GTH II.

Another modulating factor involved in sex-steroid specific feedback regulation of GTH II release, is the neurotransmitter γ-aminobutyric acid (GABA). In contrast to its predominantly inhibitory gonadotropic function in mammals, GABA-ergic neurons in the pituitary (afferent terminals) have proved to exert stimulatory effects on GTH II release in goldfish (Kah et al. 1992, Trudeau et al 1993b, c), though this stimulating effect *in vivo* varies with the stage of seasonal gonadal development. Obviously, there seems to be a correlation between GABA-induced GTH II release and the seasonal variations in (plasma and/or gonadal) sex steroid levels. Kah et al. (1992) observed this stimulating effect of GABA on GTH II release only in regressed or early maturing goldfish but not in mature individuals. However, the stimulating effect of GABA on GTH II results from an action on GnRH release and inhibition of dopaminergic activity (Trudeau et al. 1993c). The interacting role of sex steroids on this GABA responsiveness was further investigated in these studies and demonstrated opposing effects of either testosterone or estradiol. While testosterone treatment increased the stimulatory effect of GABA, estradiol decreased GTH II release (Kah et al. 1992; Trudeau et al 1993c). Furthermore, a modulating capacity of estradiol on GABA-ergic inhibitory control of growth hormone release (GH) was demonstrated in goldfish (Trudeau et al. 2000). This observation is especially interesting as GH stimulates estradiol synthesis in turn. Based on these observations, estradiol seems to

have an autoregulative capacity by positive (estradiol is able to increase DA turnover) as well as negative feedback regulation (by induction of GABA-ergic inhibition) of GH release. All these data given for goldfish clearly verify the previous indication of a sensitivity of GABA-ergic neurons to changes in circulating sex steroid levels. This indication is further supported by the observation of a direct modulating effect of testosterone, progesterone and estradiol already on the GABA synthesis level in brain and pituitary. (Trudeau et al. 1993c)

Two other GTH II release-stimulating factors in the brain were identified in goldfish that are positively influenced by sex steroids. This is either the amino acid taurine, and also its precursor compound hydrotaurine, which is able to stimulate GTH II release directly as well as indirectly, through activation of GnRH release or by inhibition of dopaminergic neurons (Sloley et al. 1992; Trudeau et al. 1993b). Testosterone treatment potentiated this taurine-associated effect, implying a functional role of taurine in the control of reproductive processes in fish. The other stimulating factor of GTH II release from the pituitary identified in the goldfish is the neuropeptide Y (NPY). Peng et al. (1993, 1994) showed that testosterone and estradiol both are able to enhance the NPY-induced GnRH mediated GTH II stimulation. Furthermore, both sex steroids indicated here an indirect (i.e. not pituitary directed), brain site directed action by stimulating already the NPY mRNA expression (Peng et al. 1994) in the preoptic area.

Besides GTH II, there are increasing evidences of a gonadotropic function of GH in fish. This was first indicated by studies in the killifish (*Fundulus heteroclitus*) (Singh et al. 1988), in which gonadal steroidogenesis was induced by GH administration. Several studies were able to demonstrate a direct correlation between GH release and gonadal growth and development and vice versa, this correlation is strongly connected with an enhancement of gonadal steroid synthesis. Modulating action of GH on gonadal steroidogenesis can be either direct, or the stimulation of estradiol synthesis is mediated via GTH II induction (Le Gac et al. 1993). An additional mechanism of GH action was detected in spotted seatrout (*Cynoscion nebulosus*), where GH directly increased ovarian aromatase activity, supposedly by adenylyl cyclase-cAMP-dependent pathways (Singh and Thomas 1993). GH-induced stimulation of steroidogenesis is probably part of a positive feedback loop as the GH pituitary content and secretion is in turn provably enhanced by steroid action (Melamed et al. 1995; Zou et al. 1996 etc.). The molecular background behind these stimulating effects of gonadal steroids is still rather uncertain, but a direct

steroid action on GH transcription is quite unlikely as there are no indications for EREs in the so far sequenced GH genes in fish or even in any vertebrate class at all.

Aromatase gene (CYP19) expression in zebrafish

As already mentioned before in this chapter, cytochrome P450 aromatase (P450arom) in mammalian species, apart from the remarkable exception of the pig (Corbin et al. 1995, Graddy et al. 2000), is encoded by one single gene in the genome, differentially regulated by multiple, tissue-specific promoters. Among fish species, exhibiting a high diversity of reproductive strategies, CYP19 gene structure, as so far investigated, does not always correspond to the common pattern in mammals. Some teleost species studied have already shown to diverge from the typical mammalian strategy as they posses several, differentially regulated CYP19 genes. The existence of several CYP19 genes, which are differentially regulated and encode for different proteins with different enzymatic properties, is already confirmed in the goldfish (Gelinas et al. 1998; Tchoudakova and Callard 1998), tilapia (Chang et al. 1997; Kwon et al. 2001) and zebrafish (Chung 2000; Kishida and Callard 2001).

In zebrafish a 3.8 kb cDNA of a brain-derived form of the P450arom was cloned and the open reading frame (ORF) of 1533 bp was confirmed between nt 74 and nt 1607. The deduced amino acid sequence based on this ORF was composed of 511 amino acids, representing a protein of a calculated molecular weight of 58.1 kDa. A second gene transcript could be derived from the ovary and a 1.6 kb cDNA was isolated and cloned. The ORF of this second transcript could be determined between nt 11 and nt 1562 and encoded a protein of 517 amino acids with the calculated molecular weight of 58.3 kDa (Kishida and Callard 2001). These cDNA sequences were aligned with several other P450arom sequences of other vertebrates, including other fish species. Both transcripts showed 88 % of sequence identity with the A- and B-isoforms of the goldfish, respectively. CYP19A and CYP19B of zebrafish, however, only shared 61 % of overall sequence identity. The existence of two distinct gene loci was confirmed and furthermore, results of comparative gene mapping studies (Chiang et al. 2001; Trant et al. 2001) identified two different P450arom gene loci in the zebrafish genome. CYP19a was detected in linkage group 18 (LG 18), which is syntenic to LG 15 and 9 of human and mouse, CYP19b otherwise was mapped to linkage group 25 (LG 25). These two gene loci are related to two different chromosomes (Chiang et al. 2001a) and they obviously originate from the same ancestral chromosome and either developed by chromosome duplication (the currently

more favored hypothesis) or by tandem duplication and a subsequent gene translocation event (Chiang et al. 2001b).

Expression patterns of these two P450arom genes during the early development of the zebrafish and the tissue-specific transcript distribution in adult fish is under intensive investigation. CYP19A and CYP19B mRNA transcripts could already been detected in unfertilized eggs and at the onset of early embryogenesis, wherein CYP19A is the dominant transcript species. But this observation could be explained by maternally transferred mRNA as the own embryonic transcription does not start before 12-24 hpf (hours post fertilization). At this time, a second increase in CYP19 expression could be detected in embryos, characterized by a strong increase in CYP19B to a five-fold maximum level around 72 hpf, whereas the CYP19A expression first only gradually increased till 72 hpf, then slightly more intensified till 120 hpf, but never reaching ¼ of the expression intensity of CYP19B (Kishida and Callard 2001). These observations are not entirely in consistence with the studies of Trant et al. (2001) as the latter author observed a predominantly high expression of CYP19A not only in the first 24 hpf but in the first 4-5 days post hatch, whereas the CYP19B transcript could not be detected before day 3 or 4 post fertilization. In contradiction to the studies of Kishida and Callard (2001), who investigated the CYP19-expression in pooled samples, Trant and colleagues used single larvae. Thus, Trant also differentiate in the expression of CYP19B between a low and a high abundance group. These two groups differ in their expression pattern in the early larval development, and with the onset of gonadal differentiation they obviously diverge more and more. This strongly indicates a significant role of CYP19B in sexual differentiation and presumably either determination. Trant and colleagues (2001) focused their study on the changes in CYP19 expression in the early development of the zebrafish till the onset of gonadal differentiation into either testes or ovary around day 40 pf.

Investigations on the presumed dictating role of CYP19 gene expression throughout the period of sexual gonad differentiation in zebrafish have to be further intensified. The study of Trant et al. (2001) was the starting-point for the investigations presented in the second part of the work.

III. Outline of the present work

Chapter 1

- Development and validation of an homologous competitive enzyme-linked immunosorbent assay (ELISA) for zebrafish vitellogenin (z-VTG).
- Validation of the assay, verification of sensitivity and applicability of the assay to different types of samples.

Chapter 2

- Assessment of adverse effects of exposure to two model chemicals of different estrogenic capacity, 17α-ethinylestradiol (EE_2) and bisphenol A (BPA), in developing fish. Performance of two full life-cycle studies, using the zebrafish (*Danio rerio*) as test organism. Measurement of VTG at different developmental stages and after different periods of exposure. Investigation of reproductive organismic parameters (fertility, fecundity ⇒ assessed by Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Schmallenberg, Germany) and sub-organismic parameters (gonad histology ⇒ assessed by G. Maack, UFZ Centre for Environmental Research Leipzig-Halle, Germany).
- EE_2 has been used to explore the importance of the developmental stage of exposure for the induction of estrogenic effects. With BPA, the concentration dependency of estrogenic effects has been studied.
- Correlating VTG response with reproductive data and gonad histology with the aim to evaluate the significance and predictive function of VTG as a biomarker of estrogenic endocrine disruption in fish.

The main issue under discussion arising from this chapter is:

? What is the relationship between VTG induction and reproductive impairment/gonadal disruption? Is VTG only an indicator of estrogenic exposure or may the induction of VTG be interpreted also as a prediction of reproductive disruption?

Chapter 3

- Investigation of gene expression of the P450 aromatase (P450arom) genes, CYP19A and CYP19B, in cephalic/trunk and brain/gonad samples, respectively, at different developmental stages of the zebrafish by semi-quantitative RT-PCR.
- Interference with the aromatase system throughout the critical period of gonad differentiation by (a) application of an aromatase inhibitor and (b) exposure to the aromatizable androgen 17α-methyltestosterone. Assessment of effects on the CYP19 mRNA expression and analysis of implications on gonad differentiation by histology (⇒ assessed by Prof. H. Segner, University of Bern, Switzerland).
- Discussion of the functional implication of the differential expression of CYP19 during gonad sexual differentiation of zebrafish, as well as of the significance of the aromatase system as potential target of endocrine disrupting chemicals (EDCs).

The chapter addresses the following issues:

? Is P450arom involved in the gonadal sex differentiation of the protogynic zebrafish, *Danio rerio,* and therefore, is a potential target for endocrine disruption?

? Are P450arom-mediated disturbances or alterations of sexual differentiation only transient or permanent and thus organizational? What are the consequences on the CYP19A and CYP19B mRNA expression level?

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Chapter 1

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Development and validation of a homologous zebrafish (*Danio rerio* Hamilton–Buchanan) vitellogenin enzyme-linked immunosorbent assay (ELISA) and its application for studies on estrogenic chemicals

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Abstract

Vitellogenin (VTG) was isolated by anion exchange chromatography from plasma of female zebrafish (*Danio rerio*) induced with 17α-ethinylestradiol (EE2). The purity of the VTG isolate was confirmed by polyacrylamide gel electrophoresis (SDS-PAGE). Purified VTG was used to raise polyclonal antibodies in rabbits and the specificity of the antisera for VTG confirmed by Western blot analysis of plasma proteins separated by SDS-PAGE. The antibodies cross-reacted with two proteins in the plasma of female zebrafish, with molecular masses of approximately 142 and 171 kDa. No cross-reactivity was observed with any other plasma proteins. A competitive enzyme-linked immunosorbent assay (ELISA) was developed using the polyclonal zebrafish VTG (z-VTG) antibodies and purified z-VTG as ligand and standard, respectively. The z-VTG ELISA was sensitive with a detection limit of between 2.0 and 3.0 ng purified VTG/ml, and a working range between 3 and 500 ng/ml (30–85% binding). The ELISA demonstrated precision, with inter- and intra-assay variations of 7.5 ± 2.7 and $4.9 \pm 1.4\%$, respectively. Plasma from adult zebrafish and whole body homogenates from juvenile zebrafish diluted parallel with the z-VTG standard in the ELISA, validating the assay for quantifying z-VTG in both of these tissues. Exposure of adult male zebrafish to EE2 via water induced a concentration-dependent induction of VTG with a lowest observed effect concentration (LOEC) ≤ 1.67 ng EE2/l (for a 21-day exposure). The homologous z-VTG ELISA provides a valuable tool for the study of environmental estrogens in zebrafish.

Keywords: Enzyme-linked immunosorbent assay (ELISA); Environmental estrogens; 17α-Ethinylestradiol (EE2); Homologous antibody; Vitellogenin (VTG); Zebrafish

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1. Introduction

Vitellogenin (VTG) is synthesized in liver parenchymal cells of female oviparous vertebrates under the control of estrogens (Wallace, 1978; Lazier and McKay, 1993). From the liver, VTG is transported to the ovaries via the bloodstream, where it is selectively sequestered by receptor-mediated endocytosis into the developing oocytes (Tyler and Lancaster, 1993). In the oocyte, VTG is proteolytically cleaved into yolk products, including lipovitellin, phosvitin and phosvettes (Wallace, 1978; Ng and Idler, 1983; Mommsen and Walsh, 1988), and these reserves provide the main nutritional sources for the developing embryo.

In female fish, concentrations of plasma VTG increase rapidly at the onset of vitellogenesis and, in the normal reproductive cycle, high plasma levels coincide with the period of most pronounced growth of the oocyte (Tyler et al., 1990; Tao et al., 1993; Bon et al., 1997; Heppell and Sullivan, 1999). Male fish are also capable of synthesizing VTG, and exposure to estrogens induces VTG in the plasma. Low levels of VTG have been measured in the plasma of male fish maintained in the laboratory (Le Guellec et al., 1988; Goodwin et al., 1992; Kishida and Specker 1993, 2000; Tyler et al., 1996), as well as in fish undergoing seasonal reproductive cycles in the field, e.g. the Japanese flounder (*Pleuronectes yokohamae*) (Bessho et al., 2000). Whether these low concentrations of VTG in males result from the presence of endogenous estrogen, or are a consequence of environmental exposure to estrogens, is not yet established.

Vitellogenin has proved to be a core endpoint to assess exposure of fish to environmental estrogens (Sumpter and Jobling, 1995; Korsgard and Pedersen, 1998). Indeed, induction of VTG is now the most widely used indicator for estrogenic exposure in fish. Vitellogenin can be measured by determining the alkaline-labile phosphorous content of the plasma (Wallace and Jared, 1968; Rinchard et al., 1997; Kramer et al., 1998), or by means of immunoassays, including competitive ELISAs (Specker and Anderson, 1994; Tyler et al., 1999). More recently, VTG induction has been quantified by measuring the amount of VTG mRNA (Bowman and Denslow, 1999; Korte et al., 2000; Ota et al., 2000).

The zebrafish (*Danio rerio*) is a species used extensively in ecotoxicology. It has a short generation time, which makes it particularly suitable for partial or full life-cycle studies. The scope of these subchronic and chronic toxicity tests could be extended for the assessment of hormonally active compounds with the addition of endocrine-specific endpoints, such as VTG induction. For the measurement of VTG in the zebrafish to date, only heterologous immunoassays with non-homologous antibodies have been used (Tyler et al., 1996). The primary structures of the VTG molecule, however, generally differ between fish species, even between closely allied species (Lee et al., 1992). As a consequence of this, interspecies cross-reactivity of anti-VTG antibodies with other VTGs from other species is often limited (Norberg and Haux, 1985; So et al., 1985; Copeland et al., 1986; Benfey et al., 1989; Tyler and Sumpter, 1990). Therefore, the value of heterologous assays for quantification of VTG is limited. Even if the heterologous antibodies show specific cross-reactivity, they may be less sensitive than their homologous counterparts, thereby restricting their utility for measuring weak estrogenic responses. The more sensitive and robust assays for quantifying VTG are homologous assays.

The objective of the present study was to develop a homologous, competitive enzyme-linked immunosorbent assay (ELISA) for VTG and validate its use for the detection of estrogenic responses in zebrafish. The zebrafish is a species routinely employed in the Organization of Economic Cooperation and Development (OECD) toxicity tests. The suggested use of zebrafish as test species to assess the estrogenic activity of chemical compounds will depend on the availability of a sensitive and robust method for quantification of zebrafish VTG.

2. Materials and methods

2.1. Experimental animals

Zebrafish were obtained from the breeding stock of the Center for Environmental Research, Leipzig. Between 100 and 200 fish were reared in 120-l glass tanks in a temperature-regulated laboratory with a 12-h dark:12-h light photoperiod. The temperature in the tanks was maintained between 26 and 27°C and the water was

permanently aerated. Prior to entering the tanks, the water was charcoal-filtered and pre-heated to approximately 26°C in a reservoir basin. Each day, the tanks were cleaned of feces and uneaten food and approximately 20–30% of the tank water was replaced. The fish were fed three times a day, twice with a commercially available flaked fish food (Tetra, Germany), and once with live food (brine shrimps, *Artemia* sp.).

2.2. Dosing of fish for vitellogenin induction

Vitellogenin was induced in zebrafish by exposure to the synthetic estrogen 17α-ethinylestradiol EE2 (Sigma Aldrich) at a nominal concentration of 10 ng/l. Fish were exposed in 25-l glass aquaria under semi-static conditions. Every second day, 80% of the water in the aquaria was replaced to ensure persistence of the test substance concentrations in the water column. The replacement water was charcoal-filtered, aerated and heated to 26°C. The required amount of the stock EE2 solution was added before the aquaria were refilled to ensure an even distribution of the test chemical. All test aquaria were permanently aerated and heated to a temperature between 26 and 27°C. The stock solution of EE2 was prepared in acetone, diluted in water and kept at 4°C under constant mixing, until required, for the duration of a test. The concentration of the acetone in the aquaria was less than 0.001‰. After up to 66 days of exposure to EE2, fish were anesthetized in an aqueous solution of benzocaine (Sigma Aldrich) at a concentration of ≤ 0.4 g/l and blood samples taken (adult fish) or whole body homogenates prepared (juvenile fish), as described below.

2.3. Tissue sampling

2.3.1. Blood sampling and plasma preparation

Blood samples were taken by cardiac puncture using a 1-ml syringe with a fixed needle of 0.33×12.0 mm^2. The syringe was heparinized and prefilled with approximately 100 μl of 0.01 M phosphate-buffered saline (PBS) buffer (pH 7.3) containing the protease inhibitor aprotinin (500 TIU/ml; Merck). This amount of buffer in the syringe was necessary to avoid agglutination of the collected blood and to completely elute the minute blood volumes from the syringe which can be drawn from an individual zebrafish. Otherwise, aliquots of the plasma sample would be lost in a non-reproducible way in the syringe. The diluted blood samples were then transferred directly into an ice-cold microfuge tube. In order to elute as much of the blood from the syringe as possible, the syringe was washed once afterwards with a small amount of buffer, and this was added to the previously collected blood fraction. The sample was centrifuged at $2900 \times g$ for 25 min at 4°C, and the plasma was collected and immediately used for purification of z-VTG. The protein content of the plasma and homogenate samples (below) were quantified by a detergent-compatible (DC) protein assay from Bio-Rad, (an assay based on Lowry et al., 1951), using bovine serum albumin (Serva) as reference standard protein.

2.3.2. Whole body homogenate preparation

For the preparation of whole body homogenates, juvenile zebrafish were snap-frozen in liquid nitrogen. The average (wet) body weight of the fishes used for homogenization was < 100 mg. The frozen tissue was subsequently homogenized in ice-cold PBS-T ELISA buffer in a 1:2 or 1:3 ratio wet weight/buffer volume (depending on the developmental stage/size of the individual fish) with a glass homogenizer (motor-driven). A higher dilution of 1:3 in buffer was used for the homogenization of fishes with a presumably higher content of vitellogenin to adjust different vitellogenin concentrations in the homogenates to the ELISA conditions. The homogenates were centrifuged at 15 000 rev./min for 3 min, and the supernatants directly analyzed in the ELISA or stored at −80°C until required.

2.4. Vitellogenin purification

Vitellogenin was isolated from plasma samples of EE2-treated adult females by anion-exchange liquid chromatography. The isolation procedure was performed according to the protocol of Silversand and Haux (1989) and Silversand et al. (1993), modified by the use of a stepwise NaCl gradient, rather than a linear one, for the elution of vitellogenin. The diethyl aminoethyl (DEAE) anion-exchange system used was a Mono-Q HR5/5 column (50×5 mm ID, Amersham Pharmacia), linked with a fast protein liquid chromatography (FPLC) system comprising a Bio-Sys 520 system, photodiode array (PDA) detector and a sample collector (all Beckman Coulter). For the

z-VTG purification, the diluted plasma from three or four females had to be pooled to give a total plasma volume of approximately 100 μl (containing on average 5–10 mg protein/ml). Before application to the column, the plasma was first diluted 10-fold with 20 mM Tris–HCl (pH 8.0) and 1.0 ml was loaded onto the column, which had been pre-equilibrated with three volumes of 20 mM Tris–HCl, pH 8.0. Unbound substances were eluted from the column with 5 ml of 20 mM Tris–HCl buffer. Subsequently, adsorbed plasma proteins were eluted using a 17-ml stepwise NaCl gradient of 0.3, 0.5 and 1.0 M NaCl (dissolved in 20 mM Tris–HCl, pH 8.0). The flow rate through the column was 1 ml/min. The elution profile was monitored in the PDA detector at an absorbance of 280 nm. To reduce proteolysis, the chromatographic procedure was performed at 4°C, and all solutions used contained the trypsin inhibitor aprotinin. The fractions containing VTG were collected and pooled from several column runs. This vitellogenin solution was concentrated by size-exclusion ultrafiltration using a Centricon® concentrator (Centricon-100, Amicon, USA) and the VTG protein aliquoted, frozen in liquid nitrogen and stored at −80°C until required. The protein content of the concentrated VTG fractions was quantified by a DC protein assay, as described above.

To confirm the purity of the z-VTG, the isolate was subjected to SDS-PAGE, according to Laemmli (1970), under reducing conditions with pre-cast 4–15% gradient mini gels (Tris–HCl, pH 8.8, Bio-Rad). The purified VTG was run in parallel with plasma samples from adult female and male zebrafish. In addition, a plasma sample from an estrogenized female rainbow trout was run for comparison. The plasma samples and the purified z-VTG were diluted 1:1 in sample buffer [0.25 M Tris–HCl, pH 6.8; 20% glycerin; 4% SDS (both Merck); 10% -mercaptoethanol; 0.01–0.02% bromophenol blue (both Serva) and 20 μl per sample (with a protein concentration between 2 and 4 μg for the plasma samples and < 1.0 μg for the purified z-VTG)] and applied to the gel. Standard proteins were applied to the gel to determine the molecular mass of the separated plasma proteins. Electrophoretic separations of plasma protein were carried out for 60–70 min at 130 V. Gels were stained in a 0.5% aqueous solution of coomassie brilliant blue R-250 (Merck) or in a 0.02% solution of the fluorescent dye Sypro® Orange protein stain (Bio-Rad) in staining buffer (25 mM Tris–HCl, 292 mM glycine, 20% methanol) prior to Western blotting. Analysis of the gels was carried out using a gel documentation system combined with an image-analysis computer program (Bio-Rad). For their subsequent use in Western blots to characterize the specificity of antisera raised against z-VTG, gels were destained completely in double-distilled water. If the gels were Sypro® Orange stained, no destaining was necessary.

2.5. Production of polyclonal z-VTG antibodies

Specific antiserum against purified z-VTG was raised in two rabbits. The immunization program was conducted by Eurogentec (Bel S.A., Belgium). In this standardized program, the rabbits were injected with 100 μg of z-VTG in adjuvant and then boosted on three subsequent occasions with 30 μg of z-VTG adjuvant after 14, 28 and 56 days. The rabbits were killed for final bleeding on day 80 after the first antigen injection. Serum from each rabbit was collected and kept on dry ice 5 days after bleeding. The antisera from both rabbits were aliquoted and stored at −80°C until required.

The specificity of the antibodies raised against z-VTG was examined by Western blotting of plasma proteins separated by SDS-PAGE. Plasma samples from EE2-induced fish and non-induced male and female mature zebrafish, and plasma from an estrogenized female rainbow trout, were run on SDS-PAGE, and transferred from the gels onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Serva) by semi-dry blotting. The transfer was performed in a semi-dry blotting chamber (Trans-blot SD cell, Bio-Rad), at 0.8 mA/cm^2 for 1 h using a discontinuous transfer buffer system consisting of three different Tris/methanol buffers. After the transfer, membranes were stained in a 0.25% ponceau S staining solution (0.25% ponceau S, 15% conc. acetic acid, 40% methanol) for 5 min. Non-specific binding sites were blocked by incubating the membrane in a solution of 5% low-fat powdered milk in phosphate buffered saline (PBS)–Tween (pH 7.4, with 0.1% Tween) buffer (PBS-T) overnight at 4°C. This was followed by several washings of the membrane with PBS-T. For the immunochemical detection, the primary polyclonal antiserum against zebrafish vitellogenin was di-

luted 1:45 000 and 1:50 000 in blocking buffer. The membrane was incubated with the polyclonal antibody under continuous agitation for 120 min at room temperature. After several washing steps in PBS-T, the membrane was incubated in a blocking buffer solution of a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit, diluted 1:2000 in blocking buffer, DAKO) for 60 min with slight agitation. Antigen–antibody complexes were visualized by the peroxidase reaction with 3,3'diaminobenzidine-tetrachloride DAB (DAB substrate kit, metal-enhanced; Boehringer). The reaction was stopped when the antibody–antigen complexes were clearly visible. Documentation and analysis of the membranes was carried out using a gel documentation system combined with an image-analysis computer program (Bio-Rad).

2.6. Development of a homologous competitive ELISA for zebrafish vitellogenin

The development of this assay was based on the protocol published for the competitive ELISA for carp vitellogenin (Tyler et al., 1999), with assay conditions optimized for the z-VTG. The assay is based on a competition for the z-VTG antibody between z-VTG coated on the wells of a microtiter plate and free VTG molecules in the sample solutions. The antigen–antibody complex bound to the plate is detected by a secondary antibody directed against the primary VTG antibody. This secondary antibody is conjugated with the enzyme horseradish peroxidase. The enzyme activity is revealed by adding a suitable substrate and hydrogen peroxide, and is measured colorimetrically.

2.7. General ELISA protocol

2.7.1. Coating the plates

z-VTG was thawed on ice and diluted in coating buffer (0.05 M sodium carbonate buffer, pH 9.6). The wells of 96-well microtiter plates (Nunc F96 Maxisorp™ Immuno Plate) were coated with 100 µl of a z-VTG solution (150 ng z-VTG/ml coating buffer), sealed and incubated overnight at 4°C. For determination of non-specific binding effects, three wells/plate were treated with coating buffer only.

2.7.2. Preincubation of samples / standards

For the standards, freshly thawed z-VTG was diluted in PBS-T blocking buffer [0.01 M phosphate-buffered physiological saline solution with 0.05% Tween 20 (Serva) and 1% fatty acid-free BSA] to a concentration of 2000 ng z-VTG/ml. From this stock solution, serial dilutions were prepared in PBS-T blocking buffer. In parallel, samples with an unknown VTG content were diluted in PBS-T blocking buffer. The VTG standards and unknown samples (60 µl/well) were incubated in non-coated 96-well microtiter plates (Greiner) with z-VTG antibody (60 µl/well, 1:20 000 in PBS-T blocking buffer). For the non-specific binding (NSB), 60 µl/well of blocking buffer only was mixed with 60 µl of the antibody solution. The incubates were mixed on a rotary shaker, and the plates were sealed and incubated overnight at 4°C.

2.7.3. Antibody incubation

The coated plates were washed three times with PBS-T washing buffer (0.01 M phosphate-buffered physiological saline solution with 0.05% Tween 20, adjusted to pH 7.4). To reduce background, the plates were blocked with 150 µl of PBS-T blocking buffer/well for 30 min at 37°C. After this blocking step, the plates were washed another three times with PBS-T, before 100 µl of the sample/antibody or standard/antibody incubates were pipetted into the wells. The plates were sealed and incubated for 120 min at 37°C. The first antibody incubates were then removed and the plates were washed three times with PBS-T. A 125-µl aliquot of second antibody against rabbit IgG (goat anti-rabbit IgG, whole molecule, peroxidase conjugate; Sigma) was added to each well at a dilution of 1:2000 in PBS-T blocking buffer and the plates were sealed and incubated at 37°C for 120 min.

2.7.4. Revelation

The plates were washed three times with PBS-T and then 125 µl of the enzyme substrate solution was added to each well. This solution was prepared by dissolving 0.5 mg of *o*-phenylenediamine dihydrochloride (OPD) (Sigma) in 0.05 M phosphate–citrate buffer, pH 5.0 (0.051 M dibasic sodium phosphate, 0.024 M citric acid). After addition of 0.5 µl/ml of H_2O_2 (30%; Merck), the substrate solution was immediately pipetted into

the plates (125 μl/well). The enzyme reaction was allowed to proceed for 30–40 min in the dark, at which point the color reaction was stopped by the addition of 30 μl of 3 N H_2SO_4. The absorbance of the reaction product was read at 490 nm in a microtiter plate reader (SpectraMax 250).

In the development of the assay concentrations of the first and second antibody were optimized. To this end, different concentrations of the homologous VTG antisera and the second antibody were applied in a two-dimensional titration. The VTG coating concentrations were kept fixed for the titration. Having established the optimal dilutions of the primary and secondary antibody for the assay, a range of VTG coating concentrations was evaluated (25, 50, 100 and 200 ng/ml) for optimizing the ELISA.

2.8. Quantification of vitellogenin

The absorbance values obtained in the ELISA were inversely proportional to the amount of VTG in the sample. Vitellogenin content in plasma samples and/or homogenates of zebrafish was quantified from the standard curve that had been log-transformed. The concentrations of z-VTG measured in plasma or homogenate samples were normalized to the total protein content of the corresponding sample. This standardization of the samples has proved to be the most convenient method for plasma samples. For technical reasons, and based on the principal problem in taking blood from small fishes, the blood samples were diluted in heparinized PBS buffer. The buffer was pre-filled into the syringe and, as consequence, the dilution of the collected blood varied to some extent, depending on the actual volume of the blood sampled. This variability refused the common normalization of plasma VTG to the sample volume; instead, we normalized to plasma protein content. As VTG can be present in fish plasma at high concentrations, it may influence plasma protein concentrations and thereby lead to a bias in the results. However, when comparing plasma protein concentrations of mature, reproducing males and females, significant sex-related differences of plasma protein levels could not be detected, except for a few individuals showing exceptionally high plasma VTG levels (> 500–600 μg VTG/mg plasma protein). In those cases, we corrected the elevated plasma protein levels for average values as obtained in non-estrogenized controls. Tissue samples were homogenized in definite buffer volumes, and therefore these VTG values can either be normalized to the protein content or to the sample volume.

Plasma and homogenate protein concentrations were determined by a Lowry-based protein assay as described above.

2.9. Validation of the z-VTG ELISA for the measurement of z-VTG in plasma samples and whole body homogenates

The robustness of the ELISA was assessed through measurements of inter- and intra-assay coefficients of variation, where inter-assay variation was measured as differences in binding between z-VTG standard replicates at 80, 50 and 30% binding, analyzed in independent assays, and intra-assay variation was measured as the difference at 50% binding between replicate z-VTG standards on different plates run in one assay. The sensitivity of the assay was evaluated as the lowest reliably detectable z-VTG concentration within the linear response range of the assay.

The ability of the ELISA to quantify z-VTG in plasma and whole body homogenates across a wide range was determined through running serial dilutions of plasma and whole body homogenates and comparing the dilution curves with the z-VTG standard. Dilution curves were run for plasma from male, female, estrogenized male and estrogenized female samples, and a whole body homogenate from an estrogenized female.

2.10. Vitellogenic responses in male zebrafish exposed to ethinylestradiol

Sexually mature adult male zebrafishes were exposed to EE2 at nominal concentrations of 20, 10, 7.5, 3.0 and 1.67 ng/l over a period of 21 days under semi-static test conditions, as described above. The population used in this experiment was composed of individuals of the same age and reared under similar laboratory conditions. The main focus of this experiment was on the effects of EE2 exposure on plasma vitellogenin level. Effects on health or body weight were not determined. For each treatment concentration, there were two aquaria, each containing a group of 15 individuals. Two groups in non-contaminated water were run as control. Based on previous test

results, estrogenic effects of the solvent could be ruled out. The acetone concentration in the aquaria was below 0.001‰.

At the end of the experiment, fishes from all treatments were anaesthetized and blood samples were taken as described above. The plasma samples collected were frozen immediately in liquid nitrogen and stored at −80°C until required for ELISA analysis.

2.11. Expression of results and statistical analysis

Results for the VTG analysis were log-transformed for linear regression. z-VTG standard curves were plotted as relative maximum binding (B/B_0) vs. $\log_{10}$[z-VTG]. To fit dose–response curves to the data, a four-parameter logistic equation $\{y = y_0 + a/[1 + (x/x_0)^b]\}$ was chosen. The steepness of the z-VTG standard curves was evaluated by linear regression $y = -a(\ln x) + b$ and the slope value 'a' was used as the parameter to review reproducibility of z-VTG standard curves of different assays. Parallelism between standard curve replicates was verified by analysis of covariance (F test on mean square). VTG data from the EE2 exposure experiment were assessed for normality and equal variance. As normality test failed, the data were analyzed by ANOVA on ranks (Kruskal–Wallis test) or by a rank sum test (Mann–Whitney U test) to determine any differences between treatments.

3. Results

3.1. Purification of zebrafish vitellogenin

VTG, purified from plasma of mature, EE2-exposed female zebrafish by anion-exchange liquid chromatography on a DEAE Mono-Q column, eluted with approximately 0.6 M NaCl at a retention time of approximately 15 min. Examples of elution profiles for plasma from EE2-induced and non-induced mature females are shown in Fig. 1a,b, respectively. In the elution profile of unexposed female fishes, the size of the protein peak presumed to be VTG was considerably lower (approx. 20-fold) than that in EE2-exposed fish. SDS-PAGE of the protein presumed to be z-VTG produced two fractions, eluting with molecular weights of 142 (the major protein component) and 171 kDa (see lane 5 in Fig. 2). These proteins were prominent in the plasma of mature female zebrafish, but were absent in the plasma of mature, untreated males (Fig. 2, lane 4).

3.2. Characterization of z-VTG antibody

The specificity of the polyclonal antisera (SA 7390 and SA 7391) raised against the purified z-VTG protein was evaluated by means of Western blots of plasma proteins separated on SDS-PAGE. The Western blot results are presented for one of the batches of antiserum only (SA 7390), as both were very similar in titer and specificity. Only this antiserum SA 7390 was employed for the routine assay. In the Western blots, there were hybridization signals for plasma samples from adult female and from EE2-treated males (Fig. 3, lanes 2, 3 and 5). There was no reaction product visible for any plasma proteins from non-exposed male zebrafish (lane 4). The main immunoreactive band in females and in EE2-treated males corresponded with the estrogen-inducible 142 kDa protein, tentatively identified as VTG. There was no hybridization signal for the second estrogen-inducible protein (171 kDa) on the DAB-stained membranes; however, this was a function of its relatively low concentration, and on gels loaded with a higher amounts of plasma protein, ≥ 10–15 μg total protein/lane, there was a hybridization signal with this plasma protein (results not shown). Faint immunopositive reactions also occurred with a series of proteins that were smaller than 142 kDa. No positive reaction signal was detectable in the lane with plasma proteins of estrogenized female trout (Fig. 3, lane 6).

3.3. Development and validation of a competitive ELISA for z-VTG

The two-way titration of the first and second antibody is shown in Fig. 4. The VTG coating concentration for this titration was 200 ng/ml. Dilutions of the homologous antiserum (SA7390) of between 1:20 000 and 1:40 000, together with a second antibody titer of 1:2000 or 1:4000, produced workable assay conditions. The effect of different VTG coating concentrations (25, 50, 100 and 200 ng/ml) in the assay is shown in Fig. 5. Maximal absorbance within the linear range occurred at z-VTG coating concentrations between 150 and 200 ng/ml. For routine applications of

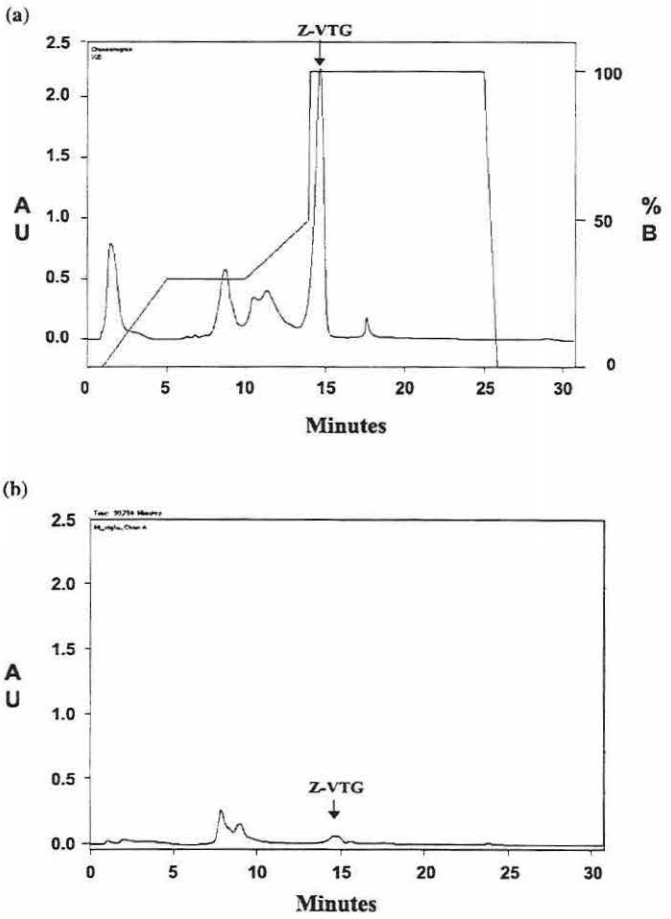

Fig. 1. (a) Purification of zebrafish vitellogenin (z-VTG) from plasma of estrogenized female fishes by anion-exchange chromatography on a DEAE Mono-Q HR5/5 column: elution profile (blue line) of proteins at a detection wavelength of 280 nm. Gradient of elution buffer (1 N NaCl) is shown by the upper line as percentage of total flow rate (% B; right *x*-axis). Fractions were collected every 30 s (AU, absorbance units). (b) Elution profile of plasma proteins from non-estrogenized female zebrafishes. Elution buffer (1 N NaCl, pH 8.0) gradient and all chromatographic conditions were as in (a). The vitellogenin peak in this chromatogram at a retention time of approximately 15 min is much smaller than in vitellogenesis-induced fish, as observed in (a) above.

the assay, therefore, a primary antibody dilution of 1:20000, second antibody dilution of 1:2000 and a VTG coating concentration of 150 ng z-VTG/ml was optimal, and this produced a maximum absorbance of approximately 2.0. A typical standard z-VTG curve for these conditions is shown in Fig. 6. In view of the fact that high quantities of VTG are difficult to obtain from a small fish species like the zebrafish, it is desirable to use as little purified VTG as possible for each assay. In an attempt to limit the amount of purified z-VTG used in an assay, the effect of lowering the VTG coating concentration from 150 to 100 ng/ml in the routine assay was investigated. Fig. 6 shows the standard curves for assays carried out with VTG coatings of 150–100 ng/ml.

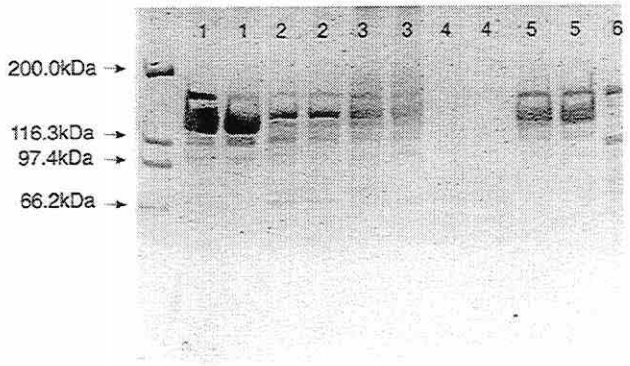

Fig. 2. SDS-PAGE of plasma proteins from zebrafish, trout and z-VTG. The gel was stained with the fluorescent dye Sypro® Orange (BioRad). Lane 1, purified z-VTG; lane 2, female zebrafish; lane 3, estrogenized female zebrafish; lane 4, male zebrafish; lane 5, estrogenized male zebrafish; and lane 6, estrogenized female trout. Molecular weight size standards are indicated on the right side.

The resulting standard curves were similar, with the lower coating concentration producing the more sensitive assay (as expected). The working range for the assay was between 30 and 85% binding for 100 ng/ml z-VTG coating, and between 40 and 95% for the 150 ng/ml z-VTG coating. For the remaining work, a z-VTG plate coating of 100 ng/ml was used throughout. Using a plate coating of 100 VTG ng/ml, the lowest reliably detectable concentration of z-VTG standard within the linear response range of the assay (85–90% binding) was between 2.0 and 3.0 ng/ml ($\equiv$ 120–180 pg/well). For the routine ELISA, the slopes of the standard curves were between (-0.1) and (-0.12). The inter-assay variation (variation in binding of the z-VTG standard between different assays) determined for 30, 50 and 80% of z-VTG standard, were 7.6 ± 2, 7.5 ± 2.7 and $5.1 \pm 3.3\%$, respectively, calculated for 26 standard curves. The intra-assay variation (variation within one assay conducted on different plates), calculated at 50% binding was $4.9 \pm 1.4\%$ ($n = 8$ z-VTG standard replicates).

Serial dilutions of plasma samples from adult

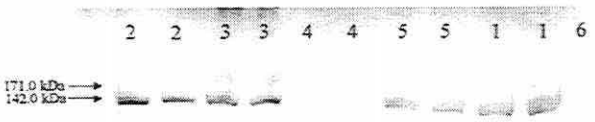

Fig. 3. Immunoreactivity of zebrafish vitellogenin (z-VTG) antiserum with purified vitellogenin and with plasma proteins of zebrafish and female trout, analyzed by Western blot. Proteins were separated by SDS-PAGE in gradient (4–15% gradient) mini-gels under reducing conditions prior to Western blotting. The proteins were transferred onto PVDF membranes and the immunopositive reaction of the antiserum was revealed by a second antibody-mediated horseradish peroxidase (hrp)–substrate reaction (see Section 2). Lane 1, purified z-VTG; lane 2, female zebrafish; lane 3, estrogenized female zebrafish; lane 4, male zebrafish; lane 5, estrogenized male zebrafish; and lane 6, estrogenized female trout. Molecular weight of the immunoreactive bands are indicated on the left side.

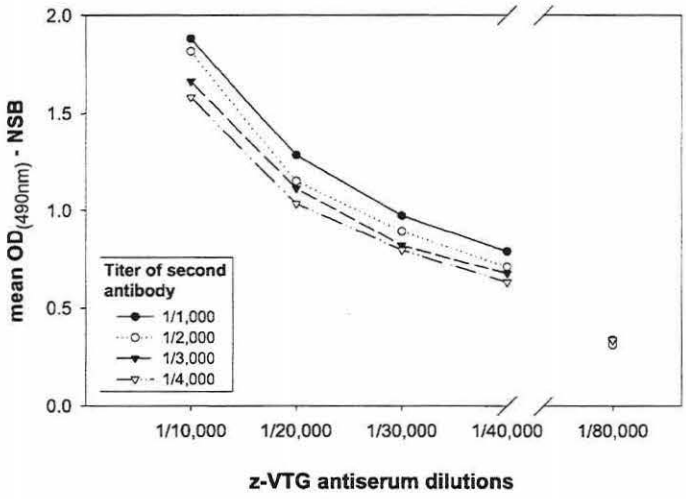

Fig. 4. Determination of optimal concentrations of second antibody complex and specific (primary) zebrafish vitellogenin (z-VTG) antiserum by two-dimensional titration.

female zebrafish and estrogenized male and female zebrafish showed a good parallelism with the standard z-VTG within the working range of the assay (Fig. 7). The dilutions adopted for some of the samples did not always cover the whole range of the z-VTG standard curve. Compared to the slope of the z-VTG standard curve, the slope values of the linearized plasma dilution curves were quite similar and ranged between −0.09 and −0.11. Plasma of male zebrafish showed lit-

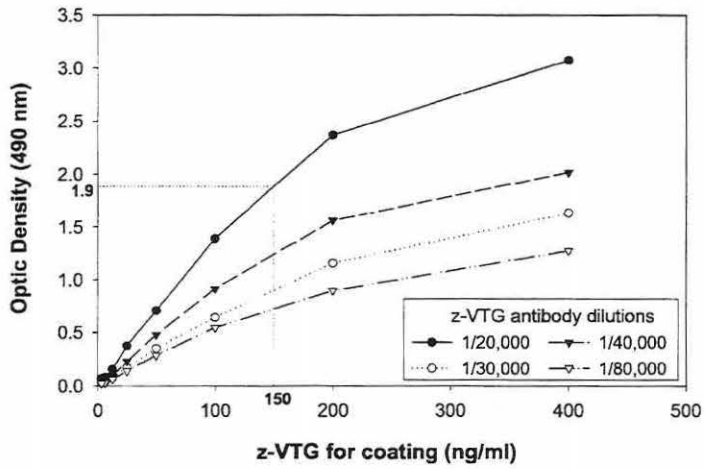

Fig. 5. Optimization of zebrafish vitellogenin (z-VTG) coating concentration and primary antiserum concentration. Selected conditions for B_0 in the routine ELISA protocol are indicated by dashed lines. All other steps were performed as described for the ELISA in Section 2.

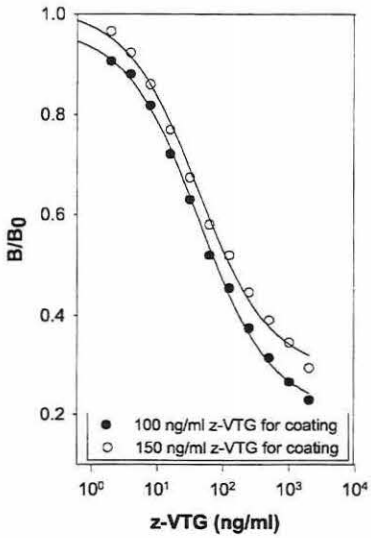

Fig. 6. ELISA results of serial dilutions of zebrafish vitellogenin (z-VTG) standard from 2000 to 0.06 ng/ml at two different vitellogenin coating concentrations. Dilutions for both curves were prepared in the same plate, each dilution in three replicate wells. The *x*-axis is log-scaled.

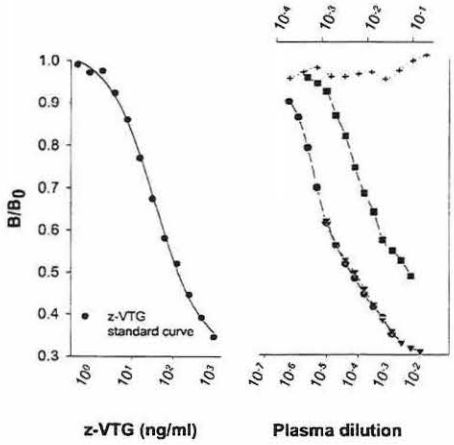

Fig. 7. Binding curves of serial dilutions of plasma samples from mature zebrafish, compared with the zebrafish vitellogenin (z-VTG) standard curve: fish were either untreated (female –■–; male –+–) or treated with 17α-ethinylestradiol (EE2) for 17 days (–●– female, 17 days EE2) or 36 days (–▼– male, 36 days EE2). The dilutions of the plasma sample from untreated male are represented on the upper *x*-axis. All *x*-axes are log-scaled.

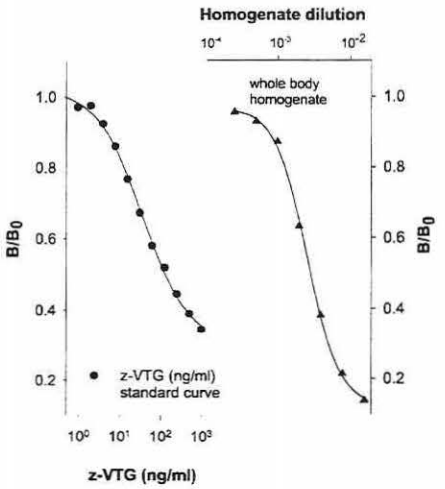

Fig. 8. Binding curves of serial dilutions of whole-body homogenate of a 66-day-old zebrafish after chronic exposure to 10 ng/l of 17α-ethinylestradiol (EE2), compared to the zebrafish vitellogenin (z-VTG) standard curve. Dilutions of the body homogenate are indicated on the upper *x*-axis. Both *x*-axes are log-scaled.

tle, if any, cross-reactivity in the z-VTG ELISA. At very low dilutions (1:10 and more concentrated) there were some 'plasma' effects that resulted in the inhibition of binding of the z-VTG coated on the plates with the z-VTG antibody. Whole body homogenates from immature estrogenized zebrafish had comparable slopes (between −0.13 and −0.14) across the working range of the assay (Fig. 8), but the parallelism of the homogenate dilution curve with the z-VTG standard proved to be not as good as for the plasma samples.

3.4. Vitellogenic response in male zebrafish exposed to EE2

Fig. 9 shows the plasma concentrations of VTG in adult male zebrafish after a 21-day exposure to EE2. Plasma VTG concentrations are expressed in μg/mg plasma total protein to normalize the VTG response data for any differences in total plasma protein content between fish. After 3 weeks, there was a clear dose-dependent induction of VTG on exposure to EE2 ($P < 0.05$). The lowest observed effect concentration (LOEC) for

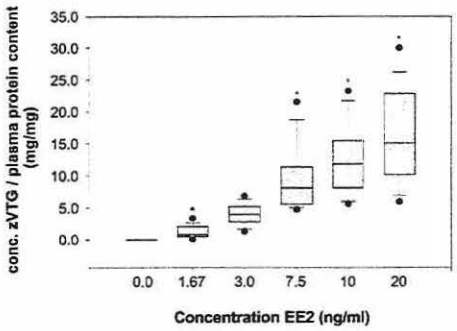

Fig. 9. Concentrations of plasma vitellogenin (VTG) in mature male zebrafish, exposed to different concentrations of 17α-ethinylestradiol (EE2) over a period of 21 days. Results are illustrated as box plots. Median (line within box) and percentiles (lower and upper boundary of box, 25th and 75th percentiles; lower and upper whiskers, 10th and 90th percentiles, respectively) are plotted as vertical boxes with error bars. The 5th and 95th percentiles are indicated as black circles, and asterisks specify significant differences ($P = < 0.001$) compared to the control group.

VTG induction by EE2 was ≤ 1.67 ng/l ($P < 0.001$, compared with the controls). The concentration of VTG in the fish of the control groups was 0.84 ± 0.73 μg/mg. The highest EE2 exposure concentrations (20 ng/l) induced more than a 30000-fold increase in plasma VTG above the controls. There was a considerable variation in the responses of individual fish to the EE2, especially at lowest EE2 exposure doses, where there was more than a 13-fold difference in the concentration of VTG between the male fish.

4. Discussion

The study aimed to develop a sensitive and robust homologous ELISA for z-VTG and to validate its use for quantifying estrogenic responses in zebrafish. Zebrafish VTG was successfully purified by means of a one-step anion-exchange liquid chromatography procedure (Silversand and Haux, 1989; Silversand et al., 1993) with a short processing time. The rapid purification procedure for z-VTG was important because of the structural lability of fish VTGs, and in view of the fact that we had to pool samples from several column runs in order to obtain a sufficient amount of z-VTG for the further procedures. The purification yielded two proteins with a molecular mass of 142 and 171 kDa, which we consider to be z-VTG for the following reasons: (a) the proteins could be detected (by SDS-PAGE) only in the plasma of females, and not of males; (b) the proteins were inducible by estrogen treatment; and (c) the chromatographic and electrophoretic characteristics observed for the proteins were typical for fish VTGs (DeVlaming et al., 1980; Silversand and Haux, 1989; Silversand et al., 1993). The size of the intact VTG molecule and its subunits can vary markedly between different fish species, even between species of the same taxonomic group (e.g. Lee et al., 1992). The molecular masses of z-VTG on SDS-PAGE, 171 and 142 kDa, are within the size range described for other teleostean VTG subunits: 140–147 kDa for *Carassius auratus* (DeVlaming et al., 1980), 176–180 kDa for *Oncorhynchus mykiss* (Tyler and Lancaster, 1993; Bon et al., 1997), 170 kDa for *Morone saxatilis* (Kishida et al., 1992), 180 kDa for *Sparus auratus* (Mosconi et al., 1998), 183 kDa for *Mycteroperca microlepis* (Heppell and Sullivan, 1999), and 158 kDa for *Salvelinus alpinus* (Johnsen et al., 1999). In *Oreochromis mossambicus*, two immunologically distinct VTGs do exist, one of 130 and one of 200 kDa (Kishida and Specker, 1993). The 142-kDa protein of zebrafish may represent a degradation product of a larger VTG molecule, particularly since VTG in many fish is a rather unstable, and even the addition of protease inhibitors to a plasma sample cannot fully prevent its proteolysis (Tyler and Sumpter, 1990; Silversand et al., 1993). However, the 142-kDa protein may also represent a VTG form different to the 171-kDa protein, as zebrafish possesses several genes coding for VTG. Only two gene transcripts seem to be translated into proteins though, a predominantly expressed phosvitin-containing (thus of higher molecular weight) form and a 10-fold lower expressed phosvitinless form (Wang et al., 2000).

The homologous antiserum prepared against z-VTG did not cross-react with any plasma proteins in non-estrogenized male zebrafish, suggesting its specificity for z-VTG. Alternatively, the proteins detected by the antibody may represent chorionic (eggshell) proteins, since these proteins are also female-specific and estrogen-inducible (Arukwe et al. 1997a). However, the hitherto characterized eggshell proteins of fish have size ranges of < 100 kDa (Hyllner et al., 1991, 1994;

Arukwe et al., 1997b, 1998, 2000; Shimizu et al., 2000), i.e. they are much smaller than the proteins detected by our antibody. Incidentally, two egg envelope proteins were recently isolated from zebrafish with a size of 44 and 49 kDa (Mold et al., 2001). Also, the observation that a polyclonal antibody prepared against yolk proteins of dechorionatedzebrafish eggs did cross-react with the 142- and 171-kDa proteins of zebrafish plasma (data not shown) argues for the VTG nature of these two proteins. Finally, the specificity of the z-VTG antibody is indicated from the Western blot results, which show no positive reaction of z-VTG antiserum with plasma proteins of estrogenized female rainbow trout.

The ELISA established for z-VTG is sensitive, with a detection limit for the purified VTG protein between 2.0 and 3.0 ng/ml (at 85–90% of binding). This makes it suitable for studies on the physiology of very young female fish, where VTG concentrations are low (10s to a few 100 ng/ml plasma) and for use in the study of weak estrogens, where vitellogenic responses are minimal. The good sensitivity and broad working range of the assay (between 3 and 500 ng/ml) and relatively low inter- and intra-assay variation compare favorably with those reported for other good teleost VTG ELISAs, e.g. carp (*Cyprinus carpio*) (Tyler et al., 1999), the Arctic charr (*Salvelinus alpinus*) (Johnsen et al., 1999) and rainbow trout (*Oncorhynchus mykiss*) (Bon et al., 1997). A peculiar feature of the z-VTG standard curve was that the minimal binding was between 20 and 30%. We evaluated a series of other assays conditions, including the use of different types of BSA in the working assay buffer, in order to lower this minimal binding level, but without success. At present we do not have any explanation for this phenomenon, but it did not compromise our study.

The dilution curves obtained for the z-VTG standard and plasma from non-estrogenized and both estrogenized male and female zebrafish were parallel. This confirms that the z-VTG antibody recognized z-VTG in the plasma in a similar manner to the purified standard. Thus, the ELISA can be used to quantify z-VTG in the plasma of zebrafish. Furthermore, we observed partial parallelism of whole body homogenates from juvenile zebrafish. This opens the possibility of using the assay for measurement of VTG levels in juvenile zebrafish. Since the parallelism between the standard curve and the homogenate dilutions is not perfect, assay results of homogenate samples have to be interpreted cautiously. However, they give an approximation of VTG responses at those life stages of fish which are still too small for blood sampling.

The low level of VTG (0.84 μg/mg) measured in males from the controls of the EE2 exposure study is comparable to that reported in other 'laboratory'-maintained fish. This may be a consequence of estrogenic substances in the feed, or of small amounts of natural steroids in the water, secreted from females in the stock tanks (see Kramer et al., 1998; Harries et al., 2000), and/or it may be a reflection of low levels of endogenous estrogens in the males themselves. Contrary to the z-VTG ELISA, which detected measurable quantities of VTG in male zebrafish, the Western blot failed to show VTG-positive proteins in the plasma of male zebrafish. This is likely to be due to a lower sensitivity of the Western blot compared to the ELISA method.

Exposure of adult male zebrafish to EE2 induced a concentration-dependent induction of VTG, with a LOEC of < 1.67 ng/l. The potency of EE2 for VTG induction in the zebrafish compares favorably with previous studies on the vitellogenic responses of other fish species exposed to EE2. For example, exposure of male rainbow trout to only 0.1 ng/l for 3 weeks induced a significant elevation in plasma VTG (Purdom et al., 1994). Furthermore, in some unpublished studies (conveyed by Tyler at al.), exposure of fathead minnow (*Pimephales promelas*) to 1 ng EE2/l for 3 weeks induced a 1000-fold increase in plasma VTG. It is worth emphasizing that the effective EE2 concentration for VTG induction is well within the EE2 concentrations reported to occur in treated sewage plant effluents and in European surface waters (reviewed in Tyler and Routledge, 1998).

An interesting observation from the EE2 exposure study was the degree of variability in the response between the individual fish. At the exposure concentration 1.67 ng EE2/l, there was a 13-fold difference in the concentrations of plasma VTG between individual males, and at the highest exposure dose, this difference was six-fold. This difference suggests that males within a population have quite different sensitivities to steroidal estrogen. The differences in the sensitivity (and dynamics) of the vitellogenic response between individuals has received little attention, yet has

implications for developing the optimal in vivo screen for maximizing the chances of detecting estrogenic effects of chemicals.

In conclusion, a sensitive and robust homologous z-VTG ELISA has been developed and validated for use in quantifying VTG in both plasma samples and whole body homogenates. The assay has potential applications for studies on the basic reproductive physiology of this species and for detecting exposure to estrogenic chemicals using both adults and juveniles. The sensitivity of the zebrafish to EE2 suggests that the z-VTG ELISA will be useful in endocrine disruptor testing with the zebrafish.

Acknowledgements

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Chapter 2

An approach to evaluate vitellogenin as a predictive biomarker of estrogen-related reproductive disruption in zebrafish, *Danio rerio*.

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Abstract

The aim of this study was to evaluate if vitellogenin (VTG), beyond its value as a biomarker of current estrogen exposure of fish, can also be used as an effect marker that predicts an organizational impact of prolonged estrogen exposure on sexual differentiation and reproduction of fish. To this end, the induction of VTG in zebrafish (*Danio rerio*) by environmental estrogen exposure was related to the estrogenic impact on the sexual differentiation and reproduction. Zebrafish were exposed post-fertilization (pf) to 3.0 ng/L of the highly potent synthetic estrogen 17α-ethinylestradiol (EE_2), either (1) continuously from fertilization until the reproductive stage, (2) during different periods of the early stages of gonad differentiation, i.e., for 14, 21,and 42 days pf (dpf) or (3) from fertilization until final bisexual gonad differentiation at 75 dpf. After developmental exposures, fish were reared in non-contaminated water until the reproductive age. With the weakly estrogenic substance bisphenol A (BPA), a life cycle exposure was performed to evaluate the concentration-dependency of chronic estrogen effects. VTG was measured by ELISA different time points of the larval (21 dpf), juvenile (42, 75 dpf) or adult, reproducing (> 100 dpf) stage. Fecundity and fertilization success were assessed for 19-22 consecutive days of continuous constant spawning. In larval zebrafish, VTG was inducible by EE_2 already on day 21 pf. In adults, VTG was significantly elevated only in chronically exposed fish, while in fish that have been removed from exposure, VTG levels were comparable to control fish. Only females that were exposed during the early life-stage still showed elevated VTG levels at the adult stage. After developmental EE_2 exposure, fish showed normal gonad morphology whereas after chronic exposure to 3.0 ng/L of EE_2, only ovarian-like gonads were histologically found. These fish did not spawn during exposure. After 50 days of recovery from exposure, the fish started to spawn, but fertilization did not exceed 30 %. Life cycle exposure to BPA led to qualitatively similar effects as observed for EE_2. The lowest observed effect concentration (LOEC) of BPA for reproductive impairment was at 375 µg/L, which agrees with the LOEC for VTG induction in adult males, whereas the LOEC for VTG induction in females was at 94 µg/L, the lowest concentration of BPA tested. A significant correlation between VTG induction and reproductive impairment was found only to a limited extent. It is concluded that in zebrafish, VTG is a reliable indicator of current estrogenic exposure, but it is not suitable to predict reproductive effects of developmental exposure to estrogens.

Keywords: Bisphenol A (BPA), 17α-Ethinylestradiol (EE_2), Gonad differentiation, Vitellogenin (VTG), Zebrafish

Introduction

The induction of vitellogenin (VTG) synthesis in oviparous vertebrate species has become a widely recognized and accepted biomarker of exposure to endocrine disrupting compounds (EDCs) or, more specifically, environmental estrogens. While the correlation between estrogen exposure and vitellogenin response is reasonably characterized, the relationship between environmental estrogen-induced VTG synthesis and adverse alterations of the endocrine system is poorly understood yet. VTG is a sensitive and highly specific biomarker for current estrogen exposure, especially in fish (Sumpter and Jobling 1995), but if it also indicates organizational or persistent adverse effects, arising from the exposure is still unknown. Investigations of physiological influences of EDC exposure so far have mainly focused on activational effects like temporary changes in morphology or behavior or indeed on increased VTG levels. Activational responses to exogenous endocrine-active substances are easy to measure and they may imply prospective profound activational or even organizational effects, like alterations of gonad morphology. Both, activational and organizational effects of EDC exposure can exert a profound impact on the individual fitness of organisms. Whereas the correlation between activational effects and exposure and its physiological background is fairly understood, a direct association between this activational 'biomarker' effects and persistent or even irreversible organizational effects is considerably demanding. Transitory exposure to EDCs, e.g., can either cause only transient (and therefore activational) effects or the exposure affects an organism persistently or possibly only when the exposure has already been stopped long before. The cause and the effect of endocrine disruption may not have to inevitably concern the same phase in life. In the case of VTG, this would demand for a predictive capability of this biomarker, i.e., presence or absence of EDC-induced VTG synthesis should therefore at any rate be indicative of prospective detrimental effects such as reduced fertility, or of no effects.

There is the need for a better understanding of the impact of endocrine-active exposure on the organizational level and particularly regarding consequences for the reproductive function. This is of immense ecological concern as the disruption of the reproductive function of wild species may endanger whole populations. Alterations of sexual differentiation and reproductive impairments are of decisive relevance for the fitness and

survival of wildlife species. To provide advances for a more profound assessment of endocrine disrupting processes, it is indispensable to give increased attention to the relative sensitivity and effectiveness of biomarkers in regard to development processes and reproductive fitness. It is implicitly proposed in many studies so far that measurement of activational parameters like hormone levels or estrogen-induced protein synthesis is sufficient for a prediction of potential organizational responses. The significance of the time of exposure, the concentration of the substance exposed and the specific endocrine-active characteristics of the substance is often neglected. The most critical exposure period covered the development, and the sexual development in particular, of fish. This process highly depends on targeted endocrine regulation and therefore is most susceptible to disrupting effects. For this reason, it is the aim of this study to address the link between exposure-derived vitellogenin induction, sexual differentiation and subsequent reproductive capabilities. As such, it focuses on the particular effect of developmental exposure to EDCs in fish and it will compare these results with short-term or life-time exposure effects in zebrafish. To this end, zebrafish was exposed temporarily during larval development or permanently until adulthood to 3.0 ng/L of the synthetic hormone ethinylestradiol (EE_2). In our previous studies, this concentration of EE_2 has shown to be effective for VTG induction in pubescent and in adult male zebrafish (see. chapter 1) as well as to alter reproductive performance causing, e.g., a retardation in the onset of spawning or a significant reduction of fertility (Schäfers et al. submitted 2001; own unpublished results). To consider different mechanisms of effect of estrogen mimics, our experiments did not only focus on the strong estrogenic compound EE_2 but was qualitatively compared with a high production volume industrial chemical of weak estrogenic activity like BPA. Comparing the spectrum of effects of both a weak and a strong estrogenic chemical in regard to their inducibility of VTG and adverse consequences for the reproductive success in fish aimed at evaluating how chemical-specific or general the results obtained are.

Material and methods

Two life cycle tests were performed with the zebrafish (*Danio rerio*) at the Fraunhofer Institute for Molecular Biology and Applied Ecology (IME) in Schmallenberg, Germany. In the first life cycle experiment conducted, fishes were exposed to a concentration of nominal 3.0 ng/L of 17α-ethinylestradiol (EE_2). The experimental design of this study was based on results from a previously performed multigeneration study in which zebrafish were exposed to different water concentrations of EE_2 over a time period of two generations. The study revealed a lowest observed effect concentration (LOEC) value for EE_2 effects on zebrafish reproduction of nominal 1.1 ng EE_2/L, and a complete abolition of reproduction at 10 ng EE_2/L. These findings led to the selection of 3.0 ng EE_2/l as exposure concentration for the present work, in order to obtain a significant but not complete disruption of zebrafish sexual differentiation and reproduction. Additionally these relevant findings needed to be linked with the sub-organismic endpoints vitellogenin and gonad histology. As it has been shown in adult male zebrafish (Fenske et al. 2001, see chapter 1) that the lowest observed effect concentration (LOEC) of EE_2 for the induction of plasma vitellogenin was at nominal 1.67 ng/l, the concentration of 3.0 ng EE_2/L suggested the most suitable concentration to link definite reproductive impairments with sub-organismic responses.

The second life-cycle study was conducted with different concentrations of the xenoestrogen bisphenol A (BPA). Nominal concentrations of BPA used in this test were 94, 188, 375, 750 and 1500 µg/L, in addition to controls which were clear-water controls. The choice of this concentration series was based on previous range-finding and acute 96 h toxicity tests in *Pimephales promelas* (Alexander et al.1988) and adapted for the zebrafish.

Culture and maintenance of fish

Zebrafish (*Danio rerio*) were derived from the breeding stock of the test performing Fraunhofer Institute IME (Institute for Molecular Biology and Applied Ecology) in Schmallenberg, Germany. The zebrafish strain descend from progeny of the West Aquarium GmbH in 37431 Bad Lauterberg, Germany. Breeding fish were cultured in activated charcoal-filtered and tempering water in glass aquaria (total volume: 107 L). Water in the aquaria was continuously filtered by aquarium filter pumps (Eheim, Germany), aerated by air supply and the temperature in the aquaria was kept between 25 and 27 °C by heating elements. Breeding and all exposure experiments were conducted in

temperature controlled rooms, keeping the temperature between 24 and 26 °C. For spawning, spawning trays were placed into the aquaria, covered by a lattice lid (stainless steel) with attached artificial "spawning-trees" as spawning substrate. The introduction of suitable spawning substrate is indispensable to initiate spawning, without zebrafishes do not spawn. Onset of spawning occurs as soon as the light was switched on.

Under experimental conditions fishes were maintained in (standardized) test facilities composed of temperature-controlled shallow water basins (water bath) in which the test vessels were placed in to provide for a consistent water temperature (for control of temperature) in all vessels during test conduction. All vessels were aerated by separate air supplies. During the first period of test, until day 35 to 42 (early life stage according to the definition of the OECD (Organization of Economic Cooperation and Development) test guideline 210, OECD 1993), glass aquaria of 29 x 22 x 21 cm (length x depth x height; total volume=13.4 L) with cages of 20 x 9 x 9 cm (length x depth x height) for the eggs were used as test vessels. These aquaria with a total volume of 13.4 L were placed into glass aquaria with a total volume of 29 L (40 x 27 x 27 cm; length x depth x height). Volume of test solution was 25 L per vessel. After 2-3 weeks the larvae were transferred from the cages into the 13.4 L-glass aquaria. At the end of the early life stage period, juvenile fish were transferred from the smaller vessels (13.4 L) into the 29 L-tanks where they were kept until the end of the study.

All fishes and fry were held under the same photoperiodic regime of 12-h light : 12-h dark. Fishes were fed three times a day, two times with TetraMin®, dry flaked food (Tetra, Germany) ad libitum and once with *Artemia* sp. nauplii as live food supplement. Shortly after hatch, larvae were supplementary fed daily twice with a commercial larval food AZ 100 (Tetra, Germany) until the end of the yolk-sac stage. Subsequently, larvae were fed three times a day, in the morning and in the evening, with AZ 100 ad libitum. Dry food was supplemented with freshwater rotifers (*Brachionus calyciflorus*; Rotifera) once daily at noontime.

Table 1. Experimental design for the life cycle study in zebrafish (Danio rerio) with 3.0 ng/L of EE_2

Stage of fish development	**Test phase**	**Course of study**	**Time scale of treatment**	**Endpoints/ parameters measured**
Embryonic phase	Fish early life stage toxicity study (FELS) F_0-generation	start with 100 fertilized eggs per vessel	day 0	
Larval phase		hatching of larvae	day 2-3	hatching rate
		feeding with breeding food	day 5	
		feeding with *Artemia* sp. nauplii	day 9	
		stop of exposure in two replicates; flow-through continued with uncontaminated water	day 14	
Embryonic and larval phase		stop of exposure in two replicates; flow-through continued with uncontaminated water; taking of samples (10 fishes per vessel i.e., 20 fishes per treatment/control	day 21	vitellogenin in whole body samples
Juvenile phase	Reproduction F_0	stop of exposure in two replicates; flow-through continued with uncontaminated water; end of FELS; reduction to 50 individuals per vessel	day 42	survival, length
			day 42	vitellogenin in (decapitated) body samples
		end of juvenile growth, reduction to 30 individuals per vessel	day 75	survival, length
			day 75	plasma vitellogenin gonad histology
Adult phase		sexual maturation fecundity assessment	day 75-100	onset of spawning
			day 92-114	fecundity, fertilization
			day 118	plasma vitellogenin gonad histology
		end of exposure	day 125	
		end of study	day 176	plasma vitellogenin gonad histology

Experimental protocol, exposure

The test substances, 17α-ethinylestradiol (17α-ethinyl-1,3,5(10)-estratriene-3,17β - diol; 98 % purity) and bisphenol A (4,4'-isopropylidenediphenol; ≥ 99 % purity), were obtained from Sigma-Aldrich. For the dosage of chemicals during exposure, primary stock solutions of 1 mg/ml EE_2 acetone was prepared in acetone (100 %), further diluted to 1 mg/L in sterile distilled water and delivered to the diluter. Due to degradation processes of the substances, EE_2 stock solutions were renewed regularly every week. Stock solutions of BPA for dosing were prepared in 10 ml of acetone (100 %) each and had to be renewed every second to third day. BPA concentrations of all stock solutions were analyzed to confirm respective dosing concentrations. Chemical analysis of test substances and concentrations had been established in the Fraunhofer Institute (Schmallenberg, Germany). Performance of chemical analyses was carried out by W. Böhmer (EE_2-analysis) and J. Bruckert (BPA-analysis) of the Department of Ecological Chemistry, FhG Schmallenberg, as described below.

Water temperature, pH-value, ammonia and dissolved oxygen were monitored throughout the studies. Temperature, dissolved oxygen content and pH in all test vessels were measured every third day during the whole test periods with portable digital pH- and oxygen-meters (WTW Weilheim, Germany). Ammonia was determined by the use of a colorimetric ammonia test kit (Aquamerck®, Merck Germany).

Table 2. Experimental design for the life cycle study in zebrafish (*Danio rerio*) with bisphenol A (BPA)

Stage of fish development	**Test phase**	**Course of study**	**Time scale of treatment**	**Endpoints/ parameters measured**
Embryonic phase	Fish early life stage toxicity study (FELS) F_0	start with 100 fertilized eggs per vessel	day 0	
Larval phase		hatching of larvae	day 2-3	hatching rate
		feeding with breeding food	day 6	
		feeding with *Artemia* sp. nauplii	day 9	
		taking of samples: 10 fishes per vessel i.e., 20 fishes per concentration	day 21	vitellogenin in whole body samples
		end of FELS; reduction to 50 individuals per vessel	day 35	survival, length
Juvenile phase	Reproduction F_0	end of juvenile growth, reduction to 30 individuals per vessel; taking of samples	day 75	survival, length
			day 75	vitellogenin in body homogenates (decapitated samples), gonad histology
Adult phase		sexual maturation	day 75-100	onset of spawning
		fecundity assessment	day 88-109	fecundity
		end of exposure; taking of samples	day 110	plasma vitellogenin gonad histology
		end of F_0 study	day 170	length
	Fish early life stage toxicity study (FELS) F_1	see above	day 170 - 205	hatching rate, survival, length

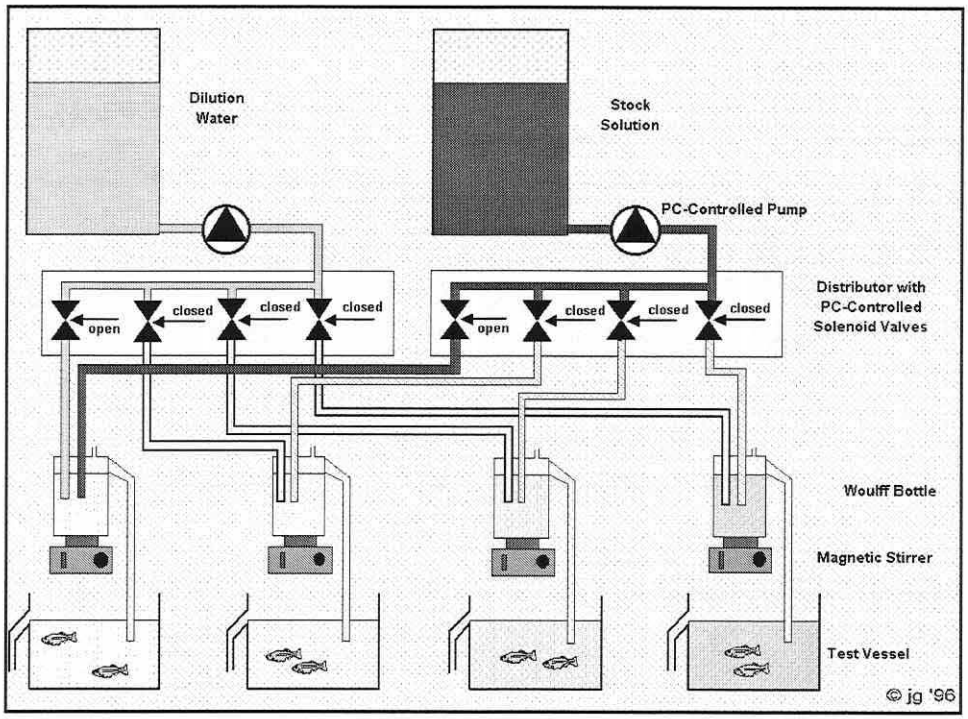

Fig. 1. Schematic illustration of the setup of flow-through system for the life cycle study as conducted with EE_2 (17α-ethinylestradiol) at the Fraunhofer Institute Schmallenberg

EE_2 experiment

The life cycle test with EE_2 was carried out in a flow-through system. Dilution water was fed from an elevated reservoir tank (temperature-regulated by heating elements) via flow control devices to the mixing/dilution vessels (Woulff bottles). Also the inflow dosage valve from stock solutions met these vessels. Inflow and distribution of dilution water and stock solution to the mixing/dilution vessels were electronically controlled by solenoid valves (see Fig. 1). Flow rates were maintained to provide two tank volume changes each day. Prior to introduction of test specimens the exposure system was run several days to saturate all surfaces with the test substance and to monitor degradation.

During the course of the study, the temperature in the test vessels was between 25.0 and 26.3 °C (until the end of reproductive performance on day 125 pf), the mean pH-value ranged between 7.6 and 8.1. Dissolved oxygen in the test vessels was between 6.0 and 8.6 mg/L.

The mean measured concentrations of EE_2, analyzed regularly during the course of the experiment, ranged between 80 % and did not exceed 120 % of the nominal 3.0 ng/L (see Fig. 2).

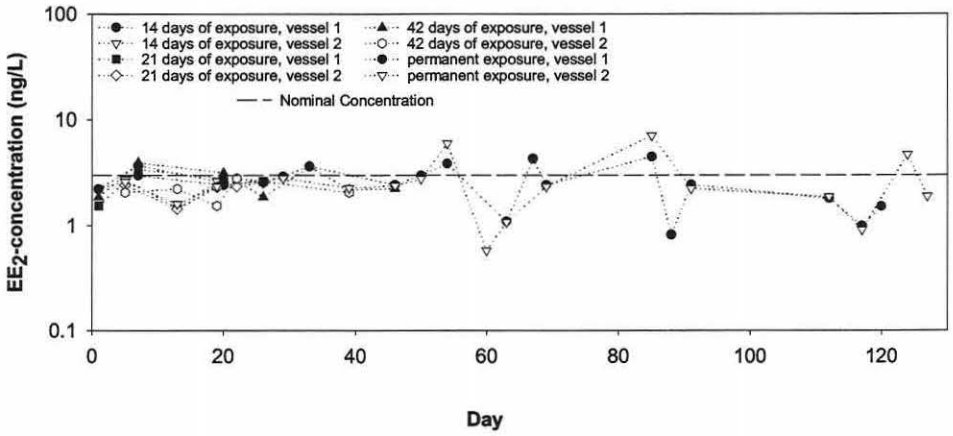

Fig. 2. Measured concentrations of 17α-ethinylestradiol (EE_2) during the exposure period of 14, 21, 42 and125 days post fertilization. The nominal concentration was 3.0 ng/L of EE_2 (dashed horizontal line). EE_2 was measured by ion trap GC/MS/MS (Böhmer and Kurzawa 2001) at the Fraunhofer Institute Schmallenberg, Germany.

The experimental design for the life cycle exposure of zebrafish to 3.0 ng/L of EE_2 is outlined in Table 1 above. At the beginning of the study 100 fertilized eggs were transferred to each test vessel. Each treatment period and the control approach consisted of two replicate vessels each. In the course of the life cycle experiment, early life stage exposure was conducted according to the OECD guideline 210 (OECD 1993). Hatching rate was recorded for each vessel. During fish early life, exposure in two replicates of EE_2 treatment vessels was stopped each time after 14, 21 and 42 days of exposure and fish were maintained in flow-through of uncontaminated (EE_2-free) water. These early life stage-specific exposure fish were raised until the reproductive stage without being sampled. At the age of 42 d after fertilization, the number of fish in each vessel were reduced to 50 fish. Thereby survival rate and body length was determined. The individual body size of fish was measured by digital image analysis. In the following period, test was continued during puberty until day 75 pf and survival rate was recorded.

Number of fish per vessel again was reduced to 30. Fish were not randomly selected from the test vessels, but it was tried to keep a male to female ratio of approximately 2:1 in each vessel to achieve suitable and similar conditions for reproduction. (Before termination of sexual differentiation, sex of individual fish can hardly, if at all, be estimated by external secondary sex characteristics.) Body length of all fish, including the selected fish, was measured and the average juvenile growth between day 42 and 75 pf determined per vessel and fish. The test was continued until sexual maturity and the onset of mating behavior and the subsequent occurrence of first spawning success were monitored. Reproductive performance was assessed between day 92 and day 114. Fecundity and fertilization capacity was recorded daily over a period of 19 – 22 consecutive days when spawning was observed constantly in all test vessels. Each day spawning trays, covered by lattice lids (stainless steel) with attached artificial "glass trees", were placed into the vessels, including the control vessels, to induce spawning behavior of the females. The "glass trees" served as spawning substrate. One to two hours after the light has switch on in the test facility, spawning trays were removed from the vessels, laid eggs were collected and counted, transferred to glass beakers with clean dilution water and incubated for 24 h at 27 °C to record fertilization. The eggs were inspected visually under a dissecting microscope and distinguished between fertilized and unfertilized eggs. Infertile eggs are opaque or the precipitated yolk in visible as a white clot inside the egg. Egg production and fertilization rate were measured daily for each vessel, total fecundity (egg production) was also determined for the whole reproductive test period for each vessel. After sexing of breeding fish by means of histological analysis of gonads, number of daily spawned eggs per vessel were referred to the number of reproducible females as number of eggs per female and vessel. For one replicate each of exposure/control groups, the assessment of fecundity was continued between day 140 and 159 pf, after the end of exposure on day 125 pf. The study was terminated on day 176 pf.

BPA experiment

Due to its specific physico-chemical characteristics BPA showed a very fast biodegradation in the test vessels and stock solutions (short half-life of BPA, 2.5 – 4 days; Dorn et al. 1987). Therefore, life cycle performance with BPA was conducted in a semi-static system. 80 – 90 % of water in test and control vessels was exchanged three times a week. Pre-dilutions of test solutions for each vessel were prepared in 10-L glass bottles, dosed with the appropriate concentration of BPA and mixed by continuous stirring over night. During experiment performance, the temperature in the test vessels was at 25.3 °C ±

0.3 °C (mean value ± SD; n= 57), the pH-value ranged between 7.4 and 8.9, and the mean value of dissolved oxygen was between 7.2 mg/L ± 0.9 mg/L and 7.5 mg/L ± 0.7 mg/L (mean value ± SD; n= 57). The measured concentrations in water samples from all test vessels throughout the exposure periods of the study corresponded (predominantly) to the nominal concentrations of 94, 188, 375, 750 and 1500 µg/L of BPA. The mean concentrations did not fall below 80 % or did exceed 120 % of the aspired values (see Fig. 3).

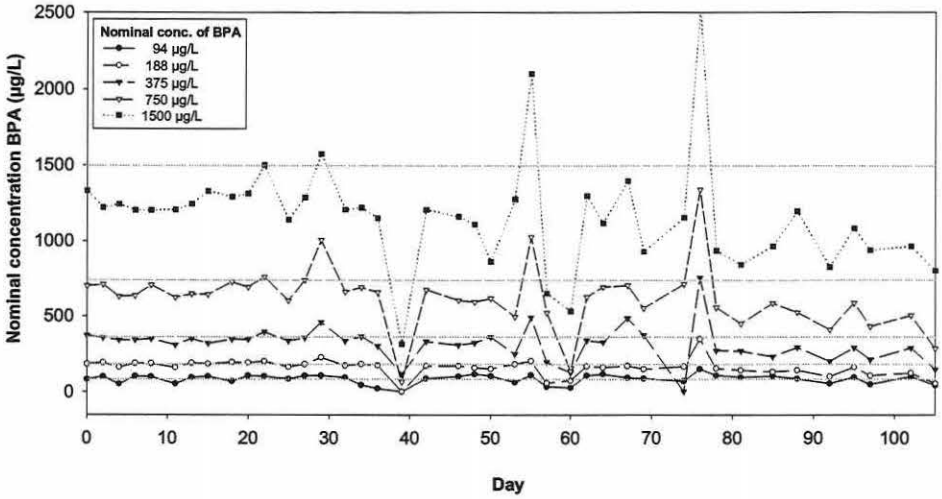

Fig. 3. Mean concentrations of BPA in test solutions (n = 2 vessels per concentration) during the exposure period of the F0 study; analytic samples were taken every second/third day from a newly prepared test solution each. Degradation processes of BPA during the following day are not diagrammed. Standard deviation values observed between the vessel were 0.3 – 14.8 µg/L in the lowest and 0.2 and 720 µg/L in the highest BPA concentration. Analyses were performed at the Fraunhofer Institute Schmallenberg, Germany by capillary GC/MS and BPA quantified in comparison to the internal calibration standard.

The outline of the experimental design for the life cycle study with BPA is given in Table 2. Conduction (Test procedure) and time flow of test widely corresponded with the previously described EE_2 study, except that the first period (FELS) of the test ended earlier on day 35 pf. Moreover, this study was supplemented by a following FELS study in the F_1-generation to assess transgenerational effects on fecundity, hatchability and survival of

fry. Fish were exposed permanently in two sets of replicate test vessels to nominal 0.0, 94, 188, 375, 750 and 1500 µg/L of BPA. Short-time exposure studies were not performed in this BPA test. The timing of assessing reproductive success (egg production = fecundity) varied from the EE_2 test as the specimens of this study reached sexual maturity earlier. Therefore fecundity was assessed already between day 89 and 110. The exposure was stopped subsequently on day 110. The study terminated on day 170 pf for the F_0 generation, but was continued until day 205 in the F_1 generation. In this third test period, fertilized eggs from the F_0 generation were retained and randomly introduced into control and replicate BPA treatment vessels. F_1-larvae were exposed to the same BPA concentrations as the F_0 fish, until day 35 pf at the end of early life stage. Hatchability and survival rate of fry were observed, body length of all specimens measured.

Sampling

In the course of the EE_2 experiment, after 21, 42, 75, 118 days of exposure and at the end of study on day 176 pf, fishes were sampled from all treatment groups for the quantification of vitellogenin and for the analysis of gonad histology. Due to the small size of developing, pubescent fish, fishes were taken as whole body samples or, depending on size, separated into head and the trunk part. After 21 and 42 days of exposure, whole fishes were anaesthetized in ice water, each specimen was measured and weighed, transferred to 2.0 ml Eppendorf tubes and subsequently snap frozen in liquid nitrogen and stored at until required. On day 42, fishes were separated into head and body sections which were frozen separately. At 75, 118 and 176 dpf fishes were anaesthetized in an aqueous solution of benzocaine (Sigma-Aldrich) (≤ 0.4 g/L) and body length and weight were recorded. Blood samples were collected from the heart by cardiac puncture into chilled heparinized 1.0 ml syringes. Blood was diluted with 0.01 M PBS (phosphate-buffered saline) buffer (pH 7.3) containing the protease inhibitor aprotinin (0.7 – 1.0 TIU/ml; Merck), centrifuged at 2900 x g for 25 min at 4 °C, the supernatants were withdrawn and snap frozen in liquid nitrogen. Plasma samples were stored at – 80 °C until analyzed for vitellogenin. After blood sampling on days 118 and 176 pf, the same fishes were dissected and gonads were fixed for histology.

During exposure to BPA, fishes were taken for vitellogenin analysis after 21, 75 and 110 dpf. On day 21 whole fishes were snap frozen, head and body samples were taken on day 75. On day 110 pf blood samples were collected and the gonads were dissected out from the same fishes as well, fixed for histological analysis or were snap frozen in liquid nitrogen. For each sampling, body length and weight of all fishes taken were recorded.

Pubescent and adult fish that already reached an adequate body size, were sexed by their external appearance (males, e.g., have a smaller body shape, the coloration is more intensive) and during the dissection procedure, by the external morphology of the differentiated gonads.

VTG measurement

Vitellogenin was measured in homogenate or plasma samples using a homologous competitive vitellogenin enzyme-linked immunosorbent assay (ELISA) for zebrafish. The method was described by Fenske et al. (2001). Plasma samples were directly applied to the assay at appropriate dilutions. Tissue samples otherwise were homogenized in ice-cold PBS-T ELISA buffer in an 1:2 or 1:3 ratio wet weight : buffer volume (depending on the developmental stage/size of the individual fish) with a glass homogenizer (motor driven) and centrifuged at 15,000 rpm for 3 min. The supernatants were collected and analyzed in the ELISA for vitellogenin. Vitellogenin concentrations measured were normalized to the volume (ml) of the corresponding sample. Relative VTG induction was calculated as the ratio of normalized VTG sample concentration (µg/ml) to the normalized mean VTG concentration of the control (µg/ml). These calculations allowed the comparison of VTG data of homogenate samples with plasma samples and vice versa.

Due to the high degree of data variation in the VTG titers measured in larval and subadult fish, VTG concentration values were divided into two different subgroups in which the mean VTG levels differed statistically significant from each other. This categorization was applied to fish that were too small to be sexed.

Pubescent and adult fish i.e., fishes that already finalized the period of sexual differentiation (at least after 70/75 dpf), featured sex-specific levels of homogenate or plasma VTG. The mean VTG titers of male fish never reached comparable levels of females. In control as well as in exposed fish, there were always two distinctly different subgroups in which the VTG concentrations were either in the 'typical' range of male or female fish. Whereas male fish did hardly ever exceeded VTG concentrations of 100 µg/ml, the VTG concentrations of females were always > 100 µg/ml, even in control fish. These VTG-based sexual differences were often not in accordance with the morphologically or histologically determined sex of individual fish.

Chemical analysis

As described previously, chemical analyses during test performance was carried out by members of the Department of Ecological Chemistry, at the Fraunhofer Institute Schmallenberg, Germany based on standardized analytical methods. Samples from each test replicates, including controls, were taken every second (for BPA) or fourth day (for EE_2) respectively, either acidified (for EE_2) (100 µL of 30 % hydrochloric acid/L) or made alkaline (with 1 N NaOH to pH 11.0 - 12.0) (for BPA) for preservation and extracted (by solid-phase extraction SPE in the case of EE_2, with dichloromethan in the case of BPA). Analysis of EE_2 was performed by ion trap GC/MS/MS (gas chromatography/mass spectrometry) (Böhmer and Kurzawa 2001), analysis of BPA by capillary-GC/MS, for both after derivatization with N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA; Sigma-Aldrich). Quantification was achieved by comparison to internal calibration standards of EE_2 (EE_2-D_4; CDN Isotopes, Canada) and BPA (BPA-d_{16}; Sigma Aldrich).

Statistical analysis

The determination of No Observed Effect Concentrations (NOEC) and Lowest Observed Effect Concentrations (LOEC) was performed by using appropriate statistical methods (e.g. ANOVA, followed by Dunn's test, etc.). As vitellogenin data commonly have a non-normal distribution, they were analyzed by Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks, followed by Dunn's Method multiple comparison test. Additionally, different groups of exposure were pairwise compared by a Mann-Whitney rank sum test. Statistical analyses of vitellogenin data were carried out in SigmaStat® 2.03 (SPSS).

Reproductive performance data were first checked for assumptions of normality and homogeneity of variance and then transformed when necessary. Survival rates (as p-values 0-1) were z-transformed (arc sin (square root p)) and analyzed for significant differences by ANOVA performance, followed by Dunett's test and t-test (to find out which mean rate values were significantly lower than the mean value found for the control). Values of mean fish length and of the 'pseudo' specific growth rates (i.e. individual growth compared to the mean initial length of the respective test vessel population) were also subjected to ANOVA and Dunnett's test, followed by t-test performance. In cases of inhomogeneous variances Dunnett's T3 was performed. Fertilization rate values (0-100%) were z-transformed (arc sin (square root x)), egg numbers log-transformed prior to ANOVA and the subsequent Dunett's test conduction. Additionally, all data were also subjected to the

appropriate statistical tests, at any rate, as untransformed raw or mean values. In case of insufficient data homogeneity, nonparametric statistical comparisons were conducted using the Kruskal-Wallis one-way ANOVA on ranks, followed by Dunn's test and complemented by Mann-Whitney U-tests. χ^2 analysis was applied to test differences in the distribution of gender compared to the controls. Statistical analyses of reproductive data were carried out either in SigmaStat® or by using the SPSS program package.

Results

Effects of EE_2 and BPA on vitellogenin

EE_2 exposure:

Vitellogenin (VTG) measured in whole body homogenates of control zebrafish on day 21 post fertilization (pf) ranged between 1.1 µg/ml and values at the lower nanogram range, just above the detection limit of the VTG assay (Fig. 4). VTG concentration values measured in chronically to 3.0 ng/L of 17α-ethinylestradiol (EE_2) exposed fish, showed a high degree of variability with a standard deviation (SD) from the mean VTG content of ± 12.0 µg/ml. A high variability in VTG titers was found in all our studies to be characteristic for developmental stages of still undifferentiated gender or for fishes that could not be sexed due to their small size. Statistic analysis of data indicated an allocation of raw data into two (statistically significant) different VTG responder groups, one group of "lower" responsiveness (group 1), composed of concentration data ≤ 1.0 µg/ml, and a second of "higher" responsiveness (group 2) with concentrations > 1.0 µg/ml. A VTG concentration above 1.0 µg/ml was clearly beyond the 95 % confidence interval of the control and higher than the maximum value of VTG found in the control group. A division of the control group was omitted. In the resulting "high"-responder group (group 2), the mean VTG homogenate content was increased significantly to the 60-fold level of the control group. Therefore, vitellogenin in whole body homogenates of almost 50 % (43 %) of 3.0 ng EE_2/L exposed zebrafish was significantly elevated above the control level already at the early age of 21 dpf. The mean homogenate VTG content of the "low"-responder group was 171 ng/ml (0.17 µg/ml) and hence even lower than the 229 ng/ml (0.23 µg/ml) determined for the controls.

In fish sacrificed after 42 days of EE_2 exposure (Fig. 5), there was again a high degree of variability in the homogenate VTG concentrations. At this developmental age, both in the controls as well as in the exposed fish, two groups of either low (≤ 1.0 µg/ml VTG) or of higher (> 1.0 µg/ml VTG) response levels differed significantly from each other. 1.0 µg/ml of VTG was clearly beyond the 95 % confidence interval of the control. These groups were again categorized as group 1 and group 2. A nine-fold increase of VTG above the control group 1, and therefore significant, in exposed fish of group 2, resulted from this

approach (or categorization). And even if compared with the mean homogenate VTG content of all control fish, this rise in VTG production to 3.4 µg/ml still proved to be significant. The percentage of treated fish in which vitellogenin synthesis was significantly enhanced was 35 %. It was surprising given that the EE_2-induced increase of VTG, measured in homogenates of 42-day old fish, did not reach a comparable or even higher level than observed in 21-day old fish.

The body size reached by the fishes on day 75 pf allowed to take plasma samples for the analysis of VTG (Fig. 6). Similar to what has been found for the whole body homogenates, a high interindividual variability of VTG concentrations was observed also in the plasma samples. Accordingly, plasma VTG values of control and exposed fishes were categorized into "low"- and "high"- responder groups. Within each of the two categories or groups of (significantly) different VTG responsiveness, VTG was significantly higher than in the corresponding control. The mean plasma VTG concentration in the "low"-responder group (group 1) increased to 37.8 µg/ml, which is a more than 2000 times higher than observed in the group 1 controls. The rise in plasma VTG in the "high"-responders (group 2) was lower, but still statistically significant. If compared with the mean plasma VTG titer of all control fish (group 1 and group 2) measured, VTG synthesis in both VTG response groups was significantly elevated. The highest mean VTG concentration observed in the plasma samples of exposed fish was 614 µg/ml.

In sexually differentiated and reproducing adult zebrafish, plasma vitellogenin was measured in male and female specimens which were either exposed chronically or had been exposed only temporarily for 14 days, 21 days or 42 days during the embryonic and larval period. After early life stage-restricted EE_2 exposures fish were raised in uncontaminated water, from the post-exposure time until the reproductive stage, without being sampled. Adult fish of all treatment groups (short-term and chronic treatment pf) were sampled for VTG analysis at first on day 118 pf, after the assessment of fecundity had been finished. Afterwards, on day 125 pf, the EE_2 treatment in the remaining exposure group was stopped as well and all fishes were continuously reared in uncontaminated water until the end of the experiment on day 176 pf. At this time, VTG was measured again in all remaining fish.

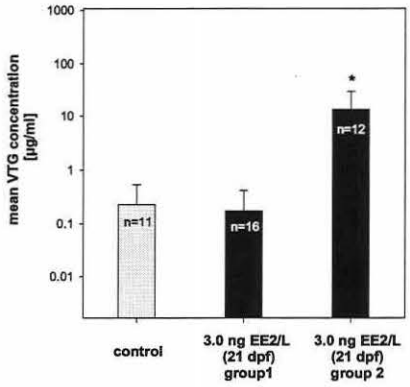

Fig 4. 3.0 ng/L EE_2 experiment:
Vitellogenin (VTG) concentrations in whole body homogenate samples of zebrafish on day 21 pf, chronically exposed to 3 ng/L of 17α-ethinylestradiol (EE_2). Values are means + S.D. Data of EE_2-exposed fish were subdivided into two different groups (group 1 and group 2) as described in the text. Total mean value ± S.D. of both exposure groups 1 and 2: 5.9 ± 12.3 µg/ml. Number of samples (fish) analyzed (= n) are indicated in the bars. Asteriks (*) indicate mean values that were significantly different from the control mean ($*P$ = <0.001). Y-axis is log-scaled.

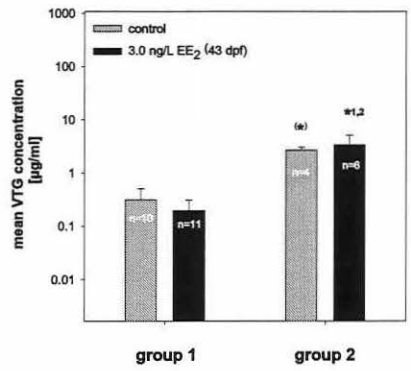

Fig 5. 3.0 ng/L EE_2 experiment:
Vitellogenin concentrations in (decapitated) body homogenate samples of zebrafish on day 42 pf, chronically exposed to 3 ng/L of 17α-ethinylestradiol (EE_2). Values are means + S.D. Data of control and EE_2-exposed fish were subdivided into two different groups (group 1 and group 2) as described in the text. Total mean values ± S.D. of control/exposure groups 1 and 2 together were: 1.0 ± 1.2 µg/ml (control) 1.3 ± 2.1 µg/ml (EE_2). Number of samples (fish) analyzed (= n) are indicated in the bars. Asteriks (*) indicate mean values that were significantly different from the control group 1 ($*^1P$ = 0.001) or from the total control mean ($*^2P$ = 0.015). (*) indicates the significant difference between the control groups 1 and 2 (P <0.01). Y-axis is log-scaled.

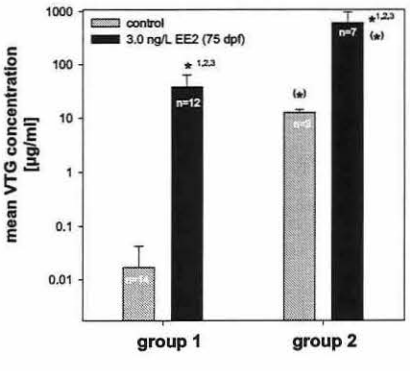

Fig 6. 3.0 ng/L EE_2 experiment:
Vitellogenin concentrations in plasma samples of zebrafish on day 75 pf, chronically exposed to 3 ng/L of 17α-ethinylestradiol (EE_2). Values are means + S.D. Data of control and EE_2-exposed fish were subdivided into two different groups (group 1 and group 2) as described in the text. Total mean values ± S.D. of control/exposure groups 1 and 2 together were: 2.3 ± 5.1 µg/ml (control) 250 ± 371 µg/ml (EE_2). Number of samples (fish) analyzed (= n) are indicated in the bars.Asteriks (*) indicate mean values of exposure groups 1 or 2 that were significantly different from the control group of the other VTG induction group ($*^1P$ <= 0.01) or from the same control group of VTG induction group ($*^2P$ <= 0.01)or from total control mean ($*^3P$ <= 0.001). (*) indicates the significant difference between the control groups 1 and 2 (P <0.01) and the treatment groups 1 and 2. Y-axis is log-scaled.

VTG plasma titers of adult male and female zebrafish, which were continuously (≤ 125 dpf) exposed to EE_2 until day 125 pf, were examined after 118 and 176 dpf; the mean VTG values obtained were compared with the VTG plasma titers of only transiently – 0–14 dpf, 0-21 dpf or 0-42 dpf – exposed fish (Fig. 7). In male fish after 118 days of chronic exposure pf, plasma VTG turned out to be significantly elevated. The mean VTG plasma concentration of 1.54 µg/ml was 13 times higher than the 1.23 µg/ml found in the control males. On day 176 pf, after 50 days of EE_2 treatment, plasma VTG in male fish was no longer significantly increased over the control titer of males at this age (Fig. 7a). In comparison to the mean plasma titer of chronically EE_2-exposed males, VTG in those fish that were only temporarily exposed during the early developmental period, did not show significant differences towards the controls. At both sampling dates, 118 dpf and 176 dpf, the mean VTG titers of short-term exposed fish were not affected by the EE_2 treatment: Plasma VTG levels in males on day 118 pf were less than 2-times higher than the controls, with mean values between 0.166 µg/ml (14 dpf) and 0.232 µg/ml (21 dpf). On day 176 pf, the mean VTG plasma concentrations varied between 0.11 and 0.6 µg/ml, wherein the highest VTG plasma levels were found in the control group. Neither for male fish, being exposed for the 125-day period nor for the transient exposure groups, any EE_2 induced enhancement of VTG synthesis was detectable. Obviously, exposure to 3.0 ng/L of EE_2 affected VTG in males only after chronic treatment.

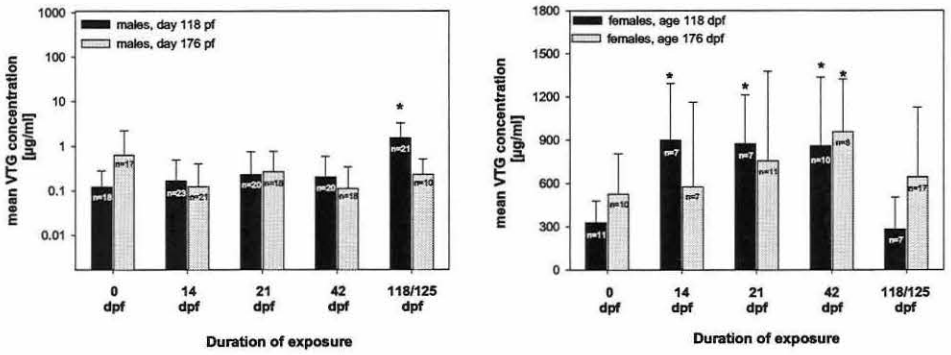

Fig 7. 3.0 ng/L EE_2 experiment:
Vitellogenin (VTG) plasma concentrations in adult male (a) and female (b) zebrafish on day 118 pf and 176 pf, exposed to 3 ng/L of 17α-ethinylestradiol (EE_2) for different time periods. Values are means + S.D. Number of samples (fish) analyzed (= n) are indicated in the bars. Asteriks (*) indicate mean values that were significantly different from the control mean (*$P < 0.05$) . Y-axis in Fig. (a) is log-scaled.

Contrary to males, plasma VTG of female zebrafishes was not induced by chronic or long-term exposure (until 125 pf) to EE_2 in comparison to the control groups of the corresponding age. On day 118 pf, chronically exposed females showed the lowest VTG plasma titer among all exposure groups of this age, with 279 µg/ml even 15 % lower than in the controls. In females on day 176 pf, exposed during 125 dpf, mean plasma VTG concentrations measured turned out to be at least 20 % higher than in the respective control, reaching 643 µg/ml ± 499 µg/ml. In contrast to the results found in males on day 118 pf, plasma VTG in females of all temporary early-life exposure groups were significantly elevated above the control group. The mean VTG titers measured in these plasma samples were rather consistently between 860 µg/ml and 899 µg/ml. At the end of the experiment on day 176 pf, the mean plasma VTG titers in these transiently exposed females were still higher than the control level, but only the mean VTG level of 957 µg/ml (± 390 µg/ml) of the 42-day exposure group was significantly different from the control level.

BPA-exposure:

In the bisphenol A (BPA) study, a series of BPA concentrations ranging from 94 µg/L to 1500 µg/L was applied. After 21 day of exposure of zebrafish to the different concentrations of BPA mean values of whole body homogenate VTG levels (Fig. 8, legend) were below 1.0 µg/ml. In control fish of this age, VTG homogenate concentrations reached not more than 0.6 µg/mL and were often in the lower nanogram range and thus below the detection limit of the assay. In comparison to the control fish of the EE_2 study at this age, the average level of homogenate VTG level was slightly lower. Due to high standard deviation values, as already observed for the early life stage fish of the EE_2 experiment, a categorization of VTG data into groups of different VTG responsiveness was done accordingly to the EE_2-study. Hence, mean VTG concentrations of whole body homogenates were divided into two different groups, either showing "lower" VTG responsiveness (< 0.1 µg/ml VTG) or with VTG concentrations proving "higher" responsiveness (≥ 0.1 µg/ml). The dividing line between the groups was a tenth part lower than in the EE_2 study, because the VTG homogenate concentrations measured were generally lower in the 21-day BPA exposed fish. A VTG concentration above 0.1 µg/ml was clearly beyond the 95 %-confidence interval of the control and higher then the maximum value of VTG found in the control group. In this figure (Fig. 8), neither the concentration groups of the "low" (group 1) VTG responders nor the concentration groups of "high" VTG responders (group 2) showed an indication of a BPA-dependent increase of

vitellogenin. Only the mean VTG concentration in the 94 µg/L BPA group of the "high"-responders (group 2) was significantly higher than the control group 1, giving the highest value of 0.90 µg/ml (± 0.92 µg/ml). Apart from this 94 µg BPA/L exposure group 2, the mean VTG contents of 21-day old fish was not influenced by the BPA treatment, independently of the category of VTG responsiveness. Also without categorizing the VTG concentration data (see legend of Fig. 8), BPA exposure-related effects on VTG were not indicated. Although the mean VTG homogenate concentration of 0.61 µg/ml in the 188 µg BPA/l exposure group was almost three times higher than the control level, this VTG increase proved to be not significant due to the high standard deviation (± 1.63 µg/ml).

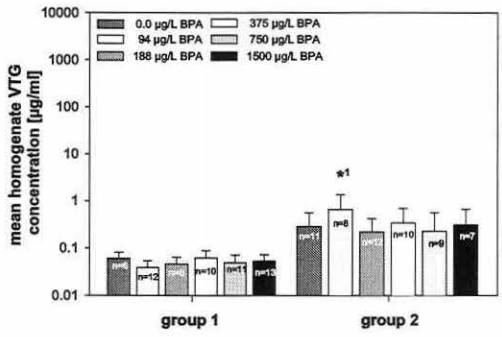

Fig 8. BPA experiment:
Vitellogenin concentrations in whole body homogenate samples of zebrafish on day 21 pf, chronically exposed to different concentrations of bisphenol A (BPA) (nominal: 94, 188, 375, 750 and 1500 µg/L). Values are means + S.D. Data of controls and of each BPA treatment were subdivided into two different groups (group 1 and group 2) as described in the text. Total mean values ± S.D. of controls/each treatment group were: 0.29 ± 0.34 µg/ml (control); 0.39 ± 0.71 µg/ml (94 µg BPA/L); 0.61 ± 1.63 µg/ml (188 µg BPA/L); 0.27 ± 0.39 µg/ml (375 µg BPA/L); 0.17 ± 0.33 µg/ml (750 µg BPA/L); 0.19 ± 0.33 µg/ml (1500 µg BPA/L). Number of samples (fish) analyzed (= n) are indicated in the bars. Asteriks (*) indicate mean values that were significantly different from the control group 1 ($*^1P = < 0.001$). Y-axis is log-scaled.

At the age of 75 dpf , mean VTG concentrations measured in homogenates of decapitated fish, indicated a concentration-dependent elevation of VTG in BPA treated fish in comparison to the controls (Fig. 9, legend). At 188 µg/l of BPA, the mean VTG homogenate concentration was 89.0 µg/ml (± 26.8 µg/ml) and reached 210 µg/ml (± 467.4 µg/ml) at the highest BPA concentration, whereas the control VTG concentration was 42.3 µg/ml (± 110.9 µg/ml). The increase in homogenate VTG in these 75-day old BPA exposure fish turned out to be significant towards the control fish already at the lowest BPA concentration of 94 µg/L.

The high degree of interindividual variation of VTG levels revealed to be even more pronounced than at the earlier developmental stage of 21 dpf. The categorization of vitellogenin concentrations in "low" (< 100 µg/ml) and "high"- (>100 µg/ml) responders (according to the 3.0 ng/L EE_2 study) blurred the impression of a concentration-dependent increase as it would be shown by the total mean vitellogenin contents per concentration

(Fig. 9, legend). All BPA "low"-responder groups (group 1) showed a considerable increase in mean body homogenate VTG concentrations compared to the control group 1. At exposure concentrations ≥ 188 µg BPA/L the elevation of vitellogenin was significant. The highest value of 78.8 µg VTG/ml (± 16.0 µg/ml) within this responder group 1, reached at 188 µg/L of BPA, was almost eight times higher than the corresponding control. In contrast to this, a BPA-induced elevation of VTG above the group 2 control level within the "high"-responders (group 2) was not observed. Although, the group 2 control was not a representative group, composed only of one fish. When alternatively compared with the average VTG concentration of both control groups, group 1 and group 2, together, VTG in the "high"-responder groups differed significantly from the control at concentrations from 375 µg BPA/L up (Fig. 9, legend).

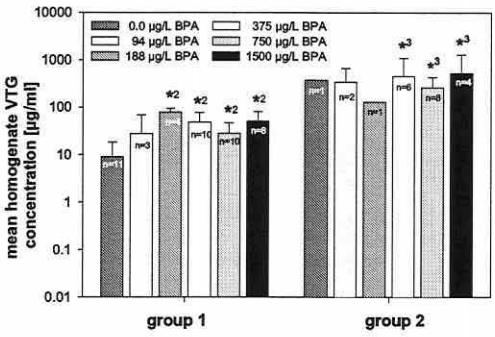

Fig 9. BPA experiment:
Vitellogenin concentrations in whole body homogenate samples of zebrafish on day 75 pf, chronically exposed to different concentrations of bisphenol A (BPA) (nominal: 94, 188, 375, 750 and 1500 µg/L). Values are means + S.D. Data of controls and of each BPA treatment were subdivided into two different groups (group 1 and group 2) as described in the text. Total mean values ± S.D. of controls/each treatment group were: 42.3 ± 110.9 µg/ml (control); 153.7 ± 235.7 µg/ml (94 µg BPA/L); 89.0 ± 26.8 µg/ml (188 µg BPA/L); 200.5 ± 418.9 µg/ml (375 µg BPA/L); 130.0 ± 161.2 µg/ml (750 µg BPA/L); 210.3 ± 467.4 µg/ml (1500 µg BPA/L). Number of samples (fish) analyzed (= n) are indicated in the bars. Asteriks (*) indicate mean values that were significantly different from the controls within one VTG induction group ($*^2P < 0.05$) or from the total control mean (control group 1 and 2 together) ($*^3P < 0.05$). Y-axis is log-scaled.

In adult reproducing zebrafish, vitellogenin was measured in plasma samples on day 110 pf, at the end of a fecundity assessment period of this BPA study (Fig. 10). Mean plasma VTG titers clearly indicated a positive concentration response to BPA exposure in males as well as in females. In males (Fig. 10a), only the mean VTG plasma titer of 0.265 µg/ml (± 0.63 µg/ml) in the control group deviated from the general concentration-response tendency and proved to be exceptionally high. In females (Fig. 10b), the 94 µg/L BPA exposure group deviated from the concentration-response tendency in that it showed a disproportionate elevation of mean VTG plasma concentration to 2842 µg/ml (± 1579 µg/ml). This was more than twice as high as the control, almost reaching the mean VTG plasma titer of the 375 µg/L BPA treatment group. In male zebrafish, the lowest observed

effect concentration (LOEC) of BPA that caused a significant elevation of plasma VTG above the control was at 375 µg/L BPA. At this exposure concentration, the mean VTG plasma titer amounted to 0.823 µg/ml (± 0.96 µg/ml), a three times higher level than measured in control male fish. The highest VTG plasma content of 230 µg/ml (± 354 µg/ml) in males was observed at the highest BPA exposure concentration of 1500 µg/L and corresponded to an almost 1000-fold increase of plasma VTG over the male control level. In female fish at the age of 110 dpf, the mean plasma VTG content at all BPA exposure concentrations was significantly higher than in unexposed females. Therefore, the lowest observed effect concentration (LOEC) of BPA for vitellogenin induction in adult female zebrafish was 94 µg/L. The highest mean plasma VTG titer reached was 5915 µg/ml ± 3290 µg/ml in the 1500 µg/L-BPA treatment group, representing a six-fold increase of plasma VTG level compared to adult control females. Generally, the mean plasma VTG concentrations of BPA exposed males were between 26-times (at 1500 µg BPA/L) and 370000-times (at 94 µg BPA/L) lower than in the corresponding females.

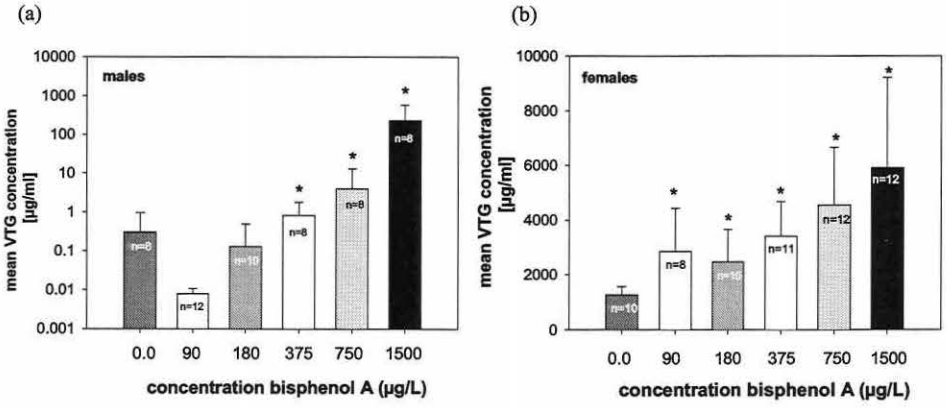

Fig 10. BPA experiment:
Vitellogenin (VTG) plasma concentrations in adult male (a) and female (b) zebrafish on day 110 pf, chronically exposed to different concentrations of bisphenol A (BPA) (nominal: 94, 188, 375, 750 and 1500 µg/L). Values are means + S.D. Number of samples (fish) analyzed (= n) are indicated in the bars.
Asteriks (*) indicate mean values of treatments that were significantly different from the control mean (*$P < 0.05$) .
Y-axis in Fig. (a) is log-scaled.

VTG was measured in different sample matrices, either in homogenates or in plasma. In order to facilitate a direct comparison of VTG levels throughout the developmental period, from 21-day-old to adult fish, we introduced the 'relative VTG induction' (i.e.,

ratio of normalized VTG sample concentration (µg/ml) to the corresponding mean VTG concentration of the control (µg/ml)). In the course of zebrafish development, the 'relative induction' of VTG increased four-fold between day 21 and day 75 pf in chronically to 3.0 ng EE_2/L exposed zebrafish (Fig. 11). The highest VTG relative induction of EE_2 exposure (110.7 ± 126.1) during development was reached after 75 days. In adult zebrafish (day 118 pf), exposure to 3.0 ng EE_2/L induced VTG still by more than 10 times above the control level in male zebrafish, but hardly caused an induction in female fish.

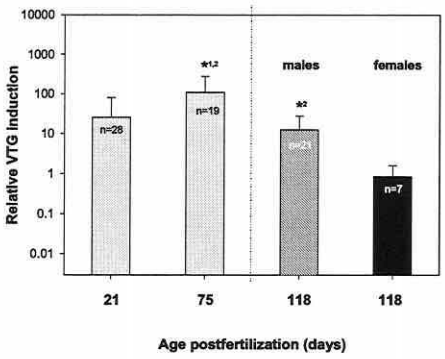

Fig 11. 3.0 ng/L EE_2 experiment: Relative vitellogenin induction in chronically to 3 ng/L of 17α-ethinylestradiol (EE_2)-exposed zebrafish after 21, 75 and 118 days pf. Relative VTG induction was calculated as the ratio of normalized VTG sample concentration (µg/ml) of individual fish to the normalized mean VTG concentration of the controls (µg/ml). Values are means of relative induction + S.D. Number of samples (fish) analyzed (= n) are indicated in the bars. Asteriks (*) indicate mean values that were significantly different from the control mean (*P = <0.001). Statistics were based on VTG concentration data, not on the relative VTG induction values. Y-axis is log-scaled.

Considering the 'relative VTG induction' in chronically BPA exposed zebrafish in relation to the course of development and duration of exposure (Fig. 12), the applied concentrations of BPA result in a significant induction of vitellogenesis for the first time in 75 d-old fish. The relative induction of VTG by chronic BPA exposure in 75-day old fish was 2.7 times (in the 94 µg BPA/L treatment group) to 7.5 times (in the 1500 µg BPA/L treatment group) higher than in the younger fishes. In 75-day old zebrafish, all BPA concentrations tested, significantly increased VTG over the control levels. The induction values varied between 2.1 ± 0.47 at 188 µg/L of BPA and 5.0 ± 5.8 at the highest BPA concentration. However, the relative VTG induction was not directly correlated with the BPA concentration applied. Comparable or even lower levels of vitellogenin augmentation were observed in adult females on day 110 pf. All BPA exposed female zebrafish on average reached significant elevated levels of VTG, and a slight concentration-dependency was indicated. The relative VTG induction in females, however, did never exceed 5.0. In the highest exposure concentration of BPA, females showed a maximum level of VTG induction of 4.7 ± 2.0. In comparison to the chronically EE_2 treated female fish, the BPA-

caused relative induction of plasma VTG in females was even at the lowest BPA concentration higher than caused by EE_2. On the contrary to the results of females, the VTG induction in adult male zebrafish was positively correlated with the BPA concentration. At an exposure concentration of 375 µg/L of BPA the LOEC was reached for the VTG induction in male zebrafish, and the relative VTG induction increased greatly to almost 1000 (867 ± 903) in the highest BPA concentration. At the highest BPA concentration of 1500 µg/L, the relative induction of plasma VTG in adult males was more than 180-times higher than observed in females. The relative plasma VTG induction in chronically EE_2 exposed male fish was just above 10, and compared with the BPA-exposed fish, the relative induction capacity at 750 µg/L approximately equaled this value.

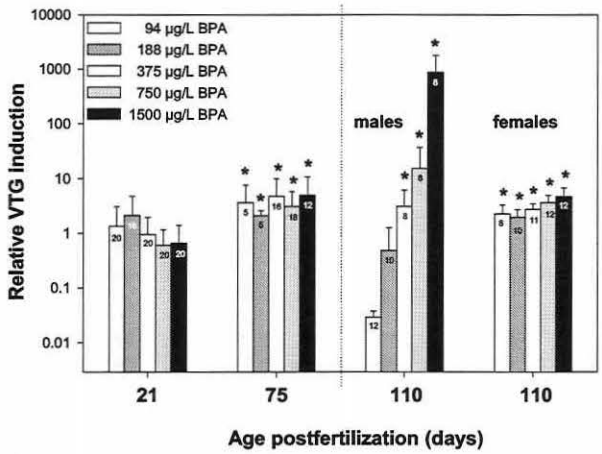

Fig 12. BPA experiment:
Relative vitellogenin induction in chronically to different concentrations of bisphenol A (BPA) (nominal: 94, 188, 375, 750 and 1500 µg/L) exposed zebrafish after 21, 75 and 110 days pf. Relative VTG induction was calculated as the ratio of normalized VTG sample concentration (µg/ml) of individual fish to the normalized mean VTG concentration of the controls (µg/ml). Values are means of relative induction + S.D. Number of samples (fish) analyzed (= n) are indicated in the bars. Asteriks (*) indicate mean values that were significantly different from the corresponding control mean for the same sampling time (*$P < 0.05$). Statistics were based on VTG concentration data, not on the relative VTG induction values. Y-axis is log-scaled.

The 'relative induction of VTG' in adult zebrafish of the 3.0 ng EE_2/L-exposure was also investigated in relation to the duration and the developmental stage of exposure (Fig. 13). Significant induction levels were only reached by females or by chronically exposed males. Transiently at the early life-stage exposed male zebrafish (Fig. 13a) did show relative induction levels > 1.0 still on day 118 pf. Although, this induction level of 1.0 could not be exceeded any more on day 176 pf, when fish were already out of exposure for at least 134 days. Even the 125-day-exposed males showed no longer an induction of

vitellogenesis. Unlike in males, a significant relative induction of vitellogenin in female fish (Fig. 13b) was caused only by early-life EE_2 exposure but not by chronic or long-term exposure (until day 125 pf). The relative VTG induction in early life-stage EE_2 exposed females reached rather consistently 2.7 ± 0.99 on day 118 pf (at the end of the fecundity period). A prolonged exposure recovery time until day 176 pf, further reduced the VTG induction, dependent on the duration of exposure. Only a 42-day pf exposure to EE_2 still resulted in a significantly increased relative induction of VTG (1.82 ± 0.55) in 176-day old female zebrafish.

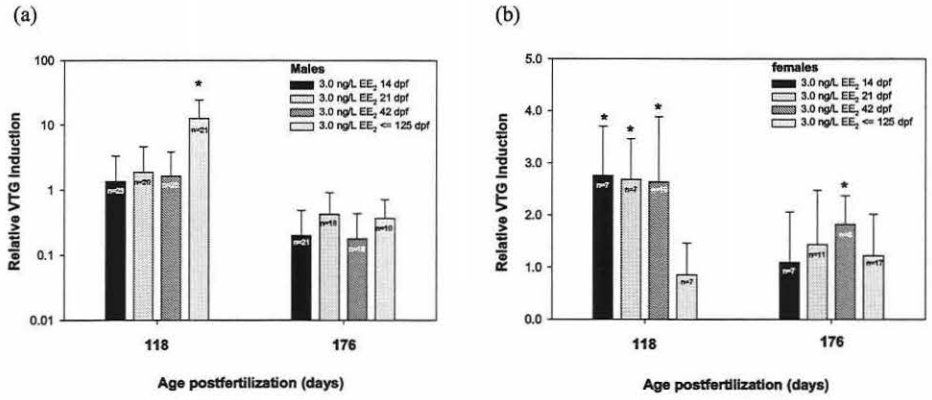

Fig 13. 3.0 ng/L EE_2 experiment:
Relative vitellogenin (VTG) induction in adult male (a) and female (b) zebrafish on day 118 pf and 176 pf, exposed to 3 ng/L of 17α-ethinylestradiol (EE_2) for different time periods. Relative VTG induction was calculated as the ratio of normalized VTG sample concentration (µg/ml) of individual fish to the normalized mean VTG concentration of the control (µg/ml). Values are means of relative induction + S.D. Number of samples (fish) analyzed (= n) are indicated in the bars. Asteriks (*) indicate mean values that were significantly different from the corresponding control mean for the same sampling time (*$P < 0.05$). Statistics were based on VTG concentration data, not on the relative VTG induction values. Y-axes are log-scaled.

Effects of EE_2 and BPA on survival, growth and reproductive success

EE_2 exposure:

Embryo hatchability (data not presented) and survival (Table.1) of the early life stages of all EE_2 treatment groups were not affected by 3.0 ng/L. Survival of exposed fish in the vessels varied between 50 % and 82 % within the first 28 days post fertilization (dpf). With 78 % ± 5.66 % the highest survival rate of all exposure and the control groups was recorded in the chronic exposure group.

Table 1: Effects of 3.0 ng/L EE_2 on survival and juvenile growth in the F_0 generation

		Exposure time post fertilization (days)									
		0		14		21		42		≤ 118	
	Vessel	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
Day 28 pf	Survival (%)	68	58	70	79	50	76	76	51	82	74
	Mean (%) ± SD	63 ± 7.07		74.5 ± 6.36		63 ± 18.39		63.5 ± 17.68		78 ± 5.66	
	Length (mm) ± SD	7.44 ± 2.49	6.80 ± 2.56	7.79 ± 2.42	7.61 ± 2.23	*** 8.82 ± 2.56	7.45 ± 2.32	7.40 ± 2.41	*** 8.35 ± 2.21	*** 8.00 ± 2.24	*** 8.19 ± 2.39
	Mean length (mm) ± SD	7.15 ± 2.53		7.69 ± 2.31		8.00*** ± 2.50		7.78*** ± 2.37		8.09*** ± 2.31	
Day 42 pf	Survival (%)	58	58	60	57	60	59	60	60	60	59
	Mean ± SD	58 ± 0.00		58.5 ± 2.12		59.5 ± 0.71		60 ± 0.00		59.5 ± 0.71	
	Length (mm) ± SD	13.11 ± 3.43	12.98 ± 3.55	11.90* ± 3.29	12.39 ± 2.77	12.25 ± 3.19	11.94* ± 3.11	13.09 ± 3.80	12.47 ± 3.35	12.00* ± 2.65	12.81 ± 3.05
	Mean length (mm) ± SD	13.04 ± 3.48		12.14* ± 3.04		12.10* ± 3.14		12.78 ± 3.58		12.40 ± 2.87	
Day 75 pf	Length (mm) ± SD	26,85 ± 3,81	27,70 ± 3,05	25,31* ± 3,07	24,95* ± 3,13	26,38 ± 3,78	25,31* ± 3,38	26,25 ± 3,43	26,60 ± 3,36	25,96 ± 3,45	25,66 ± 3,47
	Mean length (mm) ± SD	26,50 ± 3,47		25,14* ± 3,09		25,86 ± 3,61		26,42 ± 3,38		25,81 ± 3,45	
Day 28–75 pf	Juvenile growth (mean increase of body length) (mm) ± SD	19.35 ± 0.06		17.45 ± 0.13***		17.86 ± 0.2***		18.64 ± 0.42		17.71 ± 0.35***	

Based on data recorded by Fraunhofer IME, Schmallenberg, Germany.
Statistics: * = significantly different from the control (t-test)
** = significantly different from the control (ANOVA followed by Dunett's post hoc comparison; P<0.05)
*** = significantly different from the control (ANOVA + t-test)

On day 28 pf, the 42-day exposure group was still under exposure conditions like the chronic exposure group. The mean survival of 64 % (± 17.7 %) in this group, however, was lower than in the chronic exposure group. On day 42 pf, survival stabilized between 58 % in the controls and 60 % in the exposure groups.

The increase of mean body length of fish within the first 28 days was higher in those treatment groups that were exposed for 21 dpf or were still under exposure to EE_2. Between day 28 and 75 pf, juvenile growth was significantly lower in all exposure groups compared with the control, except for the 42- day-exposure group.

On day 75 pf, the mean body length of fish in the EE_2 treatment groups, however, did not differ significantly from the controls. Only in the 14-day-exposure group, mean fish length was almost 20 % lower than recorded in the controls (25.14 mm ± 3,09 mm).

The mean fertilization success of mature fish between day 92 and day 114 pf (during the period of reproductive assessment) (Fig. 14) was not affected by temporary exposure to EE_2 during early life (until day 42 pf). Fertilization remained in all temporary exposure groups above 85 %. In chronically EE_2-exposed fish, however, no fertilization of eggs could be measured during this period because these fishes did not spawn at all as long as the exposure was continuing. However, when the fishes were transferred into uncontaminated, EE_2-free water for almost two months (between day 125 and day 176 pf), a partial recovery of courtship and mating behavior was observed. The first spawning success was recorded on day 130 pf. This corresponds to a delay in the onset of spawning in chronically EE_2-exposed fish of at least six weeks, in comparison to the temporary early-life exposure fish. Recovery of reproductive parameters was quantified between day 148 and day 175 pf (Fig. 15). At the end of the study on day 176 pf, the mean fertilization of spawned eggs exceeded 30 %.

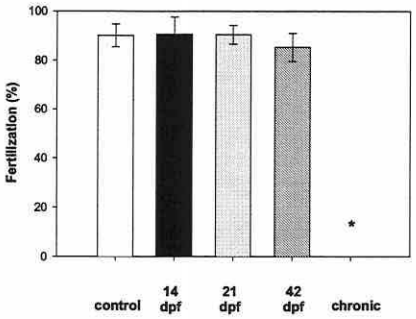

Fig 14. 3.0 ng/L EE_2 experiment:
Mean proportion of fertilized eggs (expressed as percentage) between day 91 and day 114 pf in zebrafish treatment groups, exposed to 3 ng/L of 17α-ethinylestradiol (EE_2) for different time periods. Values are means (%) ± S.D. Asteriks (*) indicate mean values that were significantly different from the control mean (*P = <0.001). Based on data recorded by Fraunhofer IME, Schmallenberg, Germany.

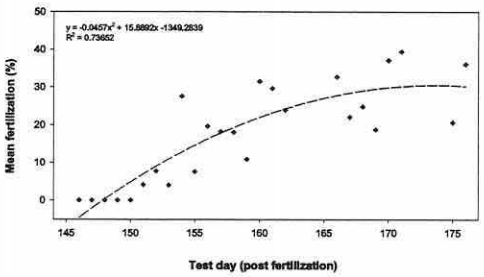

Fig 15. 3.0 ng/L EE_2 experiment:
Observation of recovery of reproductive capacity of zebrafish, chronically exposed to 3 ng/L of 17α-ethinylestradiol (EE_2) until day 125 pf, expressed as the time-dependent increase of the proportion of fertilized eggs (expressed as percentage) between day 146 and day 176 pf. Based on data recorded by Fraunhofer IME, Schmallenberg, Germany.

The first spawning success in all other exposure replicates was observed around day 83 pf, directly following the control replicates. The very first spawning days, slightly reduced numbers of spawned eggs and poor fertilization rates occurred in the short-term exposure groups (general observations, data not shown). These differences to the control, however, were compensated within a few days. During the assessment of reproductive parameters between day 92 and day 114 pf, the calculated mean number of fertilized eggs spawned by

each female that was exposed for 21 dpf (Fig. 16), was significantly higher ($P<0.001$) than in all other test treatments. Though, this was due to the exceptionally high number of daily spawned eggs in one of the two replicate vessels (data not shown). Apart from this exception, the number of spawned eggs as well as the proportion of fertilized eggs in the temporary early-life treatments did not differ from the control. The significantly higher number of eggs in the 21-day exposure group therefore can not be referred to as an EE_2 exposure related positive effect on fecundity.

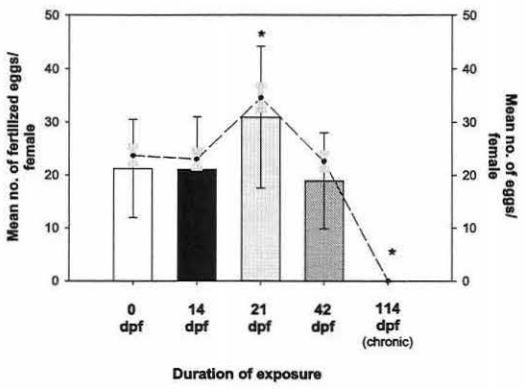

Fig 16. 3.0 ng/L EE_2 experiment: Mean number of fertilized (bars)/spawned (dashed line) eggs per spawning day between day 91 and day 114 pf in zebrafish treatment groups, exposed to 3 ng/L of 17α-ethinylestradiol (EE_2) for different time periods. Values are means ± S.D.Asteriks (*) indicate mean values that were significantly different from the control mean (*P = <0.001). Based on data recorded by Fraunhofer IME, Schmallenberg, Germany.

BPA-exposure:

No significant effects of BPA exposure on fish survival and growth were observed in any of the applied test concentrations (Table 2) during early life stage and juvenile exposure. Due to unknown reasons, unexpectedly high mortality was observed in all exposure groups and in the control groups during the first 28 days post fertilization. Mean survival rates were between 38 % ± 12.7 % in the 188 µg/L and 59 % ± 4.2 % in the 750 µg/L BPA group. On day 42 pf, survival of fish in the 94 µg BPA/L- and the 188 µg BPA/L-treatment groups was significantly reduced to 34 % and 36.5 %, respectively versus the control.

A delay in the onset of spawning was statistically not verified as only one parallel of the two highest BPA-concentration of 750 µg/L and 1500 µg/L each, laged several days behind in time of first spawning. In 75 % of all test vessels, including control, fish simultaneously started with their spawning activity around day 77 pf. During the quantification of reproductive performance between day 89 and day 110 pf, the fertilization of daily spawned eggs of chronically BPA treated fish was significantly

reduced at an exposure concentration of ≥ 375 µg/L (Fig. 17). While the fertilization success was already affected by 375 µg/L of BPA, the egg production (fecundity; mean number of spawned eggs per day and female, Fig. 18) was significantly reduced only at the highest BPA concentration of 1500 µg/L. The significant reduction of the mean number of daily spawned egg per female, as recorded in the 94 µg BPA/L-exposure group, has not to be attributed necessarily to the BPA treatment. The total mean number of eggs spawned per female (data not shown) at least did not indicate any significant BPA-exposure derived effect on the fecundity.

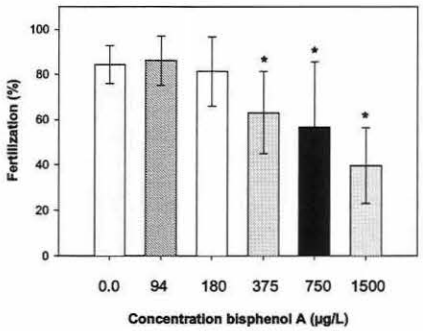

Fig 17. BPA experiment:
Mean proportion of fertilized eggs (expressed as percentage) between day 89 and day 110 pf in zebrafish treatment groups, exposed to different concentrations of bisphenol A (BPA) (nominal: 94, 188, 375, 750 and 1500 µg/L). Values are means (%) ± S.D. Asteriks (*) indicate mean values that were significantly different from the control mean (*P = <0.01). Based on data recorded by Fraunhofer IME, Schmallenberg, Germany.

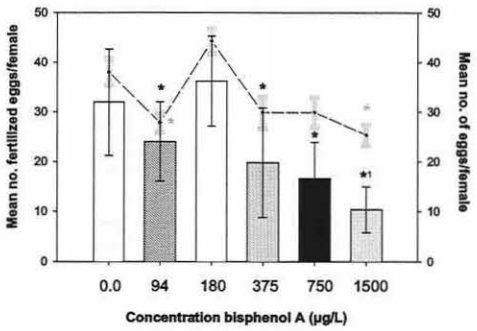

Fig 18. BPA experiment:
Mean number of fertilized (bars)/spawned (dashed line) eggs per spawning day between day 89 and day 110 pf in zebrafish treatment groups, exposed to different concentrations of bisphenol A (BPA) (nominal: 94, 188, 375, 750 and 1500 µg/L). Values are means ± S.D. Asteriks (*) indicate mean values that were significantly different from the control mean (*P < 0.05; *[1]P = <0.01). Based on data recorded by Fraunhofer IME, Schmallenberg, Germany.

Table 2: Effects of BPA on survival and juvenile growth in the F_0 generation

		Nominal exposure concentrations of BPA (µg/L)											
		0		94		188		375		750		1500	
	Vessel	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2	5/1	5/2
Day 28 pf	Survival (%)	51	44	39	45	29	47	68	40	56	62	54	59
	Mean (%) ± SD	48 ± 4.9		42 ± 4.2		38 ± 12.7***		54 ± 19.8		59 ± 4.2		57 ± 3.5	
	Length (mm) ± SD	7.97 ± 2.19	8.10 ± 1.74	8.34 ± 2.38	7.45 ± 2.08	8.81 ± 2.52	7.76 ± 1.87	8.01 ± 1.86	*** 9.02 ± 1.71	7.85 ± 2.22	8.33 ± 1.94	8.23 ± 1.87	7.70 ± 2.20
	Mean length (mm) ± SD	8.03 ± 1.98		7.87 ± 2.26		8.16 ± 2.18		8.38 ± 1.86		8.10 ± 2.08		7.95 ± 2.06	
Day 42 pf	Survival (%)	42	44	34	34	37	36	44	42	43	43	43	43
	Mean ± SD	43 ± 1.41		34 ± 0.0 ***		36.5 ± 0.71**		43 ± 1.41		43 ± 0.0		43 ± 0.0	
	Length (mm) ± SD	13.55 ± 2.58	12.80 ± 2.61	14.55 ± 3.11 ***	13.45 ± 3.58	14.79 ± 3.66 ***	13.18 ± 3.70	13.47 ± 2.86	13.95± 2.89	13.10 ± 3.42	13.66 ± 2.92	13.28 ± 2.89	12.66 ± 3.98
	Mean length (mm) ± SD	13.17 ± 2.61		14.00 ± 3.37		14.00 ± 3.74		13.70 ± 2.87		13.38 ± 3.17		12.97 ± 3.47	
Day 75 pf	Length (mm) ± SD	24.17 ± 2.43	22.40 ± 3.00	22.31 ± 3.05	24.99 ± 3.08 ***	22.85 ± 2.60	23.65 ± 3.00	23.47 ± 2.19	23.54 ± 2.50	21.66 ± 4.12 ***	23.09 ± 2.88	23.33 ± 2.29	22.71 ± 3.41
	Mean length (mm) ± SD	23.28 ± 1.25		23.65 ± 1.90		23.25 ± 0.57		23.51 ± 0.05		22.37 ± 1.01		23.02 ± 0.44	
Day 28–75 pf	Juvenile growth (increase of body length) (mm) ± SD	15.25 ± 1.34		15.75 ± 2.53		14.97 ± 1.31		14.99 ± 0.66		14.28 ± 0.67		15.05 ± 0.07	

Based on data recorded by Fraunhofer IME, Schmallenberg, Germany.
Statistics:** = (ANOVA followed by Dunett's post hoc comparison; P<0.05)
*** = significantly different from the control (ANOVA + t-test)

Correlation between VTG and reproductive parameters in BPA exposed fish

The LOEC of 375 µg/L BPA determined for VTG induction in male fish on day 110 pf completely agreed with the LOEC for the fertilization capacity in these fish (Fig. 17). Thus, the effective concentration (LOEC) of 94 µg/l of BPA for VTG induction in females showed good agreement with the LOEC for a reduction of the mean number of fertilized eggs per day and female, recorded between day 89 and day 110 pf (Fig. 18).

Mean VTG plasma titers in adults (110 dpf) showed a negative correlation with the number of fertilized eggs per female as well as with the mean fertilization capacity (VTG ↔ fecundity-fertility) found in each BPA-concentration parallel (Fig 19a,b). A significant relationship (Fig. 19a) between the mean plasma VTG titers in all applied exposure concentrations of BPA and the corresponding mean fertilization level was found and could be described by linear regression using log transformed VTG concentration data. Both parameters were highly correlated in male (R_{male}= 0.942; P_{male}= 0.0049) as well as female fish (R_{female}= 0.850; P_{female}= 0.032). Furthermore, the concentration-dependent increase of VTG after BPA exposure (Fig. 19b) was significantly related to the number of fertilized eggs per female (in each of the BPA-concentration test group) whereas the correlation with males was, as expected, inferior (R_{male}= 0.717; P_{male}= 0.109) to those observed for female fish (R_{female}= 0.852; P_{female}= 0.031). The fecundity of female fish is only indirectly influenced by male fertility and mating behavior.

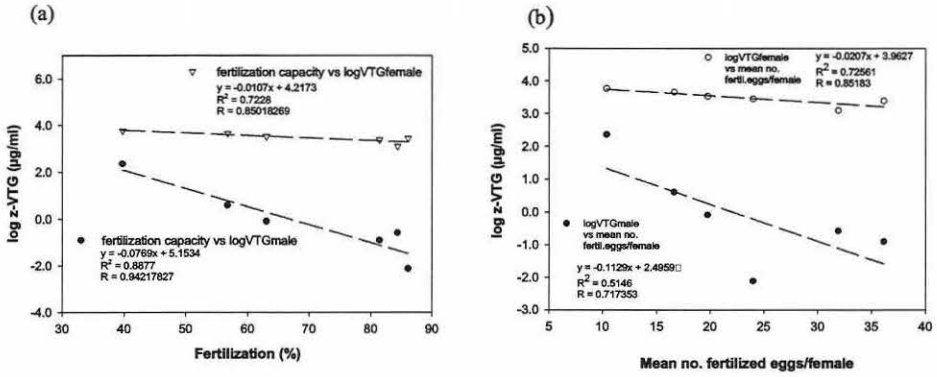

Fig 19. BPA experiment:
Relationship between the mean proportion of daily fertilized eggs (fertilization capacity) (a) or the mean number of daily fertilized eggs per spawning female (b) and the mean plasma VTG titers of zebrafish, after 110 days of exposure pf to different concentrations of bisphenol A (BPA) (nominal: 94, 188, 375, 750 and 1500 µg/L). Values are means of pooled data of each BPA treatment and the control. Fecundity (number of spawned eggs) and fertility was assessed between day 89 and day 110 pf (n=42; 21 spawning days × 2 treatment parallels). VTG values were pooled data of n females/males in each treatment or the control (n =8-12). Log-transformed VTG titers in adult, BPA exposed zebrafish were correlated with the reproductive parameters fertilization capacity and number of daily fertilized eggs/female by linear regression. (a): P_{male} = 0.005; P_{female} = 0.032; α 0.05. (b): $P_{mal\ e}$= 0.109; P_{female} = 0.031; α = 0.05. zVTG = zebrafish vitellogenin; vs = versus.
Based on data recorded by Fraunhofer IME, Schmallenberg, Germany.

Hatching, survival and early-life development of zebrafish larvae was recorded in the following F_1 generation (Table 3). A BPA exposure dependent effect could not be observed on the hatchability of these larvae on day six pf. Hatching ranged between 81 % (± 11.7 %) in the 750 µg/L BPA exposure group and 94.1 % (± 1.4 %) in the 188 µg/L BPA group (Table 2). Survival recorded on day 35 pf, at the end of the early life stage test, ranged widely between the vessels, from 27 % in the controls to 72 % in the 750 µg/L concentration. However, this high variability could not be proved as a BPA derived effect.

Table 3: Effects of BPA on hatching, survival and growth in the early life stage of the F_1 generation (F_0 offspring)

	Nominal exposure concentrations of BPA (µg/L)											
	0		**94**		**188**		**375**		**750**		**1500**	
Vessel	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2	5/1	5/2
Hatch-ability (day 6 pf) %	93.2	90.2	93.1	87.1	93.1	95.1	89.1	** 72.5	90.2	80.4	** 73.1	92.2
Mean (%) ± SD	91.7 ± 2.1		90.1 ± 4.2		94.1 ± 1.4		80.8 ± 11.7**		85.3 ± 6.9		82.7 ± 13.5**	
Survival after 35 dpf (%)	27.1	66.3	36.8	38.6	28.7	49.5	44.4	51.4	45.7	72.0	40.8	70.5
Mean (%) ± SD	46.7 ± 27.7		37.7 ± 1.3**		39.1 ± 14.7		47.9 ± 4.9		58.8 ± 18.6**		55.7 ± 21.0	
Total weight (g)	0.94	1.81	2.07	1.16	1.41	2.11	2.20	2.38	2.80	2.14	2.39	2.09
Calculated average weight/fish (g) ± SD (35 dpf)	0.033 ± 0.005		0.047 ± 0.018		0.048 ± 0.006		(***) 0.059 ± 0.006		(***) 0.051 ± 0.022		(***) 0.054 ± 0.032	

Based on data recorded by Fraunhofer IME, Schmallenberg, Germany.
Statistics:** = (ANOVA followed by Dunett's post hoc comparison; P<0.05)
*** = significantly different from the control (ANOVA + t-test)

Effects of EE_2 and BPA on gonad histology

EE_2 exposure:

Gonad histology of EE_2-exposed test fish was investigated in detail after 75 days, 118 days and 176 days pf. Determination of sex, based on the appearance of external secondary sexual characteristics (e.g. intensity of coloration or shape) or by the macroscopic gonad structure, however, could not be performed for certain until 75 pf. The histological analysis of gonads was included in this study as an additional parameter for the evaluation of exposure effects on gonad development and differentiation and it was a valuable tool to partially overcome the problem of sexing fish. For a detailed description of the gonad histology and pathology of fish from these studies refer to Maack (2002).

At all three sampling dates, histological analysis of gonad structure of control fish revealed either complete differentiation of germ cells into fully developed testes or ovary (on day 118 and day 176 pf respectively) or a high proportion of germ cells in the ongoing process of differentiation was observed. The stage of differentiation reached by most of the germ cells allowed a classification of the corresponding gonad to testis or ovary in most cases. Except if a gonad was composed of ovarian-like as well as testis-like germ cells, it was referred to an 'undefined' gonad type. Determination of sex, based on the appearance of external secondary sexual characteristics (e.g. intensity of coloration or shape) or by the macroscopic gonad structure, however, could not be performed for certain until 75 pf.

Table 4: Gonadal sex in 3.0 ng/L of EE_2 exposed zebrafish on day 75 pf

	Gonad type						
Duration of EE_2 exposure (dpf)	**Testis**		**Ovary**		**Undefined**		**Total no. of Individuals (n)**
	n	%	n	%	n	%	
0 (control)	10	41.7	14	58.3	0	0.0	24
21	10	35.0	17	59.0	2	6.0	29
42	9	37.5	15	62.5	0	0.0	24
75	0	0.0	20	100***	0	0.0	20

Data from histological analysis, performed by G. Maack, UFZ-Leipzig
Statistics: *** = significantly different from the control (χ2-test)

On day 75 pf, the proportion of fish with gonad structures, containing male germ cell types varied between 42 % in the controls and 0.0 % in the chronically EE_2 exposed fish (Table 4). Permanently exposed zebrafish in this study did only show female type gonads, predominantly composed of early developmental stage oocyte types (Maack 2002). In all temporarily exposed groups the ratio of male to female fish was between 40 % (± 3.4 %) and 60 % (± 2.3 %). Two fish in the 21-day exposure group could not unambiguously be sexed since a few oocytes were found within their otherwise unaltered testicular tissue. These results indicate that only by chronic treatment with EE_2, the ratio of fishes containing ovaries or testes shifted to female-type gonads.

In adult fish on day 118 pf, the gonadal sexes, determined by histology, showed an almost 50 % male-to-female ratio in the controls (Fig. 20a). With the duration of EE_2 exposure, the proportion of fish with female gonads increased. In fish that had been exposed for the first 42 days after fertilization, the proportion of female-gonad fish reached 57 % and elevated to almost 100 % in fish exposed chronically to EE_2. Only in one fish, some male germ cells occurred within the ovarian-like tissue.

In this study, gonad histological sex of one fish often appeared to differ from the sex as determined on the basis of 1) external morphological characters and/or the macroscopic gonad structure or 2) the plasma VTG level. As already described in 'material and methods' (VTG measurement), VTG (homogenate or plasma) titers of pubescent or adult zebrafish were usually characterized by a dimorphic pattern that allows to differentiate between the sexes due to the individual VTG level. Since pubescent or adult male zebrafish with elevated VTG levels due to estrogen exposure, never reached comparable levels of plasma VTG as usually measured in non-induced control females, it could unambiguously be differentiated between the sexes. Compared with the histological results, the proportion of fish showing male levels of plasma VTG, was generally higher in all EE_2 treatment groups and in the controls (Fig. 20a). Independently of the EE_2-treatment, the proportion of males ranged between 62 % in the controls and 77 % in the 14-day exposure group. Similar to the "vitellogenic" sex-ratio, a male-biased sex-ratio (> 60 % males) was found in temporarily early-life exposed fish, determined by their external appearance and additionally by the macroscopic structure of dissected gonads. In the 14-day and 21-day treatment groups the shift to more male fish was even significant versus the control group. The proportion of morphological males, however, dropped to 14.3 % in the chronic exposure group. Over 50 % of these permanently EE_2-treated fish did not

display unequivocally external sexual characteristics, like the characteristic rounded body shape of females, nor did they show clearly defined gonad types. These fish were defined as undistinguishable. 'Morphological' sex ratio in control fish of 40 % females to 60 % of males was approximately the opposite of the control VTG sex ratio.

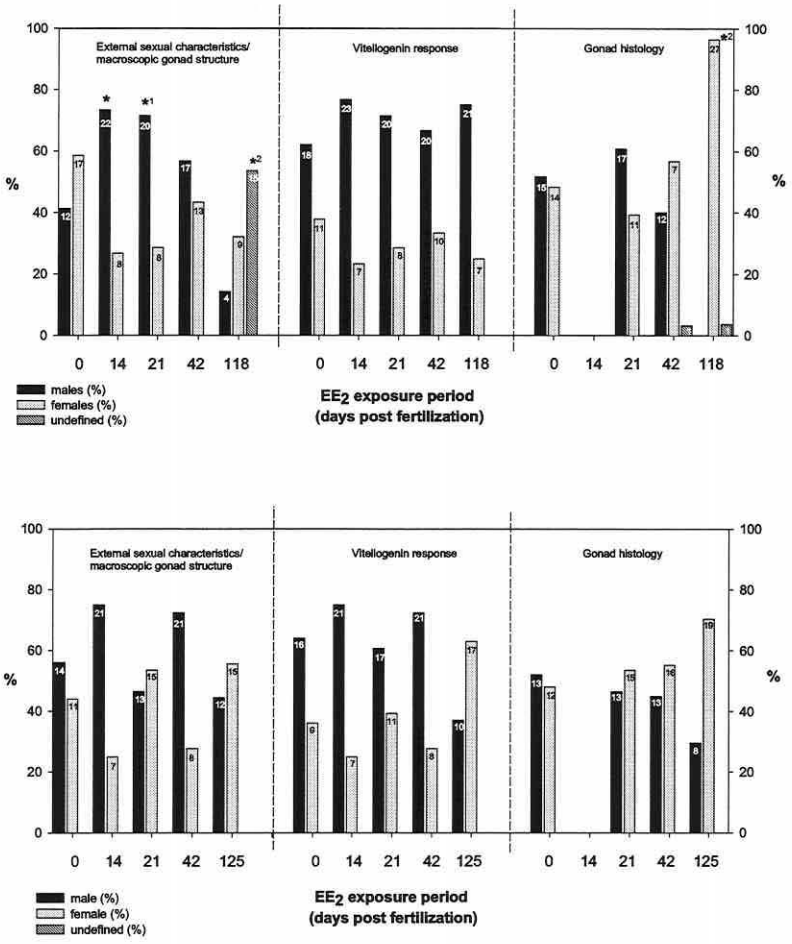

Fig 20. 3.0 ng/L EE_2 experiment:
Sex-ratio of adult zebrafish on day 118 pf (a)and 176 pf (b), exposed to 3 ng/L of 17α-ethinylestradiol (EE_2) for different periods of time. Proportions (expressed as percentage) of males and females were established on the basis of: 1) external sexual characteristics and gonad structure (left half of figure) or 2) vitellogenin response (middle of figure) or 3) gonad histology (right half of figure) (data adopted from Maack 2002). Number of samples (fish) analyzed (= n) are indicated in the bars. The three different approaches of sex determination were carried out with the same fish. 'Undefined' denotes fish that could not unambiguously be identified as male or female. Asteriks (*) indicate sex-ratios that were significantly different from the control ratio (*$P = 0.019$; *$^1P = 0.032$; *$^2P = <0.001$) (χ^2-test).

The same comparison of sex ratios was performed with the fishes that were already removed from the treatment for more than 50 days. The sex ratios of these 176 day-old fish were still different (Fig. 20b) Gonad histology still recorded an exposure time-related slight increase in the proportion of females compared to the control. In controls, the proportion of sexes was almost 1:1. Vitellogenin and morphology indicated male-biased sex ratios in the control and in the temporary treatment groups, whereas in the chronic treatment more females than males were found. At this date, the external morphological characteristics were more distinct than on day 118 and the sampled fish could be sexed easier by their morphological appearance. Therefore, the differences between the 'vitellogenic' and the 'morphological' sex-ratios were smaller than before on day 118 pf.

BPA exposure:

Effects of long-term exposure to BPA post fertilization on gonad structure was investigated histologically after 75 days and 110 days. Samples taken from the two lowest BPA concentrations of 94 µg/L and 188 µg/L were not analyzed due to technical problems occurring during fixation. In all BPA treatment groups on day 75 pf or day 110 pf respectively, fish possessed either fully differentiated testes and ovaries or an 'undefined' gonad type.

Table 5: Gonadal sex in BPA exposed zebrafish on day 75 pf

BPA concentration (µg/L) (nominal)	Gonad type: Testis n	Testis %	Ovary n	Ovary %	Undefined n	Undefined %	Total no. of individuals (n)
0.0 (control)	3	37.5	5	62.5	0	0.0	8
375.0	3	21.4	11	78.6	0	0.0	14
750.0	3	23.0	10	77.0	0	0.0	13
1500.0	1	8.0	12	92.0***	0	0.0	13

Data from histological analysis, performed by G. Maack, UFZ-Leipzig
Statistics: *** = significantly different from the control (χ2-test)

Thereby, the proportion of testicular gonads in 75-day old fish appeared to be negatively correlated with increasing BPA concentrations. However, only in the highest BPA concentration of 1500 µg/L, this "feminizing" tendency was significant compared to the controls. In the unexposed fish, the proportion of testes were 38 % whereas in the highest BPA concentration this proportion was reduced to 7.7 %.

Similarly, ovary-skewed gonadal sex ratios were found also in adult 110 day-old fish (Fig. 21). Compared with the younger fish on day 75, no BPA exposure-related effect on sex-ratios were observed. The proportion of female fish did slightly increase from 67 % in the controls to 80 % at 750 µg/L and 77 % at 1500 µg/L of BPA, though.

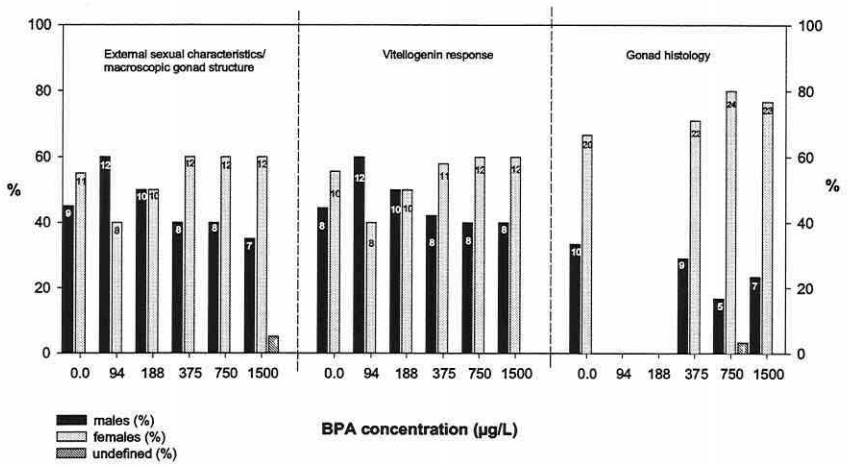

Fig 21. BPA experiment:
Sex-ratio of adult zebrafish on day 110 pf, chronically exposed to different concentrations of bisphenol A (BPA) (nominal: 94, 188, 375, 750 and 1500 µg/L). Proportions (expressed as percentage) of males and females were established on the basis of: 1) external sexual characteristics and gonad structure (left half of figure) or 2) vitellogenin response (middle of figure) or 3) gonad histology (right half of figure) (data adopted from Maack 2002). Number of samples (fish) analyzed (= n) are indicated in the bars. The three different approaches of sex determination were not carried out with the same fish. 'Undefined' denotes fish that could not unambiguously be identified as male or female. Sex-ratios were not significantly different from the control ratio (χ^2-test).

While in EE_2-exposed fish gonad histology and VTG were analyzed in the identical animals, in the BPA study, the two parameters were measured in two individuals. This means that fishes, analyzed for VTG, were not the same as were histologically examined. Nevertheless, the sex-ratio of fish, corresponding to the vitellogenin plasma level, was opposed to the histological sex ratios (Fig. 21). The VTG-measured fish were sexed beforehand during sampling by their external morphology and/or by the macroscopic structure of dissected gonads. The 'morphological' and the "vitellogenic" sexes were equivalent. At the three highest BPA concentrations and in the controls, the male-to-female ratios were almost 40 % (± 3.0 %) to 60 % (± 2.0 %). At 94 µg/L of BPA however, 60 % of fish were male and at 188 µg/L of BPA there was equality of sexes. Compared with the histological data, the proportion of vitellogenic and morphologic female fish was on the average 20 % lower than those identified by gonad histology.

Table 6: Summary of life-cycle effects in zebrafish exposed to EE_2 and BPA

		Parameter affected by 3.0 ng EE_2/L exposure (+)									
		Vitellogenin		Fertilization		No. of daily fertilized eggs		No. of daily eggs		Gonadal feminization (histology)	
Sampling days (pf)	Exposure days (pf)	M or (gr.1)	F or (gr.2)	M or (gr.1)	F or (gr.2)	M or (gr.1)	F or (gr.2)	M or (gr.1)	F or (gr.2)	M or (gr.1)	F or (gr.2)
21	21	-	+	-	-	-	-	-	-	-	-
42	42	-	+	-	-	-	-	-	-	-	-
75	75	+	+	-	-	-	-	-	-	+	-
118 (92-114 fecundity)	14	-	+	-	-	-	-	-	-	-	-
	21	-	+	-	-	+	+	-	-	-	-
	42	-	+	-	-	-	-	-	-	-	-
	≤ 118	+	-	+	+	+	+	-	-	+	-
176	14	-	-	-	-	-	-	-	-	-	-
	21	-	-	-	-	-	-	-	-	-	-
	42	-	+	-	-	-	-	-	-	-	-
	125	-	-	-	-	-	-	-	-	(+)	-

		Parameter affected by BPA exposure (µg/L)									
		Vitellogenin		Fertilization		No. of daily fertilized eggs		No. of daily eggs		Gonadal feminization (histology)	
Sampling days (dpf)	Exposure days (pf)	M or (gr.1)	F or (gr.2)	M or (gr.1)	F or (gr.2)	M or (gr.1)	F or gr.2)	M or (gr.1)	F or (gr.2)	M or (gr.1)	F or (gr.2)
21	21	-	-	-	-	-	-	-	-	-	-
75	75	188	375	-	-	-	-	-	-	1500	-
110	110	375	94							-	(1500)
89-110	110	-	-	375	375	375	375	-	1500	-	-

M denotes 'male' and **F** denotes 'female'
(gr.1) / (gr.2) indicate the "low"- and "high"- VTG-responder groups 1 and 2 (for definition see 'Results')

Discussion

Vitellogenin response and reproduction

As one central feature of reproductive success, fecundity (number of laid eggs) serves as an estimate of estrogen-exposure derived impairments of reproductive fitness. Fecundity in fish is considered to be a key parameter for the survival of fish populations (Arcand-Hoy and Benson 1998). The number of laid eggs could either be affected by estrogenic influences on oocyte development and egg production in females or, indirectly, by estrogen exposure-induced changes in mating behavior and/or pheromone release of males. The fertilization of eggs, however, depends on male as well as on female fertility. Effects on the reproductive output, as evaluated in our studies by the mean fertilization success and by the mean number of fertilized eggs per female and day, are the most relevant endpoints in the assessment of endocrine disruption and they define the ecological significance of such incidents. However, such reproductive parameters, like other organismic endpoints as well, are subjected to pronounced biological variances and are highly dependent on individual fitness. They have to be judged carefully.

In this study, the reproductive parameters clearly showed concentration-dependent impairments in BPA-exposed zebrafish whereas the reproductive parameters were impaired by EE_2 exposure in an exposure time- depended manner. With increasing BPA concentrations, the fertilization success (Fig. 17) and the number of fertilized eggs per female decreased significantly (Fig. 18). At the three highest BPA exposure concentrations $\leq$ 375 µg/L the reduction in fecundity and fertility definitely proved to be statistically evidenced. An inhibitory effect on the reproductive output (average number of daily spawned eggs per female), however, was only indicated at the highest BPA exposure concentration of 1500 µg/L (Fig. 18). As the number of spawned eggs per female is only reduced by the highest BPA concentration, the lower LOEC of BPA for a diminished fertilization may argue for an impaired fertility of male zebrafish, e.g., caused by disturbances of gonadogenesis or spermatogenesis (Shioda and Wakabayashi 2000). At least a higher sensitivity of males for BPA exposure-derived reproductive effects than females may be supposed. Though it is difficult to correlate the reduced fertility and spawning success directly with the gonad structure and germ cell type proportions. In adult, sexual mature fathead minnow, an inhibitory effect on egg production was found at a

comparable BPA exposure concentration of nominal 1280 µg/L (Sohoni et al. 2001). In contrast to our study, fathead minnows were exposed only during adult stage.

While permanent exposure of zebrafish post fertilization to 3.0 ng EE_2/L suppressed any indication of reproductive activity, at least as long as fish were still exposed, short-term exposure to EE_2 during the early life-stage did not affect reproduction of the adult fish. Early life-stage exposure of zebrafish to a strong estrogen like EE_2 did not indispensably imply a negative impact on reproductive parameters in the post-exposure time. This obviously demands for a longer period of estrogen exposure, as demonstrated in this study for chronic exposure. Apart from BPA and EE_2 exposure-derived organismic effects in zebrafish alone, it has been evidenced that an estrogenic-induced enhancement of the vitellogenin synthesis has not to be indispensably correlated with reproductive impairments. Whereas in the case of EE_2 exposure a complete abolition of reproduction was recorded after chronic treatment with 3.0 ng/L, the relative induction of VTG synthesis was significantly increased over the control level only in the males (Fig. 13a) of these chronically exposed fish. On the other hand, the BPA exposure concentration-dependent elevation of plasma VTG in chronically exposed adult fish was correlated with a reduction of the fertilization success (Fig. 19a). This dependency between VTG induction and fertilization was even stronger correlated in male than in female zebrafish. However, fertility was disturbed in both males and females, because the total number of spawned eggs per female and day was reduced as well (Fig. 18), although the physiological influence of vitellogenin on this parameter is still unclear.

The suppression of reproduction after chronic exposure to 3.0 ng EE_2/L was not unexpected and agrees with the findings of a preceding study on EE_2 (Wenzel et al. 2001; final EU-report of IDEA project, ENV4-CT97-0509). In this study we exposed zebrafish to different concentrations of EE_2 ($\leq$ 10.0 ng/L) for the whole life-cycle period (post fertilization). The lowest observed effect concentration (LOEC) of EE_2 for an impairment of the fertilization success in this study was at 1.1 ng/L. At 10 ng/L of EE_2, similar effects on reproductive success and behavior were observed as in the 3.0 ng/L-EE_2 study presented here. Likewise, these fishes did neither mate or spawn at all. Chronic exposure to EE_2 correspondingly induced VTG to significant levels above the controls only in the adult male fish with a LOEC of nominal 1.67 ng EE_2/L (on day 165 pf). VTG synthesis in female fish was not affected, similar to the findings of the 3.0 ng-EE_2 study. Contrary to this study, they were not able to regain their reproductive ability. Even after a recovery

time of 110 days out of exposure, fertilization success remained below 5 %, whereas in the 3.0 ng-EE_2 study fertilization almost reached 30 % already after 50 days of recovery (Fig. 15). Plasma vitellogenin in morphological and histological differentiated males at the end of the study on day 286 pf was found to be still significantly increased compared to unexposed males of the controls. In the 3.0 ng EE_2 study though, VTG titers of previously exposed male fish, did not show any differences to control male fish anymore, neither after short-term or long-term exposure to EE_2 (Fig. 7a). Admittedly, in female zebrafish the mean VTG titer in the 42-day exposed specimens was still significantly increased at the end of the study (on day 176 dpf), though fishes had recovered from the EE_2-exposure for at least seven weeks. This raises the question if short-term exposure performances, especially at very early developmental stages (in this experiment already from the embryonic stage), exert a persistent or even irreversible effect on (female) vitellogenesis. For the exposure period between day 0 to day 21 pf, comparable data for whole body homogenate VTG levels of adult zebrafish (on day 160 pf) that were exposed to E_2 (17β-estradiol), however, did not indicate a lasting induction of VTG. Full clearance of VTG from male fish was observed even at the highest E_2 concentration of 100 ng/L (Brion et al. submitted 2001). Critically mentioned, the ELISA-measurement of VTG in whole body homogenates of adult fish holds the risk that VTG is below the detection limit due to the high content of numerous proteins lipids within this sample matrix which demands for high dilutions. Moreover, the induction capacity of E_2 at 100 ng/L in this study was not directly comparable with 3.0 ng/L of EE_2, indeed, although the increase of homogenate VTG above the control level was significant in 21-day old fish like in our study.

From the findings of these two studies on BPA and EE_2, a direct correlation between estrogen-exposure induced VTG synthesis and reproductive impairment was not evidenced. A correspondence of LOEC values for VTG and fertility was indeed observed in chronically BPA-exposed adult zebrafish. Although, a physiological linkage of both parameters, VTG and reproduction, is most unlikely. There is only one direct physiological connection between these parameters as they were both responsive to estrogen action. Both processes, the regulation of reproduction and the regulation of vitellogenesis, are subjected to estrogen control, and therefore any kind of exposure to estrogen mimics interfere with both processes. It is highly speculative whether the "overproduction" of vitellogenin in female fish may disturb the production of ripe fertile oocytes and/or the spawning process. Though this would disagree with the VTG results, found for adult, chronically exposed females of 3.0 ng EE_2 study. In male fish, higher levels of vitellogenin may either directly

or indirectly affect sperm quality or motility (Kime et al. 1999). If alterations in courtship behavior, as they were usually observed in correlation with reduced fertility/fecundity in all our studies, were directly or indirectly influenced by vitellogenin remained highly speculative as well. Anyway, also male fish have to cope with abnormally high levels of plasma vitellogenin, especially as they do not have the option to incorporate the protein into developing oocytes. Physiological consequences of high amounts of VTG in male fish are only partly understood. Pathological impairment of the kidney in the cause of estrogenic exposure was described in immature trout (Hermann and Kincaid 1988) and a sequestration and storage of VTG in peripheral tissues like the kidney, was also considered most recently in this species (Schultz et al 2001).

Vitellogenin response and sexual differentiation

An appropriate evaluation of estrogenic effects in fish demands for a careful and critical examination of the test organism itself, as well of the physiology and the morphology. In using the zebrafish as a test species, the problem has to be faced that in this species there is no distinct external sexual dimorphism, like in some other small test fish species. With the zebrafish we encountered two particular complications as a) apart from the absence of distinct external morphological sex-characteristics, the genetics of sexual determination in zebrafish is still unclear, and sex-linked genetic 'markers' are not characterized yet b) the sexual differentiation of zebrafish is not investigated in detail yet, though gonadogenesis passes through a protogynic stage. Additionally, one specific problem in assessing estrogenic or generally endocrine disrupting effects is that morphological and/or histological modifications in the cause of the estrogen exposure always have to be considered.

To cope with the problem to sex zebrafishes, a new approach was chosen in this study to differentiate between the sexes in developing zebrafish on the basis of distinct (individual) differences in VTG response. Furthermore, it was attempted to transfer this concept of (supposed) sex-related VTG responsiveness to adult zebrafishes, especially when severe estrogen-induced impairments and changes in gonad histology were expected. Based on the observations made in regard to vitellogenin in this study, it is obvious that the appearance of individual gonadal sex, especially in chronically exposed fish, often did not agree with the vitellogenic response (Fig. 20a/b, Fig. 21). In sexually fully developed and reproducing adult zebrafish, we demonstrated that the estrogenic induction levels of VTG synthesis were unequivocally distinguishable between males and females. In several

studies with other fish species similar differences were observed. Länge et al. (2001) also described two distinctly different subgroups with VTG levels either in the range of control females or control males in fathead minnow. In result of chronic exposure to 4.0 ng/L of EE_2, adult fathead minnow could not be sexed anymore. Gonad histology of these fish only identified ovarian tissue, similar to our findings in zebrafish. Indeed, histological evaluation of adult zebrafish (day 110 pf), which were chronically treated with 3.0 ng/L of EE_2, showed only female-type gonads (Fig. 20a). Externally, they could not be sexed doubtlessly. VTG levels observed in these fish (Fig. 7) however, were in 75 % of specimens in the range of male fish. And even after they had recovered from the EE_2-treatment for seven weeks, male-to-female ratio, based on gonad histology still differed from the "vitellogenic" (Fig. 20b) sex-ratio, but to a lower extent. Two histological female fish showed male-type VTG levels. Comparable gonadal "feminization" tendencies were consequently also expected in chronically BPA exposed fish, at least at 1500 µg/L. Although the histological analyses and VTG measurements were unfortunately not performed on the same specimens, as practiced in the EE_2-study, the differences between gonadal and vitellogenic sex ratio were as expected. Astonishingly, at the three highest exposure concentrations of BPA, the proportion of fish with histologically female gonads clearly outnumbered the number of fish with female-type levels of VTG. Even in the BPA treated fish a "feminization" of gonads was indicated, whereas in the VTG response it was not.

The gonadal alterations in BPA-treated fish turned out to be not as well-defined as in the EE_2 experiment. BPA exposure did not result in a significantly ovarian-skewed gonadal sex-ratio but in changes of the gonad structure (Maack 2002). Similar observations were reported from other small fish species. In medaka (*Oryzias latipes*), after 60 day of BPA exposure post hatch the sex ratio was shifted to a higher number of females, and at the highest concentration of nominal 2000 µg/L of BPA no male fish could be identified anymore, neither by external appearance nor by gonad histology (Yokota et al. 2000). As indicated in our studies also, the differentiation of gonads in zebrafish was adversely affected by the BPA treatment, but this obviously do not change the gonadal sex-ratio of adult (i.e. at the age of 110 dpf) fish (Fig. 21) significantly. At nominal BPA concentrations > 375 µg/L alterations of the morphology and cellular structure of the gonads mainly concerned the abundance and distribution of germ cells and the pathological changes.

The "feminizing" effect of developmental exposure to EE_2 on the gonads of zebrafish found in this study are in accordance with the findings of Weber et al. (2001). The group around this author depicted a concentration-related suppression of the gametogenesis process in female as well as in male fish, accompanied by a skew in sex-ratios to females. In agreement with our findings, a significant retardation in gonadal cell development with increasing proportions of pre-vitellogenic ovarian follicles in females and spermatogonia and acellular tissue in males was described. The effects of EE_2-exposure on gonad development were attributed by Weber et al. (2001) to a retardation of the gonadal developmental process. However, this interfered with and thus retarded the development of males, passing through the proposed transitory intersexual stage in gonad differentiation (Takahashi 1977). Prospective males persisted in the female phase they are usually going through. As demonstrated by the results of the present study these feminizing or retarding effects on gonadogenesis in consequence of estrogenic exposure obviously did not affect VTG synthesis. Moreover, at the end of the 3.0 ng EE_2 study, the feminizing effect of exposure proved to be only transient and reversible and the gonadal sex-ratio on day 176 pf, when fish recovered from EE_2 exposure since day 125 pf, came definitely closer to the vitellogenic sex-ratio (Fig. 20b). These findings may support the hypothesis of an estrogen exposure-derived retardardation of gonad differentiation. We suggest, developmental estrogenic exposure did transiently change phenotypic and gonadal sex, but not genotypic and physiological sex of zebrafish. Our hypothesis of a gonadal sex-independent but genetically sex-related VTG response in zebrafish is definitely supported by the experimental results, although the underlying physiological regulation and development of this protein expression pattern is unclear. In zebrafish, this process is presumably highly correlated with the protogynic mode of sexual differentiation (Takahashi et al. 1977). Our results indicated estrogen sensitivity, at least with strong estrogens like EE_2, and inducibility of VTG expression already in the early life stage of 21-day old fish (Fig. 4). On the basis of the results of histological analyses and description of gonadogenesis, this time coincides with the pre-differentiating phase of gonad development in the zebrafish strain used in this studies. All gonads of fish on day 21 pf were expected to be either still undifferentiated or at an early protogynic stage. It is speculated that the higher levels of VTG in fish at the age of 21 dpf may reflect the early manifestation of sexual differences. However, this assumption demands for further careful evaluation, especially as such kind of response patterns of VTG synthesis was not indicated in BPA-exposed early life stage zebrafish yet. It may be hypothesized, whether the responsiveness of the VTG synthesis to

estrogenic exposure in several fish at the early-life stage (≤ 42 dpf) reflects either sex-related genotypic differences in gene expression or does only indicate the early onset of the protogynic stage. If the latter assumption would turn out to be true, a positive VTG induction response would have to be expected in all fish that just pass through the protogynic stage. In 42-day old fish, exposure to 3.0 ng EE_2/L did not induce VTG synthesis in all fish, though they were all expected to be at the protogynic stage. Moreover, the fishes in both of the studies could definitely not be sexed on the basis of secondary phenotypic-morphological sexual characteristics before day 80 pf, though sex-related response pattern of vitellogenin induction ought to be given. In particular this holds for fishes where the general development was and/or should have been far progressed, at least according to body size, age and normal gonad development. Sexual gonad differentiation takes place in the developmental period between day 30 and 75 pf, as described for the zebrafish strain used in this study (Maack. 2002).

The developmental stage-dependent induction of vitellogenesis in response to an exogenous estrogen stimulus is a very important aspect in the discussion about the essential claims to be satisfied by VTG as an estrogenic biomarker. EE_2 as a "strong" estrogenic compound ensured the induction of estrogenic effect even in the early life stage, when applied at concentrations above the induction threshold of nominal 1.67 ng/L (Wenzel et al. 2001; final EU-report of IDEA project, ENV4-CT97-0509). We did show in this study that even at the early age of 21 days post fertilization (pf) 3.0 ng/l of EE_2 was able to induce VTG synthesis in 43 % of the exposed fish. After termination of the early life-stage exposure on day 42 pf, the VTG levels in EE_2-treated fish was significantly elevated versus the control in 35 % of the individuals. The significant increase of the VTG body content in the cause of the EE_2 exposure, at least in a certain proportion of larval zebrafish, argues for a differential estrogenic responsiveness of individual fish. It was already referred to this point earlier in the discussion.

In the progression of development, VTG became inducible by estrogenic exposure in all fish but with differential susceptibilities. After 75 days of EE_2 exposure, VTG in all fish was significantly higher than the control VTG level.

Effects of BPA on VTG synthesis in zebrafish early life stage were not revealed in our study. Compared with the EE_2-treated fishes of the same age of 21 dpf, the VTG levels recorded in whole body homogenate samples of chronically BPA treated fish, generally proved to be expectably lower. The mean homogenate VTG concentration of 13.5 µg/ml

measured in the EE_2-induced fish was almost fifteen times higher than the highest mean VTG concentration that could be determined in BPA treated fish of the same age. This corresponded to an at least nineteen times higher induction capacity than found in the BPA treated fish. (though BPA-fishes were markedly larger than the EE_2 fishes; see Tables 2 and 3). This observation certainly can not be explained by an undeveloped susceptibility of the estrogenic system. The expression of the estrogen receptor subtypes in zebrafish has been proven very early in the development (Kishida et al. 2001; Legler et al. 2000) and could even be detected in the early embryo stage due to maternal gene transfer. The induction of the vitellogenin gene expression and therefore the synthesis of the vitellogenin protein, in the early life of zebrafish was clearly demonstrated in our 3.0 ng/L EE_2 study and had previously been observed in other zebrafish studies, e.g., 100 ng E_2/L (17β-estradiol) effectively induced vitellogenesis in 21-day old fish (Brion et al., submitted 2001). Furthermore, these findings are consistent with what is noticed in other small fish species like the fathead minnow (Tyler et al. 1999). Early life-stage exposure to weak estrogenic chemicals with poor potencies like BPA, was inadequate to affect VTG synthesis in zebrafish at the applied sub-lethal concentrations (Fig. 8). Only after prolonged periods of exposure, as in our study until day 75 pf, the effective concentration for VTG induction could be reached at 94 µg BPA/L (Fig. 9). The findings of this study clearly demonstrate a partial responsiveness to exogenous estrogens of the VTG system in zebrafish larvae younger than 42 dpf, at least when treated with strong estrogenic compounds. It is presumed that the induction of VTG in early-life fish was due to a low sensitivity to estrogenic stimuli, already developed in some of these larvae, but in some it was not though. The VTG response in the early life stages of zebrafish may depend on the physiology of the VTG system, the time- and dose-dependent susceptibility of the VTG gene expression in the liver and the estrogen receptor availability and sensitivity. These prerequisites widely depend on interindividual differences, first of all according to the developmental stage and/or also on the genotype. On the basis of our results, it seems reasonable to assume that the VTG responsiveness to estrogenic exposure in the zebrafish, and presumably in most fish species, is strongly correlated with the sexual development. A 'real' estrogenic induction of vitellogenesis in developing zebrafish could not be observed before day 75 pf in this study. This age widely corresponds to the final stage of gonadal sex differentiation in zebrafish of the strain, used in these studies. Only at this developmental stage, estrogen exposure caused a VTG response in all exposed fish, as well to the weak estrogenic compound BPA as to EE_2. A physiological system like

vitellogenesis, needs to be fully developed to give a real, graduate response to an exogenous stimulus. At 75 dpf, a (sexual?) dimorphic, compound- and concentration-dependent response pattern of zebrafish VTG was definitely indicated, in the EE_2 and in the BPA study.

Vitellogenin response to estrogenic exposure – a prediction of reproductive impairment?

Our data clearly show that VTG in zebrafish is a reliable indicator of current exposure to estrogenic chemicals. Even weak estrogenic substances like BPA were able to enhance vitellogenin synthesis in both sexes of zebrafish in a concentration-dependent manner, at least in specimens of post juvenile age (> 75dpf) and after a prolonged duration of treatment. The concentration of BPA that affected VTG and reproduction of chronically exposed adult zebrafish was 375 µg/L and hence was orders of magnitude higher than environmental relevant concentrations measured in moderately polluted areas of Europe (Spengler et al. 1999). An exposure concentration of 3.0 ng/L of EE_2, however, corresponds to real concentrations in the riverine environment (Stumpf et al. 1996). At current chronic treatment conditions VTG production proved not to be more or less sensitive than organismic effects like fertility or fecundity. Especially in males where the induction of vitellogenin is clearly an indicator of estrogenic exposure, effective concentrations for VTG induction generally corresponded well with the appearance of reproductive impairments in both of our studies. An adequate correspondence between the sensitivites of vitellogenin, as a biomarker of current estrogenic exposure, and of lasting or even permanent (and often organizational) organismic effects was found only in sub-adult and adult zebrafish ≥ 75 dpf that had been exposed chronically and were still under current exposure. Nevertheless, the question was raised whether VTG can also serve as a predictive indicator of organizational effects, especially on gonad morphology and reproductive success. Moreover, there was the need to know if the induction of vitellogenesis was only a transitory or a lasting response to estrogen exposure. To be not only a marker of estrogenic exposure, VTG has to comply with several requirements:

1) The claim to predictive qualities demands for a sensitivity of biomarker response appropriate to the sensitivity of the predicted effects, in this case reproductive impairments. Our data show that VTG in zebrafish fulfills this requirements to some extent. At least in sub-adult (pubescent; non-reproducing) and adult zebrafish, chronically exposed to environmental estrogens from fertilization onwards, the LOEC values for VTG induction correspond with the LOEC values of the resulting, population-relevant reproductive

parameters of fecundity and fertility. This is fairly independent of the estrogenicity of the chemical applied. A correspondence of the LOEC values, however, and thus a correspondence of the sensitivity of the biomarker response with the sensitivity of organizational effects on reproduction, that is limited to specific developmental stages or even gender of fish only insufficiently meet the demands on a biomarker with predictive function.

2) A 'predictive' biomarker ought to meet the requirement of an accurate and reliable sensitivity of response, fairly independently of the duration or time of exposure. Our data clearly show that this did not hold unrestrictedly for VTG in zebrafish. Chronic or long-term exposure assumedly caused a certain metabolic acclimation of vitellogenesis to exogenous estrogenic stress, at least in female zebrafish. Permanent developmental treatment to the 'strong' estrogen EE_2 resulted in a decrease of the mean VTG titers (Fig. 7b) as well as in a drop of the relative induction capacity (Fig. 11, Fig. 13b). In females, high levels of blood VTG occur naturally at certain stages of sexual differentiation and during pre-spawning periods, and the metabolism is accordingly capable to cope with (abnormal) alterations in VTG content by regulative mechanisms. Though this hardly explains, why this mechanisms obviously did not regulate vitellogenesis in the BPA-treated female fish.

Another aspect is the affinity of an estrogenic compound to the estrogen receptor. Only the efficiency of receptor binding and complexation triggers estrogenicity and its subsequent (endocrine) regulative feedback loops. It is speculated that such regulative processes are missing in male zebrafish, though this would disagree with the results we observed in male fish. The mean plasma VTG concentration in chronically EE_2 exposed males at the age of 118 dpf was 25-times lower than on day 78 pf in the "low" responder group. It remains to be discussed whether the attenuation of VTG production in 118-day old males compared to younger male fish (day 78 pf) indicates a certain adaptation to the EE_2 treatment similar to females. VTG in adult males, however, was significantly induced even after long-term, chronic exposure to EE_2 post fertilization, whereas in the corresponding females it was not (Fig. 11). Concluding from these observations of an attenuation of the VTG response after long-term exposure, in our study to 3.0 ng/L of EE_2, adaptive physiological processes are indicated in males and in females. Consequently, the reliability of the biomarker VTG is diminished when applied to long-term exposure studies or field studies, in which fish are chronically exposed to estrogenic contaminants. Low

levels of VTG consequently could falsely referred to a low or moderate degree of contamination or would increase effective concentrations.

3) Apart from chronic exposure effects caused either by a 'strong' estrogenic compound like EE_2 or a 'weak' xenoestrogen like BPA, this study specifically aims at enlightening the reversibility of the VTG response after short-term developmental exposure of zebrafish. This is an important aspect for the evaluation of a biomarker as an indicator of organizational effects. A fast reversibility of VTG response after termination of exposure implies an increase in the probability of false negatives and consequently negotiate every claim of a predictive quality of VTG. Temporary developmental exposure of zebrafish was therefore investigated by a treatment with the "strong" synthetic estrogen EE_2, applied at an effective concentration for organizational reproductive effects. Our results demonstrate that short-term exposure to 3.0 ng/L of EE_2 for 14, 21 or 42 days pf did not result in a lasting elevation of vitellogenin in adult male zebrafish (Fig. 7a). Only in female fish, a significant elevation of the VTG plasma titer persisted even after more than ten weeks out of treatment (Fig. 7b). After an extended period of recovery from day 125 pf until the end of study on day 176 pf, this was still expressed in the 42-day-treatment females. The persistence of the vitellogenic response presumably correlates with the duration of developmental exposure. However, the effect definitely proved to be reversible. It is speculative, whether a longer exposure time or exposure at another developmental stage would probably cause irreversible changes in female vitellogenesis. An indication of irreversible disturbances of VTG synthesis in male zebrafish was shown in a previously performed life cycle experiment with EE_2 (Wenzel et al. 2001; final EU-report of IDEA project, ENV4-CT97-0509). In this study (data not shown) adult male zebrafish had still significantly elevated levels of VTG after 166 days out of treatment. As these fishes were exposed for the same duration of time (120 dpf) as in this study but to the higher concentration of 10 ng/l of EE_2, it may be argued whether persistence of VTG response depends on the estrogen concentration and/or the estrogenic capacity, respectively. Discrepancies in the estrogenic potency and effectiveness of estrogen mimics are presumably also due to differences in bioaccumulation, bioavailability and metabolism of the estrogenic compounds in fish. This would explain the absence of a persisting VTG effect in males as observed in this study. Our results recorded a lasting VTG induction not even in long-term exposed males after a shorter period of recovery of only 58 days (Fig. 7a). In conclusion, short-term ($\leq$ 42 dpf) developmental exposure to 3.0 ng/L of EE_2 did neither induce reproductive impairment nor did it result in persisting disturbances of

vitellogenesis in male zebrafish. At the same time a persistent enhancement of VTG production in female zebrafish appears to be not indicative of an reduction of fecundity (Fig. 16).

The results presented in this study provide clear evidence that VTG is in fact a reliable indicator of current estrogenic exposure of fish, but does not meet the requirement to have a predictive capability for a subsequent impairment of the reproductive function, at least not in zebrafish. We observed both, time- and dose-dependent effects of exposure to the estrogen mimics EE_2 and BPA, as well of activational quality (and therefore usually transient) as of organizational (and usually permanent or lasting) quality. Long-term exposure of zebrafish post fertilization to a strong estrogen like EE_2 at an effective concentration can have profound detrimental impacts on the reproductive health. The exposure derived effects on sexual development and reproduction, as demonstrated by our findings, were clearly organizational. Whereas the VTG response to the estrogen exposure was mostly activational, and lasting disturbances of VTG synthesis were presumed only after long-term exposure periods and high treatment concentrations. Results of a previous life-cycle study with zebrafish, e.g., indicated a lasting effect on VTG in males after chronic exposure to 10 ng/L of EE_2. Based on the findings here, it is evident that the estrogenic induction of VTG is only a transient effect. The absence or the presence of a VTG response does not inevitably imply the occurrence or nonoccurrence of environmental estrogen exposure-derived impairment of organizational parameters, like the reproductive fitness. A predictive function of VTG for subsequent reproductive effects was not indicated, at the most an indicative function for an increased probability of effects. In regard to profound organismic, persistent effects, VTG turned out to be less sensitive to environmental estrogen exposure than the reproductive capacity (or gonad differentiation), at least if fish have not been exposed recently. And this evaluation of VTG as an estrogenic biomarker widely agrees with the findings of a similar study with the Japanese medaka (*Oryzias latipes*) by Cheek et al. (2001). They exposed medaka to the environmental estrogen *o,p'*-DDT throughout the period of sex differentiation and examined the effects on VTG induction, gonadal differentiation and the reproductive success. In the end, they ranged the effects of exposure according to their relative sensitivity in the following order: hatching success > fertility >> gonad differentiation >> VTG production. Although the order of ranking the effects correspond widely to our study results, the relative sensitivity

of VTG in response to estrogenic action showed not to be always and also not very much lower than the gonad development.

Conclusion

This study investigated EDC-caused effects in zebrafish in order to evaluate the quality and the significance of vitellogenin, the well-established and commonly used biomarker for estrogenic exposure, to predict developmental and/or reproductive impairments. Special attention was paid on alterations of fertility and the sexual differentiation because these are the most relevant parameters defining the fitness of individuals. Moreover, resulting changes in the sex ratio and disturbances of reproduction have a crucial impact on the structure of natural populations and may endanger the survival. The potential hazards of reproductive disruption is of far-reaching ecological relevance as it affects not only single individuals but populations or even species. Very few studies have yet addressed the link between vitellogenin, sex differentiation and reproductive success of exposed fish. Particularly not in the zebrafish. One crucial question in this approach was, if VTG induction can act as a predictive biomarker for subsequent alterations of sexual development or reproductive impairment. Or is the induction of vitellogenin synthesis a transitory effect which can only be interpreted as indication of acute or shortly before terminated EDC exposure? Concluding from our results we suggest that VTG definitely act as a reliable and moderately sensitive indicator of EDC exposure and therefore has to be assessed as a biomarker of exposure and/or as a biomarker of susceptibility (Adams 1990; Barrett et al. 1997). Induction or enhancement of VTG synthesis is only a transitory, activational response to environmental estrogen exposure but it is not inevitably correlated with resulting permanent and often organizational effects. However, as a reliable indicator of current estrogenic exposure, VTG calls attention to a potential hazard of ecologically relevant impairment in animals exposed.

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Chapter 3

Effects of aromatase inhibitor and 17α-methyltestosterone treatment on the expression of cytochrome P450 aromatase genes (CYP19A and CYP19B) and sexual gonad differentiation in the zebrafish (*Danio rerio*)

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Abstract

Differential expression of the ovarian-derived and brain-derived mRNA transcripts (CYP19A and CYP19B) of the P450arom gene (CYP19) was assessed in developing and adult zebrafish (*Danio rerio*) by semi-quantitative RT-PCR. CYP19A and CYP19B mRNAs transcripts were found to be expressed both as well in head and in trunk of larval or sub-adult fish as in gonad and brain of adult fish. The highest transcript abundance of each CYP19 gene was recorded in the corresponding body part and organ respectively: for CYPA in trunks or gonads, for CYP19B in heads or brains. In adult fish, CYP19A mRNA showed a sexual dimorphic expression pattern in gonads. CYP19B mRNA expression in the brain, however, did not reveal sexual differences. The primary purpose of this study was to provide evidence for the theory that the gonadal sex differentiation of zebrafish is determined by estrogens and androgens and therefore depends on the P450arom regulation. It was shown that treatment with the non-steroidal aromatase inhibitor fadrozole and 17α-methyltestosterone (MT), respectively, during the critical period of sexual differentiation adversely affected CYP19 mRNA expression and gonad differentiation in zebrafish. Application of fadrozole (500 µg/g of food) between day 35 and day 71 post fertilization (pf) suppressed CYP19A mRNA expression in trunk parts of zebrafish and induced testicular gonad differentiation. After treatment, CYP19B mRNA was upregulated in the brain of adult, all-male fish. MT treatment (10 µg/L) during the same period tremendously increased CYP19B mRNA expression in the heads of 71-day-old fish. Only ovarian-like gonads were identified by histological examinations. In adult fish CYP19B mRNA expression in brains remained to be elevated only if the MT exposure had been continued until the end of study on day 161 pf. CYP19A mRNA in gonads of fadrozole or MT treated fish appeared to be expressed in a sexual dimorphic pattern, independently of the gonadal sex. The results provide evidence that manipulations of the aromatase system may cause irreversible effects on gonadal sex differentiation of the protogynic zebrafish. Therefore, gonad differentiation is correlated with the differential expression of aromatase genes and thus depends crucially on estradiol synthesis and estradiol-mediated processes. These findings reveal the P450arom regulative system in fish as an important and highly sensitive target for environmental pollutants with endocrine disrupting potencies.

Keywords: Fadrozole, Gene expression, 17α-Methyltestosterone (MT), P450 aromatase, Zebrafish

Introduction

Disturbances of sexual differentiation and the related hazard for wild fish reproduction and survival of populations has become a central issue in the discussion about endocrine disrupting compounds (EDC) in the aquatic environment (Harries et al. 1997; Arcand-Hoy and Benson 1998; Jobling et al. 1998; Allen et al. 1999). Most attention has been focused on compounds of presumed estrogenic activity, because many of the reported effects in wildlife (e.g., occurence of intersexuality, induction of vitellogenin) strongly suggest the exposure to estrogens or estrogen mimics. Many of these environmental estrogens appear to exert their effect through interaction with the estrogen receptor (ER). However, the endogenous estrogenic system may be disrupted by environmental substances through bpathways and mechanisms other than ER-mediated, for instance they may become effective by disturbing steroidogenesis. Interference of known or unknown EDCs with the steroid synthesizing hormones or with signalling pathways of hierarchically higher endocrine levels, e.g., in the hypothalamus and/or the pituitary, has to be presumed.

In his pioneering studies Yamamoto (reviewed 1969) demonstrated that in gonochoristic fish, like the medaka, a complete reversal in sex differentiation is possible provided that sex steroid hormones were applied consistently, from the stage of undifferentiated gonad and throughout the period of sex differentiation. Based on the findings in medaka, Yamamoto postulated the sex steroid theory of natural inducers: "Estrogens act as female inducers and androgens function as male inducer." Since then, hormonal induced sex-reversal has been intensively investigated in numerous teleost species, as reviewed by Hunter and Donaldson (1983) or Pandian and Sheela (1995) and most recently with special focus on feminization, by Piferrer (2001). Nevertheless, the mechanisms of estrogen and androgen actions and their significance in the differentiation process are still poorly understood. Only few studies have addressed the physiological implication of sex steroid hormones in gonadal sex differentiation of fish and the potential impact of environmental factors (Baroiller et al. 1998, 1999; Nakamura et al. 1998; Guiguen et al. 1999). Moreover, most of the studies center on the differentiation of gonads and the gonadal steroids, but lack the consideration of the link to the sexual differentiation of the neuroendocrine system Like in the gonads, steroid hormones have proven to be key players in neuroendocrine processes in the brain, and the regulative axis hypothalamus-pituitary-gonad is of central importance for all aspects of reproduction, from sexual differentiation to the sexual behavior (Liley and Stacey 1983; Balthazart 1989; Balthazart

and Ball 1998; Habibi and Huggard 1998). All these processes highly depend on the labile hormonal balance between estrogens and androgens, and this again relies on the availability and activity of the steroid synthesizing enzymes. A key enzyme in the steroidogenic pathway is the aromatase enzyme complex (also called estrogen synthase), a member of the cytochrome P450 enzyme family, catalyzing the enzymatic transformation of androgens (androstenedione, testosterone) to estrogens (estrone, estradiol). The physiological importance of aromatase in gonadal sexual differentiation in fish is strongly evidenced by the fact that inhibition of the aromatase enzyme activity during the appropriate developmental period caused genetic female fish to develop as phenotypic males. This was impressively demonstrated by Piferrer et al. (1994) with chromosomally all-female stocks of the gonochoristic chinook salmon (*Oncorhynchus tshawytscha*), by Kitano et al. (2000) in genetically all-female Japanese flounders (*Paralichthys olivaceus*) or in the protogynous blackeye goby (*Coryphopterus nicholsii*) or (Kroon and Liley 2000).

With this study, it was aimed at investigating whether a) P450arom is involved in the gonadal sex differentiation of the protogynic zebrafish, *Danio rerio,* and b) interference of environmental substances with the P450arom function leads to transient or lasting disturbances or alterations of the gonadal organization of zebrafish. Insight into the regulative task of aromatase in sexual differentiation of zebrafish was gained by targeted manipulations of the P450arom function. To this end, zebrafish was on the one hand treated with the aromatase inhibitor fadrozole (CGS16949A, Novartis) and on the other hand induced by the exogenous supply of the substrate 17α-methyltestosterone (MT). Fadrozole, a non-steroidal competitive inhibitor of aromatase activity, belongs to the second generation of postmenopausal hormone-responsive breast cancer drugs. Besides its pharmaceutical use, fadrozole has evidenced in several studies to reduce the aromatase enzyme activity and therefore the estradiol formation in gonad (predominantly ovary) and brain of mammals (Steele et al. 1987; Harada et al. 1999), birds (Foidard et al .1995, Wade et al. 1994;) amphibians (Chardard and Dournon 1999; Miyata and Kubo 2000) and fish, like coho salmon (*Oncorhynchus kisutch*) (Afonso et al. 1997, 1999, 2000) or the protandrous black porgy (*Acanthopagrus schlegeli*) (Lee et al 2001).

MT is a strong androgen receptor agonist and its application is quite common in fish culture to control hormonal determination of sex and to promote masculinization (van den Hurk et al 1989; Feist et al. 1995; Papoulias et al. 2001; Kitano et al. 2000). Masculinization after developmental exposure to MT was also described for developing

zebrafish (Westerfield 1995). However, at higher concentrations and/or longer periods of exposure, MT has been reported to favor the development of phenotypic females, a phenomenon which is described as 'paradoxical feminization' (Hackman 1971; Hackman and Reinboth 1974). This effect might be ascribed to a substrate-dependent elevation of aromatase expression and activity and the consequent increase of endogenous estrogen levels.

In zebrafish two different forms of the cytochrome P450 aromatase genes (CYP19) have been identified and assigned to two distinct aromatase gene loci (Kishida and Callard 2001; Chiang et al. 2001a,b; Trant et al. 2001). The function of the expression of both CYP19 genes in the main reproductive organs, brain and gonads, in the process of sexual differentiation of the zebrafish is still unknown. Hence, this study investigated the mRNA expression of both CYP19 genes, CYP19A and CYP19B, in gonad and brain tissue (or head and body samples, in case of earlier developmental stages) of zebrafish, during and after sexual differentiation. Abundance of both CYP19 transcripts was analyzed by a specific semi-quantitative RT-PCR method. For the zebrafish, the period of gonad sexual differentiation occurs between week five (day 35 to 40 pf) and week 11 (day 70 to 75), as indicated by histological observations (Maack 2002). Within this period, protogynic gonads do either change into testes, or they continue differentiation from the 'early ovary' stage to reproductive ovaries.

Material and methods

The exposure experiments to fadrozole (CGS 16949A; Novartis) and 17α-methyltestosterone (MT) were conducted with zebrafish. Fish were exposed during the developmental period of gonad differentiation, characterized for the zebrafish strain kept in our laboratory between day 30/35 and day 70/71 post fertilization (pf) (Maack 2002).
For the choice of effective but sublethal concentrations of methyltestosterone and fadrozole, it was referred to several published data available either for zebrafish or other fish species (Afonso et al. 1999, 2000, 2001; Guiguen et al. 1999; Kitano et al. 2000; Kwon et al. 2000; Örn et al., TemaNord 2000, etc.). Especially in the case of fadrozole, test conditions could not simply be adopted to the zebrafish and to the existing test conditions in our laboratory. Comparable data of inhibitory effective fadrozole concentrations, applied to small fish species like the zebrafish, were not available. Therefore, short-term range finding experiments were conducted to identify suitable, inhibitory active but discernibly sublethal concentrations.

Culture and maintenance of fish

Zebrafish used in these two studies were obtained from the own breeding stock at the Umweltforschungszentrum (UFZ) Leipzig, Germany. Breeding fish were cultured in activated charcoal-filtered and tempering water in glass aquaria (total volume: 107 L). Tank water was fed from temperature-controlled and aerated header tanks via separate water supplies. Water in the aquaria was continuously filtered by external aquarium filter pumps (Thermocontrolled Filters, Eheim, Germany), aerated by air supply and the temperature in the aquaria was kept between 25 and 27 °C by heating elements of the pumps. Breeding as well as all exposure experiments were conducted in temperature controlled rooms where the temperature was kept between 24 and 26 °C. For spawning, spawning trays were placed into the aquaria, covered with stainless steel lattice lids. Artificial "spawning-trees" (plastic water plants) were attached onto the lids as spawning substrate. The introduction of suitable spawning substrate is indispensable to stimulate spawning. Onset of spawning occurs as soon as the light is switched on in the morning.

All fishes were held under the same photoperiodic regime of 12-h light : 12-h dark. Fishes were fed three times a day, two times with TetraMin®, dry flaked food (Tetra, Germany) ad libitum and once with *Artemia* sp. nauplii as live food supplement. Shortly after hatch, larvae were supplementary fed daily twice with a commercial larval food AZ

100 (Tetra, Germany) until the end of the yolk-sac stage. Subsequently, larvae were fed three times a day, in the morning and in the evening, with AZ 100 ad libitum. Dry food was supplemented with freshwater rotifers (*Brachionus calyciflorus*; Rotifera) once daily at noontime.

Normal breeding of fry for replenishment of breeding stock or planned experiments was carried out in 25 L glass aquaria, which were covered on the ground with artificial sediment, composed of ground peat soil, quartz sand and kaolinite. These "breeding tanks" were warmed to 26 °C / 27 °C by automatic water-heating elements and aerated by air supply. Freshly spawned eggs were directly transferred into these tanks and the fry was left to its own resources until most of the larva were hatched. The larvae were fed as already described above.

Chemicals

The aromatase inhibitor fadrozole (4-(5,6,7,8-tetrahydroimidazole [1,5-a] pyridin-5-yl)-benzonitrile monohydrochloride; CGS16949A) was kindly provided by Novartis Pharma AG, Basel (Switzerland). A stock solution of fadrozole was prepared in ethanol (abs.; Merck) to obtain a concentration of 1.67 mg/ml. 3.0 ml of stock solution were sprayed and as uniformly dispersed as possible by a vaporizer onto 10 g of thoroughly minced flaked TetraMin® food, to reach a final dose of 500 µg fadrozole per g food. Another 3.0 g of TetraMin® was subjected to the same treatment with pure, fadrozole-free ethanol (abs.), prepared as solvent control diet. The treated food was dried carefully until the ethanol has volatilized completely. Control and fadrozole-treated food was stored in a closed glass vessels at 4°C for the whole experimental period.

17α-methyltestosterone (17β-hydroxy-17α-methyl-4-androsten-3-one, MT) was obtained from Sigma-Aldrich (Germany). A stock solution of 2.0 mg/ml MT was prepared in 70 % ethanol (Merck) and stored at 4 °C under continuous and vigorous stirring. Apart from short time periods during dosage, the solution was kept in the dark to prevent photooxidative degradation of the substance.

Fadrozole experiment

Progeny of breeding stock on day 30 post fertilization (pf) were randomly divided into groups of 80 fish and each group was introduced into one 30-L test vessel (whole-glass tanks), filled with 20 L of dilution water. Each test vessel was heated up to 25 – 27 °C and aerated by air supply. One set of two replicate vessels each (2 x 80 fish) was used either as control or as fadrozole treatment. After fish had been acclimatized for several days, the

onset of experiment was on day 35 pf. From this day forth, fish were fed twice daily either with control ethanol-treated for or with fadrozole-treated food. All fish were supplementary fed with *Artemia* sp. nauplii as live food daily at noontime. The water in the test vessels was renewed completely every third day during the treatment period. In all test vessels, temperature, pH-value, dissolved oxygen and ammonia were controlled weekly. Temperature, pH and dissolved oxygen was measured in the test vessels by digital pH- and oxygen-meters (WTW Weilheim, Germany), ammonia was determined in water samples with a commercial ammonia-kit (Aquamerck®, Merck Germany). After 35 days of fadrozole treatment, remaining fish were transferred to two sets of identical "holding" vessels and continuously reared under unexposed conditions. All fish were fed with untreated TetraMin® and the water in the tanks was changed twice weekly. Individual body length of all test fish were recorded digitally at the beginning of the experiment on day 36 pf and at the end of fadrozole treatment period on day 71 pf by image analysis. For this purpose, all fish of each vessel were photographed with a digital camera and each fish was measured on the basis of the digital image and a suitable image analysis computer program. With the first observations of courtship behavior in test fish, glass spawning trays, covered with lattice lids (stainless steel) with artificial "spawning trees" (plastic water plants) attached on top, were regularly placed into the vessels to stimulate mating and spawning. Spawning success was recorded on a daily basis in each vessel until the end of experiment. The experiment was finished on day 161 pf after normal and regular mating behavior and spawning had been observed in the control vessels for several weeks. Fecundity and fertility were not assessed quantitatively.

Methyltestosterone experiment

According to the fadrozole experiment, larvae on day 30 pf were randomly selected and introduced into 30-L test vessels, containing 20 L of dilution water, in groups of 80 fish each for acclimatization. At the same time two additional vessels were filled with MT treated water of the provided test dose of 10 µg/L of MT, to saturate all surfaces with the test substance prior to the beginning of exposure. With the onset of the experiment on day 35 pf, two groups of 80 fish each were transferred into one set of replicate MT treatment vessels. One pair of replicate tanks was used as solvent controls. Every third day water in all test vessels was replaced completely. To this end, all fishes were netted from their tank, the tanks were exhausted and refilled at first to one quarter. Each vessel received the required amount of either the MT stock solution or of 70 % ethanol and was immediately

top up to 20 L with dilution water. This procedure of water exchange ensured appropriate and uniform distribution of the substance. Substance dosage was carried out manually with 10 – 100 µl adjustable-volume pipette (Eppendorf Reference®). Fish were fed TetraMin® and *Artemia* in the schedule described above. Water temperature, pH-value, dissolved oxygen and ammonia were recorded regularly in all vessels during the entire test period. On day 70 pf after 35-d MT treatment, exposure was finished in one replicate vessel and remaining fish were introduced into a new, untreated tank of similar size to be continuously reared in dilution water. In the second replicate MT treatment vessel, exposure was continued until the end of experiment on day 160 pf, running in parallel with one control replicate vessel, as solvent control. Fish of the second control replicate therefore were transferred into dilution water on day 70 pf.

As soon as the onset of spawning was observed in control fish, spawning success was recorded on a daily basis until the end of experiment; however, fertility and fecundity were not quantified.

Somatic growth was monitored and individual body sizes of all fish were measured by means of digital image analysis (as described previously) before the onset of treatment on day 35 pf and at the end of 35-d exposure on day 70 pf.

Sampling procedure

In both studies, with fadrozole and with MT, fish were sampled for the first time at the beginning of exposure on day 35 and 36 respectively, then after 35-d exposure period and finally at the end of the experiments around day 160 pf. At the beginning of test periods on day 35/36 pf (t_0 samples) and after 35 d of exposure (t_{end} samples), 30 - 40 fish each were sacrificed for RT-PCR analysis as well as for gonad histology. For this purpose, fish were anaesthetized in ice water. Before the fishes were sacrificed by decapitation, specimens were dried on tissue paper prior to weighing, then the body length was determined. Fish sampled for RNA-extraction were divided into head and body sections which were separately snap frozen in liquid nitrogen, then stored deep-frozen at –80 °C. Prior to histological processing, abdominal cavity of decapitated fish was opened and specimens pre-fixed in Bouin´s fixative for 1-2 h. Gonads or, depending on the size or developmental stage of fish, gonads together with all adjacent organs, were dissected out and continued to be fixed. At the end of fixation, samples were dehydrated through a grade series of alcohol, embedded in paraffin wax, sectioned at 7 µm and stained by hematoxylin-eosin or methylene blue.

At the end of the reproductive period (end of experiments) after 160/161 dpf, all remaining fish were sacrificed and sampled ($t_{reproEnd}$ samples). For RNA-extraction, fishes were anaesthetized again in ice water before body length and wet weight were measured. Thereafter, gonads and brain were dissected out and immediately submerged in RNA*later*™ (Ambion Europe, UK). RNA*later*™ (patent pending) is an aqueous, non-toxic tissue storage reagent that immediately inactivates RNases and stabilizes and protects cellular RNA in intact, unfrozen tissue samples. The tissue samples were stored at 4 °C prior to further processing.
For histological analyses gonad samples were processed and stored as described before.

Zebrafish do not have distinct secondary sexual characteristics that would allow unequivocal sex identification, at least not before the late-pubescent, sub-adult age of ≥ 70-75 dpf. In the case of exposed fish, it has to be presumed that the development of phenotypic sexual characteristics was impaired by the treatment. Hence, gonad histology was used to sex fish of both, the fadrozole and the MT study.

Semi-quantitative RT-PCR expression analysis of aromatase (CYP19) genes

Total RNA was extracted from frozen or RNA*later*™ -fixed tissue by a one-step RNA isolation method with TRIzol® Reagent (Life Technologies) or TRI Reagent® (Sigma-Aldrich). Both reagents are based on a monophasic mixture of guanidine isothiocyanate and phenol, which effectively dissolves DNA, RNA and proteins from tissue and cells.
1000 µl of TRIzol® or TRI Reagent® were added to 50 – 100 mg of tissue (tissue samples <50 mg were submerged in 800 µl). Samples were immediately homogenized in 2.0-ml RNase-free reaction tubes with an electric power homogenizer (Ultra-Turrax) and applied to further processing according to the manufacturer protocol. Due to traces of contaminating genomic DNA, especially in brain samples, all RNA samples were DNase treated prior to RT-PCR analysis. A DNase I Kit, DNA-*free*™ (Ambion Europe, UK), was used which does not require organic extraction, (phenol extraction followed by alcohol precipitation) EDTA addition or heat inactivation. Small sample sizes usually obtained from small fish species like the zebrafish result in low RNA recovery, thus careful treatment is needed to minimize RNA losses. Yield and purity of extracted total RNA was determined by UV spectrophotometrical absorbency readings at 260 nm (λ_{max} of nucleic acids) and 280 nm (λ_{max} of aromatic nucleic acids) (A_{260}/A_{280}). For roughly assessing RNA quality, 1 µg of isolated RNA from each sample was run on a native 1.5 % agarose gel

stained with ethidium bromide. cDNA was synthesized from 1 µg of total RNA from either head and body or brain and gonad samples using SuperScript™ II RT (Life Technologies), an M-MLV (moloney-murine leukemia virus) RNase H⁻ reverse transcriptase, according to the manufacturer's instructions. mRNAs were specifically transcribed by priming with oligo(dT)$_{12-18}$ (Life Technologies) primers in 20 µl reactions.

Following RT reaction, 2 µl (10 %) of first-strand reaction were applied to PCR. cDNA was amplified with gene specific primers for zebrafish CYP19A and CYP19B gene fragments and zebrafish beta-actin. The constitutively expressed "housekeeping" gene beta-actin was used as internal control to normalize each sample for variations in the amount of starting RNA. This validates comparisons of the mRNA being studied across the samples. Design of gene specific primers were based on reported GenBank sequences for zebrafish P450aromatase (accession numbers AF004521, complete cds and AF120031, partial cds for brain and ovarian derived cytochrome P450 aromatase cDNA (CYP19) respectively) and beta-actin genes (accession number AF057040 for complete beta-actin cDNA sequence). Choice of suitable primer pairs was supported by the application of web-based primer-picking software Primer3 (Rozen S, Skaletsky HJ 1998: **Primer3**; Code available at:
http://www.genome.wi.mit.edu/genome_software/other/primer3.html.)

Table 1. Primers used for PCR amplification of aromatase (CYP19A and CYP19B) and beta-actin (ACT) genes of *Danio rerio*:

Primers	Sequence (5′→ 3′)
zfCYP19A (forward)	CTCCAGCACTCGCATCTGTC
zfCYP19B (forward)	CAGGAGATACAGTCTCAGATAGGC
zfACT (forward)	GCCAACAGAGAGAAGATGACACAG
zfCYP19A (reverse)	CAGCAGAGCCACCAGAATAGAC
zfCYP19B (reverse)	CGAATGGCTGGAAGTAACG
zfACT (reverse)	CAGGAAGGAAGGCTGGAAGAG

zf = zebrafish; ACT = beta-actin

All oligonucleotide primers used were synthesized by and purchased from Metabion (Martinsried, Germany).

Table 2. PCR fragments amplified:

Amplicon	Fragment size (bp)
zfCYP19A (CYP19A)	330
zfCYP19B (CYP19B)	292
zfACT (βACT)	462

The identity (or: authenticity) of amplified fragments was verified by commissioned sequencing of PCR products (SEQLAB Göttingen, Germany) and subsequent sequence alignment performed with BLAST® (Basic Local Alignment Search Tool) programs (Altschul et al. 1990).

For each fish/sample PCR reactions were separately performed to determine transcript abundance for each of the three target sequences. PCR conditions for each of the three primer pairs were optimized considering all relevant reaction parameters for optimal amplicon yields and specific experimental conditions. Annealing temperature of primers were optimized by temperature gradient cycling ($T_{Gradient}$ Thermocycler, Biometra). PCR reactions were performed in duplicates for 34 cycles (for CYP19) and 30 cycles (for βACT) respectively, in 20 µl reaction volume. The PCR reaction mixture for the amplification of CYP19 fragments contained 2.0 mM of $MgCl_2$ and 0.8 mM of dNTP, 0.6 mM (CYP19A) and 0.8 mM (CYP19B) respectively of each primer, finally 0.03 u/µl (CYP19A) or 0.05 u/µl (CYP19B) of Taq DNA polymerase. Each reaction was filled up to a final volume of 20 µl with 2.0 µl of 10x reaction buffer and DEPC-(diethyl pyrocarbonate) treated water. All PCR chemicals were purchased by Sigma-Aldrich (Germany). For PCR amplification of zfACT (βACT), 1.5 mM of $MgCl_2$ and 0.8 mM of each primer with 0.04 u/µl of Taq polymerase was used. Amplification reactions of all three gene fragments were performed under standard PCR conditions in 0.2-ml PCR tubes using a multiblock PCR Thermocycler (T3 Thermocycler, Biometra). In the first cycle, DNA was denatured at 96 °C for 2 min, in the following cycles only at 94 °C for 1.0 min. Primers were annealed at 61.8 °C (for CYP19) and 57.8 °C (for βACT) respectively, for 0.5 min and extension was performed at 72 °C for 1.0 min, with a final extension at 72 °C for 10 min. Three µl of each PCR reaction product was loaded onto 1.5 % agarose gel, containing ethidium bromide, and separated by electrophoresis. PCR product duplicates of the CYP19A and CYP19B fragments were always loaded in parallel to their corresponding βACT reaction onto the same gel to minimize gel-to-gel variations and inconsistencies in fluorescence measurement between different gel images. Gels were recorded in digitized

form by using a software-controlled Gel Doc 1000 Documentation System (Bio-Rad) and subsequently analyzed applying Multi Analyst®, version 1.1 (Bio-Rad). Intensity of gel bands was quantified by calculations of relative pixel densities (counts) of the corresponding band area (cm x counts). Relative expression rate of CYP19 mRNAs to βACT mRNA expression was normalized by calculating the ratio of the CYP19 transcript band signal intensity area (= integral of the relative pixel density (counts) distribution of gel band area) to the βACT transcript band signal intensity area.

Data analysis and statistics

Due to the well-known measuring inaccuracies of semi-quantitative RT-PCR methods, it was refrained from statistically analyzing data on variance and significant differences. Variations of band intensities, caused by minor discrepancies in gel loading quantities as well as reduced efficiencies in band staining, already result in considerable variances between gels or even between lanes. Moreover, inconsistencies in RNA-isolation procedures and RT-PCR performance efficiency, non-quantitative methods of RT-PCR mask or may change ,e.g., exposure concentration- or time-dependent effects. Therefore, the evaluation and subsequent discussion of the RT-PCR results derived from these studies, were based on a qualitative rather than a quantitative approach.

Critical remarks on the RT-PCR method

"Housekeeping" genes like β-actin, are usually expressed at moderate levels making them easy to detect, but their expression level is not always constant. It is often affected by experimental treatments, stage of development and cell type. Moreover, β-actin generally was expressed vastly stronger than CYP19A or B. Typically the β-actin mRNA signal overwhelmed the relative RT-PCR reaction and the rare CYP19-specific products went undetected or underestimated, though it was not performed as a multiplex PCR. It is a RT-PCR-specific methodological disadvantage that the expression of the most commonly used 'internal standard' genes often strongly exceeds the expression of the gene investigated. To gain control of this problem, the amount of amplified cDNA or the PCR-product volume for β-actin, which was run on an agarose gel, was reduced by half.

Results

CYP19A and CYP19B mRNA expression in juvenile and adult zebrafish

CYP19A and CYP19B mRNA expression in juvenile, untreated fish

Sexual gonad differentiation in the zebrafish strain used in our experiments takes place between week four (≥ day 30/35) and week eleven (< day 75/80) after fertilization. This is indicated from the histological analysis of normal gonad development (Maack, 2002; unpublished). Early in gonadal differentiation at the end of week five (protogynic stage), before the onset of treatment, both CYP19 forms were expressed in the head and body samples of zebrafish at this early age of 35/36 dpf (Fig. 1, Fig. 2). A characteristic feature was the prominent transcript abundance of CYP19B in heads. The expression of CYP19A in heads was considerably lower than CYP19B, even up to nine times lower, as shown in Fig. 1. In the decapitated body samples transcript abundance of both CYP19 forms was found to be highly variable among the fishes. In 36-day-old fish, sampled before fadrozole treatment, CYP19A and CYP19B were rather equally expressed in the bodies, though the expression of CYP19A was slightly higher than CYB19B. The results given by 35-day-old fish, analyzed before MT treatment, disagreed with these findings, showing a predominant

Fig.1

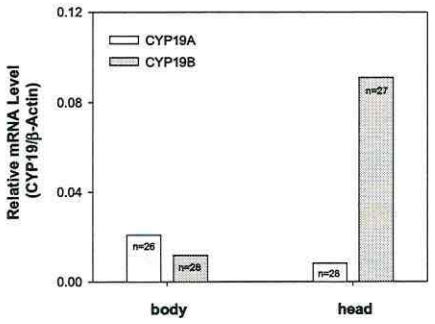

Fig.2

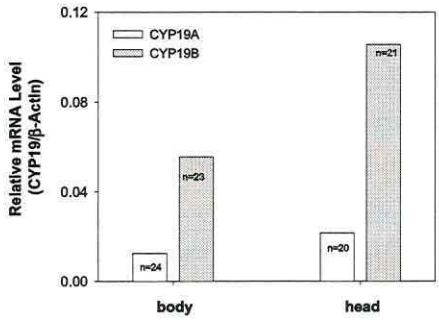

Fig.1 and 2. Relative transcript abundance of CYP19A and CYP19B genes, expressed in head and body of zebrafish (**Fig 1.**) on day 36 post fertilization (pf) (t_0 samples of the Fadrozole experiment) and (**Fig 2.**) on day 35 post fertilization (pf) (t_0 samples of the MT-experiment).
Each bar represents mean transcript abundance of n samples, normalized to the abundance of β-actin gene transcript in the corresponding sample each. The number of samples analyzed (n) are indicated in the bars. For each sample, independent RT-PCR reaction duplicates were measured for CYP19A, CYP19B and β-actin.

expression of the (brain-derived) CYP19B gene in body samples. The CYP19B transcript abundance almost reached half of the expression level of CYP19B mRNA in heads (Fig. 2).

On day 71 pf (at the end of exposure period), transcript abundance of both CYP19 forms in unexposed control zebrafish changed in comparison to the pattern of expression observed in 35-day-old fish (Fig. 3). Contrary to the earlier stage of development, the predominantly expressed aromatase mRNA form in fish at this time of development (71 dpf) was CYP19A in body samples. In the heads, the abundance of the corresponding CYP19B mRNA transcript was still the highest, but in proportion to the relative expression of CYP19A in the bodies less prominent than in 35-day-old fish. Transcript abundance of the non-corresponding CYP19 forms, CYP19B in body samples and CYP19A in head samples respectively, were considerably lower than the corresponding transcripts.

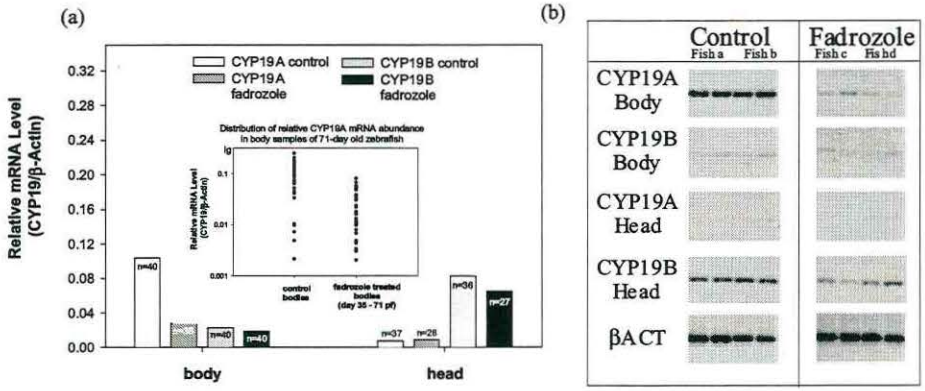

Fig. 3. Fadrozole experiment:
(a) Relative transcript abundance of CYP19A and CYP19B genes, expressed in head and body of zebrafish on day 71 post fertilization (pf) after 36 days of fadrozole treatment (500 µg fadrozole/g of food) (t_{end} samples). Each bar represents mean transcript abundance of n samples, normalized to the abundance of β-actin gene transcript in the corresponding sample each. The number of samples analyzed (n) are indicated in the bars. For each sample, independent RT-PCR reaction duplicates were measured for CYP19A, CYP19B and β-actin. (b) RT-PCR analysis of CYP19A, CYP19B and β-actin mRNA expression, depicted for representative control (left column) and fadrozole-treatment samples (right column).

Effects of fadrozole treatment

After continuous feeding of zebrafish with fadrozole-treated food from day 35 pf to day 71 pf, transcript abundance of CYP19A and CYP19B in head and body samples of 71-day-old (pf) fishes (at t_{end}) was clearly affected by the treatment, as shown in Fig. 3. In body samples of fadrozole treated fish CYP19A mRNA expression was considerably diminished compared to the control fish. Expression of CYP19B mRNA in the body parts however,

remained unaffected by the fadrozole administration. In head samples, the CYP19A expression was not influenced by fadrozole, but the CYP19B transcript expression was attenuated compared to the control head samples.

All fish sacrificed at this time (71 dpf) presumably had already reached an advanced stage of sexual gonad differentiation (Maack 2002). Nevertheless, they could not be sexed by (secondary) sexual characteristics, like the coloring or the macroscopic structure of the gonads. At least in the control fish, the abundance of CYP19A mRNA could be divided into two distinctly different subgroups, in which the CYP19A transcript levels in the body was either lower than 0.03 (n=5) or high than 0.03 (n=35) expression. The distribution of CYP19A mRNA levels measured in body samples of these 71-day-old control fish is displayed in the inset of Fig. 3. 0.03 corresponded to the 95 %-confidence interval of data distribution.

On day 161 pf, almost eight weeks after (control) fish had reached the adult, reproductive stage, CYP19A and CYP19B mRNA expression was measured in gonad and brain of control as well as of previously fadrozole exposed fish (Fig. 4). The application of fadrozole-treated food had been stopped on day 71 pf and from this day on treated fish were fed with untreated food until day 161 pf (end of test). In adult, reproducing control fish (Fig 4a), CYP19A mRNA expression in gonads showed a sexual dimorphic pattern. In all fish that were sexed as males by their external morphology and by their well-developed testes, the average gonadal CYP19A transcript abundance was significantly lower (< 0.1) than in gonads of control females, in which the CYP19A mRNA levels were always > 0.1 (Fig. 4a). According to these sex-specific expression levels of CYP19A found in gonad tissue of control fish, fadrozole-treated fish were differentiated on the basis of the abundance of CYP19A mRNA in their gonads. The treated fish were assigned to two different groups, composed of fish that show either low ($\leq 0.1 \Rightarrow$ group 1) or high ($> 0.1 \Rightarrow$ group 2) gonadal CYP19A mRNA expression. This categorization of CYP19 mRNA expression data was applied because all adult fish that were fed with fadrozole between day 35 and day 71 pf, could either not be sexed by their outward appearance and/or gonad morphology or they showed definitely male phenotype. These fish did not show courtship behavior or any reproductive activity. The anatomic structure of gonads of these fish could not unambiguously be defined as ovaries or testes. In spite of this, mean expression levels of CYP19A in gonads of fadrozole-treated fish could be divided into the two different subgroups, in which the CYP19A transcript abundance corresponded either to the range of control female or the range of control male gonads. The assignment of the fadrozole

treated fish achieved in this way for group 1 or group 2 was also conferred to the CYP19A and B mRNA transcript levels in brain of the same fish.

In correspondence with the findings in 35-day-old and 71-day-old fish, for each of the two CYP19 gene transcripts in control fish, the strongest expression was recorded in the corresponding organ, for CYP19A in gonads and for CYP119B in brain samples. The highest transcript abundance in control male and female fish, was measured in brain samples for the CYP19B transcript (Fig. 4b). The mean abundance of CYP19B was in the range of 0.4 and equally in both sexes. The expression of the non-corresponding CYP19 transcripts (CYP19B in gonads and CYP19A in brain) however, was below 0.1 and did not indicate sexual differences.

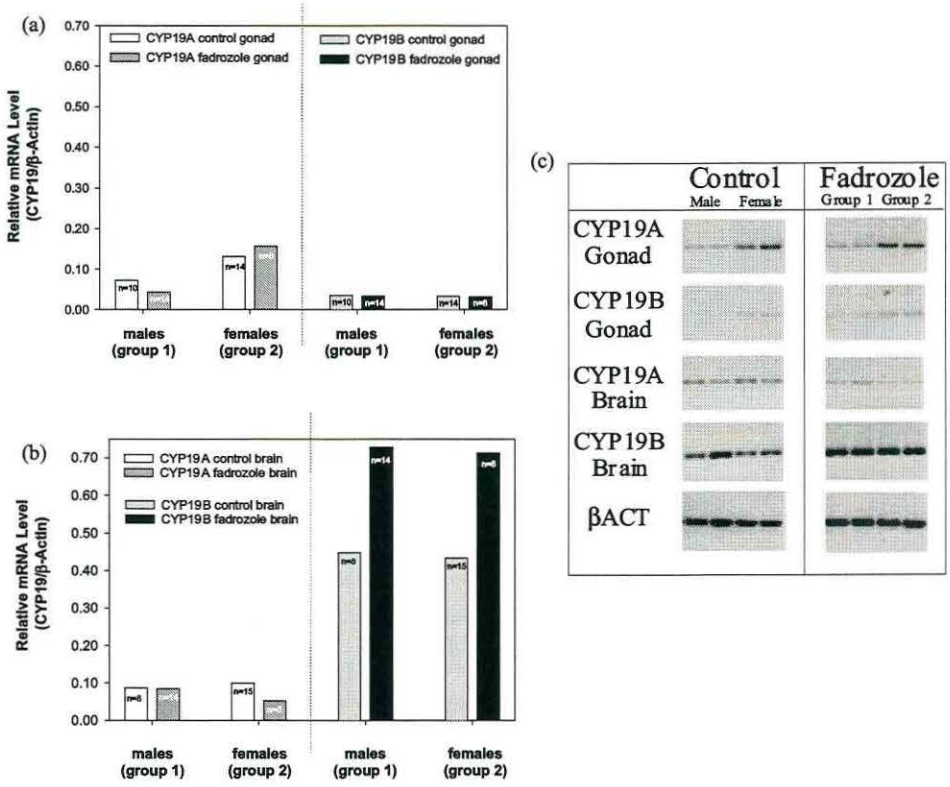

Fig 4. Fadrozole experiment:
Relative transcript abundance of CYP19A and CYP19B genes in gonad (a) and brain (b) of adult zebrafish on day 161 post fertilization (pf) (treproEnd samples). Fadrozole-treated fish were categorized into two differential sub-groups, according to the CYP19A mRNA abundance measured in the gonads. Each bar represents mean transcript abundance of n samples, normalized to the abundance of β-actin gene transcript in the corresponding sample each. The number of samples analyzed (n) are indicated in the bars. For each sample, independent RT-PCR reaction duplicates were measured for CYP19A, CYP19B and β-actin. (c) RT-PCR analysis of CYP19A, CYP19B and β-actin mRNA expression, depicted for representative control (left column) and fadrozole-treatment samples (right column).

In fadrozole treated fish, the expression of CYP19B in gonad tissues appeared to be unaffected by the fadrozole treatment (Fig. 4a). Relative transcript abundance was below 4 % in both sexes and both groups, respectively, and there were no differences between both groups and the corresponding female and male control gonads. CYP19A transcript abundance in group 1-gonads indicate a reduction of expression in fadrozole treated fish, whereas the expression of CYP19A in group 2-gonads were enhanced compared to the control samples. Generally, the abundance of CYP19A transcript in control gonads was twice as high in females as in males, in fadrozole-treated group 2 even more than three times higher than in group 1. In the brain the expression patter of both CYP19 transcripts was almost identical in group1 and group 2 fish (Fig 4b). CYP19A mRNA expression did not show a strong fadrozole-derived effect, only in group 2 brain samples the expression was reduced by approximately 50 % versus the control females. The fadrozole administration, however, caused an induction of CYP19B expression in the brain and the mean transcript abundance of all fadrozole-treated adult fish increased about 60 % above the abundance in brains of the control fishes. Evidently, after treatment with fadrozole between day 35 and day 71 pf, the expression of the tissue-specific CYP19 genes, CYP19A in gonads and CYP19B in the brain, was still altered compared to the controls. 35 days of fadrozole treatment during the period of gonad differentiation attenuated the mRNA expression of both CYP19 genes, but after more than three months of recovery time from the fadrozole treatment the mRNA expression of both genes, CYP19A and CYP19B, was higher than in untreated fish.

Effects of methyltestosterone treatment

In marked contrast to the inhibitory effect of fadrozole on CYP19 expression, exposure to 17α-methyltestosterone (MT) between day 35 and day 71 pf increased CYP19B transcript abundance in head samples of 71-day-old zebrafish (Fig. 5). The CYP19B expression in head samples of MT-treated fish was more than five times higher than in the controls. On the contrary, the CYP19A transcript abundance in body samples of treated fish was more than 50 % lower as observed in control body samples. The abundance of the non-corresponding CYP19 transcripts in head and body samples, respectively, remained mostly unaffected by the MT treatment, though the mean expression level of CYP19B in decapitated bodies was slightly increased. The CYP19A expression in heads was below 1.0 % and thus even lower than in heads of control fish of the same age in the fadrozole study (Fig 3). This corresponded to an opposing effect of MT in head samples as caused by the fadrozole treatment. In control fish however, the abundance of both CYP19 transcripts in

head and the in decapitated body parts showed good agreement with the expression of CYP19 transcripts in the control samples of fishes the same age of the fadrozole test.

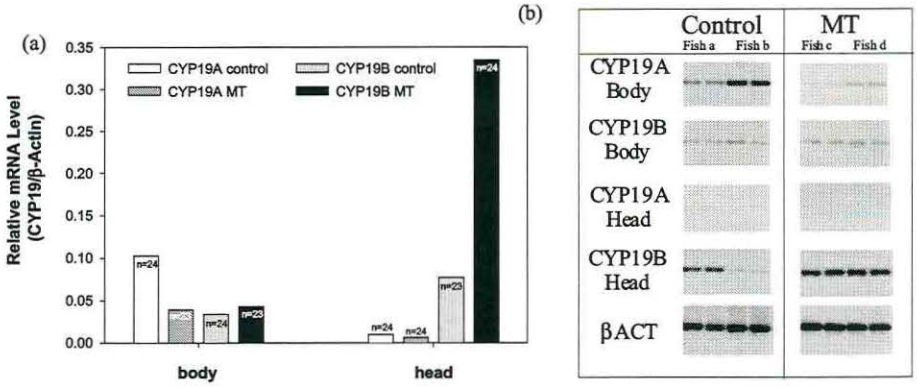

Fig 5. MT experiment:
(a) Relative transcript abundance of CYP19A and CYP19B genes, expressed in head and body of zebrafish on day 71 post fertilization (pf) after 36 days of MT treatment (10 µg/L) (t_{end} samples). Each bar represents mean transcript abundance of n samples, normalized to the abundance of β-actin gene transcript in the corresponding sample each. The number of samples analyzed (n) are indicated in the bars. For each sample, independent RT-PCR reaction duplicates were measured for CYP19A, CYP19B and β-actin.(b) RT-PCR analysis of CYP19A, CYP19B and β-actin mRNA expression, depicted for representative samples control (left row) and MT-treated fish (right row).

At the end of the MT study on day 160 pf, fish in one replicate had been exposed to 10 µg/L MT only between day 35 and day 71 pf, fish of the second replicate were continuously exposed to 10 µg/L of MT. Up to the reproducing age, all MT-treated fish could not be sexed by their secondary sexual characteristics or the macroscopic gonad structure. Consequently, according to the approach in the fadrozole experiment, MT treated fish were categorized by their abundance of CYP19A expression in gonads and assigned to two different subgroups. In group 1, the expression levels of CYP19A in gonads of MT treated fish corresponded to the range of expression levels, measured in gonads of control male fish (≤ 0.1) and in group 2, the CYP19A expression corresponded to the range as found in the gonads of control females (< 1.0). In gonad tissue of adult, 160-day-old control fish, the transcript abundance of CYP19A was six times higher in females than in males and reached a mean expression level of 0.2 (Fig. 6, Fig. 7). The expression of CYP19B in control gonads corresponded roughly with the expression of CYP19A in males and did not show consistent differences between male and female. In brain samples of control fish, the transcript abundance of CYP19B exceeded the value of 0.3 in both sexes,

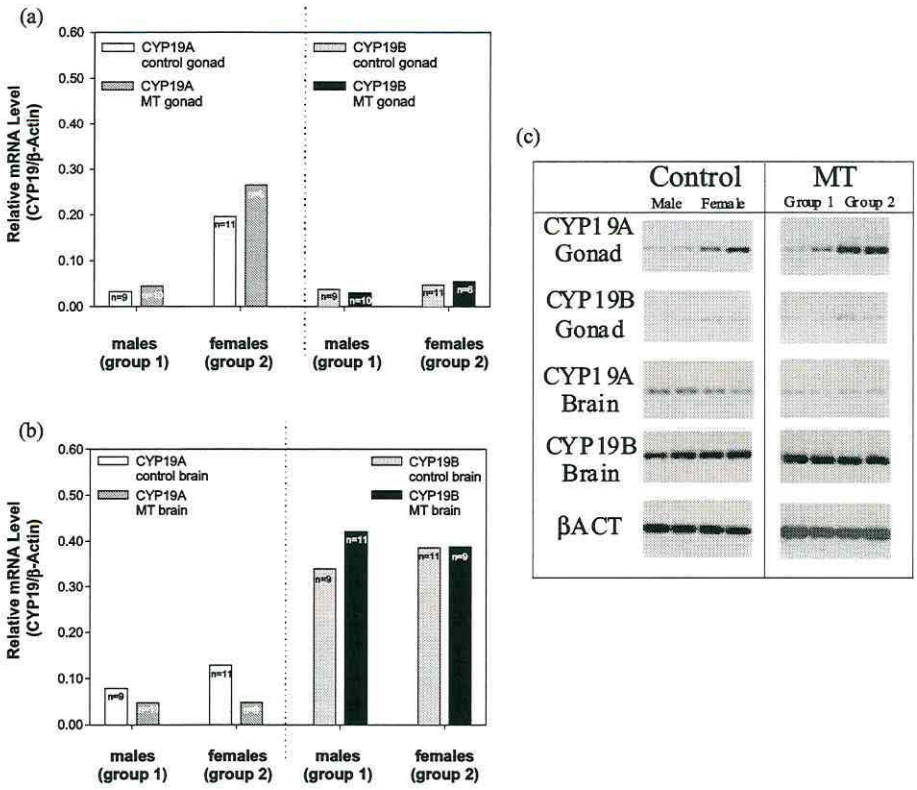

Fig 6. MT experiment:
Relative transcript abundance of CYP19A and CYP19B genes in gonad (a) and brain (b) of adult zebrafish on day 161 post fertilization (pf) ($t_{reproEnd}$ samples). Fish were treated with 10 µg/L of MT between day 35 and day 71 pf. MT-treated fish were categorized into two differential subgroups, according to the CYP19A mRNA abundance measured in the gonads. Each bar represents mean transcript abundance of n samples, normalized to the abundance of β-actin gene transcript in the corresponding sample each. The number of samples analyzed (n) are indicated in the bars. For each sample, independent RT-PCR reaction duplicates were measured for CYP19A, CYP19B and β-actin. (c) RT-PCR analysis of CYP19A, CYP19B and β-actin expression, depicted for representative control and MT-treatment samples.

without showing pronounced sex-specific differences. This hold for the expression of CYP19A in the brain of control fish as well.

The MT exposure between day 35 and day 71 pf altered persistently only the CYP19A mRNA expression. The CYP19A expression in gonads was slightly but distinctly (by approximately 30 %) enhanced in group 2-fish (Fig. 6a) compared to the controls. The CYP19A mRNA expression in gonads (Fig. 6b) was reduced after the MT treatment as well in group 1 as in group 2 brain samples. Expression of CYP19B transcript in brain samples on the one hand remained almost unaffected by the MT exposure in group 2-fish. In group 1-fish, on the other hand, the expression of CYP19B in the brains was increased

over the control level. In gonads, however, the CYP19B mRNA transcript was less intensively expressed in group 1- than group 2-fish.

In the group of fish which was continuously exposed to MT between day 35 and day 161 pf, the expression pattern of CYP19A and CYP19B mRNA corresponded roughly to that observed in fish, only temporarily exposed to MT between day 35 and day 71 pf. In the continuously MT-exposed fish the abundance of the CYP19 transcripts in gonad samples was changed by the MT treatment only in group 1-fish (Fig. 7a); the expression rate of CYP19B was more than doubled compared with the controls. CYP19A transcript abundance, however, did not indicate any effect of MT treatment, neither in the gonad nor in the brain samples of both treatment groups (group 1 and group 2). The highest expression levels of CYP19 in these fish were measured in brain samples for CYP19B (Fig. 7b). The transcript abundance of CYP19B in MT treated adult fish exceeded 0.5 in both groups and therefore was 30-40 % higher than in brain samples of control male or female fish.

As a general observation in brain samples, the expression pattern of both CYP19 transcripts did not reveal any unequivocal sexual differences, though in all adult fish that were either exposed to fadrozole or to MT, a twice as high expression of CYP19A mRNA in the brain of group 2 fish as in the control fish was observed (Fig. 4 and Fig. 6b, Fig. 7b).

Long-term exposure until day 161 pf to 10 µg/l of MT particularly influenced the CYP19 gene expression in the brain. CYP19B transcript abundance was notably increased above the control level in all MT exposed fish, while the abundance of the CYP19A transcript was reduced in group 2 fish only (Fig. 7). In gonad tissue, however, the expression of CYP19B was doubled versus the control and may indicate a MT exposure effect.

None of the fishes continuously exposed to MT between day 35 and day 161 pf could be sexed undoubtedly by their macroscopic gonad structure or the phenotypic appearance. However, two distinct groups of fish were distinguishable, having CYP19A mRNA expression levels in gonads in the range of either control female or control male fish. In accordance with this categorization on the basis of the CYP19A mRNA expression in the gonads, these fish would consist of 63 % (n=12) of "male CYP19A expresser" and 37 % (n=7) of "female CYP19A expresser" (data not shown in the figures; proportions were

calculated on the basis of the number of samples/fish (n) from the MT treatment group assigned to CYP19A expression group 1 or 2, as indicated in Fig. 7a).

Adult (on day 161 pf), fadrozole treated fish could not be sexed by external sexual characteristics and were assigned to two differential groups of distinct expression levels of CYP19A in gonads as well. Thus, 36 % (n=8) of these fadrozole treated fish showed control female-like expression levels of CYP19A in the gonads, 64 % (n=14) control male-like levels (data not shown in figures; proportions were calculated on the basis of the number of samples/fish (n) from the fadrozole treatment group assigned to CYP19A expression group 1 or 2, as indicated in Fig.4a).

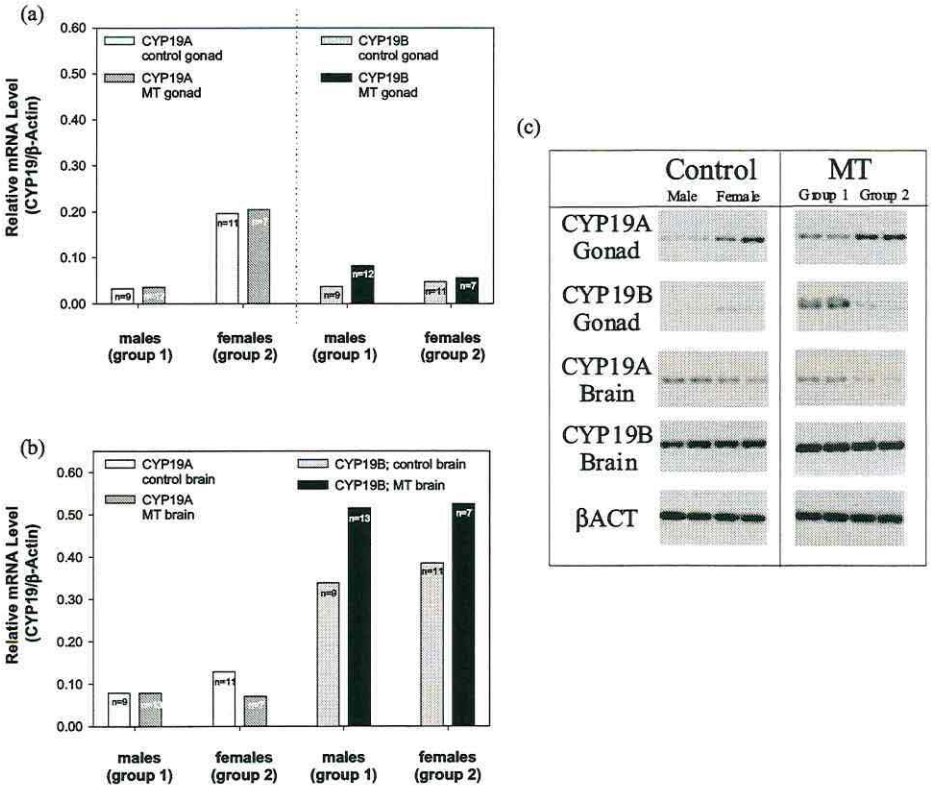

Fig 7. MT experiment:
Relative transcript abundance of CYP19A and CYP19B genes in gonad (a) and brain (b) of adult zebrafish on day 161 post fertilization (pf) ($t_{reproEnd}$ samples). Fish were treated with 10 µg/L of MT between day 35 and day 161 pf.. MT-treated were categorized into two differential subgroups, according to the CYP19A mRNA abundance measured in the gonads. Each bar represents mean transcript abundance of n samples, normalized to the abundance of β-actin gene transcript in the corresponding sample each. The number of samples analyzed (n) are indicated in the bars. For each sample, independent RT-PCR reaction duplicates were measured for CYP19A, CYP19B and β-actin. (c) RT-PCR analysis of CYP19A, CYP19B and β-actin mRNA expression, depicted for representative samples.

Gonad histology of fadrozole and MT treated zebrafish

Before the onset of treatment, the gonads of 35/36-day-old fish showed either a protogynic ovary or an undifferentiated gonad. Gonads were classified to be undifferentiated when they contained early germ cells only that could be not unambiguously identified as oogonia or spermatogonia, as the distinction between male- and female-specific germ cells in developing fish is difficult solely on the basis of cell morphological characteristics (e.g. Parmentier and Timmermans 1985). The majority (96 %) of fish examined displayed oocytes of the perinucleolar stage, in which the relative fraction of the gonad tissue occupied by perinucleolar oocytes showed pronounced inter-individual variations. Three major forms of ovary-like gonads could be described in these five-week-old fish. The gonad tissue in some fish was predominantly composed of densely packed perinucleolar oocytes; only with some nests of undifferentiated germ cells in the periphery of the gonadal tissue. In other individuals, the dorsal part of the gonads contains densely packed large perinucleolar oocytes, some with basophilic ooplasm. The basal parts of theses gonads contained rows with small-sized early stages of perinucleolar oocytes as well as undifferentiated germ cells. Then, there were some fish with only few, small perinucleolar oocytes in the gonadal tissue, accounting for not more than 10 – 20 % of gonadal volume, while the rest of the gonad consisted of undifferentiated germ cells or groups of non-germ cells. Only in one 35-day-old fish, there were first indications of transforming processes of germ cells to testicular cell types. Overall, all gonads examined were of a protogynic type at the age of 35/36 dpf.

At the end of the exposure period on day 71 pf, which corresponded roughly with the end of gonadal differentiation, most control fish showed either well-differentiated ovaries or early stages of testes (Fig. 9). In 22 % of 71-day-old control fish, the gonads were in an undifferentiated or in the transformation process from the ovarian into the testis structure. In five of these fishes, the gonads were still composed of undifferentiated germ cells, but theses cells were arranged in cyst-like formations and may therefore referred to early stages of testes. In three fishes, no cyst-like structures were found in the gonads and hence they were defined as undifferentiated (Fig. 8). As result of this histological assessment, control fish on day 71 pf were composed of 31 % males and 61 % females, while 8 % of fish were still in the process of sexual differentiation.

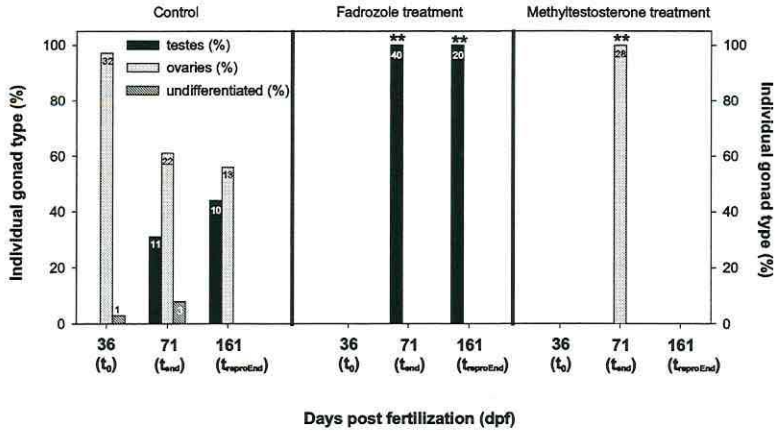

Fig 8. Fadrozole and MT experiment:
Distribution of gonadal sex types based on histological analysis. Gonad sex ratios of control fish at three different time points during sexual differentiation are indicated in the left half of the figure, in the middle of figure, gonadal sex ratio of fadrozole treated (500 µg/g of food) fish at the end of the exposure period on day 71 pf and at the end of the experiment on day 161 pf is shown. In the right half of figure, gonadal sex ratio of 17α-methyltestosterone treated (10 µg/L) fish at the end of the exposure period on day 71 pf is shown. Number of samples (n) analyzed are indicted in the bars.
** Significantly different from the control (χ^2 test, $p<0.001$)

The gonad structure of fadrozole treated fish of the same age (71 dpf) was clearly different to control fish. Fadrozole treated fish had either testicular gonad structures (Fig. 9d) or undifferentiated gonads with cyst-like arrangement of the germ cells. No ovaries or even oocytes were found. 100 % of fadrozole treated fish were male at the end of the exposure period on day 71 pf. When, after termination of the treatment, fish were reared until the reproductive age in non-contaminated water, no spawning took place. This was in contrast to the control where normal courtship and mating behavior occurred from day 83 pf on. Spawning success was not quantified, but showed continuity in both replicate vessels since day 108 pf and the fertilization of eggs was on average above 80 % (data not shown). The assessment of gonad histology of these reproducing control fish at the end of the study, on day 161 pf, did not reveal any unusual features. 44 % of fish had normally developed and differentiated testes, 56 % of fish had inconspicuous ovaries. Fish, that were treated with 500 µg/g of fadrozole between day 35 and day 71 pf, on the other hand did also show well-developed inconspicuous gonads, but only testes. Thus, application of 500 µg fadrozole/g of food during the sexual differentiation period caused a complete and persistent gonadal "masculinization" in zebrafish. Not surprisingly, reproduction could not be recorded in these fish.

Fig. 9

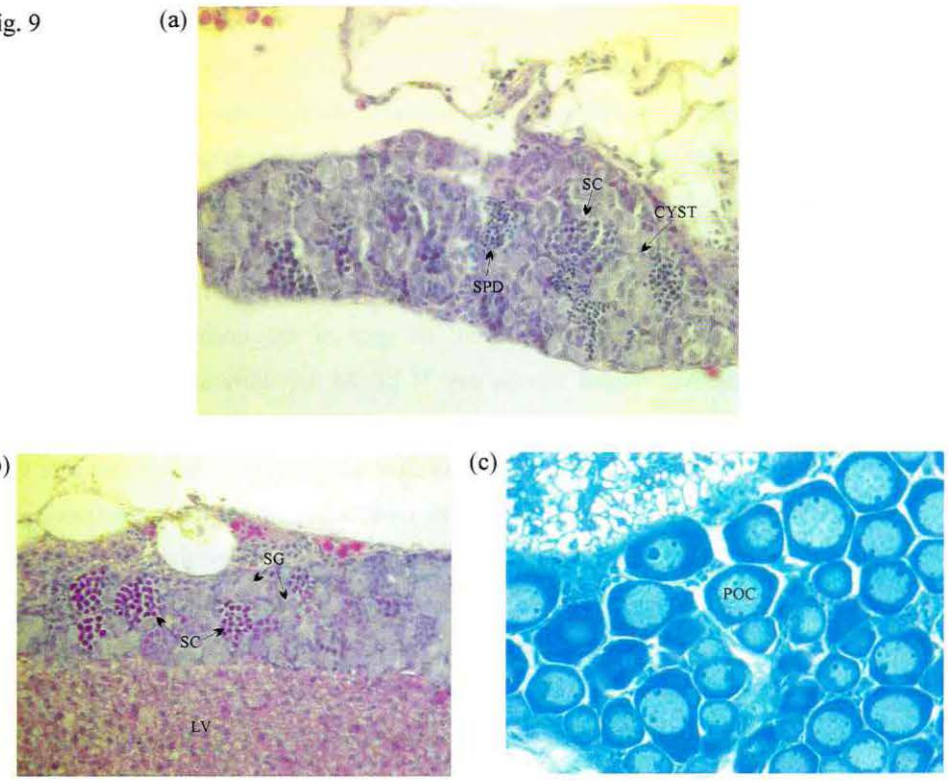

Fig. 9.
Fadrozole-test:
Histological sections of an ovary and testes of 71-day-old zebrafish (X 400), HE stained:

(a)This is an early testis of a control fish with gonadal tissue containing still larger areas of spermatogonial cysts (CYST), but also groups of spermatocytes (SC) as well as spermatids (SPD)

(b) Another testis of control fish: The right and the left parts of the testis are found in a dorso-caudal position in the body cavity, attached to the peritoneum from where they extend to the liver (LV). Chromatin granules of spermatogonial nuclei are stained intensively; several groups of spermatocytes (SC) are visible, but no spermatids.

(c) Part of an ovary of a control fish (stained with methylene blue): The ovary is filled with perinucleolar oocytes (POC) of rather homogenous size. The morphology of this ovary corresponds to the normal stage of differentiation at this age, as described for the gonad development of zebrafish (Maack 2002).

(d) Testis of a fish that was treated with fadrozole between day 35 and day 71 pf. In the fadrozole-treated fish at day 71 pf, the gonads are of the same stage of differentiation and show the same histological features as do control testes - i.e., smaller or larger areas of the tissue are occupied by cysts (CYST) with spermatogonia, then there are spermatocytes (SC) and in some indivduals already spermatids (SPD). (Here on the micrograph a developing seminiferous tubule is visible).

In the MT-study, gonad histology of fish was assessed only for the end of the treatment period between day 35 and day 71 pf, but not at the adult, reproducing stage. However, quite the opposite to the findings of the fadrozole study, 71-day-old fish that were treated with 10 mg/l of MT since day 35 pf, showed only ovary-type gonads. The ovaries were relatively large, almost entirely filled with densely packed perinucleolar oocytes of homogenous size distribution. Ooplasm area of most oocytes was enlarged and stained strongly basophilic. At the cranial and caudal periphery of most gonads, some nests with germ cells or oogonia were observed. In spite of the unambiguous ovarian gonad morphology, the MT treated fish on day 71 pf did not show unequivocally female-like external characteristics.

In the course of the MT study, MT exposure was continued in one replicate vessel of the treatment parallels, whereas in the other replicate vessel the treatment was omitted. In the control duplicates, development of courtship and mating behavior was observed and the onset of spawning was recorded on day 121 pf. The fertilization of spawned eggs was on average above 80 % (data not shown). After several weeks of constant reproductive activity and successful output (fecundity) in the control fish groups, the study was completed on day 161. Until that day, no reproductive activity nor spawning had been recorded in the MT treatment parallels, neither in the transiently MT exposed fish (during sexual differentiation) nor in the long-term exposed fish.

Growth effects

Lengths and weights of control and fadrozole/MT treated fish, recorded on the days 35/36, 71 and 161 pf, are shown in Fig. 10, in the appendix. In both experiments, effects on growth (length and weight) were observed in all treatment groups at the adult stage. Fadrozole treatment (500 µg/g of food) and MT treatment (10 µg/L) between day 35 and day 71 pf had a positive effect on growth. In fadrozole treated fish, mean body length and weight was significantly increased compared to control fish only in adult, 161-day-old fish. In MT treated fish, however, mean length and weight were significantly higher that in the controls already on day 71 pf. Contrary to this, the long-term treatment with MT (10 µg/L) until day 161 pf suppressed significantly the growth of fish.

Discussion

Sexual differentiation in lower vertebrates including fish is a very labile process, and in many cases a high degree of plasticity of phenotypic sex persists throughout the whole life span. In contradiction to mammals, sexual differentiation in fish takes place after hatching, and in several species only after years (Blazquez et al. 1998). Thus, during the developmental period when gonads are still sexually undifferentiated, fish remain especially susceptible to the action of endogenous as well as exogenous hormonal factors. Therefore an especially high susceptibility of teleost fish to environmental endocrine-active or endocrine disrupting compounds (EDCs) is presumed within this critical developmental window. Most studies on endocrine disruption in fish so far, focused on steroid hormone receptor-mediated, mainly estrogenic, effects. However, endocrine modulating effects that affect sexual differentiation and reproduction of fish, are not only estrogenic, they interfere also with non-steroid receptor mediated processes like the steroidogenesis and its participating enzymes. Due to the high variability of mechanisms of action of EDCs, interference may appear most probably on all hierarchical levels of steroidogenesis, and particularly aromatase, control.

In this study, the differential expression of both CYP19 mRNA transcripts, the ovary-derived CYP19A and the brain-derived CYP19B, were investigated in zebrafish in relation to the process of sexual differentiation. In all fish examined during this study, CYP19A mRNA as well as CYP19B mRNA were detected in head and trunk samples of young fish or in brain and gonad samples of adults. The highest expression of each transcript was found in the corresponding body part or organ, i.e., for CYP19A in trunks or in the gonads, for CYP19B in heads or in the brains. In the ontogeny on day 35 pf, which coincides with the protogynic stage in gonad differentiation, the predominantly expressed CYP19 mRNA transcript was CYP19B in the heads. On day 71 pf, however, the highest transcript abundance was observed for CYP19A in the trunks. At the end of the reproductive period on day 161 pf, a sexually dimorphic pattern of CYP19A mRNA expression was evident in the gonads of adult fish in which the CYP19A expression is distinguishably higher in females than in males. However, a sex-related expression of the CYB19B mRNA transcript in the brain was not revealed.

Treatment of zebrafish with fadrozole and 17α-methyltestosterone (MT) respectively, during the period of sexual differentiation, altered aromatase gene expression in the brain

and the gonad and adversely affected gonad differentiation. In the case of fadrozole, CYP19A mRNA expression in the trunks samples was shown to be markedly suppressed after the treatment. Then, in the cause of the fadrozole treatment, an upregulation of the CYP19B transcript was recorded in the brain of adults. Histological examination of the gonads of the fadrozole treated fish only showed differentiated testes. Temporary treatment with MT between day 35 and day 71 pf, mainly affected the CYP19B expression in heads which was tremendously upregulated directly after treatment. This effect of MT was maintained when the treatment was continued until the adult stage. In the MT treated fish a 'feminizing' effect of the treatment on the gonad differentiation was confirmed by the histological findings, at least for 71-day-old fish. These results clearly provide evidence that gonadal sex differentiation in the protogynic zebrafish is correlated with the differential expression of aromatase genes.

Gonadal development and expression of CYP19A and CYP19B mRNA in unexposed zebrafish

Apart from one single study that examined the expression of both types of CYP19 genes in the very early developmental time directly after fertilization and then until day 40 pf (Trant et al. 2001), this study here is one of the first approaches that describes the differential expression of CYP19A and CYP19B in the cephalic and non-cephalic region of (unexposed) zebrafish during and after the period of gonadal sex differentiation.

In sexually undifferentiated zebrafish around day 35 pf both brain and gonadal type aromatase mRNA were found in head and in body samples. The abundance of both CYP19 mRNA forms in head and body showed a dimorphic pattern of expression (Fig. 1, Fig. 2): CYP19A was most abundant in the body and CYP19B in heads, respectively, at least in one of the two studies. The strongest expressed CYP19 mRNA was CYP19B in heads. This was an interesting finding, because in the other few recent studies on CYP19 expression in zebrafish, the organ-specific expression of both genes has been discussed controversial. These studies (Kishida and Callard 2001, Trant et al. 2001) consistently confirm a predominant expression of the CYP19A transcript in ovarian tissue and of the CYP19B transcript in brain tissue. The highest transcript abundance found in both studies in any kind of tissue was of CYP19B in the brain of female as well as male zebrafish. Regarding the expression of CYP19 in gonadal tissue, however, only Trant et al. (2001) confirmed an expression of both CYP19 genes in ovary as well as in testis tissue. In testes, the expression levels of both forms were shown to be quite similar, whereas the CYP19A

transcript clearly dominated in the ovaries. This largely agrees with the findings of our study. On the contrary, Kishida and Callard (2001) did not confirm a detection of CYP19A and CYP19B mRNA in testis tissue. They observed an expression of both forms only in the eyes.

While the expression of CYP19A mRNA in body samples of 35-day-old fish was comparably low, CYP19A in 71-day-old control fish of the fadrozole and the MT study, however, clearly indicated high gene activity in the body. As it was shown by the histological evaluation of the gonads (Fig. 8), all fish on day 35/36 pf were still at the protogynous gonadal stage. Most fish displayed 'early ovary' gonads that were mainly composed of perinucleolar oocytes, with no indications of transforming processes. Day 71 pf, however, corresponded with the period of ongoing bisexual differentiation of gonads. Histology identified testicular and ovarian germ cells that were still in the process of differentiation. Identified testes were described as 'early testes' as there occurred generally no spermatids. As shown by the high expression of CYP19A in these fish, the developmental period was obviously characterized by a high gonadal aromatase activity. At the still undifferentiated, protogynic gonadal stage on day 35 pf, on the other hand, expression of CYP19A in gonads is detectable but at a very low level. This may indicate to a low aromatase enzyme activity also. Hence, the process of sexual gonad differentiation coincides with a substantial change in aromatase gene activity, and a pronounced estrogen synthesis in gonads of prospective female zebrafish has to be presumed. A correlation between ovarian differentiation and the aromatase gene expression in ovarian cells has already been demonstrated in many fish species, although in most cases in gonochorists. In rainbow trout, Guiguen et al. (1999) demonstrated that the aromatase mRNA in female gonads was detectable by RT-PCR first, two to three weeks before the first sign of histological sex differentiation and then was continuously expressed at high level during ovarian differentiation. Similarly, in whole body extracts of Japanese flounders (*Paralichthys olivaceus*),changes of E_2 levels during sexual differentiation were found to correspond with the abundance of aromatase mRNA recorded in the gonads (Kitano et al. 1999). Both parameters increased in a time-dependent manner during sexual differentiation of females and dropped at the end of this period.

The high expression of CYP19B in 71-day-old zebrafish is considered to indicate a dictating function of CYP19B expression in the brain in the initiation of the gonad differentiation process. Trant et al. (2001) demonstrated a dichotomic divergence of CYP19B expression in zebrafish larvae with the onset of gonadal differentiation and it was

assumed that this was sex-linked. Trant and colleagues suggested a dictating function of CYP19B in sex differentiation of the zebrafish, correlated with a sexual dimorphic pattern of CYP19B expression. This assumption is highly speculative and demands for further investigation. In our study, we did not recognize any sex-specific significant differences in the (cerebral) CYP19B expression. CYP19B was found to be expressed at high levels at any time measured in this study during zebrafish development, as well in the heads of younger fish as in the brain of adult fish, and equally abundant in both gender. This was not an unexpected result as teleost fish are well-known for their exceptionally high levels of neural aromatase (Pasmik and Callard 1985; Callard et al. 2001). The concrete function of high aromatase levels in the brain of teleost fish has still to be clarified. Due to the high complexity of aromatase expression in the brain, it can only be speculated cautiously about the reasons as this would demand for a cellular localization of expression. In the contrary to the findings in mammals, the brain proved to be the predominant estrogenic organ in fish , and not the ovary.

Gonadal development and expression of CYP19A and CYP19B mRNA in zebrafish exposed to fadrozole

An important aim of this study was to explore the implication of zebrafish exposure to an aromatase inhibitor on gonadal and cerebral expression of the CYP19 genes, and on gonadal differentiation. It was hypothesized that the application of fadrozole during the period of sexual gonad differentiation may skew this process in a male direction. And indeed, all fadrozole treated fish clearly showed male phenotype at the end of the study and they also gave no indication to a certain mating behavior or even spawning. In spite of this unambiguous result, CYP19A transcript abundance in gonads was segregated into two categories of either "low" or "high " gonadal CYP19A mRNA abundance (Fig. 4a). These two subgroups of CYP19A expression levels were in the range of "normal" male or female CYP19A transcript levels in the gonads of control fish of the same age. This is an amazing result as, apart from the all-male phenotype, the acute impact of fadrozole had already been stopped almost three months earlier. It can be assumed that the administration of fadrozole, and therefore the inhibition P450arom enzyme activity, has taken place within a critical period of gonad development in which a precise regulation of steroid synthesis is absolutely indispensable for a directional differentiation of the genetically determined gonad morphology and sex. Gonadal differentiation in fish is a very labile process, which is highly susceptible to disturbances of sex steroid metabolism and homeostasis.

Consequently, this developmental period is responsive to a switch in the gonadal sex of fish. As the application of fadrozole interfered in the process of sex differentiation by modifying aromatase enzyme activity (and therefore reduced estradiol synthesis), this developmental stage-specific treatment in fact affected the development of the phenotypic sex, while the sex-specific, genetically based regulative processes for the maintenance of genetic sex persisted. After the treatment with the aromatase inhibitor had stopped, genetic sex-derived regulative loops might gradually be reactivated. However, to gain insight in the regulative mechanisms of sex-derived gene expression was not the aim of this study and requires further investigations. At least it can be concluded from our findings, that the aromatase inhibitor was able to alter sex differentiation but not sex determination. And the maintenance of female sex, also in fish, is strongly correlated with the synthesis of estradiol.

Nevertheless, by the findings of this study the question was raised why the inhibition of aromatase in zebrafish by the application of fadrozole did also affect (directly or indirectly) the aromatase mRNA expression, in the gonads as well as in the brain. At least in mammals, non-steroidal aromatase inhibitors like fadrozole have proven to inhibit aromatase activity by binding competitively to the enzyme binding site. The inhibitors form tight complexes with the aromatase molecule (K_i value of fadrozole for aromatase = 1.6 nM; Steel et al. 1987). This may stabilize and protect the enzyme against protein degradation. The administration of fadrozole during the critical period of gonad differentiation in zebrafish predominantly suppressed the non-cephalic CYP19A mRNA expression (Fig. 3). We suggest that the suppression of CYP19A expression was a consequence of the inhibition of aromatase enzyme activity and thus the reduction of E_2 synthesis. As one result of the reduced or absent synthesis of E_2 in the gonads, a masculinizing tendency in gonad development was recorded at the end of treatment. Finally, a complete (gonadal) masculinization was caused, even though the treatment was stopped three months earlier (Fig. 8). And moreover, the CYP19B expression in the brain of these phenotypic male fish was elevated above the control level. This implies a consequent effect of the fadrozole treatment on the neuroendocrine regulative level of the CYP19 gene expression. The transcription and presumably also the translation of CYP19B was intensified. The gonadal CYP19A mRNA expression in the gonads, however, gradually returned to the control level (Fig. 4). A direct linkage between both processes, the P450arom enzyme activity and the differential regulation of CYP19A and CYP19B expression, via E_2 appears to be most likely. However, a direct ER-mediated estrogen

responsiveness of the CYP19A gene is unlikely. The promoter regions of zebrafish CYP19 genes were characterized recently (Kazeto et al. 2001; Tchoudakova et al. 2001), but EREs in the promoter region of CYP19A gene were not identified. Although the detection of three putative cAMP responsive elements (CREs) in the 5'-flanking region of CYP19A1 in zebrafish (Kazeto et al.2001) offers the potential that transcription in ovary may be regulated by gonadotropins via cAMP, like in mammals.

The treatment-derived alterations of CYP19 mRNA expression in zebrafish shown in this study are most certainly not a direct cause of the fadrozole or even the MT application. E.g., there are no published data yet, describing a possible (receptor-mediated or non-receptor-mediated) interaction of non-steroidal aromatase inhibitors with gene expression. It is of great importance in this regard to distinguish between cause and effect. First of all, fadrozole (and MT) interfere with the aromatase enzyme protein whereas the object of investigation in this study was CYP19 mRNA expression. We investigated the CYP19 mRNA gene expression, but we look at the implication of alterations of aromatase enzyme activity and consequently the changes of E_2 synthesis in the developing and adult zebrafish. This is a very important aspect of this study, especially in regard to the conceivable impact of endocrine-active compounds on steroidogenesis and P450arom expression/activity. Together with other several studies (Tanaka et al. 1995; Kitano et al. 2000; Scholz and Gutzeit 2000; Chiang et al. 2001) we corroborated the direct and/or indirect influence of E_2 on the aromatase gene expression in fish. In conclusion, an estrogenic control of at least the gonadal CYP19 mRNA in fish appears highly probable and it is also assumed for CYP19B in the brain, as discussed below.

Gonadal development and expression of CYP19A and CYP19B mRNA in zebrafish exposed to 17β-methyltestosterone (MT)

Since in consequence of the inhibition of the P450arom activity a masculinization of gonads was evident, it seemed reasonable to assume that induction of the P450arom activity by the application of substrate would skew the gonad differentiation into the opposite direction. Based on findings of Örn et al. (2000; TemaNord), showing an excess of females in zebrafish in the cause of exposure to MT since fertilization at concentrations > 1.0 µg/L, it was supposed to achieve a similar effect in zebrafish by the application of 10 µg/L of MT during the period of sexual differentiation.

It is not a unknown phenomenon, that the application of androgens at higher concentrations or for extended periods can cause 'paradoxical feminization' in fish

(Hackmann 1971; Reinboth 1980; Piferrer and Donaldson 1991). The first description of total 'paradoxical' sex inversion in fish was described by Müller (1969). He treated juvenile *Hemihaplochromis multicolor*, a tropical freshwater gonochoristic fish species (perciformes, cichlidae), with MT (≥ 50 µg/L) from the beginning of sexual differentiation in this species over a period of 80 to 100 days. At the end, all treated fish were of gonadal female sex. The challenging question behind this approach was, to provide evidence for a central feature of P450arom within this 'paradoxical' effect.

Our study results came close to expectations. Neither the transiently (between day 35 and day 71 pf) nor the continuously MT treated zebrafish could be sexed by their outward appearance or the macroscopic gonad structure until adulthood. Reproductive activity in MT treated fish was never observed. Although gonad histology was only assessed on day 71 pf, the 'feminizing' tendency at this date was definitely confirmed by the exclusive identification of ovary-like gonads. Unfortunately, the gonads of the adult, 161-day-old fish were not assessed histologically, but at least in those fish exposed to MT until the end of study, the gonads appear macroscopically either unambiguously as ovaries or were undefinable, neither as ovaries nor testes.

Concerning CYP19 mRNA expression, the most impressive effect of the MT treatment was the massive induction of CYP19B mRNA expression in the heads of fish on day 71 pf (Fig. 5). These fish had all female gonads (Fig. 8). Moreover, even after long-term exposure to MT from the beginning of gonad differentiation (day 35 pf) until the reproductive stage (day 161 pf), CYP19B mRNA abundance in all treated adult fish was still elevated above the control level. In fish exposed only transiently to MT until day 71 pf, a persistence of CYP19B mRNA expression enhancement in the brain, however, was not recorded (Fig. 6b). These data strongly suggest that the elevation of the CYP19B transcript abundance in the brain of adult zebrafish can be ascribed to a direct consequence of the MT treatment. Although this raised the question whether this stimulating effect on CYP19B expression was an estrogenic or an androgenic effect. Due to its aromatizable structure, MT may undergo aromatase conversion to E_2, but obviously in dependency of the concentration applied. This has consistently been presumed in all fish studies yet, in which MT exposure caused 'estrogenic' effects (Nakamura et al. 1975; Piferrer et al. 1993). That MT indeed undergoes aromatization was only indirectly shown, e.g., by Hori et al (1979) who demonstrated the capability of massive doses of MT to induce vitellogenesis in yearling goldfish, *Carassius auratus*. Nevertheless, the concrete

mechanism of MT action in fish has not been clarified yet. Based on the findings in fish so far, we assume that MT is converted to E_2 at a concentration of 10 µg/L as applied. Provided that this turns out to be true, the upregulation of CYP19B expression in the brain during MT treatment might probably be due the control of CYP19B in the brain via an autoregulatory feedback loop that is driven by E_2. The identification of this autoregulative loop of CYP19B expression was first described for adult goldfishes (Callard et al. 2001; Pasmik et al. 1988) but meanwhile, it has been demonstrated also for zebrafish embryos (Kishida et al.2001; Kishida and Callard. 2001). The identification of ERE's in the promoter region of the CYP19B gene of the zebrafish most recently (Kazeto et al. 2001; Tchoudakova et al. 2001) supports the assumption of a direct estrogen responsiveness of the CYP19B gene. This is an important finding in regard to disruptive effects on the P450arom system, revealing a potential target for a direct interaction of environmental estrogens with the CYP19B gene expression.

Regarding the CYP19A mRNA expression in MT-treated fish, an attenuation of expression was noticed in the trunks of 71-day-old fish at the end of the gonadal differentiation (Fig. 5). In the gonads of adult fish (Fig. 6a, Fig. 7a), CYP19A was not unambiguously affected by MT, only in the temporary MT exposed fish a certain elevation of CYP19A mRNA abundance (Fig. 6a) above the control levels was shown in both expression groups 1 and 2. A stimulation of the aromatase gene expression in the gonads of genetic females after termination of exposure would be reasonable as MT is supposed to suppress oocyte development (Müller 1969) And this process demands for the pronounced synthesis of estradiol to induce vitellogenesis.

Amazingly, in agreement with the findings of the fadrozole study, the CYP19A mRNA levels in the gonads of adult fish again showed sexual differences. Phenotypically, these fish could not be sexed due to the MT treatment. At least on day 71 pf, feminization of gonads was established histologically in treated fish. Nevertheless, in 65 % of gonads of continuously MT exposed adult fish, the relative abundance of CYP19A mRNA corresponded to male control levels (Fig. 7a). In the transiently MT exposed adult fish, a sexual dimorphic pattern of CYP19A mRNA expression was found as well, in which 63 % of fish showed 'male-like' CYP19A expression. It was referred to this important finding already earlier in the discussion of effects of fadrozole exposure.

CYP19 mRNA expression as a potential target for endocrine disruption in zebrafish

The results of this study provide evidence that the manipulation of the aromatase system during the critical period of sexual differentiation can alter or even reverse, i.e., disrupt the process of gonadal differentiation of the protogynic zebrafish. Alterations of gonadal sex differentiation were always accompanied by changes in the CYP19 gene expression, and not only in the gonads but also in the brain. The fact that the CYP19B expression in the brain was generally affected as well, further supports our assumption of a crucial role of CYP19B in the sexual development of zebrafish. But the confirmation of this theory demands further investigations and a more reliable quantification and precise localization of aromatase gene expression in the brain in particular. The expression of CYP19B in the brain of larval and adult zebrafish, investigated by whole mount in-situ hybridization, has already been demonstrated to occur in the olfactory bulb, the ventral telencephalon and the hypothalamus (Chiang et al. 2001a; Tong et al. 2001). Estrogen synthesis in these brain areas are correlated with neuroendocrine functions, sexual behavior and sexual differentiation, at least in mammals (reviewed by Lephart et al. 1996) or birds (Balthazart 1997). Similar studies in fish are still scarce. Regulative pathways, genomic- as well as non-genomic-based that regulate aromatase gene expression in the brain and in the gonads of fish have to be considered when assessing exogenous factors that interfere with sexual differentiation. Especially in regard to the potential impact of EDCs in the process of gonadal sex differentiation of fish, numerous potential molecular targets have to be taken into consideration, even if referring to the disruption of the estrogenic enzymes and their activity only. A possible direct ER-mediated influence of estrogenic compounds on the CYP19B gene expression has already been mentioned before. An indirect effect of estrogens on the CYP19 gene expression via the activation of other estrogen-responsive genes that are related with the P450arom system just reveals another potential site for EDC interference.

Neuroendocrine-active chemicals that may interfere with, e.g., gonadotropin release, also affect aromatase expression and activity and therefore reproduction. Of remarkable importance in this context is the identification of an aryl hydrocarbon (Ah) receptor/AhR nuclear translocation factor (ARNT) in the promoter region of the CYP19A gene of zebrafish most recently (Kazeto et al. 2001). The presence of AhR and its responsive element implies that AhR-active xenobiotics like PAHs (polycyclic aromatic hydrocarbons) may directly regulate CYP19A expression and therefore steroidogenesis

(predominantly in gonads) of zebrafish. However, most of the potential EDC targets in fish are still unknown.

Conclusions

We investigated in this study the consequences/effects of fadrozole or MT treatment on the CYP19A and CYP19B mRNA expression in zebrafish. The cause of these effects was either the inhibition of the P450arom enzyme and thus the suppression of E_2 synthesis by fadrozole or the activation of the P450arom enzyme activity by the application of MT as substrate. The final result of these treatments was the alteration of the gonadal sex in the course of the differentiation process. Consequently, interference with or the disruption of estrogen synthesis during development of zebrafish impairs or even changes gonad sexual differentiation. The actual cause of these organizational, permanent effects was the deficiency or excess of E_2. Changes in the E_2 synthesis influenced the estrogenic regulative pathways, including the P450arom enzyme complex with its corresponding genes CYP19A and CYP19B of zebrafish, as shown by the results. Vice versa, the consequent effects on the gonadal organization of zebrafish obviously influence the CYP19 mRNA expression and thus the synthesis of E_2. Our findings suggest that the influence of fadrozole and MT affect the expression of CYP19A and CYP19B mRNA expression, but differentially in head or body and brain or gonad respectively. The highest responsiveness, however, was always shown by the corresponding, organ-specific gene.

The results of these two studies undeniably confirm the assumption of a functional role of CYP19A and CYP19B mRNA expression in sexual gonad development in zebrafish. This corroborates the pivotal role of aromatase activity and thus the synthesis of E_2 in gonad differentiation of zebrafish. The theory that the interaction of estrogens and androgens is the crucial force for gonadal sex differentiation in gonochoristic fish (Yamamoto 1969) had been formulated a very long time ago, but among several other recent studies, our results clearly provide evidence that this theory seems to hold for a general feature in fish, independently of the mode of differentiation.

Moreover, the identification of the aromatase regulative pathways as important targets for EDCs in zebrafish was an important step to support the need for a better physiological understanding of EDC action in fish and subsequent organismic effects. With this study, an idea was given of how many regulative pathways may potentially be affected by the disruption of aromatase activity, and there are not only responsive to estrogens.

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IV. General Discussion

Introduction

The background of this thesis is the investigation and evaluation of endocrine disrupting effects in fish, particularly the effects on sexual development and reproductive function. In recent years there has been a surge in scientific effort to identify and assess environmental contaminants that may interfere with the endocrine system of fish and cause adverse reproductive effects. With the study presented here is was aimed to focus on two different aspects of endocrine disruption, and reproductive disruption in fish in particular, by:

- Evaluating the usefulness and significance of the widely applied biomarker for estrogenic effects, vitellogenin (VTG), in relation to 'disrupting' effects on the reproductive function. More specifically, it was tried to explore whether VTG induction is only an indicator of current estrogenic exposure or may be also used as an effect marker, predicting reproductive failure resulting from estrogen exposure.
- Investigating the function of differential P450 aromatase gene (CYP19) expression in sexual differentiation of the zebrafish as a central regulative switch in the steroid hormone synthesis in the gonads and the brain. The assessment of consequences of external interference with the P450arom system, and subsequently with the synthesis of estradiol may contribute to a better understanding of the sexual differentiation process in fish. Moreover, it advances the knowledge of potential molecular targets for endocrine disruptive effects that interfere with the differentiation process.

Vitellogenin (VTG) in zebrafish

The first two chapters of this work focus on the well-known and commonly applied biomarker of estrogenic environmental effects, especially in fish, the egg-yolk precursor protein VTG. The synthesis of VTG is an estrogen-dependent process that is usually restricted to female fish, but is also inducible in male fish, as both sexes carry the VTG genes. The detection of VTG in the plasma of male fish has been proven to be a reliable and useful indicator of occurence of environmental estrogens (Purdom et al. 1994;. Sumpter and Jobling 1995; Harries et al. 1997; Kime et al. 1999) In the first chapter, the development of a sensitive and specific assay for the detection and quantification of VTG in the zebrafish was described in detail. A polyclonal, homologous antibody against zebrafish plasma VTG was raised and a competitive enzyme-linked immunosorbent assay

(ELISA) for zebrafish VTG (zVTG) was established and validated. The detection limit (2.0 to 3.0 ng z-VTG standard per ml) and the working range (3.0 to 500 ng VTG/ml) of the assay corresponded well with other VTG ELISAs for other fish species using polyclonal antibodies (Bon et al. 1997; Johnson et al. 1999; Tyler et al. 1999). Thus, detection limit and working range argued well for the sensitivity and quality of the established assay. The assay proved to be very useful and accurate for the quantification of VTG in plasma samples of adult or sub-adult zebrafish, but was applicable to homogenate samples of larvae as well, though at a slightly reduced accuracy and sensitivity.

A species-specific, reliable assay for the measurement of VTG in zebrafish was the prerequisite for an assessment of VTG and its use as a biomarker of exposure to estrogens and possible estrogenic effects in this species. This was the aim of the second chapter of this work and thus, one of the few recent approaches that not solely focuses on VTG response as a biomarker of exposure but tries to correlate the VTG response with organizational effects (i.e., profound and usually persistent changes of physiological functions and/or morphology). Exposure of fish to estrogen mimics, or hormonal-active substances in general, may have activational (i.e., usually transient changes of physiological functions and/or morphology) acute physiological effects, but may also cause effects that will manifest only a long time after exposure or after prolonged exposure periods. Chapter 2 of this work shows that the manifestation of adverse exposure effects of fundamental significance, that is, on the reproductive function, was dependent on the time (i.e., the life stage) and the duration of exposure and on the hormonal potency of the substance applied. Two estrogenic chemicals of different potency but both of potential concern to aquatic wildlife, were used in the studies. The pharmaceutical synthetic estrogen 17α-ethinylestradiol (EE_2) was applied at an effective concentration of 3.0 ng/L, which can be referred to as environmentally relevant (Stumpf et al. 1996; Ternes et al. 1999). A previous full life-cycle exposure of zebrafish to EE_2 (data not presented in this work) resulted in a lowest observed effect concentration (LOEC) value for an impairment of the fertilization (success) of 1.1 ng/L. The LOEC for VTG induction in male zebrafish, derived from chronic exposure post fertilization (pf), was at nominal 1.67 ng EE_2/L. This LOEC value was similar to that established for VTG induction in adult male zebrafish (for a 21-day exposure), shown in the first chapter of this work. A longer duration of exposure to EE_2, including the embryonic and larval developmental period, apparently did not influence the sensitivity of the VTG response in male fish. Due to the results of other fish

studies, e.g., with the fathead minnow, a lowering of the effective threshold concentration for VTG induction with an increasing duration of exposure to estrogenic compounds (Sohoni et al. 2001) hence would have been expected. In spite of that, the present studies provided evidence that chronic exposure of zebrafish pf to 3.0 ng EE_2/L severely affected reproduction and even prevented spawning completely. Early life stage exposure of zebrafish to EE_2 at this concentration, however, did not result in adverse effects on the reproductive output of adult fish, though a persistent impairment of VTG synthesis in females was indicated, at least after a 42-day exposure (pf).

Indeed, the young (juvenile) life stages of vertebrates like fish, appear to be especially sensitive to hormonal exposure derived effects. It is well documented that, e.g., in salmonid fish an exposure period of only two hours post hatch (ph) is needed to induce either male or female sex differentiation, providing that appropriate concentrations of androgens and estrogens, respectively, were applied (Piferrer and Donaldson 1992; Piferrer et al. 1993) .The non-polar, lipophilic nature of steroids allows penetration through lipid membranes. Therefore they may be accumulated already in the fertilized egg, hence representing a potential hazard for the developing embryo. The expression of the estrogen receptor subtypes in zebrafish was demonstrated already very early in the development (Kishida et al. 2001; Legler et al. 2000) and could even be detected in the early embryo stage due to maternal gene transfer. A responsiveness of the VTG synthesis in 43 % of exposed fish, at least for the exposure to 3.0 ng/L of EE_2, was shown in this work already at the early age of 21 dpf. A persistent effect of estrogen exposure during early life stages of fish can be assumed, but it is most speculative to conclude from the findings of this work that six weeks (42 days) of EE_2 exposure pf persistently impaired vitellogenesis in female fish.

A lasting but presumably reversible profound effect of organizational quality was recorded after long-term exposure to 3.0 ng EE_2/L pf until adulthood. The development of reproductive behavior was completely suppressed in these fish under continuous exposure conditions. Histology of gonads revealed the impression of a retardation of gonad differentiation, skewing sex ratio to a female predominance. It appears that the observed 'feminization' derived from a reversal of gonadal sex in the cause of estrogen exposure. Due to the specific mode of gonad differentiation, i.e., the zebrafish passes through a protogynic developmental stage (Takahashi 1977), exposure to exogenous estrogens during

this critical developmental window may delay the initiation of testes differentiation. In accordance with Weber et al. (2001), the finding of a "feminizing" effect of developmental exposure to EE_2 in the gonads of zebrafish was ascribed to an exposure concentration-related suppression of the gametogenesis process in female as well as in male fish. Final maturation of oocytes or the spermatogonial proliferation either depends on 17α-20β-dihydroxy-4-pregnen-3-one (Nagahama and Adachi 1985) or testosterone and ketotestosterone (Miura et al. 1991), respectively. However, the onset of the final maturation of germ cells would demand for a switch in the steroidogenic pathways in both sexes, but was omitted by the exogenous supply of estrogens.

The second test substance used in this work was bisphenol A (BPA) as a high-volume industrial chemical. In spite of its non-steroidal structure, BPA has proven to be 'estrogenic'. It binds to the estrogen receptor *in vitro* with an affinity more than 10,000 times less than EE_2, as shown in the Yeast Estrogen Receptor Assay (Yeast-Screen; Gaido et al. 1997; Wenzel et al. 2001). *In vivo* studies in mammals revealed opposing results but BPA mostly indicated only weak estrogenicity (e.g., von Saal et al. 1998; Nagao et al. 1999). Indeed, the effective concentrations that were found in this work were orders of magnitude higher than environmental relevant concentrations measured at moderately polluted areas of Europe (Spengler et al. 1999). Adult zebrafish that were chronically exposed to BPA concentrations of ≥ 375 µg/L showed a significantly diminished fertilization success while the number of spawned eggs per female and day (fecundity) was affected only at a BPA exposure concentration of 1500 µg/L. In comparison to the effects observed after or during the exposure to 3.0 ng/L of EE_2, the quality of effects recorded in BPA exposed zebrafish was different in degree and severity. Fertilization was decreased up to a maximum of 50 % only at the highest concentration. Histological evaluation of gonad morphology of BPA exposed fish did not indicate a complete feminization of gonads like in the EE_2 study. Abnormalities in morphology and cellular structure of gonads, mainly concerning the abundance and distribution of germ cells and the pathological changes, were observed at nominal BPA concentrations > 375 µg/L (Maack 2002). This, however, did not significantly affect gonadal sex-ratio. Whether the reproduction of zebrafish would have been impaired when fish were exposed only during the early life stage, remains to be investigated. It appears, however, most unlikely, when considering the results of the EE_2 study, showing no impairment of the reproductive function of fish transiently exposed during early life. At the concentrations applied in this study, BPA was not capable of

inducing the estrogen receptor (ER) -mediated hepatic synthesis of VTG during early life stage of zebrafish on day 21 pf. In spite of this finding, the impact of BPA on any ER mediated physiological processes can not be excluded. The particularly high sensitivity of early life stages of vertebrates like fish, to estrogenic exposure has to be emphasized again. BPA-induced alterations of sexual differentiation, including the induction of testis-ova, were demonstrated in Japanese medaka (*Oryzias latipes*) after 60 days of exposure to BPA in the early life stage, at least at the highest exposure concentration of 1820 µg/L (Yokota et al. 2000). In consistency with most other fish studies on exposure effect of BPA, this work corroborated the weak estrogenic potency of BPA, especially in regard to organizational effects. The study results demonstrated a detrimental impact of BPA at concentrations ≥ 375 µg/L on the reproductive capacity and gonad differentiation at least after chronic, long-term exposure periods.

Most interestingly, significant reproductive effects after chronic BPA exposure were recorded at the same treatment concentration of BPA than found for a significant VTG induction response in the adult male fish. The lowest effective concentration for the induction of VTG in male fish was obtained at 375 µg BPA/L in this study, which corresponded well with the LOEC of fertilization effects. In adult female fish as well as in fish at the earlier developmental stage round day 75 pf, however, VTG was significantly elevated above the control level at all exposure concentrations of BPA. The lower effectiveness of BPA to induce VTG synthesis in males compared with females raises the question, whether the sensitivity to estrogenic effects of compounds like BPA is different between the sexes and generally lower in males. In adult fathead minnow, *Pimephales promelas,* the opposite effect on VTG synthesis was observed after 164 days of exposure to BPA. The effective concentration for a significant induction of VTG in male fathead minnows was already reached at 160 µg/L but in females only at 640 µg/L. And moreover, BPA was not effective in females after shorter exposure period, whereas the LOEC for VTG induction in males after 43-day-exposure was 640 µg/L (Sohoni et al. 2000). These findings for the fathead minnow deviates from most other studies, as the estrogen sensitivity in most cases and most species is higher in female than in male fish. Nevertheless, sexual differences in the sensitivity or susceptibility to estrogenic effects may be in fact due to the duration of exposure as well as to the estrogenic substance applied. Species-specific differences have to be considered as well.

The proposed 'sexual dimorphic' character of VTG synthesis demands for an in-depth discussion. In this work, it was tried to elaborate individual difference in vitellogenesis as a

potential distinctive feature to sex zebrafishes at early life (developmental) stages. A specific problem in assessing estrogenic or generally endocrine disrupting effects is that they adversely affect hormonal regulation and thus, sexual differentiation and development in particular. Estrogen exposure derived changes in morphology and/or histology, including phenotypic sex are most likely. Moreover, the zebrafish lacks distinct secondary sexual characters, and only at the reproductive stage, females are distinguishable for their rounded shape and males differ in their slender appearance and darker coloring (while performing courtship display). Facing this problem, VTG responses to estrogenic exposure in the course of zebrafish development were investigated for statistical distribution patterns and the formation of subgroups. Indeed, already at the early age of 21 dpf, two differential subgroups of different VTG levels in body homogenates were found in 3.0 ng/L EE_2 exposed fish. However, it demands for further investigations and discussions, whether at this early developmental stage different VTG response levels do not only reveal the asynchrony of the developmental progress among the fish groups examined. Nevertheless, at least during and after the period of gonadal sex differentiation, the formation of two subgroups of distinct VTG synthesis levels, also in control fish, became more evident and (statistically) verified. At the termination of sexual differentiation of zebrafish, that is ten to twelve weeks after fertilization, a sexual dimorphic VTG response pattern to estrogenic exposure was verified for adult zebrafish. Adult, reproducing zebrafish did either synthesize VTG intrinsically, showed VTG plasma titers of several hundred micrograms per milliliter and were phenotypically identified as females. Or they produced only very low to non and hardly detectable amounts of VTG and were unambiguously male. Treatment with exogenous estrogens, like EE_2 or BPA as in these studies, induced and elevated the VTG synthesis level in both sexes, as expected. Although the relative induction was on the one hand several times higher in male than in female fish, the mean VTG levels measured in plasma or homogenates of males on the other hand never reached comparable levels to that measured in females. The distinction of two different subgroups with VTG levels either in the range of control females or control males was described by Länge et al. (2001) for adult fathead minnow as well. They recognized this sexual dimorphic VTG pattern in fathead minnow that could not be sexed anymore, due to chronic exposure to 4.0 ng/L of EE_2 post hatch. In the studies presented here, the chance was taken to go one step further. Adult specimens at the age of 118 and 176 dpf, taken from the 3.0 ng EE_2/L study, were analyzed for plasma VTG and gonad histology in parallel, and therefore VTG plasma titers were directly compared with the corresponding

gonadal sex of individual fish. Amazingly, individual gonadal sex, predominantly in chronically exposed fish, did hardly agree with the vitellogenic response. Although chronic exposure to 3.0 ng/L of EE_2 caused complete feminization of gonadal sex, three quarters of fish showed VTG levels in the range of exposed males. In consequence, it was differentiated between the 'vitellogenic' and the 'histological' sex/phenotype of individual fish, in cases possible within these studies. It can be suggested that estrogenic exposure of zebrafish indeed affects the sexual phenotype, and in dependency on duration and time of exposure, is capable of distorting the phenotypic sex-ratio towards a predominance of females. Obviously, the impact of exogenous estrogens did not irreversibly change genotype-regulated physiological processes. The results achieved in this work, never generated irreversible estrogenic effects in zebrafish under the exposure condition applied. Chronic EE_2 exposure, e.g., induced a complete gonadal feminization, thus preventing reproduction. However, after terminating the exposure, gradual recovery of reproductive strength of the previously exposed fish could be observed. It was beyond the scope of the present investigation to address the question, whether the exposure to environmental estrogens may persistently impair or even change irreversibly genetic sex-related gene expression. This issue ought to be the objective of further studies, apart from the fact, that the regulation of expression (and the identification) of sex-linked genes, especially in correlation with sexual determination, in most fish species including zebrafish, is still unknown.

The main conclusion drawn from the results of these studies were first of all that VTG, as a biomarker of estrogenic exposure, acts as a reliable and moderately sensitive indicator of current exposure but does not involve necessarily a predictive function of subsequent organizational effects, and vice versa. The induction of VTG synthesis in fish definitely indicates the presence of estrogen active contaminants in the environment, but this does not inevitably cause an effect on individual fitness and the reproductive function in particular. Abnormally high levels of VTG (as well as abnormally low levels, e.g., in females (Kime 1996)) have to be interpreted with caution and related to other effected parameters, always being aware of the complexity of the reproductive endocrine system. The more profound and far-reaching consequences of environmental estrogen exposure concern organizational effects i.e., profound and persistent changes of physiological functions and/or morphology.

Apart from the difficulties in interpolating between laboratory studies and the situation in the field, impairment of the reproductive function of wildlife species may severely endanger the survival of populations, implying ecological consequences for an ecosystem

as a whole. Endocrine disruption in wildlife has evolved as a global concern, and estrogenic responses in fish have already been described for many wild species from various ecological habitats all over the world (e.g., UK: Jobling et al. 1996, 1998, 2001; van Aerle et al. 2001; France: Flammarion et al. 2000; Sweden: Larsson et al. 1999; USA: Munkittrick et al. 1998; Sépulveda et al. 2001a,b; Japan: Hashimoto et al. 2000; Africa: Okoumassoun et al. 2001).

VTG synthesis induction in male fish is a reliable indicator of the presence of environmental estrogens, indeed. In female fish, however, the expression of VTG genes in the liver is induced first of all by endogenous E_2, primarily produced by the follicle cells in the ovary (Ng and Idler 1983; Wallace 1985). The increase of E_2 plasma level triggers vitellogenesis in the liver, and the specific sequestration of VTG from the blood stream into growing oocytes is the principal event responsible for the enormous growth of teleost oocytes. Plasma levels of E_2 and vitellogenesis are positively correlated. The parallelism between the circulating E_2 and the plasma VTG concentration during oocyte growth has been shown for many teleost species, *in vitro* as well as *in vivo* (DeVlaming et al. 1980; Wallace and Selman 1980; Trant and Thomas 1989; Nagler et al. 1994; Tyler and Sumpter 1996; Pavlidis et al. 2000). Therefore, regulation of vitellogenesis basically depends on the hormonal control of steroidogenesis via the hypothalamus-pituitary axis and the subsequent secretion of gonadotropins. From studies in rainbow trout (*Oncorhynchus mykiss*), an additional regulative pathway of oocyte growth control has been proposed in which VTG is directly involved as a regulative factor in the steroidogenic activity of vitellogenic oocytes (Reis-Henriques et al. 1997, 2000). It was demonstrated, that the synthesis of E_2 *in vitro* in trout vitellogenic oocytes concentration was directly influenced by the VTG concentration. Therefore, VTG may alter its own synthesis rate in the liver in an autoregulative manner. And in fact, the injection of a vitellogenic fraction in female trout during the vitellogenic period significantly reduced the plasma levels of E_2 (Reis-Henriques et al. 1997). Together with the findings *in vitro*, these results strongly support the assumption, that VTG plays a pivotal role in ovarian steroidogenesis control. It is supposed that the attainment of a threshold level of VTG in oocytes triggers the activation of a very short feedback loop directly from the oocytes to the steroidogenic enzymes in the follicle cells, suppressing E_2 synthesis. This would imply a direct involvement of VTG in the regulation of 17α-hydroxylase/17,20 lyase (P450c17) and P450arom enzyme activities.

Cytochrome P450arom genes (CYP19A and CYP19B) in zebrafish

The molecular mechanisms of interference of anthropogenic pollutants or especially endocrine disrupting compounds (EDCs) with developmental processes, especially in fish, are poorly understood. The hormonal activities of EDCs of greatest concern are presently most notably estrogenic (or estrogenic modulating) activities, as steroid estrogens play a crucial role in sexual differentiation and development and in the control of reproduction in all vertebrate species. Several studies have shown that the same goes for lower vertebrates like amphibians or birds and also in fish, the sexual differentiation seems to depend on steroid hormones. The female phenotype, e.g., only develops under estrogen control. Estrogens and androgens exert a crucial impact on the labile process of sexual differentiation in these vertebrate classes. Brain and gonad are well-known target organs for estrogens in all vertebrates and the developmental differentiation of these organs is of major importance for the expression of sexual phenotype and the reproductive capabilities. Estrogens and androgens exert their regulative function on organism development and physiological homeostasis predominantly through steroid receptor-mediated activation of target genes, e.g., for VTG, and their expression. These estrogen or androgen responsive genes represent principal molecular targets for EDCs with estrogenic or androgenic as well as anti-estrogenic or anti-androgenic activity. Interference of EDCs with steroidogenic pathways may lead to altered reproductive development and/or output. This does not only refer to EDC activities that are estrogen receptor-mediated but may also concern other endocrine targets, particularly on the hypothalamus-pituitary-gonadal axis or within the steroid hormone synthesis. One potential molecular target to be considered in this respect, is the aromatase (P450arom) enzyme complex.

P450arom is the key enzyme in the synthesis of estradiol from the precursor androgens androstenedione and testosterone. In spite of the crucial function of estradiol synthesis in sexual development and reproduction, an insight into either the regulatory function of CYP19 expression or the enzyme activity is missing as well as the knowledge about the consequences of (tissue-specific) P450arom activation and/or inhibition on processes such as sexual differentiation and reproductive capabilities in fish. What is known is that CYP19 in fish has a common function with that in mammals, in having a pivotal role in the control of sexual development and behavior, but the gene structure is often more complex than in mammals. Some teleost species studied so far, posses several, differentially regulated CYP19 genes (Gelinas et al. 1998; Ijiri et al. 2000; Kwon et al. 2001). In zebrafish, the existence of two CYP19 genes, which are differentially regulated and encode for different

proteins with different enzymatic properties, has been proven and assigned to two distinct aromatase gene loci on different chromosomes (Chung 2000, Chiang et al. 2001b; Kishida and Callard 2001; Trant et al. 2001). Consistently with other recent studies (Chiang 2001a; Trant et al. 2001), the present results confirm a predominant expression of the one CYP19 gene (CYP19A) in ovarian tissue, whereas the highest expression of the other gene (CYP19B) transcript occurred in brain tissue. This finding argues for a functional separation of these two CYP19 genes. As a common feature among teleost fish species and most lower vertebrates, a tremendously high expression of CYP19 in the brain is described (Pasmik and Callard 1985; Gelinas and Callard 1997; Pasmik et al 1988) and the results presented in this study confirm this observation. In this work, it was focused on the P450arom gene (CYP19) mRNA expression in the zebrafish and on the susceptibility of this process to exogenous impacts during the critical period of gonadal sex differentiation. The physiological importance of aromatase in gonadal differentiation in fish is demonstrated in the present study by the fact, that inhibition of aromatase activity during the sexual developmental period caused the development of all-male phenotype zebrafish. The application of the enzyme substrate methyltestosterone (MT), however, had a paradoxical 'feminizing' effect on gonad differentiation.

Several studies have shown that the inhibition of E_2 synthesis in fish may cause masculinization (Piferrer et al. 1994; Guiguen et al. 1999; Kitano et al. 2000; Kroon and Liley 2000; Kwon et al. 2000; Afonso et al. 2001) or that the administration of aromatizable androgens promotes either masculinization (Yamamoto 1958; Piferrer et al 1993; Gale et al. 1999; Kitano et al. 2000; Papoulias et al. 2000; Tsai et al. 2000) or, at higher doses, may have the paradoxical effect to feminize fish (Müller 1969; Hackmann 1971; Nakamura 1975; Reinboth 1980; Piferrer and Donaldson 1991. The plasticity and lability of the sexual differentiation process in lower vertebrates like fish, has already been used for a long time, mainly in fish culture populations, to control sex by the application of hormones (Berkowitz 1937; Yamamoto 1958; Hunter and Donaldson 1983; Piferrer 2001). Hormonal sex control studies served as the fundamental basis for the more recent studies, focussing on the physiological-molecular processes triggering sexual differentiation (Kitano et al. 1999; Tsai et al. 2000; Gen et al. 2001; Kwon et al. 2001).

Due to the lack of knowledge concerning the role of the endogenous steroid hormones estradiol and testosterone and their synthesis as presumable determinants in the sexual differentiation, the specific function of aromatase activity and therefore the synthesis of estradiol in fish is not well characterized yet. Most of the recent studies focus on the

aromatase activity or even CYP19 expression in gonadal differentiation, but often in the context of exogenous sex control or sex-reversal events in gonochoristic teleosts. However, the gonadal development in fish is not a uniform process but varies between taxonomic groups or even species.

With the study presented here, a particular attention was directed to the role of steroid hormones, and especially of estradiol and its synthesis, during the process of sexual differentiation of a fish species that passes through a protogynic, pre-differential stage, a phenomenon called 'juvenile hermaphrodism' (Takahashi 1977). The findings of this study strongly suggest that P450arom activity and thus the synthesis of estradiol plays a pivotal part in the sexual differentiation of the zebrafish. It is presumed that E_2 is a natural determinant of ovarian differentiation, whereas the absence of estradiol and therefore the suppression of aromatase during gonadal differentiation induces the development of testes. Corresponding to other studies on hormone-induced sex-reversal in gonochoristic or protandrous fish species, a critical and hormone-sensitive period of gonadal sex differentiation was confirmed also for the (juvenile) protogynic hermaphrodite zebrafish. It can be concluded, that the undifferentiated 'early ovary' in zebrafish consists of bipotential germ cells that are highly susceptible to exogenous factors like the steroid hormones, acting as determinants of sexual differentiation. Although it remains unclear if the bipotentiality of germ cells is perhaps maintained in the mature gonad.

The study cannot clarify, whether the steroid hormones are the only decisive factor in gonad sexual differentiation of zebrafish or are only one among others. There are other endogenous and environmental factors that may affect sexual differentiation of fish and cause sex-reversal or unisexual development. The influence of temperature on sexual differentiation has been intensively studied, e.g., in tilapia species (Baroiller et al. 1999; Kwon et al. 2000; Wang and Tsai 2000; D'Cotta et al. 2001) or the Japanese flounder, *Paralichthys olivaceus* (Yamamoto 1995, 1999; Kitano et al. 1999). Most recent results of these studies clearly provide evidence for a direct correlation between temperature-induced sex-reversal and the aromatase activity and/or CYP19 gene expression. D'Cotta et al. (2001), e.g., showed that high-temperature induced masculinization of differentiating, genetically all-female tilapia (*Oreochromis niloticus*) was coincident with a repression of CYP19 expression in the gonads and a considerable reduction of the aromatase activity in the brain. In other non-mammalian vertebrates like reptiles, temperature-induced sex differentiation or even determination is much more common. In several reptile species it was shown, that temperature-induced female differentiation is accompanied by a reduction

of aromatase gene expression in the ovaries, and Crews (1996) showed that steroid hormones serve as the direct trigger for sex determination in reptiles. Together with the findings in tilapia or the Japanese flounder, the theory that female differentiation in zebrafish highly depends on CYP19 expression and the synthesis of E_2 is definitely corroborated. The results from this study provide evidence for the determinant function of E_2 synthesis in exogenously induced sex differentiation. Moreover, the fact that the inhibition of the aromatase activity throughout the period of gonad sexual differentiation caused the development of only male zebrafish indicate that the endogenous synthesis of E_2 is obviously a determinant factor in sex differentiation of zebrafish as well.

Nevertheless, the results could not answer the question, whether and how the endogenous steroids act as the definite sex inducer in zebrafish. Like in most other fish species, this study lacks a direct evidence for an endogenous synthesis of estradiol or testosterone already in undifferentiated zebrafish. Though, a high mRNA expression level of CYP19B in the cephalic region of undifferentiated, 35-day-old fish was demonstrated. An expression of CYP19A in the trunks of these fish was also indicated, thus arguing for the occurrence of E_2-synthesis. Furthermore, the expression of both CYP19 genes in the zebrafish was already observed in early embryonic stages after 24 hours pf (Kishida and Callard 2001). Study results on sex hormones as natural sex inducers in other fish species are really controversial. In most salmonid species, the identification of steroid-producing cells (Nakamura and Nagahama 1993), the histochemical detection of steroid enzymes (van den Hurk et al. 1982) or the expression of steroid enzyme genes (Govoroun et al. 2001; Liu et al. 2001) in undifferentiated gonads support the assumption that steroid hormones are already produced prior to sexual differentiation. On the other hand, in several non-salmonid species the results were negative or indefinite. As the synthesis of P450arom in undifferentiated zebrafish is most likely, a sex determinant function of E_2 is presumed, but the natural inducer of testicular development in zebrafish was unambiguously identified in our study. Based on the present findings concerning CYP19 expression before and at the end of sexual differentiation, it is hypothesized that CYP19A mRNA is expressed in a sexual dimorphic pattern in gonadal tissue of zebrafish, at least at the sex-differentiated stage. It is presumed that the sexual dimorphism in gonadal CYP19A expression is based on genetic sex-specific gene expression. It demands for further investigations to extent basic knowledge about regulative processes and factors affecting CYP19 gene expression in the zebrafish, especially during the period of sexual

differentiation, indeed. In most fish species studied so far, a sexual dimorphic aromatase expression and/or activity pattern during or at least at the end of sex differentiation has been confirmed (Guiguen et al. 1999; Scholz and Gutzeit 2000; Kwon et al. 2001; etc.), though to a different extend and often with contradictory results due to differences in the methods used and/or the molecular level examined, that is gene or protein.

Although it was not the primary goal of this thesis to unravel the mechanisms of sexual differentiation in zebrafish, the results still promote the understanding of the function of CYP19 gene(s) expression in gonadal sex differentiation of zebrafish. The synthesis of E_2 and correspondingly the expression of CYP19, could be demonstrated to be a determinant factor in the differentiation process. E_2 is indispensable for the development of ovaries, whereas the lack of E_2 throughout differentiation induces the development of testes. The phase of sexual differentiation has turned out to be a critical and hormone-sensitive period and therefore, a high susceptibility to environmental endocrine-active chemicals is especially presumed within this critical developmental window. Chemicals that may interact with steroidogenic enzymes like the P450arom, hold a hazard potential comparable to or even higher than estrogenic chemicals. This is particularly due to the lack of knowledge about the quality and structure of compounds that may be capable of either interaction with the enzymes themselves or with gene expression on transcriptional or translational levels. It has already been shown that nitrogen heterocyclic compounds, such as imidazole derivatives, have aromatase-inhibiting activity in rainbow trout (Monod et al. 1993). Fadrozole, e.g., belongs to the same compound group as agricultural fungicides like prochloraz or antimycotic drugs like clotrimazole. Also for polycyclic aromatic hydrocarbons (PAHs) evidence has been provided for their capability to disrupt reproductive function in fish by direct inhibition of steroidogenic enzymes, at least *in vitro* in ovarian tissue of the flounder, *Platichthys flesus* (Monteiro et al. 2000).

General conclusion and future aspects

It is nowadays well established that all organisms, animals as well as humans, are exposed to and potentially impaired by endocrine acting chemicals. A consolidated knowledge on effect-related molecular mechanisms is indispensable for the establishment and validation of new, more functional endpoints, diagnostic biomarkers and tests for the assessment of endocrine disruption in wildlife.

For VTG, as an established and often applied biomarker of estrogen exposure, this study provides clear evidence that it is of only limited value as a predictive marker of adverse effects. The induction of VTG alone cannot serve as definite indicator of subsequent reproductive impairment of the exposed organism. VTG can act as an adjunct parameter to evaluate reproductive (and developmental) disruption.

The findings of this work concerning the differential CYP19A and CYP19B mRNA expression in developing zebrafish definitely contribute to a better understanding of the implication of changes in the expression pattern of these two genes on the regulation or even determination of the sexual differentiation of gonads in zebrafish. It could be shown that interference with the P450arom system modulates the sexual differentiation of zebrafish and, in turn, this clearly confirms a determinant function of E_2 within this process.

There is still a need for a better understanding of the reproductive endocrine system and its complex regulative pathways at different hierarchical levels. A detailed knowledge of the reproductive endocrine system in fish is indispensable for a solid and reliable interpretation of endocrine or reproductive disruption. Both processes, vitellogenesis as well as the P450arom catalyzed conversion of androgens to estrogens, are integrated into complex regulative pathways of the endocrine system, thus offering multiple targets for disruptive action of chemicals with diverse and multiple modes of reactivity, and not simply estrogenic. There is still a huge lack of knowledge concerning the mechanisms of endocrine disruption, especially in fish that are most intensively used as study organisms. The aims of future studies demand for a more complex, comprehensive evaluation of effects, considering different causative variables in the processes that result in the endocrine disruption observed. This includes on the one hand the reactivity and interaction of the chemicals considered as hormone mimics, that is with each other (in the case of mixtures), with the environment and with the organism studied. On the other hand, the direct molecular interaction of the chemical with specific hormonal targets in the organism can cause a wide variety of physiological impacts that do not only affect the reproductive system. Therefore, an important task of future research on endocrine disruption has to be, turning away from the restricted view on reproductive disruption only. There is the need to give more attention to other physiological processes that depend on the endocrine system and determine the health and fitness of organisms, e.g., the thyroid hormone system.

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Summary

In the present work, molecular and organismic aspects of environmental endocrine disruption in teleost fish were investigated, especially the interference with the sexual (gonad) development. The thesis was focused in its first part on an established biomarker that is intensively used in the assessment of estrogenic endocrine effects in fish, *in vitro* as well as *in vivo*, in laboratory and / or field studies (chapter 1 and chapter 2): The egg-yolk precursor protein vitellogenin (VTG). The second part of this thesis deals with the expression of genes of the cytochrome P450 aromatase (P450arom), the enzyme complex that is responsible for the synthesis of estrogens from androgens in all vertebrates, including fish. (chapter 3). The aim of these studies was not only to detect endocrine disrupting effects in fish that cause developmental and/or reproductive disorders and associate these effects with the exposure to hormone mimics, but also to pay closer attention to the relationship between molecular disturbances (here: of the VTG and the P450arom/CYP19 system) and adverse organismic impairments (here: disturbances of the gonadal differentiation and the reproductive health). The knowledge of those relations will advance a more functional and mechanistic understanding of endocrine reproductive disruption in fish.

The zebrafish (*Danio rerio*) was chosen as test species as it represents a recommended and widely used test organism for standardized assay protocols and guidelines for the assessment of endocrine disruptive effects in fish, especially in European countries. It was confirmed by the results of the thesis that the zebrafish passes through a protogynic stage in the course of gonad differentiation and can therefore be referred to as a juvenile protogynous hermaphrodite.

Vitellogenin

To investigate the induction of vitellogenin synthesis in developing (during ontogeny) and adult zebrafish, a reliable and sensitive assay for zebrafish VTG was established and validated (chapter 1). In using this specific VTG assay, response of VTG to estrogenic exposure during different periods of the early development and the sexual differentiation of zebrafish was studied The results for VTG were correlated with reproductive effects, including fecundity, fertility and the gonadal sex ratio. The synthetic estrogen 17a-

ethinylestradiol (EE_2), a high estrogenic potency compound and bisphenol A (BPA), a high production volume chemical of the plastic producing industry and weakly potent xenoestrogen, were applied in this study as model endocrine disruptors.

EE_2 as a "strong" estrogenic compound, ensured the induction of estrogenic responses already in the early developmental stage of zebrafish at the environmentally realistic concentration of 3 ng EE_2/L. In 43 % of zebrafish exposed during the first 21 days after fertilization, EE_2 was capable to elevate VTG synthesis above the average VTG level found in control animals. A VTG induction response of the early life stages to the weak estrogen BPA, however, was not indicated in our study. Compared with the EE_2-treated fishes of the same age of 21 dpf, VTG levels in whole body homogenates of chronically BPA-treated fish were up to fifteen times lower and not significantly different to control at any exposure concentration. The relative VTG induction capacity (i.e., ratio of normalized VTG sample concentration (µg/ml) to the corresponding mean VTG concentration of the controls (µg/ml)) of BPA on average was hardly over 1 whereas the relative induction of EE_2 was higher than 25. This finding provides evidence for a susceptibility of the vitellogenic system in the early life-stage of zebrafish, at least in response to exposure to highly potent estrogenic compounds.

BPA became effective in inducing VTG after 75 days of continuous exposure after fertilization. A significant induction of VTG in body homogenate samples was reached by the lowest concentration tested, 94 µg BPA /L, and the relative induction of VTG in the BPA treatment groups ranged between 2.1 and 5. The relative VTG induction after 75 days (pf) of exposure to 3.0 ng EE_2/L, however, was 111 and corresponded to a mean VTG plasma titer of 250 µg/ml.

At the adult, reproductive stage, the lowest observed effect concentration (LOEC) for the significant induction of VTG after chronic exposure was 375 µg/L of BPA for male and 94 µg/L of BPA for female fish. The LOEC of 375 µg BPA/L for the induction of VTG in males equaled the LOEC for a significant reduction of the fertilization of daily spawned eggs.

Chronic exposure of zebrafish to 3.0 ng EE_2/L until the adult stage resulted in a complete suppression of reproduction which recovered only partly after the exposure was omitted from day 125 pf onward. Histological evaluation of the fish of 118 days of continuous EE_2 exposure showed that they all had ovary-type gonads. After fish had been removed from exposure from day 125 until day 176 pf, almost 30 % of formerly exposed fish were found

to have testes at the end of the study. This finding correlates with the observation that the mean fertilization capacity was regained at almost 30 %.
In the case of BPA, interpretation of results on gonad histology proved to be more difficult as they were not as unambiguously as in the EE_2 study where only ovaries were found. Nevertheless, at the three highest exposure concentrations of BPA, the proportion of fish with histologically female gonads clearly outnumbered the proportion of male gonads (see Maack 2002 for details).

A most intriguing result of these studies was that a dimorphic pattern in the VTG induction response among the fishes analyzed was found throughout zebrafish development. This dimorphic pattern was obvious even when the histological analysis indicated the presence of an all-female or all-male population. Already on day 21 pf, a group of individuals among the exposed fish, having higher response levels of VTG, could clearly be differentiated from a group of fish with lower response levels of VTG. In fishes older than 21 dpf, this dimorphic distribution in individual VTG levels were found in control fish as well. In adult, reproducing fish, plasma VTG titers were sexually dimorphic and the individual height of the VTG concentration could be unambiguously assigned to males or females, at least in control fish. In EE_2- or BPA-exposed fish the vitellogenic response was found not to correspond with the appearance of individual gonadal sex and/or external morphology. From this finding it was hypothesized that the exposure to estrogenic compounds during the sexual developmental period of zebrafish, can modulate or even change the differentiation process. It is not capable, however, of changing sexual determination and its corresponding, genetically regulated pathways that directly or indirectly also affect VTG synthesis. It was found that the exposure to a 'strong' estrogenic compound like EE_2 was able to change sexual gonad differentiation, causing a complete gonadal feminization. Nevertheless, the results of VTG analysis clearly demonstrated that the change of gonadal sex is not indispensably accompanied by a corresponding change in VTG synthesis according to the gonadal sex. This finding strongly suggested a genetic sex-related VTG response in zebrafish, though this assumption still demands for further mechanistic investigations. However, a strong correlation between the formation of this sexual dimorphic VTG synthesis and the protogynic mode of sexual differentiation in zebrafish is presumed.

Apart from the investigation of the induction response of VTG to chronic estrogenic exposure, this study particularly focused on the question of the reversibility of the VTG

response. This is an important aspect for the evaluation of VTG as a predictive indicator of organizational effects. The main result in this regard was that short-term exposure to 3.0 ng/L of EE_2 for 14, 21 or 42 days pf had no impact on vitellogenin in adult male zebrafish. Only in female fish, a significant elevation of the VTG plasma titer was observed even after ten to fifteen weeks out of treatment. However, after further eight weeks of recovery until day 176 pf, the VTG plasma levels remained elevated in the 42-day treatment females. Therefore, the persistence of the treatment effect on the vitellogenic response was supposed to be correlated with the duration of developmental exposure.

In consideration of former study results on EE_2, a dependence of the VTG response on the estrogen exposure concentration and the estrogenic potency, respectively, may be presumed as well. Intriguingly, even long-term exposure until the reproductive stage did not result in a lasting VTG induction in male zebrafish. In conclusion, short-term ($\leq$ 42 dpf) developmental exposure to 3.0 ng/L of EE_2 did neither induce reproductive impairment nor did it result in persisting disturbances of vitellogenesis in male zebrafish. The persistence of enhanced VTG synthesis as observed in female zebrafish, however, was not indicative of a reproductive impairment.

In conclusion, the study provides strong evidence that VTG is in fact a reliable indicator of current estrogenic exposure of fish, but does not meet the requirement to have a predictive function of subsequent reproductive and organizational effects, at least not in zebrafish. Under current treatment conditions, VTG induction proved to be not more or less sensitive than organizational organismic effects like fertility or fecundity. Especially in males, in which induction of vitellogenin is directly linked to environmental estrogen exposure, effective concentrations for VTG induction and reproductive impairment generally corresponded well, fairly independently of the estrogenic strength of the chemical applied. The fast reversibility of the VTG response after termination of exposure implies an increased probability of false negatives in the interpretation of EDC effects and consequently negotiates every claim of a predictive quality of VTG, at least in zebrafish.

Aromatase

In chapter 3 of this work, the differential expression of the ovarian-derived and the brain-derived P450arom gene transcripts, CYP19A and CYP19B, was investigated in juvenile and adult zebrafish by semi-quantitative RT-PCR assays. With this study it was aimed to improve the understanding of the implication of estradiol synthesis in the process

of sexual (gonad) differentiation of the zebrafish. In the intention to interfere with the aromatase system in order to look at possible consequences regarding sexual differentiation, the non-steroidal aromatase inhibitor fadrozole and the aromatizable androgen 17α-methyltestosterone (MT) were applied during the gonadal differentiation period. Changes in the expression of CYP19A and CYP19B mRNA were recorded at the end of exposure on day 71 pf and at the adult reproductive stage and compared with the expression of both CYP19 mRNA transcript in untreated fish. Effects of fadrozole or MT on gonad differentiation were assessed by the analysis of gonad histology.

In sexually undifferentiated zebrafish around day 35 pf brain and gonadal type of CYP19 mRNA were found in both head and body samples. The abundance of both transcripts, CYP19A and CYP19B, showed a dimorphic pattern of expression in head and body. CYP19A was more abundant in body samples and CYP19B in heads, respectively, at least in one of the two studies. The highest expression was recorded for CYP19B in heads. At the end of the treatment period, on day 71 pf, the expression pattern of CYP19A and CYP19B mRNA in head in body of control fish largely corresponded with the day 35 pf, Both transcripts, however, clearly indicated higher gene activity in head and body samples respectively.

Application of 500 µg of fadrozole per gram of food between day 35 and day 71 pf strongly reduced the CYP19A mRNA expression in the body of 71-day-old fish. The transcript abundance of CYP19B in heads was attenuated compared to the control fish as well, but to a lower degree. Gonad histological analysis revealed only testes in these 71-day-old fish. And although the fadrozole treatment was stopped after day 71 pf, all treated fish were definitely of male phenotype at the end of the study on day 161 pf. These fish showed neither courtship nor spawning behavior. CYP19A and CYP19B mRNA transcript abundance in brain and gonads of the fadrozole-treated fish in the adult stage was characterized mainly by an enhanced CYP19B expression in the brain.

Treatment of zebrafish with 10 µg/l of MT since day 35 pf resulted in a massive induction of CYP19B mRNA expression in head samples of 71-day-old fish. CYP19A mRNA expression in the body samples, however, was lower than in control fish. Gonad differentiation of the MT treated fish was changed on day 71 pf. In contradiction to control fish of 71 dpf, gonad histology of these fish only revealed female gonads. A permanent 'feminizing' effect of the MT treatment on gonad differentiation is presumed because at least in the cause of the continuation of MT exposure until the reproductive stage, no

reproducing males could be identified among these fish. In these continually MT treated adult fish (until day 161) pf, the expression of CYP19B in the brain was noticeably elevated over to control level. In adult fish of the same age that were only exposed to MT between day 35 and day 71 pf, the expression of CYP19B in the brain was not enhanced. Only CYP19A in the gonads was slightly increased over the control level. Before, on day 71 pf, MT treatment mainly affected the CYP19B mRNA expression in the head samples which was clearly elevated over the control level. CYP19A transcript abundance in bodies of 71-day-old fish, however, was attenuated after MT treatment.

The most remarkable finding in this study was that the relative expression value of the CYP19 mRNA transcript in gonads of adult, exposed zebrafish segregated into two different groups in which the mean expression levels did either correspond to that of control male or of control female fish of the same age. Although the gonad histology identified only male (at the end of the fadrozole study) or female individuals (at the end of the MT study), a sexual dimorphic pattern of the CYP19A mRNA expression in gonads of adult zebrafish was identified.

The results of chapter 3 demonstrate that the inhibition of the aromatase by fadrozole and thus the suppression of estradiol synthesis, or the induction of the aromatase activity by the application of the enzyme substrate MT, are able to adversely disturb the sexual differentiation of zebrafish. It can be assumed that the administration of fadrozole or MT, and therefore the interference with the P450arom enzyme system, has taken place within a critical period of gonad development in which a precise regulation of steroid synthesis is absolutely indispensable for a directional differentiation of the genetically determined gonad morphology and sex. Due to the pronounced changes in the expression of the CYP19B mRNA transcript in head/brain samples in the cause of treatment, especially in adult fish, a determining, regulatory function of the cerebral P450arom in sexual differentiation may be suspected.

The findings of this thesis strongly support the assumption of a pivotal role of aromatase and its gene expression in sexual gonad development of zebrafish. Moreover, this implies a crucial involvement of the aromatase system in environmental hormone-induced reproductive disruption in fish. To realize the aromatase system as a potential target for disruptive effects of hormonal-active chemicals in the aquatic environment

increases the awareness of the close interrelation between the aromatase activity and the estrogenic pathways concerned, including vitellogenesis.

In conclusion, it is worth emphasizing, that the investigations and results of this thesis go beyond the state-of-the-art in the field of endocrine disruption in that they

(1) provide a thorough characterization of the response of two central molecular targets for environmental estrogens, VTG and CYP19 (P450arom genes), in zebrafish under different exposure regimes (short-term developmental exposure versus life cycle exposure),
(2) evaluate the correlation of the molecular responses with activational as well as organizational alterations of the phenotypic sexual differentiation and the reproductive capability of the zebrafish,
(3) demonstrate that the consequences of disturbing gonadal sexual differentiation are different when the effects were induced by estrogens or by aromatase modulators (reversible versus irreversible alterations),

and finally

(4) provide clear evidence that the relationship between molecular effects of endocrine-active substances and organismic changes is not necessarily a linear, straightforward relationship but can be rather complex.

The last statement is clearly corroborated by this thesis in showing, e.g., the discrepancy between the VTG response and the gonadal sexual differentiation in EE_2-exposed zebrafish, or the differential effects of fadrozole and MT on the cerebral and gonadal CYP19 expression.

It is still not much that is known about the molecular processes of endocrine disruption, not only in fish. Therefore the relation of these processes with alterations of the reproductive fitness or other physiological functions of organisms that are biologically and ecologically significant as well, are difficult to realize and understand. This thesis makes an important contribution to a better understanding of reproductive disruption in fish.

Zusammenfassung

In der vorliegenden Arbeit werden molekulare und organismische Grundlagen von endokrinen Störungen in Fischen (Teleostei) untersucht, die durch hormonell wirksame Umweltschadstoffe hervorgerufen werden und insbesondere die sexuelle (gonadale) Entwicklung beeinträchtigen. Derartige schadstoffbedingte Störungen des endokrinen und des reproduktiven Systems von Organismen sind in den letzten Jahren unter dem Begriff ‚Endokrine Disruption' heftig diskutiert worden. Im Mittelpunkt des ersten Teils dieser Arbeit stand die Betrachtung eines bereits etablierten Biomarkers, der häufig bei der Bewertung von östrogenartigen, endokrinen Effekten in Fischen angewendet wird, und zwar sowohl bei Untersuchungen *in vitro* als auch *in vivo*, im Labor so wie im Freiland (Kapitel 1 und 2): Das Eidotterprotein Vitellogenin (VTG).
Der zweite Teil der Arbeit beschäftigte sich mit der Expression der Gene der Cytochrom P450 Aromatase (P450arom), dem in allen Vertebraten gleichermaßen zuständigen Enzymkomplex für die Synthese von Östrogenen aus Androgenen (Kapitel 3).

Das Ziel der Untersuchungen war nicht allein der Nachweis von endokrinen Effekten, die zu Störungen in der Entwicklung und/oder der Reproduktion von Fischen führen und somit auf eine Belastung mit hormonell wirksamen Substanzen zurückgeführt werden können. Es ging vielmehr um eine detailliertere Betrachtung der Beziehungen von molekularen Störungen (in diesem Fall von VTG und dem P450/CYP19 System) und ungünstigen organismischen Beeinträchtigungen (hier speziell die Beeinträchtigung der Gonadendifferenzierung und der Fortpflanzungsfähigkeit). Die Kenntnis dieser Beziehungen wird ein mehr funktionales und mechanistisches Verständnis von endokrinen und reproduktiven Störungen bei Fischen vorantreiben.

Als Testart wurde der Zebrabärbling (*Danio rerio*) gewählt, da er als weitverbreiteter und empfohlener Testorganismus in Standard-Testprotokollen und Richtlinien für die Bewertung von endokrin-wirksamen Substanzen und deren Effekte im Fisch gilt und als solcher insbesondere innerhalb Europas eingesetzt wird. Wie sich anhand der Ergebnisse dieser Arbeit herausstellte, ist der Zebrabärbling eine juveniler Hermaphrodit, der im Verlauf seiner gonadalen Differenzierung ein protogynes Stadium durchläuft.

Vitellogenin

Für die Untersuchung der Induktion der Vitellogeninsynthese in sich entwickelnden und in adulten Zebrabärblingen wurde zunächst eine verlässliche und sensitive Testmethode entwickelt und validiert (Kapitel 1). Unter Anwendung dieser Testmethode konnte daraufhin die Vitellogenin-Antwort auf östrogene Belastung untersucht werden. Die Belastung erfolgte zum einen während unterschiedlicher Zeitspannen im Laufe der frühen Entwicklungphase und zum anderen während der geschlechtlichen Differenzierung des Zebrabärblings. Die Ergebnisse für VTG wurden mit reproduktiven Effekten wie dem Laicherfolg, der Fertilität (Befruchtungserfolg) und dem gonadalen Geschlechterverhältnis korreliert. Als Modellsubstanzen für endokrine Disruptoren eingesetzt wurden das synthetische Östrogen 17α-Ethinylöstradiol (EE_2) – eine Verbindung von sehr hoher, östrogener Potenz – und Bisphenol A (BPA) – eine in grossen Mengen von der Kunststoffindustrie produzierte Chemikalie, die als ein schwaches Xenoöstrogen wirkt.

EE_2 führte als ein starkes Östrogen, bei einer umweltrelevanten Konzentration von 3.0 ng/L, bereits im frühen Entwicklungsstadium des Zebrabärblings zu östrogenartigen Reaktionen. In 43 % der Fische, die direkt nach der Befruchtung für 21 Tage an EE_2 exponiert waren, lag der VTG-Spiegel in Gesamtkörperhomogenaten über dem durchschnittlichen Wert der Kontrolltiere. Dagegen zeigte sich in den frühen Entwicklungsstadien, die mit dem schwacher wiksamen BPA behandelt wurden, keine VTG-Antwort. Die durchschnittlichen VTG-Spiegel in den Homogenaten dieser Fische am Tag 21 nach der Fertilisation (post Fertilisation, pf) waren gegenüber den Kontrollen nicht signifikant erhöht und bis zu fünfzehn Mal niedriger als in den EE_2-behandelten Tieren. Die relative VTG-Induktionskapazität (definiert als Verhältnis von normalisierter VTG-Konzentration einer Probe zur mittleren VTG-Konzentration der entsprechenden Kontrollen) von BPA war durchschnittlich kaum höher als 1. Die relative Induktion von EE_2 dagegen lag über 25. Diese Befunde belegen, dass das VTG Systems bereits in der frühen Entwicklungsphase des Zebrabäblings zumindest auf eine Exposition mit stark potenten östrogen Verbindungen empfindlich reagiert.

BPA wurde erst nach 75-tägiger ununterbrochener Exposition ab Fertilisation bei der VTG-Induktion wirksam. Eine signifikante Erhöhung des VTG in Körperhomogenaten bestätigte sich zu diesem Zeitpunkt bereits bei der geringsten Testkonzentration von 94 µg/L und die relative Induktion des VTG in den BPA-Belastungsgruppen lag zwischen 2.1 und 5. Nach 75 Tagen pf Belastung mit 3.0 ng/L EE_2 wurde dagegen eine relative VTG-

Induktion von 111 bestimmt was einem mittleren VTG-Plasmatiter von 250 µg/ml entsprach.

Im adulten, reproduktionsfähigem Stadium der Fische ergaben sich als lowest observed effect concentrations (LOECs) bei den Männchen ein Schwellenwert von 375 µg/L BPA und bei den Weibchen von 94 µg/L BPA. Die LOEC von 375 µg BPA/L für eine signifikante Erhöhung von VTG in den Männchen entsprach der LOEC für eine signifikante Verminderung der Befruchtung bei den im Mittel pro Tag abgelaichten Eiern.

Chronische Belastung der Zebrabärblinge, von der Befruchtung bis zum Adultstadium, an 3.0 ng/L EE_2 hatte ein vollständiges Ausbleiben der Reproduktion zur Folge. Ein Wiedererlangen der Reproduktionsfähigkeit nach Beendung der Exposition konnte nur in eingeschränktem Maße beobachtet werden. Bei der histologischen Untersuchung dieser kontinuierlich mit EE_2 belasteten Fische am Tag 118 pf konnte ausschließlich ein ovarieller Gonadentypus gefunden werden. Nachdem diese zuvor dauerbelasteten Fische aus der Belastung zwischen Tag 125 und Tag 176 pf herausgenommen waren, konnten in 30 % der 176 Tage alten Fische wieder Testes gefunden werden.

Im Fall von BPA war die Interpretation der gonadenhistologischen Ergebnisse wesentlich schwieriger als im Fall von EE_2. Nach chronischer EE_2-Belastung war das Ergebnis eindeutig, denn es wurden nur Ovarien in den Fischen gefunden. In den BPA-belasteten Fischen konnte aber zumindest in den drei höchsten Belastungskonzentrationen ein deutlich höherer Anteil an histologisch identifizierbaren Ovarien festgestellt werden (siehe Maack 2002 für weitere Details).

Das bemerkenswerteste Ergebnis dieser Studie war allerdings, dass während der gesamten Entwicklung des Zebrabärblings ein dimorphes Verteilungsmuster bei der VTG-Induktionsantwort der untersuchten Fische zu beobachten war. Selbst wenn aufgrund der histologischen Analyse der Gonaden von einer Fischpopulation auszugehen war, die nur aus Männchen oder nur aus Weibchen bestehend, so zeigte sich auch bei diesen Fischen das dimorphe VTG-Antwortmuster. Bereits in dem frühen Entwicklungsstadium von 21 Tagen pf konnte bei den EE_2-belasteten Fischen eindeutig eine Gruppe abgegrenzt werden, in der die Fische ein signifikant höheres Antwortniveau von VTG zeigten als es in den übrigen Fischen unter EE_2-Belastung der Fall war. In Fischen, älter als 21 Tage pf, bestätigte sich diese dimorphe Verteilung der VTG-Konzentrations-Messwerte auch bei den Kontrollfischen. In den adulten, reproduktionsfähigen Fischen zeigten die individuellen VTG-Plasmatiter geschlechtsspezifische Niveaus, woran eindeutig zwischen

einem Männchen oder einem Weibchen unterschieden werden konnte. Dies galt zumindest uneingeschränkt für die Kontrollen. In den EE_2-belasteten oder BPA-belasteten Fischen stellte sich heraus, dass die individuelle VTG Antwort meist nicht mit dem gonadalen Geschlecht oder dem morphologischen Geschlecht übereinstimmte. Aufgrund dieses Befundes wurde die These aufgestellt, dass östrogene Exposition während der geschlechtlichen Entwicklungsphase des Zebrabärblings zwar zu einer Modulation oder Veränderung des Differenzierungsprozesses führen kann. Allerdings kann die östrogene Belastung nicht die Geschlechtsdetermination und die daran beteiligten, genetisch regulierten Reaktionswege verändern und damit direkt oder indirekt die VTG-Synthese beeinflussen. Die Exposition an ein starkes Östrogen wie EE_2 vermochte die geschlechtliche gonadale Differenzierung zu verändern und bewirkte eine vollständige gonadale Verweiblichung, Die Ergebnisse der VTG-Analyse zeigten nichtsdestotrotz eindeutig, dass diese Veränderung des gonadalen Geschlechts nicht zwangsläufig von einer dem gonadalem Geschlecht entsprechenden Veränderung der VTG-Synthese begleitet sein muss. Dieser Befund untermauert stark die Hypothese einer genetisch geschlechtgekoppelten VTG-Antwort im Zebrabärbling, wobei diese Annahme weitere, mechanistische Untersuchungen verlangt. Eine enge Verknüpfung zwischen der Ausbildung der geschlechtsdimorphen VTG-Synthese und der protogynen Geschlechtsdifferenzierung des Zebrabärbling ist jedoch sehr wahrscheinlich.

Nicht nur die Effekte chronischer Östrogenbelastung wurden in dieser Studie untersucht, sondern insbesondere auch die Effekte zeitlich begrenzter Exposition in Hinblick auf die Frage nach der Reversibilität der VTG-Antwort. Das wichtigste Ergebnis in Bezug auf diese Frage war, dass eine kurzzeitige Exposition für 14, 21 oder 42 Tage pf an 3.0 ng EE_2/L keine Auswirkungen auf VTG in den adulten männlichen Fischen zeigte. Lediglich bei den Weibchen war VTG noch signifikant über das Kontrollniveau erhöht, auch nachdem die Fische bereits zehn bis fünfzehn Wochen aus der Belastung heraus waren. Nach weiteren acht Wochen ohne Belastung war VTG nur noch in den Weibchen erhöht, die für 42 Tage pf exponiert waren. Aus diesen Befunden leitet sich die Vermutung ab, dass als Voraussetzung für eine längere Fortdauer der Effekte auf VTG die Dauer der Belastung während der Entwicklung entscheidend sein könnte.

Abschliessend kann gesagt werden, dass die Ergebnisse dieser Studie recht eindeutige Beweise dafür liefern, dass VTG tatsächlich ein verlässlicher Indikator für eine aktuelle

östrogene Belastung ist. Allerdings kann VTG keine vorhersagende Funktion – zumindest nicht beim Zebrabärbling – für expositionsbedingte, reproduktive und organisatorische Effekte zugeschrieben werden. In der aktuellen Belastungssituation erwies sich die VTG-Induktion als nicht mehr oder weniger sensitiv als organisatorische, organismische Effekte wie die Fertilität oder die Fruchtbarkeit. In Männchen, in welchen die VTG-Induktion in direkter Verbindung mit einer östrogenen Belastung steht, stimmten die effektiven Konzentrationen für die Induktion von VTG und für reproduktive Störungen gut überein. Dies war sogar weitestgehend unabhängig von der östrogenen Stärke der exponierten Substanz. Die schnelle Reversibiltät der VTG-Antwort nach Abschluss der Exposition bedingt eine wesentlich höher Wahrscheinlichkeit für eine falsche Interpretation von Effekten endokrin wirksamer Substanzen. Dies widerspricht jeglichem Anspruch auf eine vorhersagende Qualität des VTG, zumindest für den Zebrabärbling.

Aromatase

Im 3. Kapitel dieser Arbeit stand die Untersuchung der unterschiedlichen Expression der Ovarien-abgeleiteten und der Gehirn-abgeleiteten P450arom-Gentranskripte, CYP19A und CYP19B, mittels semi-quantitativer RT-PCR im Mittelpunkt der Betrachtungen. Das Ziel der Untersuchungen war, zu einem besserem Verständnis der Bedeutung der Östradiol-Synthese bei der geschlechtlichen (Gonaden-) Differenzierung beim Zebrabärbling zu gelangen. Um mögliche Folgen einer Störung des Aromatasesystems auf die Geschlechtsdifferenzierung untersuchen zu können, wurden während der gonadalen Differenzierungsphase der Fische der nicht-steroidale Aromatase-Inhibitor Fadrozole und das aromatisierbare Androgen 17α-Methyltestosteron (MT) zur Behandlung eingesetzt. Veränderungen bei der CYP19A- und CYP19B-mRNA-Expression wurden zum einen am Ende der Belastung am Tag 71 pf und zum anderen im adulten Fortpflanzungsstadium der Fische analysiert und mit der CYP19-mRNA-Expression nicht-behandelter Fische verglichen. Die Effekte der beiden Substanzen, Fadrozole und MT, auf die Gonadendifferenzierung wurden mittels Gonadenhistologie untersucht.

In geschlechtlich noch undifferenzierten, unbelasteten Zebrabärblingen, im Alter von 35/36 Tagen pf konnten beide CYP19-mRNA-Transkripte, das zerebrale als auch das gonadale, im Kopf sowie im Körper der Fische nachgewiesen werden. Die Häufigkeitsverteilung beider Transkripte, CYP19A und CYP19B, im Kopf und im Körper offenbarte jeweils ein dimorphes Expressionsmuster. CYP19A war stärker in den Körperproben, CYB19B dagegen stärker in den Kopfproben exprimiert, eindeutig

erkennbar zumindest in einer der beiden Studien. Die höchste Expressionrate überhaupt wurde für CYB19B in den Köpfen registriert. Am Tag 71 pf, entsprechend dem Ende der Belastungzeit, zeigte sich ein sehr ähnliches mRNA-Expressionsmuster von CYP19A und CYP19B wie am Tag 35 pf. Beide Transkripte ließen allerdings auf eine höhere Genaktivität in den Köpfen beziehungsweise Rümpfen der Fischen schließen.
Die Applikation von 500 µg Fadrozole pro Gramm Futter zwischen Tag 35 und Tag 71 pf, hatte eine starke Reduktion der CYP19A-mRNA-Expression im Körper der 71 Tage alten Fische zur Folge. Die CYP19B-mRNA-Expression in den Köpfen war im Vergleich zu den Kontrollen zwar ebenfalls abgeschwächt, allerdings weniger stark als CYP19A im Körper. Die histologische Analyse ergab, dass in diesen 71 Tage alten Fischen ausschließlich Testes entwickelt waren. Auch nachdem die Behandlung mit Fadrozole am Tag 71 pf abgesetzt worden war, waren alle zuvor Fadrozole-belasteten Fische im Adultstadium (Tag 161 pf) von männlichem Phänotyp. Diese Fische zeigten weder ein Balzverhalten noch Laichaktivität. Die Expression der CYP19A- und CYP19B-mRNA-Transkripte im Gehirn und in den Gonaden dieser Fadrozole-behandelten Fische war insbesondere charakterisiert durch eine verstärkte CYP19B-Expression im Gehirn.

Die Behandlung der Zebrabärblinge ab Tag 35 pf mit 10 µg/L MT führte zu einer massiven Erhöhung der CYP19B-mRNA-Expression in Köpfen 71 Tage alter Individuen. Die CYP19A mRNA-Expression in den Körperproben war dagegen niedriger als in den entsprechenden Kontrollproben. Die Gonadendifferenzierung dieser MT-belasteten Fische erwies sich als verändert, und am Tag 71 pf konnten lediglich weibliche Gonaden identifiziert werden. Ein dauerhaft ‚verweiblichender' Effekt durch die MT-Behandlung ist zu vermuten, denn bei der Fortsetzung der MT-Behandlung bis zum fortpflanzungfähigem Stadium der Fische am Ende des Experiments konnten keine reproduzierenden Männchen unter den Fischen identifiziert werden. Die CYP19B-mRNA-Expression in diesen kontinuierlich bis zum Adultstadium (Tag 161 pf) MT behandelten Fischen war in den Gehirnen deutlich über das Kontrollniveau erhöht. Bei den adulten Fischen, die lediglich zwischen Tag 35 und Tag 71 pf mit MT belastet waren, war im Vergleich dazu die CYP19B-mRNA-Expression in den Gehirnen nicht verstärkt. Nur die CYP19A-mRNA-Expression in den Gonaden zeigte eine leichte Erhöhung gegenüber den Kontrollen an. In den jüngeren Fischen (Tag 71 pf), zeigte sich ein Effekt der MT-Behandlung bei der CYP19B-mRNA-Expression in den Köpfen, wobei die CYP19B-Transkripthäufigkeit deutlich über das Kontrollniveau hinaus erhöht war. Die CYP19A-Transkripthäufigkeit in

den Rumpfproben dieser 71 Tage alten Fische hingegen war nach der MT Behandlung abgeschwächt.
Der bemerkenswerteste Befund der Studie war, dass sich die relative Expressionstärke des CYP19A-mRNA-Transkripts in Gonaden adulter, belasteter Zebrabärblinge in zwei klar unterscheidbare Gruppen unterteilt. Dabei entsprach die mittlere Expressionsrate entweder der eines Kontroll-Männchens oder der eines Kontroll-Weibchens des gleichen Alters. Obwohl die Gonadenhistologie ausschließlich Männchen (am Ende der Fadrozole-Studie) oder ausschließlich Weibchen (am Ende der MT-Studie) identifizierte, war ein geschlechtsdimorphes Expressionmuster von CYP19A in den Gonaden weiterhin erkennbar.

Die Ergebnisse des 3. Kapitels zeigen, dass die sich Inhibition der Aromatase durch Fadrozole und damit die Unterdrückung der Östradiolsynthese, oder die Induktion der Aromataseaktivität durch die Applikation des Enzymsubstrats MT, nachteilig und störend auf die Geschlechtsdifferenzierung des Zebrabärblings auswirken können. Die Störung des P450arom-Enzymsystems erfolgte vermutlich zu einem kritischen Zeitpunkt der gonadalen Entwicklung, nämlich genau dann, wenn eine präzise Regulation der Steroidsynthese absolut unerlässlich ist, um eine gerichtet Differenzierung der Gonaden und des Geschlechts entsprechend der genetischen Voraussetzungen gewährleisten zu können. Die betont starken Veränderungen der CYP19B mRNA-Expression im Kopf/Gehirn der Fische infolge der Behandlung, insbesondere bei den Adulttieren, lässt auf eine bestimmende, regulatorische Funktion der zerebralen P450arom bei der Geschlechtsdifferenzierung schließen.

Die Befunde dieser Arbeit untermauern stark die These einer bestimmenden Rolle der Aromatase und ihrer Genexpression bei der geschlechtlichen gonadalen Entwicklung des Zebrabärblings. Darüberhinaus implizieren sie eine entscheidende Beteiligung des Aromatasesystems bei den durch Umwelthormone verursachten reproduktiven Störungen (Disruption) in Fischen. Das Erkennen des Aromatasesystems als ein mögliches Angriffsziel für hormonartige Chemikalien in der aquatischen Umwelt und für ihre störenden Wirkung verbessert das Verständnis der engen Zusammenhänge zwischen der Aromataseaktivität und allen damit verknüpften, östrogenen Stoffwechselwegen, einschließlich der Vitellogenese.

Abschließend ist noch einmal herauszustellen, dass die Untersuchungen und Ergebnisse dieser Arbeit über den aktuellen Stand der derzeitigen Forschung im Bereich der Endokrinen Disruption hinausgehen, und zwar weil die Ergebnisse

(1) eine ausführliche Charakterisierung des Antwortverhaltens zweier zentraler, molekularer Angriffsziele für Umweltöstrogene, VTG und CYP19 (P450arom), im Zebrabärbling liefern, und dies unter unterschiedlichen Belastungsregimes (kurzzeitige Belastung während der Entwicklung und Life-Cycle- Belastung),

(2) die molekularen Antworten sowohl mit funktionalen, und meist temporären, Störungen als auch mit organisatorischen Veränderungen bei der phänotypischen Geschlechtsdifferenzierung und der Reproduktionsfähigkeit des Zebrabärbling korrelieren und bewerten,

(3) Unterschiede bei der Qualität der Effekte, die durch eine Störung der gonadalen Geschlechtsdifferenzierung hervorgerufen wurden, aufzeigen, wenn diese entweder durch Östrogene oder durch Aromatase-Modulatoren hervorgerufen werden (reversible gegenüber irreversible Folgen),

und schließlich

(4) klare Belege dafür liefern, dass der Zusammenhang zwischen den molekularen Effekten von endokrin-wirksamen Substanzen und organismischen Veränderungen nicht notwendigerweise ein geradliniger und einfacher monokausaler Zusammenhang seon muss, sondern äußerst komplex sein kann.

Dieses letzte Argument wird beispielsweise durch die Darstellung der Diskrepanz zwischen der VTG-Antwort und der gonadalen Geschlechtsdifferenzierung in EE_2 belasteten Fischen oder anhand der Verschiedenartigkeit der Effekte von Fadrozole und MT auf die zerebrale und gonadale CYP19 Expression eindrucksvoll mit dieser Arbeit untermauert.

Es ist immer noch sehr wenig, was über die molekularen Prozesse endokriner Störungen, nicht nur bei Fischen, bekannt ist. Daher kann auch die Verknüpfung dieser Prozesse mit Veränderungen der Reproduktionsfähigkeit oder mit anderen physiologischen Funktionen von Organismen, die von essentieller biologischer und auch ökologischer Bedeutung sind, nur schwer verstanden werden. Diese Arbeit leistet einen wichtigen Beitrag für ein besseres Verständnis von reproduktiven Störungen in Fischen.

Appendix

Fig. 10. Length and weight data of zebrafish at different ages, exposed to fadrozole or 17a-methyltestosterone (MT) ⇒ Data belong to chapter 3.

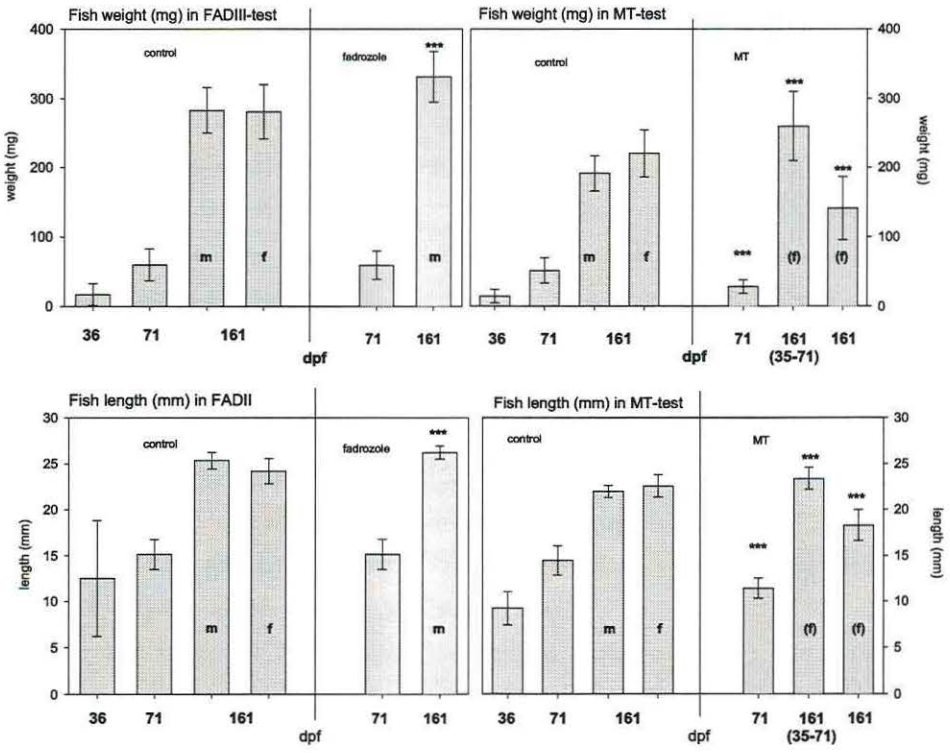

Fig 10. Fadrozole and MT experiment:
Body length (mm) and weight (mg) of zebrafish exposed to fadrozole or MT after 36, 71 and 161 dpf. Significant differences (pooled data from replicate controls and each treatment) from the controls at the same sampling time were indicated (***, t-test, $P \leq 0.001$). Values are means ± SD.

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List of publications

Manuscripts independent from this thesis

Fenske, M., Maack, G., Schäfers, C., Schmitz, A. and Segner, H., 2000. Development of bioanalytic tools for the evaluation of endocrine-modulating effects on the development and reproduction of zebrafish, *Danio rerio*. In: Proceedings of the 6th International Symposium on the Reproductive Physiology of Fish. Bergen, 1999, pp. 389

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Fenske, M., Maack, G., Schäfers, C., Segner, H., 2001. Assessment of estrogenic effects on reproduction and sexual differentiation in zebrafish (*Danio rerio*): Alterations in vitellogenesis and reproductive physiology.

Poster at the Second Statusseminar 'endocrine disrupters' of the Umweltbundesamt (UBA), 2nd – 4th April 2001, Berlin.

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Eidesstattliche Erklärung

Hiermit erkläre ich, daß ich die vorliegende Arbeit selbständig verfasst, keine anderen als die angegebenen Hilfsmittel verwendet und alle Stellen, die im Wortlaut oder dem Sinn nach anderen Werken entnommen sind, mit Quellenangaben kenntlich gemacht habe.

Mit der vorliegenden Arbeit bewerbe ich mich erstmals um den Doktorgrad.

Leipzig, den 15.04.2002

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Martina Fenske